



Plasma biomarkers in Alzheimer's disease – variability, prognostic and disease tracking abilities



PhD thesis
Frederikke Kragh Clemmensen

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Preface and acknowledgments

I wish to dedicate this work to my beloved grandfather, Professor Dr. Phil. Kristian Knud Jeppesen, who taught me that *a lack of interest often stems from a lack of knowledge*, and whose fascinating and impressive life and career were ultimately affected by dementia. It is my hope that this work may, in some way, contribute to a better understanding of this puzzling and devastating disease, affecting so many patients and families around the world.

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On February 1st 2016, after completing my fourth year in medical school, I began my research year at the memory clinic under Professor Steen Hasselbalch. This marked the beginning of my ambitions of one day entering a PhD program and continuing my growing interest in neurology, specifically in dementia disorders. While finishing medical school and beginning internships, I was fortunate to keep attached to the Danish Dementia Research Centre expanding my collaboration to include associate professor Kristian Steen Frederiksen and senior researcher Anja Hviid Simonsen. During my first year in clinical neurology this PhD-project took its form.

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List of studies

This PhD thesis consists of a synopsis based on the following four original studies (see Appendices).

Study 1 Short-term variability of Alzheimer’s disease plasma biomarkers in a mixed memory clinic cohort.

Frederikke Kragh Clemmensen, Mathias Holsey Gramkow, Anja Hviid Simonsen, Nicholas J. Ashton, Hanna Huber, Kaj Blennow, Henrik Zetterberg, Gunhild Waldemar, Steen Gregers Hasselbalch & Kristian Steen Frederiksen

*Published in Alzheimer’s Research & Therapy, January 2025*¹

Study 2 The plasma p-tau217/BD-tau ratio improves biomarker short-term variability in memory clinic patients.

Frederikke Kragh Clemmensen, Fernando Gonzalez-Ortiz, Mathias Holsey Gramkow, Cristiano Santos, Henrik Zetterberg, Kaj Blennow, Steen Gregers Hasselbalch, Kristian Steen Frederiksen & Anja Hviid Simonsen

*Published in Alzheimer’s & Dementia: Translational Research & Clinical Interventions, August 2025*²

Study 3 Prognostic value of plasma biomarkers in early Alzheimer’s disease: a longitudinal clinical and neuroimaging study

Frederikke Kragh Clemmensen, Mathias Holsey Gramkow, Fernando Gonzalez-Ortiz, Andrea Lessa Benedet, Kubra Tan, Wiebke Traichel, Ulrich Lindberg, Otto Mølby Henriksen, Ian Law, Henrik Zetterberg, Kaj Blennow, Anja Hviid Simonsen, Kristian Steen Frederiksen & Steen Gregers Hasselbalch.

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Study 4 Tracking clinical and neuroimaging progression of Alzheimer’s disease by longitudinal trajectories of plasma biomarkers

Frederikke Kragh Clemmensen, Mathias Holsey Gramkow, Andrea Lessa Benedet, Fernando Gonzalez-Ortiz, Kubra Tan, Wiebke Traichel, Ulrich Lindberg, Otto Mølby Henriksen, Henrik Zetterberg, Kaj Blennow, Ian Law, Anja Hviid Simonsen, Kristian Steen Frederiksen & Steen Gregers Hasselbalch.

*Under revision in Alzheimer’s & Dementia: Diagnosis, Assessment & Disease Monitoring, November 2025*⁴

Abbreviations

A β	Amyloid beta
ACE	Addenbrooke's Cognitive Examination
AChEI	Acetylcholinesterase inhibitor
AD	Alzheimer's disease
APOE4	Apolipoprotein E ϵ 4 allele
APP	Amyloid precursor protein
AUC	Area under the curve
BD-tau	Brain-derived tau
BMI	Body Mass Index
CAA	Cerebral amyloid angiopathy
CDR-SB	Clinical Dementia Rating – Sum of Boxes
CI	Confidence interval
CNS	Central nervous system
CSF	Cerebrospinal fluid
CT	Computed tomography
CU	Cognitively unimpaired
CV _A	Coefficient of variation - assay variability
DLB	Dementia with Lewy bodies
EDTA	Ethylenediaminetetraacetic acid
eGFR	estimated Glomerular Filtration Rate
EMA	European Medicines Agency
FDA	U.S. Food and Drug Administration
[¹⁸ F]FDG-PET	[¹⁸ F]-fluorodeoxyglucose positron emission tomography
FTD	Frontotemporal dementia
GFAP	Glial fibrillary acidic protein
HbA1c	Hemoglobin A1c
HC	Healthy Controls
LATE	Limbic-predominant age-related TDP-43 encephalopathy
LMM	Linear mixed-effects models
MCI	Mild cognitive impairment
MMSE	Mini-Mental State Examination
MRI	Magnetic resonance imaging
NfL	Neurofilament light chain

p-tau	Phosphorylated tau
PSEN1 & PSEN2	Presenilin 1 and Presenilin 2
ROC	Receiver Operating Curve
QC	Quality control
RC	Relative change
RCV	Reference change value
SCD	Subjective Cognitive Decline
SHORT-ND	Short-term variability in novel biomarkers for neurodegenerative diseases
Simoa	Single molecule array (ultrasensitive immunoassay platform)
sTREM2	soluble Triggering Receptor Expressed on Myeloid Cells2
SUVr	Standardized uptake value ratio
TRACK-AD	Tracking Alzheimer's disease: a study of disease trajectory and development of diagnostic and disease stage biomarkers in AD
t-tau	Total tau
VaD	Vascular dementia

Summary

Alzheimer's disease (AD) is a progressive neurodegenerative disorder characterised by gradual cognitive and functional decline, ultimately resulting in institutionalization and death. Traditionally, diagnosis has relied on clinical assessment, supported by the detection of neuropathological hallmarks of AD, amyloid plaques and tau tangles. Measurements of these have been limited to neuroimaging and cerebrospinal fluid analysis, both methods invasive, costly, and restricted to specialised tertiary units. Advances in technologies have enabled the detection of AD biomarkers in blood, representing a significant step toward more widespread accessibility. These novel blood biomarkers may have the potential to identify individuals at risk of AD, confirm AD pathology, predict rate of disease progression, and monitor disease progression and treatment effect. The latter two applications are less studied, especially in memory clinic patients, yet increasingly essential as disease modifying treatments become available. Valid and robust prognostic and disease tracking biomarkers should ideally identify patients at risk of a more rapid disease course, allocate to treatment, and monitor the effect and safety.

This thesis aims to investigate AD plasma biomarkers as novel prognostic and disease tracking biomarkers in memory clinic patients with mild cognitive impairment (MCI) or mild to moderate dementia due to AD (Studies III-IV). This work builds on the understanding of the variability of biomarker levels in blood – essential to ensure correct interpretation of changes as disease-related. Therefore, it also aims to examine within- and between subject variability in AD plasma biomarkers (Study I-II).

In Study I and II, we conducted a test-retest variability study to assess variability across plasma biomarkers of core AD pathology, neurodegeneration, and neuroinflammation. We included 27 patients with AD and 20 patients with other neurodegenerative, cognitive or affective disorders and collected three consecutive blood samples within approximately one month. We observed acceptable within subject variabilities, supporting their use to monitor longitudinal changes on an individual level. However, using within-subject variability together with the biomarkers' specific assay variability, we found considerable broad reference change values – i.e. the threshold needed between two repeated blood samples to distinguish disease related changes from physiological fluctuations. Moderate to high between-subject variability was also observed, potentially causing diagnostic and disease staging challenges. Normalising phosphorylated tau to a brain-derived form of tau reduced between-subject variability, which was further improved when adjusting for confounders such as kidney dysfunction and body mass index.

In Study III and IV, we performed a longitudinal observational study where patients with MCI and dementia due to AD were followed for up to two years with repeated blood sampling, cognitive tests and neuroimaging. In total, 94 patients completed one year follow-up, and 85 patients further completed two-year follow-up.

In Study III we evaluated the prognostic value of baseline and short-term changes in plasma biomarkers in clinical and imaging-based progression, using fluorodeoxyglucose positron emission tomography (^{18}F]FDG-PET) and magnetic resonance imaging (MRI). Baseline levels of neurofilament light significantly predicted up to two-year future decline in the Clinical Dementia Rating scale (CDR-SB). Short-term changes in neurofilament light (NfL) may predict changes in regional glucose metabolism measured by ^{18}F]FDG-PET, which suggests that short-term plasma biomarker changes may offer novel prognostic potential, although these changes should be interpreted with caution and always in context with their baseline level.

Study IV examined longitudinal changes in AD plasma biomarkers, revealing a significant steeper increase in phosphorylated tau 217 (p-tau217) among patients who clinically progressed to more severe dementia stages compared with clinically stable individuals. We found no clear relationship between individual rate of change in biomarker levels and individual rate of change in CDR-SB or neuroimaging measures.

In conclusion, the findings from this thesis suggest that AD plasma biomarkers may demonstrate distinct potential: plasma NfL could be a reliable predictor of disease progression, whereas plasma p-tau217 shows promise as a disease tracking marker. Moreover, variability in plasma biomarker levels should be carefully considered when interpreting plasma biomarker levels and may influence the understanding of their prognostic value and of longitudinal changes.

The development of robust blood biomarkers may prove valuable for informing treatment decisions, enabling individualised prognostic counselling, and optimizing healthcare resource allocation. Ultimately, blood biomarkers may become essential for monitoring treatment efficacy and safety in an expanding population potentially eligible for disease-modifying therapies – thereby supporting more precise and efficient dementia care in the future.

Resume

Alzheimers sygdom (AD) er en fremadskridende neurodegenerativ sygdom, karakteriseret ved gradvis tab af kognitive og funktionelle evner, der med tiden gør det svært at opretholde en selvstændig hverdag og som ultimativt resultater i døden. Traditionelt har diagnosen været baseret på en klinisk vurdering, understøttet af påvisning af AD's neuropatologiske kendetegn; ophobningen af amyloide plaques og tau-filamenter. Måling af disse har indtil for nylig krævet hjerneskanninger og analyse af cerebrospinalvæske, begge invasive og omkostningstunge metoder, der er forbeholdt specialiserede udredningsenheder.

Teknologiske fremskridt har muliggjort påvisning af AD biomarkører i blodet, hvilket er et vigtigt skridt mod mindre invasive og lettere tilgængelige biomarkører. Biomarkører skal ideelt kunne identificere personer i risiko for AD, bekræfte AD-patologi, forudsige sygdomsprogression og monitorere sygdomsudvikling og behandlingseffekt. Særligt blod-biomarkørers evne til at forudsige og følge sygdomsudvikling er endnu ikke velundersøgt hos patienter i de tidlige faser af AD. Dette bliver fortsat mere relevant i takt med, at sygdomsmodificerende behandlinger bliver tilgængelige – at udvikle blod-biomarkører, der kan identificere patienter i risiko for et mere hastigt progredierende sygdomsforløb samt monitorere behandlingseffekt.

Formålet med denne afhandling er at undersøge om AD blod-biomarkører kan forudsige og følge sygdomsudviklingen hos patienter med let kognitiv svækkelse (MCI) eller demens forårsaget af AD (studie III-IV). Grundlaget for at vurdere ændringer i blod-biomarkører over tid ligger i det fundamentale arbejde at undersøge variationen af biomarkørers niveauer i blodet (studie I-II). Det er essentielt at forstå tilfældige fysiologiske udsving i biomarkør-niveauerne for at sikre en klinisk relevant fortolkning af en biomarkør og ændringer i denne.

I studie I og II udførte vi en test-retest-undersøgelse for at vurdere variationen i AD specifikke blodmarkører, samt i biomarkører for neurodegeneration og neuroinflammation. Vi inkluderede 27 patienter med AD og 20 patienter med anden neurodegenerativ, kognitiv eller affektiv lidelse, og indsamlede 3 gentagne blodprøver inden for ca. en måned. Vi fandt acceptabel intraindividuel variation, hvilket er lovende med henblik på at monitorere sygdomsudvikling på individniveau. Ved at kombinere intraindividuel variation med assay-specifik variation fandt vi dog brede reference-ændringsværdier: den tærskel, der skal overskrides mellem to blodprøver for at skelne reelle sygdomsrelaterede ændringer fra fysiologiske udsving. Vi observerede også en moderat til høj inter-individuel variation, hvilket potentielt kan komplicere diagnostik og stadieinddeling.

Kombinationen af et fosforyleret tau med en hjerne-specifik form af proteinet tau reducerede inter-individuel variation, og justering for blandt andet nyrefunktion og BMI reducerede den yderligere.

I studie III og IV udførte vi et longitudinalt observationsstudie, hvor patienter med MCI og demens pba. AD blev fulgt i op til to år med gentagne blodprøver, kognitive tests og hjerneskaninger. I alt gennemførte 94 patienter ét års opfølgning og 85 patienter to års opfølgning.

I studie III undersøgte vi den prognostiske værdi af baseline- og korttidsændringer i blod-biomarkørerne i forhold til klinisk og billeddiagnostisk progression, målt ved fluorodeoxyglucose positron emissions tomografi ($[^{18}\text{F}]\text{FDG-PET}$) og MR-skanning. Baseline-niveauer af neurofilament light (NfL) kunne signifikant forudsige op til to års udvikling på skalaen Clinical Dementia Rating (CDR). Korttidsændringer i NfL kunne forudsige ændringer i regional glukosemetabolisme målt ved $[^{18}\text{F}]\text{FDG-PET}$, hvilket kunne tyde på, at korttidsændringer i blod-biomarkører kan indeholde nyt prognostisk potentiale. Dette bør tolkes med forsigtighed og altid i kontekst af baseline blod-niveauet.

Studie IV undersøgte longitudinelle ændringer i AD blod-biomarkører. Vi fandt en signifikant stejlere stigning i fosforyleret tau hos patienter, der klinisk progredierede til mere alvorlige demensstadier, sammenlignet med klinisk stabile individer. Vi fandt dog ingen klar sammenhæng mellem individuelle ændringer i blod biomarkørniveauer og ændringer i CDR eller billeddiagnostiske mål.

Sammenfattende resultater fra denne ph.d. viser at AD blod-biomarkører muligvis har flere potentialer: at NfL i blod kan være en pålidelig markør for sygdomsprogression, mens fosforyleret tau i blod viser sig lovende som markør for sygdomsudvikling. Det er afgørende at tage højde for variation i blod-biomarkør ved fortolkning af blod-niveauer – variation kan påvirke forståelsen af blod-biomarkørers prognostiske værdi og af ændringer over tid.

Udviklingen af robuste blod-biomarkører kan understøtte behandlingsvalg, muliggøre individualiseret prognostisk rådgivning og optimere ressourceanvendelsen i sundhedsvæsenet. I sidste ende kan blod-biomarkører blive essentielle værktøjer til monitorering af behandlingseffekt samt bivirkninger hos den voksende patientgruppe, der er potentielle kandidater til sygdomsmodificerende behandlinger, og dermed bidrage til mere præcis og effektiv behandling af AD i fremtiden.

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Introduction

Throughout medical history, identification of biological markers that reflect underlying pathophysiological mechanisms has transformed disease diagnosis and management. In oncology, the shift from a morphological to molecular defined classifications have enabled targeted therapies and personalised treatment strategies. A comparable paradigm shift is now emerging in the field of dementia research. Traditionally relying on clinical assessment and neuroimaging, dementia diagnosis is now being revolutionized by advances in fluid biomarkers, particularly those measurable in blood. This advancement has enabled the detection of specific pathological processes in vivo and holds the potential of a biological understanding of dementia disorders, thus paving the way for earlier detection, improved prognostication, and novel therapeutic opportunities.

1. Background

1.1 Dementia & Mild cognitive impairment

The term dementia stems from the latin *demens*, *de-* meaning “without” and *mens* meaning “mind”, denoting “out of mind”. Throughout the 18th century, the term dementia was broadly used to describe psychiatric and neurological disorders with psychosocial consequences. During the 19th century, however, *senile* (from Latin ‘of old age’) dementia began to be recognized as a distinct medical condition ⁵. In 1906, the German psychiatrist and neuropathologist Alois Alzheimer identified amyloid plaque and neurofibrillary tangles in the brain of a young female patient suffering from memory loss, hallmarks of what was later recognised as Alzheimer’s disease (AD) ⁶. This discovery prompted a shift toward differentiating dementia into different neuropathological conditions.

Today, dementia is understood as an umbrella term for a syndrome, characterised by persistent cognitive impairment affecting activities of daily living ^{7,8}. Dementia progresses from mild to moderate and severe stages, reflecting the gradual cognitive and functional decline and loss of daily independence.

The functional decline distinguishes dementia from mild cognitive impairment (MCI), which represents an intermediate stage between normal cognitive functioning and dementia. MCI is defined by objective signs of cognitive dysfunction that does not impact independent living ^{9,10}.

A range of cognitive domains may be affected in MCI and dementia, including memory, language, executive functions, visuospatial abilities and behaviour, depending on the underlying etiology. The underlying etiologies of dementia include neurodegenerative disorders (e.g. AD, Dementia with Lewy

Bodies (DLB), and Frontotemporal dementia (FTD)), cerebrovascular disease and various non-neurodegenerative conditions. While some non-neurodegenerative causes (e.g. subdural hematoma, normal pressure hydrocephalus, and metabolic conditions) may be reversible, neurodegenerative diseases are progressive and ultimately fatal. AD is the leading cause of dementia, accounting for up to seventy percent of dementia cases ¹¹.

1.2 Alzheimer's disease

1.2.1 Clinical characteristics

AD is typically characterised by a gradual onset, but with persistent progressive memory loss, especially episodic memory loss. As the disease progresses, other cognitive domains are affected causing disorientation in time and space, language difficulties, impaired problem solving, planning, judgement, and visuospatial impairment ⁷. A minority of patients suffer from a non-amnesic presentation of AD with predominantly impaired language (logopenic primary progressive aphasia) ¹², behavioural dysfunction (frontal variant of AD) ^{13,14}, impaired visual processing (posterior cortical atrophy) ^{15,12}, or with corticobasal symptoms (the corticobasal variant) ^{16,17}.

With advancing disease, the patients suffer from functional decline where carrying out independent tasks of daily living, finances, and home and hobbies become increasingly difficult. At severe stages of AD dementia, the patients are fully dependent on caregivers for daily functioning and personal hygiene. Ultimately, AD leads to institutionalization and death.

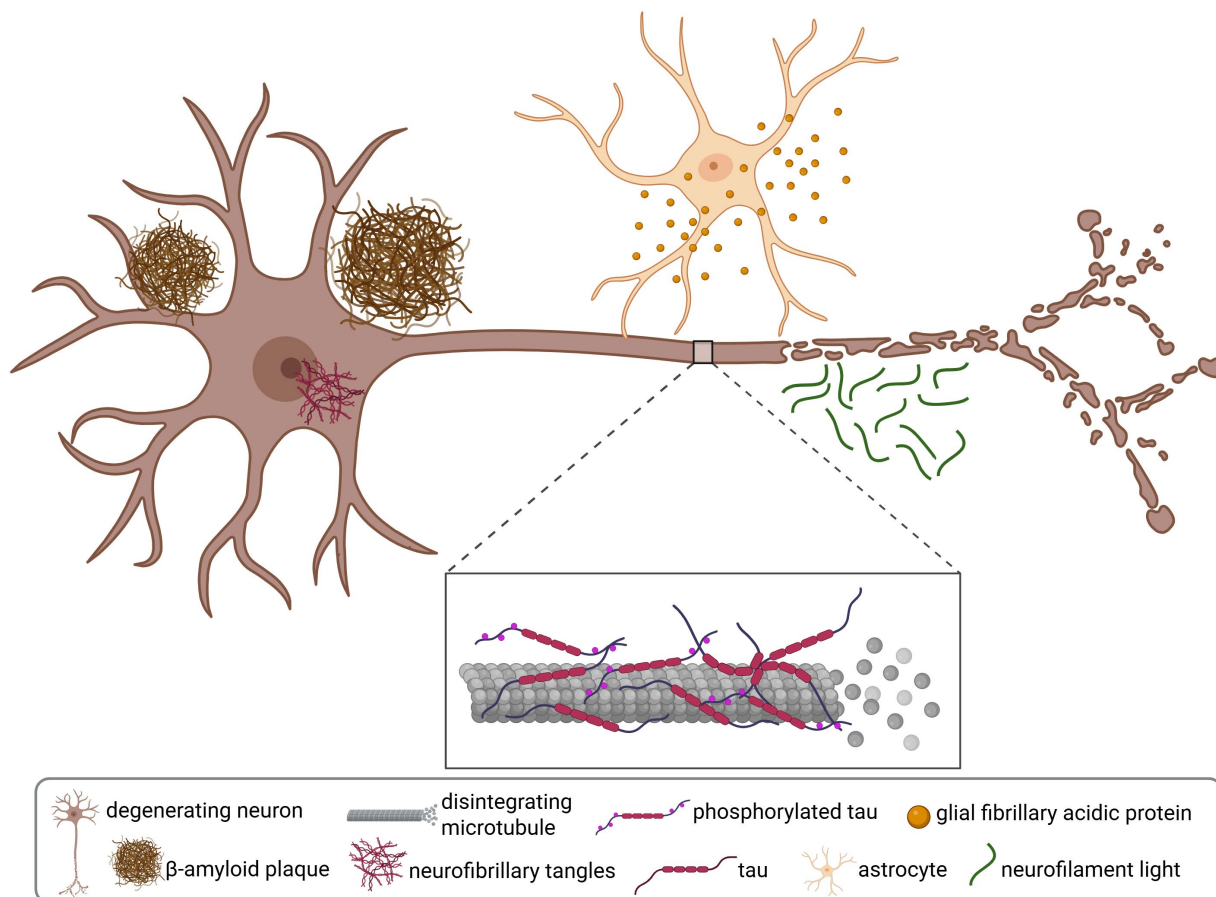
1.2.2 Neuropathological hallmarks

Since Alois Alzheimer in 1906 described the presence of amyloid plaques and tangles in the brain, decades passed before the amyloid- β peptide and tau protein were identified in the 1980's. This led to the proposal of the amyloid cascade hypothesis by J. Hardy and G. Higgins in 1992 ¹⁸. The amyloid cascade hypothesis suggested that the initial pathological changes are the extracellular accumulation of the 42 amino acid peptide amyloid- β (A β 42) in amyloid plaques. Downstream consequences hereof are intracellular hyperphosphorylation of tau and accumulation of neurofibrillary tau tangles, causing axonal death, neurodegeneration, and consequently activation of glial cells causing a neuroinflammatory response, see Figure 1.2.2.

Less than 1% of AD cases have an underlying genetic cause ¹⁹. The known genetic forms of autosomal dominant AD have mutations in *APP*, *PSEN1*, and *PSEN2*, causing overproduction of A β 42 and early onset AD (under the age of 65 years), which to some extent corroborate the amyloid

cascade hypothesis²⁰. Most AD cases are considered sporadic, (often late onset, over the age of 65 years), meaning caused by environmental exposures, unknown factors and genetic risk factors, where the strongest common genetic risk factor is the apolipoprotein E variant $\epsilon 4$ (*APOE* $\epsilon 4$). Homozygous *APOE* $\epsilon 4/\epsilon 4$ carriers have a substantially higher risk of developing AD^{21,22}.

Figure 1.2.2 AD neuropathological hallmarks



Visualisation of extracellular accumulation of amyloid plaques and intracellular hyperphosphorylation of tau, leading to the formation of neurofibrillary tangles. These tangles contribute to axonal degeneration and subsequent release of tau, phosphorylated tau, and neurofilaments. The extracellular processes trigger astrocytic activation and release of glial fibrillary acidic protein.

Created in <https://BioRender.com> by FK Clemmensen.

Over the past decades, several modifications of the amyloid-cascade hypothesis have been proposed, suggesting a more complex interplay between the several pathogenic factors contributing to AD²³.

Frisoni et al.²⁴ recently challenged the amyloid cascade hypothesis by proposing a probabilistic model of AD, in which, rather than a single deterministic cascade, multiple variants of the disease may exist. This model outlines three variants of AD: autosomal dominant AD, *APOE* $\epsilon 4$ -related sporadic AD, and *APOE* $\epsilon 4$ -unrelated sporadic AD, in which the degree of penetrance of the amyloid pathway differs and the contributions of stochastic and environmental factors vary. In

genetically driven autosomal-dominant cases, the cascade is more robust and predictable, whereas in sporadic forms, the amyloid pathway plays a more modest role, and non-amyloid influences are more prominent.

Recognizing this probabilistic and heterogeneous model may better account for the clinical and biological variability observed in AD, including why some individuals with amyloid pathology do not progress to dementia and why amyloid-targeting therapies have not been uniformly successful.

The following section provides a more detailed description of AD neuropathological hallmarks.

1.2.2.1 Amyloid

Under normal physiological conditions, the amyloid precursor protein (APP), a transmembrane protein highly expressed in neurons, is cleaved by α -secretase and γ -secretase through the non-amyloidogenic pathway. APP and the non-amyloidogenic fragments are believed to play important roles in synaptic regulation, neuroprotection, and neuronal plasticity ^{25–27}.

However, in AD, APP undergoes amyloidogenic processing, cleaved by β - and γ -secretase, generating β -amyloid peptides in varying lengths, most predominantly 40 (A β 40) and 42 (A β 42) amino acids. The A β 42 peptides have a hydrophobic aggregation prone nature and through successive molecular events, they accumulate into amyloid plaques ²⁸. Deposition of A β plaques occurs in the cerebral cortex starting in neocortical areas, spreading throughout the cerebral cortex in later stages ^{29,30}.

Aggregation of A β has been found to increase with age, and more than 40% of cognitively unimpaired (CU) individuals over the age of 85 being amyloid positive, suggesting, in line with the previous section, that many amyloid-positive individuals may remain asymptomatic and that amyloid deposition alone does not inevitably lead to cognitive decline ³¹.

The deposition of extracellular amyloid plaques may disrupt synaptic communication and activate an immune response resulting in neuroinflammation, which both contribute to neuronal dysfunction and neurodegeneration.

1.2.2.2 Tau

The tau protein is a microtubule-associated protein that plays an important role in the axonal cytoskeleton, as it stabilises the microtubule and ensures vesicle transport to the synapses. Posttranslational modifications such as phosphorylation are important to regulate tau's interaction with the microtubules. Tau undergoes phosphorylation at various phosphorylation sites along the tau protein, most studied in AD at amino acid 181, 217, and 231 ³².

Abnormal hyperphosphorylation of tau causes its detachment from the microtubule. In AD, phosphorylated tau aggregates into dimers and oligomers which form filaments that cluster to intracellular neurofibrillary tangles (see Figure 1.2.2.2). This leads to disintegration of the cytoskeleton and neuronal dysfunction. Accumulation of phosphorylated tau starts in the entorhinal cortex and spreads across the neocortex. The spread of neurofibrillary tangles is used to stage the degree of AD pathology postmortem (Braak stages) ^{33,34}.

Figure 1.2.2.2 Tau phosphorylation, tangle formation and neurodegeneration

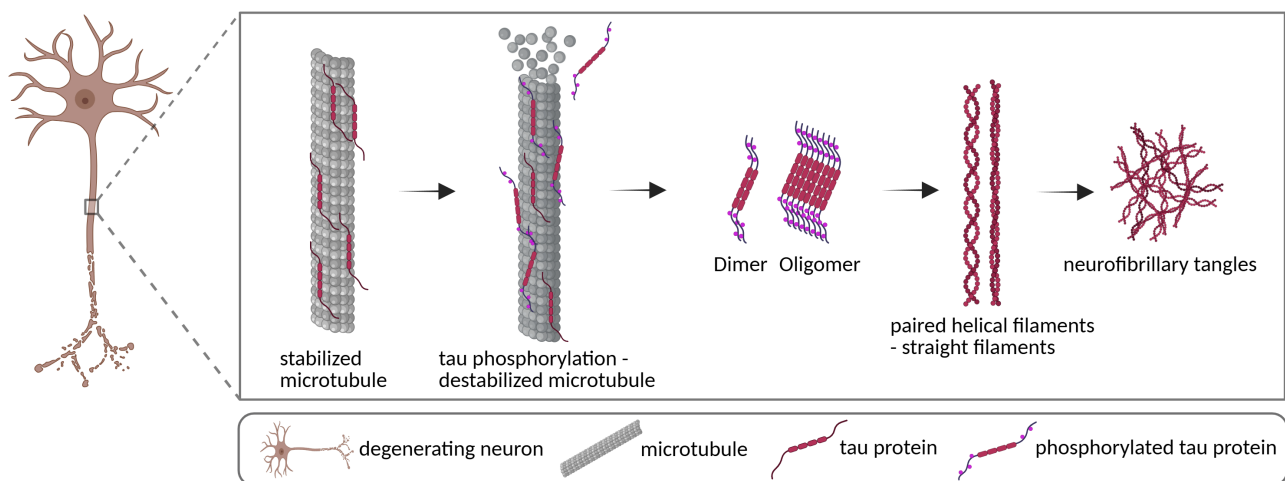


Figure illustrating the intracellular phosphorylation of tau which leads to destabilization of microtubules and detachment of phosphorylated tau proteins. These phosphorylated tau proteins aggregate into dimer, oligomers, and paired helical and straight filaments, ultimately resulting in the formation of neurofibrillary tangles. Created in <https://BioRender.com> by FK Clemmens.

1.2.2.3 Neurodegeneration

As AD progresses, neurodegeneration is considered a downstream effect of amyloid and tau pathology. Neurodegeneration refers to the progressive dysfunction of synapses, loss of neurons and eventually atrophy, which correlate with clinical symptoms. When neurons degenerate, several proteins, including neurofilament light chain (NfL) and tau, are released into the extracellular space.

NfL belongs to the group of intermediate filaments, that contain three subunits according to their molecular weight: neurofilaments light (NfL), medium, and heavy. Intermediate filaments are found predominantly in myelinated axons and play an important role in the structural integrity of axons. NfL is released from degenerating axons upon injury, regardless of the underlying cause of injury. Thus, NfL is considered a non-specific marker of neurodegeneration.

Tau is, as previously mentioned, a microtubule-associated protein, that is released into the extracellular space as the neurons degenerate. Elevated levels of total-tau (t-tau) may indicate neuronal damage. However, like NfL, t-tau is not specific for AD, but rather reflects general neuroaxonal injury across a range of neurological conditions.

1.2.2.4 Neuroinflammation

As the extracellular environment is altered by the primary AD pathologies, glia cells (microglia and astrocytes) become activated, creating an immune response to eliminate these neuropathological changes. Initially, the microglial and astrocyte activation serve as protective mechanisms by enhancing the release of pro-inflammatory mediators while anti-inflammatory molecules are reduced. However, in AD it is believed that the regulators that should normally resolve inflammation are reduced, resulting in chronic neuroinflammation. This chronic phase prompts increase in pro-inflammatory cytokines, complement activation, and oxidative stress, all of which accelerate synaptic loss and neurodegeneration ³⁵.

The extracellular deposits of amyloid plaques and axonal damages activate astrocytes and microglial cells which then release molecules, including glial fibrillary acidic protein (GFAP) and soluble Triggering Receptor Expressed on Myeloid Cells2 (sTREM2). GFAP is predominantly expressed in astrocytes and upregulation of GFAP is considered a response to astrocyte activation triggered by amyloid plaque accumulation, and has been linked to early neuropathological changes in AD ³⁶. TREM2 helps regulate microglial functions, and the soluble form, sTREM2, is considered a marker for microglial activation in various neurodegenerative diseases including AD, and has been linked to amyloid aggregation ³⁷ and tau phosphorylation ³⁸.

