Faculty of Pharmaceutical, Biomedical and Veterinary Sciences
Department of Biomedical Sciences

ALZHEIMER'S DISEASE CEREBROSPINAL FLUID BIOMARKERS FOR DIFFERENTIAL DEMENTIA DIAGNOSIS

LIMITATIONS AND OPPORTUNITIES

HET GEBRUIK VAN ALZHEIMER BIOMARKERS IN CEREBROSPINAAL VOCHT VOOR DIFFERENTIËLE DEMENTIE DIAGNOSTIEK: BEPERKINGEN EN KANSEN

Dissertation for the degree of doctor in Biomedical Sciences
at the University of Antwerp to be defended
by

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3R	3 repeat tau isoform
4R	4 repeat tau isoform
[^{11}C]PiB	[^{11}C]Pittsburgh Compound-B
[^{18}F]FDG	[^{18}F]fluorodeoxyglucose
Aβ	amyloid-β protein
$A\beta_{1-42}$	Aβ isoform of 42 amino acids length
AD	Alzheimer's disease
ANOVA	analysis of variance
APOE	apolipoprotein E
APP	amyloid precursor protein
AUC	area under the curve
BIODEM	Reference Center for Biological Markers of Dementia
CAA	cerebral amyloid angiopathy
CBD	corticobasal degeneration
CI	confidence interval
CJD	Creutzfeldt-Jakob disease
CRM	certified reference material
CSF	cerebrospinal fluid
CT	computed tomography
CV	coefficient of variation
CVD	cerebrovascular disease
DLB	dementia with Lewy bodies
DTI	diffusion tensor imaging
ELISA	enzyme-linked immunosorbent assay
ES	Erlangen Score
FTD	frontotemporal dementia
FTLD	frontotemporal lobar degeneration
GRN	Progranulin gene
IBB	Institute Born-Bunge
IMR	immunomagnetic reduction
IP	immunoprecipitation
IQR	interquartile range
IWG	International Working group

LBD	Lewy Body disease
LP	lumbar puncture
MCI	mild cognitive impairment
MMSE	Mini-Mental State Examination
MRI	magnetic resonance imaging
MS	mass spectrometry
MxD	mixed dementia
NFL	neurofilament light
NFT	neurofibrillary tangles
Ng	neurogranin
NIA-AA	National Institute on Aging/Alzheimer's Association
Non-AD	dementia not attributed to Alzheimer's disease
NP	neuritic plaques
PD	Parkinson's disease
PDD	Parkinson's disease dementia
PET	positron emission tomography
PMCA	protein-misfolding cyclic amplification
PrP	prion protein
PSP	progressive supranuclear palsy
P-tau	phosphorylated tau protein
QC	quality control
ROC	receiver operating characteristic
RT-QuIC	real-time quaking-induced conversion
sAPP	soluble amyloid precursor protein
SCD	subjective cognitive decline
SD	standard deviation
Simoa	single-molecule array
TDP-43	TAR DNA-binding protein 43
TLPD	time between lumbar puncture and time of death
T-tau	total tau protein
VaD	vascular dementia

Over the last couple of decades, cerebrospinal fluid (CSF) biomarkers have been studied thoroughly for their potential to closely reflect neuropathological processes in the brain. To date, three pathological hallmarks of Alzheimer's disease (AD), including amyloid plaques, neurofibrillary tangles and neurodegeneration, can be reliably used for its biochemical diagnosis as they are reflected in the changes of CSF biomarker levels of the 42 amino acid long amyloid-beta peptide ($A\beta_{1-42}$), total tau protein (T-tau), and tau phosphorylated at threonine 181 (P-tau_{181}). Despite their well-established accuracy for picking up AD pathology, these biomarkers still show distinct overlap between AD and non-AD neurodegenerative disorders. Therefore this PhD thesis hypothesizes that the early differential diagnosis of AD needs further improvement and aims to do so by overcoming the overlap of neurochemical biomarkers measured in CSF.

By correlating the core AD CSF biomarkers with recently updated criteria for neuropathological diagnosis of AD to assess how well the CSF biomarkers actually reflect AD pathology, CSF P-tau_{181} was found to be the most specific factor in the CSF biomarker profile for AD diagnosis as it was found to correlate with both amyloid and neurofibrillary pathology in *APOE* ε4 non-carriers late-stage AD pathology. In contrast, limited correlations were found for CSF T-tau which supports its use as neurodegeneration marker rather than an AD-specific neurofibrillary marker, while for CSF $A\beta_{1-42}$, lack of correlations probably reflect the ceiling effect of amyloid plaque pathology but does not exclude its value as an early AD biomarker.

Despite its role in the early differential diagnosis of AD, it is also the biomarker showing the most overlap. By reviewing other potential biomarker candidates of the Aβ metabolism, CSF $A\beta_{1-42}$ was shown to remain the most accurate AD biomarker, as it proved to be consistently altered in AD compared to controls. However, in the early differential diagnosis of AD versus non-AD disorders, the CSF biomarker panel may benefit from the addition of the $A\beta_{1-42}/A\beta_{1-40}$ ratio to increase the CSF biomarker concordance with AD neuropathological findings in the brain. Validating the use of the $A\beta_{1-42}/A\beta_{1-40}$ ratio both for its differential accuracy in autopsy-confirmed AD and non-AD disorders as well as for its potential to detect clinical AD over the disease spectrum, showed that the CSF $A\beta_{1-42}/A\beta_{1-40}$ ratio outperformed CSF $A\beta_{1-42}$, also when mixed pathology was present. In addition, although the $A\beta_{1-42}$/P-tau_{181} ratio was found to perform equally well as the $A\beta_{1-42}/A\beta_{1-40}$ ratio, the stability of the Aβ ratio and its cut-points over the disease spectrum is advantageous. The use of certified reference material (CRM) calibrated $A\beta_{1-42}$ assays enables the determination of a cut-off value that could be introduced worldwide in order to improve the interpretation of the CSF biomarker results.

As prion aggregation is the main pathological hallmark of Creutzfeldt-Jakob disease (CJD), the potential role of total prion protein (PrP-t) in CSF for the discrimination of AD and CJD with overlapping CSF T-tau levels was determined. Although CSF PrP-t has shown its value in the biomarker-based differential diagnosis of AD, the more so in the specific scenario of differentiation between atypical forms of AD with very high T-tau levels and CJD, the T-tau/P-tau_{181} ratio as well as the PrP real-time quaking-induced conversion (RT-QuIC) assay was found to outperform PrP-t.

Besides biological factors causing overlapping CSF biomarkers levels, pre-analytical and analytical factors have been shown to influence CSF biomarker measurements, preventing the comparison of biomarker data. To improve the harmonization of absolute CSF biomarker values, a diagnostic-relevant interpretation algorithm, namely the Erlangen Score (ES), was validated for the AD versus non-AD differentiation. The ES was a significant predictor for the probability to having AD pathology post-mortem in an autopsy-confirmed cohort of AD and non-AD disorders, making it a useful diagnostic tool for comparing neurochemical diagnoses between different labs or methods used, independently of their specific cut-offs, pre-analytical handling procedures, and applied analytical methods.

As no single biomarker has yet been found to fully and specifically diagnose AD along its progression spectrum due to its high complexity and overlap with other neurodegenerative disorders, a panel including different biomarkers is crucial. The addition of new biomarkers may therefore improve its differential performance, either by detecting AD or non-AD specific pathology, preferably in its earliest stages while also eliminating the invasiveness of a lumbar puncture (LP) or the high costs and limited access of PET imaging. Such biomarkers would have a critical value in the screening for AD and related disorders, patient selection for inclusion and monitoring drug responses in clinical trials with frequent repetitive measures.

Het gebruik van biomarkers in cerebrospinaal vocht (CSV) zijn door de decennia heen uitgebreid bestudeerd geweest op hun potentieel om neuropathologische processen in de hersenen te weerspiegelen. Drie CSV biomarkers worden momenteel gebruikt voor de biochemische diagnose van de ziekte van Alzheimer, namelijk het amyloïde peptide bestaande uit 42 aminozuren ($A\beta_{1-42}$), het totaal tau proteïne (T-tau), en het tau proteïne gefosforyleerd op threonine 181 (P-tau_{181}). Deze kunnen betrouwbaar gemeten worden en weerspiegelen de pathologische kenmerken van de ziekte van Alzheimer, respectievelijk amyloïde plaques, neurofibrillaire kluwens, en neurodegeneratie. Hoewel deze biomarkers gekend zijn om accuraat de pathologie op te pikken, bestaat er een overlap tussen de concentraties gemeten in de ziekte van Alzheimer en andere niet-Alzheimer gerelateerde neurodegeneratieve aandoeningen. Deze doctoraatsthesis focust zich daarom op het verbeteren van de vroege differentiële diagnostiek van de ziekte van Alzheimer door deze overlap van neurochemische biomarkers te adresseren.

In welke mate deze 'kern' CSV biomarkers voor de ziekte van Alzheimer de pathologie werkelijk weerspiegelen werd beoordeeld in een autopsie-geconfirmeerde cohorte om hun klinische relevantie beter te karakteriseren. Hiermee werd gevonden dat in vergevorderde pathologische stadia CSV P-tau_{181} correleert met zowel de amyloïde als de neurofibrillaire pathologie in patiënten die het *APOE* ε4 allel niet dragen. Dit benadrukt de rol van P-tau_{181} als surrogaat marker in het CSV biomarker pofiel voor de diagnose van de ziekte van Alzheimer. Daarentegen werden slechts geringe correlaties gevonden voor T-tau, hetgeen zijn gebruik als neurodegeneratie marker ondersteunt eerder dan zijn reflectie van Alzheimer-specifieke neurofibrillaire pathologie. Het gebrek aan correlaties voor $A\beta_{1-42}$ kan toegeschreven worden aan plafondeffecten van de amyloïd pathologie, hetgeen de waarde van deze biomarker in de vroege diagnostiek niet uitsluit.

CSV $A\beta_{1-42}$ is de biomarker waarvoor veranderingen het snelst gezien worden, maar die ook de meeste overlap vertoont. Hierdoor werd het Aβ metabolisme gescreend op nieuwe potentiële biomarker kandidaten op basis van de bestaande literatuur. Hoewel CSV $A\beta_{1-42}$ consistent verlaagd is in de ziekte van Alzheimer in vergelijking met controles en hiermee superieur blijkt ten opzichte van andere Aβ peptiden of amyloïde precursor proteïne fragmenten, kon geconcludeerd worden dat de toevoeging van de $A\beta_{1-42}/A\beta_{1-40}$ ratio als meest belovende kandidaat de differentieel diagnostiek positief kan beïnvloeden. Ter validatie van de diagnostische accuraatheid in autopsie geconfirmeerde ziekte van Alzheimer ten opzichte van andere definitieve neurodegeneratieve hersenaandoeningen alsook van het potentieel om klinische AD te detecteren, overtrof de $A\beta_{1-42}/A\beta_{1-40}$ ratio het gebruik van $A\beta_{1-42}$ alleen, zelfs wanneer gemengde pathologie van Alzheimer met niet-Alzheimer gerelateerde aandoeningen aanwezig was. Daarbij kwam de $A\beta_{1-42}$/P-tau_{181} ratio naar boven als evenwaardig aan de $A\beta_{1-42}/A\beta_{1-40}$ ratio, hoewel de stabiliteit van de Aβ ratio en zijn cutoff waarden doorheen het gehele ziekteverloop van de ziekte van Alzheimer een sterk voordeel zijn. Door het gebruik van CSF $A\beta_{1-42}$ assays die werden gekalibreerd met behulp van gecertifieerd referentie materiaal kan de bepaling van wereldwijd geïntegreerde cutoff

waarden mogelijk gemaakt worden om zo de interpretatie van CSV biomarker resultaten te optimaliseren.
Aggregatie van het prion protein (PrP) is een neuropathologisch kenmerk van de ziekte van Creutzfeldt-Jakob. De potentiële rol van de totale PrP concentratie in CSV werd daardoor bepaald voor de onderscheiding van de ziekte van Alzheimer en de ziekte van Creutzfeldt-Jakob met overlappende CSV T-tau waarden. De totale CSV concentratie van PrP bleek hierbij waarde te hebben in de biomarker-gebaseerde differentieel diagnose, des te meer in het specifieke scenario om het onderscheid te maken tussen atypische vormen van de ziekte van Alzheimer met heel hoge T-tau waarden en de ziekte van Creutzfeldt-Jakob. Echter, de totale PrP concentratie werd overtroffen door de T-tau/P-tau$_{181}$ ratio, alsook door de PrP real-time quaking-induced conversion (RT-QuIC) methode.
Niet enkel biologische factoren zijn verantwoordelijk voor de biomarker overlap. Ook pre-analytische en analytische factoren zijn gekend CSV biomarker metingen te kunnen beïnvloeden en dit verhindert de vergelijkingen van ruwe biomarker data. Ter standaardisatie van de interpretatie van de ruwe CSV biomarker waarden, wat kan leiden tot een verlaagde diagnostische accuraatheid wanneer niet optimaal uitgevoerd, werd de Erlangen Score gevalideerd als algoritme voor de differentieel diagnostiek. Dit algoritme bleek een significante voorspeller te zijn voor de kans op Alzheimer pathologie, postmortaal, in een autopsie-geconfirmeerde cohorte met patiënten met een ziekte van Alzheimer en patiënten met een niet-Alzheimer gerelateerde hersenaandoening. De Erlangen Score is hierdoor een geschikt diagnostisch middel om neurochemsiche diagnoses tussen verschillende labo's of methodes te kunnen vergelijken, en dit onafhankelijk van labo-specifieke cutoff waarden, pre-analytische behandelingen, of gebruikte analytische methode.
Door zijn complexiteit en overlap met andere neurodegeneratieve aandoeningen, is het nog niet mogelijk om met slechts één biomarker de ziekte van Alzheimer specifiek en over zijn complete ziekteverloop te diagnosticeren, waardoor een biomarker panel opgesteld uit verschillende biomarkers cruciaal is. Daarom kan de toevoeging van nieuwe biomarkers de differentiële accuraatheid verbeteren, door ofwel de detectie van Alzheimer of juist van niet-Alzheimer specifieke pathologie te detecteren, en dit liefst in de vroegste fases van de ziekte, zonder de invasiviteit van een lumbale punctie of de hoge kosten en beperkte toegankelijkheid van PET beeldvorming. Deze biomarkers zouden van onschatbare waarde zijn om te screenen naar de ziekte van Alzheimer of verwante aandoeningen, om patiënten te selecteren voor inclusie en opvolging van reacties op therapieën in klinische studies met frequente metingen.

GENERAL INTRODUCTION

"The time to fix the roof is when the sun is shining" – John F. Kennedy

Accounting for 60-80% of the population older than 60 years, Alzheimer's disease (AD) covers the most common cause of dementia[1]. Dementia is the general description of a syndrome, with a wide range of cognitive symptoms varying by underlying diseases like AD or other (neurodegenerative) brain disorders such as frontotemporal lobar degeneration (FTLD), Lewy Body disease (LBD), cerebrovascular disease (CVD) and Creutzfeldt-Jakob disease (CJD). Prevalence of dementia is known to increase with age, currently estimated at 5-7% in the population of 60 years and older, and is expected to rise even further as life expectancy goes up and mortality goes down[2].

Recommendations for the clinical diagnosis of AD have been described by the Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association, originating from 1984 (NINCDS-ADRDA; table 1.1)[3, 4]. A clinical diagnosis of AD can be established by a full clinical workup[5], which incorporates a neuropsychological examination and brain imaging, resulting at best, in a diagnosis of probable AD, mainly by the exclusion of other disorders that could account for cognitive deficits.

A typical course of AD (figure 1.1) is represented by an insidious onset and a progressive nature. Because of the involvement of the hippocampal area in the beginning of the disease course, first symptoms usually include episodic memory problems[6]. Frequently, the cognitive impairment is initially only noticeable by the patient and cannot yet be objectified by neuropsychological testing. This is a preclinical phase of subjective cognitive decline (SCD), later progressing to a prodromal phase of mild cognitive impairment (MCI) upon objective assessment. Symptoms progress gradually and only until the final phase, when symptoms are severe enough to interfere with a person's everyday activities, the term of dementia can be applied[4, 7].

The most prevailing theory on the pathophysiological trigger of AD proposes an imbalance in the production and/or clearance of the amyloid-β peptide (Aβ)[8]. The Aβ peptide is produced by the enzymatic cleavage of the membrane spanning amyloid precursor protein (APP; figure 1.2). Cleavage by the enzymes β-and γ-secretase will lead to the formation of Aβ isoforms of varying lengths. It is known that the overload of the very sticky Aβ isoform of 42 amino acids length ($A\beta_{1-42}$) accompanying the Aβ imbalance, provokes an aggregation process leading to the formation of extracellular amyloid plaques. Surprisingly, Aβ deposition does not show strong correspondence with neurodegeneration nor with cognitive dysfunction[9], indicating the involvement of other downstream mechanisms. It has been found that a small fraction of soluble Aβ oligomers is particularly bioactive[10]. Although no consensus on the matter yet exists, it was suggested that this Aβ fraction, before aggregating into plaques, drives neurotoxicity and cognitive dysfunction subsequently[11]. Following this theory, Aβ-mediated toxicity may induce oxidative stress, inflammation, synaptic degeneration, dysfunction of the brain glucose metabolism, and tau pathology. These processes are likely involved in a second pathological hallmark of AD causing the dysregulation of phosphatases and kinases that leads to increased phosphorylation of microtubule binding protein tau. Hyperphosphorylated tau is prone to aggregation, forming paired helical filaments which accumulate into neurofibrillary tangles (NFT)[12]. The

presence of NFT, particularly in the medial temporal lobe, has been proven to correlate with neuronal loss and cognitive dysfunction [13–15].
These hallmarks are useful to accomplish a definite diagnosis of AD which is based on the neuropathological examination of disease-vulnerable brain regions. Macroscopically, atrophy caused by neuronal degeneration can be observed and is the most pronounced in the temporal end parietal lobes of both brain hemispheres. Microscopic examination of brain slices brings to light the other pathological hallmarks of AD: amyloid plaques, NFT, and neuronal degeneration. Both amyloid plaque and neurofibrillary hallmarks follow a specific topological timing in AD, amyloid pathology initiating in the neocortical regions, and spreading into the deeper regions[16, 17], whereas neurofibrillary pathology is known to arise in the hippocampus, gradually finding its way into the cortical regions[17, 18]. Determining the spread and density of the AD-related pathological changes will allow for specific staging of the disease process which will provide evidence on the sufficiency of the pathology to have caused symptoms[19]. This is especially of concern as both in cognitively healthy elderly as in other neurodegenerative diseases, although in limited amounts, AD pathological hallmarks have been observed[20, 21]. Staging of the pathology will therefore differentiate AD as primary diagnosis, as co-pathology, or as part of normal aging.
Although the initiating factor in AD pathology is unknown for the majority of cases (more than 95%), a small subset of patients with a disease onset before the age of 60 years shows mutations of genes such as *APP*, *PSEN1* and *PSEN2*[22–25]. These genes are mostly involved in the processing of Aβ and their mutations will cause an imbalance in the Aβ metabolism. Patients developing disease at a later age are of sporadic onset, without mutational causation. One gene has been particularly well studied and found to be highly associated with the occurrence of AD pathology. This gene, *APOE,* encoding for apolipoprotein E, is involved in processes such as the aggregation and clearance of Aβ, synapse formation, neuronal signaling, and phospholipid transport[26–28]. *APOE* consists of three allele variances (ε2, ε3, and ε4) of which a genotype of ε3/3 has the highest prevalence. However, the presence of the ε4 allele, accounting for 15-20% in the normal population[29, 30] and for 50-60%[31–33] in patients with AD, was found to be strongly associated with Aβ pathology[34] and was suggested to act as a disease onset modifier[35]. A genotype of ε3/4 was found to increase the risk of AD with 20-30% and a genotype of ε4/4 increased this risk to over 50%[36]. The ε2 allele, on the other hand, was found to have protective properties[37, 38].

WHY THE DIFFERENTIAL AD DIAGNOSIS IS CRUCIAL

Despite clinical recommendations, dementia diagnosis only reaches a diagnostic accuracy of 82-84%[5, 39]. This is a limitation that may be attributed to atypical cases, where symptoms may diverge and diagnosis may even become more difficult such as in cases with a sudden disease onset, when the progressive nature cannot be determined, or when the presentation is mixed with features of other neurodegenerative disorders. Such atypical cases may present as frontal (behavioral) variants of AD, biparietal (visual) variants of AD such as posterior cortical atrophy, and logopenic (language) variants of AD[40–42]. Other scenarios may arise

in the form of fast progressive AD, which may be confused with CJD, or in cases with cerebrovascular disease which can frequently lead to an overdiagnosis of VaD[43]. Misdiagnosis therefore occurs in 16% of cases when distinguishing AD from dementias not attributed to AD (non-AD) and another 16% of these cases remain doubtful[39, 43, 44].
Additionally, in the AD continuum, pathology will already be present in its preclinical phase up to ten to twenty years before any symptoms have arisen[20, 45]. Later on, in the SCD and prodromal MCI phases, symptoms show large heterogeneity and remain mostly unspecific for AD. It is especially difficult, not to say impossible, to define an underlying AD pathology in these phases. In a cohort of SCD and MCI, of which 25% developed dementia after a follow-up period of 6 years, only 42% was found to have dementia due to AD[46].
Depending on the underlying pathology of dementia, disease course and prognosis varies. Distinguishing amongst different causes of dementia is therefore clinically crucial, as this affects the care of patients and their future planning. The relevance of correct diagnosis will increase even more once disease-modifying drugs for treating AD come available. Besides its clinical relevance, inclusion of the correct target population into research studies and clinical trials focusing on AD is a critical step towards positive outcomes in finding new screening methods and therapies. Especially the prodromal phases, in which patients progress to dementia at a rate much higher than seen for cognitive healthy elderly[47, 48], are of exceptional interest for progression studies and clinical trials to evaluate for instance anti-amyloid therapies.

BIOMARKERS FOR PRECLINICAL AND CLINICAL DIAGNOSTIC SUPPORT

To increase the diagnostic certainty in cases with suspected AD and to define preclinical and prodromal phases of dementia due to AD, additional evidence can be gained with biomarkers, providing a biological surrogate measure of the underlying pathology. As core symptoms of AD may also be shared with other neurodegenerative diseases, especially in concomitant pathologies, and AD may present itself in an atypical form, biomarkers significantly raise the diagnostic accuracy and confidence in these cases where the clinical diagnostic work-up may not be able to discriminate between different causes of dementia[43, 44, 49, 50]. The use of biomarkers in research is of particular interest in the earliest phases of the AD continuum (preclinical and prodromal AD)[51, 52], when no or unspecific symptoms are present. The more so, as previously mentioned, these phases are the optimal window for intervention with disease-modifying drugs and provide an excellent study population. On top of that, biomarkers also come to use as outcome measure or as a way to monitor drug efficacy in clinical trials, or may serve as progression markers[53].
The International Working group (IWG) as well as the NIA-AA have proposed the use of these biomarkers into the research diagnostic criteria in 2007 and 2011, respectively, as an addition to the clinical NIA-AA guidelines (table 1.1)[4, 7, 20, 54, 55]. This has expanded the coverage of the full disease spectrum from preclinical to end-stage dementia, as well as advanced the diagnostic criteria for atypical forms of AD and mixed cases with concurrent pathology by providing evidence for AD pathology. According to the latest A/T/N model,

biomarker evidence for AD pathology can be split three ways, each focusing on a specific hallmark of AD pathology[56]. Markers for amyloid pathology (A) contain the neurochemical measurement of Aβ-isoforms in cerebrospinal fluid (CSF) and of brain amyloid plaque load by positron emission tomography (PET) imaging. These biomarkers have been found to change first and are therefore identified as early markers for AD (figure 1.3). NFT burden (T) is reflected in CSF in the form of free phosphorylated tau protein (P-tau)-isoforms or can be visualized by tau PET imaging, and CSF measurements of total tau protein (T-tau) reflect neurodegeneration (N) of the brain. As these markers have been found to correlate with cognitive dysfunction they are thought of as progression markers. Structural imaging by magnetic resonance imaging (MRI) or computed tomography (CT) and imaging of the brain glucose metabolism with [^{18}F]fluorodeoxyglucose ([^{18}F]FDG)-PET are other ways to measure neuronal loss in the brain.

CSF has been suggested to be the window of the brain, reflecting pathophysiological changes happening in the brain, even at the earliest stages of disease. It is present around the brain to fulfill several functions: 1) physical support and safety of the neuronal structure of the central nervous system, 2) "sink" and excretory actions, 3) as a vehicle for intracerebral transport, and 4) the maintenance of an optimal chemical environment of the brain. The major sources of CSF are the choroid plexuses located in the brains ventricles, open cavities holding and transporting the CSF. Formation meets a stable rate of 350-370 µl/min and CSF is circulated into the subarachnoid space covering the brain. Under physiological circumstances, the CSF is isolated from the rest of the body and the systemic circulation, maintained by the blood-CSF barrier, in order to tightly control the brains environment. During its circulation, brain metabolites are free to flow into the CSF. Eventually CSF, fluid as well as constituents, will be adsorbed by a unidirectional process that takes CSF directly into the venous system.

CSF is obtained by the process of lumbar puncture (LP), a technique with the disadvantage of being regarded as invasive and of having a risk of complications. The most common complications include post-LP back pain and post-LP headache[57]. Other, more severe but rare complications include post-LP infections, spinal and subdural cerebral hematoma, and cerebral venous thrombosis[58]. In a multicenter LP feasibility study, mostly mild post-LP complaints were reported in 31% of its population, with respective proportions for back pain, headache and post-LP headache of 17%, 19%, and 9%[59]. Factors that can increase the risk of complication are patients history of headache and fear of complications, as well as type and diameter of the puncture needle, and the number of puncture attempts[59]. This risk, however, has been found to be age-related, decreasing significantly in elderly[60–62], making CSF an exceptional attractive source of a range of biomarkers for AD pathology.

The past two decades, much effort has been put in developing CSF biomarkers for AD[39, 52, 63–66]. The first detection of tau proteins in CSF was accomplished in 1987[67], for which only six years later increased T-tau levels were found in AD dementia by the introduction of the first ELISA for its measurements[68, 69]. The detection of Aβ peptide in CSF was only accomplished in 1992 with observations of decreased levels of $A\beta_{1\text{-}42}$ in AD dementia being established in 1995[70]. In that same year, increased CSF P-tau levels were also defined in AD dementia[69]. This resulted in a panel including three core CSF biomarkers for AD diagnostics, for which decreased CSF levels of $A\beta_{1\text{-}42}$ combined with increased T-tau and/or

increased tau phosphorylated at threonine 181 (P-tau$_{181}$) CSF levels is suggestive for AD pathology[71].

Table 1.1 - Historical summary of the diagnostic (research) criteria of AD

	Published in	Diagnostic criteria
NINCDS-ADRDA	McKhann et al. 1984[3]	**Probable AD** can be diagnosed in patients of age 40-90 years with progressive dementia by the exclusion of other disorders that could account for cognitive deficits. **Definite AD** can only be diagnosed after neuropathological examination.
IWG	Dubois et al. 2007[54]	**Prodromal AD** can be diagnosed in patients with mild cognitive deficits, before the dementia stage, by incorporating biomarkers (CSF $A\beta_{1-42}$, CSF tau proteins, volumetric MRI, amyloid PET). A CSF biomarker paned is considered "suggestive for AD" when $A\beta_{1-42}$ and one or both tau proteins are abnormal or "not suggestive for AD" in all other cases.
NIA-AA	Sperling et al. 2011[20] Albert et al. 2011[7] McKhann et al. 2011[4]	**Preclinical AD and MCI or dementia due to AD** can be defined by a CSF biomarker profile with "high likelihood of AD" if both amyloid and neuronal injury markers are abnormal or with "intermediate likelihood of AD" if only one of both is abnormal. A CSF biomarker profile is defined as "low likelihood of AD" when both are normal.
IWG2	Dubios et al. 2014[55]	CSF biomarkers (CSF $A\beta_{1-42}$ and CSF tau proteins) and amyloid PET have a more central role, while topographical biomarkers (volumetric MRI and FDG-PET) have a role in monitoring neurodegeneration and disease course.
NIA-AA update	Jack et al. 2016[56]	AD pathological process can be diagnosed a biological definition identified primarily by biomarkers based on the **A/T/N model** (A: Aβ pathology, T: tau pathology, N: neuronal injury).

Abbreviations: $A\beta_{1-42}$, amyloid-β peptide with a length of 42 amino acids; AD, Alzheimer's disease; CSF, cerebrospinal fluid; FDG-PET, fluorodeoxyglucose positron emission tomography; IWG, International Working Group; MCI, mild cognitive impairment; MRI, magnetic resonance imaging; NIA-AA, National Institute on Aging and Alzheimer's Association; NINCDS-ADRDA, Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association; PET, positron emission tomography.

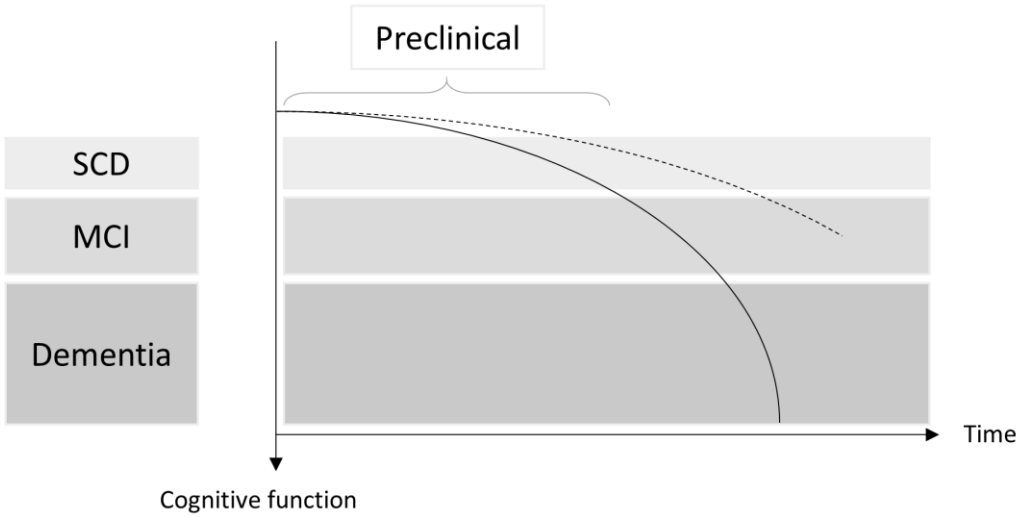

Figure 1.1 - Clinical disease course of AD (modified from Sperling et al. 2011[20]). Normal aging is represented by a dotted line.

Abbreviations: AD, Alzheimer's disease; MCI, mild cognitive impairment; SCD, subjective cognitive decline

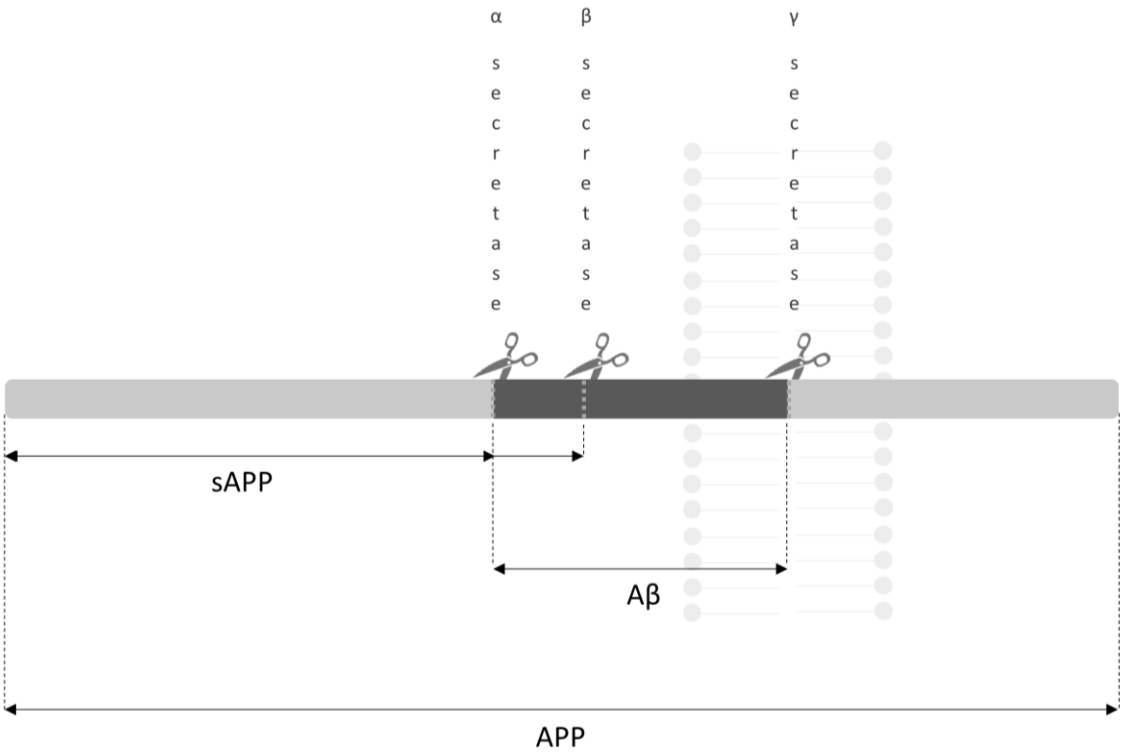

Figure 1.2 - The Aβ metabolism (modified form Rang et al. 2008[72]).

Abbreviations: Aβ, amyloid-β peptide; APP, amyloid precursor protein; sAPP, soluble amyloid precursor protein

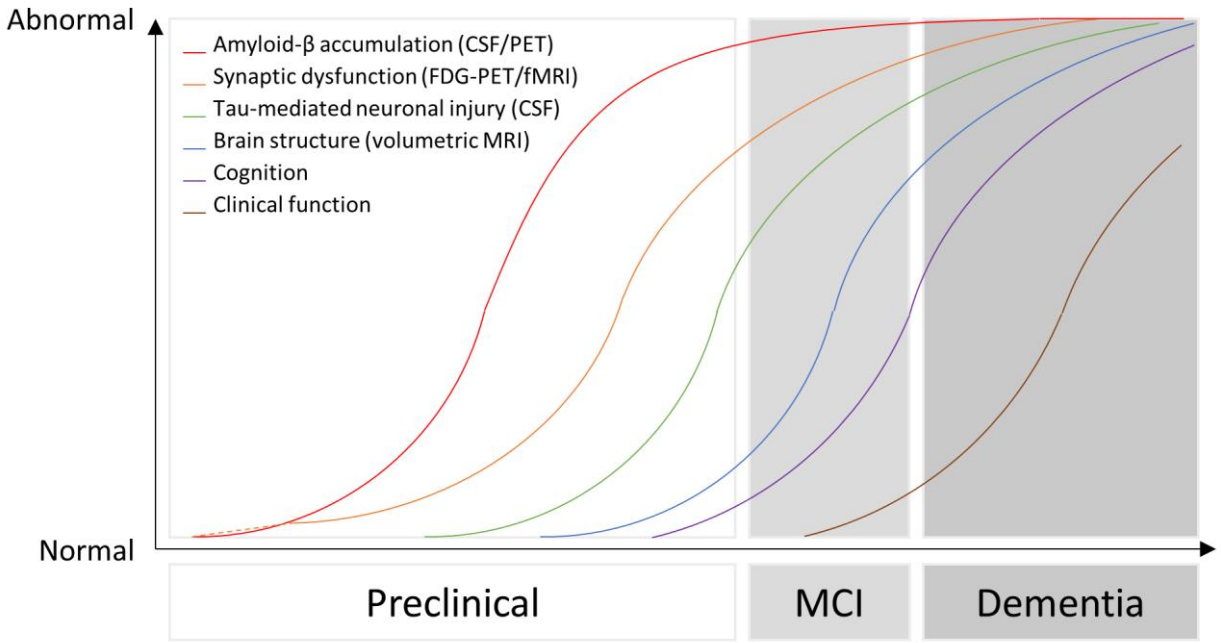

Figure 1.3 - Hypothetical model of biomarker changes in AD over its clinical disease stage (modified from sperling et al. 2011[20])

Abbreviations: AD, Alzheimer's disease; CSF, cerebrospinal fluid; FDG-PET, fluorodeoxyglucose positron emission tomography; fMRI, functional magnetic resonance imaging; MCI, mild cognitive impairment; MRI, magnetic resonance imaging; PET, positron emission tomography.

A DECADE OF CSF BIOMARKERS FOR AD IN BELGIUM

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ABSTRACT

During the past ten years, over 5000 cerebrospinal fluid (CSF) samples were analyzed at the Reference Center for Biological Markers of Dementia (BIODEM), UAntwerp, for core Alzheimer's disease (AD) CSF biomarkers: amyloid-β peptide of 42 amino acids ($A\beta_{1-42}$), total tau protein (T-tau) and tau phosphorylated at threonine 181 ($P\text{-}tau_{181}$). CSF biomarker analyses were performed using single-analyte ELISA kits. In-house validated cutoff values were applied: $A\beta_{1-42}$ <638.5 pg/mL, T-tau >296.5 pg/mL, $P\text{-}tau_{181}$ >56.5 pg/mL. A CSF biomarker profile was considered to be suggestive for AD if the CSF $A\beta_{1-42}$ concentration was below the cutoff, in combination with T-tau and/or $P\text{-}tau_{181}$ values above the cutoff (IWG2 criteria for AD). Biomarker analyses were requested for following clinical indications: 1) neurochemical confirmation of AD in case of clinical AD, 2) neurochemical confirmation of AD in case of doubt between AD and a non-AD dementia, 3) neurochemical diagnosis of prodromal AD in case of mild cognitive impairment, 4) neurochemical confirmation of AD in case of psychiatric symptoms (like depression, psychosis) or 5) other clinical indications. During these ten years, the number of yearly referred samples increased by 238% and clinical indications for referral showed a shift from neurochemical confirmation of AD in case of clinical AD to differential dementia diagnosis in case of doubt between AD and a non-AD dementia. Four percent of the patients also had a postmortem neuropathological examination. Together, these biomarker data were the basis for several research papers, and significantly contributed to the validation of these biomarkers in autopsy-confirmed subjects.

INTRODUCTION

The past two decades, much effort has been put in developing biomarkers for AD[39, 51, 64–66, 73]. This resulted in three core CSF biomarkers for AD diagnostics, namely the $A\beta_{1\text{-}42}$, T-tau, and P-tau_{181}[71]. Aβ and both tau proteins can reliably be measured in CSF by the use of several analytical techniques including single-analyte enzyme-linked immunosorbent assays (ELISA)[74].
Biomarkers have been incorporated into research diagnostic criteria for AD[4, 6, 7] and, although the clinical examination (including full neuropsychological evaluation) is still the basis for AD diagnosis[5], these biomarkers are being introduced in daily clinical dementia practice as *in vivo* surrogate markers for the confirmation of AD neuropathology. The core CSF AD biomarkers increase the diagnostic accuracy for diagnosing AD (including cases with atypical presentations), also in its prodromal phase (MCI due to AD)[51, 73], and are able to differentiate between AD and psychiatric disorders. The CSF biomarkers are useful to diagnose AD in patients with ambiguous clinical dementia diagnoses[75, 76] and in cases with mixed brain pathology like AD with cerebrovascular disease[43, 49, 50].
At the Reference Center for Biological Markers of Dementia (BIODEM), UAntwerp, CSF samples from referring clinical centers have been analyzed for the core AD CSF biomarkers since 2004. In what follows, an overview of a decade of CSF biomarker analyses at BIODEM will be given. This overview includes over 5000 neurochemical analyses. Four percent of the patients also had a postmortem neuropathological examination. Together, these biomarker data were the basis for several research papers. This overview forms a basis for revealing limitations and providing new insights to improve the neurochemical diagnosis of AD.

METHODOLOGY

CSF samples from referring clinical centers

CSF samples from patients with presumed cognitive deterioration were referred to the BIODEM lab. Since 2004, clinical centers from all over Belgium as well as some international centers have been referring samples (figure 2.1). CSF biomarker analyses were requested by the physicians in the context of a diagnostic workup. The physicians were asked to fill out a request form consisting of the patient's age, gender, mini-mental state examination (MMSE) score[77], sampling date, and the clinical indication for referral, that consisted of one of the following options (tick boxes):

- Neurochemical confirmation of AD in case of clinical AD. CSF biomarkers were used to increase the diagnostic accuracy.
- Neurochemical confirmation of AD in case of doubt between AD dementia and a non-AD dementia, including atypical clinical presentations of AD, possible DLB, possible FTLD, possible VaD, CJD etc.
- Neurochemical confirmation of prodromal AD in case of MCI.

- Neurochemical confirmation of AD in case of psychiatric symptoms (like depression, psychosis).
- Other clinical indication: the referring clinician was asked to provide additional clinical information.

In case of CJD confirmation or AD versus CJD differential diagnosis, additional analysis of 14-3-3 protein could be requested[78–80].

CSF sampling and handling

All CSF sampling and handling steps are represented in figure 2.2. In short, CSF was obtained by LP at the L3/L4 or L4/L5 interspace[81]. Referring centers were asked to perform CSF sample handling and processing according to the standard procedure as described in the BIODEM lab guidelines (www.bornbunge.be). This includes collection of minimal 2 ml CSF in a labeled polypropylene tube to avoid adsorption of Aβ to the wall of the vial. In case of a hemorrhagic LP detected by macroscopic inspection, CSF should be centrifuged for ten minutes at 3000 rpm within four hours after LP. The supernatant should thereafter be transferred to an unused polypropylene vial. CSF samples should be shipped to the BIODEM lab, unfrozen if sent within twenty-four hours after LP (shipment at room temperature) or frozen if sent later (shipment on dry ice). Samples were stored at -80°C until analysis.

CSF analysis

CSF concentrations of $A\beta_{1-42}$, T-tau, and P-tau_{181} were determined with commercially available single-analyte ELISA kits (INNOTEST® β-Amyloid($_{1-42}$), INNOTEST® hTau-Ag, and INNOTEST® Phospho-Tau($_{181P}$), respectively; Fujirebio Europe, Ghent, Belgium). With each assay the clinical samples, together with a blank (sample diluent), calibrator solutions, and appropriate quality controls, were analyzed according to the manufacturer's instructions as described previously[39]. The 14-3-3 protein analyses were performed using Western blot[82].
As described in the IWG2 criteria for AD, a CSF biomarker profile was considered pathological and suggestive for AD if the analysis resulted in a low CSF $A\beta_{1-42}$ value in combination with increased levels of T-tau and/or P-tau_{181}[6]. Cutoffs used for the determination of normal values are $A\beta_{1-42}$ >638.5 pg/ml, T-tau <296.5 pg/ml, and P-tau_{181} <56.5 pg/ml as determined in a cohort of autopsy-confirmed AD patients and cognitively healthy elderly[83].
As a part of the Alzheimer's Association Quality Control (QC) Program for CSF biomarkers, the performance of the assays was monitored with CSF QC samples provided by the program. Two different longitudinal samples were included in the routine ELISA analyses since 2010. Sample characteristics and procedures have been described previously by Mattsson et al.[34].

Informed consent for research purposes

Whenever possible, patients were asked to give written informed consent to use CSF leftovers for scientific research as well as to undergo brain autopsy after death[39, 75]. In case consented patients died, a brain autopsy was performed according to a standard protocol[39, 75]. Neuropathological examination was performed on the right hemisphere of the brain at the biobank of the Institute Born-Bunge (IBB; Antwerp, Belgium). Patients were neuropathologically diagnosed as definite AD, DLB and VaD using the criteria of Montine[19]. Definite FTLD was diagnosed through the criteria of Cairns[84] and Mackenzie[85, 86], while definite CJD was diagnosed in agreement with the criteria of Markesbery[87].

Statistical analyses

The variables were assessed for normal distribution using the Kolmogorov-Smirnov test. Since variables were not normally distributed, non-parametric tests were performed. For comparisons across groups, Kruskal-Wallis and Mann-Whitney tests were applied. Chi-Square tests were performed to explore gender distributions. Spearman's ρ was calculated to determine correlations. Confidence intervals (CI) were established at 95%. The level of significance was set at $P<0.05$. The diagnostic accuracies of the autopsy-confirmed patients was obtained by receiver operating characteristics (ROC) curve analysis for the determination of the area under the curve (AUC), sensitivities, specificities and the optimal cutoff for the differentiation of AD and non-AD dementia diagnosis. The optimal cutoff values were determined by calculation of the maximal sum of the sensitivity and the specificity. All statistical analyses were done using SPSS statistical package of IBM Statistics, version 22.

RESULTS

Description of the population

Table 2.1 shows the demographic and clinical characteristics of the patients. The database contains samples of a total of 5022 patients with an equal distribution of males and females. The mean age at time of CSF sampling was 71 years. A proportion of the population (4%) received an autopsy-confirmed diagnosis. For a subgroup of 1710 patients, the MMSE scores at time of CSF biomarker analysis request were available. Patients who were referred for neurochemical confirmation of AD or for differential AD versus non-AD dementia diagnosis showed significantly lower MMSE scores as compared to the prodromal AD group, and the patients who were referred for a neurochemical diagnosis of AD in case of psychiatric symptoms and other indications ($P<0.001$). During the past ten years, the mean MMSE scores remained unchanged (results not shown). Indeed, the relative number of samples from patients referred for neurochemical confirmation of prodromal AD did not increase significantly.

Referral of CSF samples from 2004-2014

Between 2004 and 2014, the analyses of unique samples per year have increased from 238 in 2004 to 804 in 2014 (figure 2.3A). Where initially only Antwerp centers referred samples in 2004, increasing to 381 in 2014, more and more centers from other regions in Belgium started referring samples throughout the years, leading to a total of 423 in 2014 (figure 2.3B). The relative amount of patients per indication (figure 2.3C) demonstrates a switch at the time point of the second quarter of 2011 from the majority of requests concerning neurochemical confirmation of AD, to most referrals being for differential dementia diagnosis. Samples referred for neurochemical diagnosis of AD in case of psychiatric symptoms also become slightly more frequent over time. The number of samples referred for other indications, such as CJD, show a decrease over time. Over the time course of ten years, the number of samples referred for diagnosis of prodromal AD in case of MCI was stable.

Results of CSF biomarker analyses

The mean biomarker concentrations are described in table 2.2. All three CSF biomarkers were analyzed in 4983 of the 5025 samples, while the remaining samples were tested only for T-tau (n=16), $A\beta_{1-42}$ (n=4) or P-tau_{181} (n=2) or two of the three biomarkers (n=20). All samples were analyzed in duplicates. These missing data are due to insufficient sample volume to analyze all three biomarkers and/or reanalyze biomarkers in case the coefficient of variation (CV; 100*standard deviation/mean) exceeded 20%. Of the entire study population, 39% (n=1956) had a neurochemical profile indicative for AD pathology, based on the IWG2 criteria [10]. Samples with a normal CSF biomarker profile accounted for 22% (n=1123). This leaves another 39% (n=1943) of the samples unclassifiable by the IWG2 criteria, including samples presenting with normal $A\beta_{1-42}$ but increased T-tau and/or P-tau_{181} concentrations (n=724) and samples presenting with low $A\beta_{1-42}$ (<638.5pg/ml) but normal T-tau and P-tau_{181} concentrations (n=1181). Thirty-eight samples could not be categorized due to missing analyses of one or two of the CSF biomarkers. Of the 261 samples with very high T-tau values (>1200 pg/ml; value higher than the upper limit of quantification of the test kits used for analysis, as described in the package inserts), 95 samples were analyzed for 14-3-3 protein of which 69 samples were positive.
In total, 170 patients had autopsy-confirmed diagnoses of which 108 with AD pathology and 62 with non-AD pathology (table 2.1). Six out of 108 subjects (6%) were autopsy-confirmed AD patients who presented with normal CSF biomarker levels of $A\beta_{1-42}$, T-tau and P-tau_{181}, three subjects (3%) with normal $A\beta_{1-42}$ but increased T-tau and/or P-tau_{181}, and 15 subjects (14%) with low $A\beta_{1-42}$ (<638.5pg/ml) but normal T-tau and P-tau_{181} levels. The biomarker profiles of these neuropathologically confirmed AD patients therefore do not comply with the IWG2 criteria for AD biomarker diagnosis. Of the autopsy-confirmed non-AD patients, 27 subjects (44%) had a CSF neurochemical profile that was in compliance with the IWG2 criteria for AD biomarker diagnosis. In 16 subjects (9% of the total number of autopsy-confirmed patients) a neurochemical diagnosis could not be established due to analysis of only $A\beta_{1-42}$ or T-tau levels. Thirteen patients with very high T-tau levels (>1200 pg/ml) were

autopsy-confirmed CJD. Four of these patients scored negative for 14-3-3 protein. Of the 70 samples with positive 14-3-3 protein analysis, one sample showed to be autopsy-confirmed hippocampal sclerosis. Inter-biomarker correlations and correlations of the biomarkers with age at LP and MMSE score are presented in table 2.3. The diagnostic accuracies and optimal cutoff values determined for the individual markers as well as the $A\beta_{1-42}$/T-tau and $A\beta_{1-42}$/P-tau_{181} ratios are shown in table 2.4.

Alzheimer's Association QC program for CSF biomarkers

The results of the Alzheimer's Association QC Program first and second longitudinal QC (QC-L) samples are shown in figure 1.4. A second longitudinal QC sample was used in succession of the first due to a supply shortage. All BIODEM results were within the 2SD range calculated from the mean value of all participating laboratories. The inter-run CV was 14%, 16% and 8% for $A\beta_{1-42}$, T-tau and P-tau_{181}, respectively, for the first QC-L and 12%, 16% and 6% for the second QC-L. No shift occurred for the three biomarkers (i.e. a systematic error, where results of six consecutive measurements lie on the same side of the mean) nor was there a visible trend for all three biomarkers (i.e. a systematic error, were the measurements move in one direction, heading towards an 'out-of-control' value).

DISCUSSION

During ten years of CSF AD biomarker analyses, the amount of samples referred to the BIODEM lab at UAntwerp from clinical centers has increased with 238%. Due to the revisions of diagnostic criteria for AD diagnosis and the herein described use of CSF biomarkers[4, 6, 7], confidence in the importance of biomarkers has grown. Not only are they used more often by clinicians, they are also useful in clinical trials as enrichment strategy or outcome measures due to their *in vivo* pathophysiological characteristics[53]. The observed increase of referrals could also have been influenced by different milestones, e.g. the publications of biomarker-based diagnostic criteria for AD. These specific milestones did not show clear associations with the time course of the number of samples referred. However, there is a general shift from samples referred for neurochemical confirmation of AD diagnosis to referrals for differential AD versus non-AD dementia diagnosis. This may be due to the growing scientific support for biomarker-based differential diagnosis between AD and non-AD dementias[75, 76, 88–90].

Several research papers found their origin in the BIODEM database of CSF AD biomarker analyses and an important contribution is ascribed to the subjects that gave informed consent for brain autopsy. Our lab was the first to demonstrate the diagnostic value of the CSF biomarkers $A\beta_{1-42}$, T-tau and P-tau_{181} in clinical AD, using the neuropathological diagnosis as a reference[39, 91]. The data demonstrated that all three biomarkers provide useful information, showing promising sensitivity and specificity values that systematically exceed the 80% threshold. The use of a biomarker-based model in patients with a clinically ambiguous diagnosis, resulted in a correct diagnosis in the majority of autopsy-confirmed AD

and non-AD cases, indicating that biomarkers have an added diagnostic value in these cases [5]. Moreover, the diagnostic value of the AD biomarkers $A\beta_{1-42}$, T-tau and P-tau_{181} is independent of the platform (single-analyte ELISA or multiplex) on which they are tested, despite the differences in absolute values, provided that appropriate cutoff points are being used[74].

Investigating autopsy-confirmed AD and non-AD dementia patients has also improved our knowledge and insights with regard to the differential diagnostic value of the existing AD CSF biomarkers. The ratio of $A\beta_{1-42}$/P-tau_{181} was shown to have a higher diagnostic accuracy (sensitivity of 73.5% and specificity 63.9%) than $A\beta_{1-42}$ (sensitivity of 90.0% and specificity 38.7%), T-tau (sensitivity of 65.5% and specificity 56.2%), P-tau_{181} (sensitivity of 65.7% and specificity of 63.9%), or the $A\beta_{1-42}$/T-tau ratio (sensitivity of 80.8% and specificity 45.9%), while P-tau_{181} was found to perform better than $A\beta_{1-42}$ or T-tau to discriminate between AD and non-AD dementias (table 2.5). This clearly signifies the importance of P-tau_{181} in the biomarker panel for differential dementia diagnosis[76]. Concomitant AD pathology in DLB, which occurred in 72% of the autopsy-confirmed DLB patients, is reflected by low CSF $A\beta_{1-42}$ values in these patients[39]. However, the differential diagnostic value against pure AD is limited due to the fact that only AD pathology will be detected because of a lack of established CSF DLB biomarkers. Very high T-tau concentrations in CSF (>1200 pg/ml) are indicative for CJD. In case of suspicion of CJD, analysis of CSF 14-3-3 protein can be useful.

The differential diagnostic value of the existing biomarker panel might be improved by the addition of $A\beta_{1-40}$, as this biomarker better reflects the total pool of Aβ. As the analysis of $A\beta_{1-42}$ may lead to false negative values in case of "high Aβ producers", normalizing the CSF $A\beta_{1-42}$ concentration to that of the most abundant and stably produced Aβ isoform (i.e. $A\beta_{1-40}$) may improve the diagnostic value for these samples. Since formation of amyloid-plaques starts up to twenty years before the onset of the clinical symptoms, the use of low CSF $A\beta_{1-42}$ and the CSF $A\beta_{1-42}/A\beta_{1-40}$ ratio opens new possibilities for the identification of 'asymptomatic at risk for AD' subjects for clinical trials[92]. Differential diagnosis of AD versus non-AD dementia may also benefit of adding other Aβ isoforms[93] as well as other biomarkers, such as α-synuclein[94, 95] and TDP-43[96], as they are specific for non-AD dementias.

Even with an elevated confidence in the use of CSF biomarkers for the clinical work-up of dementia diagnosis, multicenter trials have shown that there is a substantial center-to-center variation[97]. The different pre-analytical sample procedures used by the different centers contribute to variability of CSF biomarker concentrations. This causes an important concern as direct comparisons of measurements between laboratories and across techniques are not reliable, hampering biomarker development and their utility for clinical routine diagnosis[98]. Temperature of freezing, delay until freezing and number of freeze-thaw cycles significantly influence CSF biomarker concentrations, stressing the need for standard operating procedures for pre-analytical sample handling[99]. The differences observed in the latter study were, however, relatively small. An exploratory study in MCI patients with clinical follow-up and autopsy-confirmed AD patients provided evidence that, for a specific context of use, the impact on clinical diagnostic accuracy of biomarker concentration shifts might be lower than originally expected[98]. However, standardization of (pre)analytical sample handling as well as the cutoff thresholds should be accomplished as they influence

biomarker results[99, 100]. With this in mind, several programs for standardization and harmonization were set up, such as the QC program of the Alzheimer's Association, as well as the Alzheimer Biomarker Standardization Initiative (ABSI) and the JPND BIOMARKAPD consortium[101–104].

Conclusion

This paper provides insight in the use of CSF biomarkers for the clinical diagnostic work-up of AD and related disorders in Belgium during the past decade. Within this period, over 5000 CSF samples have been analyzed. Several research papers found their origin in the CSF AD biomarker analyses, with a significant contribution of subjects who gave informed consent for brain autopsy. Studies on autopsy-confirmed AD patients that do not comply with the criteria for a neurochemical diagnosis of AD, which accounted for 30% of the definite diagnoses, are needed to improve our insights in the boundaries of the current AD CSF biomarkers. Improving the quality of CSF biomarker analyses for AD diagnosis can be accomplished by complying with recommendations of standardization programs.

TABLES AND FIGURES

Table 2.1 - Demographic and clinical data, classified by clinical indication

	Total	Neuro-chemical confirmation of AD	Differential AD versus non-AD dementia diagnosis	Diagnosis prodromal AD in case of MCI	Differential AD versus depression or other psychiatric disorder diagnosis	Other indication
	(n = 5022)	(n = 1596)	(n = 2136)	(n = 651)	(n = 307)	(n = 332)
Age (y):						
mean	70	72	70	70	65	63
(CI)	(69.4-70,0)	(71.1-72.2)	(69.3-70.2)	(69.5-71.0)	(64.1-66.6)	(61.3-64.7)
Gender:						
female / male	2493 / 2529	890 / 706	988 / 1148	317 / 334	166 / 141	132 / 200
MMSE score:						
mean (n)	22 (1710)	20 (486)	21 (776)	25 (310)	24 (87)	24 (51)
(CI)	(21.4 –22)	(19.9-21.0)	(20.1-21.0)	(25.0-25.8)	(22.5-24.9)	(22.5-25.6)
Abnormal concentrations: n (%)						
$A\beta_{1-42}$	3140 (62)	1097 (69)	1361 (64)	374 (57)	172 (56)	135 (41)
T-tau	2630 (52)	939 (59)	1111 (52)	331 (51)	109 (36)	137 (41)
P-tau_{181}	1851 (37)	692 (43)	738 (35)	261 (40)	70 (23)	88 (26)
Biomarker results (pg/ml)						
$A\beta_{1-42}$, mean ± SD	594± 292	557 ± 266	603 ± 306	619 ± 300	611 ± 273	645 ± 301
T-tau, mean ± SD	409 ± 294	420 ± 294	418 ± 303	399 ± 292	357 ± 266	355 ± 255
P-tau_{181}, mean ± SD	58.2 ± 36.1	63.8 ± 40.0	56.9 ± 34.7	53.7 ± 32.6	51.5 ± 34.4	54.9 ± 31.6
Autopsy-confirmed diagnosis: n	170	82	71	5	1	11
AD	108	72	29	4	1	2
Non-AD	62	10	42	1	0	9
FTLD	16	3	13	0	0	0
DLB	12	1	11	0	0	0
VaD	14	5	9	0	0	0
CJD	13	0	5	0	0	8
Other	9	2	5	1	0	1

Legend: Biomarker concentrations that were found to be abnormal can be indicative for AD pathology, following the AD profile cutoff points: $A\beta_{1-42}$ <638,5 pg/ml, T-tau >296,5 pg/ml and P-tau_{181} >56,5 pg/ml. Other autopsy-confirmed diagnosis involves hippocampal sclerosis, progressive supranuclear palsy and anoxic necrosis.

Abbreviations: $A\beta_{1-42}$, amyloid-β peptide of 42 amino acids; AD, Alzheimer's disease; CI, confidence interval; CJD, Creutzfeldt-Jakob disease; DLB, Lewy body disease; MMSE, Mini-mental State Examination; FTLD, frontotemporal degeneration; P-tau_{181}, tau phosphorylated at threonine 181; T-tau, total tau; VaD, vascular disease.

Table 2.2 - AD CSF biomarker profile and biomarker levels of autopsy-confirmed AD and non-AD dementia patients

		AD	Non-AD	Significance
		(n=108)	(n=62)	
Normal	**N**	6	7	
	T-tau (pg/ml)	212 ± 49	244 ± 33	n.s.
	$A\beta_{1-42}$ (pg/ml)	914 ± 209	962 ± 188	n.s.
	P-tau_{181} (pg/ml)	41.4 ± 9.3	40.0 ± 5.2	n.s.
	$A\beta_{1-42}$/T-tau	4.38 ± 0.64	3.99 ± 0.84	n.s.
	$A\beta_{1-42}$/ P-tau_{181}	22.62 ± 5.59	24.16 ± 4.30	n.s.
	Age at LP	78 ± 6	71 ± 6	n.s.
	MMSE score	24 ± 2	17 ± 15	n.s.
Suggestive for AD	**N**	72	27	
	T-tau (pg/ml)	585 ± 216	446 ± 155	n.s.
	$A\beta_{1-42}$ (pg/ml)	402 ± 131	435 ± 134	n.s.
	P-tau_{181} (pg/ml)	84.0 ± 42.9	61.6 ± 34.8	p=0.001
	$A\beta_{1-42}$/T-tau	0.72 ± 0.42	0.88 ± 0.58	n.s.
	$A\beta_{1-42}$/ P-tau_{181}	5.61 ± 2.91	8.62 ± 4.08	P<0.001
	Age at LP	76 ± 13	74 ± 10	n.s.
	MMSE score	15 ± 7	19 ± 7	n.s. (p=0.58)
Normal $A\beta_{1-42}$ but increased T-tau and/or P-tau_{181}	**N**	3	13	
	T-tau (pg/ml)	552 ± 134	481 ± 204	n.s.
	$A\beta_{1-42}$ (pg/ml)	702 ± 57	832 ± 116	n.s. (p=0.057)
	P-tau_{181} (pg/ml)	78.9 ± 24.9	59.9 ± 23.3	n.s.
	$A\beta_{1-42}$/T-tau	1.17 ± 0.67	1.56 ± 0.77	n.s.
	$A\beta_{1-42}$/ P-tau_{181}	20.53 ± 6.38	15.83 ± 6.01	n.s.
	Age at LP	81 ± 5	67 ± 9	p=0.014
	MMSE score	18 ± 6	19 ± 7	n.s.
Low $A\beta_{1-42}$ but normal T-tau and P-tau_{181}	**N**	15	11	
	T-tau (pg/ml)	117 ± 54	166 ± 65	p=0.033
	$A\beta_{1-42}$ (pg/ml)	324 ± 117	408 ± 154	n.s.
	P-tau_{181} (pg/ml)	30.9 ± 7.6	25.7 ± 8.9	n.s. (p=0.066)
	$A\beta_{1-42}$/T-tau	1.95 ± 0.90	3.00 ± 2.07	n.s.
	$A\beta_{1-42}$/ P-tau_{181}	11.84 ± 6.10	14.44 ± 9.04	n.s.
	Age at LP	72 ± 14	69 ± 10	n.s.
	MMSE score	16 ± 7	19 ± 3	n.s.

Legend: Data presented as mean values with respective SD. Significance level set at p<0,05 for differences between AD and non-AD groups.

Abbreviations: $A\beta_{1-42}$, amyloid-β peptide of 42 amino acids; AD, Alzheimer's disease; LP, lumbar puncture; MMSE, Mini-mental State Examination; n.s., no significant difference; P-tau_{181}, tau phosphorylated at threonine 181; SD, standard deviation; T-tau, total tau.

Table 2.3 - Correlations between the three core biomarkers and correlations between the biomarkers and the clinical data of the autopsy-confirmed AD and non-AD dementia patients

		T-tau	Aβ1-42	Age at LP	MMSE score
AD	**T-tau**	-	-	n.s.	-0.320 (P=0.004)
	$A\beta_{1-42}$	n.s.	-	n.s.	n.s.
	P-tau_{181}	0.726 (P<0.001)	n.s.	n.s.	n.s.
non-AD	**T-tau**	-	-	n.s.	n.s.
	$A\beta_{1-42}$	n.s.	-	n.s.	n.s.
	P-tau_{181}	0.618 (P<0.001)	0.307 (P=0.016)	0.257 (P=0.045)	n.s.

Legend: Correlations are shown as Spearman's ρ with *p*-value between brackets. Significance level set at $p<0,05$.

Abbreviations: $A\beta_{1-42}$, amyloid-β peptide of 42 amino acids; AD, Alzheimer's disease; LP, lumbar puncture; MMSE, Mini-mental State Examination; n.s., no significant difference; P-tau_{181}, tau phosphorylated at threonine 181; T-tau, total tau.

Table 2.4 - Diagnostic accuracies for the differentiation of AD and non-AD dementia

	AUC	95% CI	Cutoff (pg/ml)	Sensitivity (%)	Specificity (%)
T-tau	0.670	0.474-0.660	414	65.5	56.2
$A\beta_{1\text{-}42}$	0.647	0.555-0.739	615	90.0	38.7
P-tau_{181}	0.676	0.591-0.760	54.5	65.7	63.9
$A\beta_{1\text{-}42}$/T-tau	0.635	0.544-0.726	1.55	80.8	45.9
$A\beta_{1\text{-}42}$/P-tau_{181}	0.734	0.655-0.813	9.05	73.5	63.9

Legend: The optimal cutoff values were determined by calculation of the maximal sum of the sensitivity and the specificity.

Abbreviations: $A\beta_{1\text{-}42}$, amyloid-β peptide of 42 amino acids; AD, Alzheimer's disease; AUC, area under the curve; CI, confidence interval; P-tau_{181}, tau phosphorylated at threonine 181; T-tau, total tau.

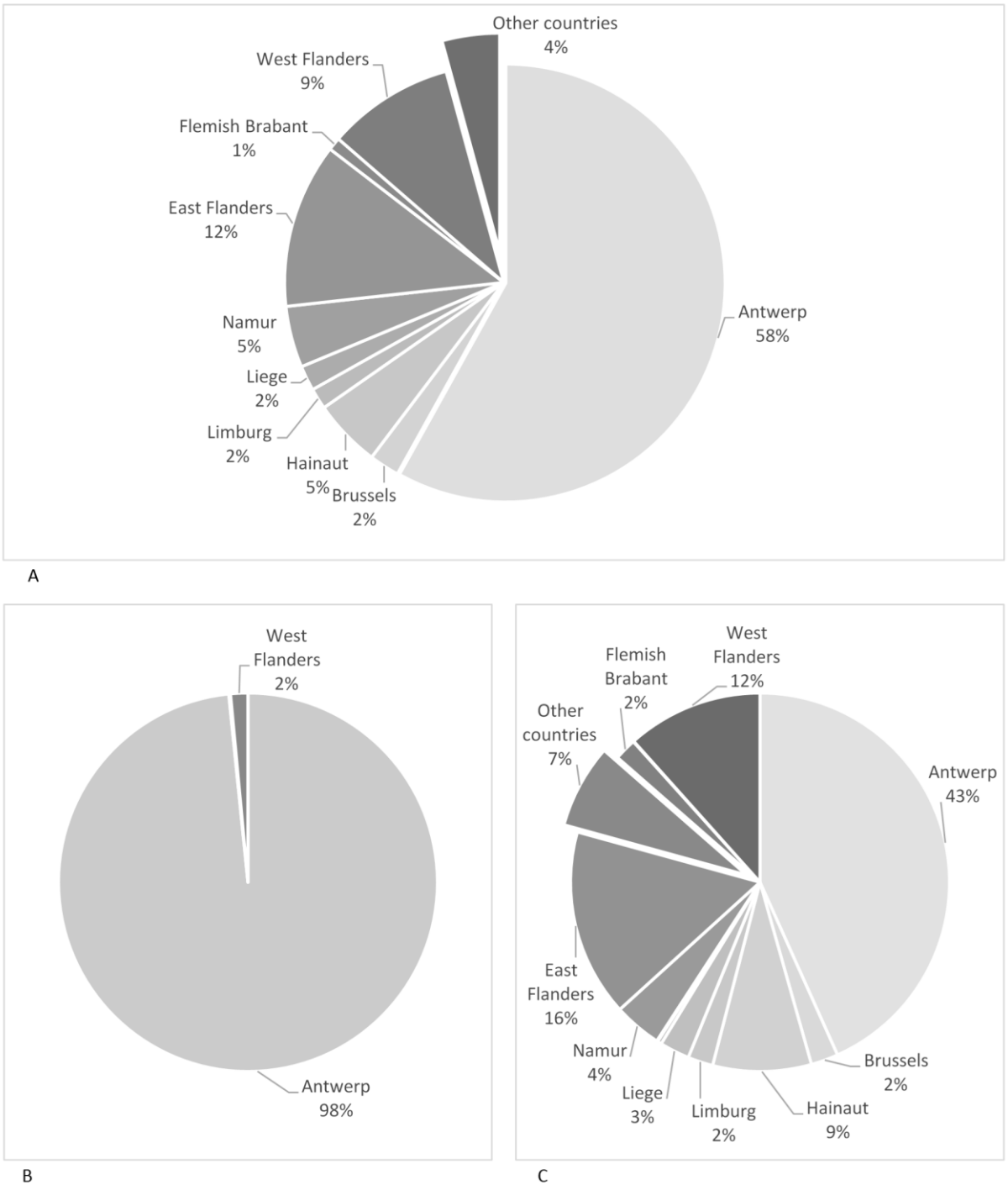


Figure 2.1 - CSF samples from referring clinical centers. National and international locations of the centers referring samples between 2004 and 2014 (A). Locations of the referring centers subdivided in a time range of 2004-2006 (B) and 2012-2014 (C).

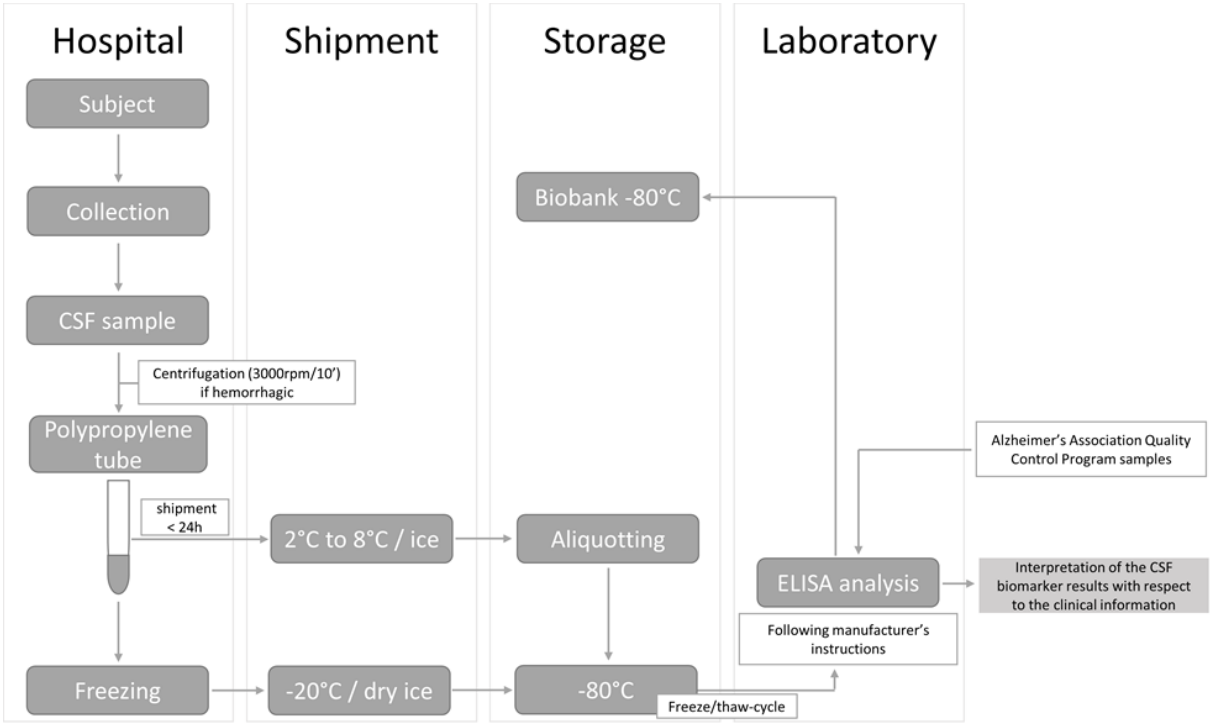


Figure 2.2 - Workflow of the pre-analytical, analytical, and post-analytical steps of the BIODEM lab for CSF biomarker analyses

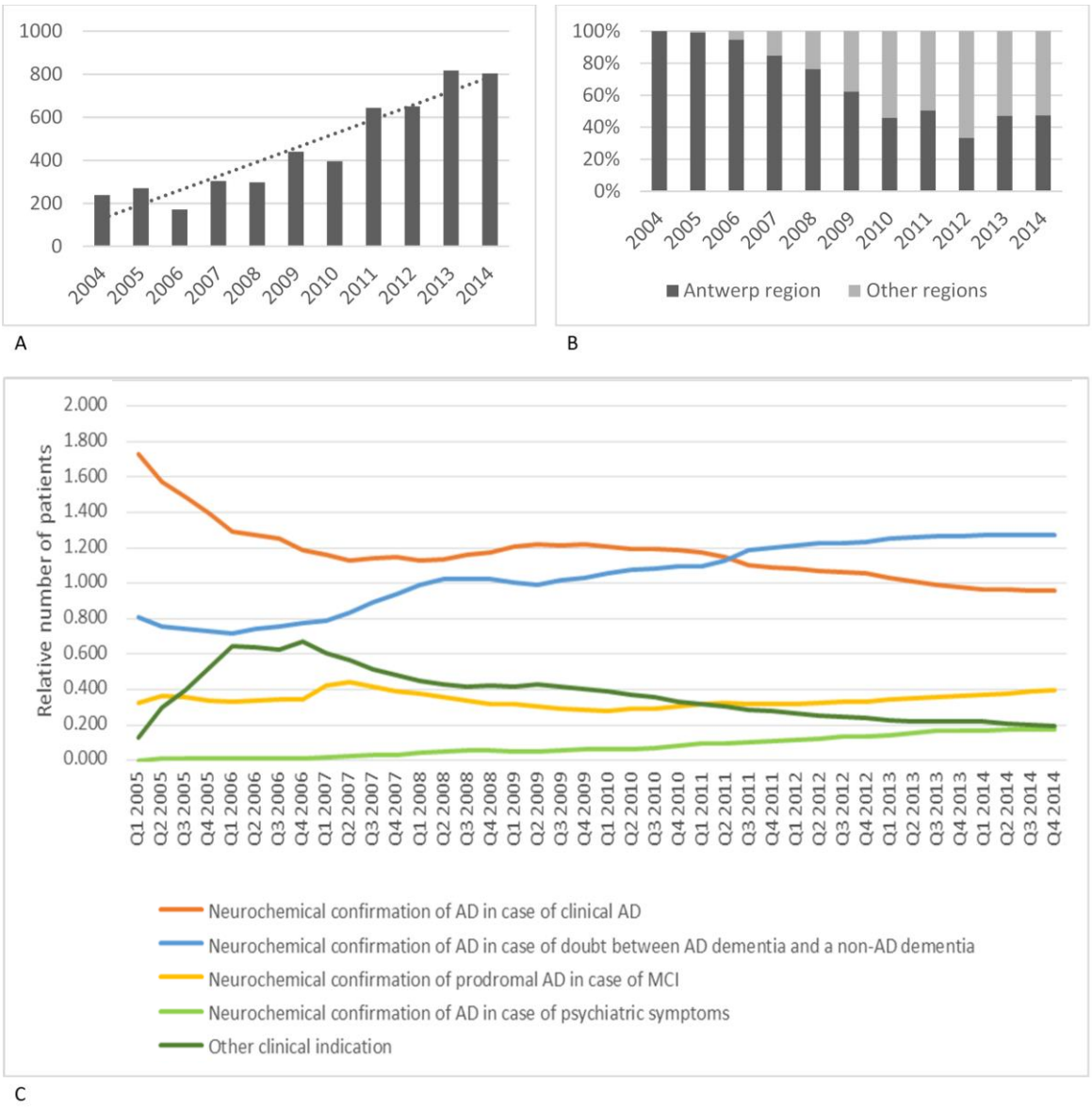


Figure 2.3 - Referral of CSF samples between 2004 and 2014**.** (A) Number of unique samples analyzed per year. (B) Number of unique samples per year, grouped by location of referring centers. (C) Representation of the number of patients for each indication relative to the total amount of patients per quartile.