1.2.3 Co-pathologies

The presence of co-pathologies such as cerebrovascular pathology, increased levels of α -synuclein, or TAR DNA-binding protein 43 (TDP-43) often co-exist with AD pathology. Up to 30% of AD cases have coexisting cerebrovascular pathology ³⁹. These vascular changes can exacerbate neuronal injury, including small vessel disease, infarcts and microbleeds which may contribute to and accelerate cognitive decline ⁴⁰. Alpha-synuclein, which reflects Lewy body pathology, has been suggested to co-exist in up to 50% of AD patients, and the coexistence with amyloid pathology may synergistically effect neurodegeneration compared with each pathology alone ^{41,42}. TDP-43 aggregates, associated with limbic-predominant age-related TDP-43 encephalopathy (LATE), are found in up to 57% of AD cases and are associated with accelerated memory loss and hippocampal atrophy ^{43,44}.

These co-pathologies contribute to the clinical heterogeneity of AD and may influence both disease severity and progression ⁴⁵⁻⁴⁷.

1.2.4 Treatment

For the past three decades, treatment of AD has been restricted to symptomatic management. In 1993, the first acetylcholinesterase inhibitor (AChEI) named tacrine was approved by the U.S. Food and Drug Administration (FDA) ^{48,49} – it was soon followed by donepezil ^{50,51}, rivastigmine ^{52,53} and galantamine ^{54,55}. These AChEIs inhibit the enzyme acetylcholinesterase, responsible for acetylcholine degradation, thereby increasing the synaptic availability of the neurotransmitter acetylcholine, thus supporting cholinergic signalling. A non-cholinergic therapy, the NMDA-receptor antagonist Memantine ^{56,57}, was introduced in 2003 and approved for moderate to severe dementia. These drugs do not modify underlying disease progression but alleviate symptoms temporarily.

More recently, the development of disease-modifying therapies targeting amyloid- β pathology marks a paradigm shift from managing symptoms to actively slowing disease progression. In 2021, the FDA approved the first disease-modifying monoclonal antibodies, Aducanumab ⁵⁸, followed by Lecanemab ⁵⁹ in 2023, and Donanemab ^{60–62} in 2024. These monoclonal antibodies bind aggregated forms of β -amyloid to facilitate plaque clearance, reduce downstream tau pathology and neurodegeneration, and should consequently slow cognitive decline. Initial real-world evidence on the safety and efficacy of Lecanemab treatment is beginning to emerge ⁶³.

In Europe, the European Medicines Agency (EMA) and the European Commission have approved Lecanemab to patients with early stages of AD who are not *APOE* ϵ 4 homozygote. Donanemab is currently (November 2025) awaiting the final approval from the European Commission, after being approved by the EMA with the same restrictions as Lecanemab.

AD currently affects more than 50 million people worldwide ¹¹. With the aging global population, this number is expected to have doubled by 2050 ⁶⁴. The growing prevalence of AD and the upcoming treatment possibilities will cause increasing pressure on health care systems, emphasising the need of accessible biomarkers supporting both timely diagnosis and treatment initiation.

1.3 What is a biomarker

A biomarker is a natural occurring substance in the body that reflects a normal biological or pathological process. Biomarkers can be any given type of entity that measures a biological signal, i.e. a protein derived from biofluids, e.g. cerebrospinal fluid (CSF) and blood, or hallmarks measured by imaging modalities.

Biomarkers can have several applications: For risk stratification, biomarkers can help identify individuals at risk of developing a disease. In a diagnostic context, biomarkers can confirm the presence of disease pathology. Prognostic biomarkers can provide insight into a likely disease course and rate of progression. Finally, by repeated longitudinal measures, biomarkers can track disease progression or monitor intervention effect. See Figure 1.3.

Figure 1.3 The use of biomarkers

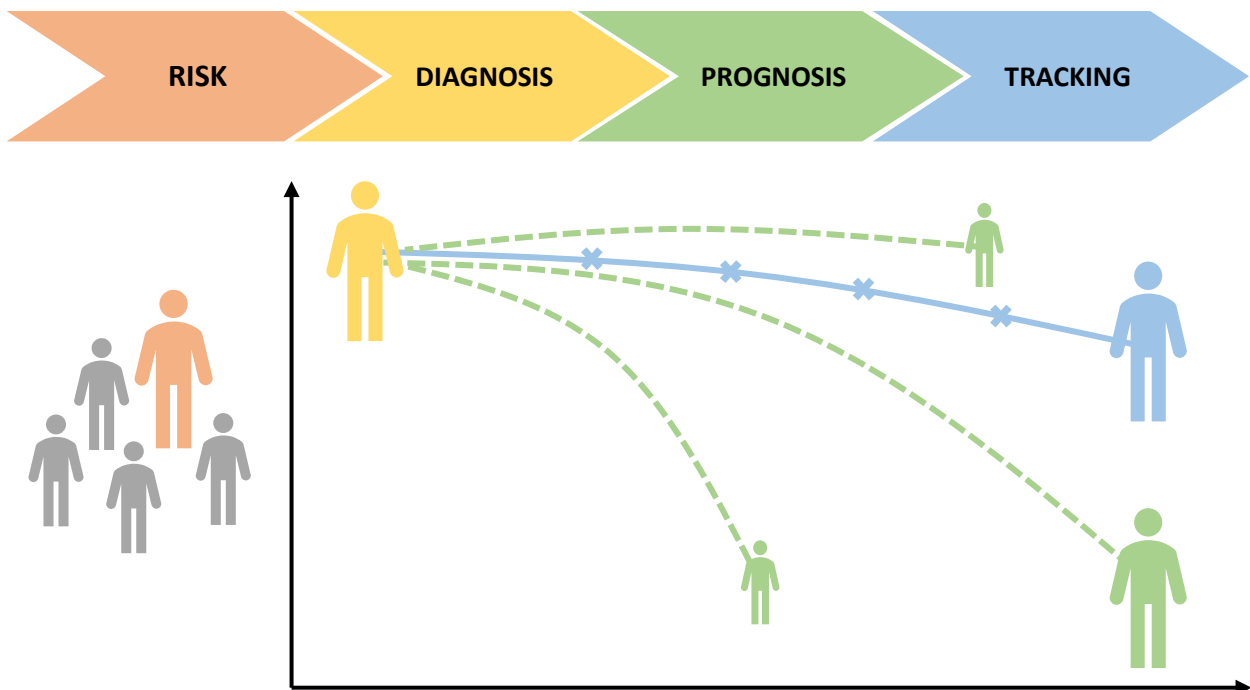


Figure illustrating the various applications of a biomarker: identify individuals at risk, confirm pathology, predict rate of and track disease progression. Created by FK Clemmensen.

In the following, the development of AD biomarkers will be presented, followed by an overview of their current applications.

1.4 Development of AD biomarkers

1.4.1 Imaging

The first use of biomarkers in AD was brain CT scans used to exclude other potential reversible diagnosis such as brain tumours, subdural hematomas, and vascular changes, rather than to confirm AD. The first commercial CT became available in the 1970's, and the first reports of CT scans describing cortical atrophy and ventricular enlargement in patients with AD were published ⁶⁵. In the 1980's, MRI became available for the diagnosis of AD ⁶⁶⁻⁶⁸, also at first to exclude other causes of cognitive problems and later to distinguish AD from other dementia disorders ⁶⁹. Currently, AD signatures on MRI scans include - among others – grey matter atrophy, hippocampal atrophy, and ventricular enlargement ^{70,71}. These changes are believed to reflect underlying neurodegeneration.

With the development of positron emission tomography (PET) tracers targeting A β and tau proteins, imaging technologies moved from displaying downstream neurodegeneration to measuring specific pathological processes. The first amyloid PET ligand was developed in the early 2000s by Klunk et al. who demonstrated increased cortical accumulation of amyloid in patients with AD compared with healthy controls ⁷², hence providing the first direct in vivo evidence of A β plaque burden in the human brain. In the 2010's the first selective tau tracers were developed ⁷³ which made it possible to map tau accumulation in patterns corresponding to the Braak stages – confirming the spatial progression of tau pathology from entorhinal cortex to neocortical regions in AD ^{34,74}. Thus, amyloid- and tau-PET allow for a more spatial reflection of amyloid and tau distribution. Together, imaging biomarkers provide in vivo evidence of structural, functional, and molecular brain changes.

1.4.2 Cerebrospinal fluid

Cerebrospinal fluid (CSF) is produced by the choroid plexus in the lateral ventricles of the brain and it circulates from the ventricular system to the subarachnoid space, where it serves as a proxy for the biochemical state of the brain parenchyma and interstitial fluid. In 1995, Motter et al. ⁷⁵ and Blennow et al. ⁷⁶ developed the first methods to measure reduced A β 42 and elevated tau and phosphorylated tau proteins in the CSF. In 1996, Rosengren et al. ⁷⁷ developed a method to measure NfL in CSF. Several studies have since then investigated and supported the diagnostic superiority of CSF A β 42/40 and p-tau181/A β 42, compared with the individual biomarkers ⁷⁸⁻⁸⁰. Today, CSF biomarkers are widely implemented in clinical practice in specialised units.

Thus, measures of AD biomarkers have long been limited to CSF and imaging modalities, both invasive, costly, associated with discomfort for the patients, and limited to specialised clinical units.

However, the advances in immunoassays and mass spectrometry have led to the discovery of AD biomarkers in blood. In 2010, the novel ultrasensitive single-molecule enzyme-linked immuno-sorbent assay (Simoa) was developed ⁸¹, followed by various assays for blood AD markers developed in the past decade ^{82–84}.

1.4.3 The single molecule array technique

The Simoa technique is an immunoassay technique that uses magnetic beads to capture single molecules, i.e. the desired protein (biomarker). The magnetic beads are coated with antibodies specific to the biomarker (e.g. NfL). The fluid (e.g. plasma, serum or CSF) is diluted to bring concentrations into the assay's dynamic range (the span between the lowest and highest measurable concentration that the assay can reliably quantify) and then added to the magnetic beads that will capture the biomarker if present, see Figure 1.4.3, step 1. Following this, a detection antibody carrying a biotin tag is added, which will bind to the biomarker creating an immune-complex “sandwich” with the biomarker attached to the antibody coated bead and the detection antibody (step 2). Then follows a washing step (to remove unbound reagent and reduce background signals), and the enzyme streptavidin-bound (β -galactosidase (SBG)) is added, forming a bond to the enzyme attached detection antibody (step 3). Finally, beads are added into tiny wells which each fit one magnetic bead. Adding a substrate (resorufin- β -D-galactopyranoside, RGP) to the array (step 4), the immuno-complex produces a fluorescent signal which is digitally counted as “on” / “off” (step 5). The number of “on” beads is then converted to biomarker concentration using a calibration curve from known standards, adjusted for dilution.

Figure 1.4.3 Simoa stepwise procedures

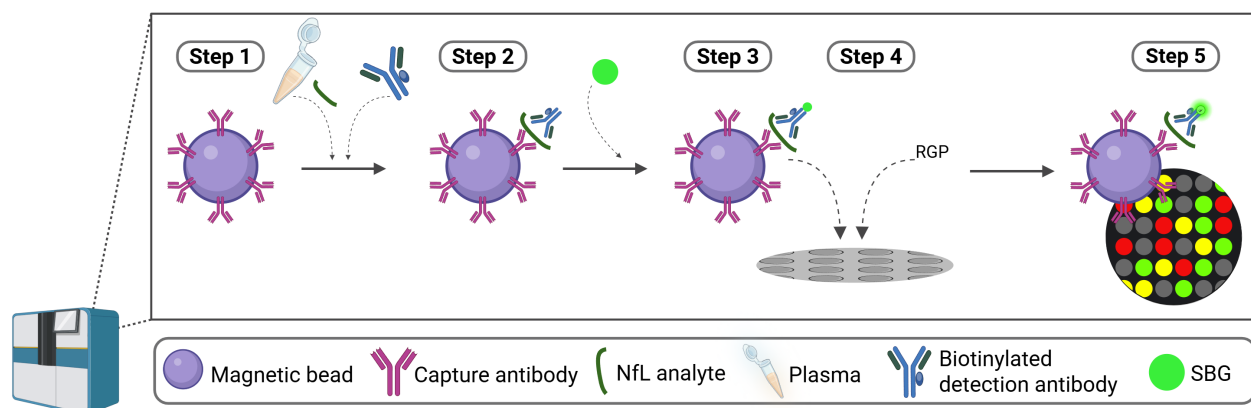


Figure illustrating step-by-step biomarker measurement using the Simoa technique. Step 1: antibody coated magnetic beads, diluted fluid and detection antibody are added together. Step 2: If the biomarker of interest, here neurofilament light (NfL), is present in the fluid, an immune-complex forms between the antibody-coated bead, the analyte, and the detection antibody. Step 3: streptavidin-bound (β -galactosidase (SBG)) enzyme is added, binding to the detection antibody. Step 4: resorufin- β -D-galactopyranoside (RGP) is added to the array. Step 5: if an immunocomplex is present in a well, a fluorescent signal is produced and digitally counted as “on” / “off”. Created in <https://BioRender.com> by FK Clemmensen.

1.5 Integrating a clinical and biological definition of AD

As mentioned, the definition of AD has until recently been based on a clinical diagnosis ^{7,10} and a confirmation of AD pathology was restricted to post-mortem pathological examinations. The abovementioned technologies have paved the way for novel strategies and possibilities which is crucial to developing cost-efficient, non-invasive, easily accessible AD biomarkers, such as blood biomarkers ⁸⁵, see Figure 1.5.

Figure 1.5 Biomarkers from CSF to blood

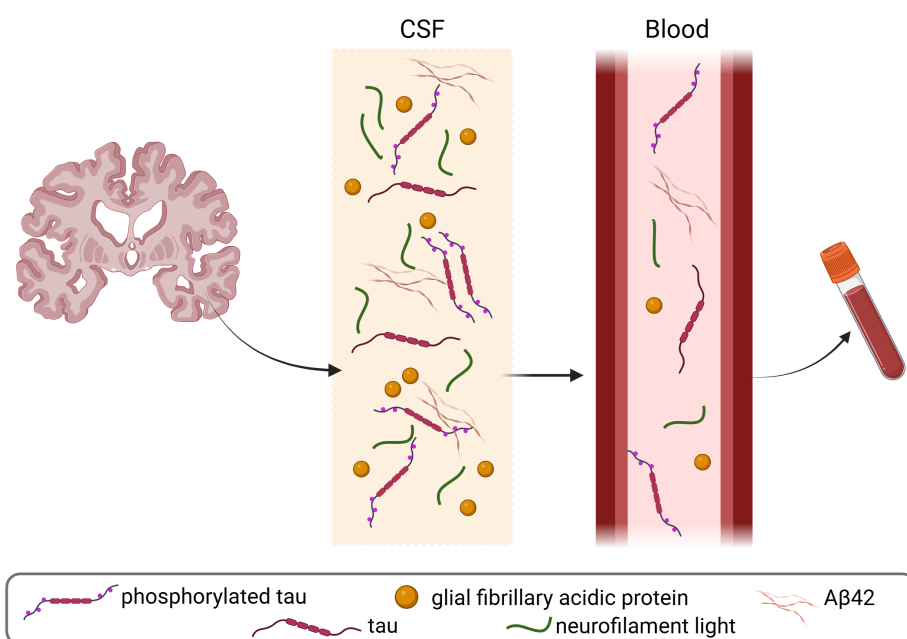


Figure illustrating how pathological proteins associated with AD can be detected in the cerebrospinal fluid (CSF) and subsequently in the blood. Created in <https://BioRender.com> by FK Clemmensen.

In 2016, the AT(N) research framework was proposed ⁸⁶ and in 2018 incorporated into the NIA-AA research framework ⁸⁷. This guideline contrasted with its predecessor by introducing the need for a biological definition of AD to ensure proper diagnosis. Latest, in 2024, the Alzheimer's Association workgroup introduced a biological definition of AD which included detection of neuropathological changes in asymptomatic patients ⁴⁷, hence a biological diagnosis of AD without clinical symptoms. Here, biomarkers are incorporated and classified as core AD biomarkers (reflecting early- (core 1) and late-changes (core 2)) and non-AD specific biomarkers, see Table 1.5.

In the following sections the ATN(I) biomarkers are described in detail across imaging modalities and fluid biomarkers. To briefly clarify, the main difference in the liquid part of blood plasma and serum is the presence of clotting factors, lacking in serum. The term blood will be used to refer collectively to plasma and/or serum, whereas plasma will be used when specifically referring to biomarkers measured in plasma.

Table 1.5 ATN(I) classification of AD biomarkers

AD Core biomarkers		
Core 1	A ($A\beta$)	CSF $A\beta$ 42/40 and p-tau181/ $A\beta$ 42, amyloid PET
	T ₁ (phosphorylated tau)	CSF and plasma p-tau217, p-tau181, p-tau231
Core 2	T ₂ (tau proteinopathy)	CSF and plasma MTBR-tau243, Tau PET
Non-AD specific biomarkers		
N (neurodegeneration, axonal injury)		CSF and plasma NfL, MRI and FDG-PET
I (inflammation)		CSF and plasma GFAP

Table displaying the A (amyloid), T (tau), N (neurodegeneration), I (inflammation) classification system for biomarkers in Alzheimer's disease. Modified from Jack. et al. 2024⁴⁷ by FK Clemmensen.

1.5.1 AD core biomarkers

Amyloid PET measures the $A\beta$ fibrils that aggregates into extracellular plaques, whereas the amyloid detected in the CSF captures the soluble monomeric form of $A\beta$ ⁸⁸. Thus the amyloid PET reflects the cortical distribution of amyloid plaques, and as $A\beta$ 42 aggregates into plaques, $A\beta$ 42 levels in CSF and blood decline, reflecting its deposition in the brain parenchyma^{31,89}. Amyloid PET ligands and assays for CSF $A\beta$ 42/40 and p-tau181/ $A\beta$ 42 are approved for use in clinical practice, see Table 1.5.

However, measuring the core 1 A biomarker, amyloid in blood has proven to be challenging^{90,91}. Several head-to-head comparison studies have showed that measuring plasma phosphorylated tau at amino acid 217 (p-tau217) outperforms plasma amyloid and other phosphorylated tau isoforms (p-tau181, p-tau231) aligning closely with amyloid-PET^{92–94}. Plasma p-tau217 is therefore considered a surrogate marker of amyloid pathology⁹⁵.

Plasma p-tau217, CSF $A\beta$ 42/40, and p-tau181/ $A\beta$ 42 are considered sensitive markers of early amyloid pathology, and altered levels are closely linked to abnormal amyloid PET⁴⁷. In early AD, some tau phosphorylation occurs concomitantly with initial amyloid plaque formation, even before it can be detected by amyloid-PET imaging^{96–101}. At later stages tau PET becomes abnormal¹⁰¹. Soluble phosphorylated tau is measured in CSF and blood, whereas tau-PET imaging detects insoluble, aggregated tau within the neurofibrillary tangles⁹⁶.

In CSF and blood, measures of e.g. the microtubule-binding region tau243 (MTBR-tau243) are elevated concomitantly with tau PET^{102,103}, both reflecting the development of neurofibrillary tangles and more closely linked to the development of cognitive symptoms. Thus, this dual reflection of tau biomarkers paved the way for suggesting two tau categories: T1 reflecting soluble tau

fragments measured by plasma p-tau217 linked to early amyloid pathology, and T2 reflecting aggregation of tau in neurofibrillary tangles at later stages as measured by tau PET ^{104,47} (see Table 1.5).

To summarise, several promising fluid markers for amyloid pathology (A and T₁) are emerging and further discussed in section 1.6.1. While MTBR-tau243 is a promising fluid biomarker that closely reflects tau pathology, tau-PET remains the only clinically approved method for detecting tau pathology (T₂). Thus, we still lack a validated and available fluid biomarker for tau tangles.

Notably, p-tau217 is not pathognomonic for AD, and elevated levels are observed in several other neurological diseases such as Creutzfeldt-Jacob's disease and Niemann Pick disease, and in newborns ^{99,100,105}.

1.5.2 Non-specific biomarkers related to AD pathology

1.5.2.1 Biomarkers of neurodegeneration

Imaging biomarkers of neurodegeneration in AD include atrophy detected by structural MRI in specific regions such as the medial temporal lobe and hippocampus, and glucose hypometabolism in cortical areas such as temporal and parietal measures by [¹⁸F]-fluorodeoxyglucose positron emission tomography ([¹⁸F]FDG-PET) ¹⁰⁶. MRI visualises brain atrophy, particularly in the medial temporal lobe and hippocampus, which correlates with neuronal loss and disease progression ^{71,107}. [¹⁸F]FDG-PET measures cerebral glucose metabolism with characteristic temporoparietal hypometabolism in AD ¹⁰⁶.

In CSF and blood, measures of NfL reflect axonal injury in AD ^{108,109}, but like the imaging markers, NfL increase is not specific to AD and altered levels also occur in other neurodegenerative diseases, vascular disease, multiple sclerosis, traumatic brain injury, peripheral neuropathy etc. ^{110,111}.

Not included in Table 1.5 are other potential neurodegenerative fluid biomarkers such as CSF and plasma total tau (t-tau) and plasma brain derived tau (BD-tau). T-tau was removed from the revised criteria ⁴⁷ and while tau measured in CSF correlates with tau-PET, plasma t-tau levels show weak or inconsistent association with tau-PET. This discrepancy may arise partly due to increasing levels of t-tau in other brain disorders (i.e. Creutzfeldt-Jacob disease, trauma and peripheral neuropathy ^{112,99}) and due to contribution of tau from peripheral tissues, such as the liver, kidney, and heart ¹¹³. Levels of blood tau are therefore not limited to the contribution from the central nervous system. The peripheral produced tau isoform is distinct from the six CNS specific isoforms as it contains an additional amino acid insert, extending the length of the peripheral produced tau. In 2023, an assay measuring brain-derived tau (BD-tau), hence measuring the six CNS specific isoforms, was

developed ¹¹⁴. BD-tau has shown promising results reflecting neurodegeneration and being less influenced by confounders, i.e. kidney dysfunction ¹¹⁵.

1.5.2.2 Biomarkers of neuroinflammation

Inflammatory biomarkers currently include markers of astrocytic and microglia activity. GFAP, released by reactive astrocytes can be measured in CSF and blood, the latter seemingly performing better in identifying A β pathology in early AD stages ¹¹⁶. Recently, a study has suggested several limitations of plasma GFAP questioning its interpretation as an astrocytic marker ¹¹⁷. Reflecting microglial activity, sTREM2 can be measured in CSF interpreted as an immediate response to amyloid aggregation in AD ³⁷.

As this thesis centres on plasma p-tau217, NfL, BD-tau, and GFAP, the subsequent sections will primarily address these biomarkers.

1.6 Using blood biomarkers in clinical context

1.6.1 Diagnosis

Working towards clinical implementation of diagnostic blood biomarker for AD, two assays have in 2025 been approved for clinical use: Roche's Elecsys p-tau181 plasma assay, by the European Medicines Agency and the FDA, and Fujirebio's Lumipulse p-tau217/A β 1-42 plasma assay by the FDA. These are the first blood biomarkers approved to rule out an AD diagnosis, just like CT and MRI scans at first. More specifically, these novel biomarkers rule out the presence of amyloid pathology. This will help guide clinicians to determine which patients require additional assessments such as PET scans or CSF analysis.

However, implementation of novel biomarkers into the diagnostic workflow in both primary care settings and specialised memory clinic units, calls for guidelines and strategies to ease this transformation, and to reduce the potentially high number of patients referred to confirmatory testing. In 2023, Brum et al. suggested the use of a two-way cut-off approach for plasma p-tau217 ¹¹⁸. Instead of relying on a single diagnostic threshold, this relies on two thresholds: a low and a high cut-off, below and above which amyloid pathology is considered unlikely and highly probable, respectively. Individuals in the intermediate zone, i.e. with p-tau217 levels within these two limits, should be referred to additional diagnostic testing, i.e. confirmatory CSF or PET. Palmqvist et al. ¹¹⁹ have reported promising diagnostic accuracy when applying this predefined two-way cut-off across several specialised memory units and in primary care, demonstrating the feasibility in diagnostic workflows.

Figure 1.6 visualises how AD biomarkers could be incorporated into clinical context, with the potential of identifying patients at risk (preclinical stage) of developing dementia prior to clinical onset. The next section focuses on how biomarkers may be used in the clinical manifest stages of AD, i.e. in individuals with MCI and dementia.

Figure 1.6 Incorporating biomarkers in the clinical AD continuum

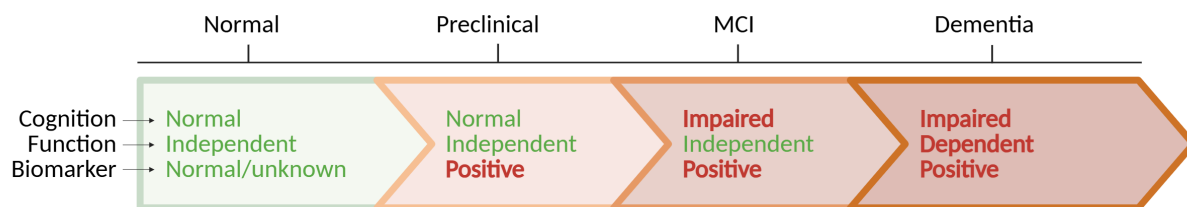


Figure illustrating the timely application of biomarkers in the clinical AD continuum. MCI, mild cognitive impairment. Created in <https://BioRender.com> by FK Clemmensen.

1.6.2 Prognosis & tracking

While several ATN(I) blood biomarkers have been studied broadly in clinical cohorts to assess their diagnostic value, studies assessing their prognostic and monitoring abilities in clinical AD cohorts remain limited and not fully elucidated. Valid prognostic markers are essential for predicting the rate of disease progression, which is crucial for guiding treatment decisions and planning individualised care. Robust biological staging of dementia, covering MCI, mild, moderate, and severe dementia, using blood biomarkers can enhance the monitoring of disease progression by objective and biological measures. And as disease-modifying treatment emerges, there is an increasing need of prognostic and disease monitoring biomarkers to track treatment effect and safety.

Ideally, prognostic and disease tracking biomarkers should reflect real-world disease progression and patient outcomes, be linked to concrete endpoints, and be robust and reproducible across the disease continuum, different populations and clinical settings.

Table 1.6.2 summarises key studies on AD blood biomarkers, with particular emphasis on their prognostic potential and utility in tracking disease progression.

The table was generated through a PubMed search for publications evaluating the diagnostic, prognostic, and disease-tracking potential of the four blood biomarkers of interest in this thesis, p-tau217, NfL, BD-tau, and GFAP. Studies included as prognostic studies required a baseline blood biomarker measurement with subsequent longitudinal clinical or neuroimaging follow-up, whereas disease tracking studies required repeated longitudinal measurements of blood biomarkers alongside corresponding cognitive data.

Table 1.6.2 Summary of AD blood biomarker key studies

	References	Diagnosis	Prognosis	Tracking	Cohort(s)
p-tau217	Mattsson-Carlgren et al. 2020 ¹²⁰			Steeper p-tau217 increase in AB+ compared to AB- over six years. p-tau217 increase correlated with worsening in cognition and brain atrophy over time.	BioFINDER CU n=150, MCI n=100
	Palmqvist et al. 2020 ⁹²	Plasma p-tau217 discriminate AD from non-AD like CSF p-tau217 and tau-PET.			Three cohorts n=1402 (CU, MCI, AD , non-AD neurodegenerative diseases)
	Milà-Alomà et al. 2022 ⁹⁸	Plasma p-tau217 change in preclinical stage of AD.			CU n=397
	Jonaitis et al. 2023 ¹²¹		Baseline plasma p-tau217 was associated with 5 years change in cognition.		CU n=173
	Mendes et al. 2024 ⁹³	Plasma p-tau217 discriminates amyloid- and tau-PET positive from negative like CSF p-tau217.			CU n=33, MCI n=67, AD n=14
	Gonzalez-Ortiz et al. 2025 ¹²²	Plasma p-tau217 identifies AB+ individuals among CU.	Plasma p-tau217 predicts cognitive decline in AB+ individuals.		DDI: SCD and MCI n=431. PREVENT-AD: CU and MCI n=190
	Kirsebom et al. 2025 ¹²³		Baseline p-tau217 was associated with up to 3 years progression in cognitive decline.	Longitudinal increase in plasma p-tau217 in rapid decliners.	AB+ CU and MCI. Two cohorts: CU/SCD n=71, MCI = 115, SCD n=63, MCI n=144
	Yakoub et al. 2025 ¹²⁴		p-tau217 could predict progression from CU to MCI within 10 years.		CU n=228, 7.7 years FU
	Sanchez et al. 2025 ¹²⁵		Baseline p-tau217 was associated with two years regional MRI changes in AD/MCI.		N = 47, AD /MCI n=126
	Insel et al. 2025 ¹²⁶		Baseline plasma p-tau217 predicts cognitive decline.	Longitudinal increase in plasma p-tau217 in AB+. A modest correlation between p-tau217 and concurrent cognitive changes.	Multicentre study CU n=1707
Plasma NFL	Ossenkoppele et al. 2025 ¹²⁷		Baseline plasma p-tau217 was associated with up to 3.8 years cognitive decline and progression to MCI.		Multicentre study CU n=1474
	Salvadó et al. 2025 ¹²⁸	Plasma p-tau can identify amyloid pathology in CU.			CU n=2916
	Mattsson et al. 2017 ¹⁰⁸	Plasma NFL discriminates AD from CU.	Baseline plasma NFL was associated with 4 years worsening in cognitive test scores, larger ventricular volume, smaller hippocampal volume, and thinner cortex.	Over 12 months, increased NFL was associated with decline in cognitive and neuroimaging measures.	ADNI CU n=193, MCI = 197, AD = 180
	Mielke et al. 2019 ¹²⁹		Baseline NFL was associated with 15-30 months change in cognitive and neuroimaging measures.	Longitudinal change in plasma NFL was associated with change in cognition and amyloid PET.	CU n=79
	Mattsson et al. 2019 ¹³⁰			NfL increased over a 4–11-year period with greater rate in AD compared to MCI and CU, and in MCI compared to CU.	ADNI CU n=401, MCI n=855, AD = 327
	Ya-Nan et al. 2019 ¹³¹			Plasma NFL increased and the rate of change was associated with cognitive and neuroimaging changes, no info on FU length.	ADNI CU n=291, MCI n=458, AD n=86
	Chatterjee et al. 2021 ¹³²			NfL increased over 12 months in AB- individuals.	CU n=76
	Marks et al. 2021 ¹³³		Baseline plasma NFL was associated with six years change in cognitive and neuroimaging measures.		CU n=995
	De Meyer et al. 2023 ¹³⁴		Baseline NFL predicted up to approx. 6 years future cognitive decline and MRI atrophy in AB+ compared to AB-.		CU n=185

	References	Diagnosis	Prognosis	Tracking	Cohort(s)
	Yakoub et al. 2023 ¹³⁵			NfL increases over time, no difference in 4-year rate of increase in plasma NfL in PET AB+ and tau+ compared to negative.	CU n=373
	Silva-Spínola et al. 2023 ¹³⁶		Higher baseline NfL in individual who progressed to dementia. NfL predicts progression from MCI to dementia over approx. 6 years.		MCI n=116
	Martino-Adami et al. 2024 ¹³⁷		Baseline plasma NfL was associated with an 8-year increased risk of progression to clinical diagnosed AD.	Plasma NfL increased more rapidly over 4 years in individuals who progressed to clinically AD.	Primary care elderly without dementia CU and MCI n=1007 (FU n=305).
	Joynes et al. 2024 ¹³⁸			Plasma NfL increased steeper in men vs. women (FU approx. 7 years).	AB+ CN n=42, AB+ MCI n=14, AB+ Dementia n=22)
	Götze et al. 2025. ¹³⁹		Baseline plasma NfL was associated with decline in MMSE score over 60months.		AD n=158, DLB n=30, FTD n=32, non-neuro-deg. n=59, SCD n=41
	Qiu. et al. 2025 ¹⁴⁰		Baseline NfL was associated with 48 weeks change in CDR-SB and in bilateral lateral ventricles and mid-temporal MRI volumes.	Increasing NfL over 48 weeks was associated with worsening in CDR-SB.	Clinically probable AD n=319 from a phase 2 CT with troriluzole.
	Musaeus et al. 2025 ¹⁴¹		Baseline NfL predicted one year decline in MMSE.		AD dementia n=247
	Sanchez et al. 2025 ¹²⁵		Baseline NfL was associated with two years regional MRI changes in AD/MCI.		HC n = 47, AD /MCI n=126
Plasma BD-tau	Gonzalez-Ortiz et al. 2023 ¹¹⁴	Plasma BD-tau discriminates AD from non-AD neurodegenerative diseases.			AD n=42, non-AD n=18 with post-mortem neuropathological examination
	Gonzalez-Ortiz et al. 2024 ¹¹⁵		Baseline BD-tau was associated with longitudinal worsening in cognitive tests and MRI atrophy.		cohort 1: CU n=221, MCI n=143, cohort 2: CN/MCI/ AD n=370, cohort 3: CN n=42, AD n=48
Plasma GFAP	Chatterjee et al. 2021 ¹³²	Plasma GFAP discriminates AB+ from AB- CU elderly.		GFAP increased over 12 months in AB+ individuals.	CU n= 76
	Cicognola et al. 2021 ¹⁴²		Plasma GFAP predicted conversion to AD dementia over approx. 5-year.	Plasma GFAP increased more in AB+ MCI patients who converted compared with AB- stable MCI.	MCI n=160
	Yakoub et al. 2023 ¹³⁵			Up to 4-year increase in plasma GFAP in CU PET AB- and tau-positive compared to negative. GFAP increase faster in woman compared to men.	CU n=373
	Silva-Spínola et al. 2023 ¹³⁶		Higher baseline GFAP in individuals who progressed to dementia. GFAP predicts progression from MCI to dementia over approx. 6 years.		MCI n=116
	De Meyer et al. 2024 ¹³⁴		Baseline GFAP predicted up to approx. 6 years future cognitive decline and MRI atrophy in AB+ compared to AB-.		CU n=185
	Martino-Adami et al. 2024 ¹³⁷		Baseline plasma GFAP was associated with an 8-year increased risk of progression to clinical diagnosed AD.	Plasma GFAP increased more rapidly over 4 years in individuals who progressed to clinically AD.	Primary care elderly without dementia: CU/MCI n=1007 (FU n=305).
	Peretti et al. 2024 ¹⁴³		Baseline plasma GFAP was correlated with more than 1-year cognitive decline.		Memory clinic subjects n=122
	*Guo et al. 2024 ¹⁴⁴	Increased levels of plasma GFAP 10 years prior to dementia.			UK Biobank adults without dementia n=52,645
	Qiu. et al. 2025 ¹⁴⁰		No association between baseline GFAP and 48 weeks change in clinical or MRI measures.	No association between 48 weeks change in plasma GFAP with changes in CDR-SB.	Clinically probable AD n=319 from a phase 2 CT with troriluzole
	Sanchez et al. 2025 ¹²⁵		Baseline GFAP was associated with two years regional MRI changes in AD/MCI.		HC n = 47, AD /MCI n=126

Table outlining studies on the diagnostic, prognostic and disease tracking abilities of AD blood biomarkers. AD, Alzheimer's disease; MCI, mild cognitive impairment; SCD, subjective cognitive decline; CN, cognitive normal; HC, healthy controls; DLB, Dementia with lewy bodies; n, number; p-tau, phosphorylated tau; NfL, neurofilament light; BD-tau, brain-derived tau; GFAP, glial fibrillary acidic protein; MRI, magnetic resonance imaging. *This study more accurately identifies increased levels of GFAP as a risk marker of future dementia, rather than a diagnostic marker.