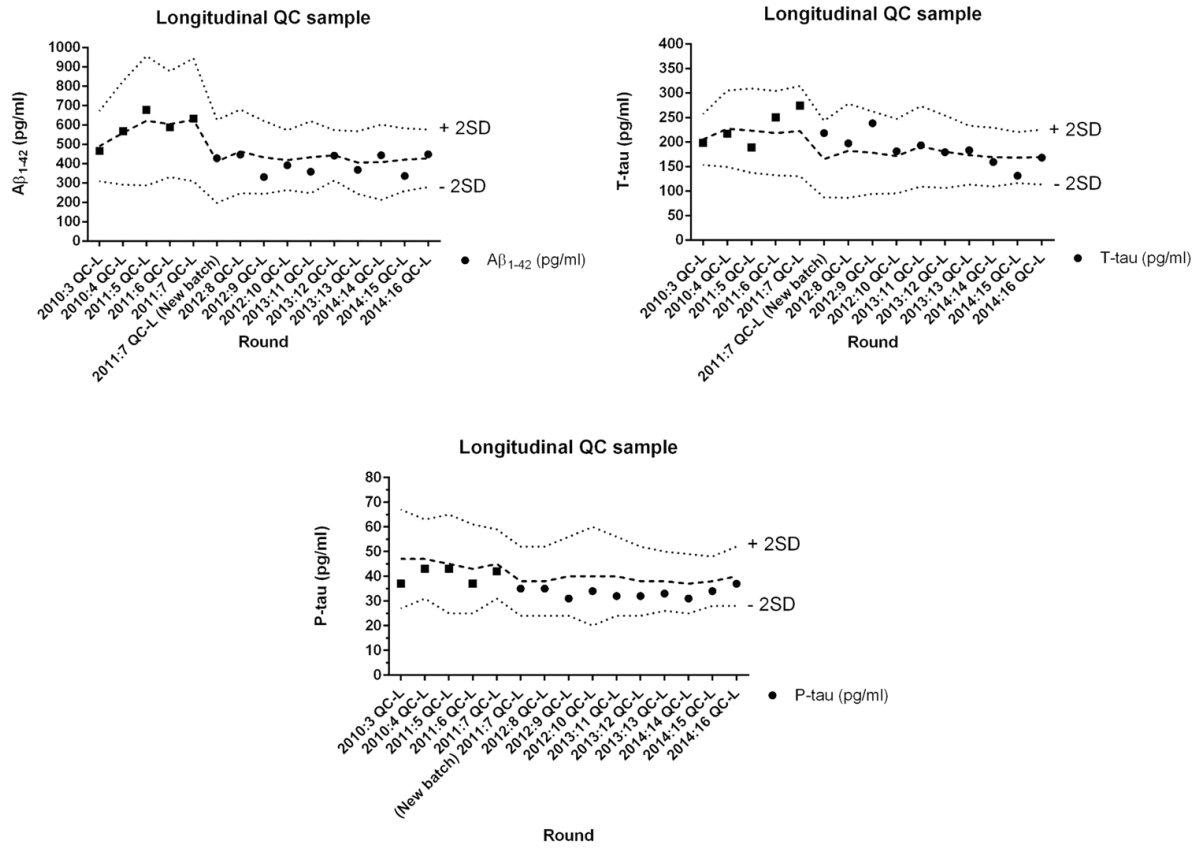


Figure 2.4 - CSF biomarker results of the first and second QC-L samples for $A\beta_{1\text{-}42}$, T-tau and P-tau_{181}. Our results are plotted (filled squares batch 1, filled circles batch 2) against the mean value (dashed line) with their 2SD ranges (dotted lines) of all participating laboratories.

AIM OF THE THESIS

THESIS AIM

With an increase in requests for CSF biomarker analyses and the shift in the indication from confirmation of AD diagnosis to differential (AD versus non-AD) diagnosis over the past ten years, the importance of the differential diagnostic value of biomarkers is highlighted. However, in neuropathologically-confirmed cases relatively high numbers of AD and non-AD patients present with a biomarker profile that does not match their primary diagnosis. Of the three core AD CSF biomarkers, P-tau$_{181}$ has been proven to be the most specific marker for AD, but CSF A$\beta_{1\text{-}42}$ and T-tau levels still show distinct overlap between AD and non-AD neurodegenerative disorders. Therefore this PhD thesis hypothesizes that the early differential diagnosis of AD needs further improvement and aims to do so by overcoming the overlap of neurochemical biomarkers measured in CSF.

THE OBJECTIVES

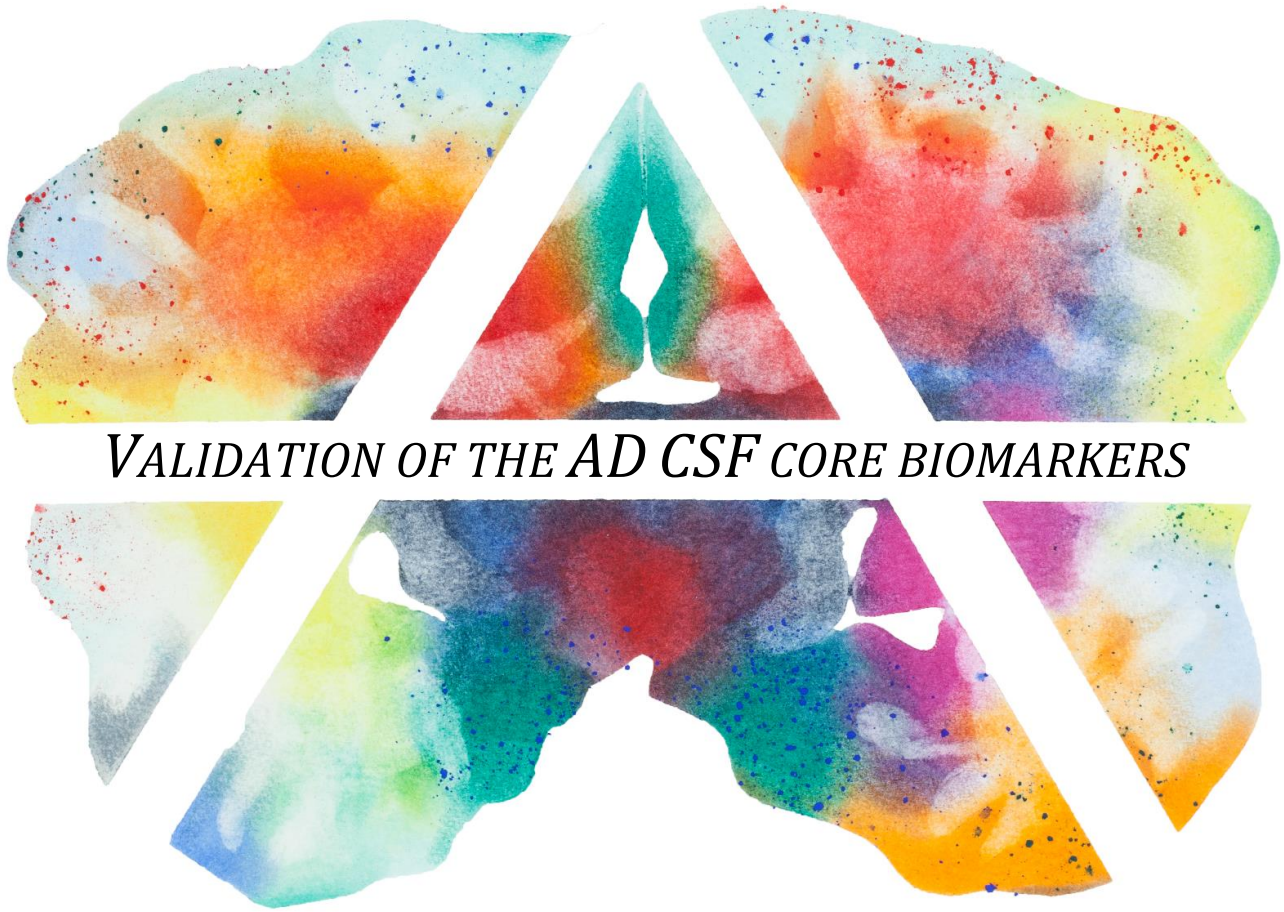
The first objective of this PhD thesis is to further characterize the core AD CSF biomarkers by studying their associations with the recently updated criteria for neuropathological diagnosis of AD in order to further improve their validation.

The second objective of this PhD thesis is to address the overlap of CSF A$\beta_{1\text{-}42}$ between AD and non-AD disorders as improving its discriminative power is critical for the early differential diagnosis. This will include reviewing the current literature on the diagnostic value of potential candidates originating from the Aβ metabolism such as CSF A$\beta_{1\text{-}40}$ and the CSF A$\beta_{1\text{-}42}$/A$\beta_{1\text{-}40}$ ratio to investigate the most promising candidates as well as their neuropathological validation and their assessment throughout the AD disease course.

The third objective of this PhD thesis is to examine the potential role of total CSF prion content in the discrimination of AD and CJD with overlapping CSF T-tau levels.

The fourth objective of this PhD thesis is to improve the harmonization of absolute CSF biomarker values by the validation of a diagnostic-relevant interpretation algorithm, namely the Erlangen Score, for the AD versus non-AD differentiation.

PART I



VALIDATION OF THE AD CSF CORE BIOMARKERS

CORRELATING ALZHEIMER'S DISEASE PATHOLOGY WITH ITS BIOMARKERS IN CEREBROSPINAL FLUID

Charisse Somers, Claire Bridel, Anne Sieben, Jean-Jacques Martin, Annemieke Rozemuller, Christine Van Broeckhoven, Peter P. De Deyn, Maria Bjerke, Charlotte E. Teunissen, Sebastiaan Engelborghs

To be submitted after internal review

ABSTRACT

The Alzheimer's disease (AD) cerebrospinal fluid (CSF) biomarkers amyloid-β peptide of 42 amino acids ($A\beta_{1-42}$), total tau protein (T-tau) and tau phosphorylated at threonine 181 (P-tau_{181}) are well validated and are sufficiently performant with regard to diagnostic accuracy to be used for daily clinical (research) practice. As these biomarkers are assumed to reflect the pathology of AD, studying their correlation with the recently updated neuropathological criteria for AD staging by Montine et al. may further improve their validation. Therefore, a multicenter study was set up including antemortem CSF samples from 114 clinically diagnosed dementia patients who had definite AD upon post-mortem neuropathological examination. Median interval between CSF sampling and death was one year. For these patients neuropathological staging was defined following the Montine criteria resulting in "ABC" scores for each patient. CSF samples of all patients were analyzed for $A\beta_{1-42}$, T-tau, and P-tau_{181} by ELISA. Including only cases with a short time interval (<4 years; *n=91*) between lumbar puncture and death, weak to mild, but significant, positive correlations were found between CSF P-tau_{181} and Montine Score A (Spearman's $\rho = 0.414$ with $p<0.001$), incorporating Thal phases for amyloid plaque staging, and Montine Score B (Spearman's $\rho = 0.321$ with $p<0.001$), which incorporates Braak's NFT staging. These correlations were ascribed to the *APOE* ε4 non-carrier subgroup (*n=27*) as in the subgroup of ε4 carriers (*n=37*) the level of statistical significance was not achieved. To conclude, in late-stage AD pathology, CSF P-tau_{181} levels correlated with both amyloid and neurofibrillary pathology in *APOE* ε4 non-carriers, highlighting its importance as a surrogate marker and its probable role as driver of AD pathology.

INTRODUCTION

The usefulness of CSF biomarkers for the purpose of improving the clinical AD diagnosis has been investigated for multiple decades[105]. Due to the disease complexity, no sole biomarker has been found to represent the pathophysiological process occurring in AD. The current AD CSF biomarkers, consisting of $A\beta_{1\text{-}42}$, T-tau, and P-tau_{181}, are considered as surrogate biomarkers of AD pathology. Low CSF $A\beta_{1\text{-}42}$ levels mirror amyloid plaque accumulation in the brain, high CSF P-tau_{181} levels represent NFT and high CSF T-tau is suggestive for neurodegeneration. In particular, the tau markers have been found to be associated with disease onset and progression of clinical symptoms [106–109]. However, few studies have been conducted to explore the direct association of the AD CSF biomarkers to AD neuropathology.

Although a high accuracy for the clinical diagnosis of AD can be obtained by the addition of the CSF biomarkers[39], a definite diagnosis of AD can only be certified through neuropathological examination of the brain. Thus, to study the direct relationship between CSF biomarkers and pathology and to better understand the association to disease mechanisms, studies on human brain tissue are critical. The latest update for neuropathological scoring of AD is described in the Montine criteria[19]. Here within, a practical assessment is set up to classify the stage of AD neuropathological change occurring in the brain. This staging focuses on the core hallmarks of AD neuropathology, the amyloid plaques and the NFT, in order to acquire "ABC" scores. The A score represents a score for Aβ plaque pathology, based on Thal phases modified to a four-point scale[16].The B score entails NFT staging according to Braak [17, 18], which was reduced into four instead of six stages in order to improve the inter-rater reliability. The C score serves to characterize the subset of amyloid plaques that is particularly associated with neuronal injury, the neuritic plaques (NP), according to the Consortium to Establish a Registry for Alzheimer's Disease (CERAD) protocol[110]. These revised consensus criteria therefore take into account the continuum of AD pathological changes and improve the relation to the clinical findings, also including a better definition of the intermediate changes.

This multicenter study aims to investigate how well core AD CSF biomarkers quantitatively reflect neuropathological changes in cases of definite "pure" AD, by exploring their correlations with the improved staging system for AD neuropathology according to the Montine criteria.

MATERIALS AND METHODS

Study population

A total of 133 CSF samples of dementia patients with neuropathologically confirmed diagnoses of AD were available from two centers. Of these cases, 19 were not considered for this study due to mixed or co-pathology, leaving a cohort of 114 pure definite AD cases. Cases that were not included presented with concomitant DLB/PD, TDP-43, CVD or CJD pathology.

The biobank of the IBB (Antwerp, Belgium) accounted for 94 cases and the VU University medical center (VUmc; Amsterdam, Netherlands) accounted for 20 cases. All patients and/or their relatives gave informed consent. The study was approved by the local ethics committees.

Neuropathological work-up

All neuropathological examinations were performed by two neuropathologists (JJM and AS) for the Antwerp cohort and by one neuropathologist (AR) for the Amsterdam cohort, who were blinded for the CSF biomarker results. Pathological diagnoses of AD were established by evaluation of AD neuropathological change, by means of the four-point scales of the "A, B and C" scores, using the Montine criteria[19]. The combination of ABC scores represent the level of explanation of AD neuropathological changes for dementia as "not", "low", "intermediate" or "high", where the "intermediate" and "high" profiles are considered to be sufficient for explaining dementia. Nine patients were found to have an "ABC" score that would not be considered sufficient to explain dementia, but were diagnosed with definite AD by the exclusion of all other causes based on neuropathology.
Brain tissue was fixated in 10% of buffered formalin and embedded in paraffin. Sections of the right hemisphere were prepared from the frontal superior gyrus, temporal superior gyrus, area striata, hippocampus, neostriatum, putamen, globus pallidus, thalamus, and of the brainstem and cerebellum. Histological staining was performed with Cresyl violet, Hematoxylin & Eosin, Bodian, and Klüver-Barrera. Immunohistochemistry was performed with antibodies against Ubiquitin (Dako, Glostrup, Denmark), AT8 (Fujirebio Europe, Ghent, Belgium), and 4G8 (Signet, Dedham, MA).

CSF biomarkers

All CSF samples were obtained between 1995 and 2014 in the context of a diagnostic dementia workup[111]. Antemortem CSF was obtained by LP at the L3/L4 or the L4/L5 interspace[81]. A minimum of 2 ml CSF was collected for each patient in a labeled polypropylene tube. In case of a hemorrhagic puncture detected by macroscopic inspection of the sample, samples were centrifuged for ten minutes at 3000 rpm within four hours after LP. After centrifugation, the supernatant was transferred to a new, labeled polypropylene tube. For the Antwerp cohort, samples were either frozen immediately in liquid nitrogen and shipped on dry ice to the IBB biobank or shipped unfrozen within 24 hours after LP. Samples were stored in the biobank of the IBB at -80°C until analysis. CSF samples obtained in Amsterdam were centrifuged within 1 hour of LP and the supernatant transferred to a new polypropylene tube. Samples were frozen immediately at -20°C and thawed within 48h for the analysis of the AD biomarkers.
Retrospective CSF biomarker concentrations of $A\beta_{1\text{-}42}$, T-tau and $P\text{-}tau_{181}$ were measured at time of LP with commercially available single analyte ELISA kits (INNOTEST® β-AMYLOID($_{1\text{-}42}$), INNOTEST® hTAU Ag, and INNOTEST® PHOSPHO-TAU($_{181P}$), respectively; Fujirebio Europe, Ghent, Belgium) following manufacturer's instructions as previously described[39].

For the Antwerp cohort, interpretation of the biomarker levels was based on cut-offs previously determined in a cohort of autopsy-confirmed AD patients and cognitively healthy elderly[83]. The cut-offs for the Amsterdam cohort were recently redefined for $A\beta_{1-42}$ to correct for the drift observed across time and earlier measurements were recalculated based on this new cut-off[112]. Levels of $A\beta_{1-42}$ <638.5 pg/ml, T-tau >296.5 pg/ml and P-tau_{181} >56.5 pg/ml and $A\beta_{1-42}$ <813 pg/ml, T-tau >375 pg/ml and P-tau_{181} >52 pg/ml were defined as abnormal in the Antwerp and Amsterdam cohorts, respectively. As the cut-off values of Antwerp and Amsterdam differed slightly, the raw data of the CSF biomarkers were categorized in order to compare between centers. Ranges were set for negative, borderline negative, borderline positive and positive results of each of the CSF biomarkers (table 4.1). Borderline results were defined as 15% around the cut-off values. Hereafter, the AD CSF biomarker profile was determined according to the NIA-AA criteria in order to evaluate the biomarker likelihood of AD etiology. A high likelihood of AD was attributed if both amyloid and neuronal injury markers were abnormal, an intermediate likelihood if only one marker was abnormal, and a low likelihood if both markers were normal[4].

APOE genotyping

DNA was extracted from total blood by following standard methods. Genotyping for *APOE* was hereafter performed as previously described[113]. Cases with an *APOE* genotype without ε4 allele (ε2/3 or ε3/3) were classified a non-carrier, cases with one or two ε4 alleles (ε3/4 or ε4/4) were classified as carrier.

Statistical analysis

Statistical testing was performed using IBM statistics program SPSS, version 24, with significance levels defined as $p<0.05$. Differences across CSF biomarker categories and the AD neuropathological change scores were evaluated by means of Chi-square statistics. As numbers in some categories were small (<5), Monte Carlo calculations were performed for Fisher's exact testing. Relations across CSF biomarkers and AD neuropathological changes are represented by Spearman's correlation (ρ).

RESULTS

Clinical, neuropathological, and biomarker data

Clinical, neuropathological, and biomarker data are represented in table 4.2. Demographics show an equal distribution of gender. Of the total of 84 patients for which an *APOE* genotype was available, slightly more *APOE* ε4 carriers (56%) than non-carriers were included. The overall population had a median age at moment of LP of 74 years, with median disease duration of 6 years. For 1 patient age of sampling was unknown and for another patient date of death was unknown. Median time between LP and time of death (TLPD) was 1 year ranging

from 1 years to 10 years. Cases of whom a Mini-Mental State Examination (MMSE) score was available within 1 year of LP (n=75) showed a median total MMSE score of 16/30 (interquartile range [IQR] 10-22). More than 80% of the cohort had a high likelihood AD CSF biomarker profile. Accordingly, for both CSF T-tau and $A\beta_{1-42}$, approximately 80% of patients were in the positive range, while for CSF P-tau_{181} only 50% of the patients had levels in the positive category. Over 90% of the cases had an AD neuropathological change profile in the intermediate or high categories. Cases with an A Score of the highest category (A3) accounted for 79%, cases with a B Score of the highest category (B3) accounted for 63%, and cases with a C Score of the highest category accounted for 49%.

Correlations

The cohort was divided into groups based on one year intervals of TLPD (i.e. TLPD of ≤1, ≤2, ≤3, etc.), in order to evaluate the effect of TLPD on the results. Comparing the separate "ABC" scores and the single biomarker categories separately (table 4.3), consistent significant correlations were found between CSF P-tau_{181} and the total ABC score, Montine Score A and Montine Score B in the cases up to TLPD ≤4 years (n=91). In this subgroup, the number of *APOE* ε4 alleles showed no significant correlations with any of the CSF biomarkers or AD neuropathological change scores (A Score Spearman's $\rho = 0.160$ with $p = 0.251$, B Score Spearman's $\rho = 0.219$ with $p = 0.092$, and C Score Spearman's $\rho = 0.084$ with $p = 0.538$, T-tau Spearman's $\rho = 0.003$ with $p = 1.000$, $A\beta_{1-42}$ Spearman's $\rho = 0.229$ with $p = 0.069$, P-tau_{181} Spearman's $\rho = 0.109$ with $p = 0.401$). Inclusion of cases with longer TLPD showed additional significant correlations as shown in table 4.3.
When separating *APOE* ε4 carriers from non-carriers (table 4.4), the abovementioned correlations between CSF P-tau_{181} and the total ABC score, Montine Score A and Score B in the cohort with TLPD ≤4 years were observed in the subset of non-carriers (n=27), but not in the carriers (n=37). In the non-carriers, an additional significant correlation was found between the AD CSF biomarker profile and the Montine Score A.

DISCUSSION

This study aimed to examine the relation between the current core AD CSF biomarkers and the improved staging system for AD neuropathology according to the Montine criteria. Including only cases with a short TLPD (≤4 years), weak to mild, but significant, positive correlations were found between CSF P-tau_{181} and the total ABC score, Montine score A, incorporating Thal phases for amyloid plaque staging, and Montine score B, which incorporates Braak's NFT staging. Upon separation of *APOE* ε4 carriers from non-carriers, observed correlations only applied to non-carriers. CSF $A\beta_{1-42}$ and T-tau did not seem to correlate with the neuropathological changes in late-stage, pure AD cases.
Although the median TLPD in the whole cohort was 1 year (IQR 0-4 years), some intervals went up to 10 years. During this period, pathology may have changed to such an extent that

the CSF composition at time of sampling does not entirely reflect the situation at the moment of pathological examination. However, more than half of the included cohort had very short TLPD within one year and the majority of cases had a relatively short TLPD up until 4 years. To account for the longer TLPD in some cases, correlation analysis was performed in a subgroup of TLPD ≤4 years as up until this interval correlations remained consistent. Increasing the inclusion of cases with longer TLPD (≤10 years) showed additional correlations of the CSF biomarkers, although these cannot be expected to reliably reflect the pathological stage at moment of death. By reflecting earlier pathological stages, these biomarkers may not yet be at ceiling levels. The additional findings of CSF T-tau correlating with Montine Score A and C and P-tau$_{181}$ correlating with Montine Score C, suggest CSF tau proteins to have reached ceiling levels closer to moment of death. Correlations for CSF A$\beta_{1\text{-}42}$ remained absent, suggesting these levels have reached ceiling levels much earlier, exceeding 10 years to moment of death. Except for one previous study of our group[91], no other research was found concerning the effect of increasing TLPD on correlations between CSF biomarkers and AD neuropathology. This study showed, despite an overlap with regard to the autopsy population (n=50), no associations between the CSF biomarkers and AD pathological staging[91].

The results in the current study with regard to CSF tau markers support the findings of previous studies[114, 115], but not those of our previous study[91]. One study showed CSF P-tau$_{231}$ to correlate with NFT burden in the frontal, temporal, parietal and hippocampal cortex, as well as with NP burden, although the latter only in frontal cortex areas[114]. In another study it was shown that CSF P-tau$_{181}$ correlates with NFT and with NP as well[115]. As hyperphosphorylated tau is the main constituent of NFT, correlations of CSF P-tau$_{181}$ may reflect the NFT formation during the progression of pathology. While NP are structured as a dense amyloid core surrounded by a ring of dystrophic neurites, these abnormal neurites are known to contain NFT and may therefore be responsible for the relation to CSF P-tau. With regard to our previous study, Braak staging for amyloid plaques was used as compared to Thal staging in both the study of Tapiola et al. and the current study. Braak staging describes the distribution pattern of amyloid plaques by differentiating amongst three stages, whereas Thal expanded this differentiation into five, specifying the sequence of amyloid deposition. This may explain the finding of correlations between CSF P-tau and amyloid plaque pathology. Interestingly, there exists a stronger correlation for P-tau$_{181}$ with Thal staging (Spearman's $\rho = 0.475$) than with Braak's NFT score (Spearman's $\rho = 0.346$). Evidence supporting a pattern of higher proportions of P-tau present in NFT than in NP contradicts this finding[114]. However, as the neuropathological staging does not control for the extent of dystrophic neurites in NP, the degree of neuritic pathology may vary[116]. The absence of correlation between CSF T-tau and NFT pathology could support the notion that T-tau reflects neurodegeneration, rather than AD-specific neurofibrillary changes[117].

As tau protein contains more than 40 phosphorylation sites[118, 119], the fraction of phosphorylated tau visualized by staining with the standard anti-tau phospho-specific antibody AT8 for phosphorylation at Ser202 and Thr205 may not fully correspond to the fraction of CSF P-tau measured with antibodies against Thr181. Although it is currently not known whether disease-specific phosphorylation of tau exists, these two antibodies may not

reflect the exact same pathological processes, accounting for relatively weak correlations between the neuropathology and CSF biomarkers.

The lack of correlations for CSF $A\beta_{1-42}$ with any of the neuropathological staging is in accordance with our previous study[91]. The results were based on a cohort containing exclusively mild to severe disease stages of AD. Similar studies that did find relations between CSF $A\beta_{1-42}$ and NP as well as NFT[115, 120] also included either non-demented or non-AD dementia subjects, therefore their cohort represents the absence and early stages of amyloid pathology as well. Another study also provided evidence of correlations between CSF $A\beta_{1-42}$ levels and amyloid pathology in the neocortex and the hippocampus, although using postmortem, ventricular CSF[120]. The authors acknowledged their CSF $A\beta_{1-42}$ levels to be lower than other studies which included ante-mortem CSF and found a correlation of $A\beta_{1-42}$ levels with the postmortem interval. This suggests a degrading effect of CSF $A\beta_{1-42}$ after death, accounting for their positive results.

The observation of limited correlation of CSF $A\beta_{1-42}$ with neuropathological scores in end-stage AD may fit the theory of amyloid plaque formation already being maximal quite early in the disease process, and the companioning CSF $A\beta_{1-42}$ levels reaching an equilibrium state of production, accumulation and clearance[121, 122]. Often co-existing with AD is the accumulation of Aβ in the cerebral vasculature seen in cerebral amyloid angiopathy (CAA). Although consisting mainly of $A\beta_{1-40}$, next to $A\beta_{1-42}$,[123, 124] CAA may also contribute to lowered $A\beta_{1-42}$ levels[120, 125–127]. However, current neuropathological criteria do not yet account for CAA, limiting the search for correlations between CSF $A\beta_{1-42}$ and the total amyloid load in the brain.

Our main finding of correlations between CSF P-tau_{181} and amyloid plaque and NFT pathology was attributed to the *APOE* ε4 non-carriers only. As *APOE* ε4 is an established risk factor for AD[128], differences in pathology between carriers and non-carriers were expected. The presence of an *APOE* ε4 allele has been proposed to mediate disease onset by facilitating amyloid plaque formation rather than its growth[129, 130], with carriers having a lower age of onset than non-carriers[131–133]. Although we possess data on the onset of symptoms of our patients, pathology starts at least 10 years before first symptoms arise[20]. As we could not estimate the onset of pathology, no analysis could be performed on correlations of the *APOE* genotype and onset of pathology. The inclusion of predominantly late-stage AD may therefore have had an impact on the negative results seen in *APOE* ε4 carriers. It can be speculated that the lack of correlations in carriers may be due to further progressed pathology, influenced by an earlier onset, leaving their CSF biomarker measurement already at ceiling levels.

Although studies including relatively large numbers of autopsy-confirmed cases are valuable and rare, the natural limitation is the exclusive inclusion of end-stage AD patients, excluding associations of biomarkers with the neuropathological changes in the early disease process or, in case of co-pathology, with changes related to other neurodegenerative disorders. Assigning the CSF biomarker data into four categories in order to compare between centers may also have introduced a limitation by methodologically reducing possible correlations in the highest or lowest ranges of biomarker levels.

CONCLUSION

In order to further investigate how well the diagnostic biomarkers for AD reflect the underlying pathology, we explored the relationship between the core AD CSF biomarkers and the current neuropathological staging criteria in a cohort of autopsy-confirmed AD patients. In a cohort of late-stage AD pathology, CSF P-tau$_{181}$ levels correlated with both amyloid and neurofibrillary pathology in *APOE* ε4 non-carriers, highlighting its importance as a surrogate marker and their role as driver of AD. Limited correlations for CSF T-tau supports its use as neurodegeneration markers rather than an AD-specific neurofibrillary marker while for CSF A$\beta_{1\text{-}42}$, lack of correlations probably reflect the ceiling effect of amyloid plaque pathology but does not exclude its value as an early AD biomarker.

Table 4.1 - Ranges of CSF biomarker categories within their center

		Antwerp	Amsterdam
T-tau	*Negative*	<252.0	<318.75
	Borderline negative	252.0 – 296.5	318.75 – 375.00
	Borderline positive	296.5 – 341.0	375.00 – 431.25
	Positive	>341.0	>431.25
$A\beta_{1-42}$	*Negative*	>735.3	>934.95
	Borderline negative	735.3 – 638.5	935.95 – 813.00
	Borderline positive	638.5 – 542.7	813.00 – 691.05
	Positive	<542.7	<691.05
P-tau_{181}	*Negative*	<48.0	<44.2
	Borderline negative	48.0 – 56.5	44.2 – 52.0
	Borderline positive	56.5 – 65.0	52.0 – 59.8
	Positive	>65.0	>59.8

Legend: All CSF biomarker data are measured in pg/ml. Borderline results were defined at 15% around the cutoff value.

Abbreviations: $A\beta_{1-42}$, amyloid-β peptide of 42 amino acids; CSF, cerebrospinal fluid; MMSE, Mini Mental State Examination (/30); LP, lumbar puncture; P-tau_{181}, tau phosphorylated at threonine 181; SD, standard deviation; TLPD, time between LP and death; T-tau, total tau.

Table 4.2 - All clinical and CSF biomarker data, as well as neuropathological scoring

		Total		Antwerp		Amsterdam	
TLPD <10 years		**n**		**n**		**n**	
Age at LP (years)		113	74 (66-83)	93	76 (71-84)	20	62 (58-67)
Disease duration (years)		84	6 (3-9)	64	5 (3-8)	20	9 (6-10)
TLPD (years)		112	1 (0-4)	92	(0-2)	20	5 (2-6)
MMSE within one year of LP		75	16 (10-22)	58	16 (10-20)	17	19 (14-25)
TLPD <4 years							
Age at LP (years)		91	75 (70-84)	82	76 (71-85)	9	67 (62-74)
Disease duration (years)		63	5 (3-6)	54	4 (3-6)	9	6 (5-8)
TLPD (years)		91	0 (0-1)	82	0 (0-1)	9	2 (1-4)
MMSE within one year of LP		56	16 (9-22)	49	16 (8-20)	7	22 (19-27)
				TLPD <10 years		**TLPD <4 years**	
				n	**%**	**n**	**%**
Gender	*Female*			55	48	46	51
	Male			59	52	45	49
APOE ε4	*Carrier*			47	56	37	58
	Non-carrier			37	44	27	42
AD CSF biomarker profile	*Low*			2	2	1	1
	Intermediate–normal Aβ			4	4	4	5
	Intermediate–low Aβ			16	14	13	14
	High			92	81	73	80
T-tau	*Negative*			17	15	14	16
	Borderline negative			3	3	2	2
	Borderline positive			6	5	3	3
	Positive			88	77	72	79
$A\beta_{1-42}$	*Negative*			2	2	1	1
	Borderline negative			4	4	4	5
	Borderline positive			21	18	15	17
	Positive			87	76	71	78
P-tau_{181}	*Negative*			26	23	22	24
	Borderline negative			11	10	9	10
	Borderline positive			12	11	9	10
	Positive			64	56	51	56
AD neuropathological change profile	*Not*			1	1	0	0
	Low			8	7	7	8
	Intermediate			37	32	32	35
	High			68	60	52	57
Montine score A	*0*			1	1	0	0
	1			3	3	3	3
	2			20	18	16	18
	3			90	79	72	79
Montine score B	*0*			0	0	0	0
	1			8	7	7	8
	2			34	30	29	32
	3			72	63	55	60
Montine score C	*0*			3	3	2	2
	1			16	14	13	14
	2			39	34	37	41
	3			56	49	39	43

(Legend on next page)

Legend: Demographic values are presented as median and respective interquartile ranges. The AD CSF biomarker profiles were determined according to the NIA-AA criteria[4] and AD neuropathological change profiles were based on the Montine criteria[19].

Abbreviations: Aβ_{1-42}*, amyloid-*β *peptide of 42 amino acids; AD, Alzheimer's disease; APOE ε4, apolipoprotein E gene allele ε4; CSF, cerebrospinal fluid; MMSE, Mini Mental State Examination (/30); n, absolute number; LP, lumbar puncture; P-tau*$_{181}$*, tau phosphorylated at threonine 181; SD, standard deviation; TLPD, time between LP and death; T-tau, total tau.*

Table 4.3 - Associations of the AD CSF biomarker profile with AD neuropathological change

	AD neuropathological change profile						
				Montine score A		**Montine score B**		**Montine score C**	
	TLPD	*Spearman's ρ*	*p-value*	*Spearman's ρ*	*p-value*	*Spearman's ρ*	*p-value*
AD CSF biomarker profile	*≤4*	0.136	0.194	0.067	0.559	0.167	0.117	0.070	0.504
	≤5	0.160	0.117	0.114	0.278	0.156	0.118	0.101	0.328
	≤10	**0.243**	**0.009***	0.181	0.062	**0.222**	**0.019***	**0.201**	**0.030***
T-tau	*≤4*	0.077	0.469	0.180	0.095	0.107	0.313	0.104	0.322
	≤5	0.127	0.209	**0.252**	**0.009***	0.120	0.239	0.163	0.109
	≤10	0.172	0.075	**0.268**	**0.005***	0.156	0.097	**0.207**	**0.027***
Aβ$_{1-42}$	*≤4*	0.069	0.525	0.067	0.513	0.053	0.614	0.025	0.814
	≤5	0.096	0.355	0.113	0.271	0.051	0.610	0.058	0.559
	≤10	0.097	0.304	0.141	0.121	0.043	0.646	0.073	0.433
P-tau$_{181}$	*≤4*	**0.297**	**0.004***	**0.414**	**<0.001***	**0.321**	**<0.001***	0.209	0.051
	≤5	**0.341**	**0.001***	**0.466**	**<0.001***	**0.339**	**<0.001***	**0.268**	**0.009***
	≤10	**0.376**	**<0.001***	**0.475**	**<0.001***	**0.346**	**<0.001***	**0.295**	**0.001***

Legend: The level of significance was set at a *p*-value below 0.05 (values in bold, *). All correlations are represented as Spearman's ρ. The AD CSF biomarker profiles were determined according to the NIA-AA criteria[4] and AD neuropathological change profiles were based on the Montine criteria[19].

Abbreviations: Aβ$_{1-42}$, amyloid-β peptide of 42 amino acids; AD, Alzheimer's disease; CSF, cerebrospinal fluid; P-tau$_{181}$, tau phosphorylated at threonine 181; TLPD, time between lumbar puncture and death; T-tau, total tau.

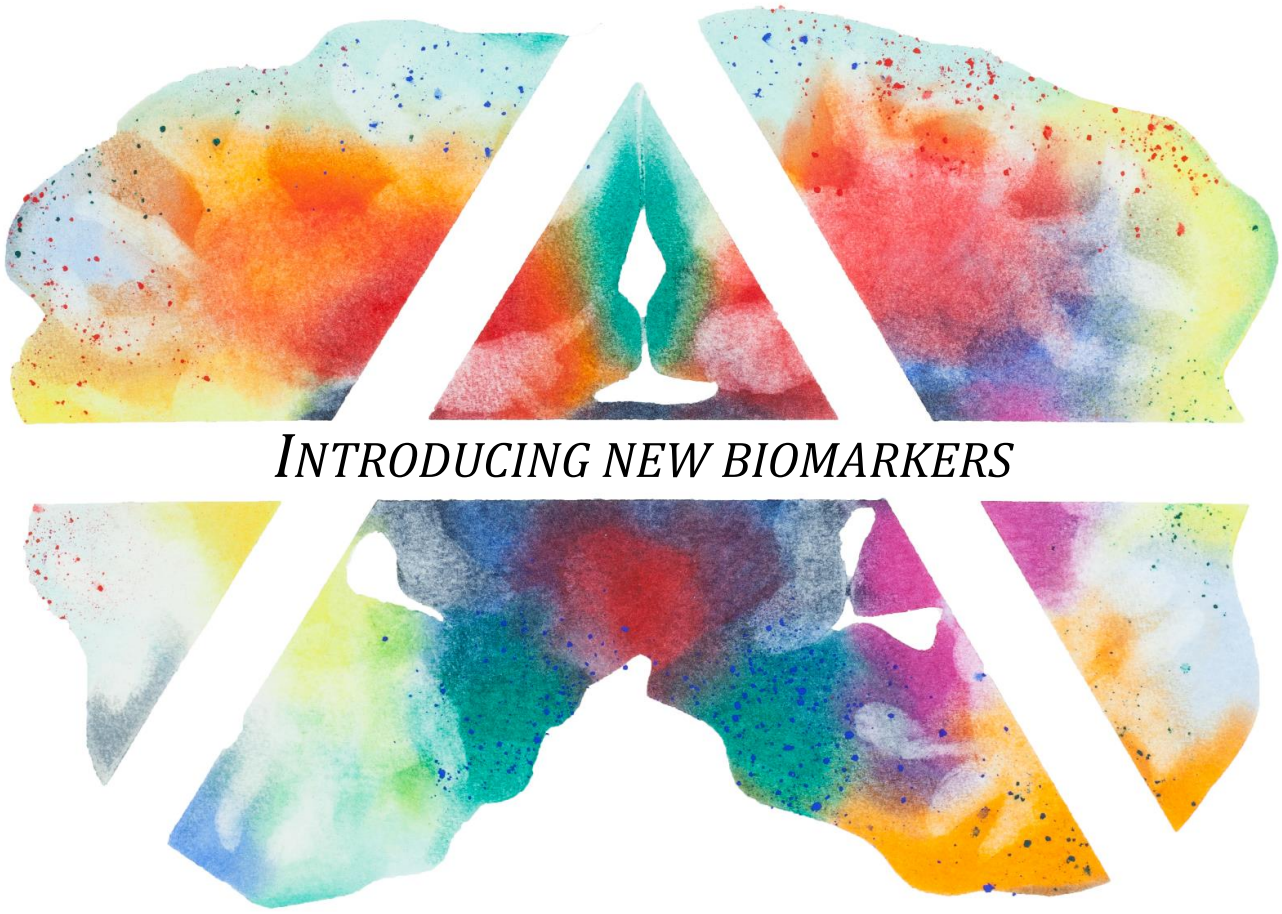
Table 4.4 - Associations of the AD CSF biomarker profile with AD neuropathological change of *APOE* ε4 carriers and non-carriers in a cohort with TLPD ≤4 years

		AD neuropathological change profile					
	APOE ε4	*Spearman's ρ*	*p-value*				
AD CSF biomarker profile	*carrier*	-0.027	1.000				
	Non-carrier	0.267	0.207				
	total	0.136	0.194				
T-tau	*carrier*	-0.078	0.680				
	Non-carrier	0.371	0.065				
	total	0.172	0.068				
Aβ$_{1-42}$	*carrier*	0.161	0.360				
	Non-carrier	-0.039	0.834				
	total	0.097	0.298				
P-tau$_{181}$	*carrier*	-0.083	0.662				
	Non-carrier	**0.496**	**0.008***				
	total	**0.376**	**<0.001***				
		Montine score A		**Montine score B**		**Montine score C**	
	APOE ε4	*Spearman's ρ*	*p-value*	*Spearman's ρ*	*p-value*	*Spearman's ρ*	*p-value*
AD CSF biomarker profile	*carrier*	-0.167	0.567	0.028	0.828	-0.111	0.552
	Non-carrier	0.388	0.055	0.267	0.207	0.220	0.286
	total	0.067	0.559	0.167	0.117	0.070	0.504
T-tau	*carrier*	0.257	0.131	-0.024	0.759	0.258	0.189
	Non-carrier	0.388	0.058	0.371	0.065	-0.096	0.588
	total	0.180	0.095	0.107	0.313	0.104	0.322
Aβ$_{1-42}$	*carrier*	0.070	1.000	0.069	0.666	0.075	0.633
	Non-carrier	0.190	0.379	-0.039	0.833	0.016	0.954
	total	0.067	0.513	0.053	0.614	0.025	0.814
P-tau$_{181}$	*carrier*	0.197	0.276	-0.094	0.567	-0.029	0.872
	Non-carrier	**0.497**	**0.007**	**0.496**	**0.010**	0.278	0.155
	total	**0.414**	**<0.001***	**0.321**	**<0.001***	0.209	0.051

Legend: The level of significance was set at a *p*-value below 0.05 (values in bold, *). All correlations are represented as Spearman's ρ. The AD CSF biomarker profiles were determined according to the NIA-AA criteria[4] and AD neuropathological change profiles were based on the Montine criteria[19].

Abbreviations: Aβ$_{1-42}$, amyloid-β peptide of 42 amino acids; AD, Alzheimer's disease; APOE ε4, apolipoprotein E gene allele ε4; CSF, cerebrospinal fluid; P-tau$_{181}$, tau phosphorylated at threonine 181; TLPD, time between lumbar puncture and death; T-tau, total tau.

PART II



INTRODUCING NEW BIOMARKERS

SELECTING Aβ-ISOFORMS FOR AN ALZHEIMER'S DISEASE CSF BIOMARKER PANEL

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ABSTRACT

Although the core cerebrospinal fluid (CSF) Alzheimer's disease (AD) biomarkers amyloid-β ($A\beta_{1-42}$) and tau show a high diagnostic accuracy, there are still limitations due to overlap in the biomarker levels with other neurodegenerative and dementia disorders. This overlap is especially concerning in the early disease stage before symptom onset when tau is not or minimally affected and performs less accurately compared to Aβ. On the other hand, Aβ is the marker showing the most overlap and this decreases the utility of the biomarker panel even more, urging the need for further improvement.

As the $A\beta_{1-42}$ metabolism has been shown to be altered decades before the clinical onset of AD, much effort has been put into finding peptides or protein fragments related to this mis-metabolism that could increase the early diagnostic accuracy of AD. The Aβ peptide is part of the amyloid precursor protein (APP) from which it is being released by enzymatic cleavage. The hydrophobic $A\beta_{1-42}$ peptide aggregates into parenchymal plaques in the AD brain, which is one of the neuropathological hallmarks of AD. During $A\beta_{1-42}$ production and clearance in the brain, several other Aβ peptides and APP fragments are formed that could potentially serve as biomarkers for this ongoing disease process. Furthermore, they could improve the *in vivo* detection of plaques as well as improve the diagnostic accuracy, since $A\beta_{1-42}$ alterations in CSF in other brain disorder most likely do not represent plaque pathology. Therefore, this review will present the current status of the findings for APP and Aβ peptide isoforms in AD and clinically related disorders.

In conclusion, adding new Aβ-isoforms to the AD biomarker panel may improve early differential diagnostic accuracy and increase the CSF biomarker concordance with AD neuropathological findings in the brain.

AD is the most common neurodegenerative disorder in elderly, leading to dementia. An accurate and early diagnosis of AD is important to select the optimal patient care and will be critical in current clinical trials. Its value will grow even more so when a disease modifying treatment is available. To date, diagnosis of AD is still based on a full clinical work-up, including neuropsychological testing[5]. However, clinical dementia diagnosis does not always correspond to the neuropathological definite diagnosis with clinical diagnostic accuracy levels ranging between 82 and 84%[39, 134]. When a clinician should discriminate AD from a non-AD dementia relying on (non-biomarker based) clinical diagnostic criteria, 16% of the patients have a doubtful AD versus non-AD diagnosis[39, 75, 135]. Therefore it is important to increase the clinical diagnostic accuracy, which will be even harder at an early stage of the disease. Since biochemical changes are believed to take place and be detectable through biomarkers around two decades before clinical symptom onset[136, 137], they will be important tools in the clinical set-up for early dementia (differential) diagnosis.

Neuropathologically, AD is characterized by the formation of extracellular cortical senile plaques[16] and intracellular neurofibrillary tangles composed of P-tau mainly found in the limbic and association cortices[17, 138]. The core structure of the senile plaques mainly comprises the Aβ peptide, which is derived from APP[139]. While many different Aβ-isoforms are present in the brain, the hydrophobic $A\beta_{1\text{-}42}$ peptide in particular is prone to self-accumulation into soluble oligomers. These oligomers further form larger fibrils and aggregate into insoluble extracellular plaques, both are believed to possess neurotoxic properties[140, 141]. The exact cause of the accumulation and deposition of $A\beta_{1\text{-}42}$ into plaques is still unknown, though it has been hypothesized that an imbalance in production and clearance may be one of the reasons[8]. An imbalance in the regulation of protein kinases and phosphatases is believed to lead to hyperphosphorylation of microtubule-associated protein tau, which causes it to dissociate and accumulate into paired helical filaments that compose the intraneuronal neurofibrillary tangles[12].

Interestingly, it has been shown that patients that are clinically diagnosed with a high likelihood of AD due to a typical AD profile with an amnestic syndrome, portrait postmortem with a neuropathological tangle intensive (as graded by Braak staging) profile that involves hippocampus[142]. On the contrary, patients with a neuropathologically confirmed diagnosis of AD with a plaque intensive profile (as graded by CERAD) were clinically diagnosed with a moderate likelihood of having AD due to an atypical clinical profile[142]. Together with the demand for early diagnosis, these findings support the need for *in vivo* biological verification of the disease[55]. Decades of research have resulted in the development of three core CSF biomarkers for the neurobiochemical diagnosis of AD[143] of which, especially in early diagnosis of atypical clinical cases, $A\beta_{1\text{-}42}$ may prove to be an invaluable tool for AD detection.

The amyloid precursor protein and amyloid-β production

The prevailing theory of AD etiology, the amyloid cascade hypothesis, postulates a central role of $A\beta_{1-42}$ as an initiator of AD pathology. The toxic Aβ oligomers and/or senile plaques cause hyperphosphorylation of tau and formation of neurofibrillary tangles, activation of glia and neuroinflammation, synaptic loss and ultimately neuronal degeneration. The $A\beta_{1-42}$ is generated through the so-called amyloidogenic pathway through sequential enzymatic cleavage of the APP protein by β-and γ-secretases. APP is a type-1 integral membrane protein and the β-secretase cleavage site (the β-site) is found in the extracellular amino terminal part of APP and thus the extracellular soluble APP (sAPPβ) fragment is also liberated, while γ-secretases cleavage occurs in the membrane domain of APP which also leads to the liberation of an intracellular domain into the cytosol. An alternative pathway, referred to as the non-amyloidogenic pathway, involves cleavage of APP within the Aβ domain by α-secretase to preclude the formation of intact full-length $A\beta_{1-42}$. This pathway also liberates a 16 amino acid longer extracellular domain compared with the amyloidogenic pathway, an APP soluble fragment that has been termed sAPPα. There are several sites in the carboxyl terminal of Aβ domain for γ-secretase cleavage as well as many other possible cleavage sites for various enzymes in the Aβ sequence that may be involved in Aβ degradation and give rise to the multitude of Aβ peptides found in the brain[8, 144].

Biomarker characteristics and cerebrospinal fluid $A\beta_{1-42}$ reflecting AD pathology

For a biomarker to be an acceptable candidate for AD diagnosis it should be precise, reliable, inexpensive and non-invasive. In addition, the biomarker should be able to detect one of the pathological hallmarks of AD and this should be validated in neuropathologically confirmed cases. These biomarker features were established in the consensus paper by the Ronald and Nancy Reagan Research Institute of the Alzheimer's Association and the National Institute on Aging Working Group[145], which also stated that biomarker candidates should have a sensitivity of at least 80% for the detection of AD and a specificity for differentiation from other neurodegenerative disorders between 75% and 85%.

As CSF is in direct contact with the brain interstitial fluid, its contents reflect the biochemical changes taking place during a disease process, making CSF biomarkers good diagnostic candidates for brain disorders. The contamination of the CSF proteome by blood proteins is prohibited by the blood-CSF/blood-brain barriers, obstructing possible degradation, binding or dilution of the biomarker of interest by other non-brain derived and non-AD pathology-specific proteins[146, 147]. The choice of CSF biomarkers over blood biomarkers, at least in the case of $A\beta_{1-42}$, is supported by studies in plasma showing conflicting results but no overall difference between AD and controls when evaluated in a meta-analysis (22 study comparisons)[143]. Also, no correlation between plasma and CSF $A\beta_{1-42}$ biomarker have been found[148, 149]. Furthermore, $A\beta_{1-42}$ in CSF has proven its potential to mirror the build-up of plaques, which is supported by the inverse correlation between the CSF $A\beta_{1-42}$ levels and the amount of amyloid plaques found at neuropathological examination of AD brains[120] as

well as the *in vivo* correlation to cortical amyloid load as measured by amyloid PET with Pittsburgh Compound B in patients with AD[150]. Moreover, the CSF $A\beta_{1-42}$ levels have also shown satisfactory sensitivity and specificity for the distinction between healthy subjects and AD cases in neuropathologically confirmed cohorts[39, 151] as well as between pathologically confirmed AD and non-AD dementia cohorts[39, 75, 76, 152]. Also, a recent meta-analysis of $A\beta_{1-42}$ in CSF shows that the sensitivity and specificity for differential diagnosis between clinical AD and non-AD dementias reached the consented level for an acceptable AD biomarker[153].

Due to its favourable characteristics and the relative inexpensive costs, much effort has been put into making $A\beta_{1-42}$ manageable as an AD biomarker worldwide to be used in daily clinical dementia practice[105] as *in vivo* surrogate marker for plaque pathology. Though the absolute measurements of $A\beta_{1-42}$ in CSF show inter-laboratory variability, mainly due to differences in pre-analytical and analytical procedures when performed on assays utilizing different calibrators, major efforts are undertaken to overcome these problems by introducing a certified reference material that can be used for value assignment of the assay calibrators[99, 100, 104, 154]. Also, the Alzheimer's Association external QC program monitors site-to-site and batch-to-batch CSF test variation for the purpose of enabling the participating laboratories to synchronize their procedures[102]. These efforts will lead to precise and reliable measurements between laboratories that will enable the introduction of a world-wide cut-off point for CSF $A\beta_{1-42}$ measurements for the purpose of clinical diagnostics and patient stratification in clinical trials.

CEREBROSPINAL FLUID $A\beta_{1-42}$ FOR EARLY AND DIFFERENTIAL AD DIAGNOSIS

The present core AD CSF biomarkers, including $A\beta_{1-42}$, T-tau, and P-tau_{181}, have recently been incorporated into the research diagnostic criteria of AD, with a CSF profile suggestive for AD being low $A\beta_{1-42}$ in combination with high T-tau and/or P-tau_{181} levels[55]. The inclusion of these biomarkers into the new criteria for AD diagnosis for research purposes is not only based on the correlation to the above mentioned imaging and neuropathology findings, but also on more than 100 studies showing the consistency of altered levels of the three core biomarkers in AD compared to controls, as well as over 10 papers showing the same alterations in patients with MCI who later develop AD dementia compared to those who remain stable[143]. It should be emphasized that early biomarkers are especially important for the selection of preclinical AD (subjects who are asymptomatic at risk for AD or subjects who suffer from SCD due to AD) and for the selection of patients in the earliest symptomatic stages of AD (prodromal AD or MCI due to AD). SCD and MCI are both very heterogeneous syndrome entities and it should be kept in mind that when solely clinically assessed, less than 50% of these subjects converted to dementia[46] even after an extensive follow-up period of 6 years. Even fewer (18%) of these SCD and MCI subjects specifically developed a dementia due to AD[46]. Particularly, CSF $A\beta_{1-42}$ is a more attractive biomarker for early AD detection than both CSF T-tau and P-tau_{181} as tau alterations seem to occur at a later time point in the disease process[155]. Early detection is likely to become even more important as soon as

disease modifying pharmacological treatment for AD will be available, since medications that halt or prevent the disease are likely to be most efficient in an early stage, when neurodegeneration has not become too severe. At present, low CSF $A\beta_{1-42}$ levels are an inclusion criterion for several clinical trials with potential disease-modifying drugs that target AD in its earliest (and even preclinical) stages. Hence, biomarkers reflecting the pathology targeted by specific clinical trials are crucial for monitoring treatment effects[156]. Although there is strong evidence for the importance of CSF $A\beta_{1-42}$ as a biomarker for AD, there are still limitations to be overcome. One such limitation is the overlap of CSF $A\beta_{1-42}$ between different neurodegenerative disorders. For instance, decreased CSF levels of $A\beta_{1-42}$ have also been observed in patients with (subcortical) VaD[88, 157–159], DLB[160, 161], CJD[162] and normal pressure hydrocephalus (NPH)[163, 164] compared to healthy individuals. Though the $A\beta_{1-42}$ levels are most often still lower in AD compared to VaD and DLB, a significant overlap nevertheless limits their discrimination[88, 157, 158, 165]. Conflicting results have been shown in FTLD were either no changes in $A\beta_{1-42}$ levels or lower levels compared to controls have been found, though these levels were also still higher than in AD[166, 167]. While AD pathology is often found to co-exist with both Parkinson's disease and DLB as well as with cerebrovascular disease[39, 161, 168, 169], decreased $A\beta_{1-42}$ levels in CSF of patients with pure VaD (related to subcortical small vessel disease), CJD, NPH or FTLD are most likely not due to plaque pathology. Proposed causes for the decreased concentrations of $A\beta_{1-42}$ are a diminished production, an increased clearance or an enhanced binding to carriers that will mask the epitopes and therefor decrease the detection of $A\beta_{1-42}$ by immunoassays. For example, CJD is characterized by presence of prion protein (PrP^{Sc}) depositions and physiological PrP^{C} has been shown to promote the aggregation of $A\beta_{1-42}$ into plaques, therefore influencing Aβ metabolism and its presence in CSF. As for subcortical VaD and NPH, the decreased levels of $A\beta_{1-42}$ may be caused by the inhibition of axonal transport of APP, causing a disturbed APP metabolism, or by the altered clearance of Aβ due to disrupted CSF dynamics or alterations in enzymatic systems.

Additional APP/Aβ mediators to increase diagnostic specificity for AD

Cerebrospinal fluid $A\beta_{1-40}$ and $A\beta_{1-38}$ in AD and other dementia disorders

Both $A\beta_{1-40}$ and $A\beta_{1-38}$ are more abundant in CSF than $A\beta_{1-42}$. They have been less extensively studied compared to $A\beta_{1-42}$ as a biomarker for AD possibly due to early discouraging findings showing no difference between AD and controls[170, 171]. Though there have been some conflicting results, the negative findings were confirmed in a recent meta-analysis showing only negligible overall decrease in $A\beta_{1-40}$ levels with a small effect size in AD compared with controls (25 comparisons). Furthermore, no significant difference was found in the same meta-analysis (three studies included) for the comparison of CSF $A\beta_{1-40}$ in patients with MCI that converted to AD at follow-up and subjects who maintained their MCI status[143]. It has also previously been shown that there was no difference between AD and non-AD neurodegenerative disorders (Mehta et al., 2000); however, the non-AD group contained

small patient numbers in each scattered patient group precluding any subanalyses. Another Aβ peptide that was assessed in the meta analyses, summarized in the AlzBiomarker database (http://www.alzforum.org/alzbiomarker), that is also highly abundant in CSF and for which no differences between AD and controls have been found is $A\beta_{1-38}$ (8 studies included)[143]. Importantly, both $A\beta_{1-40}$ and $A\beta_{1-38}$ have been further investigated for their ability to differentiate between AD and non-AD brain disorders and it has been shown in several studies that lower levels of both $A\beta_{1-40}$ and $A\beta_{1-38}$ are found in FTLD, VaD and DLB/Parkinson's disease dementia (PDD) compared to AD and controls[172–176]. The differential diagnostic added value of $A\beta_{1-40}$ and $A\beta_{1-38}$ will be addressed below.
So far, immunoassays have mainly been used for quantitative assessments of $A\beta_{1-42}$, $A\beta_{1-40}$ and $A\beta_{1-38}$ in human CSF, but it has also been shown that established quantitative methods based on mass spectrometry (MS) perform very well[177–180] and the measurements correlate highly with immunobased assays[104, 181]. The interchangeable use of the methods for Aβ measurements is advantageous and an even more important role for MS is expected in the search for new biomarkers and new possible Aβ peptides that may contribute to the differential diagnosis of AD, but for which there are no (specific) antibodies available.

Cerebrospinal fluid Aβ peptides in AD

One of the first attempts to characterize Aβ species in human CSF was undertaken in the early 1990s when there was still much doubt about its existence in CSF. The peptides identified by laser desorption MS confirmed the presence of Aβ species, all beginning with aspartic acid in the Aβ (Asp 1) carboxyl-terminus (C-terminal), containing 27, 28, 30, 34, 35, 40, 42, or 43 amino acids[182]. Through a further refined method that employed a combination of immunoprecipitation (with 6E10 and 4G8 antibodies) and MS (IP-MS) it was shown that a whole range, including the above mentioned species, of Aβ fragments (1-13 – 1-20, 1-32 - 1-34, 1-37 - 1-42) truncated in the amino-terminus (N-terminal) were captured, but also many shorter C-terminally truncated peptides (2-, 3-, 4-, 5-, 6-, -8, 11-, 12-, 14-, 15-, 16-, and 17-) of different lengths[183]. This method has since paved the way for characterization of Aβ fragments in human CSF. While CSF candidates for the differentiation between AD and controls, such as $A\beta_{1-16}$, $A\beta_{1-33}$, and $A\beta_{1-39}$, have been lifted forward using IP (antibodies 6E10 and 4G8)-MS with label free (semi) quantification[184] most of these peptides have not yet been quantitatively assessed and the results have not been verified by others. The combined levels of $A\beta_{1-15/16}$ were investigated further but no difference was found between AD and controls, while there was a significant decrease in Parkinson's disease (PD) and PDD, multiple system atrophy and progressive supranuclear palsy compared to controls[185]. One argument in support of the above $A\beta_{1-16}$ findings is if increased levels of $A\beta_{1-16}$ were masked by a concomitant decrease in the concentration of $A\beta_{1-15}$ in AD, this would render the overall change non-significant with this assay set-up. However, $A\beta_{1-15}$ was not suggested to be decreased in AD in above study. Also, no significant changes in the above peptides ($A\beta_{1-15}$, $A\beta_{1-16}$, $A\beta_{1-33}$, or $A\beta_{1-39}$) or any other Aβ peptides assessed ($A\beta_{1-13}$, $A\beta_{1-14}$, $A\beta_{1-17}$, $A\beta_{1-19}$, $A\beta_{1-20}$, $A\beta_{1-30}$, $A\beta_{1-34}$, $A\beta_{1-37}$, $A\beta_{1-38}$ or $A\beta_{1-40}$) except for $A\beta_{1-42}$ were seen between sporadic AD and healthy controls in another IP-MS attempt[186]. Another candidate that was lifted forward

by IP (antibodies 6E10, 4G8 and antibody directed against $A\beta_{21-34}$)-MS using isobaric quantification was $A\beta_{1-34}$ (slightly decreased, but not significant after Bonferroni correction), while in another MS study that also employed isobaric labelling $A\beta_{22-40}$ was found to be reduced in AD compared to controls[187]. Neither study verified the other study findings. Thus, there are so far no other Aβ peptides except for $A\beta_{1-42}$ that seem to be specific for AD versus controls.

Cerebrospinal fluid Aβ-oligomers in AD

The interest in Aβ-oligomers as a biomarker arose when it was shown that they may be toxic and cause synaptic dysfunction and inhibit long term potentiation. Since oligomers are, according to the amyloid cascade hypothesis, the preceding step to the formation of amyloid plaques it was hoped that they would serve as even earlier biomarkers than $A\beta_{1-42}$. Although significantly higher levels of high molecular weight CSF Aβ-oligomers (40-200kDa) were found in AD compared to controls[188], and in AD and MCI due to AD compared to controls[189], contradictory results have been shown by another study[190], were no differences were found for Aβ-oligomers in CSF between MCI patients that developed AD, MCI patients that remained stable or non-demented controls. This discrepancy could be due to differences in assay design and performance that favour detection of different analytes or Aβ-oligomers. It was further stated in the above study, were differences between patient groups were found, that Aβ oligomers have not potential as biomarkers due to a high degree of overlap between the diagnostic groups and the oligomers did not perform better than CSF $A\beta_{1-42}$. Furthermore, there was no correlation at all to $A\beta_{1-42}$ levels, indicating other possible mechanisms for the aggregation of $A\beta_{1-42}$ into plaques[189].

Cerebrospinal fluid sAPPα and sAPPβ in AD and other dementia disorders

As the extracellular part of the APP protein is being released by β- and α-secretases as soluble APP fragments (sAPPβ and sAPPα, respectively) these were proposed to be able to serve as upstream biomarkers for the amyloid and non-amyloid pathways in CSF. Unfortunately, it has been shown in the AlzBiomarker database meta-analysis that neither sAPPβ nor sAPPα holds the potential to serve as AD biomarkers, since the levels are not significantly different in AD compared to controls (10 publications for sAPPβ and 9 for sAPPα) or in MCI patients that developed AD at follow-up compared to those who were stable in their MCI syndrome (3 publications including sAPPβ and sAPPα)[143]. It should be noted that for the measurement of sAPPα many of the assays do not specifically capture fragments cleaved at the α-secretase cleavage site and therefore may include both fragments that are shorter and longer than intended.
Both sAPPβ and sAPPα have also been investigated for their potential as differential diagnostic tools. In one study, patients with clinical dementia disorders with either an AD supportive or an AD dismissive CSF profile were investigated for their sAPP profiles. Decreased levels of both sAPPβ and sAPPα were found in the dementia patients with a negative AD CSF profile compared to those with a CSF profile indicative of AD[191]. Further

characterization of sAPP alterations in non-AD dementias have shown sAPPβ to be significantly decreased in FTLD compared with AD[176, 192], while no difference was observed between the levels in AD and DLB or PDD[193]. Furthermore, CSF sAPPβ has been shown to be inversely correlated with white matter lesion volume in patients with cerebrovascular disease[194] and acute stroke[195] possibly indicating dysfunctional axonal transport in patients with small vessel disease. Moreover, sAPPα and sAPPβ levels have been shown to be decreased in CSF in NPH compared with healthy subjects[196] and to be strong markers for the differentiation between AD and NPH[163]. The alterations in sAPPα and sAPPβ have been shown to be independent of Aβ pathology, though it may be that changes take place much earlier in the cascade causing a disconnection to current tissue pathology, and it has been suggested that sAPP may rather reflect metabolic impairment in the brain tissue possibly caused by ischemia[197].

Aβ peptide ratios to increase diagnostic accuracy for AD

It has been shown previously that a combination of the core AD biomarkers are superior compared to the single biomarkers alone; however, it has also been shown that tau does not perform as well as $A\beta_{1-42}$ in the early stages of disease[155]. Thus if there are other amyloid metabolites in CSF that can be added to the AD biomarker panel to improve early differential diagnostic accuracy this would be immensely important.

The introduction of Aβ ratios may prove to be of importance for early differential AD diagnosis. First of all, it has been demonstrated that the $A\beta_{1-42}/A\beta_{1-40}$ ratio shows better concordance with amyloid load in the brain as assessed by PiB-PET than $A\beta_{1-42}$ alone[172, 198]. The $A\beta_{1-42}/A\beta_{1-40}$ ratio has been revealed to be decreased in AD compared with controls, FTLD, VaD, DLB and PDD, the latter 4 groups being inseparable, and was more accurate for differentiating AD from the other types of dementia than $A\beta_{1-42}$ alone[172, 173, 199, 200]. Also, the CSF $A\beta_{1-42}/A\beta_{1-40}$ ratio has been shown to be superior or equal to $A\beta_{1-42}$ alone when concerned with the distinction between MCI patients who progress to AD dementia and MCI patients who remain stable[201, 202] and the $A\beta_{1-42}/A\beta_{1-40}$ ratio has been shown to perform equally well as the combination of $A\beta_{1-42}$, P-tau181 and T-tau in differentiating between AD and other non-AD dementias[93, 200]. In another study, it was shown that adding the Aβ ratio to the core biomarkers (T-tau not included) improved the accuracy when distinguishing between AD and non-AD dementias in cases with intermediate $P\text{-}tau_{181}$[203]. Along the same line, the added value to the core biomarkers has also been assessed in a clinical setting where it was shown that in cases with a discrepancy in the AD core biomarker profile the $A\beta_{1-42}/A\beta_{1-40}$ ratio pointed in the direction in over 50% of the cases to be in agreement with the clinical diagnosis[204]. These findings speak in favour of the added value of the $A\beta_{1-42}/A\beta_{1-40}$ ratio for early dementia differential diagnosis, when alterations in CSF tau are yet to be seen. The influence of the ratio could possibly be contributed to the fact that $A\beta_{1-40}$ closely represents the total cerebral Aβ load and thus eliminates inter-individual differences in total Aβ concentrations.

Other ratios that have been less well investigated but still show potential as biomarkers are $A\beta_{1-42}/A\beta_{1-38}$ and $A\beta_{1-42}/A\beta_{1-37}$[93, 205–207]. These studies concluded that $A\beta_{1-42}/A\beta_{1-38}$ was

the best ratio for the separation between AD and DLB and that it outperformed the single AD biomarkers. Also, $A\beta_{1-42}/A\beta_{1-37}$ has been shown to have an additive value for the differentiation between AD and FTLD[93]. More studies are needed to in order to determine which Aβ peptide ratios achieve the best separation in different diagnostic setting.

CONCLUSION

In this review we summarize the possible added value of CSF APP and Aβ metabolites as biomarkers for early and differential AD diagnosis. It can be concluded that CSF $A\beta_{1-42}$ is the superior AD biomarker that has consistently proven to be altered in AD compared to controls compared to other Aβ peptides or APP fragments. Additionally, both sAPPβ and various Aβ peptides (e.g. $A\beta_{1-40}$, $A\beta_{1-38}$ and $A\beta_{1-37}$) are altered in several non-AD neurodegenerative and dementia disorders and may therefore prove to be valuable tools for differential diagnosis, most likely combined in a ratio with $A\beta_{1-42}$. However, it is far too early to state which specific Aβ peptide ratio combinations may prove to be the most accurate predictors when concerned with early differential diagnosis. Importantly, the Aβ ratio needs to capture the AD neuropathological changes taking place in the brain that leads to the build-up of plaques better than $A\beta_{1-42}$. In the case of the $A\beta_{1-42}/A\beta_{1-40}$ ratio the concordance between CSF findings and amyloid load measured by PET imaging is higher than for $A\beta_{1-42}$ alone and this seems to be a general feature for all the neurodegenerative and dementia diseases assessed. These findings are immensely important for patient selection to reach improved treatment effects when concerned with amyloid-based therapy.

FUTURE PERSPECTIVES

With the prospective of disease modifying treatment for AD becoming available in the near future, an accurate diagnosis that reflects the neuropathology will be of great importance for selecting patients that will benefit from the treatment. Though a combination of the current AD core CSF biomarkers shows high accuracy for AD, they may be less efficient in the really early phases of disease due to the time dependence of tau to symptom onset. The Aβ-ratio may be a future substitute for early detection of AD amyloid pathology, since the decrease of CSF $A\beta_{1-42}$ alone overlaps with other neurodegenerative and dementia diseases known to be less affected by plaque pathology. The concordance between CSF and amyloid PET imaging increases with the introduction of the CSF $A\beta_{1-42}/A\beta_{1-40}$ ratio, which speaks in favour of its clinical utility as an inexpensive *in vivo* biomarker of plaque pathology. Its potential of reflecting pathology should also be assessed in autopsy confirmed cases with and without plaque pathology, preferably including cases with other dementia diseases and mixed AD pathologies. Furthermore, the potential as an early differential biomarker must be evaluated in more longitudinal studies that include early MCI cases. Other Aβ ratios, with peptides such as $A\beta_{1-38}$ and $A\beta_{1-37}$, should also be included to assess their performance in comparison to $A\beta_{1-42}/A\beta_{1-40}$. The implementation of the $A\beta_{1-42}/A\beta_{1-40}$ ratio (or possibly another ratio) or the

substitution of $A\beta_{1-42}$ by the ratio will most likely increase the core AD biomarker accuracy and great focus should therefore be put on its validation in the very near future.

IMPROVED DIAGNOSTIC ACCURACY USING THE CEREBROSPINAL FLUID AMYLOID BETA RATIO

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ABSTRACT

The diagnostic accuracy of the Alzheimer's disease (AD) cerebrospinal fluid (CSF) biomarkers, amyloid beta composed of 42 amino acids ($A\beta_{1-42}$), the total amount of tau (T-tau) and tau phosphorylated at threonine 181 (P-tau_{181}), is hampered due to an overlap between neurodegenerative disorders. We hypothesize that the $A\beta_{1-42}/A\beta_{1-40}$ ratio will be more accurate than $A\beta_{1-42}$ alone in discriminating AD from controls and more importantly from non-AD dementias. We aim to establish new biomarker reference values for the core AD biomarkers, using a certified reference material (CRM) calibrated $A\beta_{1-42}$ assay, as well as for the $A\beta_{1-42}/A\beta_{1-40}$ ratio, to improve the diagnostic accuracy and the interpretation of the CSF biomarker results.

For this study, CSF samples were included from two cohorts. A first cohort consisted of 50 autopsy confirmed AD patients, 42 autopsy confirmed patients with other types of dementia (non-AD) and 30 autopsy confirmed patients with mixed AD and non-AD pathology (MxD) as well as cognitively healthy controls and a second cohort consisted of 34 clinically diagnosed mild cognitive impairment (MCI) due to AD/AD dementia patients and 36 cognitively healthy/stable MCI subjects. All samples were analysed for $A\beta_{1-42}$, T-tau, and P-tau_{181} as well as $A\beta_{1-40}$ by automated analysis, of which the $A\beta_{1-42}$ kits had been calibrated using three CRMs.

Both CSF $A\beta_{1-42}$ levels and the Aβ ratio were significantly decreased in AD as well as in MxD compared with controls and non-AD dementia patients, which was also observed in MCI-AD/AD when comparing with controls. AUC values for the discrimination of AD from controls for $A\beta_{1-42}$ levels and the Aβ ratio were 0.982 and 0.966, respectively, for the discrimination of MCI-AD/AD from controls 0.917 for both, for the discrimination of AD from non-AD 0.829 and 0.915, respectively and for the discrimination of MxD from non-AD 0.764 and 0.886, respectively. Though the $A\beta_{1-42}/A\beta_{1-40}$ and the $A\beta_{1-42}$/P-tau_{181} ratio perform equally well to detect AD pathology, cut-points of the Aβ ratio remained stable over the complete disease course.

The CSF $A\beta_{1-42}/A\beta_{1-40}$ ratio outperformed CSF $A\beta_{1-42}$ in the differentiation between AD and non-AD dementia, also when mixed pathology was present. The stability of the Aβ ratio and its cut-points over the complete disease course are advantageous.