This overview highlights a growing consensus across studies that plasma p-tau217, NfL, and GFAP show promise as biomarkers for prognostication and monitoring of AD progression. Notably, most existing studies are conducted in CU individuals, primarily assessing the risk of conversion to MCI or AD. Consequently, in preclinical cohorts, the prognostic utility of these biomarkers may more accurately reflect risk prediction rather than progression within the clinical AD continuum. Furthermore, studies involving individuals with AD dementia tend to compare biomarker trajectories across groups, i.e. CU, MCI, and dementia, rather than examining heterogeneity in biomarker dynamics within the AD dementia population itself. This highlights the first knowledge gap that this thesis aims to address:

Knowledge gap 1)

Limited data on blood biomarkers' prognostic and disease tracking abilities within the clinically manifest stages of AD.

The foundation for studying longitudinal changes in blood biomarkers lies in the fundamental work of examining the variation of biomarkers in blood, to ensure clinically relevant interpretation of changes in biomarker levels. This will be outlined in the next section.

1.7 Challenges interpreting blood biomarker levels

Measuring CNS specific proteins in the blood is a challenging task and awareness of factors influencing blood levels are important ¹⁴⁵. Variability in biomarker levels may cause misclassification of patients and challenge longitudinal measures of changes in biomarker levels.

At any given time, the concentration of a blood biomarker reflects the dynamic balance between its rate of production, its release into the blood stream, and how fast it is cleared from the blood again. Production rate is of course a consequence of the underlying pathological changes, i.e. a large cerebral infarct causes higher NfL levels compared with the more chronic nature of neurodegeneration cause by AD. The release from CNS to the blood stream is mainly influenced by the blood brain barrier's (BBB) permeability and transport system. Impaired clearance is – among other factors – caused by kidney dysfunction, resulting in altered removal of proteins and thereby build-up of the proteins in the blood.

Preanalytical factors such as sampling time point, tube type, handling and storage of the blood, as well as analytical factors (assay variability, sensitivity) may also influence blood biomarker levels ^{146–151}. Together, these factors can affect the stability of blood biomarker levels.

1.7.1 Confounding physiological factors

Several confounders, such as kidney dysfunction, body mass index (BMI), fasting status, and diurnal fluctuations, have further been reported to impact biomarker levels and should be considered in the perspective of clinical use of AD blood biomarkers ^{145,152}. The impact of these factors on biomarkers levels are summarised in Table 1.7.1 This summary is based on a PubMed search for publications examining how the abovementioned factors impact blood levels of p-tau217, NfL, and GFAP, which are of most interest in this thesis. BD-tau was not included due to the limited available studies on this novel blood biomarker. In addition, a peripheral production or release of tau have been observed in prostate cancer ^{153,154}, and increased blood NfL levels can also be caused by damaged peripheral nerves due to e.g. neuropathy or trauma ^{155,156}.

Table 1.7.1 Summary of confounders that may influence biomarker levels

Confounders	p-tau217	NfL	GFAP
Increasing age	-	↑ ^{157–159}	↑ ¹⁵⁹
Impaired kidney function	↑ ^{160–162}	↑ ^{163–167}	↑ ^{163,167}
High BMI	↓ ^{163,168–170}	↓ ^{159,163,168,170}	↓ ^{159,163,170}
Recent food intake		↑ ¹⁷¹	↑ ¹⁷¹
Diurnal fluctuation	Lowest levels morning, highest afternoon/early evening ¹⁷² No diurnal fluctuation ¹⁷³	Lowest levels morning, peak during sleep ¹⁷² No diurnal fluctuation ¹⁷³	No diurnal fluctuation ^{172,173}

Table outlining studies examining potential confounders that may influence AD blood biomarker levels. ↑ = blood levels increase as the confounder increases, ↓ blood levels decrease as the confounder increases, – no clear evidence.

1.7.2 Biological variation

Biological variation is a concept in clinical biochemistry assessing the variation of a given biomarker's level due to the individual physiology. Biological variation refers to within-subject and between-subject variability, typically based on non-affected individuals. Within-subject variability captures fluctuations in a biomarker's concentration around an individual's reliable normal value, whereas between-subject variability reflects the differences in biomarker across individuals within a group ^{174,175}.

The reference change value (RCV) defines the minimum significant difference needed between two consecutive samples from the same individual, to reliably distinguish a pathological change from within-subject and analytical variation.

Further, depending on the within-subject and analytical variation, it is possible to estimate the minimum number of samples required from an individual to determine their homeostatic set point, i.e. their reliable normal level, denoted as the Number of samples needed to obtain Homeostatic Set Point (NNHS) ¹⁷⁶.

Few studies have previously examined the biological variation of AD blood biomarkers in younger healthy individuals ^{177–180}. However, biomarker variability observed in nonaffected younger individuals may not directly translate to memory clinic patients, in which plasma biomarker levels are likely influenced by a more complex interplay of age-related changes, genetic variation, comorbidities, and the underlying neurodegenerative process itself.

In 2022, Hansson et al. ¹⁸¹ highlighted research priorities within the field of AD blood biomarkers, one being a “Study of other variables relevant for real-world implementation, such as biological variation over time (intra-day, between-day variation) in individuals with different conditions” ¹⁸¹.

Other medical fields have implied that biological variation in healthy individuals does not necessarily translate into biological variation in individuals affected by the target condition and have suggested that disease-related factors may influence biological variation ^{182,183}. Moreover, RCVs established from healthy populations may exhibit reduced range compared with RCVs derived from a disease population ¹⁸⁴. This discrepancy can lead to misinterpretation of biomarker changes as clinically meaningful, when they may instead reflect the broader biological variability present in the disease population. Factors such as disease mechanisms, treatment effects, and individual physiological responses may contribute to this increased variability, underscoring the importance of context-specific reference values. In addition, on group level, between-subject variation may be influenced by disease heterogeneity, co-pathologies, comorbidities, genetics, and lifestyle factors. These challenges underscore the need to investigate variability in individuals with AD, rather than relying on data from healthy populations.

Figure 1.7.2 highlights factors that may influence within- and between-subject variability in blood biomarkers in memory clinic patients with AD.

Figure 1.7.2 Factors influencing blood biomarker variability

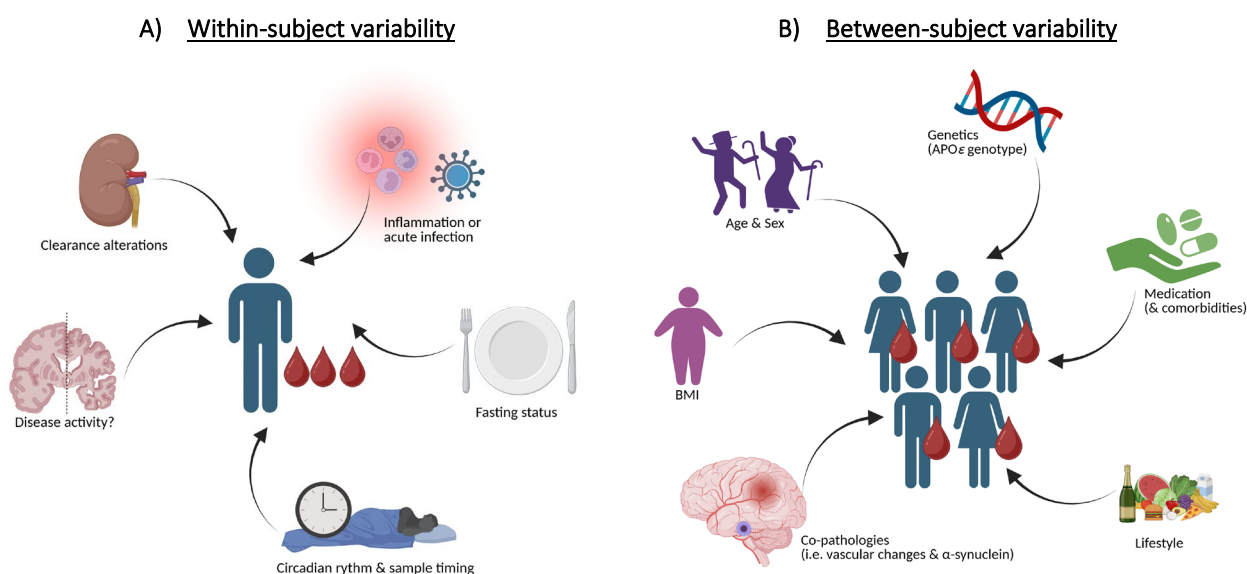


Figure A) illustration of factors that may influence variability in blood biomarker levels across repeated measures within the same individual. Figure B) illustration of factors that may influence the variability in blood biomarker levels between individuals in a group. Created in <https://BioRender.com> by FK Clemmensen.

To acknowledge the distinction regarding the conceptual meaning of biological variation, based on healthy populations, from variation in affected populations, e.g. memory clinic patients, we referred to intra- and inter-individual variability in studies I-III. However, this synopsis consistently uses within- and between-subject to enhance readability and avoid misunderstanding between the pre-fixes *intra-* and *inter-*.

Taken together, several factors may contribute to variability in blood biomarker levels, which can have important implications for clinical applications at both individual and group level:

At an individual level, variability may:

- 1) Lead to misclassification of biomarker levels as normal or abnormal.
- 2) Challenge the interpretation of changes between repeated measurements, potentially obscuring true disease-related changes.

At group level, while some degree of variation must be expected, excessive variability can:

- 3) Hinder establishment of reliable diagnostic thresholds
- 4) Obscure disease staging criteria
- 5) Challenge the ability to monitor treatment effects or interventions.

Therefore, accounting for biomarker variability is essential to prevent misinterpretation and to ensure that observed changes reflect true pathological processes rather than biological or methodological noise. This leads to the second knowledge gap of this thesis:

Knowledge gap 2)

Within- and between-subject variability of AD biomarkers in memory clinic patients is yet to be investigated, and it remains unexplored whether variability in blood biomarkers may mask true disease progression or the effects of interventions.

To summarise, blood biomarkers may exhibit distinct prognostic and disease tracking characteristics in patients within the clinically AD continuum compared with those in the preclinical phase. Moreover, blood biomarkers are susceptible to various confounders that contribute to variability in measured levels. This variability may consequently mask true signals of disease progression or treatment response, complicating both clinical interpretation and research outcomes. Ideally, a robust biomarker should be resilient to such confounders or alternatively, strategies to account for these influences are needed when interpreting biomarker data.

2. Aims

2.1 Overall aim

The overall aim of this PhD project has been to investigate the abilities of plasma biomarkers to predict and track disease progression in patients with early stages of Alzheimer's disease. The foundation for studying this relies on understanding variability in plasma biomarker levels.

2.2 Study-specific aims

Study 1: To explore short-term within- and between-subject variability of various plasma AD biomarkers in memory clinic patients with and without a clinical diagnosis of AD. And to estimate the biomarker specific reference change value.

Hypothesis: Plasma biomarker levels are stable in memory clinic patients during a short period of time.

Study 2: To improve biomarker stability by normalising plasma p-tau217 to BD-tau and to assess whether this further improves the differentiation between amyloid positive and negative memory clinic patients.

Hypothesis: The plasma p-tau217/BD-tau ratio reduces within- and between subject variability compared to p-tau217 alone.

Study 3: To investigate if baseline and short-term changes in plasma biomarker levels can predict clinical and neuroimaging progression in patients with early stages of AD over a two-year period.

Hypothesis: Baseline and short-term changes in plasma biomarkers predict clinical and neuroimaging AD progression.

Study 4: To evaluate whether plasma biomarkers can track clinical and neuroimaging progression of AD over a two-year period.

Hypothesis: Trajectories of plasma biomarkers follow concurrent changes in clinical and neuroimaging measures.

3. Methods

3.1 Study design

This thesis is based on data from two clinical studies carried out at the memory clinic, Rigshospitalet Copenhagen:

- 1) Short-term variability in novel biomarkers for neurodegenerative diseases (SHORT-ND)
- 2) Tracking Alzheimer's disease: a study of disease trajectory and development of diagnostic and disease stage biomarkers in AD (TRACK-AD)

The SHORT-ND study provided data for Study I and II, while Study III and IV were based on the TRACK-AD study. Both cohorts were recruited and characterised by the PhD student together with her co-PhD student, MD Mathias Holsey Gramkow.

The methods applied in Studies I-IV are outlined in the following section. Please refer to the original papers in the appendices Studies I-IV for further details ¹⁻⁴.

3.1.1 The SHORT-ND study

The SHORT-ND study was designed as a test-retest variability study carried out at the Memory clinic, Rigshospitalet between January 2022 and May 2023. We included patients who were referred to the Memory Clinic, Rigshospitalet and who were further scheduled for a lumbar puncture for CSF analysis of AD biomarkers, requested by a physician. Immediately following the lumbar puncture, the first blood sample was collected, and two additional blood samples were collected at visit 2 and 3, carried out 4-16 and 19-36 days after the lumbar puncture, respectively ^{1,2}.

See Figure 3.1 for inclusion- and exclusion criteria and study flow.

3.1.2 The TRACK-AD study

The TRACK-AD project was a prospective longitudinal study carried out at the Memory Clinic, Rigshospitalet, between January 2022 and November 2024 ^{3,4}.

Patients with a diagnosis of MCI or mild-moderate dementia due to AD, according to McKhann et al. and Albert et al. ^{7,10}, were included if they fulfilled the study's inclusion and exclusion criteria, see Figure 3.1. At the inclusion we collected data from the diagnostic assessments at the Memory Clinic. This included data from cognitive testing, data on comorbidities, medications, weight, blood pressure and routine blood screening. A baseline blood sample was collected at the patients' initial visit to the Memory clinic and stored in the Danish Dementia Biobank if consent to this was given.

If baseline cognitive testing was performed more than 4 months prior to inclusion, or if the blood sample was collected more than 3 months prior to inclusion, the cognitive tests and/or blood sampling were repeated at the time of inclusion. Further data from their diagnostic [^{18}F]FDG-PET/MR or [^{18}F]FDG-PET/CT scan was collected, together with data from CSF analysis, if available ^{3,4}.

The follow-up visits were carried out 3-5 months, 12, and 18-24 months after inclusion. Each follow-up visit was carried out by a study investigator and a blood sample was collected with cognitive tests including the Mini-Mental Status Examination (MMSE) and Addenbrooke's Cognitive Examination (ACE), covering global cognition. The semi-structured interview, the Clinical Dementia Rating (CDR), that covers both functional and cognitive testing, was performed by the study investigator interviewing the primary caregiver followed by testing and interviewing the patient. Based on all clinical information collected through the follow-up visit, the study investigator classified the patient as either MCI, mild, moderate or severe dementia, according to the NIA-AA and WHO's ICD-10 classification ^{8,10}. In addition, we checked blood pressure and weight, and a medication review was carried out and medication adjusted if indicated. The control [^{18}F]FDG-PET/MR was performed as part of the 12 months follow-up visit ^{3,4}.

After the study ended, patients were classified as MCI stable (patients with MCI who remained MCI), MCI converters (MCI patients who progressed to dementia), dementia stable (patients with dementia who stayed at the same severity stage: mild, moderate, or severe), and dementia progression (patients with dementia who advanced to a more severe stage). Due to the limited sample size, these groups were subsequently combined into two broader categories: *stable* and *progressors* to enhance statistical power ^{3,4}.

Similar for both studies, all patients underwent a thorough diagnostic assessment program (see Figure 3.1) and were diagnosed at a multidisciplinary team conference ¹⁸⁵. A more detailed description of the assessment program carried out in the Memory Clinic, Rigshospitalet, can be found in Studies I-IV in the appendices ¹⁻⁴.

Figure 3.1 Study flows of the SHORT-ND & TRACK-ND

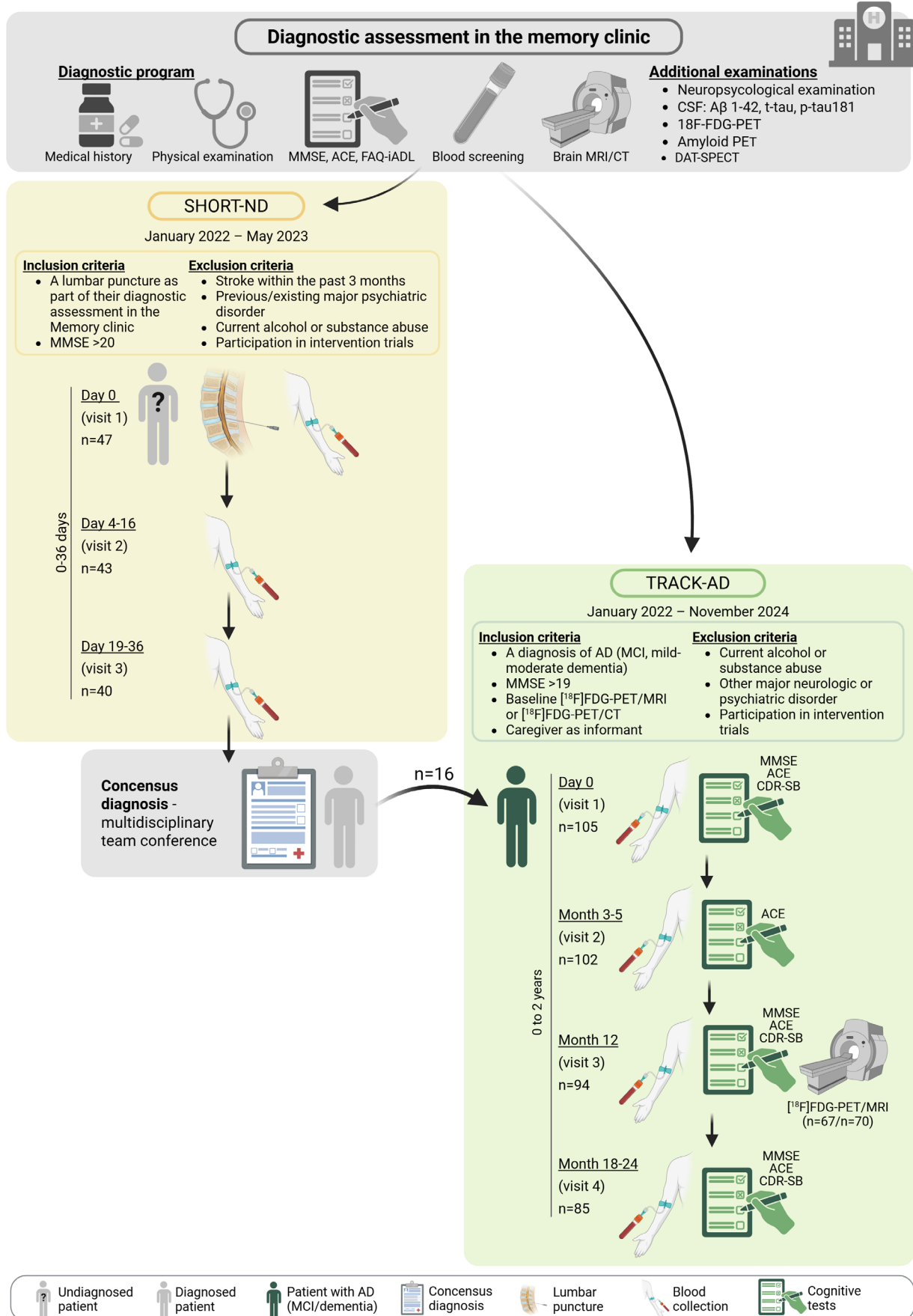


Figure of patient flow in the memory clinic, recruitment and study design of SHORT-ND and TRACK-AD. N=16 AD patients from SHORT-ND were included in TRACK-AD. Based descriptions of study designs in Studies I-IV¹⁻⁴. Created in <https://BioRender.com> by FK Clemmensen.

3.2 Blood test sampling and analysis

All blood samples collected in SHORT-ND and TRACK-AD were collected in daytime between 9AM and 3PM. Blood was collected in ethylenediaminetetraacetic acid (EDTA)- plasma tubes and within 30 to 120 minutes after collection, centrifuged at 2,000g, 4°C for 10 minutes. Plasma was aliquoted into 250µL and frozen at -80°C¹⁻⁴.

All samples were transferred to and analysed at the Department of Psychiatry and Neurochemistry, The Sahlgrenska Academy, University of Gothenburg. Samples from SHORT-ND were analysed from November 2023 to February 2024 and in December 2024. Samples from TRACK-AD were analysed from December 2024 to January 2025.

All analyses were performed with the single molecule array technique (SIMOA) by Quanterix HD-X¹⁸⁶. Samples from SHORT-ND were analysed with the multiplex Neurology 4-plex E assay from Quanterix (Aβ40, Aβ42, NfL, and GFAP), by the TAU kit by Quanterix (t-tau), the ALZpath assay by Quanterix (p-tau217), and for p-tau181 and p-tau231 by assays developed at the University of Gothenburg^{187,188}. For BD-tau we used the University of Gothenburg developed assay¹¹⁴, later commercialised by Quanterix¹⁸⁹. Samples from TRACK-AD were analysed by the two-plex Quanterix assay for NfL and GFAP, the Quanterix Alzpath assay for p-tau217, and with the University of Gothenburg developed BD-tau assay¹¹⁴.

All available samples from each patient were placed next to each other on the same plate and all samples from the respective study were analysed in one round of experiment. Each plate included the samples together with calibrators and quality controls (QCs). Calibrators were run in duplicates, and the signals were plotted to create the calibration curve where potential outliers were removed. QCs, test samples with known concentrations, were placed at the start and the end of each plate, run in duplicates and used to monitor “drift” across the plate (within-assay variability) and the intermediate precision between plates, i.e. inter-assay variability CVA%). All sample analysis were run in singlicates¹⁻⁴.

3.3 Additional CSF analysis

In SHORT-ND all patients underwent a lumbar puncture, while in TRACK-AD CSF analyses were performed when clinically requested by the physician as part of the patient’s diagnostic assessments. CSF was analysed routinely as part of the diagnostic evaluation for β-amyloid 1–42, phosphorylated tau 181 (p-tau181), and tau at the Biochemical Department at Rigshospitalet. Before June 20th 2022, CSF was analysed by the Innostest enzyme-linked immunosorbent assay (ELISA)

and after July 1st 2022, CSF was analysed by the Elecsys sandwich electrochemiluminescence-immunoassay by Roche, Cobas 8000 ¹⁻⁴. The following cut-offs were used: 0.077 (Innotest) and 0.023 (Elecsys) ^{190,191}.

3.4 Imaging procedures (TRACK-AD)

For diagnostic purposes, prior to entering the TRACK-AD study, all patients had undergone an [¹⁸F]FDG-PET and MRI or CT scan ^{3,4}. Imaging data was included for patients who had a baseline [¹⁸F]FDG-PET/MRI and who participated in the follow-up [¹⁸F]FDG-PET/MRI scan. For most patients, the baseline scan was performed on a hybrid PET/3T MRI scanner (Siemens mMR, Siemens Healthineers, Erlangen, Germany), but for 14 patients it was performed on a PET and 1.5T MRI scanner separately. All follow up scans were performed on the hybrid PET and 3T MRI scanner at the Department of Nuclear Medicine, Rigshospitalet according to guidelines ¹⁹². Approximately 200MBq [¹⁸F]FDG was administered via an antecubital vein, and patients were instructed to remain lying still for 40 minutes to allow for tracer uptake. Subsequently, patients were positioned within the hybrid scanner and PET and MRI data were recorded simultaneously, within approximately 20 minutes. The MRI scans included a T1- and T2-weighted sequence, a T2 fluid-attenuated inversion recovery (FLAIR) sequence, and a T2* weighted imaging (SWI) ^{3,4}. T1 was measured in a voxel of 1mm³ (slice thickness 1mm), whereas the other three sequences were measured using 5mm thick slices and an in-plane resolution of 0.9*0.9mm.

3.4.1 Processing of imaging data

Visual MRI rating was performed by a neuroradiologist and rated in accordance with Sheltens medial temporal atrophy (MTA) scale ¹⁹³, the global cortical atrophy (GCA) scale ¹⁹⁴, and the Fazeka scale ¹⁹⁵, grading white matter hyperintensities.

The neuroimaging software Freesurfer (version 7.4.1) was used to process the T1-weighted MRI and PET scans to extract quantitative data of the brain's anatomy and metabolism, respectively. Freesurfer is based on a predefined atlas and performs automated processing of the images. Firstly, it segments each voxel to identify the tissue types, e.g. cerebrospinal fluid, grey and white matter, then it identifies which region of the brain the voxel belongs to. Along with MRI images FDG-PET data was imported and registered to the subject specific MRI scan. Based on the predefined atlas, Freesurfer extracted the standardized uptake value ratios (SUVR) (normalised to the cerebellar cortex) for each region. For longitudinal data the recommended pipeline, PETSurfer, for longitudinal

data was used ¹⁹⁶. This ensures that the intracranial volume is the same between a subject's baseline and follow-up scan.

For Study III and IV, we used predefined AD specific regions of interest (ROIs) for analysis, selected based on findings from previous studies ^{3,4}. These included MRI total grey matter, cortex, hippocampal, and lateral ventricle MRI volumes ^{108,125}, all normalised to the within-cranial volume (ICV). Predefined SUVr measures of interest included the hippocampal, isthmus cingulate, temporal, and parietal cortices ¹⁹⁷.

Data from SHORT-ND and TRACK-AD was used in Studies I-IV, summarised in Table 3.0

Table 3.0 Key data sources used in Studies I-IV

Study 1 Biomarker variability (SHORT-ND) • AD & non-AD patients (clinical diagnosis) • Plasma Aβ40, Aβ42, p-tau217, p-tau181, p-tau231, NfL, GFAP, t-tau • CSF Aβ40, Aβ42, p-tau217, p-tau181, p-tau231, NfL, GFAP, t-tau
Study II Improvement of biomarker variability (SHORT-ND) • Aβ+ & Aβ- individuals (based on the CSF p-tau181/Aβ1-42 ratio) • Plasma p-tau217, BD-tau, t-tau • CSF p-tau217, BD-tau, t-tau
Study III Prognosis (TRACK-AD) • Patients with MCI or dementia due to AD • Plasma p-tau217, NfL, BD-tau, GFAP from baseline and 4 months follow-up • CDR-SB, MMSE, and disease severity stage from baseline and 1- and 2 year follow-up • [¹⁸F]FDG-PET/MRI from baseline and 1-year follow-up
Study IV Tracking (TRACK-AD) • Patients with MCI or dementia due to AD • Plasma p-tau217, NfL, BD-tau, GFAP from all available visits • CDR-SB, MMSE, and disease severity stage from all available visits • [¹⁸F]FDG-PET/MRI from baseline and 1-year follow-up

Table outlining the participants included and key variables used in studies I-IV ¹⁻⁴. AD, Alzheimer's disease; MCI, mild cognitive impairment; MMSE, mini mental status examination; CDR-SB, clinical dementia rating scale sum of boxes; A β , amyloid- β ; p-tau, phosphorylated tau; t-tau, total tau; NfL, neurofilament light; GFAP, glial fibrillary acidic protein; [¹⁸F]FDG-PET/MR, [¹⁸F]-fluorodeoxyglucose positron emission tomography/magnetic resonance imaging.

3.5 Statistics

3.5.1 Sample size calculation

For Study I and II, sample size estimation was based on concentrations of plasma NfL, considered one of the more variable biomarkers, across three repeated measures per participant. Assumptions included a mean plasma NfL concentration of 43.0 pg/mL, a standard deviation of 21.9 pg/mL, and a potential change of up to 20 pg/mL between time points¹⁹⁸. Based on these parameters, the sample size was conducted to appropriately model the repeated measures design. The significance level was set at $\alpha=0.05$ and statistical power at 80%. The estimated sample size required to reliably estimate within- and between-subject variability across three time points was 15 participants. As participants were recruited blinded to diagnosis, and to ensure adequate representation across diagnostic groups and account for potential drop-out, the study was designed to include 45 participants in the SHORT-ND study.

The power calculation for Study III and IV was based on the null hypothesis that there was no significant difference in our primary outcome, CDR-SB, between stable individuals and progressors. Based on previous findings, we assumed that approximately 50% will progress from MCI to dementia, or from dementia to more severe stages, defined as an annual increase in CDR-SB of 1.6 points (SD 1.8) (22,23). The significance level, set at $\alpha = 0.05$, rejects the null hypothesis if true, and a power of 80% reflects the probability of rejecting the null hypothesis if false. Based on this, we needed $n = 42$ patients in each group. Adjusted for an expected drop-out rate, the aim was to include 100 patients in the TRAKC-AD study.

3.5.2 Statistical methods

All statistical analyses were performed in R statistical software version 4.4.1 - 4.5.0. Results of statistical analyses are presented with 95% confidence intervals (CI), corresponding to a 5% significance level.

For continuous variables, baseline or group differences were assessed with two-sample t-tests or analysis of covariances and baseline values are presented as mean and standard deviations or median and interquartile ranges where appropriate. If necessary, outcome variables were log transformed to ensure normality distribution. For categorical variables, differences were assessed by Pearson's chi-squared tests and presented as count numbers and percentages¹⁻⁴.

Table 3.5.2 gives a schematic overview of the different statistical methods applied in each study.

3.5.2.1 Study I

In Study I the SHORT-ND cohort was used to examine within- and between-subject variability, in clinically diagnosed patients with and without AD ¹. We used linear mixed models with log10-transformed biomarkers as outcomes and with patients as random effects. Within-subject variability was based on the residual's standard deviation whereas the between-subject variability was based on the total variance,

$$(\sqrt{\text{variance}(\text{patients}) + \text{variance}(\text{residual})}).$$

Linear mixed models (LMM) for each biomarker were then carried out with time, estimated glomerular filtration rate (eGFR), BMI, and Hemoglobin A1c (HbA1c) as fixed effects one at a time. Confidence intervals for the within- and between-subject variability were estimated using the bootstrap method ¹. Although biomarker values were log-transformed to approximate normality, bootstrapping was applied to account for potential residual non-normality and to provide robust estimates without relying strictly on parametric assumptions.

Based on the assay variability and within-subject variability, we calculated the reference change value (RCV),

$$RCV = 100 \times (\pm \exp(z \times \sqrt{2} \times \sqrt{\log(CV_{\text{intra-individual}}^2 + CV_{\text{inter-assay}}^2 - 1)}),$$

and the number of samples needed to estimate an individual's homeostatic setpoint (NNHS),

$$NNHS = (z * \sqrt{(CV_{\text{intra-individual}}^2 + CV_A^2) / D})^2 ,$$

for each biomarker ^{1,176,180}.

Finally, to explore if biomarker variability led to changes in the biomarker classification values between AD and non-AD patients, the dataset was split 50/50 and a predictive model was built. Classification accuracy and the corresponding 95% confidence interval were then calculated using a confusion matrix ¹.

We used Spearman's correlations coefficient between plasma levels across visits, CSF measures and the abovementioned confounders.

3.5.2.2 Study II

Based on the SHORT-ND cohort, Study II focuses on improving biomarker stability by normalising plasma p-tau217 to BD-tau ². In this study we dichotomized the cohort based on CSF p-tau181/Aβ1-42 status. We used the same approach as in Study I, using linear mixed models to examine within-

and between-subject variability. However, in this study we also combined all potential confounders in one model to account for the combined effect of eGFR, BMI, and HbA1c. To assess differences in diagnostic performance between visits, we computed the receiver operating characteristic (ROC) curves and corresponding area under the curve (AUC) values, based on the entire available dataset at each visit. And we performed a sensitivity analysis on complete cases only. We used Spearman's correlations coefficient to assess the correlations between plasma and CSF markers ².

3.5.2.3 Study III

In Study III we used the TRACK-AD cohort and classified patients as either clinically *stable* or *progressors* at one- and at two-year follow up, as described previously in section 3.1.2 ³.

In this study, linear regression models were used to assess the prognostic value of each specific biomarker's baseline level and changes between baseline and 3-5 months follow-up, denoted *short-term changes* ³.

Outcomes were changes in CDR-SB and imaging ROIs, calculated as the differences between baseline and either one- or two-year follow-up, divided by baseline and adjusted for time (days). All analyses were adjusted for age, sex and creatinine. Imaging analyses were further adjusted for multiple testing correction. To account for the observed inverse relationship between baseline levels and short-term changes, which could indicate regression towards the mean, all models were tested for an interaction effect between baseline biomarker values and time. If this interaction was statistically significant, it was retained in the final model specification and in cases where the interaction was not significant, models were adjusted for baseline biomarker levels ³.

Secondary analyses assessed the biomarkers' ability to distinguish between clinically stable and progressors. Here, we calculated the AUC for each biomarker using the dataset as 80/20 training/test set.

Finally, we used the reference change values (RCVs) for p-tau217, NfL, and GFAP from Study I. Based on the within-subject variability and inter-assay variability from Study II, we calculated the RCV for BD-tau based on the same methods applied in Study I (please see RCV calculation, section 3.5.2.1) ^{1,2}.

Based on these RCVs, we grouped patients into either within, above or below each marker's RCV. This categorical variable was included in the regression model with short-term changes to better address the influence of within-subject and assay variability on annual changes in CDR-SB and imaging measures ³.

3.5.2.4 Study IV

In Study IV, based on the TRACK-AD cohort, participants were classified as either *stable* or *progressors* according to the last available follow-up visit ⁴.

Linear mixed models, adjusted for time, age, sex, and baseline creatinine, were used to examine group specific biomarker trajectories in clinically *stable* and *progressors*. To examine differences between the group specific biomarker trajectories, we included their clinical status (i.e. *stable* or *progressor*) and time as an interaction term in the LMMs ⁴.

To investigate correlations between individual biomarker changes with concurrent changes in CDR-SB and MMSE, we extracted the random slopes for each patient from the LMMs. These were used to calculate Pearson's correlations coefficient between trajectories of biomarkers and CDR-SD.

For imaging data, only two datapoints were available, we calculated the annual relative change for each imaging measure and correlated it with the annual relative change in biomarker levels, between baseline and one-year follow-up ⁴.

Finally, we used the RCVs for p-tau217, NfL, GFAP from Study I ¹, and BD-tau from Study II and III ^{2,3}. We then calculated the number of changes in plasma biomarker levels exceeding their biomarker specific RCV ⁴.

Table 3.5.2 Statistical methods used in the studies

	Study I	Study II	Study III	Study IV
Linear mixed models	X	X		X
Linear regression			X	
ROC AUC		X	X	
Classification accuracy	X			
Spearman's correlations	X	X		
Pearsons' correlations				X

Table outlining the different main statistical methods applied in studies I-IV ¹⁻⁴. ROC, Receiver Operating Curve; AUC, Area under the curve.

3.6 Ethics and approvals

The SHORT-ND and TRACK-AD protocols were approved by the Regional Research Ethics Committee (H-21044863) and (H-21040317), respectively and registered at clinicaltrials.gov (NCT05175664). Prior to enrolment all participants gave written informed consent. Only patients with the ability to understand the written and oral information were invited to participate and, preferably, they had a caregiver willing to participate in the consent process. The study followed the tenets of the 1975 and 2024 Declaration of Helsinki. Any personal information regarding participants is protected according to the national legislation, specifically the Databeskyttelsesforordningen and Databeskyttelsesloven. The projects were reported to Videnscenter for Dataanmeldelser in Region Hovedstaden, as required by law.

In general, blood sampling is associated with few and insignificant risks. The TRACK-AD study was designed to align with patients' routine clinical visit at the Memory Clinic, which we considered would enhance compliance to study participation. The [^{18}F]FDG-PET scan is associated with an increased risk of cancer (an absolute increase of 0.2% compared to the general population) due to the radiation dose from the examination. All participants were informed about this, both orally and by written information, prior to participating in the additional scan.

Participants were informed about the results of their control PET-MRI scans, and additional interventions were initiated if medically indicated. Furthermore, participants were provided, if interested, with information regarding the overall findings from the SHORT-ND and TRACK-AD studies; however, they were not given access to their individual plasma biomarker levels or changes over time. This approach was taken to maintain confidentiality and to avoid potential misinterpretation of personal health data outside a clinical context.

4. Summary of results

In the following section the main findings from Study I to IV will be outlined study by study. Please refer to the original papers in the appendices Studies I-IV for further details ¹⁻⁴.

Table 4.0 summarises baseline demographic characteristic of the populations in studies I-IV ¹⁻⁴.

Table 4.5 summarises outcomes and selected key findings from studies I-IV ¹⁻⁴.

Table 4.0 Baseline demographics in studies I-IV

Cohort	SHORT-ND				TRACK-AD			
Study	Study I		Study II		Study III		Study IV	
	AD	Non-AD	AB+	AB-	Stable year 1	Progressors year 2	Stable in FU	Progressors in FU
Number, n	27	20	29**	18	54	40	41	53
Age, mean SD	71 (5)	72 (6)	72 (5)	72 (7)	74 (7)*	78 (6)*	74 (7)*	77 (6)*
Gender, Male, n %	15 (56%)	16 (80%)	15 (52%)	16 (89%)	28 (52%)	21 (53%)	22 (54%)	23 (43%)
MMSE, mean SD	27 (3)	27 (2)	27 (2)	27 (2)	26 (3)	26 (2)	26 (2)	26 (3)
Disease severity MCI/dementia, %*	48/52%	60/40%	55/45%	50/50%	26/94%**	30/70%	17/83%	36/64%
CSF Aβ1-42, pg/mL	665 (245)	1124 (465)	p-tau181/Aβ1-42		703 (586,848)	649 (579,857)	704 (634,883)	649 (465,760)
CSF p-tau181, pg/mL	33 (15)	21 (12)	0.05 (0.03)	0.01 (0.004)	40 (27,51)	38 (23,60)	36 (26,52)	41 (30,48)
CSF t-tau, pg/mL	317 (129)	235 (123)	287 (191)***	134 (71)***	337 (263,466)	324 (231,422)	333 (249,480)	392 (248,434)
eGFR, mean SD	83 (14)	80 (26)	82 (18)	82 (23)	79 (14)*	71 (18)*	78 (15)	74 (17)
BMI, mean SD	25 (3)	27 (5)	25 (4)	28 (5)	25 (4)	26 (4)	25 (4)	25 (4)

*one patient in TRACK-AD had moderate dementia at baseline. **the two patients who “shifted” from non-AD to AB-positive were diagnosed with DLB. *** CSF analysis by Simoa (the other CSF results are from analysis performed by Elecsys or Innnotest at Rigshospitalet). CSF values are in SHORT-ND presented as mean with SD, whereas in TRACK-ND, CSF values are presented as median with IQR. FU, follow-up period; MMSE, mini mental status examination; MCI, mild cognitive impairment; CSF, cerebrospinal fluid; eGFR, estimated glomerular filtration rate; BMI, body mass index. Based on results presented in Studies I-IV ¹⁻⁴.

4.1 Study I

In total, 47 patients were included in this study of whom 27 had a clinical diagnosis of AD while 20 had a non-AD diagnosis (affective disorder (n=1), amyotrophic lateral sclerosis (n=1), cerebral amyloid angiopathy (n=2), DLB (n=6), FTD (n=2), head trauma (n=1), kidney failure (n=1), normal pressure hydrocephalus (n=1), other tauopathy (n=1), unclassified patients (n=3), and vascular dementia (n=1)) ¹. 43 patients completed Visit 2, and 40 patients completed Visit 3. The AD group had significantly higher CSF Aβ 1-42, tau, and p-tau compared to the non-AD, but no other

group differences regarding cognitive status, disease severity stage, BMI, HbA1c, eGFR, creatinine were observed ¹.

Plasma levels of A β 40, A β 42, p-tau181, p-tau217, p-tau231, t-tau, NfL, and GFAP were investigated. In Table 4.1, within-and between subject variabilities for all plasma markers are presented. Across all biomarkers, within-subject variability was low and there were no differences in within-subject between the AD and non-AD group – the only exception was that within-subject variability of A β 42 was significant lower in the AD group. No notable changes were observed when adjusting for time and peripheral confounders, see Study I ¹.

Between-subject variability was lowest for plasma A β 40, A β 42, moderate for p-tau variants, T-tau, and GFAP, and highest for NfL. Higher between-subject variability was observed for A β 40 and p-tau181 in the non-AD group compared with AD patients, see Table 4.1. Adjusting for BMI decreased the between-subject variability with <3%; no other notable changes were observed when adjusting for time or peripheral confounders, see Study I ¹.

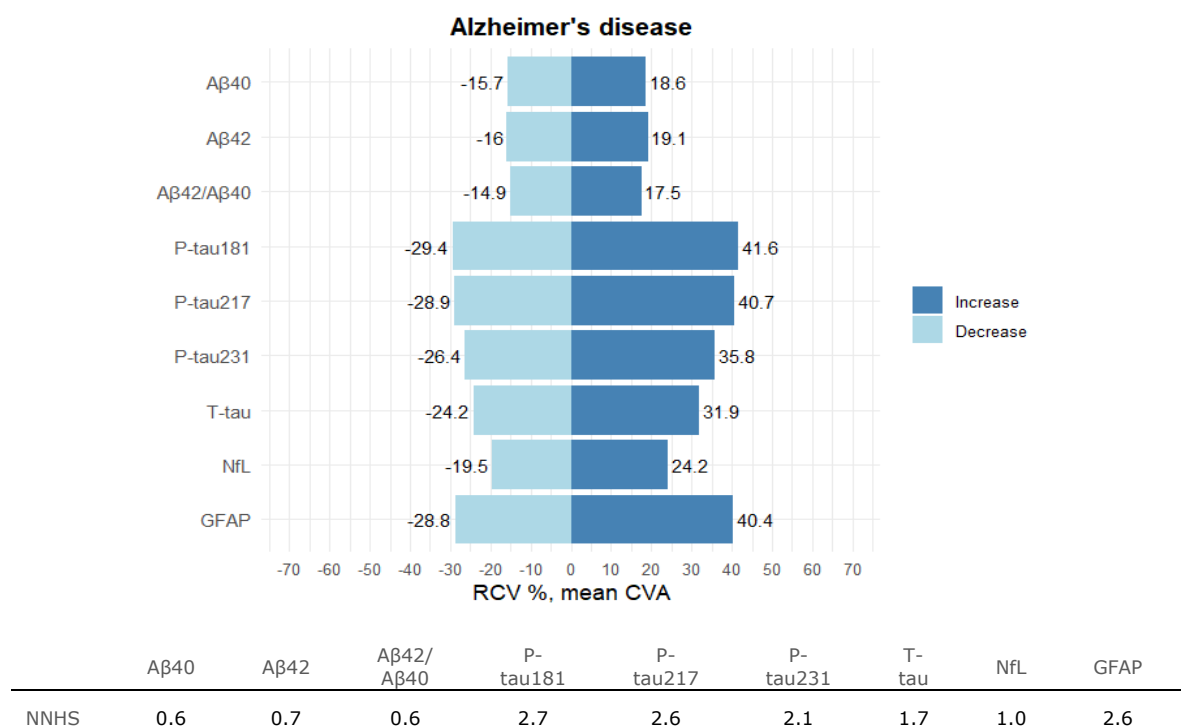
Table 4.1 Plasma markers within- & between-subject variability

Biomarker	Population	No. of Samples	Within-subject variability CV (%; 95% CI)	Between -subject variability CV (%; 95% CI)
Aβ40	AD	71	4.1 (3.3; 5.0)	7.1 (5.5; 8.7)**
	non-AD	57	6.2 (4.9; 7.5)	12.5 (9.3; 16.2)**
Aβ42	AD	71	4.5 (3.6; 5.5)**	12.2 (9.2; 15.3)
	non-AD	57	7.2 (5.4;8.8)**	16.9 (12.2; 21.8)
Aβ42/Aβ40	AD	71	3.4 (2.7; 4.1)	11.2 (8.3; 14.1)
	non-AD	57	3.2 (2.5; 3.9)	8.4 (5.9; 10.8)
Ptau181	AD	71	6.9 (5.6; 8.5)	15.1 (11.5; 18.8)**
	non-AD	57	7.2 (5.5; 8.8)	26.3 (18.4; 34.0)**
Ptau217	AD	71	10.5 (8.3;12.8)	19.7 (15.5; 24.5)
	non-AD	57	7.8 (6.0; 9.5)	30.9 (21.3; 40.7)
Ptau231	AD	71	7.0 (5.6;8.5)	13.4 (10.2; 16.3)
	non-AD	55*	8.8 (6.8; 10.9)	21.7 (15.6; 28.0)
T-tau	AD	68*	9.6 (7.4; 11.6)	17.4 (13.3; 21.7)
	Non-AD	57	12.5 (9.7; 15.5)	18.7 (14.3; 23.2)
NfL	AD	71	7.1 (5.5;8.6)	29.6 (21.6; 37.2)
	non-AD	57	7.5 (5.8;9.2)	39.4 (27.1; 51.1)
GFAP	AD	71	11.4 (9.2; 13.9)	19.6 (15.3; 23.9)
	non-AD	57	10.9 (8.2; 13.5)	21.5 (16.4; 26.6)

Within- and between-subject variability for each biomarker is presented. Blood samples from two patients obtained at V1 were lost due to technical issues before freezing. N-missing: AD: V1 n=2, V2 n=3, V3 n=5. Non-AD: V1 n=0, V2 n=1, V3 n=2. *<LOQ (under limit of quantification): Plasma p-tau231 <LOQ n=2 in the non-AD group, plasma T-tau <LOQ n=3 in the AD group. **Linear mixed models showed significant difference between groups. ***Non-overlapping 95% CI. CI: confidence interval, A β : amyloid- β ; p-tau: phosphorylated tau; t-tau: total tau; NfL: neurofilament light; GFAP: glial fibrillary acidic protein. Modified from Clemmensen et al ¹.

Reference change values (RCV) were narrowest in plasma A β 40, A β 42, whereas plasma p-tau181 showed the widest RCV. We observed no difference in RCVs between AD and non-AD. Our results indicate that the number of repeated measurements required to reliably estimate an individual's homeostatic set-point (NNHS) varied across biomarkers. For NfL, a single sample was sufficient, whereas three samples were needed for p-tau217 and GFAP, see Figure 4.1.

Figure 4.1 Reference change values



The figure shows the amount of change for each biomarker to overcome within-subject variability and assay variation, named Reference Change Value (RCV). The table shows the NNHS; number of samples needed to obtain homeostatic setpoint with 15% proximity to true value for each plasma biomarker. Calculations are based on the mean intermediate CVA. A β : amyloid- β ; p-tau: phosphorylated tau; t-tau: total tau; NfL: neurofilament light; GFAP: glial fibrillary acidic protein. Modified from Clemmensen et al ¹.

Finally, across all markers we observed no differences in classification accuracies between the three time-points, see Study I ¹.

4.2 Study II

In this study, we included the same 47 individuals from SHORT-ND as in Study I, but dichotomized the cohort based on their CSF p-tau181/A β 42 status (A β + n=29, A β - n=18) from their routine CSF analysis ². We observed significantly more females in the A β + group, which also exhibited higher baseline levels of CSF t-tau and BD-tau, and lower BMI and HbA1c, compared to the A β - group ². See Table 4.0.

Plasma and CSF measures included p-tau217, t-tau and BD-tau in this study.

Normalising p-tau217 to BD-tau we observed a reduced variability, both within- and between subjects, see Table 4.2, model 1. In particular, the between-subject variability was reduced. We observed a between-subject variability in the A β + group on 19% in p-tau217 alone, compared to 15% in the p-tau217/BD-tau ratio. Further reduction in between-subject variability was observed when adjusting for eGFR, HbA1c, and BMI ², see Table 4.2, model 2.

Furthermore, we found no correlations between plasma BD-tau levels and baseline data on eGFR, HbA1c, or BMI, whereas t-tau significantly correlated with eGFR and HbA1c. Plasma p-tau217/BD-tau levels showed a stronger correlation with CSF p-tau217 compared with plasma p-tau217 alone, see Study II ².

Across visits, the classification performance did not differ for p-tau217 or the p-tau217/BD-tau ratio, see Study II ².

Table 4.2 Within- and between-subject variability

		Within-subject Variability CV (%, 95%CI)		Between-subject Variability CV (%, 95%CI)	
		Model 1: Time-adjusted	Model 2: Fully adjusted	Model 1: Time-adjusted	Model 2: Fully adjusted
p-tau217	A β +	9.4 (7.4;11.5)*	9.5 (7.6;11.3)	19.1 (15.0;23.4)	13.1** (10.5;15.5)*
	A β -	5.9 (4.5;7.3)*	6.2 (4.5;7.9)	27.1 (18.4;36.0)	27.7 (16.1;39.5)*
BD-tau	A β +	7.5 (5.97;9.1)	7.5 (6.0;9.1)	12.1 (9.8;14.7)	10.7 (8.4;12.7)
	A β -	6.6 (5.0;8.2)	6.5 (5.0;8.6)	20.1 (14.1;26.2)	16.8 (10.5;23.3)
t-tau	A β +	10.4 (8.2; 12.5)	10.4 (8.3;12.6)	15.0 (12.1;18.3)	12.7 (10.4;15.1)
	A β -	11.1 (8.3;14.0)	10.9 (7.8;14.0)	20.6 (14.8;26.7)	19.4 (13.2;26.4)
p-tau217/BD-tau	A β +	7.1 (5.6;8.4)	7.1 (5.6;8.5)	15.1 (11.7;18.7)	11.1 (8.8;13.7)
	A β -	7.3 (5.6;9.1)	7.4 (4.5;9.6)	18.4 (13.0;23.8)	18.8 (12.0;26.1)
p-tau217/T-tau	A β +	13.9 (10.9;16.8)	13.9 (11.2;16.8)	19.3 (15.6;23.1)	18.2 (14.9;21.7)
	A β -	9.6 (6.9;12.0)	9.5 (6.8;12.2)	30.2 (21.1;39.2)	32.5 (19.8;45.1)

Within- and between-subject variability for each biomarker is presented together with the corresponding confidence interval. **Bold** indicate the reduction of p-tau217 variability from the single marker to the ratio, to adjusted for confounders.

Model 1 is adjusted for sampling-time-point. Model 2 is adjusted for eGFR, HbA1c, BMI, and sampling time point. Excluding patients with eGFR<<36/min/1,73m² did not alter the results. Adjusting for sex in Model 2 did not affect the variability.

*Non-overlapping 95% CI between A β + and A β -group. **Non-overlapping 95% CI between raw and model adjusted for all covariates.

CV: Coefficient Variant; CI: Confidence Interval; p-tau: phosphorylated tau; t-tau: total tau; BD-tau: brain-derived tau.

Reprinted from Clemmensen et al. ²

4.3 Study III

In TRACK-AD we included 105 patients with MCI or mild-moderate dementia due to AD at baseline. A total of 94 patients completed one-year follow-up and 85 patients completed the two-year follow-up.

Eleven patients discontinued participation before the one-year follow-up: one due to a change in diagnosis to DLB, two were diagnosed with terminal cancer, two had terminally ill spouses, and six withdrew their consent. Nine patients did not participate at two-year follow-up, due to withdrawal or perceived burden of testing. See Figure 3.1.

The 94 patients who completed one-year follow-up were included in Study III. At one year follow-up, 54 patients were classified as clinically *stable* and 40 as *progressors*.

90 patients exhibited the amnesic variant of AD and four presented with non-amnesic variants of AD: two with posterior cortical atrophy (one MCI and one with dementia at baseline) and two with the logopenic variant of primary progressive aphasia (both with mild dementia at baseline). All four patients were followed for two years. During this period, the patient with mild dementia due to posterior cortical atrophy progressed to moderate, while the other three patients remained stable.

At baseline, the progression group was significantly older, had lower eGFR, and exhibited higher levels of plasma NfL, compared to the stable group ³. See Table 4.0. There were no group differences in imaging regional volumes or metabolism at baseline. Longitudinal data showed that CDR-SB increased significantly more in progressors compared to stable ³.

We found that baseline plasma NfL could significantly predict changes in CDR-SB at both one- and two-years follow-up. Plasma NfL could further distinguish between *stable* and *progressors* with intermediate accuracy. We found no associations between baseline p-tau217, BD-tau or GFAP with changes in CDR-SB nor with short-term changes in any of the markers ³, see Table 4.3.

Table 4.3 Plasma markers as predictors of annual changes in CDR-SB

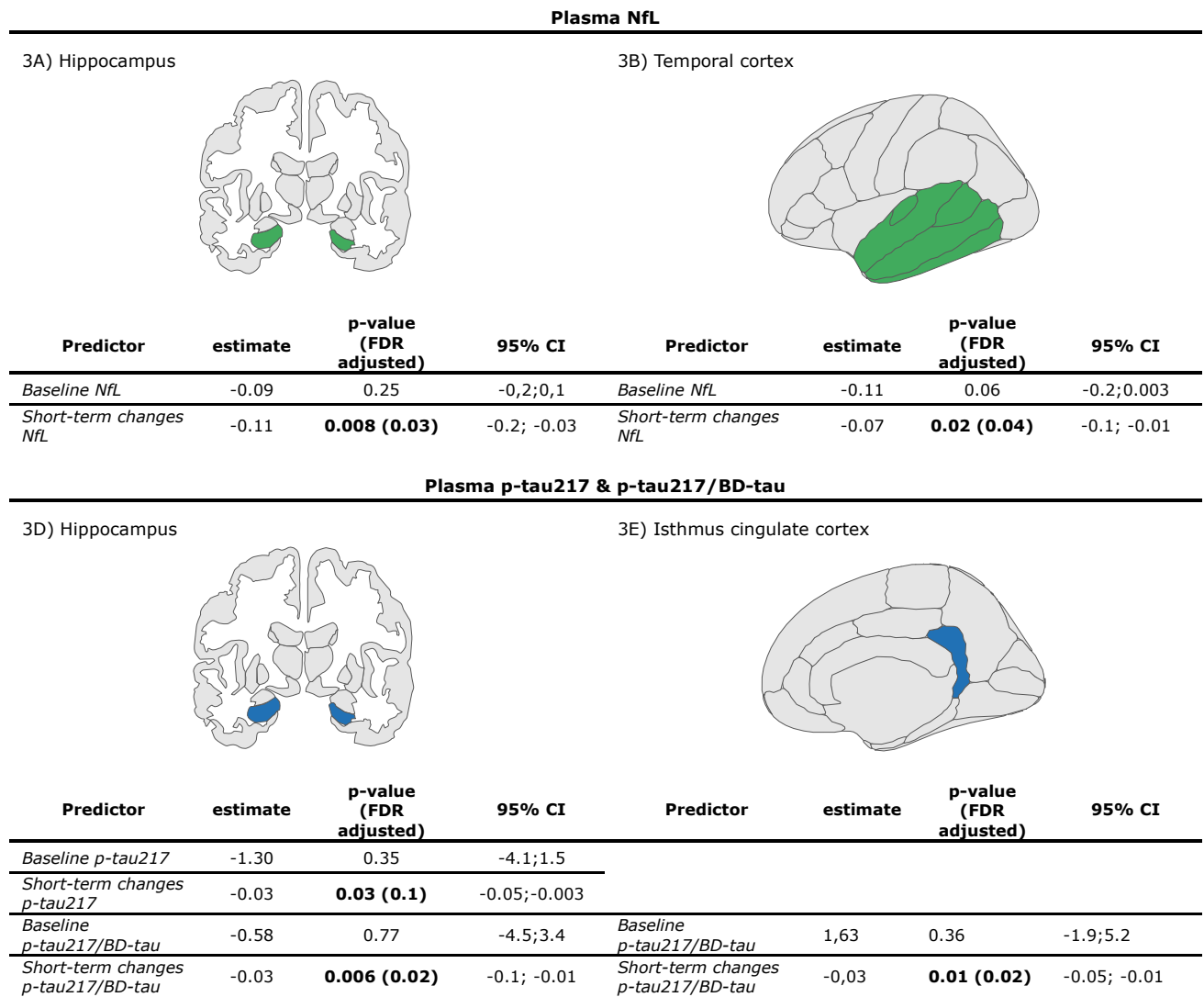
	Predictor	Estimate per 10 units increase	p-value	95% CI
CDR-SB 1Y	Baseline NfL, pg/mL	0.5	0.01	0.1;0.9
	RC NfL, %	-0.001	0.26	-0.002;0.0004
	Baseline BD-tau, pg/mL	-3.5	0.54	-14.9;7.8
	RC BD-tau, %	-0.0002	0.49	-0.001;0.0004
	Baseline p-tau217, pg/mL	-3.4	0.41	-11.3;4.6
	RC p-tau217, %	0.001	0.43	-0.001;0.002
	Baseline GFAP, pg/mL	-0.005	0.73	-0.03;0.02
	RC GFAP, %	-0.001	0.15	-0.001;0.0002
	Baseline p-tau217/BD-tau, pg/mL	-0.9	0.13	-1.8;0.3
CDR-SB 2Y	RC p-tau217/BD-tau, %	-0.01	0.01	-0.02;-0.001
	Baseline NfL, pg/mL	0.6	<0.01	0.2;1.0
	RC NfL, %	0.0001	0.84	-0.001;0.001
	Baseline BD-tau, pg/mL	5.0	0.33	-5.2;15.1
	RC BD-tau, %	0.0002	0.52	-0.0004;0.001
	Baseline p-tau217, pg/mL	3.1	0.39	-4.1;10.3
	RC p-tau217, %	-0.0001	0.90	-0.001;0.001
	Baseline GFAP, pg/mL	-0.01	0.65	-0.03;0.02
	RC GFAP, %	-0.0003	0.46	-0.001;0.0004
	Baseline p-tau217/BD-tau, pg/mL	0.1	0.8	-0.9;1.1
	RC p-tau217/BD-tau, %	0.002	0.6	-0.01;0.01

Table showing results from linear regression models with CDR-SB as outcome and predictors: 1) baseline biomarker, 2) short-term biomarker changes (adjusted for baseline), denoted relative change (RC). Both models were adjusted for age, sex, and creatinine. Age had a significant effect in all models. Sex and creatinine adjustment altered the models but were not significant associated in any of the models. All models were additionally adjusted for baseline CDR-SB, which did not alter the results (data not shown). Finally, models with p-tau217 were further adjusted for BMI, which did not alter the results (data not shown). Baseline and short-term changes of NfL, GFAP, p-tau217, and BD-tau estimates are for 10 units of increase. Reprinted from Clemmensen et al. 2025 ³.

For imaging data, 70 patients had baseline and one-year MRI scan available, while 67 patients had baseline and one-year [¹⁸F]FDG-PET scan. We found no association between any baseline biomarker levels and regional annual changes in MRI volumes or [¹⁸F]FDG-PET metabolism. We found that short-term changes in plasma NfL were significantly associated with an annual SUVR decrease in bilateral hippocampal and temporal cortex, whereas short-term changes in the plasma p-tau217/BD-tau ratio were significantly associated with annual SUVR decrease in bilateral hippocampal and isthmus cingulate cortex. These findings were significant after multiple testing correction, see Figure 4.3. For MRI we found no associations after multiple testing correction ³.