A new conceptual framework has been purposed that allows for the *in vivo* verification of pathology through biomarkers to improve the accuracy of the clinical diagnosis of AD[6, 54, 208]. Biomarker inclusion is particularly important for research as well as enrichment purposes in clinical trials and to monitor therapeutic effects. Biomarker verification will be vital for differential diagnosis of AD and non-AD neurodegenerative and/or cerebrovascular brain diseases once there is an available treatment to allow early intervention at the prodromal stage of the disease. An overlap in clinical profiles is especially prominent at this early stage, or when there is an involvement of co-pathology, which reduces the diagnostic certainty. It has been shown that the established CSF Aβ and tau biomarkers for AD are fully changed in the prodromal phase[155] and may be used to increase the diagnostic accuracy, especially in clinically ambiguous cases[39, 209]. Furthermore, it has been shown that alterations in the CSF biomarkers are related to an insidious slowing in cognitive functions and to changes in atrophy in AD related brain structures already at the preclinical stage[210, 211], highlighting the importance of the biomarkers and their diagnostic utility throughout the course of the disease.

Biomarker diagnostic accuracy and analytical variability have been addressed worldwide in order to reach to a stage where biomarkers can be implemented into clinical routine. A tremendous amount of studies have shown that the AD CSF biomarkers, $A\beta_{1\text{-}42}$, T-tau and P-tau_{181}, are consistently altered in AD and in MCI due to AD compared with controls[212]. It has also been shown that the AD biomarkers differentiate between AD, even at the prodromal stage, and non-AD neurodegenerative brain disorders with a high diagnostic accuracy when used in combination[63, 73]. Even so, on a single biomarker level, there are still improvements to be done. The most specific CSF biomarker for AD is regarded to be P-tau_{181}[76]; however, both P-tau_{181} and T-tau are considered to be late markers as changes seem to appear close to dementia onset. $A\beta_{1\text{-}42}$ on the other hand, is altered early in the course of the disease, at least a decade before dementia onset[155], though it suffers from an overlap between different neurodegenerative diseases and secondary dementias[213, 214]. This overlap can seemingly be overcome by introducing the $A\beta_{1\text{-}42}/A\beta_{1\text{-}40}$ ratio, which has been shown to more accurately reflect amyloid plaque pathology than $A\beta_{1\text{-}42}$ alone as visualized by the increased concordance with amyloid PET[172, 198, 215]. Furthermore, reducing the effect of pre-analytical issues such as adsorption to storage tubes[100], causing variability in the measurement of $A\beta_{1\text{-}42}$, also speak in favour of introducing the ratio into the AD biomarker diagnostic work-up[216]. Additionally, as the ratio reflects the overall Aβ metabolism within one person, as opposed to $A\beta_{1\text{-}42}$ alone, patients with higher baseline levels of Aβ[217] with a specific decrease in $A\beta_{1\text{-}42}$ would be detected.

To decrease the analytical variability and improve the interpretation of the biomarker results numerous efforts have been undertaken world-wide[104, 154, 218–220]. The field is moving towards automation of the measurement procedure and calibration of assays through the use of certified reference materials (CRM)[221], which should diminish or even completely eliminate the analytical variability. Furthermore, the introduction of common cut-offs throughout the world may be achieved with the availability of calibrated assays[222]. Our

aim was to establish new biomarker reference values for the core AD CSF biomarkers, using a CRM calibrated $A\beta_{1-42}$ assay, as well as for the $A\beta_{1-42}/A\beta_{1-40}$ ratio. We hypothesize that the ratio will be more accurate than $A\beta_{1-42}$ alone in discriminating AD from controls as well as from non-AD subjects. A true accuracy can only be obtained if the diagnosis is definite, we will therefore use an autopsy confirmed cohort of AD and non-AD subjects in combination with clinical controls. We also aim to determine cut-offs and assess the biomarker accuracy in an early clinical cohort consisting of patients with prodromal AD and AD dementia with positive amyloid PET scans or conversion to dementia during clinical follow-up, in relation to stable MCI and control subjects. The latter cohort was chosen for the purpose of investigating whether age or disease stage have an effect on the cut-offs. We will also investigate the effect of *APOE* genotypes on the reference values.

MATERIALS AND METHODS

Autopsy confirmed cohort

The study cohort originates from the IBB biobank (University of Antwerp, Belgium) and consisted of a total of 92 neuropathologically confirmed subjects with clinical history of cognitive impairment. More specifically, the cohort consisted of 50 subjects with definite AD and 42 with a definite non-AD diagnosis. The latter group included 7 patients with definite diagnosis of LBD, 12 patients with definite diagnosis of CVD, and 23 patients with definite diagnosis of FTLD. Furthermore, 30 patients with mixed pathology of AD and another neurodegenerative or cerebrovascular brain disease (MxD) were included, which consisted of subjects with CVD (n=18) or with LBD (n=12).
Neuropathological diagnoses were established by two neuropathologists (JJM and AS) based on macro- and microscopic examination of the right hemisphere of the brain. AD neuropathological changes were evaluated according to the Montine criteria in order to establish a definite diagnosis of AD[19]. LBD pathology was rated using the McKeith classification[223]. CVD pathology was evaluated using the Deramecourt criteria[224]. Definite diagnosis of FTLD was established according to the criteria of Cairns[84] and Mackenzie[85, 86].
There were 50 cognitively healthy control subjects included in this cohort based on the following inclusion criteria; (1) no cognitive complaint or decline; (2) no history of neurological or psychiatric antecedents; and (3) no organic disease involving the central nervous system following extensive neuropsychological exam that confirmed their normal cognitive function for age. CSF biomarker values were not included in the selection of any of the subjects in this study cohort.

Clinical cohort

The clinical cohort, published previously[215], consisted of 34 subjects with clinical suspicion of AD, either MCI due to AD or AD dementia (MCI-AD/AD). All AD dementia patients

(n=8) were positive on amyloid PET. The main part of the patients with MCI due to AD had received an amyloid PET (n=23) and were positive for amyloid pathology, 12 subjects converted to dementia due to AD within a follow-up of 2 years. The remaining part of the MCI subjects (n=3) converted to dementia due to AD within 1 year of follow-up but did not receive an amyloid PET.
A diagnosis of dementia due to AD was made based on the NIA-AA criteria[4, 6]. Diagnosis of MCI due to AD was made by applying the NIA-AA criteria[4, 7], which includes (1) objective cognitive impairment, determined by a score of more than 1.5 SD under the appropriate mean on neuropsychological sub testing; (2) a sparing of general cognitive functioning; and (3) with activities of daily living largely intact.
The control cohort consisted of 36 cognitive healthy or stable MCI (sMCI) subjects. Cognitive healthy controls (n=30) were included based on the same criteria as in the autopsy cohort, which was also confirmed by extensive neuropsychological examination, as well as having an age of 61 years or older. For two cognitive healthy controls amyloid PET imaging was available, indicating no amyloid pathology. Stable MCI subjects (n=6) with a negative result on amyloid PET and stable cognitive performance during a follow-period of 2 years were also included in the control cohort. CSF biomarker values were not included in the selection of any of the subjects in this study cohort.

CSF sampling and handling

All CSF samples were collected in the Memory Clinic of the Hospital Network Antwerp, Middelheim and Hoge Beuken (Antwerp, Belgium) and in referring centres between 1994 and 2017 in the context of a diagnostic dementia workup[111]. CSF was obtained into polypropylene tubes (Nalgene) by LP at the L3/L4 or L4/L5 interspace[81] according to standard collection protocols as previously described[105]. Samples were frozen immediately and shipped on dry ice to the lab or shipped unfrozen within twenty four hours at room temperature. The samples were stored in the IBB biobank at -80°C until and after the performance of routine biomarker analyses.

CSF biomarker analysis

The concentrations of the CSF core AD biomarkers $A\beta_{1-42}$, T-tau, and $P\text{-}tau_{181}$ as well as $A\beta_{1-40}$ were determined by automated analysis (EUROIMMUN Analyzer I-2P; Medizinische Labordiagnostika AG, Lübeck, Germany) using commercially available single-analyte ELISA kits (EUROIMMUN Beta-Amyloid (1-42), EUROIMMUN Beta-Amyloid (1-40), EUROIMMUN Total-Tau, and EUROIMMUN pTau(181), respectively; Medizinische Labordiagnostika AG, Lübeck, Germany) according to manufacturer's instructions. The $A\beta_{1-42}$ kits had been calibrated using three CRMs (ERM®-DA480/IFCC, ERM®-DA481/IFCC and ERM®-DA482/IFCC, European Commission, Joint Research Centre (EC-JRC) and the International Federation for Clinical Chemistry and Laboratory Medicine (IFCC))[221]. All CSF samples, two kit QC and two CSF QC samples (QC samples having low and high concentration values) were run in duplicates. The analyses were performed in a strict blinded manner. All Aβ

measurements were within the analytical range, while 6% of the tau values were above the highest limit of quantification. The out of range tau samples were diluted 1:4 and re-analysed. For the individual CSF samples, all CVs were below 15%, the mean CVs for $A\beta_{1-42}$, $A\beta_{1-40}$, T-tau and for P-tau_{181} were 2.1%, 1.9%, 2.3 % and 1.9 %, respectively.

APOE genotyping

Genomic DNA was isolated from lymphocytes in total blood following standard methods. Hereafter, genotyping for *APOE* was performed by Sanger sequencing as previously described[113]. Patients with an *APOE* genotype without ε4 allele (ε2/3 or ε3/3) were classified a non-carrier, cases with one or two ε4 alleles (ε3/4 or ε4/4) were classified as carriers. Genotyping was not performed for 11 AD patients, 12 non-AD patients and 1 control subject as no blood was available.

Statistical analyses

Statistical analyses were performed using SPSS statistical package of IBM Statistics, version 24. Results were reported as mean values with standard deviation (SD). Due to the adequate sample size of the groups, parametric testing was used. Chi-square testing was performed to detect differences in sex distribution. To compare clinical and biomarker data, one-way ANOVA was used for comparisons across several groups (n>2) and independent-samples T test was used for comparisons between 2 groups, applying Bonferonni post-hoc to correct for multiple comparisons. With MedCalc statistical software, version 18.9, ROC curve analysis was performed to obtain AUC values and 95% CI to evaluate diagnostic performance. Sensitivity and specificity percentages were determined at the maximal sum of sensitivity and specificity by use of the Youden's index. Delong testing was performed to compare the AUC of different variables. The level of significance was set at $p < 0.05$.

RESULTS

Demographics of the autopsy and clinical cohorts

The demographics of the autopsy cohort are presented in table 6.1. Distribution in gender differed significantly across the cohort with more women in the control group compared with the patient groups. Disease duration was significantly longer in non-AD patients compared with the MxD patients. Age at the moment of LP differed significantly, with older patients in the MxD compared with the AD group, both consisting of older patients compared with the control and non-AD groups. AD, non-AD and MxD patients scored significantly lower on the MMSE, which was time-linked within 3 months of the LP, than controls. There were no significant distribution differences across the groups for *APOE* ε4 genotype. TLPD was similar in all pathological groups with means of 1 year (± 1.5 year), 2 years (± 1.6 year), and 1 year (± 1.3 year) for AD, non-AD and MxD patients, respectively.

The demographic data of the clinical cohort is presented in table 6.2. No significant gender distribution differences were observed. Patients with AD, either MCI or dementia, were significantly older at the moment of LP than control subjects. Scores on the MMSE were lower for patients with AD compared with controls subjects. Distribution of *APOE* ε4 allele carriers versus non-carriers differed significantly, with a higher subset of carriers in the MCI-AD/AD group.

CSF biomarker results in the autopsy and clinical cohorts

All the biomarker data for the autopsy cohort can be found in table 6.1. Significantly decreased CSF $A\beta_{1-42}$ levels were found in the AD, non-AD, and MxD groups compared with controls, of which the AD and MxD groups had significantly lower levels compared with the non-AD group. CSF $A\beta_{1-40}$ levels were significantly decreased in the AD group compared with controls and the MxD group, as well as in the non-AD group compared with controls. CSF T-tau levels were significantly increased in all patient groups compared with controls, of which the MxD group had significantly higher levels than the non-AD groups. CSF levels of P-tau_{181} were significantly increased in the AD and MxD groups compared with controls and the non-AD group. For the $A\beta_{1-42}/A\beta_{1-40}$ ratio, significantly lower values were obtained in the AD and MxD groups compared with controls and the non-AD group (figure 6.1). The ratio of $A\beta_{1-42}$/P-tau_{181} was significantly decreased in all patient group compared with controls, of which significant lower levels were observed in the AD and MxD groups compared with the non-AD group. The tau ratio was significantly decreased in the AD group compared with controls and the non-AD group, and in the MxD group compared with the non-AD group.
CSF biomarker levels differed significantly between *APOE* ε4 carriers and non-carriers only in the control group, for which decreased CSF $A\beta_{1-42}$ and $A\beta_{1-42}/A\beta_{1-40}$ levels were observed in the carrier subgroups (supplementary table 6.1).
The biomarker data for the clinical cohort are presented in table 6.2. A significantly decreased concentration of CSF $A\beta_{1-42}$ was observed in the MCI-AD/AD group compared with the control group, while CSF $A\beta_{1-40}$ was unaltered. Also, the $A\beta_{1-42}/A\beta_{1-40}$ ratio was significantly decreased in the MCI-AD/AD group (figure 6.2). CSF T-tau and P-tau_{181} levels were significantly increased in the AD group, while the $A\beta_{1-42}$/P-tau_{181} and T-tau/P-tau_{181} ratios were significantly decreased compared with controls.
Significant differences for the CSF biomarker levels between *APOE* ε4 carrier and non-carriers were only observed in the control group, with decreased CSF $A\beta_{1-42}$ and $A\beta_{1-42}/A\beta_{1-40}$, $A\beta_{1-42}$/P-tau_{181} and T-tau/P-tau_{181} ratios and increased CSF T-tau and P-tau_{181} levels in carriers (supplementary table 6.2).

Biomarker diagnostic performance in the autopsy and clinical cohorts

In the autopsy cohort, the Aβ ratio significantly outperformed $A\beta_{1-40}$, T-tau and the tau ratio (AUC values of 0.966 versus 0.783, 0.876, and 0.882, respectively) and performed equally well or not significantly different from the other biomarkers in differentiating AD from controls. The best performing markers to differentiate between AD and controls were $A\beta_{1-42}$

and the $A\beta_{1-42}/A\beta_{1-40}$ and $A\beta_{1-42}$/P-tau_{181} ratios. $A\beta_{1-42}$ showed an AUC of 0.982 with a sensitivity of 92% and a specificity of 98%. The Aβ ratio performed with an AUC of 0.966 with a sensitivity of 96% and a specificity of 88%, while $A\beta_{1-42}$/P-tau_{181} ratio presented with an AUC of 0.988 with a sensitivity of 96% and a specificity of 96%. All values including cut-offs for the respective comparisons are presented in table 6.3.

For the comparison between AD and non-AD, the Aβ ratio performed significantly better than both Aβ isoforms individually and T-tau (AUC values of 0.915 versus 0.829, 0.513, and 0.639, respectively) while almost reaching significant difference compared with P-tau_{181} (AUC of 0.861). The best performing markers to differentiate between AD and non-AD were the $A\beta_{1-42}$/ $A\beta_{1-40}$ and $A\beta_{1-42}$/P-tau_{181} ratios. The Aβ ratio showed an AUC of 0.915 with a sensitivity of 92% and a specificity of 85%, while $A\beta_{1-42}$/P-tau_{181} ratio presented with an AUC of 0.922 with a sensitivity of 96% and a specificity of 85%.

The Aβ ratio significantly outperformed $A\beta_{1-40}$ alone, T-tau and the tau ratio in the differentiation of MxD from non-AD (AUC values of 0.886 versus 0.629, 0.692, and 0.740, respectively), almost reaching significant difference compared with P-tau_{181} (AUC of 0.772). The best performing markers to differentiate between MxD and non-AD were the $A\beta_{1-42}$/ $A\beta_{1-40}$ and $A\beta_{1-42}$/P-tau_{181} ratios. The Aβ ratio showed an AUC of 0.886 with a sensitivity of 94% and a specificity of 73%, while $A\beta_{1-42}$/P-tau_{181} ratio presented with an AUC of 0.858 with a sensitivity of 89% and a specificity of 83%.

For differentiating MxD from controls, the Aβ ratio significantly outperformed $A\beta_{1-40}$ and the tau ratio (AUV values of 0.943 versus 0.596 and 0.698, respectively), and performed equally well as all other biomarkers. The best performing markers to differentiate between MxD and controls were $A\beta_{1-42}$ and the $A\beta_{1-42}/A\beta_{1-40}$ and $A\beta_{1-42}$/P-tau_{181} ratios. The $A\beta_{1-42}$ showed an AUC of 0.951 with a sensitivity of 94% and a specificity of 92%. The Aβ ratio performed with an AUC of 0.943 with a sensitivity of 97% and a specificity of 80%, while $A\beta_{1-42}$/P-tau_{181} ratio presented with an AUC of 0.967 with a sensitivity of 89% and a specificity of 94%. The $A\beta_{1-42}$/P-tau_{181} ratio was on the border to being significantly better than the Aβ ratio.

For the differentiation between the non-AD group and the controls, the Aβ ratio performed equally well as all biomarkers except for $A\beta_{1-42}$ alone and its ratio with P-tau_{181}, which significantly outperformed the Aβ ratio (AUC values of 0.649 versus 0.800 and 0.811, respectively).

In the clinical cohort, the Aβ ratio performed equally well compared with $A\beta_{1-42}$, the $A\beta_{1-42}$/P-tau_{181} and T-tau/P-tau_{181} ratios. The $A\beta_{1-42}$ showed an AUC of 0.971 with a sensitivity of 94% and a specificity of 92%. The Aβ ratio performed with an AUC of 0.971 with a sensitivity of 100% and a specificity of 86%, while $A\beta_{1-42}$/P-tau_{181} ratio presented with an AUC of 0.995 with a sensitivity of 96% and a specificity of 100%. The T-tau/P-tau_{181} ratio reach an AUC of 0.976 with a sensitivity of 93% and a specificity of 94%. All values including cut-offs for the respective comparisons are presented in table 6.4.

DISCUSSION

The most important added value for a biomarker in the clinical diagnostic work-up is to detect ongoing pathological processes as early as possible and to be able to differentiate between clinically overlapping diseases leading to cognitive dysfunction. CSF $A\beta_{1-42}$ has proven to reflect the core neuropathological hallmark of amyloid plaques in the AD brain *in vivo*; however, there is overlap between AD and brain disorders with no or little plaque pathology. We showed that the Aβ ratio performed equally well as $A\beta_{1-42}$ to detect definite AD compared with controls and outperformed $A\beta_{1-42}$ in the distinction between definite AD and non-AD subjects. Furthermore, it is also the most accurate marker for discriminating between cases with a MxD (AD plus non-AD) neuropathological diagnosis and definite non-AD diagnosis. The cut-off determined in the autopsy confirmed cohort also remained stable in the clinical cohort and there were no differences between *APOE* ε4 carriers and non-carriers in their ratio values in either of the neurodegenerative disease groups, neither in the autopsy cohort nor in the clinical cohort, speaking in favour of its stability.

The numbers of studies showing that the concordance is higher between amyloid PET and the Aβ ratio than compared with $A\beta_{1-42}$ alone is steadily increasing[172, 198, 215, 225], supporting the superiority of the ratio to reflect the actual pathology. The two discordant groups found in clinical amyloid PET studies, when comparing imaging to CSF $A\beta_{1-42}$ alone, are represented by positive CSF $A\beta_{1-42}$ in combination with negative PET and by negative CSF $A\beta_{1-42}$ matched with positive PET, both groups believed to be true to their imaging conclusion with regard to AD pathology[226]. It has been shown in clinical cohorts that both groups decrease when applying the ratio[198, 215], the former especially in frontotemporal and vascular dementia as the Aβ values are normalized using the ratio signifying no existing plaque pathology[172, 198]. Some studies also indicate that CSF is an earlier marker of AD pathology when compared with amyloid PET[227], which may explain parts of the results. The inconsistent results of negative CSF $A\beta_{1-42}$ findings while amyloid PET remains positive seems to be mainly found in MCI (due to AD; PET positive) and in AD, possibly indicating a small proportion of patients with higher overall production of Aβ; however, it may also depend on the stringency of the cut-off value[198, 215]. Our results in the autopsy-confirmed cohort confirms the findings of previous studies done in clinical populations, that the ratio has a diagnostic value when it comes to differential diagnosis between AD and other neurodegenerative or secondary dementias such as FTLD, LBD and CVD. We can show with certainty that the Aβ ratio has an added value, outperforming $A\beta_{1-42}$, to differentiate between AD and non-AD diseases that often in the clinical practice overlap both based on the clinical profile as well as individual biomarker values. Care was also taken to separate subjects with MxD pathology to show that such ambiguous cases may be discriminated from non-AD dementias by applying the Aβ ratio, as they too carry AD pathology. It was furthermore shown that the ratio was the best performing CSF biomarker in this setting. Thus, a concomitant pathology of CVD, FTLD or LBD does not seem to alter the value of the ratio, while $A\beta_{1-42}$ performed worse also in this sub-cohort.

Comparing the CSF $A\beta_{1-42}/A\beta_{1-40}$ ratio to the other members of the AD biomarker panel, the $A\beta_{1-42}$/P-tau_{181} ratio was performing equally well in all comparisons. Both $A\beta_{1-42}$ and T-tau

are known to be non-specific markers of AD, while P-tau$_{181}$ is regarded to be the most specific CSF biomarker[100, 228]. However, it has previously been shown that changes in CSF Aβ metabolism occur at least a decade before a clinical dementia diagnosis may be established, while changes in tau occur closer to clinical conversion[155]. This may negatively affect the biomarker utility of tau for early AD differential diagnosis and speaks in favour of the Aβ ratio. Interestingly, in the MxD group, T-tau and P-tau$_{181}$ performed less well than Aβ$_{1-42}$ in separating MxD from non-AD subjects leading to a slight reduction also in the performance of the Aβ$_{1-42}$/P-tau$_{181}$. Moreover, T-tau, which is a general marker of neuronal degeneration, is highly correlated with P-tau$_{181}$ in AD and its decrease in performance may be explained by a longitudinal decrease in biomarkers of neuronal injury that has been observed both in familial as well as sporadic AD[229, 230]. The biological reason is unclear. It has been proposed that the decrease in neuronal injury biomarkers reflect a general slowing of acute neurodegenerative processes with symptomatic disease progression and/or neuronal death, leading to a smaller number of neurons that remain and contribute to the pool in CSF. However, as P-tau$_{181}$ and the Aβ$_{1-42}$/P-tau$_{181}$ ratio as well as Aβ$_{1-42}$ and the Aβ$_{1-42}$/Aβ$_{1-40}$ ratio are performing equally in the separation between AD and non-AD, one would assume that in AD the neurodegenerative process has not advanced as far as in MxD and as such worse cognitive performance would be expected in the latter group. However, there were no differences in MMSE scores between the MxD and AD groups. The only significant difference in demographic variables between the two groups, is the increased longevity of the MxD group. Another explanation for the findings may be that there is a slight difference in the stage of the AD pathology between 'pure' AD and the MxD, that goes undetected by the MMSE. The biomarkers may indicate less AD tangle pathology in the MxD group in combination with a slight increase in neurodegeneration, possibly affected by the concomitant pathology. Anyhow this may not have any effect on the early clinical diagnostic work-up. It will be important to clarify the clinical implication of the time relation in changes in tau to clinical conversion to AD, which may render the tau markers to be less accurate for early diagnosis. It is of outmost importance that the AD biomarkers function equally well to detect ambiguous clinical diagnoses, including MxD, especially once there is an available therapeutic option for the treatment of AD. Whether the Aβ$_{1-42}$/Aβ$_{1-40}$ will outperform the Aβ$_{1-42}$/P-tau$_{181}$ ratio for early differential AD diagnosis remains to be clarified.

There was no major difference between the cut-offs for the Aβ$_{1-42}$/Aβ$_{1-40}$ or Aβ$_{1-42}$/P-tau$_{181}$ ratios with regard to if they were established between AD and controls or AD and non-AD subjects. The Aβ$_{1-42}$/Aβ$_{1-40}$ ratio cut-off also remained unchanged when compared between the autopsy cohort and the clinical cohort. The latter was not the case for the Aβ$_{1-42}$/P-tau$_{181}$ ratio, which decreased, indicating a longitudinal impact on the P-tau$_{181}$ marker rendering its values higher in a clinical population. When concerned with the effect of *APOE* genotype, we did not observe significant differences between the autopsy confirmed AD, MxD or non-AD subjects with regard to the biomarkers in carriers versus non-carriers of the ε4 allele. Neither was there any significant difference between the clinical carrier and non-carrier patient groups. However, there were significant differences in both the controls groups belonging to the autopsy confirmed and the clinical cohorts. In the clinical cohort, these findings also hold true for the Aβ$_{1-42}$/P-tau$_{181}$ ratio. As our control groups were significantly younger compared

with all other groups and the possession of the *APOE* ε4 allele has been proposed to modify the age at which Aβ accumulation in the brain starts[35, 132, 133], up to ten years compared with ε4 non-carriers[35], the finding of lower CSF $A\beta_{1-42}$ levels in ε4 carriers in our youngest group is not surprising. However, CSF $A\beta_{1-42}$ and Aβ ratio values in both *APOE* ε4 carrier and non-carrier controls remained above the clinical cut-off value. Therefore *APOE* genotype does not seem to affect the discriminative power of the biomarker levels, which is in accordance with previous findings[231].

This study is not without limitations. Our autopsy confirmed cohort did not include an autopsy confirmed healthy control population, but a clinical control population consisting of thoroughly investigated cognitively normal subjects. This could mean that some of them may have age related changes with minor plaques or tangles or even an incipient AD pathology that may have gone unnoticed. This would impact the results in a negative way. On the other hand, both the controls in the autopsy confirmed cohort and in the clinical cohort were significantly younger, which could be regarded as a drawback or in a positive light with regard to the notion that they are less likely to be affected yet by AD or any other age related primary or secondary dementia pathology.

Conclusion

The CSF $A\beta_{1-42}/A\beta_{1-40}$ ratio outperformed CSF $A\beta_{1-42}$ in the differentiation between non-AD and AD, also when mixed pathology is present. Though the $A\beta_{1-42}/A\beta_{1-40}$ and the $A\beta_{1-42}$/P-tau_{181} ratio perform equally well to detect the AD pathology, the stability of the Aβ ratio and the cut-points over the complete disease course may prove to be advantageous.

Table 6.1 - Demographics and biomarker data of the autopsy cohort

	Controls	AD	Non-AD	MxD	*p*-value
N	**50**	**50**	**41**	**31**	
Gender					
Female	32	19	10	12	0.001
(%)	64	38	24	39	
Disease duration					
Mean (n); years		5 (10)	8 [c] (10)	4 (8)	0.012
SD		2.9	8.1	2.6	
Age at LP					
Mean (n); years	62	74 [a*, b*, c]	66 [c*]	80 [a*]	<0.001
SD	5.7	8.4	10.8	7.1	
MMSE					
Mean (n)	29 (6)	14 [a*] (15)	17 [a*] (8)	16 [a*] (11)	<0.001
SD	1.2	8.0	8.9	8.3	
***APOE* ε4**					
Non-carrier	31	18	21	11	0.200
Carrier	18	21	9	9	
TLPD					
Mean (n); years		1 (1)	2	1 (1)	0.138
SD		1.5	1.6	1.3	
$A\beta_{1\text{-}42}$					
Mean; pg/ml	1383	475 [a*, b*]	903 [a*, c*]	573 [a*]	<0.001
SD	441	206	393	269	
$A\beta_{1\text{-}40}$					
Mean; pg/ml	7187	5056 [a*, c]	5196 [a*]	6336	<0.001
SD	1926	1944	1979	2604	
T-tau					
Mean; pg/ml	390	758 [a*]	613 [a, c]	843 [a*]	<0.001
SD	114	332	314	389	
P-tau_{181}					
Mean; pg/ml	36	109 [a*, b*]	51 [c*]	94 [a*]	<0.001
SD	18	44	42	55	
$A\beta_{1\text{-}42}/A\beta_{1\text{-}40}$					
Mean	0.19	0.09 [a*, b*]	0.18 [c*]	0.10 [a*]	<0.001
SD	0.04	0.02	0.05	0.04	
$A\beta_{1\text{-}42}$/P-tau_{181}					
Mean	49	5.2 [a*, b*]	26 [a*, c]	8.7 [a*]	<0.001
SD	29	3.9	17	7.9	
T-tau/P-tau_{181}					
Mean	12	7.6 [a*, b*]	15 [c]	10	<0.001
SD	4.5	3.2	7.7	4.0	

Legend: Data presented as mean values with respective SD. Missing data is shown between brackets (n). [a] Significantly different from controls; [b] Significantly different from non-AD; [c] Significantly different from MxD; Significance level set at $p < 0.05$, * p-value < 0.001.

Abbreviations: $A\beta_{1\text{-}42}$, amyloid-β peptide of 42 amino acids; AD, Alzheimer's disease; APOE ε4, apolipoprotein gene allele ε4; LP, lumbar puncture; MMSE, mini mental state examination (/30) within 90 days of LP; MxD, mixed pathological diagnosis of AD and non-AD; SD, standard deviation; P-tau_{181}, tau phosphorylated at threonine 181; TLPD, time between LP and death; T-tau, total tau.

Table 6.2 - Demographics and biomarker data of the clinical cohort

	Controls/sMCI	MCI-AD/AD	*p*-value
N	**36**	**34**	
Sex			
Female	21	14	0.232
(%)	58	41	
Disease duration			
Mean (n); years		3 (8)	0.006
SD		1.9	
Age at LP			
Mean (n); years	66	74	<0.001
SD	4.2	6.6	
MMSE			
Mean (n)	29 (3)	23 (3)	<0.001
SD	1.6	4.3	
***APOE* ε4**			
Non-carrier	23	8	0.002
Carrier	13	26	
$A\beta_{1-42}$			
Mean; pg/ml	1433	627	<0.001
SD	459	185	
$A\beta_{1-40}$			
Mean; pg/ml	7815	8264	0.378
SD	2014	2212	
T-tau			
Mean; pg/ml	434	850	<0.001
SD	116	215	
P-tau_{181}			
Mean; pg/ml	41	131	<0.001
SD	16	38	
$A\beta_{1-42}/A\beta_{1-40}$			
Mean	0.19	0.08	<0.001
SD	0.04	0.02	
$A\beta_{1-42}$/P-tau_{181}			
Mean	41	5.4	<0.001
SD	19	2.8	
T-tau/P-tau_{181}			
Mean	11	6.7	<0.001
SD	2.3	1.3	

Legend: Data presented as mean values with respective SD. Missing data is shown between brackets (n). Significance level set at $p < 0.05$.

Abbreviations: $A\beta_{1-42}$, amyloid-β peptide of 42 amino acids; AD, Alzheimer's disease; APOE ε4, apolipoprotein gene allele ε4; LP, lumbar puncture; sMCI, stable mild cognitive impairment; MCI-AD/AD, mild cognitive impairment due to AD and dementia due to AD; MMSE, mini mental state examination (/30) within 90 days of LP; P-tau_{181}, tau phosphorylated at threonine 181; SD, standard deviation; T-tau, total tau.

Table 6.3 - Diagnostic performance of the CSF biomarkers in the autopsy cohort

	Biomarker	AUC	Sensitivity	Specificity	Cut-off	*p*-value
AD vs controls	$A\beta_{1-42}$	0.982 (0.961-1.003)	98	92	827.5	0.256
	$A\beta_{1-40}$	0.783 (0.692-0.873)	58	94	4913	<0.001
	T-tau	0.876 (0.808-0.943)	70	92	536	0.007
	P-tau_{181}	0.950 (0.911-0.990)	90	88	57.5	0.446
	$A\beta_{1-42}/A\beta_{1-40}$	0.966 (0.934-0.997)	96	88	0.143	
	$A\beta_{1-42}$/P-tau_{181}	0.988 (0.972-1.004)	96	96	12.68	0.147
	T-tau/P-tau_{181}	0.882 (0.808-0.957)	82	90	8.97	0.030
AD vs non-AD	$A\beta_{1-42}$	0.829 (0.741-0.917)	78	78	635.5	0.024
	$A\beta_{1-40}$	0.513 (0.393-0.634)	46	66	4368	<0.001
	T-tau	0.639 (0.534-0.754)	62	66	598.5	<0.001
	P-tau_{181}	0.861 (0.779-0.943)	94	68	48	0.061
	$A\beta_{1-42}/A\beta_{1-40}$	0.915 (0.849-0.980)	92	85	0.121	
	$A\beta_{1-42}$/P-tau_{181}	0.922 (0.856-0.989)	96	85	12.25	0.636
	T-tau/P-tau_{181}	0.885 (0.814-0.955)	84	83	9.165	0.450
MxD vs non-AD	$A\beta_{1-42}$	0.764 (0.649-0.878)	77	76	640	0.090
	$A\beta_{1-40}$	0.629 (0.493-0.766)	55	76	6397	0.002
	T-tau	0.692 (0.550-0.835)	78	61	580	0.009
	P-tau_{181}	0.772 (0.643-0.902)	89	68	46	0.078
	$A\beta_{1-42}/A\beta_{1-40}$	0.886 (0.811-0.962)	94	73	0.165	
	$A\beta_{1-42}$/P-tau_{181}	0.858 (0.752-0.964)	89	83	14.21	0.714
	T-tau/P-tau_{181}	0.740 (0.602-0.878)	56	88	8.73	0.039
MxD vs controls	$A\beta_{1-42}$	0.951 (0.898-1.005)	94	92	823.5	0.755
	$A\beta_{1-40}$	0.596 (0.458-0.733)	32	98	4453	0.005
	T-tau	0.917(0.821-1.013)	89	90	527	0.786
	P-tau_{181}	0.881 (0.772-0.990)	72	96	68	0.309
	$A\beta_{1-42}/A\beta_{1-40}$	0.943 (0.898-0.988)	97	80	0.177	
	$A\beta_{1-42}$/P-tau_{181}	0.967 (0.929-1.004)	89	94	14.37	0.061
	T-tau/P-tau_{181}	0.698 (0.524-0.872)	50	98	7.84	0.004
Non-AD vs controls	$A\beta_{1-42}$	0.800 (0.709-0.891)	66	86	1038	0.007
	$A\beta_{1-40}$	0.775 (0.677-0.874)	61	88	5340	0.128
	T-tau	0.754 (0.650-0.858)	54	92	542	0.139
	P-tau_{181}	0.579 (0.458-0.699)	29	96	66.5	0.301
	$A\beta_{1-42}/A\beta_{1-40}$	0.649 (0.535-0.763)	61	70	0.196	
	$A\beta_{1-42}$/P-tau_{181}	0.811 (0.719-0.903)	83	76	35.05	<0.001
	T-tau/P-tau_{181}	0.611 (0.487-0.735)	46	84	14.1	0.698

Legend: Performance of all CSF biomarkers and their ratios in the differentiation between groups in cohort 1 described by an AUC value and respective CI at 95% between brackets as well as by the sensitivity and specificity at a cut-off at maximum sensitivity and specificity (Youden's index). The performance of $A\beta_{1-42}/A\beta_{1-40}$ was compared to all other biomarkers by Delong testing. Significance level set at $p < 0.05$.

Abbreviations: $A\beta_{1-42}$, amyloid-β peptide of 42 amino acids; AD, Alzheimer's disease; AUC, area under the curve; CI, confidence interval; MxD, mixed pathological diagnosis of AD and non-AD.

Table 6.4 - Diagnostic performance of the CSF biomarkers in the clinical cohort

Controls/sMCI vs MCI-AD/AD	Biomarker	AUC	Sensitivity	Specificity	Cut-off	*p*-value
	$A\beta_{1-42}$	0.971 (0.940-1.003)	94	92	989	0.805
	$A\beta_{1-40}$	0.566 (0.428-0.704)	59	69	7835	<0.001
	T-tau	0.965 (0.927-1.003)	93	91	551.5	0.713
	P-tau_{181}	0.988 (0.969-1.006)	89	100	80	0.274
	$A\beta_{1-42}/A\beta_{1-40}$	0.971 (0.941-1.002)	100	86	0.13	
	$A\beta_{1-42}$/P-tau_{181}	0.995 (0.984-1.006)	96	100	9.16	0.106
	T-tau/P-tau_{181}	0.976 (0.946-1.006)	93	94	8.54	0.833

Legend: Performance of all CSF biomarkers and their ratios in the differentiation between groups in cohort 2 described by an AUC value and respective CI at 95% between brackets as well as by the sensitivity and specificity at a cut-off at maximum sensitivity and specificity (Youden's index). The performance of $A\beta_{1-42}/A\beta_{1-40}$ was compared to all other biomarkers by Delong testing. Significance level set at $p < 0.05$.

Abbreviations: $A\beta_{1-42}$, amyloid-β peptide of 42 amino acids; AD, Alzheimer's disease; AUC, area under the curve; CI, confidence interval; sMCI, stable mild cognitive impairment; MCI-AD/AD, mild cognitive impairment due to AD and dementia due to AD.