Finally, to interpret short-term changes in plasma biomarkers in relation to expected within-subject and assay variability, we categorised participants based on whether their changes fell within, exceeded, or were below the biomarker-specific RCV. Most short-term changes were within the expected RCV range. Notably, NfL changes exceeding the RCV were significantly associated with changes in SUVR in the bilateral hippocampus and bilateral temporal cortex, compared to those whose NfL changes remained within the RCV threshold. No other associations were observed ³.

Figure 4.3 Plasma NfL & p-tau217 association with annual changes in [18F]FDG-PET



Linear regression models, p-value adjusted for age, sex and creatinine. The NfL models with short-term changes contain an interaction term with biomarker and time. Models with plasma short-term changes in p-tau217 and the p-tau217/BD-tau ratio were adjusted for baseline. P-values in () are after multiple testing correction. Reprinted from Clemmensen et al.³

4.4 Study IV

In Study IV, the same 94 individuals from the TRACK-ND cohort used in Study III were included. Based on their last available visit (one- or two-year follow-up), patients were classified as clinically *stable* or *progressors*. The progression group was significantly older and presented with higher baseline NfL levels. No group differences regarding comorbidities (hypertension, diabetes, kidney dysfunction, FAZEKA score, etc.) were observed ⁴.

In the stable group we observed no change in any plasma biomarkers during the study period. In the progression group we found a significant increase in plasma NfL, p-tau217, and in the p-tau217/BD-tau ratio, see Figure 4.4.1. Testing differences in trajectories between groups, no differences were observed in plasma NfL. For p-tau217 and the p-tau217/BD-tau ratio, the progression group had a significantly steeper trajectory compared to the stable group. The p-tau217/BD-tau ratio was also able to distinguish MCI patients who progressed to dementia from stable MCI patients and from stable individuals with dementia ⁴.

We found no correlations between individual changes in plasma p-tau217, NfL, BD-tau, GFAP levels with changes in CDR-SB, MMSE, MRI regional volume changes and [¹⁸F]FDG-PET regional SUVR changes. We did, however, find a weak correlation between individual changes in the p-tau217/BD-tau ratio and CDR-SB (Rho 0.23, p -value: 0.025) ⁴.

Figure 4.4.1 Plasma biomarker trajectories in stable vs. progressors

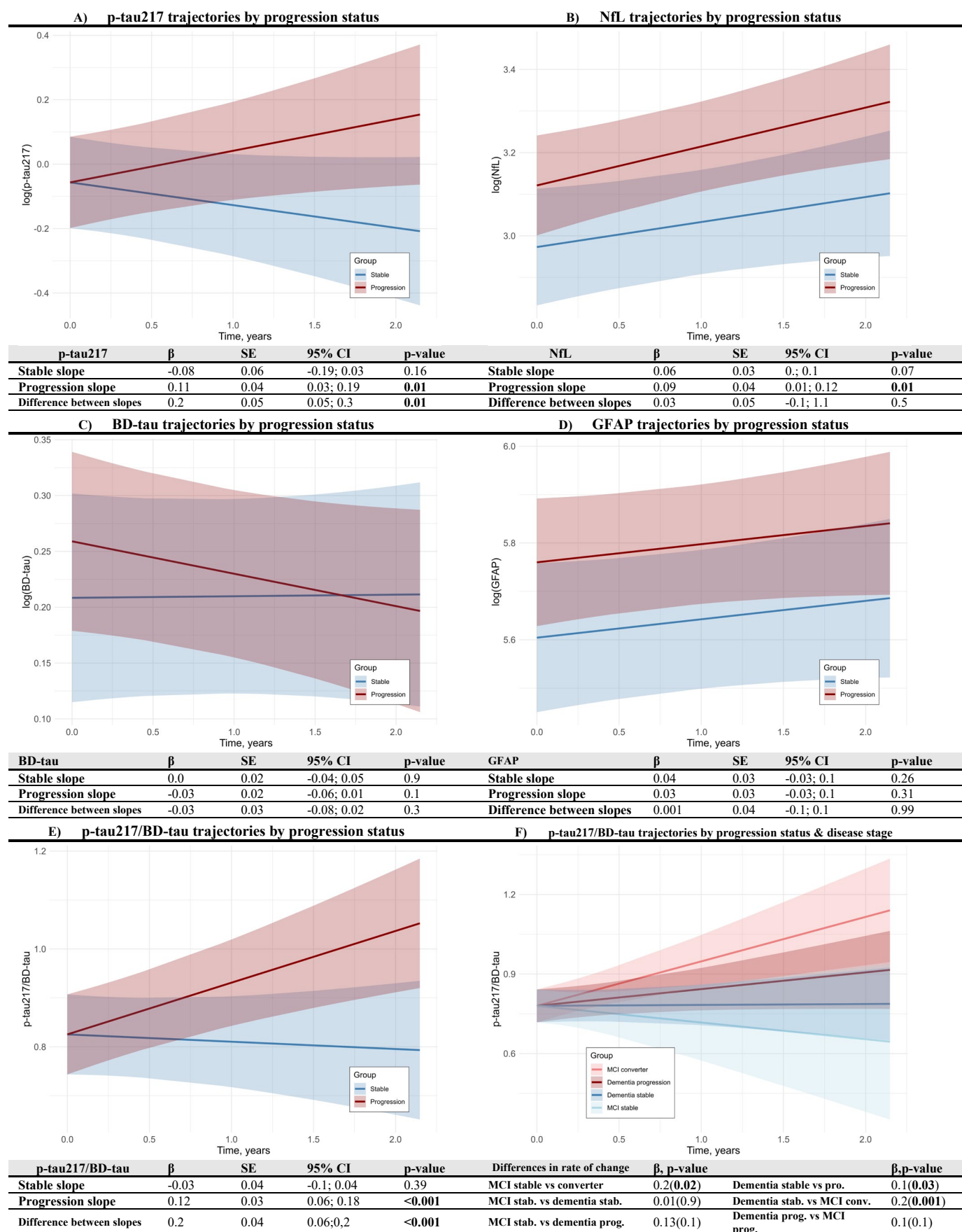
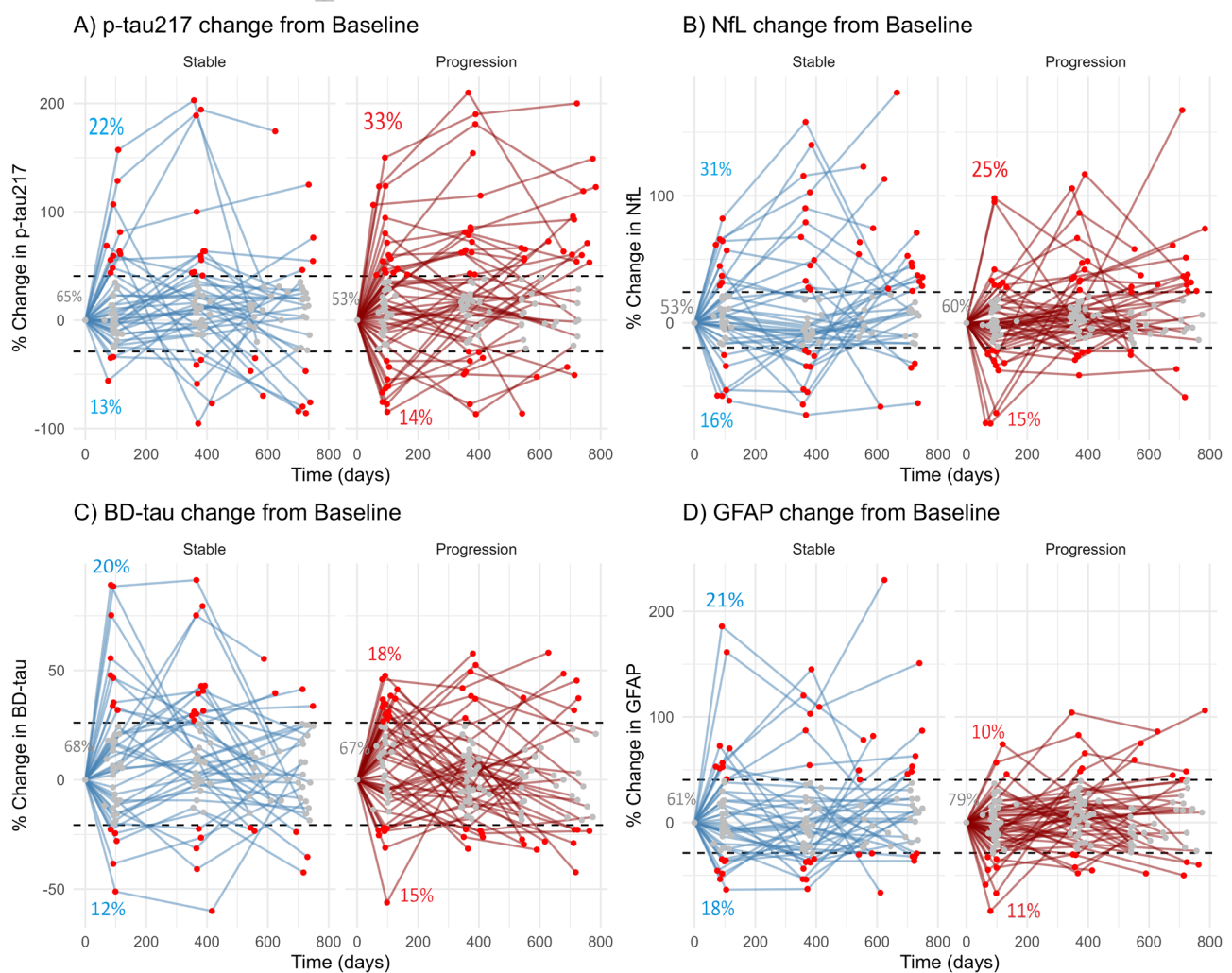


Figure 4.4.1 legend: Longitudinal plasma trajectories stratified by stable individuals vs. individuals who progressed. LMM models reflecting the change in plasma biomarker levels over time. Based on NfL, p-tau217, BD-tau and GFAP (log transformed) and p-tau217/BD-tau as outcomes, time (years) and age, sex and creatinine as fixed effect, and patients as random effects. *age was significant. For p-tau217 and p-tau217/BD-tau models, there were no evidence of baseline differences, hence for visual interpretation the model was fitted without group as the main effect, but with group:time as main effect and plotted. Reprinted from Clemmensen et al. ⁴.

Finally, we examined changes in plasma biomarker levels from baseline to follow-up visits in relation to their biomarker-specific Reference Change Value (RCV). For most plasma biomarkers, the observed percentage changes remained within the expected RCV range, see Figure 4.4.2. Here most changes above the RCV threshold were observed in plasma p-tau217 in the individuals who progressed during the study period ⁴.

Figure 4.4.2 Changes in plasma levels in the context of their reference change levels



% change from baseline that is above, within, or below the RCV. The dotted lines represent the plasma biomarkers individual upper and lower reference change values ^{1,2}. Red dots = exceeds RCV, Grey dots = within RCV. Reprinted from Clemmensen et al. ⁴.

Table 4.5 Summary of study outcomes and key findings

Study I Biomarker variability (SHORT-ND) • OUTCOME MEASURES: repeated measures of plasma Aβ40, Aβ42, p-tau181, p-tau217, p-tau231, t-tau, NfL, and GFAP • KEY FINDINGS across all plasma biomarkers: • Low within-subject variabilities • Moderate to high between-subject variabilities. • Wide reference change values.
Study II Improvement of biomarker variability (SHORT-ND) • OUTCOME MEASURES: repeated measures of plasma p-tau217, BD-tau, and t-tau • KEY FINDINGS: • Within-subject variability of plasma p-tau217/BD-tau ratio was lower than for p-tau217 alone. • Between-subject variability of p-tau217/BD-tau ratio was reduced in both Aβ and Aβ- groups, compared to p-tau217 alone. Between-subject variability in the Aβ+ group was further reduced after adjusting for eGFR, HbA1c, and BMI. • CSF p-tau217 exhibited a stronger correlation with plasma p-tau217/BD-tau compared with p-tau217 alone.
Study III Prognosis (TRACK-AD) • OUTCOME MEASURES: Annual change in CDR-SB and neuroimaging measures • PREDICTORS: baseline levels and short-term changes in plasma NfL, p-tau217, p-tau217/BD-tau, BD-tau and GFAP • KEY FINDINGS: • Baseline plasma NfL could significantly predict one- and two-year changes in CDR-SB and could distinguish between clinically stable and progressors. • Short-term changes in plasma NfL were significantly associated with annual SUVR decrease in bilateral hippocampal and temporal cortex, and the p-tau217/BD-tau ratio with annual decrease in bilateral hippocampal and isthmus cingulate cortex SUVR. • Plasma p-tau217, BD-tau or GFAP could not predict progression.
Study IV Tracking (TRACK-AD) • OUTCOME MEASURES: The trajectories of clinical and neuroimaging measures • PREDICTORS: Trajectories of plasma NfL, p-tau217, p-tau217/BD-tau, BD-tau and GFAP • KEY FINDINGS: • Plasma p-tau217 had a significantly steeper increase in AD progressors compared with stable individuals. • Plasma NfL, BD-tau or GFAP trajectories showed no differences between stable and progressors. • No correlations were found between any plasma biomarkers with individual changes in CDR-SB, changes in MRI regional volumes or with changes in [^{18}F]FDG-PET regional metabolism. • We observed that most changes in plasma biomarker levels from baseline to follow-up visits lied within their biomarker specific reference change values.

Table outlining outcome measures, predictors and key findings from each studies I-IV ¹⁻⁴. AD, Alzheimer's disease; CDR-SB, clinical dementia rating scale sum of boxes; CSF, cerebrospinal fluid spinal; A β , amyloid- β ; p-tau, phosphorylated tau; t-tau, total tau; NfL, neurofilament light; GFAP, glial fibrillary acidic protein; BD-tau, brain-derived tau; eGFR, estimated glomerular filtration rate; BMI, body mass index; HbA1c, Hemoglobin A1c; SUVR, Standardized uptake value ratio; [18 F]FDG-PET/MR, [18 F]-fluorodeoxyglucose positron emission tomography/magnetic resonance imaging.

5. Discussion

5.1 Summary of main findings in the studies I-IV

In the test-retest variability study, SHORT-ND, we found low within-subject variability across various AD plasma biomarkers – which is promising to capture individual changes. However, we found wide reference change values prompting necessary caution when interpreting changes in biomarker levels as disease-related, **Study I**. Moreover, the moderate to high between-subject variability across all biomarkers may raise concern for diagnostic and disease staging purposes. By normalizing p-tau217 to BD-tau we observed a reduced within- and between-subject variability compared to p-tau217 alone, **Study II**.

In the longitudinal study, TRACK-AD, we found that baseline plasma NfL predicted up to two years future changes in CDR-SB, whereas short-term changes in plasma NfL and in the p-tau217/BD-tau ratio were associated with future decrease in regional brain metabolism assessed by [¹⁸F]FDG-PET scans, **Study III**. Investigating the disease tracking potential of AD plasma biomarkers, we found that plasma p-tau217 increased at a significantly higher rate in individuals who progressed compared with stable individuals. However, we did not find a clear relationship between individual changes in plasma biomarkers with concurrent individual changes in clinical and neuroimaging measures, **Study IV**.

The following discussion focuses on key findings related to plasma A β 42/40, p-tau217, NfL, BD-tau, and GFAP across the four studies included in this thesis. Particular emphasis is given to Study III and IV, interpreted in the context of the findings from Study I and II. This is followed by a discussion of methodological considerations relevant to the interpretation and generalizability of the results.

5.2 Plasma A β 42/40

Results from Study I demonstrated the lowest within- and between-subject variability in plasma A β 42/40 compared with the other plasma markers. However, there are several challenges using blood A β 42/40 in clinical practice. This is partly due to the low fold change in plasma A β 42/40 between amyloid positive and negative individuals compared with their CSF levels. This causes low biomarker robustness⁹⁰ and makes blood A β 42/40 more susceptible to even minor fluctuations⁹¹, consequently increasing the risk of misclassification. The wide RCVs suggested in Study I further challenges the use of plasma A β 42/40 in clinical settings. Therefore, we did not include analyses of plasma A β 42/40 in Study III and IV.

5.3 Plasma p-tau217

We did not find supporting evidence of baseline plasma p-tau217 as a prognostic marker of clinical or neuroimaging progression, **Study III**, which contrasts with several previous studies on CU and MCI ^{121,122,126} (see Table 1.6.2). This discrepancy could be due to methodological differences, such as sample size, but may also indicate that plasma p-tau217 levels reflect distinct neuropathological changes across stages in the AD continuum. Moreover, we found that longitudinal trajectories of plasma p-tau217 differed markedly between clinical progressors and stable individuals, **Study IV**, which is in line with previous studies on CU and MCI patients ^{120,123} (see Table 1.6.2). We did not find a clear relationship between changes in plasma p-tau217 and in CDR-SB on an individual level, **Study IV**. This finding is partly in accordance with Insel et al. ¹²⁶, a large cohort study on more than 1,500 CU individuals reporting that longitudinal measures of plasma p-tau217 only moderately correlated with concurrent changes in cognitive measures.

Findings from **Study I** indicated considerable biomarker variability and suggested that approximately three repeated samples per individual (mean = 2.7) are needed to reliably estimate a stable biomarker value. This finding aligns with Dodge et. al ¹⁹⁹ emphasizing the importance of repeated blood sampling to reduce within-subject variability and improve measurement precision. Notably, this study suggested that 3-5 repeated samples could strengthen the evaluation of biological efficacy in short-duration clinical trials. In the context of findings from **Study III** and **IV**, this may support the idea that repeated sampling provides complementary information beyond what is captured by a single assessment. And that repeated measures may improve the precision, interpretability, and clinical utility of plasma p-tau217, potentially preventing misinterpretation of an individual's biomarker level. However, this hypothesis warrants further investigation in larger cohort studies to confirm its applicability and clinical relevance. In a diagnostic setting, repeated blood measures together with the two-way cut-off approach suggested by Brum et al. ¹¹⁸, may further reduce the number of patients who will need additional diagnostic assessment with CSF or PET.

Aiming to reduce the variability of plasma p-tau217, we investigated the p-tau217/BD-tau ratio, an approach conceptually similar to %p-tau217 (the proportion of phosphorylated tau217 to non-phosphorylated tau), as previously described by Janelidze et al. (2023) and Barthélemy et al. (2024) ^{161,200}. Thus, the ratio-based method may help reduce biomarker variability.

In **Study II**, we found a modest reduction in within-subject variability for the plasma p-tau217/BD-tau ratio compared to p-tau217 alone. The between-subject variability for p-tau217/BD-tau decreased notably compared with p-tau217 alone, and was further reduced adjusting for eGFR, BMI,

and HbA1c. These findings are consistent with a recently published large multi-cohort study, reporting that normalization with plasma non-phosphorylated tau reduced between-subject variability of plasma p-tau217, but had limited effect on within-subject variability²⁰¹. Furthermore, this study found that normalised plasma biomarkers correlated significantly better with tau-PET regional load compared with unnormalised biomarkers.

Given the wide RCV observed for plasma p-tau217 in **Study I**, it would have been valuable to assess whether the reduced variability in the p-tau217/BD-tau ratio from **Study II** also translated into narrower RCVs. However, this was not feasible, as the two biomarkers were measured using separate assays, preventing a reliable estimation of combined assay variability.

To validate the findings from **Study II**, we examined the p-tau217/BD-tau ratio prognostic, **Study III**, and disease-tracking, **Study IV**, abilities. The baseline ratio could not predict future cognitive or neuroimaging changes, but short-term changes in the ratio were associated with future alterations in regional glucose metabolism, **Study III**. The rate of longitudinal changes in the p-tau217/BD-tau ratio was steeper among individuals who clinically progressed compared with stable individuals, **Study IV**.

These findings correspond to those observed with plasma p-tau217 alone, but with some distinctions. Specifically, the plasma p-tau217/BD-tau ratio showed associations with two PET regions, and these associations remained significant after correction for multiple comparisons. P-tau217 alone was associated with one PET region and this association was not significant after testing for multiple comparisons, **Study III**. Furthermore, the ratio was able to differentiate trajectories between individuals with MCI who remained stable during the study period and MCI individuals who converted to dementia. We also observed a significant, albeit weak, correlation between individual changes in the p-tau217/BD-tau ratio and changes in CDR-SB, a relationship not established by plasma p-tau217 alone, **Study IV**.

Together, these results suggest that the p-tau217/BD-tau ratio may offer a more sensitive marker for prognosis and disease progression. However, these results should be interpreted with caution. Firstly, we observed that p-tau217 levels increased over time, while BD-tau levels tended to decrease in clinical progressors. This dual trend naturally leads to an increasing ratio, which may lead to an overestimation of change of the ratio – which does not necessarily reflect a true acceleration of disease progression. Secondly, while the ratio reduces between-subject variability, adjusting for confounders had a comparable effect (see Table 4.2), raising the question of whether the observed improvements in variability are driven by statistical control rather than by biological refinement. In

Study III and **IV**, all models were adjusted for age, sex, and creatinine, suggesting that the ratio's performance is not solely dependent on these covariates. While covariate adjustment and ratio-based normalization both contribute to reduce variability, they serve different purposes: the former improves statistical clarity, whereas the latter may enhance biological precision.

To summarise, based on our findings it is premature to recommend a replacement or modification of plasma p-tau217. The intention is to highlight the importance of addressing the variability of p-tau217 when estimating longitudinal changes, as this variability may contribute to misinterpretation. This warrants further investigation in larger and independent cohorts to validate our findings and to evaluate whether such ratio can improve diagnostic accuracy and reduce misclassification.

5.4 Neurofilament light

In the prognostic **Study III**, we found that baseline plasma NfL levels were higher in the group of patients who progressed and that baseline NfL could predict future cognitive decline on the CDR-SB and with intermediate accuracy distinguish between patients classified as stable or progressors at one- and two-year follow-up. These findings support several previous studies reporting blood NfL predicting future cognitive decline^{108,129,133,134,136,139–141} (see Table 1.6.2). Further, short-term changes in plasma NfL were associated with annual decrease in bilateral hippocampus and temporal cortex SUVr of [¹⁸F]FDG-PET. This may be in line with a previous study reporting an association between baseline NfL and 15-30 months changes in a global SUVr measure based on several regions¹²⁹. The lack of association between baseline NfL and future changes in MRI regions in **Study III** contrast previous findings reporting associations between baseline NfL levels and future changes in MRI regions^{108,125,129,133,134,140} (see Table 1.6.2). A further discussion on our imaging results is elaborated in section 5.7.1.

The low within-subject variability observed in individuals with AD, **Study I**, together with the narrowest reference change value (second only to amyloid), and the suggested requirement of just one sample to reliably estimate a stable biomarker level (= 1), may collectively support the use of baseline plasma NfL as a sensitive prognostic marker.

Plasma NfL levels increased significantly over time in patients who progressed, **Study IV**, supporting the hypothesis that NfL increases with advancing disease and reflect ongoing neurodegeneration. However, the rate of change was not significantly different from that observed in the stable group. This finding aligns with Yakoub et al.¹³⁵ reporting a general increase in plasma NfL over four years and found no differences in trajectory between PET A β ⁺ and A β [–] CU individuals. In contrast, Mattsson et al.¹³⁰ and Martino-Adami et al.¹³⁷ have reported a greater NfL increase over

4–11 years in AD patients compared with MCI and CU individuals, and over eight years in CU individuals who progressed to AD compared to stable CU individuals, respectively. A key difference may lie in the methodological approach: while our study examined progression across the MCI and dementia stages, Mattsson et al.¹³⁰ focused on differences between CI, MCI and CU. In addition, the considerably longer follow-up period in the Mattsson et al.¹³⁰ and Martino-Adami et al.¹³⁷ studies may have allowed for clearer separation of NfL trajectories, suggesting that our shorter follow-up may have been insufficient to detect such divergence between stable and progressors. Moreover, the high between-subject variability observed in **Study I** (approximately 30% in the AD group) may be generalizable to longitudinal settings. If so, such variability may obscure distinct trajectories between groups, as observed between stable and progressors in **Study IV**. This potential confounding effect highlights the importance of accounting for individual variability when interpreting longitudinal biomarker changes.

The absence of a relationship between individual NfL trajectories and corresponding clinical or neuroimaging changes contrasts with several previous studies reporting associations between longitudinal increases in NfL and cognitive decline^{108,129,131,140} (see Table 1.6.2). This discrepancy may partly reflect the limited sample size and study duration but could also arise from methodological differences. In **Study IV**, we derived individual trajectories of plasma biomarkers and CDR-SB scores from linear mixed models and examined the correlations between these slopes, whereas other studies, such as Mattsson et al.¹⁰⁸ and Preische et al.²⁰², modelled cognitive outcomes as dependent variables with plasma NfL as the predictor. By focusing on individual rates of change rather than modelling cognitive outcomes directly as dependent variables, we may have reduced statistical sensitivity to small but potentially meaningful correlations. Moreover, our cohort may not be as strictly selected as, e.g. participants in the ADNI¹⁰⁸ cohort and may differ from preclinical AD mutations carriers²⁰². Consequently, clinical progression observed in our study may not solely reflect AD pathology, but coexisting co-pathology, such as vascular changes, may also contribute to clinical progression and confound the interpretation of plasma NfL in relation to AD progression. Furthermore, with increasing age, NfL becomes a confounded or less specific biomarker due to age-related degenerative changes²⁰³. These changes may contribute to elevated NfL levels independent of disease progression, thereby complicating its interpretation in older populations. To address this, we accounted for potential confounding factors including age, sex, BMI, and kidney function in **Study III** and **IV**^{3,4}. Together, these factors need to be addressed to support the clinical implementation of NfL measurements in blood¹¹⁰.

5.5 Brain derived tau

Unlike plasma t-tau, influenced by peripheral produced tau, BD-tau was developed to specifically target tau isoforms from the CNS ¹¹⁴ and has been suggested as a potential novel neurodegenerative marker less sensitive to confounders, commonly affecting plasma NfL and t-tau levels. This aligns partly with results from **Study II**, in which we observed no significant correlations between plasma BD-tau and eGFR, BMI or HbA1c, whereas t-tau significantly correlated with eGFR and HbA1c ². We observed similar within- subject variabilities in BD-tau in AB+ individuals compared with t-tau, **Study II**, and with NfL in AD patients, **Study I**. However, we found a significantly lower between-subject variability in BD-tau (12%) in AB+ compared with NfL (30%) in AD patients (see Table 4.1 & 4.2). This comparison should be drawn with caution. While data is based on the same cohort, patients in **Study I** were characterised by clinical AD diagnosis opposed to the CSF based amyloid positive characterization in **Study II**. It is however promising that the variability of p-tau217 in AD vs. non-AD, **Study I**, and in AB+ vs- AB-, **Study II**, can be considered similar, see Table 4.1 & 4.2.

While technically promising, data on the clinical prognostic and disease tracking utility of this novel biomarker is essential. Plasma BD-tau has recently been associated with MRI structural changes and cognitive decline, and with future MRI atrophy and decline in cognitive tests in CU and in patients with MCI and dementia ¹¹⁵. Furthermore, Gonzalez et al. ¹¹⁵, found that patients with both elevated plasma p-tau217 and BD-tau baseline levels exhibited the fastest cognitive and neuroimaging decline. Our data could not replicate these findings as we found no association between baseline BD-tau levels and future cognitive decline, **Study III**. Several factors may play a role in this discrepancy. Compared with Gonzalez et al. ¹¹⁵, our study was a single centre study with less power, shorter follow-up duration and without CSF biomarker confirmation available on all participants.

Regarding association with imaging changes, we observed an association between short-term changes in BD-tau and an annual decrease in cortical and total grey matter volume, supporting previous findings ¹¹⁵. However, this finding was non-significant after multiple correction testing and should be validated in future clinical larger cohort studies.

To date, we are not familiar with other studies examining repeated measures of BD-tau in longitudinal studies. We hypothesized that with advancing disease, BD-tau would likely increase reflecting ongoing and advancing neurodegenerative processes. But while we found no changes in BD-levels in clinically stable patients, we observed a non-significant but decreasing trend in clinically progressors, **Study IV** ⁴. This unexpected decrease could reflect a plateau effect in BD-tau levels once

a certain neurodegeneration threshold is reached, limiting further measurable increases. This does however seem unlikely in this cohort of patients in the early stages of AD. Additionally, it could reflect cohort-related factors, e.g. baseline disease stage heterogeneity or regression to the mean.

Finally, it may reflect biological and technical factors unrelated to true disease progression, hence it should be noted that when examining changes in BD-tau levels between baseline and follow-up visits, most individual changes (66%) fall within the reference change value (RCV). This could suggest that the apparent trajectory may be heavily influenced by within subject and assay variability, rather than reflecting neurodegenerative progression itself. However, this interpretation relies on the assumption that baseline levels accurately reflect the individuals' reliable biomarker status.

5.6 Glial fibrillary acidic protein

We hypothesized that GFAP levels would increase with advancing disease progression. However, we did not observe any changes in GFAP levels during the study period, **Study IV**, nor did GFAP baseline levels predict future disease progression, **Study III**. These findings align with one previous study by Qiu et al.¹⁴⁰, but contrast with several studies reporting a predictive value of baseline GFAP^{142,136,134,125} and longitudinal changes of plasma GFAP^{132,142,135,137}. Notably, the study by Qiu et al., who found no association between plasma GFAP and future progression and disease tracking, was based on patients with a clinically established diagnosis of AD. In contrast, the other studies that established associations between baseline plasma GFAP and future progression and plasma increase over time, were based on CU individuals and individuals with MCI. This may indicate distinct properties of plasma GFAP across the preclinical and clinical AD continuum. This may also reflect the dual-phase nature of neuroinflammation, characterised by early increase followed by a plateau or chronic activation.

Moreover, we found a notably lower within-subject variability in plasma GFAP, **Study I**, compared with studies establishing within-subject variability in younger healthy individuals^{177,180}. One might speculate that this could suggest that GFAP is a surrogate marker for decreased astrocytic activity in the more chronic disease phases. Consequently, plasma GFAP may serve better as an early risk marker in individuals at risk of AD¹⁴⁴, rather than as a progression or disease tracking marker in later disease phases.

Table 5.0 summarises the results of our overall aim of this thesis, whether plasma biomarkers can predict and track disease progression in patients with early stages of AD.

Table 5.0 Overview of plasma biomarkers prognostic and disease tracking potential

	Prognosis		Tracking	
	Clinical	Imaging	Clinical	Imaging
p-tau217	✗	✗	✓	✗
p-tau217/BD-tau	✗	✓ (FDG-PET)	✓	✗
NfL	✓	✓ (FDG-PET)	✗	✗
BD-tau	✗	✗	✗	✗
GFAP	✗	✗	✗	✗

Table summarising the key findings of this thesis. A red cross indicates no evidence supporting the biomarker's ability to predict or track clinical or neuroimaging progression, whereas a green checkmark represents a significant finding. p-tau, phosphorylated tau; NfL, neurofilament light; BD-tau, brain-derived tau; GFAP, glial fibrillary acidic protein.

5.7 General remarks

After summarizing the findings for each biomarker individually, several overarching observations emerge across biomarkers and studies.