Supplementary table 6.1 - Biomarker data of the *APOE* ε4 carriers and non-carriers in the autopsy cohort

	***APOE* ε4 non-carrier**	***APOE* ε4 carrier**	***p*-value**
Controls			
$A\beta_{1-42}$	1549 (437)	1118 (304)	0.001
$A\beta_{1-40}$	7332 (2055)	7004 (1756)	0.574
T-tau	372 (92)	413 (143)	0.230
P-tau_{181}	32 (13)	42 (24)	0.112
$A\beta_{1-42}/A\beta_{1-40}$	0.21 (0.02)	0.16 (0.048)	0.001
$A\beta_{1-42}$/P-tau_{181}	55 (26)	41 (32)	0.103
T-tau/P-tau_{181}	13 (4.5)	11 (3.9)	0.297
AD			
$A\beta_{1-42}$	538 (211)	416 (204)	0.077
$A\beta_{1-40}$	5592 (1798)	4584 (2182)	0.128
T-tau	806 (335)	703 (320)	0.336
P-tau_{181}	111 (46)	110 (48)	0.955
$A\beta_{1-42}/A\beta_{1-40}$	0.10 (0.03)	0.09 (0.02)	0.809
$A\beta_{1-42}$/P-tau_{181}	6.3 (5.6)	4.2 (2.3)	0.131
T-tau/P-tau_{181}	7.8 (3.3)	6.8 (2.7)	0.296
non-AD			
$A\beta_{1-42}$	960 (396)	950 (385)	0.949
$A\beta_{1-40}$	5666 (2063)	5054 (2093)	0.464
T-tau	723 (336)	591 (272)	0.309
P-tau_{181}	57 (51)	52 (37)	0.765
$A\beta_{1-42}/A\beta_{1-40}$	0.17 (0.04)	0.20 (0.05)	0.214
$A\beta_{1-42}$/P-tau_{181}	27 (21)	23 (9.5)	0.592
T-tau/P-tau_{181}	17 (9.4)	14 (4.4)	0.302
MxD			
$A\beta_{1-42}$	716 (365)	569 (139)	0.272
$A\beta_{1-40}$	6526 (3036)	7178 (2631)	0.618
T-tau	609 (234)	740 (382)	0.501
P-tau_{181}	64 (29)	64 (24)	0.994
$A\beta_{1-42}/A\beta_{1-40}$	0.12 (0.04)	0.09 (0.04)	0.130
$A\beta_{1-42}$/P-tau_{181}	13 (8.8)	11 (8.7)	0.764
T-tau/P-tau_{181}	10 (2.5)	12 (4.3)	0.406

Legend: Data presented as mean values and respective SD between brackets, with biomarker unit in pg/ml. Significance level set at $p < 0.05$.

Abbreviations: $A\beta_{1-42}$, amyloid-β peptide of 42 amino acids; AD, Alzheimer's disease; APOE ε4, apolipoprotein gene allele ε4; MxD, mixed pathological diagnosis of AD and non-AD; P-tau_{181}, tau phosphorylated at threonine 181; T-tau, total tau.

Supplementary table 6.2 - Biomarker data of the *APOE* ε4 carriers and non-carriers in the clinical cohort

	***APOE* ε4 non-carrier**	***APOE* ε4 carrier**	***p*-value**
Controls/sMCI			
$A\beta_{1-42}$	1612 (437)	1115 (306)	<0.001
$A\beta_{1-40}$	7823 (2166)	7802 (1797)	0.977
T-tau	403 (92)	494 (137)	0.025
P-tau_{181}	36 (13)	51 (17)	0.006
$A\beta_{1-42}/A\beta_{1-40}$	0.21 (0.02)	0.15 (0.05)	<0.001
$A\beta_{1-42}$/P-tau_{181}	48 (14)	26 (18)	<0.001
T-tau/P-tau_{181}	12 (2.4)	10 (1.8)	0.021
MCI-AD/AD			
$A\beta_{1-42}$	585 (157)	640 (194)	0.472
$A\beta_{1-40}$	7625 (2003)	8460 (2273)	0.358
T-tau	767 (215)	873 (214)	0.294
P-tau_{181}	133 (41)	131 (39)	0.880
$A\beta_{1-42}/A\beta_{1-40}$	0.08 (0.02)	0.08 (0.02)	0.950
$A\beta_{1-42}$/P-tau_{181}	4.7 (2.1)	5.6 (2.9)	0.508
T-tau/P-tau_{181}	5.9 (0.8)	6.9 (1.3)	0.066

Legend: Data presented as mean values and respective SD between brackets, with biomarker unit in pg/ml. Significance level set at $p < 0.05$.

Abbreviations: $A\beta_{1-42}$, amyloid-β peptide of 42 amino acids; AD, Alzheimer's disease; APOE ε4, apolipoprotein gene allele ε4; sMCI, stable mild cognitive impairment; MCI-AD/AD, mild cognitive impairment due to AD and dementia due to AD; P-tau_{181}, tau phosphorylated at threonine 181; T-tau, total tau.

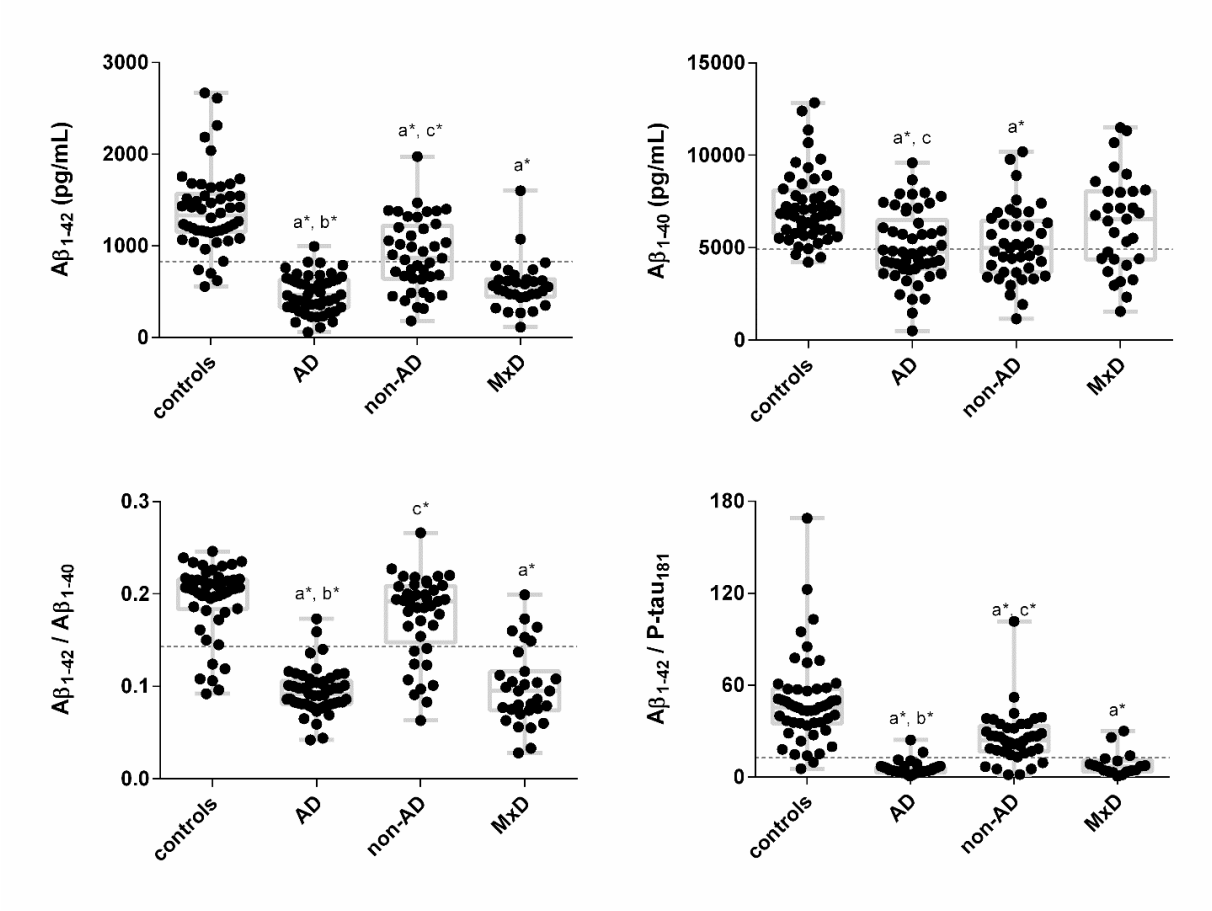

Figure 6.1 - Boxplots of CSF $A\beta_{1-42}$, $A\beta_{1-40}$, $A\beta_{1-42}$/ $A\beta_{1-40}$, and $A\beta_{1-42}$/P-tau_{181} showing the differentiation between controls, AD, non-AD and MxD patients in the autopsy cohort with cut-offs line set at 823 pg/ml, 4884 pg/ml, 0.140, and 11.45 (dotted line), respectively, derived from ROC curve analyses between AD and controls. a Significantly different from controls; b Significantly different from non-AD; c Significantly different from MxD; Significance level set at $p < 0.05$, * $p < 0.001$.

Abbreviations: $A\beta_{1-42}$, amyloid-β peptide of 42 amino acids; AD, Alzheimer's disease; MxD, mixed pathological diagnosis of AD and non-AD; P-tau_{181}, tau phosphorylated at threonine 181; ROC, receiver operating characteristic; T-tau, total tau.

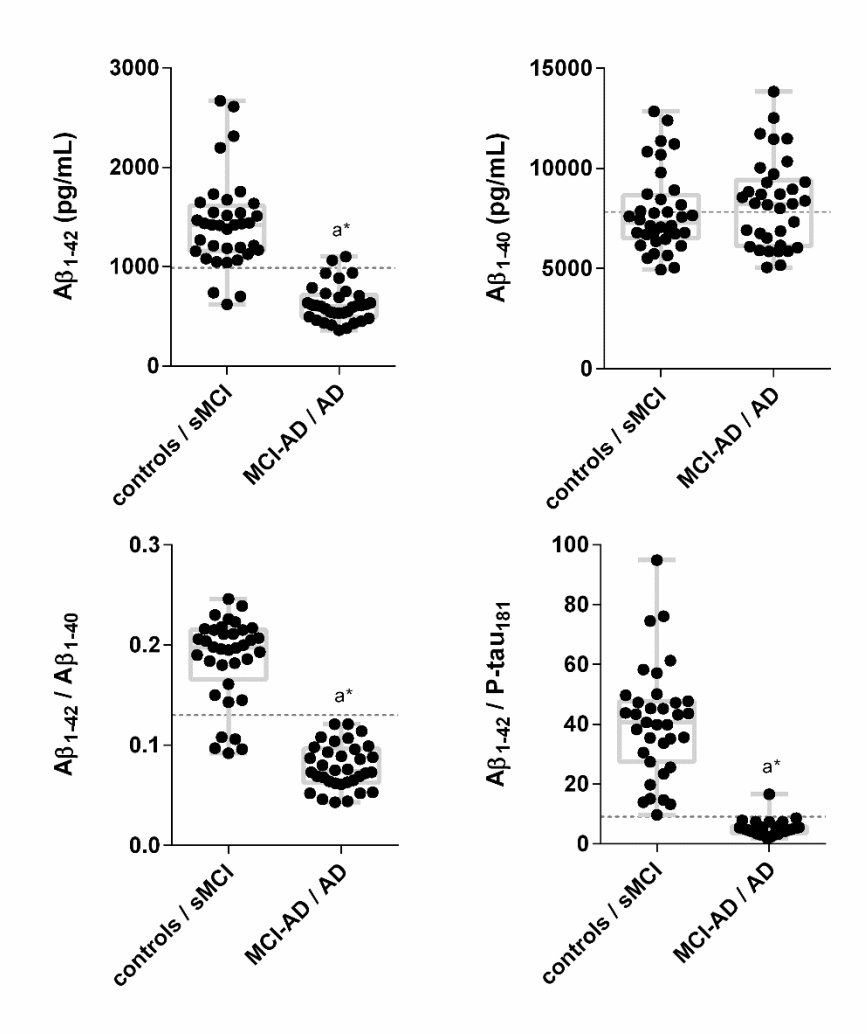

Figure 6.2 - Boxplots of CSF $A\beta_{1-42}$, $A\beta_{1-40}$, $A\beta_{1-42}$/ $A\beta_{1-40}$, and $A\beta_{1-42}$/P-tau_{181} showing the differentiation between controls/sMCI and MCI-AD/AD patients in the clinical cohort with cut-offs set at 938 pg/ml, 7866 pg/ml, 0.121, and 8.58 (dotted lines), respectively, derived from ROC curve analyses between the two groups. a Significantly different from controls; * $p < 0.001$.

Abbreviations: $A\beta_{1-42}$, amyloid-β peptide of 42 amino acids; AD, Alzheimer's disease; sMCI, stable mild cognitive impairment; MCI-AD, mild cognitive impairment due to AD; MxD, mixed pathological diagnosis of AD with non-AD; P-tau_{181}, tau phosphorylated at threonine 181; ROC, receiver operating characteristic; T-tau, total tau.

TOTAL PRION PROTEIN IN CEREBROSPINAL FLUID TO DIFFERENTIATE ALZHEIMER'S DISEASE WITH VERY HIGH T-TAU LEVELS FROM CREUTZFELDT-JAKOB DISEASE

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To be submitted

ABSTRACT

Alzheimer's disease (AD) and Creutzfeldt-Jakob disease (CJD) are both neurodegenerative disorders that are, in their typical forms, clinically distinct. However in daily clinical practice, atypical forms of both diseases show a clinical as well as biochemical overlap that hampers their differentiation. As prion aggregation is the main pathological hallmark of CJD, total prion protein (PrP-t) in cerebrospinal fluid (CSF) may prove to be an additive marker, especially in the differentiation between CJD and AD cases with an atypical presentation of very high CSF total tau protein (T-tau) levels (>1000pg/mL).

Concentrations of amyloid-β peptide with a length of 42 amino acids ($A\beta_{1-42}$), T-tau, tau protein phosphorylated at threonine 181 ($P\text{-}tau_{181}$) and of PrP-t were determined with commercially available single-analyte ELISA kits (INNOTEST®, Fujirebio, and BetaPrion® Human ELISA, Analytik Jena Roboscreen provided by IBL Int., respectively), 14-3-3 protein levels were measured by Western blot analysis and prion detection by real-time quaking-induced conversion (RT-QuIC) was performed. CSF samples of 33 definite AD and 61 definite CJD cases were included. In addition, CSF samples of 15 cognitive healthy elderly controls, 35 clinical AD patients, of which 19 presented with very high T-tau levels (>1000 pg/ml), and 20 clinical CJD patients were included. Samples were either stored in Nalgene of Eppendorf polypropylene tubes.

CSF PrP-t levels were influenced by type of tube as significantly reduced levels were seen in Eppendorf tubes. Measurements on CSF stored in Nalgene tubes showed increased PrP-t levels in clinical, high T-tau, AD cases compared with definite CJD cases. The use of CSF PrP-t showed a diagnostic performance with an area under the curve (AUC) of 0.791 for the differentiation of definite AD from definite CJD and a higher AUC (0.913) for the differentiation of clinical high T-tau AD from definite CJD. Comparing the diagnostic performance of PrP-t with other CSF biomarkers used for CJD diagnosis, including 14-3-3 protein, PrP RT-QuIC, and $T\text{-}tau/P\text{-}tau_{181}$, showed $T\text{-}tau/P\text{-}tau_{181}$ to outperform all other markers.

CSF PrP-t has shown its value in the biomarker-based differential diagnosis of AD, the more so in the specific scenario of differentiation between atypical forms of AD with very high T-tau levels and CJD, but has no added value to the current core AD or CJD CSF biomarkers as it was outperformed by the tau ratio as well as the PrP RT-QuIC assay.

AD and CJD are both neurodegenerative disorders that are, in their typical forms, clinically distinct. Typical AD cases are defined by a more gradual progressive cognitive decline, predominantly in memory function, resulting in an impairment of daily functioning. Typical cases of CJD are characterized by a rapid progressive cognitive decline and pyramidal and extrapyramidal signs and symptoms may occur as well as myoclonus, ataxia, and visual symptoms. An atypical presentations of AD may also involve a rapid disease evolution[232] and CJD may present with longer disease duration or without their characteristic symptoms[233]. These overlapping clinical features hamper the differentiation of the disorders[234, 235], which is critical for counselling and treatment purposes and will become of even more importance once disease-modifying treatments become available. Furthermore, correct diagnosis is of great concern to prevent iatrogenic transmission in case of CJD.

During the past decades, biomarkers have been introduced as a biochemical support for the diagnosis of both AD and CJD[105]. Decreased CSF levels of the amyloid-β peptide with a length of 42 amino acids ($A\beta_{1-42}$) in combination with increased levels of T-tau and/or P-tau_{181} characterize an AD biochemical profile[55]. The AD CSF biomarker panel has been shown to have a high diagnostic accuracy of 93.9% (sensitivity of 100% and specificity of 90.7%) for the detection of definite AD amongst cognitively healthy elderly, while an approximately 10 % lower accuracy was found for the differentiation between definite AD and non-AD (sensitivity of 90% and specificity of 93%)[39]. The decrease in accuracy between AD and other neurodegenerative diseases may be assigned to a partial overlap in the biomarkers. The CSF $A\beta_{1-42}$ is the least specific biomarker for AD[213] and its levels in CJD have been found to vary greatly [236–242]. Although no correlation with number of Aβ plaques has been elucidated in CJD, age-related amyloid co-pathology and a pathogenic interaction between $A\beta_{1-42}$ and prion protein (PrP) have both been suggested to lead to its decreased levels in CSF[242–244]. Furthermore, though the CSF T-tau concentration is distinctively elevated in AD as a reflection of neuronal damage, it has for the same physiological reason also been found to be increased in CJD. However, as the levels of T-tau have been shown to be extreme in CJD (>1000 pg/mL), Tau has previously been investigated for its diagnostic accuracy to differentiate CJD from non-CJD disorders. Due to its disease unspecific overlapping nature the sensitivity and specificity ranges from moderate to high values of 87-94% and 75-100%, respectively[80, 237, 242, 245, 246]. To further improve the diagnostic accuracy for CJD, the 14-3-3 protein is currently utilized as a biomarker. It has been shown that the 14-3-3 protein has a sensitivity of 89-100% and specificity of 78-92% for the differentiation of CJD versus non-CJD[80, 245–249] with findings of positive 14-3-3 testing in AD patients[248–250].Though the biomarker accuracy may seem satisfactory for typical cases of AD and CJD, it still needs improvement for atypical forms of the disorders to aid in the clinical setting. Atypical presentations of AD with a rapid disease evolution[232] may also present with very high CSF T-tau levels indicating severe neuronal degeneration[232, 251] and/or positive 14-3-3 immunotests mimicking CJD[240, 252–255]. In addition, normal CSF levels of T-tau have also been found in CJD[247] and amyloid co-

pathology may cause the CSF $A\beta_{1-42}$ levels to be decreased[162, 236–238, 256]. Besides the overlap in core AD CSF biomarkers, 14-3-3 immunoblot testing may be negative in CJD[240, 247, 252, 257] and of weak positive results less than 50% has proven to be CJD[249]. These false weak positive cases may, besides AD, be afflicted with herpes simplex encephalitis, metabolic and hypoxic encephalopathy, cerebral metastases, or paraneoplastic disease[258, 259]. As the 14-3-3 protein is a surrogate marker of neuronal damage and unspecific for CJD, just like T-tau, new CSF biomarkers that reflect the CJD neuropathology of PrP aggregates in the brain has been investigated. The prion protein is under normal physiological conditions abundantly expressed in neurons as a cell-surface glycoprotein (PrP^{C}). However, in CJD the PrP^{C} undergoes post-translational conversion, resulting in a misfolded, pathogenic scrapie isoform (PrP^{Sc}) which is prone to aggregation. The PrP^{Sc} facilitates disease proliferation through self-propagation by binding to the PrP^{C} to induce further conformational changes and continuous PrP^{Sc} accumulation[260]. One technique that utilizes the conversion of (recombinant) PrP into aggregates seeded by small amounts of PrP^{Sc} present in the CSF, is the real-time quaking-induced conversion (RT-QuIC) assay[261]. This aggregation assay has been shown in several studies to have a sensitivity and a specificity of 85-87% and 99%-100%, respectively, for the detection of CJD against non-CJD disorders[261–264]. However, the RT-QuIC assay is both time-consuming and costly, and does not give a quantitative measurement value.

The addition of a comparably non-expensive, time efficient and quantitative CJD-specific biomarker may have an added value to the current AD CSF biomarker panel. As prion aggregation is the main pathological hallmark of CJD, measuring total prion protein (PrP-t) in CSF may provide a surrogate marker similar to CSF $A\beta_{1-42}$ for brain amyloidosis[241, 265]. We set out to validate a PrP-t ELISA in a clinical and autopsy confirmed cohort with AD and CJD patients. The second aim was to compare its performance with the existing 14-3-3 and RT-QuIC assays for the detection of CJD in the definite cases as well as in clinical atypical AD patients with very high CSF T-tau levels (>1000pg/mL). An assessment of the accuracy of the combination of CSF P-tau_{181}, which has proven to be the most specific biomarker for AD[76], and T-tau as a biomarker for CJD compared with the 14-3-3 protein and the prion related assays was also performed with the hypothesis that the Tau ratio performs equally well with the RT-QuIC assay to distinguish definite and atypical AD from definite CJD.

MATERIALS AND METHODS

Study population

In this retrospective study, a cohort containing CSF samples from patients with dementia with a definite diagnosis of AD (n=33) or a definite diagnosis of CJD (n=61) were selected. Neuropathological examination was performed by two neuropathologists (JJM and AS). Definite diagnosis of AD was established by staging of AD neuropathological changes using the Montine criteria[19]. For definite diagnosis of CJD, the criteria of Markesbery[87] were applied.

In addition, CSF samples from clinical probable AD (n=35) and probable CJD (n=20) patients were included, as well as cognitively healthy elderly controls (n=15). Patients with AD were diagnosed by applying the NIA-AA criteria[4] and were hereafter selected based on a positive CSF biomarker profile, being suggestive for AD[55]. A positive CSF biomarker profile was defined by decreased $A\beta_{1-42}$ levels (<638.5 pg/mL) in combination with increased levels of T-tau (>296.5 pg/mL) and/or P-tau_{181} (>56.5 pg/mL) using cut-offs previously determined in an autopsy-confirmed cohort[39, 83]. Of the 35 clinically diagnosed AD cases, 15 had a typical CSF biomarker profile suggestive of AD with CSF T-tau levels up to 1000 pg/ml and 20 had a CSF biomarker profile suggestive for AD, but with very high CSF T-Tau levels exceeding 1000 pg/ml. All clinical CJD patients met the clinical diagnostic criteria[266], including 14-3-3 positivity on western blot immunoassay[82]. Controls were included based on following criteria: (1) no neurological or psychiatric antecedents and (2) no organic disease involving the central nervous system following extensive clinical examination. The control group consisted of patients with disorders of the peripheral nervous system (polyneuropathy; n=2), patients with urological disorders that underwent spinal anaesthesia during surgical procedure (n=9), patients with tension headache (n=2) and patients with subjective complaints who had an extensive clinical work-up to rule out disorders of the central or peripheral nervous system (n=2).
All samples were selected from the biobank of the Institute Born-Bunge (Antwerp, Belgium). The patients were recruited at the Memory Clinic of the Hospital Network Antwerp, Middelheim and Hoge Beuken (Antwerp, Belgium) and in referring centres between 1995 and 2017.

CSF sampling and handling

All CSF samples were collected according to a standard collection protocol as previously described[105]. CSF was obtained into polypropylene tubes (Nalgene (n=76) or Eppendorf (n=88)) by lumbar puncture at the L3/L4 or L4/L5 interspace. Samples were frozen immediately and shipped on dry ice to the lab or shipped unfrozen within 24 hours at room temperature. After biomarker analyses, samples were stored at -80°C.

CSF biomarker analysis

The CSF concentration of $A\beta_{1-42}$, T-tau, and P-tau_{181} was determined with commercially available single-analyte ELISA kits (INNOTEST® β-AMYLOID($_{1-42}$), INNOTEST® hTAU Ag, and INNOTEST® PHOSPHO-TAU($_{181P}$), respectively; Fujirebio Europe, Ghent, Belgium) according to manufacturer's instructions. Calibrators, QC and CSF samples were routinely analysed in duplicates and the CVs for the CSF samples were below 15% for all of the markers. The analytical ranges of the assays are described in the package inserts ($A\beta_{1-42}$: 125-2000 pg/mL, T-tau: 75-1200 pg/mL, P-tau_{181}: 15.6-500 pg/mL). Out of range values were not re-measured for the purpose of this study, in order to mimicking a clinical biomarker routine setting. Instead, patients with values exceeding the upper limit of quantification were assigned the highest calibrator value, which in the case of T-tau is 1200 pg/mL. Analyses of

14-3-3 protein in CSF by Western blot immunoassay and prion protein aggregation by RT-QuIC were routinely performed, as previously described in detail[80, 267]. To be regarded as a positive result, the RT-QuIC reaction had to be positive in at least two out of four analyses. CSF PrP-t concentration was determined using commercial BetaPrion Human ELISA Test kits (BetaPrion® Human ELISA; AJ Roboscreen GmbH, Leipzig, Germany; provided by IBL International). Calibrators, QC and CSF samples were analysed in duplicates and the intra- and inter-assay CVs were less than 10%.

Statistical analyses

Statistical analyses were performed using SPSS statistical package of IBM Statistics, version 24, and Graphpad Prism, version 6. Results were reported as median values with IQR. Due to lack of normal distribution and small sample size in some of the groups, non-parametric testing was used. Chi-square testing was performed for gender. To compare clinical and biomarker data, Kruskal-Wallis was used for comparisons across several groups (n>2), while Mann-Whitney U test (adjusted for multiple comparisons (Bonferroni) for biomarker comparisons) was used to asses pairwise comparisons. With MedCalc statistical software, version 18.9, ROC curve analysis was performed to obtain the AUC values and 95% CI to evaluate diagnostic performance. Sensitivity and specificity percentages were determined at the maximal sum of sensitivity and specificity by use of the Youden's index. Delong testing was performed to compare the AUC of different variables. The level of significance was set at p-values below 0.05 for all of the tests.

RESULTS

Effect of storage tubes on CSF PrP-t concentration

Due to the retrospective approach of this study, CSF had been collected and stored in different tubes (polypropylene Nalgene or Eppendorf) due to different analytical protocols at the referring hospitals. As the clinical cohorts suffered from a bias with regard to the tubes the CSF was collected in (all suspected AD patients had their CSF collected in Nalgene tubes, while CSF from suspected CJD patients was collected in Eppendorf tubes), the possible tube effect was investigated separately in the autopsy confirmed cohorts (table 7.1 and figure 7.1). In both the definite AD cohort and the definite CJD cohort significant differences were found between the CSF PrP-t levels in Nalgene compared with the levels in Eppendorf tubes (p<0.001). Both the AD and the CJD definite patients samples exhibited PrP-t values within the measurable analytical range for CSF stored in Nalgene, while 36% of the AD and 44% of the CJD samples were below the lowest limit of quantification when the CSF was stored in Eppendorf tubes. Based on the PrP-t results, the discrimination between the AD and CJD patients is less reliable when CSF is stored in Eppendorf tubes. This is reflected by significantly lower levels of PrP-t in CJD compared with AD when CSF was stored in Nalgene tubes (p<0.05) and barely significantly lower concentration in CJD (p<0.05) when CSF was

stored in Eppendorf tubes. Consequently, subsequent statistical analyses were only performed in the cohort for which the CSF has been collected and stored in Nalgene tubes. All of the samples from the clinical CJD patients were collected in Eppendorf tubes; therefore, the whole cohort was excluded from further analyses.

Study population demographics

The demographical data is shown in table 7.2. No significant distribution differences were observed for gender. The clinical AD patients, both the typical and the very high T-tau cases, were significantly older than the controls and definite CJD patients (p<0.05 for both). The definite AD patients had significant longer TLPD compared with definite CJD patients (p<0.05; medians of 0.5 and 0.1 year for AD and CJD respectively).

Core AD CSF biomarker performance

The biomarker results are presented in table 7.2. The CSF $A\beta_{1-42}$ levels were significantly lower in clinical, both typical and very high T-tau patients, and definite AD patients compared with controls (p<0.001) and definite CJD patients (p<0.05).
A high proportion of the patients (60% of clinical AD with very high T-tau, 36% of definite AD, and 80% of definite CJD) were assigned the highest calibrator value for CSF T-tau, which was set as the upper limit of quantification (1200 pg/ml). Increased CSF T-tau levels compared with controls were found for clinical AD patients, only for high T-tau cases (p<0.001), definite AD patients (p<0.05), and definite CJD patients (p<0.001). The clinical, typical AD cases had significant lower CSF T-tau levels than the clinical high T-tau AD cases and definite CJD patients (both p<0.05).
The CSF P-tau_{181} concentration was significantly higher in clinical, both typical and high T-tau cases (p<0.05 and p<0.001, respectively), and in definite AD patients (p<0.05) compared with controls, as well as higher levels in clinical AD patients, only for high T-tau cases (p<0.05), compared with CJD. Of the clinical AD patients, high T-tau cases had significantly higher P-tau_{181} levels compared with typical cases (p<0.05).

PrP-t performance

The CSF PrP-t concentration was significantly increased in clinical AD patients, only in the high T-tau cases, compared with definite CJD patients (p<0.001) (table 7.2 and figure 7.2A). Establishing a cut-off (234 ng/ml) between controls and definite CJD would mean that 95% of the clinical high T-tau AD patients have normal PrP-t levels, while 5% would have an abnormal PrP-t concentration.

Core CJD biomarker performance

In clinical AD patients with high T-tau, none had a positive 14-3-3 immunoblot, while in definite AD cases 36% were positive. In definite CJD, 53% were positive. None of the clinical

high T-tau AD patients were positive for RT-QuIC, nor any of the definite AD cases. In the definite CJD patient group, 86% were positive for RT-QuIC. Assessments of 14-3-3 and RT-QuIC were not performed in controls nor in the clinical typical AD patients.

Tau ratio performance

The CSF tau ratio was significantly increased in clinical AD patients, both in the typical AD as well as in the high T-tau cases, compared with controls ($p<0.05$) (table 7.2 and figure 7.2B). No significant increase was found in definite AD compared with controls. The tau ratio was significantly increased in definite CJD compared with both clinical, typical ($p<0.05$) as well as high T-tau cases ($p<0.001$), and definite AD patients ($p<0.05$) as well as controls ($p<0.001$). Establishing a cut-off (9.9) between controls and definite CJD would mean that 85% of the clinical high T-tau AD patients have normal tau ratio values, while 15% would have an abnormal tau ratio.

Correlation between biomarkers

No correlations were found between PrP-t and the core AD CSF biomarkers in the control group. In the clinical and definite AD group, PrP-t correlated significantly with T-tau ($\rho=0.433$ with $p<0.05$ and $\rho=0.4637$ with $p<0.05$, respectively) as well as with P-tau$_{181}$ ($\rho=0.478$ with $p<0.05$ and $\rho=0.645$ with $p<0.05$, respectively). In the definite CJD group, PrP-t correlated significantly only with P-tau$_{181}$ ($\rho=0.543$ with $p<0.05$).

Comparison of biomarker diagnostic performance

Differentiating between definite AD and definite CJD patients based on CSF PrP-t levels rendered an AUC of 0.764 with a high sensitivity (91%) and a low specificity (53%). An AUC of 0.913 was obtained for the distinction between clinical high T-tau AD patients and definite CJD with a high sensitivity (85%) and a high specificity (87%) (table 7.4).
For the differentiation between definite AD and definite CJD, 14-3-3 resulted in a low AUC of 0.585 and a low sensitivity (64%) and specificity (53%). The AUC increased to 0.767 for the separation between clinical AD patients with high T-tau and definite CJD, the sensitivity was high (100%) but the specificity remained low (53%) (table 7.4).
Based on RT-QuiC, an AUC of 0.929 with a high sensitivity (100%) and high specificity (86%) was obtained for the discrimination between definite AD and definite CJD as well as for the discrimination of between clinical AD patients with high T-tau and definite CJD.
For the differentiation based on the T-tau/P-tau$_{181}$ ratio, an AUC of 0.988 was acquired accompanied by a high sensitivity (91%) and high specificity (100%) for the differentiation between definite AD and definite CJD. The AUC remained high (0.957) with a high sensitivity (85%) and a high specificity (100%) for the distinction between clinical AD patients with high T-tau and definite CJD.
In differentiating between definite AD and definite CJD, the tau ratio had a significant higher AUC value compared with PrP-t ($p<0.05$) and 14-3-3 ($p<0.001$), but not compared with RT-

QuIC. The AUC value of PrP-t did not significantly differ from AUC values of 14-3-3 and RT-QuIC, although the latter had a significantly higher AUC value compared with 14-3-3 (p<0.001).
For differentiating between clinical high T-tau AD patients and definite CJD PrP-t, RT-QuIC, and the tau ratio performed equally well. RT-QuIC and the tau ratio had a significant higher AUC value compared with the AUC of 14-3-3 (p<0.05 for both).

DISCUSSION

In the present study, CSF PrP-t was assessed for its diagnostic value for the differential diagnosis of AD, especially to distinguish between AD patients presenting with very high T-tau levels (>1000 pg/mL) and CJD patients that often exhibit high T-tau levels. In this specific scenario, CSF PrP-t demonstrates a high discriminative power. However, both RT-QuIC and the T-tau/P-tau$_{181}$ ratio perform with a higher diagnostic accuracy to discriminate between clinically diagnosed AD patients with high T-tau levels and definite CJD, as well as to discriminate definite AD from definite CJD.
Two earlier studies have been conducted on the diagnostic value of CSF PrP-t, which demonstrated similar results for the differentiation between clinical AD (typical as well as atypical clinical presentations of AD) and CJD and between autopsy-confirmed AD and CJD subjects [241, 265]. Our study partly confirms their findings. In our cohort, a decreased PrP-t concentration was found in definite CJD compared with AD, although this was only observed in the clinical AD cohort and could solely be attributed to the AD patients with high CSF T-tau levels. Furthermore, while the previous studies reported a decreased CSF PrP-t concentration in CJD compared with controls, we could not confirm this finding in our study. Previous studies also reported AUC values for the differentiation between AD and CJD that were slightly higher (0.825 and 0.886) than in our study (0.764). The most pronounced difference is a seemingly larger overlap in the PrP-t levels between our definite CJD subjects and our controls. One explanation for the diverging results could be that in our study we controlled for tube type by excluding further analyses on CSF originating from Eppendorf tubes. We could see that the different tubes had a major effect on the results on PrP-t, rendering much lower concentration values for CSF originating from Eppendorf tubes compared with Nalgene. As previous studies do not mention tube type, no conclusion can be drawn on how much of the lower levels in CJD could be due to the used tubes as samples originating from different centres were included. Given that PrP-t could have similar binding properties as A$\beta_{1\text{-}42}$, which would not be unlikely given their known interactions for which evidence has been provided by several studies [268, 269], it may be possible that PrP is adsorbed in the same way to certain types of tube materials in different degrees [100, 270], making tube standardization for PrP-t measurement of critical importance. Another plausible explanation for the PrP-t overlapping levels in several of our definite CJD subjects is that it could be caused by a lower seeding activity in CJD subjects with longer disease durations [267, 271], data that was not available for our study, which may possibly contribute to slightly higher PrP-t levels.

CJD is characterized by rapid progressive neurodegeneration leading to an excessive release of neuronal proteins such as T-tau and PrP-t into the interstitial fluid. However, in the case of CJD the PrP-t levels likely reflects the PrP^{C} conversion into abnormal pathological PrP^{Sc} and possibly reflects the burden of the deposition in the brain, in a similar fashion as CSF $A\beta_{1-42}$ in AD[260]. Neuronal damage in AD could drive the same release of PrP-t as T-tau into CSF, and without any subsequent modifications that would lead to aggregation, resulting in elevated levels of PrP-t in AD. The finding of significantly higher CSF PrP-t levels in clinical AD cases with very high T-tau levels support this theory as well as the link between the findings of CSF PrP-t and neuronal degeneration in AD, represented by both CSF T-tau and P-tau_{181}, which were found to moderately correlate with the levels of PrP-t in AD subjects, the more so when the diagnosis was definite. In one study, significantly increased PrP-t levels were shown in AD, both typical and atypical, compared with controls, possibly reflecting differences in patient populations compared with our cohort[241]. Another study has shown decreased PrP-t levels in AD compared with controls, which was attributed to the lack of specificity of the assay[272]. What the contribution was of pre-analytical confounders (such as effect of storage tubes) in these previous studies remains unknown and to be investigated. Of the core CJD biomarkers, our results showed 14-3-3 to be an unspecific marker, with specificity of 64% for CJD diagnosis, and RT-QuIC to be a highly specific marker, with specificity of 100% for CJD diagnosis, which is in agreement with previous studies[262, 273–275]. Besides its high specificity, sensitivity of RT-QuIC reaches 86%. Variances in prion pathology have been brought forward between different subtypes of CJD[276], of which some subtypes were not able to be picked up by RT-QuIC[242, 277]. Seeking out specific subtypes with differently altered PrP-t levels by histotype classification may have improved segregation from AD even further, however, explanations for the variability of seeding activity are not yet well determined. Latest setbacks in clinical trials for development of AD therapeutics increase the search for new potential drug target candidates. As PrP^{C} has been a protein of interest [278], the use of PrP-t ELISA analysis may aid further characterization and serve as outcome measure in clinical trials of possible drug candidates targeting PrP.