5.7.1 Imaging

The lack of association between plasma biomarkers and MRI changes should not be interpreted as evidence that no structural changes occur during the study period, **Study IV**; rather, it may suggest that the plasma biomarkers investigated in our study could not predict nor track such changes. Previous longitudinal studies, such as Mattsson et al. ¹⁰⁸, reported associations between baseline plasma NfL and subsequent changes in hippocampal, cortical, and ventricular volumes over four years. Similarly, Sanchez et al. 2025 ¹²⁵, found that baseline plasma GFAP, NfL, and p-tau217 levels were associated with ventricular and grey matter volume changes over two years. These studies, however, differ from ours both in sample size and in statistical methodology: they employed linear mixed-effects models to test for statistical associations between baseline biomarker levels and longitudinal MRI change, whereas we in Study III applied linear regression models to test the *predictive value* of baseline plasma biomarkers for future neuroimaging changes. This distinction is important, an observed statistical association does not necessarily translate into predictive utility at the individual level. However, our approach may be too stringent to detect subtle associations, particularly in a limited sample size.

5.7.2 Clinical heterogeneity in disease progression

Clinical heterogeneity remains a central challenge in AD ¹²³. The variability in clinical presentation and disease course across individuals complicates the assessment of progression at the group level, particularly when aiming to develop a biologically grounded staging system. The 2024 revised

criteria from the Alzheimer's Association acknowledge this complexity, emphasizing that a shared underlying AD pathology may manifest in diverse clinical phenotypes⁴⁷. Moreover, Frisoni et al.²⁴, suggests that variability in clinical onset, pathology, and progression of neurodegeneration depends on the interplay between various stochastic and genetic factors.

Although advancing tau pathology and neurodegeneration are generally associated with clinical deterioration, emerging research underscores the importance of identifying co-existing pathologies, such as α -synuclein, cerebrovascular disease, and TDP-43, which may influence disease severity or progression^{45–47}. These co-pathologies may influence both biomarker levels and clinical outcomes, contributing to the observed misalignment between clinical presentation and biological stage. Thus, especially in a diagnostic context, a biomarker should, for now, never stand alone and always be interpreted in relation to clinical symptoms.

In TRACK-AD, we aimed to capture disease progression broadly by employing multiple cognitive and functional assessments, including MMSE, ACE, CDR-SB, and clinical staging. This approach acknowledges that clinical heterogeneity may impact the sensitivity and specificity of individual tests. Recent findings have demonstrated a misalignment between biomarker changes and clinical symptoms, with some patients showing significant biomarker alterations despite minimal cognitive decline, and vice versa²⁰⁴. This imbalance may reflect cognitive resilience, comorbidities, or the influence of co-pathologies, and highlights a fundamental challenge in clinical trial design: plasma biomarkers reflect underlying biological processes that may not directly correlate with functional or cognitive decline. The heterogeneity of memory clinic cohorts – as ours – further complicates interpretation, as patients often present with mixed pathologies and varying degrees of comorbidity. This variability must be considered when evaluating biomarker-based outcomes and designing trials.

5.7.3 Biomarker variability

Our findings of biomarker variability should overall be interpreted with caution. It is a novel topic that until recently was only investigated in healthy individuals^{177,178,180}. Recently, studies have examined how biomarker variability may impact on clinical decision-making^{161,205} and designing clinical trials¹⁹⁹.

However, we must acknowledge some methodological considerations of our study design in SHORT-ND. It is difficult to estimate a robust sample size when designing a test-retest study aiming to apply linear mixed model. The sample size calculation was based on findings from a study on NfL levels in MCI and AD patients. While designing the study, we chose NfL because we

considered it the least stable biomarker. However, whether another sample size would have been required had we based the calculation on plasma p-tau217, remains unknown.

While we consulted relevant guidelines, such as the *standard for reporting biological variation data studies* by the European Federation of Clinical Chemistry and Laboratory Medicine, it could be questioned whether the length of the study and the number of samples per participant were appropriate, and whether the lack of a strict sampling time point during the day, lack of fasting status, and seasonal variation have impacted our results. In addition, it remains unknown whether other requirements should apply when estimating variation in a population affected by disease, rather than biological variation limited to healthy individuals. Moreover, a statistical assumption was applied when measuring biological variability to ensure that the biomarker was in steady state – meaning no systematic increase or decrease occurred during the study period. While this assumption was met for all biomarkers, except for GFAP, one may argue that biomarker levels in patients with a progressive neurodegenerative disease are never in a steady state. Thus, a critical interpretation of our results regarding plasma biomarkers' variability is important.

However, some of our findings are consistent with other studies on biomarker variability. As mentioned, Dodge et al.¹⁹⁹ suggest the same number of required samples to ensure a stable biomarker level of plasma p-tau217 and demonstrate improved precision of within-subject measures. Karlsson et al.²⁰⁵ demonstrate reduced between-subject variability, but limited effect on within-subject variability by normalizing plasma p-tau217 to non-phosphorylated tau, which is consistent with our limited reduction of plasma p-tau217 normalised to BD-tau or when adjusted for confounders. This may suggest that beyond assay variation, further reduction of within-subject variability is limited. Moreover, Karlsson et al. demonstrate that normalization of plasma biomarkers further improved association with amyloid- and tau-PET²⁰⁵. Biomarker variability can ultimately cause misclassification and obscure interpretation of longitudinal changes or intervention efficacy. For the individual patient it is crucial not to misdiagnose the presence of AD pathology or misinterpret changes in biomarker levels caused by treatment or other types of intervention, when it could be physiological variation interfering.

5.8 Methodological considerations

5.8.1 Strengths

The main strength of the studies is the well-characterised cohort of patients from an everyday memory clinic. The SHORT-ND study was designed with flexible intervals between visits to enhance study feasibility and reduce participant dropout. This approach was particularly important

given that the study was conducted during a period when patients were undergoing the stress of diagnostic evaluation in the memory clinic. The TRACK-AD study was designed to mimic the routine follow-up carried out in the memory clinic, minimizing additional visits to the hospital, and increasing the likelihood of participant retention. The prospective longitudinal study design in TRACK-AD was an optimal setup to test the plasma biomarkers in an actual clinical setting with memory clinic patients. Finally, we aimed to evaluate the predictive value of plasma biomarkers using the validated measure, changes in CDR-SB, as primary outcome. While we considered using an annualised CDR-SB change cut-off for grouping patients, we aimed at strengthening our findings by validating them against a broader clinical classification. Therefore, patients were classified based on their disease severity stage at each study visit: *Stable* (MCI stable + dementia stable) & *Progressors* (MCI converted to dementia + dementia progressed).

5.8.2 Limitations

Both SHORT-ND and TRACK-AD suffer from limited sample size and ethnic diversity, limiting our studies generalizability. Preferably a longer study duration would have allowed improved statistical power and strengthened longitudinal observations. The dropout rate in TRACK-AD was consistent with expectations. However, we cannot rule out the possibility of bias if participants who discontinued exhibited greater disease progression, potentially obscuring important findings.

Applying results derived from the relatively small SHORT-ND cohort to evaluate plasma biomarker changes in the TRACK-AD cohort may introduce some limitations, as differences in sample size and demographic composition could affect the generalizability of findings.

In TRACK-AD, CSF confirmation of amyloid pathology was available for approximately half of the patients. However, the study aimed to apply plasma biomarkers, developed to reflect underlying biological processes, to detect disease progression. While clinical and biological classification are expected to align, confirming biomarker status in all participants would have strengthened the study ability to interpret plasma biomarkers and changes in relation to disease stage and progression. Furthermore, *APOE* genotyping would also have been desirable as previous studies have established different disease trajectories according to *APOE* genotype and to elaborate the probabilistic AD model proposed by Frisoni et al.²⁴. Finally, four patients presented with a non-amnesic variant of AD, which could potentially influence disease progression and interpretation of our findings. However, only one patient with posterior cortical atrophy progressed during study period and the impact of this single patient on overall findings is considered minimal.

In **Study III** and **IV**, we included specific PET and MRI regions of interest. We aimed to balance sensitivity to early disease processes with robustness and generalizability of the findings, while also limiting the number of comparisons to reduce the risk of type I error. For both imaging modalities, we included regions that have previously been associated with longitudinal changes in AD. We acknowledge that smaller regions such as the precuneus would have been relevant and that our regional selection represents a limitation and should be extended in future studies with larger sample sizes.

Pre-analytical conditions were kept as consistent as possible by adhering to standardized hospital procedures for blood handling and processing. However, information on fasting status was not available in either of the studies, which we acknowledge as a limitation. Exact blood collection times were not recorded, and all blood samples were collected according to clinical routine between 9:00 AM and 3:00 PM. While both diurnal and short-term physiological fluctuations may contribute to within-subject variability (see Table 1.7.1), we aimed to mitigate these factors by restricting sampling to daytime hours, consistent with real-life clinical practice. Nonetheless, residual variability due to unstandardized sampling times may remain.

Finally, we thoroughly discussed the advantages and disadvantages of running all plasma analysis simultaneously versus continuously. While running samples continuously more closely reflects everyday clinical practice, we opted to process all samples simultaneously to minimize pre-analytical and analytical variability. Additionally, given the rapid development of biomarker assays, we were able to include novel markers, such as BD-tau in our panel. This would not have been feasible had we conducted analyses continuously. Moreover, it is important to note that the Simoa technique, while highly sensitive, is a research tool not validated for clinical purposes.

5.8.3 Statistical considerations

In **Study I**, biomarker concentrations are presented as mean and SD, but as these were non-normal distributed it would have been more correctly to report the raw plasma concentrations as median with IQR. Log transformation was applied to ensure normal distribution prior to conducting the linear mixed models.

As we in **Study II** observed a combined impact of confounders on between-subject biomarker variability, we must acknowledge that for **Study I** the same method should have been applied, here we adjusted for each confounder separately with the aim to assess their individual impact on the variability. For some markers, this approach resulted in a further reduction of variability.

In **Study I**, classification accuracy was estimated using a 50/50 train-test split due to the limited sample size. This approach was chosen to ensure that both training and testing sets retained enough cases to enable meaningful model fitting and evaluation, while avoiding overfitting. This approach prioritizes conservative and interpretable results, acknowledging the constraints of the available data. However, we later consulted Frank Harrell’s recommendations outlining pitfalls of data splitting in small datasets ²⁰⁶. Thus, for Study II, we abandoned the 50/50 train/test split and reported the area under the ROC curve (AUC) using the full dataset. AUC is a threshold-independent metric that reflects the biomarker’s overall discriminative ability, is less sensitive to class imbalance or prevalence, and tends to be more robust in small datasets. To address potential biases due to missing data, we further performed AUC analyses on complete cases across all visits. This adjustment did not alter AUC values. Lastly, these analyses were not the primary outcome of the **Study I and II**, but rather an exploratory investigation into whether biomarker variability impacts classification performance.

Linear mixed-effects models were used to analyse within- and between-subject variability in **Study I and II**. LMMs allow for including participants with incomplete observations and handle missing data under the assumption that data is missing at random. LMMs use maximum likelihood estimation, hence they are robust to unbalanced data structures and do not require complete cases, thereby increasing statistical power compared to traditional methods that exclude incomplete cases. No imputation was performed.

To account for variability between sampling time point, time was included as a covariate in the statistical models throughout **Studies I-IV**.

In **Study IV**, the outcomes in the LMMs were plasma biomarkers, which were log-transformed to approach normality. The p-tau217/BD-tau ratio, however, was not log transformed. It is mathematically illogical that although both individual biomarkers approached normality after log transformation, the ratio did not benefit. In our case, the distribution of the ratio did not warrant transformation and applying it was neither appropriate nor necessary.

6. Conclusion and perspectives

6.1 Conclusions

In conclusion, this thesis suggests that different AD plasma biomarkers may have different applications in patients with clinically manifest AD. Plasma NfL may be a reliable marker predicting future disease progression, while plasma p-tau217 may serve as a disease tracking marker. Moreover, plasma biomarkers may provide different information in preclinical and clinical AD. Furthermore, our findings suggest that biomarker variability plays an important role in our interpretation of biomarker plasma levels and that variability in plasma biomarkers may mask true disease progression.

The following outlines future perspectives derived from findings of this thesis. They are hypothetical and rely on the limitations regarding biomarker variability highlighted in section 5.7.3 and 5.8.3.

6.2 Clinical implications

Prognosis

Improved prognostication is crucial to improve treatment decisions and to help guide patient and relatives navigating on the difficult path of dementia. When informing patients of the diagnosis I have always been met with a form of these questions: *How will this affect me? How many good years do I have?* – and not once have I been able to give a useful answer. While no doctor can foresee the future, other medical fields, e.g. oncology, have markers to risk stratify patients and guide towards more aggressive treatments if indicated. Thus, the goal would be, beyond treatment itself, to predict who will likely experience a rapid decline and who will have a slower disease path.

While our findings support previous findings suggesting baseline levels of NfL as a possible prognostic marker for disease progression, it remains unclear to what extent these findings can be translated into predicting an individual's rate of progression. How do we meaningfully communicate an annual risk of, e.g. a specific point increase in CDR-SB or another relevant scale to the patient? Should thresholds for high, intermediate or low NfL levels be established? And if so, how can we ensure that elevated baseline levels reflect neurodegenerative processes due to AD, rather than co-pathologies? All these question warrants further investigation prior to clinical implementation.

Tracking

Tracking AD progression with plasma biomarkers may initially seem redundant, especially when clinical symptoms are already evident – it is not clearly beneficial for patient and relatives with information like *the blood sample indicate disease progression in line with what you are experiencing*, or opposite, *we cannot detect a change in your blood sample, but we can clinically confirm there has been a progression since the last visit*. But as disease-modifying therapies become

available, there is a pressing need for robust biomarkers that truly measure treatment effect, here blood biomarkers serve as a crucial tool to meet the demands implementation of disease-modifying treatments will require from health care systems.

Figure 6.2. illustrates a hypothetical case of a patient initiating disease-modifying treatment with an anti-amyloid drug. Prior to the first drug administration, three blood samples are drawn to measure the patient's reliable level of plasma p-tau217. After 12 weeks a new blood test reveals a decrease in p-tau217 levels. However, based on our findings, this difference lies within the RCVs, and thus establishing a clear disease-related change is not possible. Following additional treatment, the 36-week follow-up reveals a further decrease in plasma p-tau217 level, exceeding the lower limit of the RCV. This may more likely reflect target engagement, indicating that the therapeutic intervention is interacting with its intended molecular target, in this case, plasma p-tau217.

Figure 6.2. Conceptual changes in plasma p-tau217 levels following treatment

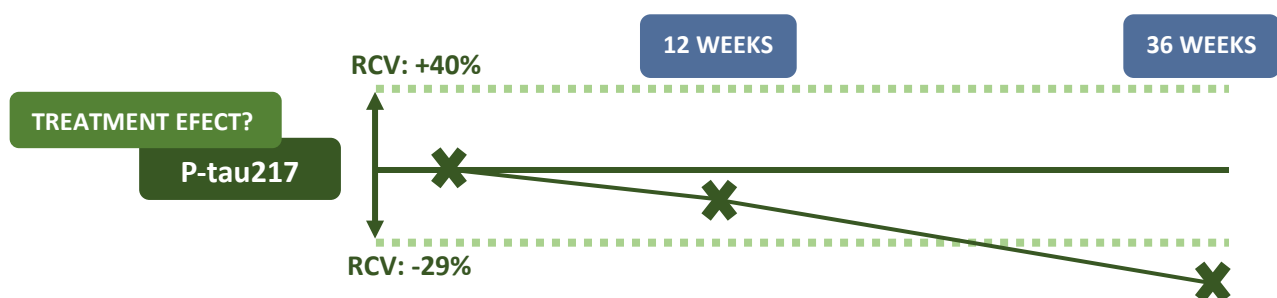


Figure outlining a hypothetical case tracking treatment response in a patient receiving an anti-amyloid drug, by assessing changes in plasma p-tau217. The dotted lines represent the estimated upper and lower reference change value, RCV. The green crosses indicate measures of plasma p-tau217 before treatment and after 12 and 36 weeks of treatment. Created by FK Clemmensen.

Furthermore, blood biomarkers may become valuable tools for monitoring treatment safety. For instance, they could help identify the presence of amyloid-related imaging abnormalities, edema or haemorrhage (ARIA-E and -H), and thereby identify which patients require further evaluation by MRI. This would prevent unnecessary scans, improve compliance, lower costs, and ultimately allocate resources to patients in need for more closely monitoring or treatment adjustment.

Finally, high between-subject variation may warrant some considerations when estimating treatment response between placebo and treatment arm. By comparing baseline between-subject variability in a blood biomarker with the magnitude of change in that biomarker during treatment, it could be possible to assess whether the effect exceeds physiological variation and/or placebo effect. Further, if post-treatment between-subject variability is remarkably lower in the treatment group compared to placebo, this may indicate a uniform response to the treatment. Such a reduction in variability could indicate that treatment have a consistent effect across individuals in the treatment group, strengthening the evidence for its efficacy.

6.3 Future research implications

In pursuit of accurate and accessible diagnostic tools, as well as improved care and treatment options for individuals suffering from AD, we are eager to implement the novel blood biomarkers in clinical practice. However, as outlined in this thesis, several challenges remain.

Firstly, the modest relationship we found between changes in biomarker levels with concurrent clinical and neuroimaging changes highlights the need to identify co-existing pathologies as either confounders or contributors to biomarker levels, or as independent drivers of disease progression. The development of promising tools, such as a blood α -synuclein seed amplification assay and an assay for TDP-43, presents a significant step forward in disentangling these complex pathological processes, which should be investigated in future large-scale longitudinal studies.

Secondly, acknowledgement of variability in plasma biomarker levels should be further validated in larger cohorts, ideally with a discovery and validation cohort of biomarker confirmed preclinical individuals, and individuals with MCI and dementia, both mild, moderate, and severe. Examining short-term variability across different AD stages and implementing these to longitudinal changes could provide important insight into how biomarker variability may change and should be interpreted according to disease stage. Moreover, it is important to test biomarker variability on other assay platforms, such as mass spectrometry, nucleic acid linked immuno-sandwich assay. While assay sensitivity is likely to improve over time, inherent physiological and technical variability will persist. Therefore, it is essential to develop strategies that account for and mitigate the impact of biomarker variability, especially in contexts where precision is critical. The importance of variability may differ depending on the specific biomarker applications and its relevance should be evaluated based on the specific clinical or research setting.

Finally, clinical trials have to some extent begun to use plasma biomarkers as surrogate endpoints for treatment efficacy^{207–210}. It is therefore crucial to further study plasma biomarkers' ability to track disease progression on an individual level. Ideally such studies should be multicentred and sufficiently powered. Prior to study enrolment, patients should undergo repeated blood measures to reduce variability and enhance measurement precision, and changes in biomarker levels should be interpreted with respect to their reference change values.

To summarise, this thesis suggests that blood biomarkers hold promise as novel tools for AD prognosis and monitoring. As these tools are likely to become essential in the future landscape of dementia diagnosis, care and treatment, clinicians and decision-makers must be aware of their current limitations and strategies must be developed to overcome these challenges.

7. References

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8. Appendices: Studies I - IV

8.1 Study I

Short-term variability of Alzheimer's disease plasma biomarkers in a mixed memory clinic cohort

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RESEARCH

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Short-term variability of Alzheimer's disease plasma biomarkers in a mixed memory clinic cohort

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Abstract

Background For clinical implementation of Alzheimer's disease (AD) blood-based biomarkers (BBMs), knowledge of short-term variability, is crucial to ensure safe and correct biomarker interpretation, i.e., to capture changes or treatment effects that lie beyond that of expected short-term variability and considered clinically relevant. In this study we investigated short-term intra- and inter-individual variability of AD biomarkers in the intended use population, memory clinic patients.

Methods In a consecutive sample of memory clinic patients (AD $n=27$, non-AD $n=20$), blood samples were collected on three separate days within a period of 36 days and analysed for plasma A β 40, A β 42, p-tau181, p-tau217, p-tau231, T-tau, neurofilament light (NfL), and glial fibrillary acidic protein (GFAP). We measured intra- and inter-individual variability and explored if the variability could be affected by confounding factors. Secondly, we established the minimum change required to detect a difference between two given blood samples that exceeds intra-individual variability and analytical variation (reference change value, RCV). Finally, we tested if classification accuracy varied across the three visits.

Results Intra-individual variability ranged from ~3% (A β 42/40) to ~12% (T-tau). Inter-individual variability ranged from ~7% (A β 40) to ~39% (NfL). Adjusting the models for time, eGFR, Hba1c, and BMI did not affect the variation. RCV was lowest for A β 42/A β 40 (~15%/+~17%) and highest in p-tau181 (~30/+~42%). No variation in classification accuracies was found across visits.

Conclusion We found low intra-individual variability, robust to various factors, appropriate to capture individual changes in AD BBMs, while moderate inter-individual variability may give rise to caution in diagnostic contexts. High RCVs may pose challenges for AD BBMs with low fold changes and consequently, short-term variability is important to take into consideration when, e.g., estimating intervention effect and longitudinal changes of AD BBM levels.

Trial registration Clinicaltrials.gov (NCT05175664), date of registration 2021–12-01.

Keywords Alzheimer's disease biomarkers, Blood-based biomarkers, Memory clinic cohort, Short-term variability, Intra- and Inter-individual variability, Reference change value (RCV), Plasma A β 42/A β 40 ratio, Phosphorylated tau (p-tau), Neurofilament light (NfL), Glial fibrillary acidic protein (GFAP)

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Background

Blood-based biomarkers (BBMs) for Alzheimer's disease (AD) are emerging and have potential to revolutionize diagnostics and prognostication of the disease. As new disease-modifying drugs are on the horizon, there is an urgent need for easily available biomarkers to detect disease pathology, select patients for advanced diagnostic work-up or treatment, and to track treatment effects in AD patients. Advances in measuring AD BBMs [1, 2] may supplement and in some cases replace lumbar puncture procedures and PET scans when a two-step diagnostic workflow is used [3], and have opened the door for repeated measurements monitoring AD pathophysiological processes. This study investigates short-term variability of AD BBMs to ensure correct interpretation of changes in biomarker concentrations as clinically relevant.

At present, AD BBMs include amyloid beta ($A\beta_{40}$, $A\beta_{42}$ and the $A\beta_{42}/A\beta_{40}$ ratio) [4, 5], phosphorylated tau variants (p-tau181, p-tau217, p-tau231) [6], total-tau (T-tau), neurofilament light (NfL) [7] and glial fibrillary acidic protein (GFAP) [8]. These constitute a panel of BBMs reflective of amyloid pathology, neurodegeneration, and inflammation, valuable as diagnostic and prognostic biomarkers and as markers of target engagement in clinical trials [9]. However, several factors such as pre-analytical and confounding factors, assay variability and short-term variability must be considered before implementation of AD BBMs. Preanalytical factors have been investigated to ensure stable biomarker concentrations [10–15] and confounding factors such as age, kidney dysfunction and body mass index are known to affect levels of BBMs [16–18]. Recently also food intake have been suggested to alter concentrations of AD BBMs [15]. Furthermore, efforts to optimize assays and minimize assay variability are ongoing [5, 19]. Indeed, low fold change and large overlap in i.e. plasma $A\beta_{42}/A\beta_{40}$ ratio values result in a too low robustness to withstand these factors in clinical routine, making this biomarker unsuitable for diagnostic use [20, 21].

However, only few studies have investigated short-term variability in AD biomarkers. One study has examined test–retest stability between two time points of plasma $A\beta_{42}/A\beta_{40}$, p-tau217, NfL and GFAP in 38 participants from a memory clinic with a 6–10 weeks interval [22]. In healthy individuals biological variation of $A\beta_{42}/A\beta_{40}$, p-tau181, p-tau217, p-tau231, NfL and GFAP have been estimated in previous studies [23–26]. Thus, there is a need, recently highlighted by an expert group, to examine biological variation (BV), of AD BBMs in patients with and without AD [27]. The term "biological variation", determined by a patient's physiology, is a fundamental concept in clinical chemistry and is usually estimated in

a reference population of healthy individuals. However, BV may be impacted by underlying pathological disease process [28], necessitating estimation of BV in the target population. Within-subject and between-subject BV quantify different aspects of BV and may be influenced by age, sex, genetic factors, chronic diseases, lifestyle factors etc. Within-subject BV is a measure of the variation of a biomarker's concentration around an individual's homeostatic set-point. Between-subject BV is a measure of the variation across individuals' homeostatic set point in a group, i.e. how much the concentration differs from one person to another within the group [26, 29]. However, when studying a disease population, biological variation, especially between-subjects, may be influenced by disease heterogeneity and varying disease severities within the group and may therefore not directly represent the inherent biological variation of the marker as in a healthy population. Therefore, throughout this article we refer to intra-individual and inter-individual variability, since the contribution of disease-related factors cannot be dismissed. Thus, intra-individual variability can be used to assess whether changes in biomarker levels in individual patients are beyond physiological variation whereas inter-individual variability can be applied to investigate whether within group physiological or disease-related variation is favorable towards e.g., application of the biomarker in a diagnostic role.

Thus, it is of great importance to examine short-term variability of AD BBMs in patients with AD to examine the impact of intra- and inter-individual variability on the interpretation of the biomarker results. Knowledge on the expected variability of BBMs is crucial to establish potential diagnostic cut-offs and to capture longitudinal changes or treatment effects that lies beyond that of intra- and inter-individual variability and thereby should be considered as clinically relevant changes.

Here we examined short-term variability of several AD BBMs at three different time points within 36 days in a real-life memory clinic setting. We measured intra- and inter-individual variability of AD BBMs in patients with and without AD. Secondly, we explored if short-term variability was affected by confounding factors. Also, we established the minimum change required to detect a difference between two given blood samples. Finally, for more clinical reliable use we tested if classification accuracy of the biomarkers differed between the three time points.

Methods

Study design and participants

This is a test–retest variability study with repeated blood sampling performed three times on the same individual within 36 days, see Fig. 1. Participants were patients with cognitive complaints, referred to the Memory Clinic

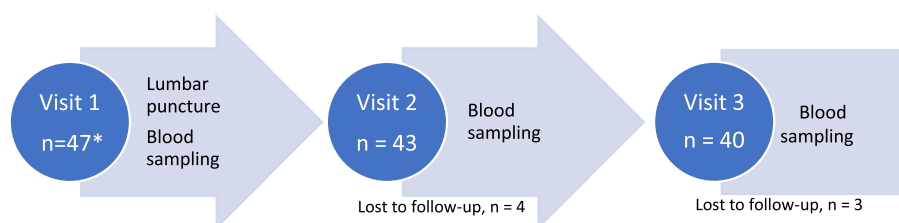


Fig. 1 Study design

Visit 1 (baseline), Visit 2 (4–16 days post LP), Visit 3 (19–36 days post LP). *Two blood samples from visit 1 were lost due to technical issues. Lost to follow-up was due to personal reasons for the participants

at Rigshospitalet, Copenhagen where physicians had requested CSF analysis of AD biomarkers as part of the routine diagnostic evaluation for suspected AD. Participants were consecutively and prospectively recruited, and the inclusion criteria were 1) a lumbar puncture as part of the diagnostic assessment and 2) Mini-Mental State Examination (MMSE) ≥ 20 . Exclusion criteria were 1) stroke within the past 3 months, 2) previous or existing major psychiatric conditions such as schizophrenia, bipolar affective disorder, or psychosis, 3) alcohol or substance abuse within the last two years, or 4) participation in intervention studies. Study inclusion was conducted between January–March 2022 and between August 2022–May 2023. The study was approved by the Danish Research Ethics Committee (H-21044863) and followed the tenets of the 1975 Helsinki Declaration. The study was registered at clinicaltrials.gov (NCT05175664). Written informed consent was obtained from each participant prior to enrolment.

Study procedure

A blood sample (baseline – denoted V1) was drawn via venipuncture immediately following the lumbar puncture procedure. The blood sampling was repeated on two separate occasions. The second blood sampling (visit 2 (V2)) occurred 4–16 days after baseline and the third (visit 3 (V3)) 19–36 days after V1. Blood samples were carried out between 9:00 AM and 15:00 PM. No information about last food intake was collected.

Sample processing

Blood was collected in ethylenediaminetetraacetic acid (EDTA)–plasma tubes according to hospital guidelines. Plasma and CSF, collected according to standard operating procedures for lumbar puncture, were centrifuged at 2,000 g, 4 °C for 10 min within 30 to 120 min after collection. Plasma and CSF were then redistributed into 250 μ l aliquots and stored at -80 °C until further analysis.

Biomarker assays

All samples were analysed at the Department of Psychiatry and Neurochemistry, the Sahlgrenska Academy, University of Gothenburg, in November 2023 to February 2024. Biomarker concentrations in both plasma and CSF were measured by Single molecule array (Simoa) technology by Quanterix HD-X [30]. The multiplex, Neurology 4-plex E assay from Quanterix was used to analyse plasma and CSF A β 42, A β 40, NfL, and GFAP. Plasma and CSF p-tau217 were measured using Simoa singleplex assay from Quanterix, ALZpath p-tau217. To analyse plasma and CSF p-tau181 and p-tau231 Simoa assays developed at University of Gothenburg were used [31, 32], while the Quanterix Simoa singleplex TAU kit was used for T-tau.

For all BBMs, calibrators were run in duplicates and obvious outlier calibrator replicates were masked before curve fitting. Plasma samples for A β 40, A β 42, NfL, GFAP and total-tau analysis were diluted fourfold, p-tau217 samples were diluted threefold, and p-tau231 and p-tau181 were diluted twofold. Results have been compensated for the dilution. A β 42/ A β 40 ratio was calculated. All samples were run in singlicates, and analyses were performed according to Simoa instructions [30]. Two QC levels were run in duplicates in the beginning and the end of each plate and determined the intermediate precision between plates, labelled inter-assay variability, CV_A . All samples were analysed in one round of experiments and all three plasma samples from each participant were run on the same plate. Concentrations lower than the calibration curve were labelled under limits of quantification (<LOQ).

Cognitive tests and diagnoses

Cognitive test results were extracted from medical files and stored in an electronic database (REDCap, Vancouver). Cognitive test results included the Mini-Mental State Examination (MMSE) [33] and Addenbrookes Cognitive Examination (ACE) [34] administered by a trained nurse and medical doctor, respectively. Patients were diagnosed based on criteria for MCI or dementia

due to AD [35, 36], dementia with Lewy bodies [37] frontotemporal dementia [38], AD with cerebrovascular pathology [36], vascular dementia [39], and cerebral amyloid angiopathy [40].

Demographics & biochemical data

Demographic data included age, sex, weight, height, body mass index (BMI), blood pressure, heart rate, medication status, and comorbidities, all extracted from medical files from the patients' visits to the Memory clinic and stored in REDCap. Biochemical data included results from the blood sample taken as part of the patient's diagnostic work-up program in the Memory clinic. Blood results included C-reactive protein, hemoglobin, hemoglobin A1c (HbA1c), alanine aminotransferase, high-density lipoprotein, low-density lipoprotein, cholesterol, triglycerides, and estimated glomerular filtration rate (eGFR). For a few patients with eGFR > 90, an exact eGFR was calculated based on age, gender, and level of creatinine, using the Modification of Diet in Renal Disease (MDRD) Study Equation [41]. CSF biomarkers used for diagnosis were analysed as part of routine analysis at Rigshospitalet for β -amyloid 1–42, tau and phosphorylated tau 181 (p-tau181) proteins. The assay used at Rigshospitalet changed during study inclusion: before June 30th 2022 the Innatest enzyme-linked immunosorbent assays (ELISA) by Fujirebio, Ghent were used, and after July 1st 2022 the Elecsys sandwich electrochemiluminescence-immunoassay by Roche, Cobas 8000, was used. $N=8$ patients CSF samples were analysed by Innatest.

Statistical analysis

Demographics and baseline characteristics are presented as means with standard deviations. Differences in baseline characteristic variables between AD and non-AD were examined with two-way sample t-tests for continuous variables and with Pearson's Chi-squared tests for categorical variables. Differences in total BBM concentration between AD and non-AD were analysed using linear mixed models with diagnosis as fixed effect and an unstructured covariance pattern to account for repeated measurements, using the *lmm* function from the *LMMstar* R package. Correlations between BBM concentrations between visits, CSF and possible confounders were examined with Spearman's correlation coefficients. We further investigated correlations between BBM and age, MMSE, ACE and all above-mentioned biochemical data.

Biological variation was examined by fitting linear mixed models (LMM) with patients as random effects, using the *lmer* function in the *lme4* package in R. The biomarkers' steady state (meaning no systematic trend (increase or decrease) in the biomarker concentration

during the study period) was evaluated with linear regression models on each biomarker with the mean concentration of the biomarker and the day of sample collection, and tests for variance of homogeneity were performed on log-10 scale-transformed data with Brown-Forsythe test. Outlier observations were detected using the interquartile range criterion. All LMM were based on log-10-transformed biomarker values. Coefficient of variation for intra-individual variability was based on the residual's standard deviation. Coefficient of variation for inter-individual variability was calculated from the square root of the total variances ($CV = \sqrt{(\text{variance}(\text{patients}) + \text{variance}(\text{residual}))}$), thereby incorporating intra-individual variability. The 95% confidence intervals for intra- and inter-individual variability were calculated from the model using the function *confint* with the *bootstrap* method. Biomarker values measured as under limit of quantification (<LOQ) (p-tau231 $n=2$ and T-tau $n=3$), were labelled as missing data (NA). The LMM models removed any observations labelled NA. All analyses were run on the total number of samples from the three visits. To investigate the effect of time and of biological confounders with a significant correlation with BBM concentrations (see Additional file 7, Figure 5), LMMs were carried out with time, eGFR, BMI, and HbA1c as a fixed effect. We considered the variables as stable over the short study period and used baseline data of the covariates. Assay-dependent analytical variation, CV_A , was based on the intermediate QC levels.

Number of samples needed to estimate an individual's homeostatic set point (n) with 15% proximity to the true value, and 95% CI was calculated by $n = (z * \sqrt{(CV_{\text{intra-individual}}^2 + CV_A^2)}) / D^2$, with $z = 1.64$ and D sat to 15%. To estimate the minimum change needed between an individual's two measurements to exceed intra-individual variability and analytical variation, CV_A , the reference change value (RCV), was calculated for each biomarker across diagnostic groups, by $RCV = 100 * (+\exp(z * \sqrt{2} * \sqrt{\log(CV_{\text{intra-individual}}^2 + CV_A^2) - 1}))$, with $z = 1.64$ (one-sided approach reflecting a unidirectional change) and CV_A based on the mean intermediate precision [42].

Classification accuracy (i.e., for the performance of each biomarker to separate AD from non-AD), was based on a predictive classification model build by splitting the dataset 50/50, using the *randomForest* package in R. Classification accuracy and corresponding 95% CI was then calculated by creating a confusion matrix, using the *caret* package, predicting the probability of an AD diagnosis at each time point.

All statistical analyses were performed using the R Statistical Software, version 4.4.1 and statistical significance

was set to $\alpha=0.05$. The R codes can be shared upon request.

Results

Participants

A total of 47 patients with cognitive dysfunction, due to AD ($n=27$) or other disorders (non-AD, $n=20$), were included in the study. Baseline characteristics for all 47 patients are presented in Table 1. In the AD group CSF A β 1–42, tau, and p-tau181 were significantly different from the non-AD group. No other significant differences in baseline demographic or clinical variables were found between the AD and non-AD group.

Concentrations of plasma & CSF biomarkers

Number of available plasma samples were V1 $n=45$, V2 $n=43$, and V3 $n=40$. The total biomarker concentrations in plasma per visit are presented in Table 2 and Fig. 2 (see Additional file 8, Figure 6 for BBMs per visit per patient). Overall, strong positive correlations of plasma concentrations between visits were found, see Additional file 9, Figure 7. A significant difference between the AD and non-AD groups was found for total plasma p-tau217 (p -value < 0.004 , 95% CI $(-0.35; -0.07)$). BBMs and CSF concentrations from each visit

are presented in Additional file 2, Table 4. CSF biomarkers were significantly different between AD and non-AD, except A β 40, NfL, and GFAP, see Additional file 2, Table 4. Correlations between CSF and plasma were only significant for NfL and p-tau217. See Additional files 5 & 10, Table 7 & Figure 8.

Intra- and inter-individual variability of plasma biomarkers

Results are presented from all participants, see Additional file 4, Table 6 for results with outliers excluded. All patients across BBMs were in steady state and no heterogeneity of variance was detected, except plasma GFAP not meeting these criteria (neither log transformed). We found a significant change in time for GFAP (p -value $= 0.02$), and the variances were not equal across samples.

Intra-individual variability

All CVs for intra-individual variability were low, see Table 2. A β markers had the lowest CV, ranging from ~ 3 to $\sim 4.5\%$ in the AD group, increasing in the p-tau variants and NfL, and was highest for T-tau and GFAP ($\sim 11\%$ in both groups). CV did not differ between the AD and the non-AD group except for

Table 1 Baseline characteristics

	AD (N=27)	non-AD (N=20)	p-value
Age, years	71.4 (5.4)	72.1 (6.2)	0.65 ⁽¹⁾
Gender, Male	15 (55.6%)	16 (80.0%)	0.08 ⁽²⁾
MMSE, Mean (SD)	26.8 (2.5)	27.0 (2.2)	0.79 ⁽¹⁾
ACE, Mean (SD)	78.8 (8.4)	78.0 (11.0)	0.77 ⁽¹⁾
Disease severity			0.42 ⁽²⁾
- MCI	13 (48.1%)	12 (60.0%)	
- Mild dementia	14 (51.9%)	8 (40.0%)	
CSF A β 1–42, pg/mL, Mean (SD) (AD $n=21$, non-AD $n=18$)*	664.8 (245.3%)	1124.2 (465.4%)	0.0004 ⁽¹⁾
CSF T-tau, pg/mL, Mean (SD) (AD $n=21$, non-AD $n=18$)*	317.2 (128.8%)	235.4 (122.7%)	0.05 ⁽¹⁾
CSF p-tau181, pg/mL, Mean (SD) (AD $n=21$, non-AD $n=18$)*	32.8 (15.2%)	20.7 (12.3%)	0.01 ⁽¹⁾
BMI, Mean (SD)	24.8 (3.4)	26.8 (5.3)	0.14 ⁽¹⁾
HbA1c, mmol/mol, Mean (SD)	40.6 (6.4)	40.8 (7.4)	0.95 ⁽¹⁾
eGFR, mL/min/1.73m ² , Mean (SD)	82.9 (13.7)	80.1 (26.1)	0.64 ⁽¹⁾
Creatinine, pmol/L, Mean (SD)	73.7 (17.7)	90.3 (42.4)	0.08 ⁽¹⁾
Diabetes II, Yes	3 (11.1%)	6 (30.0%)	0.10 ⁽²⁾

MCI mild cognitive impairment, non-AD: DLB $n=6$, Dementia with Lewy bodies, FTD $n=2$, Frontotemporal dementia, head trauma $n=1$; VD $n=1$, Vascular dementia, CAA $n=2$, cerebral amyloid angiopathy, ALS + VD $n=1$, amyotrophic lateral sclerosis, NPH $n=1$, normal pressure hydrocephalus, other tauopathy $n=1$, affective disorder $n=1$; kidney failure $n=1$; unclassified $n=3$. P-values from CSF are based on log-10-scale-transformed data. BMI 25–29.9: $n=11$, BMI > 30 : $n=8$. HbA1c > 48 mmol/mol: $n=8$. eGFR < 60 mL/min/1.73m²: $n=5$. Creatinine: Female < 46 : $n=1$, > 90 : $n=9$ & Male < 60 : $n=10$, > 105 : $n=4$

⁽¹⁾ two-way sample t-tests

⁽²⁾ Pearson's Chi-squared test

* $n=8$ patients' CSF were analyzed by Innostest (AD $n=6$ (A β 1–42 867.0 (73.1) pg/mL, T-tau 506.7 (160.2) pg/mL, p-tau 72.0 (22.1) pg/mL) and non-AD $n=2$ (A β 1–42 1176.0 (168.3) pg/mL, T-tau 255.0 (72.1) pg/mL, p-tau 39.0 (4.2) pg/mL))

Table 2 BBMs intra- & inter-variability in AD vs. non-AD

Biomarker	Population	No. of Samples	Mean plasma concentration (pg/mL, 95% CI)	Intra-individual variability CV (% 95% CI)	Inter-individual variability CV (% 95% CI)	CV _A (%)	
Aβ40	Total	128	103.2 (98.7, 107.6)	5.2 (4.4; 6.0)	10.0 (8.4; 11.9)	Low QC	4.7
	AD	71	96.9 (93.1, 100.6)	4.1 (3.3; 5.0)	7.1 (5.5; 8.7) ^c	High QC	7.5
	non-AD	57	111.0 (102.3, 119.7)	6.2 (4.9; 7.5)	12.5 (9.3; 16.2) ^c	Mean	6.1
Aβ42	Total	128	5.4 (5.1, 5.7)	5.9 (5.0; 6.7)	14.7 (12.2; 17.3)	Low QC	7.8
	AD	71	5.0 (4.7, 5.2)	4.5 (3.6; 5.5) ^c	12.2 (9.2; 15.3)	High QC	4.3
	non-AD	57	6.0 (5.3, 6.6)	7.2 (5.4; 8.8) ^c	16.9 (12.2; 21.8)	Mean	6.05
Aβ42/Aβ40	Total	128	0.052 (0.050, 0.054)	3.3 (2.8; 3.8)	10.1 (8.2; 12.0)	Mean	6.075 ^d
	AD	71	0.051 (0.049, 0.054)	3.4 (2.7; 4.1)	11.2 (8.3; 14.1)		
	non-AD	57	0.053 (0.050, 0.055)	3.2 (2.5; 3.9)	8.4 (5.9; 10.8)		
Ptau181	Total	128	8.8 (8.0, 9.6)	7.0 (6.0; 8.1)	20.5 (16.8; 24.3)	Low QC	21
	AD	71	8.6 (7.9, 9.3)	6.9 (5.6; 8.5)	15.1 (11.5; 18.8) ^c	High QC	5.8
	non-AD	57	9.1 (7.5, 10.7)	7.2 (5.5; 8.8)	26.3 (18.4; 34.0) ^c	Mean	13.4
Ptau217	Total	128	0.7 (0.6, 0.7)	9.3 (7.9; 10.8)	27.3 (21.9; 32.4)	Low QC	15.6
	AD	71	0.8 (0.7, 0.9) ^b	10.5 (8.3; 12.8)	19.7 (15.5; 24.5)	High QC	5.3
	non-AD	57	0.5 (0.4, 0.6) ^b	7.8 (6.0; 9.5)	30.9 (21.3; 40.7)	Mean	10.5
Ptau231	Total	126	7.3 (6.8, 7.8)	7.8 (6.6; 9.1)	17.4 (14.2; 20.8)	Low QC	15.6
	AD	71	7.4 (6.9, 7.9)	7.0 (5.6; 8.5)	13.4 (10.2; 16.3)	High QC	6.9
	non-AD	55 ^a	7.2 (6.3, 8.2)	8.8 (6.8; 10.9)	21.7 (15.6; 28.0)	Mean	11.3
T-tau	Total	125	1.53 (1.43, 1.66)	11.1 (9.4; 12.8)	17.7 (14.7; 20.5)	Low QC	9.2
	AD	68 ^a	1.55 (1.43, 1.66)	9.6 (7.4; 11.6)	17.4 (13.3; 21.7)	High QC	5.1
	Non-AD	57	1.50 (1.32, 1.68)	12.5 (9.7; 15.5)	18.7 (14.3; 23.2)	Mean	7.2
NfL	Total	128	31.4 (25.61, 37.23)	7.3 (6.2; 8.3)	34.1 (27.4; 41.1)	Low QC	3.2
	AD	71	24.6 (20.1, 29.1)	7.1 (5.5; 8.6)	29.6 (21.6; 37.2)	High QC	9
	non-AD	57	39.9 (28.3, 51.6)	7.5 (5.8; 9.2)	39.4 (27.1; 51.1)	Mean	6.1
GFAP	Total	128	128.9 (118.8, 139.0)	11.2 (9.3; 12.9)	20.5 (17.0; 23.8)	Low QC	5.5
	AD	71	137.3 (123.7, 150.9)	11.4 (9.2; 13.9)	19.6 (15.3; 23.9)	High QC	13.1
	non-AD	57	118.5 (103.4, 133.6)	10.9 (8.2; 13.5)	21.5 (16.4; 26.6)	Mean	9.3

Table showing the mean concentrations and 95% CI of each biomarker from all three study visits for the total number of participants as well as stratified by a diagnosis of Alzheimer's disease or other cognitive dementia diseases. Number of samples available for analyses are also presented. Intra- and inter-individual variability for each biomarker is presented together with the corresponding assay variability

Blood samples from two patients obtained at V1 were lost due to technical issues before freezing. CSF was available for those two patients. CSF from 42 patients were available for analyses collected at V1. For 5 patients there was not sufficient CSF for study analyses. In total, 38 patients had a complete set of three blood samples, and CSF was available for 33 of these patients

CI confidence interval, CV_A Assay variability (mean intermediate precision), Aβ amyloid-β, p-tau phosphorylated tau, NfL neurofilament light, GFAP glial fibrillary acidic protein

N-missing: AD: V1 *n* = 2, V2 *n* = 3, V3 *n* = 5. Non-AD: V1 *n* = 0, V2 *n* = 1, V3 *n* = 2

^a < LOQ (under limit of quantification): Plasma p-tau231 < LOQ *n* = 2 in the non-AD group, plasma T-tau < LOQ *n* = 3 in the AD group

^b Linear mixed models showed significant difference between groups

^c Non-overlapping 95% CI

^d Mean intermediate CV_A for the Aβ42/Aβ40 ratio was calculated as the mean across low and high QCs from both Aβ40 and Aβ42

a significant difference in Aβ42 CV between the two groups. Adding time as fixed effect made no notable changes to the CV, see Additional file 3, Table 5. Excluding outlier observations, Aβ42 (*n* = 6), Aβ40 (*n* = 7), T-tau (*n* = 2) and NFL (*n* = 13), in the analyses, made no change to the intra-individual variability, see Additional file 4, Table 6, for comparison.

Inter-individual variability

The lowest CVs for inter-individual variability were found in the amyloid markers ranging from ~7 to 12% in the AD group and from ~8 to ~17% in the non-AD group. CV was moderate in the p-tau variants, T-tau and GFAP and reached the highest variability in NfL. ~40% for NfL, see Table 2. There was a significant difference in CV between the AD and non-AD group for Aβ40 and

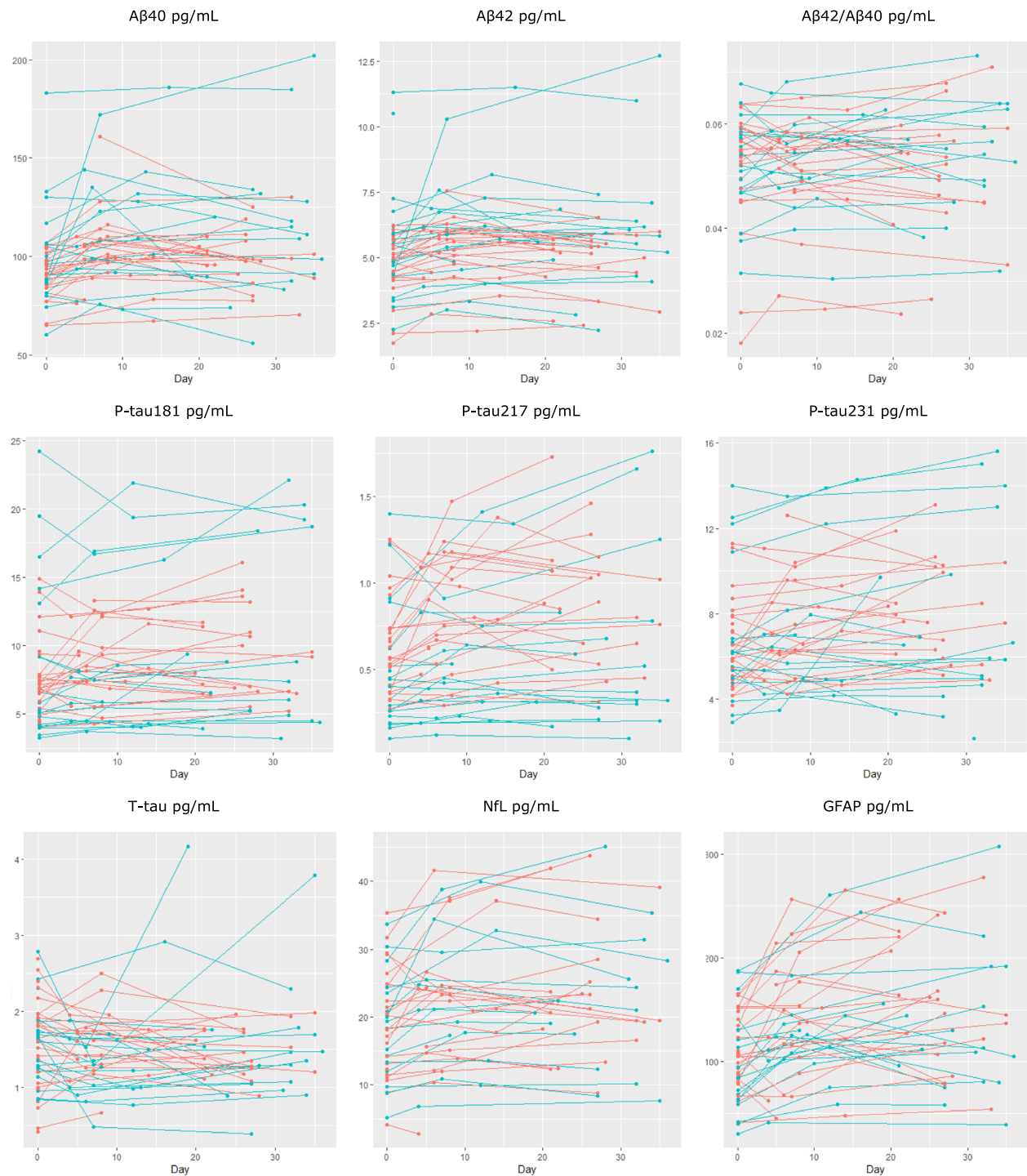


Fig. 2 BBB concentrations over time for each study participant

Spaghettiplots of all BBMs stratified by diagnosis. Outliers $n = 6$, NfL > 150 pg/mL have been removed. 

p-tau181. Adding time as a fixed effect made no notable changes to the inter-individual variability, see Additional file 3, Table 5. Excluding NfL outliers ($n = 13$), we found

a moderate effect on CV. Excluding outliers for A β 40 ($n = 7$) had a minor effect on CV, and for A β 42 ($n = 6$) a minor effect was observed in the non-AD group, and no

effect was found on CV when excluding T-tau ($n=2$) outliers, see Additional file 4, Table 6.

Inter-assay variability, CV_A

The inter-assay variability, CV_A , is based on the intermediate precision data for each biomarker and is presented in Table 2 as the low and high QC levels as well as the mean value. CV_A ranged between ~6% ($A\beta$ markers) and ~13% (p-tau181). All CV_{A_s} were lower than the corresponding intra-individual variability.

Biological confounders and its effect on intra- and inter-individual variability

No notable changes were found in intra-individual variability when adjusting for BMI, eGFR, and HbA1c. When adding BMI to the models the inter-individual variability decreased a few percent in p-tau181, p-tau217, p-tau231, NfL, and GFAP. Adjusting for eGFR, inter-individual variability in NfL and T-tau decreased a few percent. No notable differences in the variabilities were found on inter-individual variability when adjusting for HbA1c, see Additional file 3, Table 5.

Reference change value

Figure 3 shows the estimated reference change values (RCV), i.e., the minimum change (exceeding intra-individual variability and CV_A) required to detect a difference between a given patient's two blood samples. Results are based on the mean intermediate CV_A , see Additional file 6, Figure 4 for RCVs based on the lowest CV_A . The lowest RCV needed for a significant change was found in plasma $A\beta$ markers (decrease ~14% and increase ~17%). Plasma p-tau181 showed the highest RCV (increase ~42% and decrease ~-30%). Across diagnostic groups, no striking differences in RCV were observed. The number of samples needed to estimate individual homeostatic setpoints were between 1 to 3 samples per individual.

Classification accuracy across visits

Classification accuracies across visits are presented in Table 3. In general, classification accuracies were stable across visits, with T-tau and NfL showing the highest variation.

Discussion

This study examines the short-term intra- and inter-individual variability together with RCV in a large panel of AD plasma biomarkers in a memory clinic population,

including AD patients, and examined if short-term variability could be affected by known suspected confounders. We found overall low intra-individual variability and moderate to high inter-individual variability, which varied across AD biomarkers. Short-term variability was overall lowest in the $A\beta$ -markers, increasing for p-tau variants and highest in NfL and GFAP. In general, intra-individual CV was lower than inter-individual CV with comparable values across diagnostic groups. All CV estimates were robust to various confounders. Intra-individual variability was consistent regardless of outliers, however removing NfL outliers had moderate impact the inter-individual variability. RCVs were consistent across the diagnostic groups, overall lowest in the β -amyloid markers and highest in plasma GFAP.

The low intra-individual variabilities in our study are promising when tracking longitudinal changes and treatment effect for the individual patient, see Use Case 1 in Additional file 1. Inter-individual variability is important to both establish diagnostic cut-off values and to investigate if an intervention effect is consistent in the study population, and/or have different effect on different groups, see Use Case 2 in Additional file 1. Knowledge of RCV is crucial when tracking longitudinal changes and intervention effects, to estimate differences that lie beyond that of intra-individual variability and assay performance, see Use Case 1 in Additional file 1. Calculation of the patient's homeostatic set-point and use of RCVs of the given BBMs, can ensure that a given change in the plasma biomarkers is likely caused by i.e., an intervention and in fact a clinically relevant change. RCVs based on the lowest CV_A , did as expected cause narrower ranges. Though we assume the CVs for both intra- and inter-individual variability from this study to be generalizable to similar disease groups, CV_A will vary between laboratories and analytical platforms.

Furthermore, no significant variation in classification accuracies were found across visits. Some variation was observed for T-tau and NfL, which also showed high inter-individual CV, suggesting it may influence classification accuracy. In this vein, it is important to highlight, that the motivation for investigating the diagnostic ability of the biomarkers, was to examine whether the variation across a short time span, would impact these metrics which are more relatable to clinical practice.

No previous studies have investigated the short-term variability of these AD biomarkers, however few studies have examined the biological variation of some of the markers in younger healthy individuals. BV is commonly used to establish population-based reference intervals, i.e., in a non-affected population. However, previous research have addressed challenges using population-based BV and reference intervals for disease monitoring

[43] and have suggested that BV is not comparable between patients and healthy individuals, e.g. that an underlying disease may affect the variation observed in the biomarker. Therefore RCVs derived from healthy individuals may be narrower than RCVs based on the target population [28]. Meaning that if variation in a biomarker is smaller in healthy vs. the target disease, a change in a biomarker concentration could be misinterpreted as clinically relevant – whereas it is rather an effect of various physiological factors, that could include disease severity, comorbidities, medication, and clearance mechanisms in the blood. Therefore, our results of intra- and inter-individual short-term variability, which were marginally affected by confounding factors, must be seen in context of previous estimations of intra- and inter-individual variability in younger healthy individual, in which it is possible to isolate biological variation.

The low fold difference in plasma A β 42/A β 40 between individuals with or without AD pathology compared to CSF [44], makes it sensitive to even small variations [20, 21]. Plasma A β 40, A β 42, and A β 42/A β 40 showed overall low intra-individual variability, lowest for A β 42/A β 40, ~3%, consistent with results in healthy individuals [26]. Across diagnostic groups inter-individual variability and RCV were consistent and within the range of results reported in healthy individuals [26]. Therefore, despite the issues related to the low fold differences, the low variation indicates that A β measures in plasma may be relevant and affect classification into amyloid positive or negative. Furthermore, while we found significant difference in CSF A β 42/A β 40, we found no significant difference of plasma A β 42/A β 40 between groups. Whether this is an effect of underpowering, as the study was not designed to examine diagnostic performance of BBVs or poor performance of the biomarker, remains undetermined.

P-tau variants have so far shown great promise in detecting amyloid pathology in AD patients [6]. While p-tau231 may be increased in initial stages of AD [32, 45], p-tau217 seems to reflect amyloid pathology, and predict cognitive decline [46, 47], whereas p-tau181 could be indicative of more manifest tangle pathology at later disease stages [31]. Across diagnostic groups, we found the lowest intra-individual variability in p-tau181

and the highest inter-individual variability in p-tau217. In healthy individuals a previous study showed the lowest intra-individual variability for p-tau231 and the highest inter-individual variability in p-tau181 [26]. If p-tau variant concentrations are increasing at different disease stages throughout AD, an increase/decrease of inter-individual variability should be expected according to the spread of disease severity of the group. RCV for p-tau217 was consistent with results from healthy individuals [26]. The RCV for p-tau181 (–29/+42%) in the AD group was markedly lower compared to healthy individuals (–38/+62%), while RCV for p-tau231 was higher in the AD group (–26/+36%) compared to healthy individuals (–21/+26%) [26]. This difference between patients and healthy individuals could be considered to reflect that p-tau181 is more likely increased in later disease stages, narrowing the RCV index.

NfL, released following axonal damage or neuronal degeneration, has also been suggested as an early diagnostic triage biomarker [48] and can predict disease progression in AD [49–55]. Low intra-individual variability, moderate to severe inter-individual variability, and a rather wide RCV index for plasma NfL were similar across the diagnostic groups, and consistent with estimates in healthy individuals based on day-to-day and week-to-week blood samplings [24–26].

GFAP, released from activated astrocytes [8], may be elevated in pre-symptomatic AD stages [56]. Further, changes in plasma levels of GFAP following 12 weeks treatment with Donanemab were found to be decreased [9]. Our study found a reasonable intra-individual variability, consistent with results on healthy individuals [23, 26]. However, the moderate inter-individual variability was notably smaller compared to younger healthy individuals [23, 26], suggesting GFAP is a more stable marker in groups with brain pathology compared to healthy individuals. The RCV was considerably wide, ranging from –29 to +40%, consistent with RCVs based on healthy individuals [23, 26] and our results suggest at least three blood samples are needed to estimate a person's homeostatic set-point. Further, the increase over time in GFAP was unexpected and is likely to represent a spurious finding as opposed to reflecting disease progression.

(See figure on next page.)

Fig. 3 Model of reference change values, RCV %

Figures showing the amount of change for each biomarker to overcome intra-individual variability and assay variation, named Reference Change Value (RCV). The tables show the RCVs for each biomarker in patients with A) Alzheimer's disease and B) non-AD consisting of a mixed memory clinic cohort. Calculations are based on the mean intermediate CV_A, NNHS; number of samples needed to obtain homeostatic setpoint with 15% proximity to true value

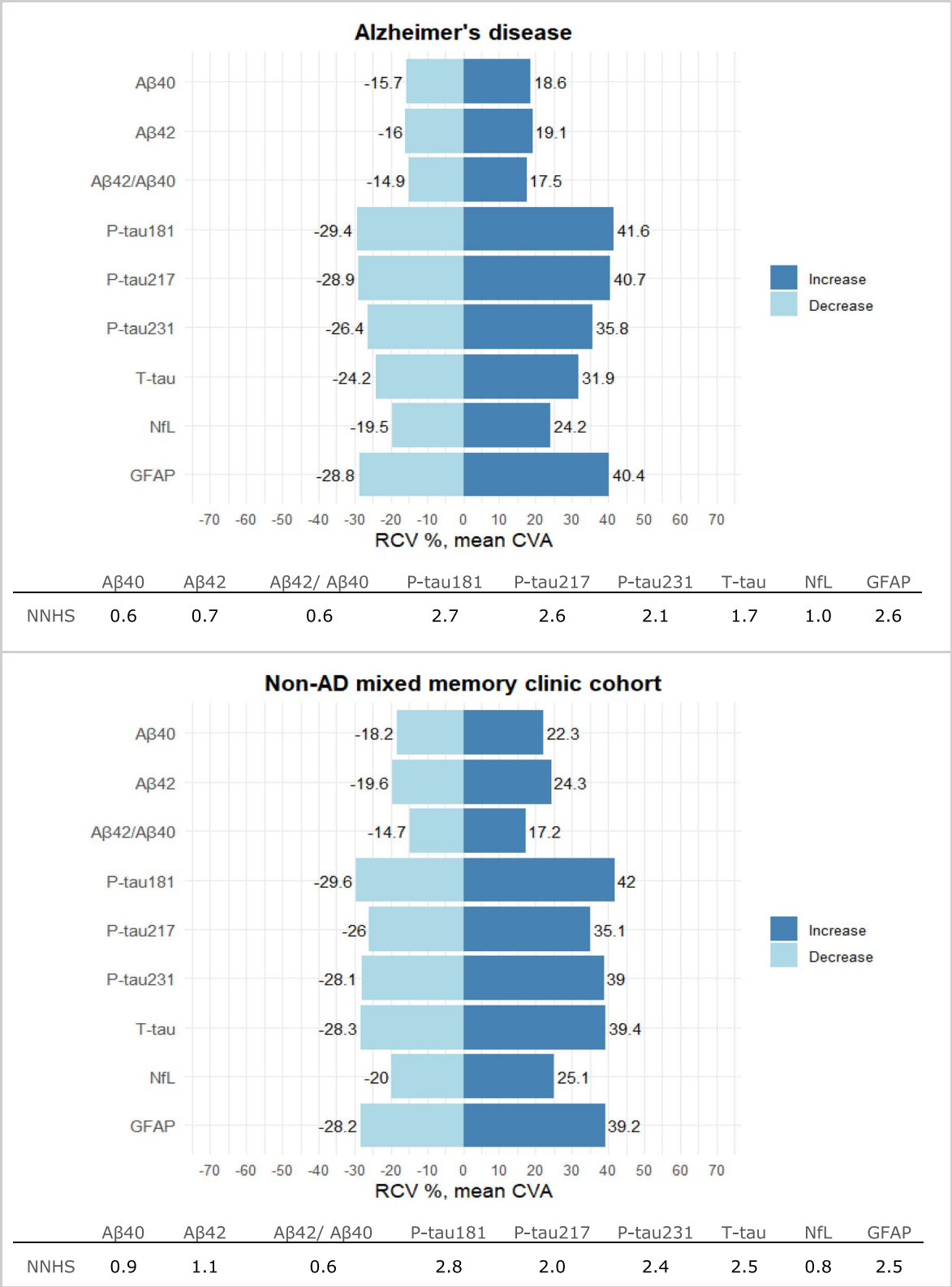


Fig. 3 (See legend on previous page.)