Due to an imbalance in the activity of cellular kinases and phosphatases, hyperphosphorylation of tau is typically seen in AD. Remarkably, P-tau_{181} levels in definite CJD did not seem to differ significantly from levels in definite AD and clinical typical AD, as CJD presented with levels above the clinical routine cut-off value (56.5 pg/ml). Although relatively low levels of P-tau_{181} generally depict CJD [241, 279, 280], other studies have shown significantly increased P-tau_{181} levels [242, 281, 282]. It has also been demonstrated that elevated CSF P-tau_{181} levels correlate with prion-specific P-tau containing deposits as well as with co-morbid AD related neurofibrillary pathology in certain subtypes of CJD [242, 283, 284]. As P-tau_{181} is normally the most specific marker for AD[76] and by combining it in a ratio with T-tau, which is especially increased in CJD, an increased diagnostic accuracy can be achieved for the discrimination between CJD and AD. We could show that even when the T-tau levels exceeded the valid measurement range of the analytical kits and the concentration of the samples was set to the highest limit of quantification, the diagnostic accuracy of the tau ratio was still high. This result is supported by earlier findings[76]. Comparing the diagnostic performance in our study to a previous study, wherein the high T-

tau samples were diluted to obtain an absolute value, it can be concluded that the diagnostic performance was highly comparable and thus that it remains highly accurate even without the dilution and the absolute analytical value[76, 279]. Furthermore, it has been shown that it is analytically more robust using biomarker ratios for markers that are affected by (pre)analytical factors such as adsorption to tube materials[270, 285].
The observation of significantly reduced PrP-t levels in CSF stored in polypropylene Eppendorf tubes was an unexpected finding, since polypropylene has been proven to show less adsorption in the case of $A\beta_{1-42}$, and highlights the importance of standardization. This reduced our sample size considerably, however, the inclusion of definite diagnosis provides a high reliability of our results.

CONCLUSION

CSF PrP-t has shown its value in the biomarker-based differential diagnosis of AD, the more so in the specific scenario of differentiation between atypical forms of AD with very high T-tau levels and CJD, but has no added value to the current core AD or CJD CSF biomarkers as it was outperformed by the tau ratio as well as the PrP RT-QuIC assay.

Table 7.1 - Tube type distributions of the study population

	Control	AD				CJD	
		Clinical			Definite	Clinical	Definite
		Total	Typical	Very high T-tau			
N	15	35	15	20	33	20	61
Tube							
Nalgene (n)	15	35	15	20	11		15
Eppendorf (n)					22	20	46

Legend: Data presented as median values with 25th and 75th quartiles between brackets (IQR).

Abbreviations: AD, Alzheimer's disease; CJD, Creutzfeldt-Jakob disease; IQR, Inter Quartile Range; MMSE, Mini-Mental State Examination; TLPD, time between lumbar puncture and death.

Table 7.2 -Demographics and biomarker results in patients with CSF stored in Nalgene tubes

	Control	AD				CJD
		Clinical			Definite	Definite
		Total	Typical	Very high T-tau		
N	15	35	15	20	11	15
Gender						
Female	7	19	8	11	6	9
(%)	(47)	(54)	(53)	(55)	(55)	(60)
Age						
Median; years	66	81 [a, b*]	82 [a, b]	81 [a, b]	73	68
(IQR)	(60-74)	(74-85)	(74-85)	(75-85)	(67-75)	(64-72)
TLPD						
Median; years					0.5 [b]	0.1
(IQR)					(0.1-1.3)	(0.0-0.2)
$A\beta_{1-42}$						
Median; pg/ml	849	431 [a*, b]	431 [a*, b]	434 [a*, b]	404 [a*, b]	673
(IQR)	(713-1010)	(338-508)	(306-527)	(339-504)	(268-496)	(537-941)
T-tau						
Median; pg/ml	227 [b*]	1039 [a*]	459 [b, c]	1200 [a*]	811 [a]	1200 [c]
(IQR)	(190-236)	(503-1200)	(418-633)	(1061-1200)	(428-1200)	(1200-1200)
$P\text{-}tau_{181}$						
Median; pg/ml	39.8	111 [a*, b]	60.0 [a, c]	149.9 [a*, b]	87.9 [a]	58.2
(IQR)	(31.5-42.8)	(60.0-156.0)	(53.0-90.0)	(119.5-68.3)	(55.0-151.1)	(47.2-73.5)
PrP-t						
Median; ng/ml	334	452 [b*]	385	496 [b*]	403	234
(IQR)	(296-399)	(307-574)	(227-545)	(409-651)	(289-533)	(114-313)
14-3-3						
Positive/total				0/20	4/11	8/15
(%)				(0)	(36)	(53)
RT-QuIC						
Positive/total				0/20	0/9	12/14
(%)				(0)	(0)	(86)
$T\text{-}tau/P\text{-}tau_{181}$						
Median; pg/ml	5.6 [b*]	7.7 [a, b*]	7.5 [a, b]	7.7 [a, b*]	7.9 [b]	20.3
(IQR)	(5.1-6.0)	(6.7-9.3)	(6.6-9.8)	(7.1-9.2)	(6.6-8.6)	(15.4-24.5)

Legend: Data presented as median values with 25th and 75th quartiles between brackets (IQR). [a] Significantly different from controls; [b] Significantly different from definite CJD; [c] Significantly different from clinical, typical AD; [*] p-value < 0.001.

Abbreviations: $A\beta_{1-42}$, amyloid-β peptide of 42 amino acids; AD, Alzheimer's disease; CJD, Creutzfeldt-Jakob disease; IQR, Inter Quartile Range; MMSE, Mini-Mental State Examination; PrP-t, total prion protein; $P\text{-}tau_{181}$, tau phosphorylated at threonine 181; TLPD, time between lumbar puncture and death ; T-tau, total tau.

Table 7.3 - Diagnostic performance of biomarkers in CSF stored in Nalgene tubes

	Biomarker	AUC	Sensitivity	Specificity
Definite AD vs definite CJD	PrP-t	0.764 (0.572 - 0.955)	91%	53%
Clinical high T-tau AD vs definite CJD	PrP-t	0.913 (0.822-1.000)	85%	87%
Definite AD vs definite CJD	14.3.3	0.585 (0.360-0.810)	64%	53%
Clinical high T-tau AD vs definite CJD	14.3.3	0.767 (0.593-0.940)	100%	53%
Definite AD vs definite CJD	RT-QuIC	0.929 (0.814-1.000)	100%	86%
Clinical high T-tau AD vs definite CJD	RT-QuIC	0.929 (0.818-1.000)	100%	86%
Definite AD vs definite CJD	Tau ratio	0.988 (0.957-1.000)	91%	100%
Clinical high T-tau AD vs definite CJD	Tau ratio	0.957 (0.894-1.000)	85%	100%

Legend: AUC values are represented together with 95% CI between brackets for the differentiation of definite AD from definite CJD and of clinical high T-tau AD from definite CJD.

Abbreviations: AD, Alzheimer's disease; AUC, area under the cure; CI, confidence interval; CJD, Creutzfeldt-Jakob disease; PrP-t, total prion protein; RT-QuIC, real-time quacking-induced conversion; T-tau, total tau.

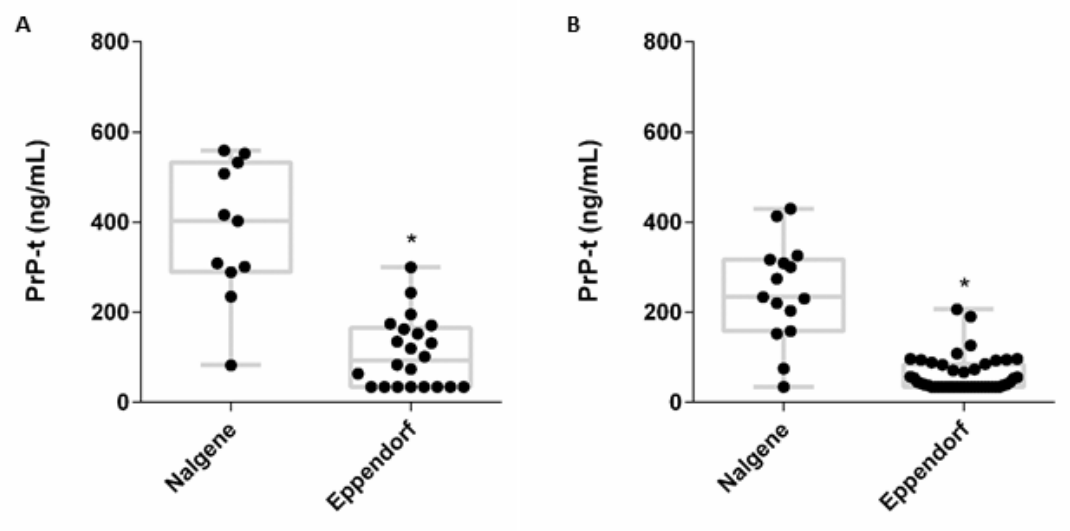

Figure 7.1 - Boxplots comparing the PrP-t levels measured in CSF originating from Nalgene and in Eppendorf tubes for A) the definite AD patients, and B) the definite CJD patients. * Significantly different from levels in Nalgene tubes; p-value < 0.001.

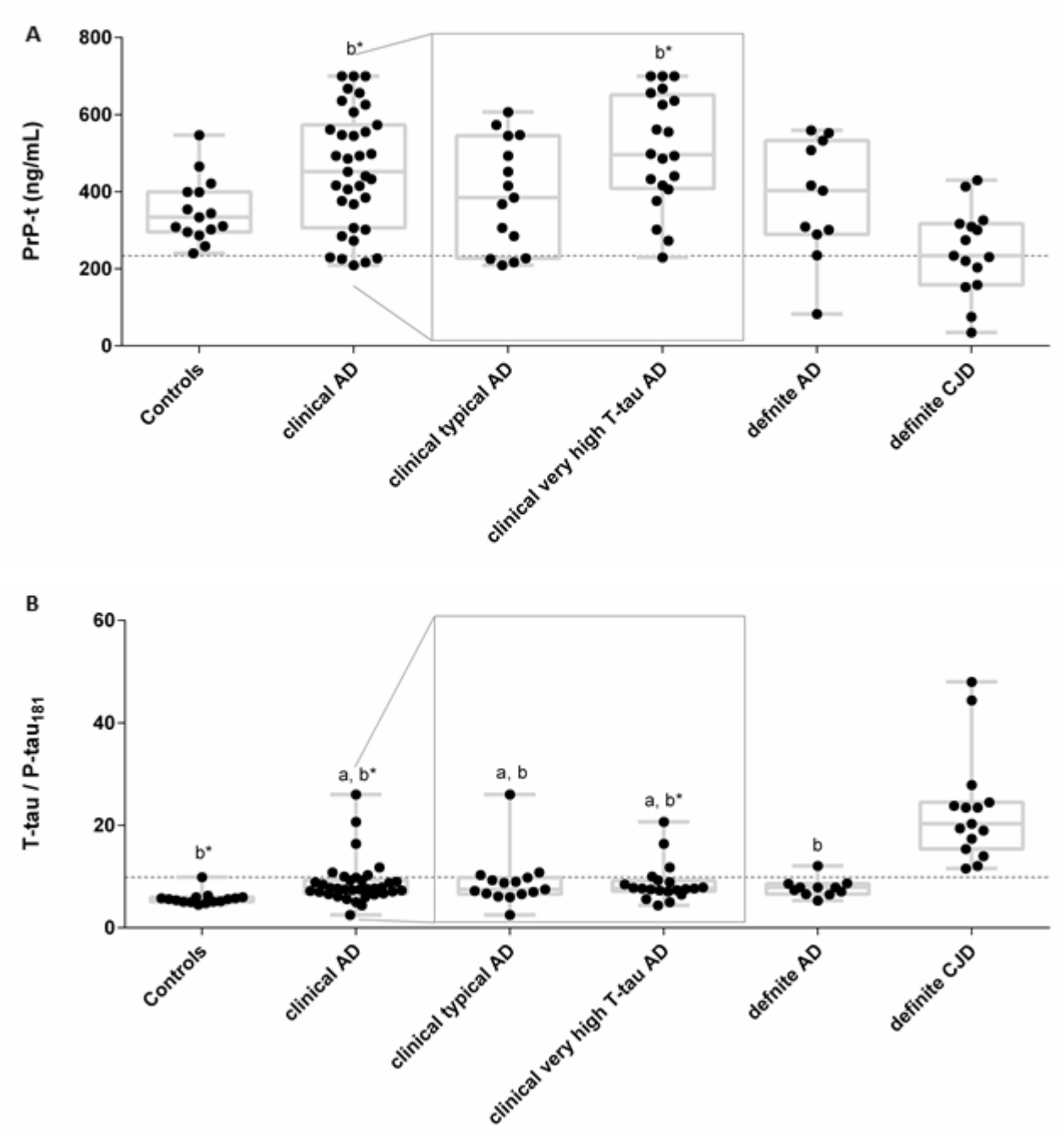

Figure 7.2 - Boxplots of A) the PrP-t CSF levels with cut-off line set at 234 ng/ml (dotted line), and B) the tau ratio with cut-off line set at 9.9 (dotted line) for the differentiation across controls, clinical AD patients, typical as well as high T-tau cases, definite AD patients, and definite CJD patients. [a] Significantly different from controls; [b] Significantly different from definite CJD; * p-value < 0.001.

PART III

HARMONIZATION

VALIDATION OF THE ERLANGEN SCORE ALGORITHM FOR DIFFERENTIAL DEMENTIA DIAGNOSIS IN AUTOPSY-CONFIRMED SUBJECTS

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ABSTRACT

Despite decades of research on the optimization of the diagnosis of Alzheimer's disease (AD), its biomarker-based diagnosis is being hampered by the lack of comparability of raw biomarker data. In order to overcome this limitation, the Erlangen Score (ES), among other approaches, was set up as a diagnostic-relevant interpretation algorithm.

This algorithm has now been validated in a cohort of neuropathologically confirmed AD (*n=106*) and non-AD dementia (*n=57*) cases. Cerebrospinal fluid (CSF) biomarker concentrations of $A\beta_{1\text{-}42}$, T-tau and P-tau_{181} were measured with commercially available single analyte ELISA kits. Based on these biomarkers, ES was calculated as previously reported.

This algorithm proved to categorize AD in different degrees of likelihood, ranging from neurochemically "normal", "improbably having AD", "possibly having AD" to "probably having AD", with a diagnostic accuracy of 74% using the neuropathology as a reference.

The ability of ES to standardize for the high variability of raw CSF biomarker data makes it a useful diagnostic tool for comparing neurochemical diagnoses between different labs or methods used, independently of their specific cut-offs, pre-analytical handling procedures, and applied analytical methods.

INTRODUCTION

AD is one of the most frequently occurring neurodegenerative disorders in the Western population and decades of research on the optimization of AD diagnosis has led to the discovery of a validated CSF biomarker profile that reflects the presence of AD pathology in the brain[39, 220]. This biomarker profile is characterized by lowered CSF levels of $A\beta_{1-42}$ in combination with elevated levels of T-tau and/or P-tau_{181} as described in the IWG-2 criteria and is being used in clinical work-up as well as for research purposes[55, 105]. Although these biomarkers demonstrate sensitivities and specificities of 100% and 91% respectively for confirmation of AD against healthy controls, sensitivity and specificity values still only reach the 80% threshold to differentiate AD against other neurodegenerative disorders (80% and 93%)[39, 76]. The optimization of the stratification of patient populations would benefit the success rate of clinical trials with potential disease-modifying drugs against AD.
However, further improvement of the biomarker-based diagnosis of AD is being hampered by the lack of comparability of raw biomarker data[286]. These raw data are subjected to inter-laboratory variances due to a lack in standardization of sample collection, handling and storage protocols, and due to laboratory-specific cut-off values or different laboratory platforms used[99, 100, 102, 287, 288]. This has already been partially addressed by providing standard operating procedures for pre-analytical sample handling[81] as well as recommendations for analytical processes to improve standardization[103, 289, 290]. Despite these steps, and provided the ongoing evolution in biomarker research, currently used methods and platforms may be modified. Therefore much could still be gained by introducing a diagnostic-relevant interpretation algorithm for raw biomarker data.
Accordingly, the Erlangen Score (ES) was set up and previously validated across different patient cohorts, different pre-analytical operating procedures and different analytical platforms as an algorithm to standardize and improve the biomarker-based diagnosis of AD[227, 291]. In order to further validate the diagnostic utility of this algorithm for its use in differential AD diagnosis, this study with a neuropathologically confirmed cohort of AD and non-AD dementia patients was set up.

MATERIALS AND METHODS

Study population

The study cohort consists of 106 patients with a definite diagnosis of AD and 57 patients with a definite diagnosis of non-AD, all confirmed by post mortem neuropathological examination. Non-AD is defined as clinical dementia with a pathological diagnosis not attributed to AD, consisting of definite FTLD (*n=28*), VaD (*n=13*), LBD (*n=8*), corticobasal degeneration (CBD; *n=1*) or other including hippocampal sclerosis, arteriosclerosis, CAA, and cases without specific neuropathological findings (*n=7*). Definite diagnosis was attained by neuropathological examination of the right hemisphere of the brain, performed at the Institute Born-Bunge (Antwerp, Belgium) by two neuropathologists (JJM and AS). AD

neuropathological changes were scored using the Montine criteria[19], whereas LBD pathology was evaluated using the McKeith classification[223]. Vascular pathology was rated using the Deramecourt criteria[224]. Definite diagnosis of FTLD was established through the criteria of Cairns[84] and Mackenzie[85, 86]. A definite diagnosis of CBD was confirmed by visual assessment of pathological hallmarks of CBD[292].
The study was conducted according to the revised Declaration of Helsinki and good clinical practice guidelines. This study was approved by the ethics committee of UAntwerp, Antwerp, Belgium (B300201420406). Informed consent was obtained from all subjects.

CSF sampling and analysis

All CSF samples were obtained following standard collection protocols as previously described[105]. CSF was collected by LP at the L3/L4 or L4/L5 interspace[81] into polypropylene vials. Samples were either frozen immediately and shipped on dry ice to the BIODEM lab or shipped unfrozen within twenty four-hours after the puncture. Samples were stored at -80°C until analysis.
CSF biomarker concentrations of $A\beta_{1-42}$, T-tau and P-tau_{181} were measured with commercially available single analyte ELISA kits (INNOTEST® β-Amyloid($_{1-42}$), INNOTEST® hTau-Ag, and INNOTEST® PhosphoTau($_{181P}$), respectively; Fujirebio Europe, Ghent, Belgium) following manufacturer's instructions as previously described[39]. The concentration ranges of the test kits, determined as the highest and lowest calibrator concentration, are described in the package inserts ($A\beta_{1-42}$: 125-2000 pg/mL, T-tau: 75-1200 pg/mL, P-tau_{181}: 15.6-500 pg/mL). Interpretation of the biomarker levels was based on cut-offs previously determined in a cohort of autopsy-confirmed AD patients and cognitively healthy elderly[83]. Levels of $A\beta_{1-42}$ <638.5 pg/ml, T-tau >296.5 pg/ ml and P-tau_{181} >56.5 pg/ml were defined as abnormal.

Erlangen Score

The ES was proposed as an algorithm taking into account the core CSF biomarkers, as previously described[291]. The ES suggests a classification into four diagnostic groups. Depending on the pattern of the biomarker alterations, the CSF results of a given patient are scored between 0 and 4 points (figure 8.1). A CSF result with all biomarkers normal is scored 0 points; a pattern with marginal alterations in one biomarkers group (either Aβ or Tau, but not both) results in the score of 1; a CSF result with the alterations in either Aβ metabolism (decreased $A\beta_{1-42}$ concentration or $A\beta_{1-42}/A\beta_{1-40}$ ratio) or tau metabolism (increased concentrations of T-tau and/or P-tau_{181}) but not both is scored 2 points; a result with clear alterations in one biomarkers' group (either Aβ or Tau) accompanied by marginal alterations in the other group is scored 3 points; clear alterations in both Aβ and T-tau/P-tau_{181} result in 4 points.

Statistical analysis

Descriptive statistics on all data were performed using SPSS of IBM Statistics, version 24, with significance level defined as $p < 0.05$. In spite of an adequate sample size, non-parametric testing was selected as the variances across the groups was heteroscedastic. Demographic data and biomarker concentrations were compared between the groups with Mann-Whitney test. To compare gender and APOE genotype distributions, Chi-square test was performed. Logistic regression was then used to model the probability of having AD pathology at the post mortem examination as a function of the ES, whereupon the score was recoded, due to the small number of observations in some categories, into: neurochemically improbable AD (ES=0 or 1, the reference category), neurochemically possible AD (ER=2 or 3), or neurochemically probable AD (ES=4), which is in agreement with the wording in the routine laboratory report presented to clinicians. The model was fitted with maximal likelihood, adjusting for gender (with female as the reference category), age, and the TLPD. After having the model fitted, marginal probabilities, odds ratios to have AD-pathology on neuropathological examination, and the ROC curve were post-estimated. Statistical modelling was performed with Stata 14.2 (StataCorp, College Station, TX, USA).

RESULTS

All demographic data and biomarker concentrations are summarized in table 8.1. Patient groups differed in age at LP ($p<0.001$), but not in gender distribution ($p=0.156$) or TLPD ($p=0.083$). All biomarkers differed significantly between the groups ($p<0.001$). Of the 106 definite AD patients, 69 were classified as neurochemically probable AD (ES=4), 34 as neurochemically possible AD (ES=2 or 3), and 3 as neurochemically improbable AD (ES=0 or 1). On the other hand, 13 of the 57 definite non-AD patients were classified as neurochemically probable AD (ES=4), 32 as neurochemically possible AD (ES=2 or 3), and 12 as neurochemically improbable AD (ES=0 or 1).

The logistic regression model is presented in table 8.2. Compared to the reference category (ES=0 or 1, i. e. neurochemically improbable), both categories, (ES=2 or 3, i. e. neurochemically possible) and ES=4 (i. e. neurochemically probable) were significant positive predictors for the probability of having AD pathology post mortem ($p<0.05$ and $p<0.001$, respectively). Compared to the reference category (ES=0 or 1), the group classified as neurochemically possible AD (ES=2 or 3) had odds 4.22 times greater to have AD pathology on the post-mortem examination, and the group classified as neurochemically probable AD (ES=4) had odds 18.6 times greater. Compared to the neurochemically possible group, the odds of the neurochemically probable group were 4.4 times greater (figure 8.2). Of the explanatory variables, only age showed significant positive effect ($p<0.001$), with every year of age increasing the odds by 8%, while the effect of gender was borderline insignificant ($p=0.054$), and the effect of TLPD was insignificant.

The ROC curve comparing the two groups, post-estimated from the above logistic model, resulted in an AUC of 0.821 [95%CI: 0.750 to 0.893], which was significantly larger ($p<0.05$)

compared to the AUC (0.737 [95%CI: 0.656 to 0.819]) of the ROC curve, resulting from the model with ES as the sole explanatory variable.

DISCUSSION

In order to enable comparison of interpretations of AD biomarkers measurements across laboratories applying different pre-analytical handling procedures, analytical methods, cut-offs or even different sets of the biomarkers, the ES interpretation algorithm was proposed[291] and validated on two large-scale multicentre cohorts[227]. In the current study, the ES algorithm enabled a correct prediction of the post-mortem neuropathological outcome on the ground of the intra vitam CSF results of three core AD biomarkers. The probabilities to have AD pathology post mortem increased almost linearly with increasing ES ordered categories. To this end, the results presented here are entirely in line with the previously published report showing prediction of the disease progression based on the ES outcome[17].

Less than 3% of the neuropathologically definite AD patients (3 out of 106) were categorized as neurochemically improbable AD (ES=0 or 1). Foremost, these patients were in the early stages of AD pathology based on the Montine criteria (data not shown). According to the amyloid cascade hypothesis, the prevailing theory of AD aetiology, $A\beta_{1\text{-}42}$ is attributed a central role as an initiator of AD pathology. This implies that $A\beta_{1\text{-}42}$ is the first biomarker to change in the CSF, before changes reflecting neurofibrillary tangles and neurodegeneration (CSF P-tau_{181} and T-tau) can be detected[155]. Also, borderline values in the "normal" range and relative longer TLPD may have contributed to lower ES than expected. Further, it should be taken into consideration that neuropathological alterations in different areas of the brain are reflected in the CSF to different extents, depending on their distance to the CSF space and the dynamic pathway the molecules need to diffuse to reach the CSF. Yet another potential explanation is that only $A\beta_{1\text{-}42}$ was included in this study as a biomarker of amyloidosis, without considering $A\beta_{1\text{-}42}/A\beta_{1\text{-}40}$, which was unavailable. Therefore, it is plausible to speculate that some cases without alterations in $A\beta_{1\text{-}42}$, and hence interpreted as not having amyloid-related alterations, may have turned into amyloid-positive if $A\beta_{1\text{-}42}/A\beta_{1\text{-}40}$ had been measured[215].

On the other hand, we observed that 23% (13 out of 57) of the definite non-AD patients, which were categorized as neurochemically probable AD (ES=4). This, in turn, is in line with the presence of concomitant AD pathology in non-AD dementia patients, as reported previously[161, 244, 293]. Indeed, many of the non-AD cases in this study that had an ES suggestive for AD pathological findings (n=7), presented with AD-related neuropathological changes that may have had a higher impact than expected. Although these cases seemingly decrease diagnostic accuracy of the CSF biomarkers, and in consequence the ES, their inclusion is most representable for the general population.

P-tau_{181} has previously demonstrated to be the most specific marker for AD, in contrast to T-tau[76, 105, 294], and hence it must be stressed that the current version of the ES, treating

all three (or four) CSF biomarkers equally weighted, shows a considerable limitation from the point of view of specificity, favouring diagnostic sensitivity.
Lack of studies on the harmonization of CSF biomarker interpretation in light of the differentiation of AD against non-AD dementias makes this study potentially interesting particularly in the scenarios where biomarker results must be compared across centres, the more so as a large cohort of neuropathologically confirmed AD and non-AD cases was included.
Despite lack of $A\beta_{1-40}$ results in this cohort, which is probably the strongest limitation of the study, the ES proved to correctly categorize the vast majority of the patients, reconfirming its utility as an interpretation algorithm. As $A\beta_{1-40}$ is the most abundant and stable isoform, its addition obviously further increases the diagnostic performance by eliminating the inter-individual variability of high or low content of total Aβ peptides[93, 172, 213, 215, 217, 295–297] and correcting for other non-AD-specific subcortical changes that may alter the overall Aβ levels in the brain[195].

Conclusion

In light of improving the early differential diagnosis of AD, this validation of the ES demonstrated the categorization of AD and non-AD subjects with reasonable diagnostic accuracy. Its ability to standardize for the high variability of raw CSF biomarker data makes the ES a useful diagnostic tool for comparing neurochemical diagnosis between different labs or methods used, independently of their specific cut-offs.

Table 8.1 - Descriptive table of demographic and biomarker data

	AD	**Non-AD**	**p-value**
N	106	57	
Gender (f/m)	47/59	18/39	0.156
Age at CSF sampling (y)	77 (72-85)	70 (60-76)	<0.001*
TLPD (y)	0.2 (0.1-1.5)	0.7 (0.1-2.1)	0.083
AD suggestive IWG-2 algorithm	84	22	<0.001*
ES			<0.001*
0	2	8	
1	1	4	
2-3	34	32	
4	69	13	
A$\beta_{1\text{-}42}$ (pg/ml)	389 (290-493)	585 (407-774)	<0.001*
T-tau (pg/ml)	570 (361-927)	336 (214-547)	<0.001*
P-tau$_{181}$ (pg/ml)	65.0 (44.6-94.3)	39.0 (27.2-55.2)	<0.001*
***APOE* ε4 (carrier/non-carrier)**	37/37	12/24	0.184

All data are presented as median values and corresponding interquartile ranges between brackets. Significant differences between groups are marked with the level of significance set at a *p*-value below 0.05 (*).

Abbreviations: AD, Alzheimer's disease; ES, Erlangen Score; TLPD, time between LP and death.

Table 8.2 - Logistic regression model of the probability to have AD-pathology on the neuropathologic examination

Predictors	**β**	**Std. Error**	**z**	**p-*value***	**95% CI**
ES (ref. 0 or 1)					
2 or 3	1.439	0.732	1.97	0.049*	0.004 to 2.873
4	2.921	0.757	3.86	<0.001*	1.438 to 4.405
Age (y)	0.074	0.020	3.73	<0.001*	0.035 to 0.114
Male gender	-0.815	0.423	-1.93	0.054	-1.643 to 0.014
TLPD (y)	0.052	0.152	0.34	0.734	-0.0247 to 0.350
Constant	-6.292	1.643	-3.83	<0.001*	-9.513 to -3.071

The logistic regression model was performed as a function of the ES, gender, and TLPD. The level of significance was set at a *p*-value below 0.05 (*). Log likelihood=-78.72; Pseudo R^2=0.2539; Wald $\chi^2(5)$= 53.57, p<0.0001.

Abbreviations: AD, Alzheimer's disease; CI, confidence interval; ES, Erlangen Score; TLPD, time between LP and death

	$A\beta_{1-42}$ normal (+0)	$A\beta_{1-42}$ in border zone (+1)	$A\beta_{1-42}$ pathologic (+2)
T-tau/P-tau_{181} normal (+0)	0	1	2
T-tau/P-tau_{181} in border zone (+1)	1	2	3
T-tau/P-tau_{181} pathologic (+2)	2	3	4

Figure 8.1 - ES classification pattern based on the CSF biomarker alterations. Points appointed to each biomarker alteration is given between brackets.

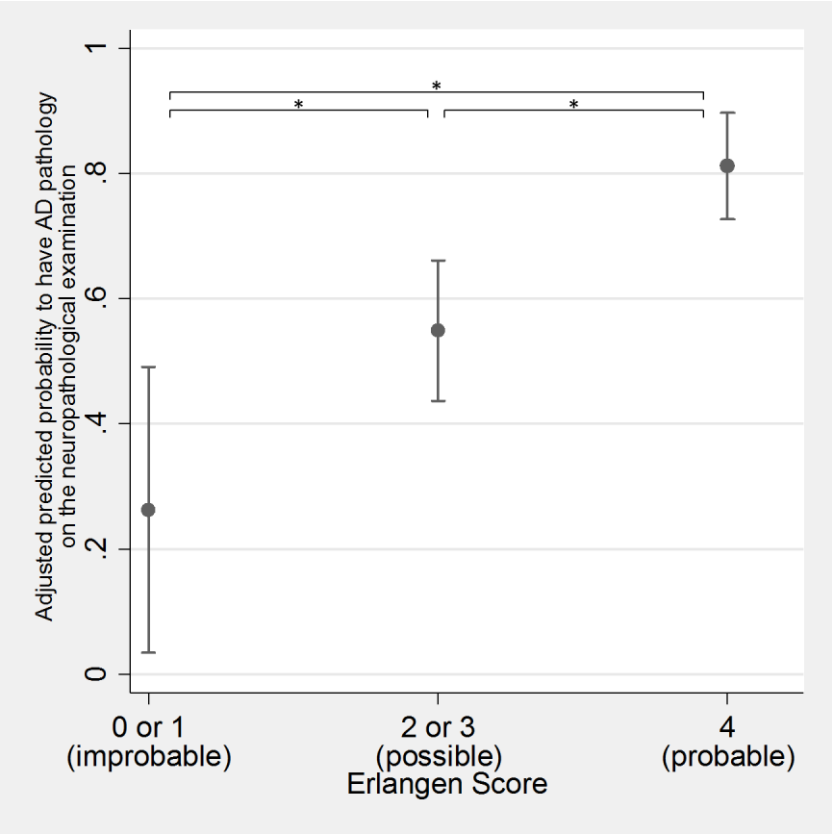

Figure 8.2 - Marginal predictions of the probability to have AD pathology at the post mortem examination. Predications were made at the fixed values of the overall average of age and TLPD and the overall proportion of females across the groups. The level of significance was set at a *p*-value below 0.05 (*).

GENERAL DISCUSSION AND CONCLUSIONS

Over the last couple of decades, CSF biomarkers have been studied thoroughly for their potential to closely reflect neuropathological processes in the brain. To date, three pathological hallmarks can be reliably used for the biochemical diagnosis of AD through the reflection of changes of CSF levels of $A\beta_{1-42}$, T-tau, and P-tau_{181}[39]. Despite the well-established precision of these biomarkers for picking up AD pathology, the overlap in CSF levels with other neurodegenerative diseases provides the basis for this thesis as it offers a window of opportunity to improve the diagnostic accuracy as well as the understanding of AD pathophysiology.

Shared pathological features, such as accumulation of amyloid or tau in the extra- or intracellular space and neurodegeneration, cause the most obvious source of overlap, and AD pathology may also simply co-exist with other primary neurodegenerative or secondary diseases as a mixed pathology. Some of the main features of AD may also arise as part of normal aging and both biomarkers and pathology have been correlated with age in healthy cognitive individuals as well[298]. CSF biomarkers are also known to be variable depending on individual baseline production rates[217] or may be altered due to interactions with disease-specific proteins, and therefore resemble AD pathology suggestive levels. Additionally to these biological features, pre-analytical and analytical issues may cause differences or shifts, especially seen in the levels of the hydrophobic Aβ peptide[100, 299].

Overcoming the overlap in AD CSF biomarkers amongst neurodegenerative disorders is a necessity in order to improve the differential AD diagnosis. In the clinical practice, correct diagnosis of underlying pathologies will affect patient management, the more so once disease-modifying therapies become available. Well-validated biomarkers with a low signal-to-noise ratio will aid drug discovery in clinical trials as biomarkers are valuable tools in constructing inclusion criteria as well as outcome/end-point measures.

This PhD thesis includes the assessment of the core AD CSF biomarkers performance to reflect AD pathology in order to better characterize their clinical utility. Focusing on the role of CSF biomarkers to correctly detect the amyloid plaque pathology, potential biomarker candidates of the Aβ metabolism were reviewed in light of the overlap in CSF $A\beta_{1-42}$ levels between neurodegenerative disorders. Furthermore, the most promising candidate, the $A\beta_{1-42}/A\beta_{1-40}$, was evaluated both for its diagnostic accuracy in autopsy-confirmed AD towards definite neurodegenerative and secondary brain disorders as well as for its potential to detect clinical AD. In order to overcome the overlap of CSF T-tau between AD and CJD, the value of CSF PrP-t was compared against the performance of the novel PrP method of RT-QuIC and the T-tau/$Ptau_{181}$ ratio. To target the interpretation of the biomarker results, which may lead to a decreased diagnostic accuracy if not done optimally, the ES was validated as an algorithm for differential diagnosis. In what follows, the overlap of the current biomarkers for differential diagnosis will be examined and possible ways to overcome this gap will be discussed.

EARLIEST CHANGES OF PATHOLOGY DISPLAYED BY $A\beta_{1-42}$

Role of $A\beta_{1-42}$ in AD diagnosis

In line with the amyloid cascade hypothesis, which states that the amyloid pathology is the initial feature in the disease process of AD, CSF $A\beta_{1-42}$ has been the proposed biomarker for early differential diagnosis of AD[155]. A decreased concentration of CSF $A\beta_{1-42}$ in AD is suggested to reflect its deposition into amyloid plaques, preventing this Aβ peptide to enter the CSF. The lack of correlation between CSF $A\beta_{1-42}$ and AD plaque pathology found in our previous study, discussed in chapter 4, was attributed to ceiling effects of the amyloid pathology. This underlined the early change of Aβ in the disease process, reaching maximal levels in later stages.

Overlapping CSF $A\beta_{1-42}$ levels hamper differential diagnosis

Decreased levels of CSF $A\beta_{1-42}$ have also been consistently demonstrated in VaD, DLB, CJD, as well as NPH, and with conflicting results in FTLD[65, 159, 167, 200, 300–303]. The biggest caveat of CSF $A\beta_{1-42}$ is that it presents with an altered concentration in the above mentioned diseases when no plaque pathology is present, as shown in our definite non-AD cases with overlapping levels with AD. Furthermore, as it is supposed to reflect AD pathology, the $A\beta_{1-42}$ biomarker by itself is unable to distinguish between 'pure' AD and mixed dementia of non-AD with AD pathology [304–306], a feature that is commonly seen in VaD and DLB[157, 161, 294, 307]. The reason for the overall altered Aβ levels in for instance VaD may be attributed to an altered clearance or metabolism, possibly a decrease in the production of these peptides, explaining the absence of amyloid plaques[172]. Additionally, AD patients may present with normal CSF $A\beta_{1-42}$ levels, although often in a borderline range, probably due to inter-individual variance of the amyloid metabolism with some having higher production rates of all Aβ metabolites.

Ways to overcome the Aβ biomarker overlap

In chapter 2 of this PhD thesis it was suggested that the Aβ metabolism might render potential candidates that add to the early differential diagnosis. In reviewing Aβ-isoforms and APP fragments for their value as AD biomarkers in chapter 5, the $A\beta_{1-42}/A\beta_{1-40}$ ratio was brought forward as the most interesting marker. The addition of CSF $A\beta_{1-40}$ combined in the ratio has recently been confirmed to better reflect amyloid plaque pathology compared with $A\beta_{1-42}$ alone[172, 297]. Another advantage with the ratio is that it corrects for the inter-individual variation, as the production of all Aβ isoforms is linked and will be equally changed in individuals[213]. In chapter 6, the diagnostic accuracy of the Aβ ratio was validated in combination with the established AD biomarkers in an autopsy confirmed AD, MxD and non-AD cohort. As both Aβ isoforms are the products of β- and γ-cleavage of APP, disease specific disturbances in the Aβ metabolism of these diseases may cause these isoforms to change concomitantly. This is in contrast with AD, were deviations are observed because of the

depositions of $A\beta_{1-42}$, while $A\beta_{1-40}$ remains largely unaltered[308]. Such changes result in a decreased $A\beta_{1-42}/A\beta_{1-40}$ ratio compared with controls[172, 215, 309], which was confirmed in our study. While CSF $A\beta_{1-40}$ levels in AD seem to be unaltered compared to controls, lower levels have been found in multiple non-AD[172, 173, 176], leading to decreased $A\beta_{1-42}/A\beta_{1-40}$ ratios in AD as compared with FTD, VaD, DLB[173, 310, 311]. We can confirm in our definite cases with AD and MxD that the Aβ ratio was decreased compared with controls and it performed with higher accuracy than $A\beta_{1-42}$ to distinguish AD and MxD from non-AD. Although the combination of CSF $A\beta_{1-42}$ with P-tau_{181} provides a similar high discriminative accuracy as the Aβ ratio, this ratio did not show the same stability over the disease-course as its cut-off values changed between the autopsy confirmed cohort and the clinical cohort. Disease stage may play an important role as slightly less unaltered CSF biomarker levels were observed in our AD and MxD cases of the autopsy cohort compared with the MCI-AD/AD subjects in the clinical cohort. It has previously been suggested that this may be due to a loss of degenerating neurons, which especially affects the tau biomarkers related to neurodegeneration[93]. However, using the ratio of the Aβ isoforms, as stated above, corrects for the intra-individual biomarker changes. Therefore, the biomarker cut-off of the Aβ remains largely unchanged during the course of the disease, which is a huge advantage for clinical routine analyses. Furthermore, using ratios based on neurodegeneration or tau pathology may prove less valuable in the early course of the disease compared with the Aβ ratio, due to their differences in relation to time of disease onset. It has previously been shown that $A\beta_{1-42}$ changes at least a decade before clinically overt AD, while changes in tau occur close to dementia conversion[155]. Thus, the biomarker accuracy and stability speaks in favour of implementing the Aβ ratio into the clinical routine.
Other biomarker candidates related to the APP/Aβ metabolism, such as $A\beta_{1-37}$, $A\beta_{1-38}$, and sAPP, have shown to be altered in several non-AD diseases. Though these markers would not provide the ability to detect AD among healthy controls or other neurodegenerative disorders as the levels seem to be unaltered[93, 175, 176, 312, 313], they may still have an added value when clinical doubt exists between AD and a non-AD disease.

Non-CSF biomarkers to improve the detection of amyloid pathology

In vivo measurements of amyloid plaque pathology can be accomplished by the use of amyloid PET. Several ligands have been studied, of which [^{11}C]Pittsburgh Compound-B ([^{11}C]PiB) is the most accurate to bind to amyloid deposits[314]. Amyloid PET imaging has already been approved in clinical (research) practice for AD diagnosis and has shown Aβ burden in the brain to inversely correlate with CSF $A\beta_{1-42}$ levels as well as with the CSF $A\beta_{1-42}/A\beta_{1-40}$ ratio[215, 315, 316]. However, studying the binding affinity of amyloid PET ligands, it has been shown that they selectively target fibrillary Aβ formations, in contrast to the pre-fibrillary Aβ that is measured in CSF[317]. Not only does amyloid PET detect amyloid pathology later than CSF biomarkers, amyloid PET positivity was found to increase with age in cognitively healthy individuals as well as in non-AD patients[298, 318]. Regarding the differential diagnostic power of amyloid PET, no discrimination between amyloid plaques and cerebral amyloid angiopathy (CAA) can be made[319–321]. On the other hand, PET

imaging may provide topological information of the regional distribution of plaques, serving as a network measure[322]. Still, PET amyloid imaging requires an expensive infrastructure, specialized personnel and uses radioactivity, as opposed to CSF that may be obtained by any trained MD in every clinic and can be sent to specialized laboratories for the analyses of the biomarkers by ELISA that remains a relative inexpensive technique.

The development of ultra-sensitive technologies such as single-molecule enzyme-linked immunosorbent arrays (Simoa) and immunomagnetic reduction (IMR)[323, 324] has made it possible to detect peripheral Aβ in blood. In recent studies, plasma $A\beta_{1-42}$ levels were found to be increased in the early stages of cognitive impairment[325], but decreased in patients that reached the stage of amyloid positivity measured by CSF[326]. Other studies indicate that changes in the periphery occur later than in the brain as the plasma Aβ levels were not altered as compared with CSF Aβ levels in amyloid PET positive subjects in the preclinical phases of the AD continuum[327, 328]. However, there are also some studies that found plasma $A\beta_{1-42}$ levels to be unchanged or even increased in AD compared with controls[329, 330]. Even so, plasma $A\beta_{1-42}$ has been found to correlate with its CSF counterpart[328, 330, 331]. Some of the disagreement may be due to differences in the biomarker alterations in the different compartments during the course of the disease and some may be due to the end-point measures to compare the $A\beta_{1-42}$ plasma levels against. Overall, plasma $A\beta_{1-42}$ remains an interesting marker for amyloid pathology, but whether its performance equals that of CSF remains doubtful and should still be determined. As in CSF, the addition of the Aβ ratio in plasma increased the biomarker diagnostic performance[326, 332]. The differential diagnostic utility of plasma Aβ was tested in a small cohort of patients with dementia due to AD and non-AD[333]. Although no differences were found for neither plasma $A\beta_{1-42}$ nor $A\beta_{1-40}$, the ratio differed significantly between the groups. However, its discriminative performance did not meet that of CSF or PET. Few studies examined plasma Aβ metabolism particularly in vascular disease, describing increased levels of both $A\beta_{1-42}$ and $A\beta_{1-40}$[327, 334]. Such elevated levels may hamper AD diagnosis if vascular co-pathology exists, but may provide differential value for their pure forms. Drawbacks of blood as a source for biomarker measurements are the attenuation of CNS derived peptides due to their passage through the blood-CSF barrier as well as their interactions with other biomolecules found in blood, leaving plasma Aβ levels about 20 times lower than found in CSF[327]. The role of peripheral-derived sources of Aβ and its degradation should still be investigated as well as pre-analytical factors for their effect on Aβ plasma levels[335] in order to set up standardization of sample handling.

Still, LP is invasive and PET expensive and limited access, which hampers their routine use and repeated measurements, as well as large scale recruitment for clinical trials[336], especially in preclinical AD. For this reason, blood biomarkers may be applicable as an early (pre-) screening tool[331, 337].

P-TAU$_{181}$ AS THE MOST SPECIFIC BIOMARKER

Role of P-tau in AD diagnosis

The use of CSF P-tau in AD diagnosis is based on its reflection of hyperphosphorylated tau accumulated into NFT in the brain. Although aggregates of P-tau are found in a wide range of other neurodegenerative diseases, called tauopathies, CSF P-tau$_{181}$ is the most specific of the established biomarkers for AD differential diagnosis[76]. It was also the only CSF biomarker found to correlate with both amyloid and neurofibrillary pathology in *APOE* ε4 non-carriers with late-stage AD pathology, highlighting its importance as a surrogate marker (chapter 4). CSF P-tau$_{181}$ has been found to best correspond with neurodegeneration in AD, as it both correlates with CSF T-tau levels and MRI atrophy as well as with cognitive decline even in the early phases of AD[106–109]. CSF P-tau$_{181}$ may also be suitable as a progression marker with a potential predictive value, estimating the chances on conversion to AD dementia[338–340]. With the growing evidence on the importance of CSF P-tau, it has now earned its spot in the A/T/N model as separate measure of hyperphosphorylated tau, in contrast to the IWG-2 criteria[55, 56]. In the latter, a change in one of two tau biomarkers in combination with abnormal A$\beta_{1\text{-}42}$ was enough to characterize an AD suggestive biomarker profile. The isolation of markers for tauopathy in this new model only boosts the attention for further improvement of AD-specific NFT markers in order to discriminate between AD and non-AD patients.

Overlapping CSF P-tau$_{181}$ levels hamper differential diagnosis

Elevated levels of CSF P-tau$_{181}$ have been shown to discriminate AD from disorders, such as FTLD, DLB/PDD, VaD and CJD[159, 166, 341–344]. However, high heterogeneity showing overlapping levels can be observed when comparing AD to other tauopathies with characteristic aggregation of P-tau, such as seen in the FTLD-tau disease entities; progressive supranuclear palsy (PSP), CBD, Pick's disease, argyrophilic grain disease and FTLD linked to chromosome 17 (FTDP-17). Additionally, DLB and VaD, due to mixed pathologies, as well as CJD may also present with elevated P-tau$_{181}$ levels [69, 159, 279, 345]. CJD is a highly heterogeneous disease and P-tau$_{181}$ levels did not significantly differ from those of AD in our study described in chapter 7, which can be explained by either the formation of prion-specific deposits containing P-tau, or by presence of AD-related NFT seen in specific subtypes of CJD[242, 283, 346].

Ways to overcome the P-tau$_{181}$ biomarker overlap

One way to improve the differential accuracy of CSF P-tau$_{181}$, is its combination with other CSF biomarkers. As CSF P-tau$_{181}$ has been shown to have high specificity for AD NFT pathology, different ratios have been investigated for their differential use in diagnosing AD. In chapter 2 of this PhD thesis, the results were presented form the analyses of the AD CSF biomarkers obtained over a time period of ten years in autopsy-confirmed cases with AD or

non-AD. In this study, we showed that CSF P-tau$_{181}$ displays the highest diagnostic performance (AUC 0.676), which was even improved further by the combination with CSF Aβ_{1-42} (AUC 0.734)[105]. The ratio of P-tau$_{181}$/Aβ_{1-42} has proven to distinguish AD from FTLD as well as VaD with a high accuracy, performing better than the single biomarkers alone[76, 176, 304, 305, 347–350].

The shared pathological features between AD and non-AD tauopathies remains a source of CSF biomarker overlap. The addition of an AD specific-tau marker may prove its value in the discrimination of AD from other tauopathies. To date it is still not known whether hyperphosphorylation of tau is a cause or a result of disease, as it is not precisely known how this mechanism is actually regulated. However, the accompanying tau-induced toxicity has been proposed to occur by three main mechanisms, including the phosphorylation, oligomerization or aggregation of tau, but other post-translation modifications such as glycosylation, nitration, truncation, acetylation, sumoylation, ubiquitination are being investigated as well.

Phosphorylation of tau at different sites regulates the assembly and stabilization of microtubules[351, 352] and a number of sites seem to be involved in the development, morphogenesis and maintenance of neuron axons[353]. Abnormal phosphorylation therefore causes a disruption of microtubules and misfolding of tau promoting them to aggregate[12, 354]. This high phosphorylation state of tau seems to be linked to the overexpression of certain kinases, possibly in combination with the unfolding and extending of its structure, making the protein easily accessible to protein kinases[355]. Although over 40 phosphorylation sites have been identified in AD[118, 119], it is not yet known whether disease-specific phosphorylation exists. Aside from the P-tau$_{181}$ isoform, tau phosphorylated at threonine 231 (P-tau$_{231}$) has been the subject of interest in some studies, as it was suggested to reflect earlier changes of neurofibrillary pathology compared to P-tau$_{181}$[114, 356–358]. CSF P-tau$_{231}$ was found to have the ability to separate AD from non-AD with high sensitivity and specificity (85% and 97%, respectively) and although P-tau$_{181}$ showed the highest performance in differentiating AD from DLB, CSF P-tau$_{231}$ performed best differentiating AD from FTD[359] and has also been found to differentiate AD from CJD[360]. However. In the scenario of AD versus CJD it was not assessed whether CSF P-tau$_{231}$ had a superior diagnostic value to that of P-tau$_{181}$[343]. Tau phosphorylated at serine 199 (P-tau$_{199}$) has also been assessed, but this isoform did not meet the discriminative power of the other isoforms[359, 361]. The fraction of tau that remains unphosphorylated (P-tau$_{rel}$) has also been evaluated for its potential as differential biomarker, but failed to provide additional power to the biomarker panel to separate AD from non-ADs grouped, FTLD, and DLB, only showing value in the differentiation from CJD[362].

Disease specific up-regulation of different kinases has been found to affect the phosphorylation level of tau, but was also found to modulate alternative splicing of the isoforms[355]. An imbalance in alternative splicing of mRNA, regulated by post-translation modifications or influenced by gene mutations, of exons 2, 3 or 10 results in the formation of six different isoforms of tau. The tauopathies have therefore been classified based on the isoform conformation of the aggregates and which cells that are affected by them. Alternative splicing of exon 10 affects the number of microtubule-binding domains that are included in

the tau protein, either resulting in 3 repeats (3R) or 4 repeats (4R)[363, 364]. Dysregulation of exon 10 splicing leads to the heterogeneous spread of tau isoforms seen in the tau aggregates among tauopathies. In AD, relative equal amounts of 3R-tau and 4R-tau are found, similarly to what is found in normal subjects[365]. What is of interest are the higher levels of one specific isoform in Pick's disease (3R-tau), CBD (4R-tau), and PSP (4R-tau)[366, 367]. The combination of tau isoforms may therefore serve as diagnostic measurements to differentiate AD from other tauopathies.
Furthermore, it has been found that tau oligomers, rather than NFT, correlate best with neuronal loss, even before onset of symptoms, suggesting oligomer-induced toxicity during early stages of AD[368]. This opens a window for the use of oligomeric tau in earlier phases of AD pathology.

Non-CSF biomarkers to improve the detection of tau pathology

Another approach would be to turn to imaging. Although tau PET has shown to be more problematic compared to amyloid PET, for abovementioned reasons of the ligand affinity towards heterogeneous tau aggregates composed of different tau isoforms, and the intracellular location of NFT, correlations with CSF measurements, especially CSF P-tau and less pronounced with CSF T-tau [369, 370], provide promising results[371]. High affinity was found for some of the tau PET ligands, such as ^{18}F-AV1451, for AD specific tau aggregates composed of both 3R-tau and 4R-tau, in contrast to the ligands binding to the different conformers found in other tauopathies with 4R-tau or 3R-tau dominance[372–377]. ^{18}F-AV1451 retention was also found to be higher in AD compared to DLB, and especially the retention in the medial lobe discriminated both diseases[320]. However, one drawback with the ^{18}F-AV1451 ligand is that it seems to show age-dependent increase of tau signals[378], which may hamper its sensitivity. Nonetheless, as most of the tracers used for tau PET specifically detect AD-related tauopathy, tau PET may have a possible differential value, which has recently been confirmed with ^{18}F-AV1451, having a sensitivity and specificity of approximately 90%[379]. However, while current promising tracers performed better than its amyloid equivalent in the AD dementia stage, they were not able to detect tau pathology in MCI subjects, as only limited pathology is still present in this stage of the AD continuum[379].
Although tau PET has not yet been validated for its integration in the diagnostic criteria, longitudinal imaging studies with amyloid PET and promising tau PET ligands may provide more insights in the interaction of AD neuropathological processes. This would also open possibilities to further validate the amyloid cascade hypothesis as this theory is still under debate[8, 380].
As the collection of CSF remains an invasive method, other body fluids have been proposed for measurements of tau proteins. Using Simoa or IMR, increased levels of plasma P-tau$_{181}$ were found in AD compared to controls[381, 382]. However, how plasma P-tau$_{181}$ performs in relation to CSF P-tau$_{181}$ remains to be determined. Unfortunately, no studies have yet been performed to measure plasma levels in non-AD.

IS A NEURODEGENERATION MARKER NECESSARY FOR DIFFERENTIAL DIAGNOSIS?

Role of T-tau in AD diagnosis

No correlations were found for CSF T-tau with any of the AD pathological changes studied in chapter 4, even though T-tau is known to be highly correlated to P-tau$_{181}$ in CSF. It is likely that CSF T-tau and/or the tau related pathology, such as neurodegeneration and NFT, have reached a plateau and therefore the associations are obscured. A concomitant increase in the levels of CSF T-tau and P-tau in AD have been suggested, by us and others, to rather represent the excessive release of intracellular tau proteins following neuronal degeneration. However, CSF T-tau levels are often somewhat increased in non-AD tauopathies, as is reflected in our non-AD cohort, while P-tau largely remains unaltered. One possible explanation for the divergences may be an imbalance in apoptotic and necrotic processes between AD and non-AD diseases[383]. In the apoptotic process less of pathological tau may be released into the interstitial fluid due to phagocytosis by microglia causing a discontinuation of the relation between T-tau and P-tau in CSF[384, 385]. T-tau may be released as part of the early neurodegenerative process, while pathological P-tau is apprehended by phagocytosis. As T-tau is a general marker of neurodegeneration, reflected by the worse performance in our autopsy-confirmed cohort of this biomarker in separating AD from non-AD compared with P-tau, the value of CSF T-tau for differential diagnosis may be questioned. As neurodegeneration occurs only at a later stage in the AD disease process, it has been stated that CSF T-tau may better reflect cognitive progression of AD[386].

Overlapping CSF T-tau levels hamper differential diagnosis

As mentioned above, the increase of CSF T-tau is a sign of neurodegeneration, also seen in traumatic brain injury and stroke[387, 388]. However in VaD, CSF T-tau levels seem to be very variable, ranging from no changes to reaching the same levels as seen in AD[159, 304, 389]. Although elevated CSF T-tau levels have been found in FTLD, these levels are usually still lower than in AD[390]. CSF T-tau levels are especially high in CJD due to its fast progressive nature[360, 391][279], characteristically higher than levels in AD although overlapping values have been established in atypical cases.

Ways to overcome the T-tau biomarker overlap

Combining CSF T-tau with P-tau$_{181}$ measurements has been proposed to increase its discriminative power and was validated in multiple studies to be specifically altered in CJD compared with AD[279, 345, 392]. Our study, described in chapter 7, confirmed these findings. In this study, the diagnostic relevance of CSF PrP-t was examined for the discrimination of AD from CJD. The validation of this biomarker was of particular interest for its use in order to discriminate ambiguous cases of AD showing overlapping levels of very high T-tau, which is characteristically seen in CJD. However, when comparing CSF PrP-t with

the core CSF tau biomarker, the ratio T-tau/P-tau$_{181}$, and the current CJD-specific biomarkers including RT-QuIC, the two latter biomarkers showed the highest discriminative power.
As a marker of subcortical axonal degeneration, neurofilament light (NFL) has been investigated broadly and was found to be increased in CSF of AD patients[393–398]. However, this again is a marker of general neurodegeneration and was also found to be increased in non-AD, including FTLD, PSP, amyotrophic lateral sclerosis, Parkinson's disease, VaD, CJD, multiple sclerosis, and stroke patients[157, 395–411]. In this regard, CSF NFL suffers the same faith as CSF T-tau of being regarded as a predictive marker for clinical progression rather than a differential marker[412].
Degeneration of synapses was found to occur before global neuronal loss and cognitive decline[413–415]. Therefore, the use of synaptic markers such as neurogranin (Ng) may provide an earlier measure of the degenerative feature of AD. CSF levels of Ng in AD have been found to be elevated, an event that was not seen in a range of other neurodegenerative disorders[416], thus highlighting its potential as an early differential biomarker. This may be due to the regional expression of Ng in neurons of the cortex, hippocampus and amygdala, regions that are specifically affected in AD[417, 418]. However, it may also just be an expression that is related to the overall decreased release of proteins in the included non-AD cases, reflected by the unaltered levels of T-tau in these neurodegenerative non-AD cases.

Non-CSF biomarkers to improve the detection of neurodegeneration

Structural brain images obtained by MRI are readily available due to their routine use in the clinical dementia work-up to exclude other, non-AD, brain diseases such as tumor or stroke. Currently the utility of MRI is shifting towards being implemented into the biomarker-based dementia diagnosis by the detection of brain specific alterations. Improving its AD-specific utility is therefore of particular interest and the development of both volumetric brain imaging of neocortical and hippocampal volumes as well as white matter integrity measurements by diffusion tensor imaging (DTI) has obtained focus. New automated tools for the segmentation of AD-affected regions such as the medial lateral lobe, including the hippocampus, but also the cerebral cortex, have been developed and can accurately confirm AD[419, 420]. Although regional differences may depict AD from other non-AD, the accuracy compared with AD CSF biomarkers needs to be investigated[421, 422]. DTI was found to detect regional AD suggestive changes in white matter and may find applications for the discrimination of AD with NPH, subcortical ischemic VaD and FTD[423–428]. However, this technique is rather advanced, making its clinical utility questionable.
Plasma T-tau levels were found not to be altered in the earliest phases of the AD continuum[331], but only in in the dementia phase[429–433]. Either T-tau measurements in plasma are not yet sensitive enough to detect changes in preclinical phases; or more likely, T-tau concentration in plasma may suffer from the same drawback as in CSF with its alterations being dependent on the closeness in time to clinical conversion to AD[155].

THE ROLE OF NON-AD SPECIFIC MARKERS IN AN AD BIOMARKER PANEL

The overlap found in the AD CSF biomarkers hamper the differential diagnostic utility, though the addition of the Aβ ratio may improve the accuracy significantly even in the preclinical phase. Another way to increase the biomarker performance to separate AD from non-AD would be the introduction of non-AD-specific biomarkers. Such markers have already been proposed for CJD, FTLD and DLB. Although the use of CSF prion protein analysis, either in the form of RT-QuIC or PrP-t levels, has been confirmed to discriminate AD from CJD[241, 265, 271], our findings proved that the combination of AD CSF tau biomarkers in the form of the P-tau$_{181}$/T-tau ratio have the highest discriminative power. This tau ratio has also been suggested in the diagnosis of FTLD as lowered values in CSF measurements have been reported compared with controls; however, compared with AD, findings are inconsistent in the reporting of equal or increased values compared with FTLD[302, 362, 434, 435]. For the differentiation between AD and FTLD with TAR DNA-binding protein 43 inclusions (FTLD-TDP) or FTLD with mutations in the progranulin gene (FTLD-GRN), CSF TDP-34 and progranulin have also been suggested as potential biomarkers, respectively. No validated assays have yet been developed for TDP-43 and the value of progranulin is limited as the FTLD-GRN patients could be detected by genetic screening[302]. Due to the high occurrence of mixed pathology of AD with DLB, DLB/PDD-specific markers would be highly valuable for the detection of the non-AD related pathology. CSF α-synuclein has been under investigation as the main constituent of Lewy bodies, the protein deposits marking DLB and PDD. With the new and promising techniques used in prion disease diagnosis, protein-misfolding cyclic amplification (PMCA) and RT-QuIC have been tested in a few, small scale studies showing high sensitivity and specificity even in early stages of DLB and PDD[436–440].

FINAL RESORT: ELIMINATING METHODOLOGICAL CAUSES OF OVERLAP

Not all of the CSF biomarker overlap has a biological source. Pre-analytical and analytical factors have been shown to influence CSF biomarker measurements, preventing the direct comparison of biomarker data across studies or laboratories[99, 101, 287, 441]. Enabling the comparisons of biomarker data amongst multiple centers can improve the validation of biomarkers by accumulating data of bigger cohorts, and will eventually aid the multi center validation of drug treatments. Interpretation-algorithms such as the ES have been validated to overcome biomarker data variances and to harmonize CSF biomarker analysis. Still, as explained in chapter 2, standardization efforts are needed to eliminate these factors and several initiatives were set up in order to accomplish this[101–103, 289, 442]. The use of fully automated methods has been of great help to increase the standardization and precision of biomarker analysis[443]. Most recently, the use of a CRM has been introduced as a way for manufacturers to calibrate their ELISA assays for CSF measurements[104, 154]. The use of these assays, in combination with standardized pre-analytical factors, will enable the determination of a general biomarker specific instead of a center-specific cut-off value. CRMs for Aβ$_{1-42}$ have already been validated in different assays[104], and are under further

development for other biomarkers as well[219]. Especially $A\beta_{1-42}$ is susceptible to pre-analytical factors, surface exposure of collection tubes having an enormous impact on its measurement values. The same pre-analytical issue was also found to significantly alter levels of PrP-t in chapter 7. In this regard, the effect of pre-analytical factors on new biomarker candidates should always be addressed during the validation to obtain reliable biomarker analyses. The use of ratios such as $A\beta_{1-42}/A\beta_{1-40}$ has been recommended to account for the adhesion of Aβ to surfaces. Although the longer, more hydrophobic, Aβ peptide is affected more than the $A\beta_{1-40}$ peptide, the latter is also prone to adsorption to some degree and therefore its combination was shown to reduce the adhesion effect with 50% compared to the use of $A\beta_{1-42}$ alone[444]. The use of CRM calibrated assays in combination with pre-analytical and analytical standardization have already and will continue to significantly improve the reliability of fluid biomarkers.

CONCLUDING REMARKS

No single biomarker has yet been found to fully and specifically diagnose AD along its progression spectrum due to its high complexity and overlap with other neurodegenerative disorders. However, a biomarker panel portraying the three AD hallmarks covers the early differential diagnosis of AD and may provide a positive prognostic value. With the inclusion of the $A\beta_{1-42}/A\beta_{1-40}$ ratio into the core AD biomarker portfolio, biological as well as methodological improvement for the detection of AD-specific amyloid pathology was accomplished. The search for AD-specific tau isoforms has been stressed, now more than ever with NFT pathology earning its place in new diagnostic criteria. Although neurodegeneration is a general feature amongst central nervous system disorders, its added value to the biomarker panel is unmistakable, as it can help set a prognosis. Especially in the clinic, measurement of amyloid pathology alone does not have much added value in the context of prodromal AD or AD dementia diagnosis and frequent occurring cases with mixed pathologies call for action on the front of non-AD-specific biomarkers. The ultimate goal would be to further develop differential biomarkers with the ability to detect AD and non-AD specific pathology in its earliest stages, while eliminating the invasiveness of a lumbar puncture and reducing the high costs and limited access of PET imaging. Such biomarkers would have a critical value in the screening for AD and related disorders, patient selection for inclusion and monitoring drug responses in clinical trials with frequent repetitive measures.

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EDUCATION AND TRAINING

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2008-2014: **Bachelor in Biomedical Sciences**
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GRANTS

2018: FWO - Travel grant for AAT-AD/PD Focus Meeting 2018, Torino, Italy, 15-18 March.

2016: Alzheimer's Association International Conference - Travel grant (registration) for Alzheimer's Association International Conference 2016, Toronto, Canada, July 24-28.

INTERNATIONAL CONFERENCE PARTICIPATIONS

2018: 2nd Meeting of the of the Society for CSF analysis and clinical neurochemistry, Amsterdam, Netherlands, June 7-8:
Oral presentation: **Somers C**, De Vil B, Cras P, De Deyn PP, Engelborghs S, Bjerke M. Total prion protein in cerebrospinal fluid to differentiate Creutzfeldt-Jakob disease from Alzheimer's disease with very high T-tau levels.
Poster presentation: **Somers C**, Lewczuk P, Sieben A, Van Broeckhoven C, De Deyn PP, Kornhuber J, Martin JJ, Bjerke M, Engelborghs S. Validation of the Erlangen Score algorithm for differential dementia diagnosis in autopsy-confirmed subjects.

2018: AAT-AD/PD Focus Meeting 2018, Torino, Italy, 15-18 March:
Poster presentation: **Somers C**, De Vil B, Cras P, De Deyn PP, Engelborghs S, Bjerke M. Total prion protein in cerebrospinal fluid to differentiate Creutzfeldt-Jakob disease from Alzheimer's disease with very high T-tau levels.

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Poster presentation: **Somers C**, Struyfs H, Goossens J, Niemantsverdriet E, Luyckx J, De Roeck N, De Roeck E, De Vil B, Cras P, Martin JJ, De Deyn PP, Bjerke M, Engelborghs S. A decade of cerebrospinal fluid biomarkers for Alzheimer's disease in Belgium. *Alzheimers Dement.* 2016, 12(7): p467-468.

MEMBERSHIP OF PROFESSIONAL SOCIETIES AND RESEARCH CONSORTIA

Member of EU Innovative Medicines Initiative (IMI): European Medical Information Network (EMIF) (EMIF-AD, WP2)

Associated member of the Belgian Neurological Society (BNS)

TEACHING ACTIVITIES

Co-promoter Master thesis Sarah Khadir: Improving the biomarker-based differential diagnosis of Alzheimer's disease. 2nd Master Biomedical Sciences, UAntwerp, Academic year 2017-2018

Co-promoter Master project Nouchine De Loose: First steps in the characterization of possible Alzheimer's disease specific tau-antibodies. 2nd Master Biochemistry and Biotechnology, UAntwerp, Academic year 2017-2018

Co-promoter Bachelor thesis Matisse Verboven: The added value of Aβ1-42/1-40 ratio for early differential diagnosis of dementia. 3rd Bachelor Biomedical Sciences, UAntwerp, Academic year 2016-2017

Guidance of practical sessions Integrated research - Neuroimaging. 2nd Master Biomedical Sciences, UAntwerp, Academic year 2016-2017 and 2017-2018

Guidance of practical sessions Neurophysiology. 3rd Bachelor Biomedical Sciences, UAntwerp, Academic year 2017-2018 and 2018-2019

PUBLICATIONS IN INTERNATIONAL PEER-REVIEWED JOURNALS

Willemse E, Sieben A, **Somers C**, Vermeiren Y, De Roeck N, Luyckx J, Van Broeckhoven C, De Vil B, Cras P, De Deyn PP, Martin JJ, Teunissen C, Engelborghs S, Bjerke M. Neurogranin in cerebrospinal fluid is not specific to Alzheimer's disease dementia. Alzheimers Dement. 2018, 14(7): p1549-1550. doi: 10.1016/j.jalz.2018.07.092.

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SCIENCE POPULARIZING PUBLICATIONS

Drs. Charisse Somers, Prof. Dr. Maria Bjerke en Prof. Dr. Sebastiaan Engelborghs namens BIODEM. Tien jaar Antwerps onderzoek naar biomarkers in lumbaalvocht voor de ziekte van Alzheimer. Alzheimer Liga Vlaanderen. 2016, Nr. 114: p21

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ABSTRACTS

Somers C, De Vil B, Cras P, De Deyn PP, Engelborghs S, Bjerke M. Total prion protein in cerebrospinal fluid to differentiate Creutzfeldt-Jakob disease from Alzheimer's disease with very high T-tau levels. 2nd Meeting of the Society for CSF analysis and clinical neurochemistry 2018, Amsterdam (Netherlands).

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Willemse E, Sieben A, **Somers C**, Vermeiren Y, De Roeck N, Luyckx J, Van Broeckhoven C, De Vil B, Cras P, De Deyn PP, Martin JJ, Teunissen C, Engelborghs S, Bjerke M. Neurogranin in cerebrospinal fluid is not specific to Alzheimer's disease dementia. Alzheimer's association international conference (AAIC) 2018, Chicago (USA).

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Somers C, Struyfs H, Goossens J, Niemantsverdriet E, Luyckx J, De Roeck N, De Roeck E, De Vil B, Cras P, Martin JJ, De Deyn PP, Bjerke M, Engelborghs S. A decade of cerebrospinal fluid

biomarkers for Alzheimer's disease in Belgium. Alzheimer 2016 Joint scientific meeting of the Belgian Neurological Society and the Belgian Dementia Counsil (Alzheimer26) 2016, Antwerp (Belgium). Acta Neurol Belg. 2017; 117:587

Niemantsverdriet E, Ottoy J, Verhaeghe J, **Somers C**, De Roeck E, Strufs H, Soetewey F, Bjerke M, Van den Bossche T, Van Mossevelde S, Goeman J, De Deyn PP, Mariën P, Versijpt J, Parizel PM, sleegers K, Van Broeckhoven C, Wyffels L, Albert A, Ceyssens S, Stroobants S, Staelens S, Engelborghs S. Concordance improves between amyloid-PET and CSF amyloid-β for diagnosing Alzheimer's disease in a clinical setting by applying Aβ1-42/Aβ1-40 ratio - COMBAD. Alzheimer 2016 Joint scientific meeting of the Belgian Neurological Society and the Belgian Dementia Counsil (Alzheimer26) 2016, Antwerp (Belgium). Acta Neurol Belg. 2017; 117:586.

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* Papers this PhD thesis was based upon

"Happiness does not lead to gratitude. Gratitude leads to happiness."
— Benedictijner monnik David Steindl-Rast

Geïnspireerd door deze quote lijkt het me ideaal deze laatste bladzijden te nemen om een aantal belangrijke invloeden naar voren te brengen. Het punt dat ik hiermee wil maken is dat, hoewel er soms periodes van sociale isolatie zijn geweest, doctoreren geen weg is die je alleen afgaat. Ik ben verwend geweest met een lab vol heerlijke collega's en ervaren leermeesters, een thuis van warme mensen, en vrienden die altijd paraat staan om plezier en af en toe ook leed te delen. Jullie hebben van deze afgelopen intensieve jaren een prachtig leerproces gemaakt, waar ik dankbaar naar terug zal kijken. Ik hoop dat ik met deze laatste woorden weer een stukje van mijn geluk met jullie kan delen!

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Jessie, schat van griet, je hebt al mijn verwachtingen voor deze prachtige cover foto overtroffen. Wat een originele uitvoering! Deze kan dan ook binnenkort bewonderd worden in zijn kader bij mij thuis. Daarbij brengt het samen plannen van onze “Tchicks going Thai” reis de nodige afleiding en geeft het vooruitzicht me emmers motivatie om nog een laatste spurtje te trekken naar de finish. Het was super om te ontdekken dat, na alle jaren dat we elkaar kennen, de meerderheid van de Ladies, Emilie, Kim, Amparo, en Hannah, we gewoon weer bij elkaar achter de hoek kwamen te wonen! Dank jullie wel voor de gezellige babbels en heerlijke etentjes, ik denk dat we best kunnen stellen dat onze vriendschappen voor het leven zijn.

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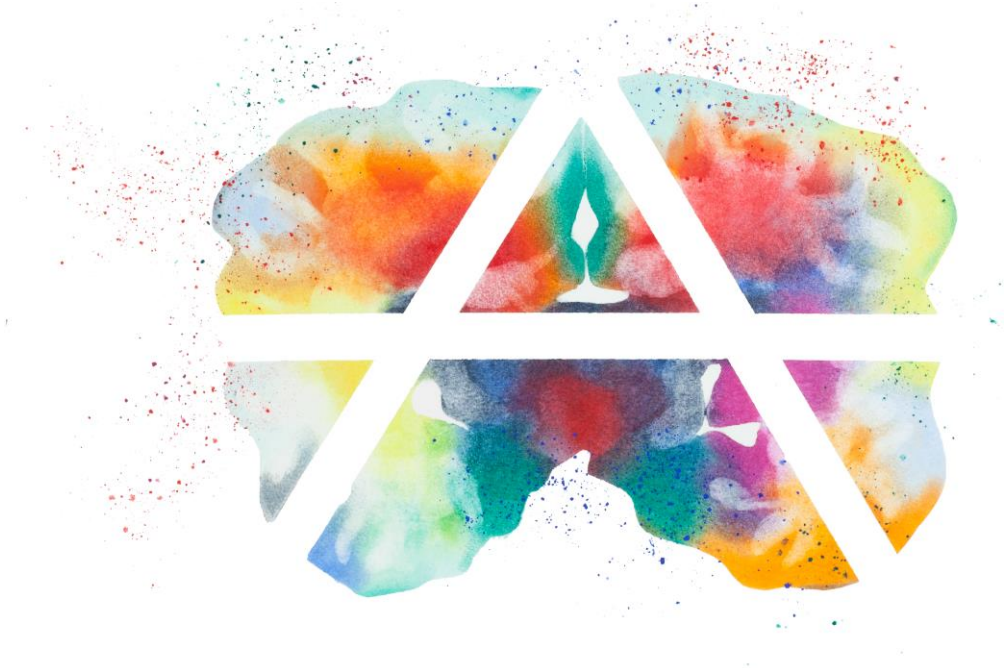

Original cover photo drawing by Jessie De Bie