Table 3 Classification accuracy across visits

Biomarker	Classification Accuracy (95% CI)		
	V1	V2	V3
Aβ40	0.68 (0.45, 0.86)	0.57 (0.34, 0.78)	0.65 (0.41, 0.85)
Aβ42	0.55 (0.32, 0.76)	0.52 (0.30, 0.74)	0.5 (0.27, 0.73)
Aβ42/Aβ40	0.55 (0.32, 0.76)	0.57 (0.34, 0.78)	0.5 (0.27, 0.73)
p-tau181	0.68 (0.45, 0.86)	0.71 (0.48, 0.89)	0.60 (0.36, 0.81)
p-tau217	0.68 (0.45, 0.86)	0.67 (0.43, 0.85)	0.75 (0.51, 0.91)
p-tau231	0.57 (0.34, 0.78)	0.52 (0.30, 0.74)	0.55 (0.32, 0.77)
T-tau	0.55 (0.32, 0.76)	0.5 (0.27, 0.73)	0.26 (0.09, 0.51)
NfL	0.32 (0.14, 0.55)	0.38 (0.18, 0.62)	0.5 (0.27, 0.73)
GFAP	0.59 (0.36, 0.80)	0.57 (0.34, 0.78)	0.55 (0.32, 0.77)

Classification accuracy, based on a predictive classification model, together with their corresponding 95% CI

CI confidence interval, Aβ amyloid-β, p-tau phosphorylated tau, NfL neurofilament light, GFAP glial fibrillary acidic protein

One study has examined test–retest variability, i.e., the stability of the biomarker over time, and we notice a similar trend to our results: lowest variability in amyloid, increasing in the phosphorylated tau variants to the highest in NfL and GFAP [22].

Several biological factors such as chronic kidney disease, diabetes, history of myocardial infarction and stroke, eGFR, BMI, as well as sex and age have been reported to impact plasma level concentrations of AD BBMs [16–18]. In this study, some correlations were found between eGFR, BMI and HbA1c and baseline levels of BBMs. These potential confounders were included one by one in the LMM without considerable impact on the variation.

This study has some limitations. Though the largest study to date in a real-world clinical setting, the sample size was moderate in size and limited by patients who failed to complete all three visits. This resulted in a rather small number of plates for Simoa analysis, 3 plates in this study, which is likely to have increased the intermediate precision between plates, CV_A. The fact that all analyses were run at the same time, with all three samples from a given patient on the same plate, with the same reagent batch and in the same laboratory limits generalisability to other circumstances in which this is not the case (i.e. to most real-world clinical settings). The smaller the assay variability, the smaller the impact on RCV. However, all CV_A were less than intra-individual variability. Blood samples were collected without information on last food-intake due to the real-life clinical setting of this study. A recent study [15] investigated the impact of food intake by monitoring BMM over a duration of 3 h in groups of postprandial or fasting healthy individuals and found significant fluctuations. The clinical impact remains

unknown and further studies should collect information on food-intake prior to blood-sampling to elaborate on its potential confounding effect on BMM variability. However, in this study, postprandial physiology is considered a part of biological variation. Further, the moderate to high inter-individual variability found in this study could be due to disease heterogeneity in the non-AD group, also explaining the moderate effect when removing the few NfL outliers or may serve as an indicator of disease severity or pathological burden within the AD group. The real-life memory clinic setting with limited inclusion criteria strengthens the diversity of the study population. Though this study was not designed to test the relationship between CSF and plasma levels of AD biomarkers, we found it important to report the correlation coefficients in the AD and non-AD group, to ensure transparent research, see Additional files 5 & 10, Table 7 & Figure 8. We consider it of great strength that we had confirmatory CSF biomarkers enabling us to evaluate the effect of AD pathology of intra- and interindividual variation, which we considered to be limited. The short time span of this study is considered a strength since no pathophysiological changes are expected in AD in such a short window of time.

Conclusion

In conclusion, this is the first study to investigate short-term variability of AD BBMs in the intended use population, memory clinic patients. Across all biomarkers investigated in this study, the low intra-individual variability, robust to various factors, is promising when considering BBMs as novel prognostic markers for tracking disease progression and treatment efficacy. Moderate inter-individual variability prompts some concern for diagnostic and disease staging purposes. The relatively high RCVs may pose challenges for AD biomarkers with low fold change (plasma Aβ42/40) when i.e., estimating intervention effect and longitudinal changes. Overall, intra- and inter-individual variability of AD BBMs should be considered in both clinical and research settings. And we suggest that further research continuously assess the impact of these metrics on AD BBMs’ interpretation as this research field evolves.

Abbreviations

AD	Alzheimer’s disease
BBMs	Blood-based biomarkers
BV	Biological variation
T-tau	Total-tau
p-tau	Phosphorylated tau
NfL	Neurofilament light
GFAP	Glial fibrillary acidic protein
RCV	Reference change value
CV _A	Inter-assay variability
EDTA	Ethylenediaminetetraacetic acid

MMSE Mini-Mental State Examination
 ACE Addenbrookes Cognitive Examination
 HbA1c Hemoglobin A1c
 eGFR Estimated glomerular filtration rate

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s13195-024-01658-7>.

Additional file 1: Use cases.

Additional file 2: Table 4. BBM concentrations in CSF & plasma V1-3.

Additional file 3: Table 5. BBM variability models with time, eGFR, and BMI.

Additional file 4: Table 6. Intra- and inter-individual variability excluding outliers.

Additional file 5: Table 7. Correlations between baseline plasma and CSF biomarkers.

Additional file 6: Figure 4. Model of reference change values, RCV %—Based on the lowest intermediate CV_A.

Additional file 7: Figure 5. Correlogram with BBM and confounders.

Additional file 8: Figure 6. Biomarker concentration pr. visit pr. Patient.

Additional file 9: Figure 7. Correlations in BBMs between visits.

Additional file 10: Figure 8. Scatterplots plasma and CSF correlations.

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Authors' contributions

Conceptualization: FKC, MHG, AHS, SGH, KSF. Methodology: FKC, MHG, AHS, SGH, KSF, HZ. Data Collection: FKC, MHG, HH. Formal Analysis: FKC, AHS, SGH, KSF. Writing—Original Draft: FKC. Writing—Review & Editing: FKC, MHG, AHS, NJA, HH, KB, HZ, GW, SGH, KSF. Supervision: AHS, SGH, GW, KSF. Funding Acquisition: FKC, KSF, SGH, AHS, GW, MHG. Resources: NJA, KB, HZ, GW, AHS, SGH, KSF. All authors read and approved the final manuscript.

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Data availability

Datasets from the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

The study was approved by the Danish Research Ethics Committee (H-21044863) and followed the tenets of the 1975 Helsinki Declaration. The study was registered at clinicaltrials.gov (NCT05175664). Written informed consent was obtained from each participant prior to enrolment.

Consent for publication

Not applicable.

Competing interests

KB has served as a consultant and at advisory boards for Abbvie, AC Immune, ALZPath, AriBio, BioArctic, Biogen, Eisai, Lilly, Moleac Pte. Ltd, Neurimmune, Novartis, Ono Pharma, Prothena, Roche Diagnostics, and Siemens Healthineers; has served at data monitoring committees for Julius Clinical and Novartis; has given lectures, produced educational materials and participated in educational programs for AC Immune, Biogen, Celdara Medical, Eisai and Roche Diagnostics; and is a co-founder of Brain Biomarker Solutions in Gothenburg AB (BBS), which is a part of the GU Ventures Incubator Program, outside the work presented in this paper. HZ has served at scientific advisory boards and/or as a consultant for Abbvie, Acumen, Alector, Alzinova, ALZPath, Amylyx, Annexon, Apellis, Artery Therapeutics, AZTherapies, Cognito Therapeutics, CogRx, Denali, Eisai, LabCorp, Merry Life, Nervgen, Novo Nordisk, Optoceutics, Passage Bio, Pinteon Therapeutics, Prothena, Red Abbey Labs, reMYND, Roche, Samumed, Siemens Healthineers, Triplet Therapeutics, and Wave, has given lectures in symposia sponsored by Alzecure, Biogen, Cellectricon, Fujirebio, Lilly, Novo Nordisk, and Roche, and is a co-founder of Brain Biomarker Solutions in Gothenburg AB (BBS), which is a part of the GU Ventures Incubator Program (outside submitted work). KF serves on a scientific advisory board for Novo Nordisk and Eisai, has given lectures in symposia for Novo Nordisk, and has served or serves as principal investigator in trials for Roche, Biogen, AbbVie, Novo Nordisk and Roche Diagnostics for which fees is paid to the institution and no personal remuneration is received. KF also serves as Editor-in-Chief for *Alzheimer's Research and Therapy* (Springer) for which personal remuneration is paid. The other authors have no conflict of interest to report.

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Supplementary material

Additional file 1

Use cases of intra-and inter-individual short-term variability of AD plasma biomarkers

1. **Intra-individual variability**: Patient X, diagnosed with AD, is allocated to receive intervention XX. Prior to intervention two blood samples are drawn to estimate the patients homeostatic set-point of plasma **P-tau217**, e.g. **0.77pg/mL** and **GFAP**, e.g. **135pg/mL**. A new blood sample is drawn after 12 weeks to measure the effect of the intervention on plasma **P-tau217** and **GFAP**. Plasma P-tau217 is decreased to **0.65pg/mL** (a difference on **0.12pg/mL**) which could be considered as an effect of intervention XX? However, based on the **P-tau217** RCV calculated in this study, a decrease in plasma **P-tau217** should be more than **-28.9%** = more than **0.22pg/mL**, to be considered a difference exceeding intra-individual and assay variability. Further, plasma **GFAP** was increased to **150pg/mL**, which could be considered as a side effect or lack of effect of XX? **GFAP** RCV in the AD group was **+40.4%**: thus, an increase should be more than **54.5pg/mL**. New samples are drawn from patient X at week 36. Plasma **GFAP** is unchanged, but **P-tau217** is further decreased to **0.50pg/mL**, i.e., a decrease exceeding RCV, indicating a change in the biomarkers concentration that could be due to the intervention.
2. **Inter-individual variability**: N=100 patients with AD are allocated to the treatment arm and n=100 to the placebo arm, in a clinical trial testing the effect of medication Y on plasma NfL. Baseline NfL is collected and the inter-individual variability in both groups are calculated at baseline. X months after treatment/placebo, a new plasma NfL is measured. Comparing the baseline variance with the changes in plasma NfL, one can determine if the change in plasma NfL is clinically relevant and should be interpreted as due to treatment effect and furthermore, one can examine if the variance in the treatment group is markedly lower compared to the placebo group, indicating a uniform response to treatment.

Additional file 2

Table 4. BBM concentrations in CSF & plasma V1-3

Biomarker pg/mL (mean (CI))	AD (N=27)	nonAD (N=20)	p-value
CSF A β 40	5364 (1714)	4560 (1567)	0.094
CSF A β 42	256 (71)	335 (135)	0.046*
CSF A β 42/A β 40	0.05 (0.01)	0.08 (0.02)	< 0.0001*
CSF P-tau217	60 (46)	29 (37)	< 0.001*
CSF P-tau181	224 (108)	92 (78)	< 0.00001*
CSF P-tau231	291 (139)	138 (147)	< 0.0001*
CSF T-tau	278 (188)	169 (132)	0.02*
CSF NfL	2597 (2710)	2328 (2383)	0.60
CSF GFAP	19159 (6988)	15434 (5799)	0.56
Plasma A β 40, pg/mL V1	90.3 (11.3)	105 (31.7)	0.049*
Plasma A β 40, pg/mL V2	101 (18.9)	115 (31.2)	0.099
Plasma A β 40, pg/mL V3	99.6 (14.5)	112 (36.3)	0.257
Plasma A β 42, pg/mL V1	4.72 (1.1)	5.57 (2.2)	0.196
Plasma A β 42, pg/mL V2	5.18 (1.2)	6.2 (2.2)	0.116
Plasma A β 42, pg/mL V3	4.97 (1.2)	6.12 (2.5)	0.137
Plasma A β 42/ A β 40, pg/mL V1	0.05 (0.01)	0.05 (0.01)	0.908
Plasma A β 42/ A β 0, pg/mL V2	0.05 (0.01)	0.05 (0.01)	0.564
Plasma A β 42/ A β 40, pg/mL V3	0.05 (0.01)	0.05 (0.01)	0.311
Plasma P-tau181, pg/mL V1	7.74 (2.8)	8.57 (6.0)	0.809
Plasma P-tau181, pg/mL V2	8.86 (2.7)	8.95 (6.0)	0.396
Plasma P-tau181, pg/mL V3	9.24 (3.1)	9.85 (6.6)	0.607
Plasma P-tau217, pg/mL V1	0.64 (0.3)	0.48 (0.4)	0.012*
Plasma P-tau217, pg/mL V2	0.84 (0.3)	0.53 (0.4)	0.002*
Plasma P-tau217, pg/mL V3	0.89 (0.4)	0.59 (0.5)	0.003*
Plasma Ptau231, pg/mL V1	6.65 (2.0)	6.79 (3.2)	0.803
Plasma Ptau231, pg/mL V2	7.46 (2.3)	7.34 (3.6)	0.548
Plasma Ptau231, pg/mL V3	8.14 (2.3)	7.59 (4.3)	0.197
Plasma T-tau, pg/mL V1	1.58 (0.6)	1.57 (0.5)	0.730
Plasma T-tau, pg/mL V2	1.58 (0.4)	1.31 (0.5)	0.04*
Plasma T-tau, pg/mL V3	1.47 (0.3)	1.62 (1.0)	0.899
Plasma NfL, pg/mL V1	24.7 (25.9)	37.5 (39.9)	0.313
Plasma NfL, pg/mL V2	24.3 (15.4)	38.9 (40.8)	0.224
Plasma NfL, pg/mL V3	24.8 (12.5)	43.7 (52.9)	0.369
Plasma GFAP, pg/mL V1	113 (35.7)	98.3 (46.9)	0.121
Plasma GFAP, pg/mL V2	141 (62.8)	128 (54.2)	0.609
Plasma GFAP, pg/mL V3	160 (63.5)	131 (65.5)	0.131

N-missing: AD: CSF n=4, pV1 n=2, pV2 n=2, pV3 n=4. Non-AD: CSF n=1, pV1 n=0, pV2 n=3, pV3 n=3.
 <LOQ (under limit of quantification): CSF A β 42 <LOQ was measured for n=2 in the AD group. Plasma P-tau231 <LOQ n=2, plasma T-tau <LOQ n=3. *significant <0.05 on logtransformed data

Table 5. BBM variability models with time, eGFR, and BMI

% (95% CI)	Model ^B TIME			Model ^C eGFR			Model ^P BMI			Model ^F HbA1c		
	Intra-individual variability CV	Inter-individual variability CV	Inter-individual variability CV	Intra-individual variability CV	Inter-individual variability CV	Inter-individual variability CV	Intra-individual variability CV	Inter-individual variability CV	Inter-individual variability CV	Intra-individual variability CV	Inter-individual variability CV	Inter-individual variability CV
Aβ40	5.1 (4.3; 5.8)	10.0 (8.3; 11.8)	5.2 (4.4; 6.0)	5.2 (4.4; 6.0)	8.4 (7.1; 9.7)	5.2 (4.4; 6.1)	5.2 (4.4; 6.1)	9.7 (8.1; 11.4)	5.3 (4.5; 6.1)	5.3 (4.5; 6.1)	10.3 (8.6; 12.3)	10.3 (8.6; 12.3)
Aβ42	5.7 (4.9; 6.7)	14.6 (12.1; 17.6)	5.9 (5.0; 6.7)	5.9 (5.0; 6.7)	13.6 (11.0; 16.1)	5.9 (4.9; 6.8)	5.9 (4.9; 6.8)	14.6 (12.2; 17.3)	6.0 (5.0; 6.9)	6.0 (5.0; 6.9)	14.8 (12.0; 17.7)	14.8 (12.0; 17.7)
Aβ42/40	3.3 (2.8; 3.8)	10.1 (8.2; 12.1)	3.3 (2.8; 3.8)	3.3 (2.8; 3.8)	10.2 (8.3; 12.2)	3.3 (2.8; 3.8)	3.3 (2.8; 3.8)	10.3 (8.2; 12.3)	3.2 (2.7; 3.7)	3.2 (2.7; 3.7)	10.1 (8.2; 12.2)	10.1 (8.2; 12.2)
P-tau181	6.6 (5.6; 7.7)	20.3 (16.5; 24.2)	7.1 (6.0; 8.3)	7.1 (6.0; 8.3)	20.6 (16.5; 25.0)	7.2 (6.0; 8.3)	7.2 (6.0; 8.3)	18.1 (14.6; 21.6)*	7.2 (6.0; 8.3)	7.2 (6.0; 8.3)	20.7 (16.5; 24.7)	20.7 (16.5; 24.7)
P-tau217	8.4 (7.1; 9.8)	27.0 (21.9; 32.3)	9.4 (8.0; 10.9)	9.4 (8.0; 10.9)	27.2 (21.9; 32.5)	9.4 (7.9; 10.9)	9.4 (7.9; 10.9)	23.5 (19.0; 27.9)*	9.5 (8.0; 11.1)	9.5 (8.0; 11.1)	26.8 (21.3; 32.2)	26.8 (21.3; 32.2)
P-tau231	7.4 (6.3; 8.5)	17.3 (14.1; 20.3)	7.9 (6.7; 9.1)	7.9 (6.7; 9.1)	17.3 (14.1; 20.4)	7.9 (6.7; 9.2)	7.9 (6.7; 9.2)	15.5 (12.7; 18.3)*	8.0 (6.8; 9.3)	8.0 (6.8; 9.3)	17.4 (14.4; 20.6)	17.4 (14.4; 20.6)
T-tau	11.2 (9.5; 13.1)	17.7 (15.0; 20.9)	11.0 (9.3; 12.8)	11.0 (9.3; 12.8)	16.4; (13.7; 19.1)*	11.3 (9.5; 13.0)	11.3 (9.5; 13.0)	17.5 (14.8; 20.0)	11.2 (9.4; 13.0)	11.2 (9.4; 13.0)	17.6 (14.8; 20.6)	17.6 (14.8; 20.6)
NFL	6.9 (5.9; 7.9)	34.0 (27.5; 40.9)	7.4 (6.2; 8.4)	7.4 (6.2; 8.4)	31.9 (25.3; 38.2)*	7.3 (6.2; 8.5)	7.3 (6.2; 8.5)	29.9 (24.0; 36.3)*	7.5 (6.2; 8.7)	7.5 (6.2; 8.7)	34.3 (27.0; 41.8)	34.3 (27.0; 41.8)
GFAP	9.8 (8.3; 11.3)	20.0 (16.4; 23.6)	11.3 (9.5; 13.0)	11.3 (9.5; 13.0)	20.1 (16.3; 23.1)	11.2 (9.3; 13.0)	11.2 (9.3; 13.0)	18.5 (15.4; 21.6)*	11.1 (9.2; 12.9)	11.1 (9.2; 12.9)	20.0 (16.6; 23.6)	20.0 (16.6; 23.6)

Table showing total intra- and inter-individual variability for each biomarker and their 95% CIs. CI: confidence interval; Aβ: amyloid-β; P-tau: phosphorylated tau; NFL: neurofilament light; GFAP: glial fibrillary acidic protein. To each model the analyses are added a potential confounder as a fixed effect. eGFR: estimated glomerular filtration rate; BMI: body mass index; HbA1c: hemoglobin A1c. *decrease in inter-individual variability compared to results without confounders, see Table 2, but no significant difference.

Additional file 4

Table 6. BBM variability excluding outliers

Biomarker	Population	No. of. Samples	Intra-individual variability CV (%; 95% CI)	Inter-individual variability CV (%; 95% CI)
Aβ40	Total	121	4.3 (3.6;5.0)	7.8 (6.5;9.3)
	AD	70	4.1 (3.2;4.8)	6.5 (5.1;8.0)
	non-AD	51	4.6 (3.5;5.7)	9.4 (6.7;11.9)
Aβ42	Total	122	4.5 (3.8;5.3)	12.6 (10.1;15.0)
	AD	71	No outliers in this group	
	non-AD	51	4.6 (3.5;5.7)	9.4 (6.6;12.0)
T-tau	Total	125	10.3 (8.6;12.0)	16.9 (14.1;19.7)
	AD	68	No outliers in this group	
	Non-AD	55	11.0 (8.4;13.6)	16.8 (13.0;20.7)
NfL	Total	115	6.8% (5.8;7.8)	22.6 (18.0;27.4)
	AD	68	7.0% (5.5;8.6)	23.1 (16.9;28.7)
	non-AD	47	6.4% (4.8;8.0)	22.3 (15.0;29.2)

The table presents the intra- and inter-individual variability for each biomarker together with outliers excluded together with their 95% CIs.

Number of samples used for analyses are also presented.

CI: confidence interval; Aβ: amyloid-β; P-tau: phosphorylated tau; NfL: neurofilament light; GFAP: glial fibrillary acidic protein.

AB40: n=1 AD, n=6 non-AD observations with plasma AB40 >150pg/mL were excluded.

AB42: n=6 non-AD observations with plasma AB42 >10pg/mL were excluded.

T-tau: n=2 non-AD observations with plasma T-tau >3pg/mL were excluded.

NfL: n=3 AD, n=10 non-AD observations with plasma NfL>60pg/mL were excluded.

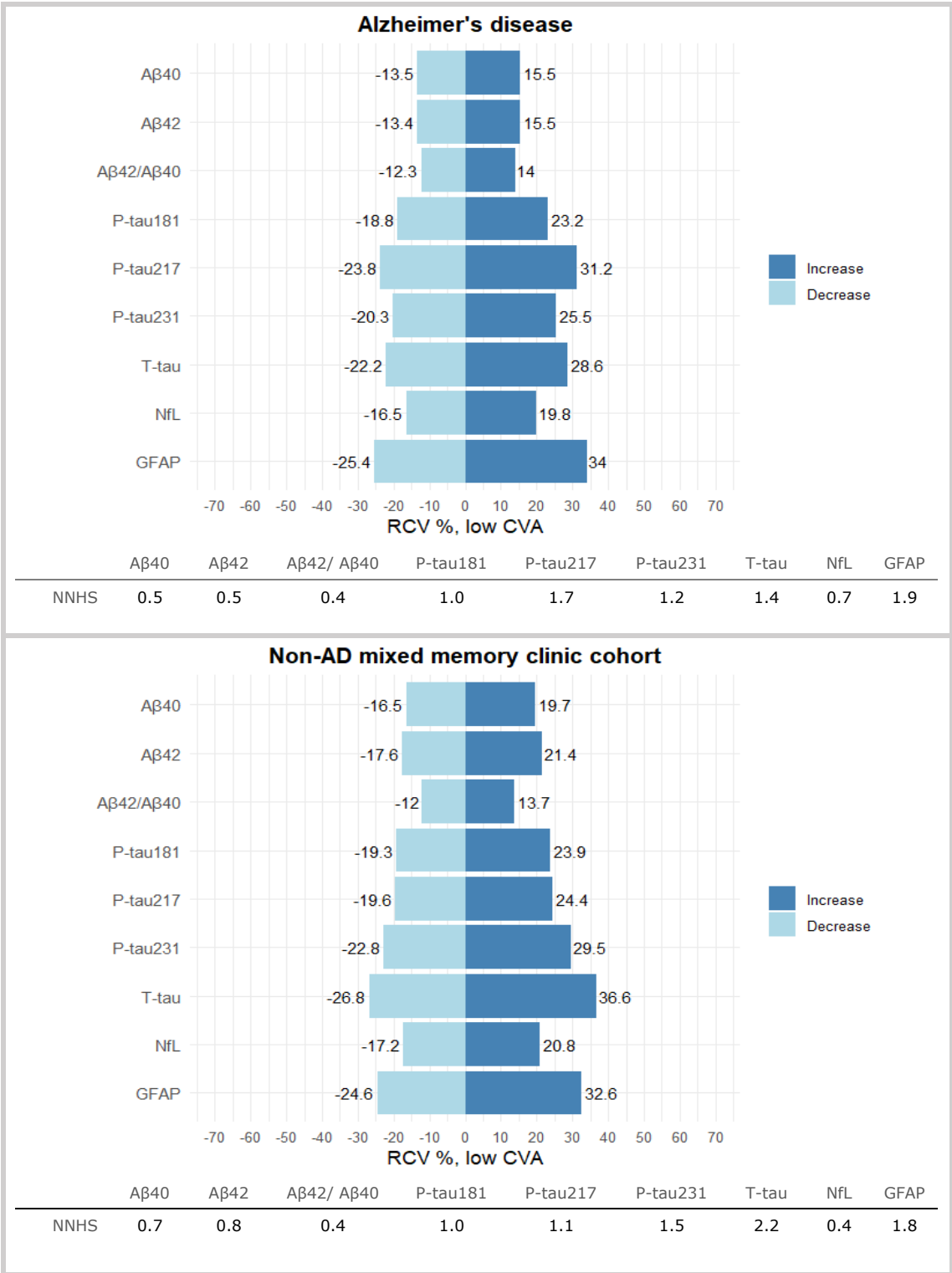
Additional file 5

Table 7. Correlations between baseline plasma and CSF biomarkers

CSF & Plasma	Aβ40	Aβ42	P-tau181	P-tau217	P-tau231	T-tau	NfL	GFAP
<i>p-value</i>	0.58	0.91	0.06	0.02*	0.31	0.64	0.002*	0.32
<i>rho</i>	0.09	0.020	0.30	0.37	0.30	0.08	0.48	0.16

Spearman's correlations coefficients between baseline plasma and cerebrospinal fluid (CSF). *Significant correlations between baseline plasma marker levels and CSF.

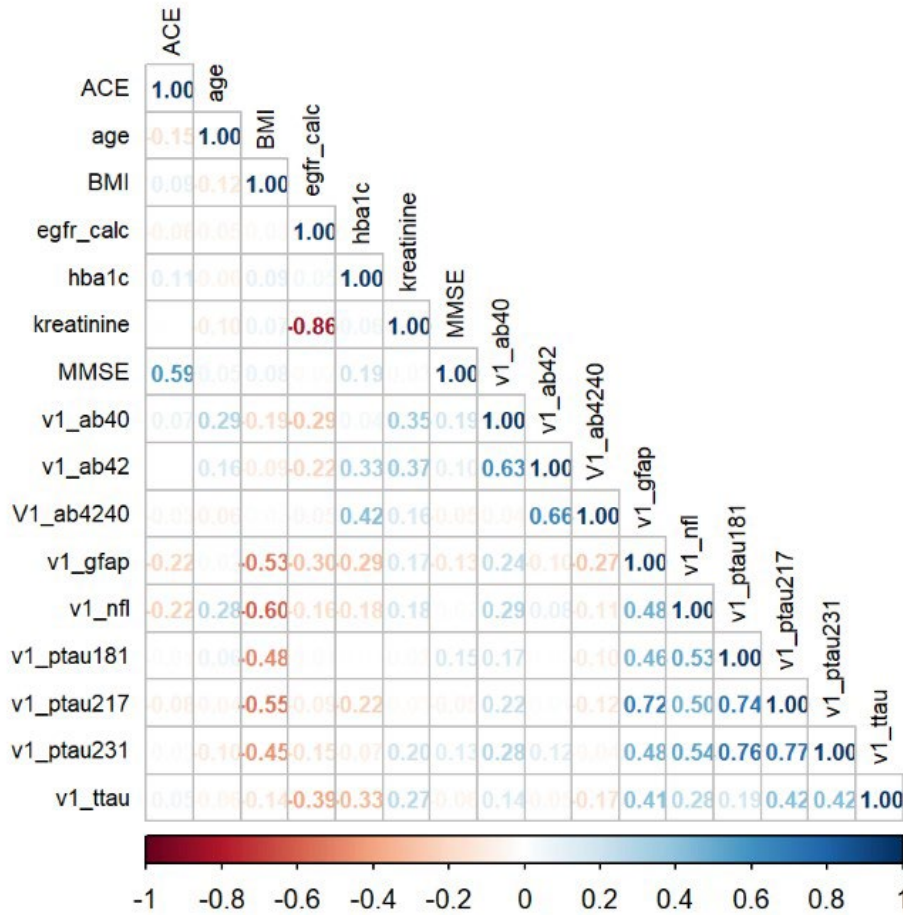
Figure 4. Model of reference change values, RCV % - Based on the lowest intermediate CV_A



Figures showing the amount of change for each biomarker to overcome that of intra-individual variability and assay variation, named Reference Change Value (RCV). The tables show the RCVs for each biomarker in patients with A) Alzheimer's disease and B) non-AD consisting of a mixed memory clinic cohort. Calculations are based on the lowest intermediate CV_A. NNHS; number of samples needed to obtain homeostatic setpoint with 15% proximity to true value.

Additional file 7

Figure 5. Correlogram with BBM and confounders



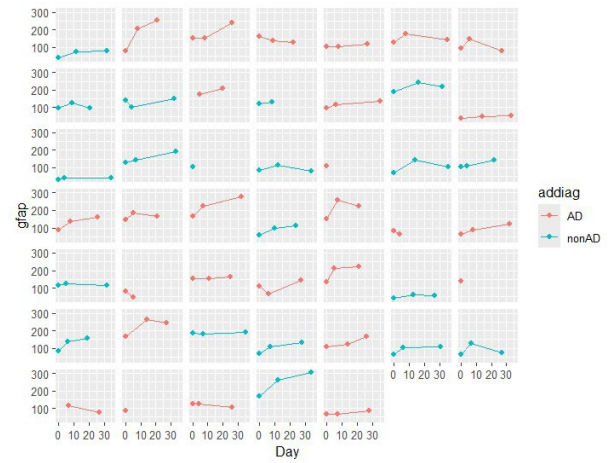
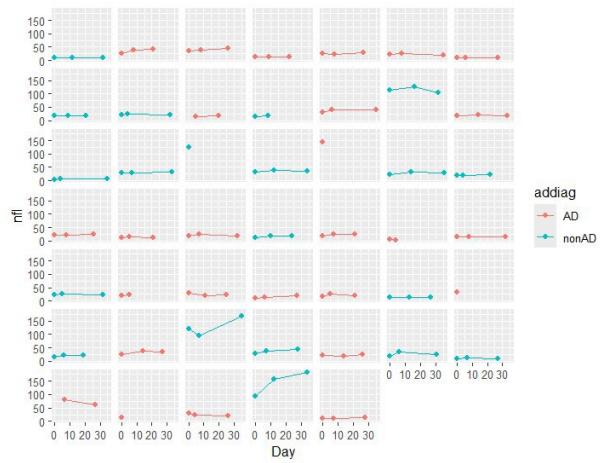
Colored scale showing the negative (red) and positive (blue) significant correlations between baseline plasma marker levels and potential confounding factors. ACE: Addenbrookes cognitive examination; MMSE: mini mental status examination; BMI: body mass index; HbA1c: Hemoglobin A1c; eGFR_calc: estimated glomerular filtration rate; Aβ: amyloid-β; P-tau: phosphorylated tau; NfL: neurofilament light; GFAP: glial fibrillary acidic protein.

Significant correlation between BBMs and confounders: HbA1c was significantly positive correlated with plasma: Aβ42 ($\rho=0.33$, $p\text{-value} = 0.03$), Aβ42/Aβ40 ($\rho=0.42$, $p\text{-value} = 0.004$), T-tau ($\rho=0.33$, $p\text{-value} = 0.029$). BMI was significantly negative correlated with plasma: GFAP. ($\rho=-0.5$, $p\text{-value} < 0.001$), NfL ($\rho=-0.6$, $p\text{-value} < 0.0001$), P-tau217. ($\rho=-0.5$, $p\text{-value} = 0.0001$), P-tau231 ($\rho=-0.4$, $p\text{-value} = 0.003$), P-tau181 ($\rho=-0.5$, $p\text{-value} < 0.001$). eGFR was significantly negative correlated with plasma: GFAP ($\rho=-0.3$, $p\text{-value} = 0.046$), T-tau ($\rho=-0.39$, $p\text{-value} = 0.008$).

Additional file 8

Figure 6. Biomarker concentration pr. visit pr. patient

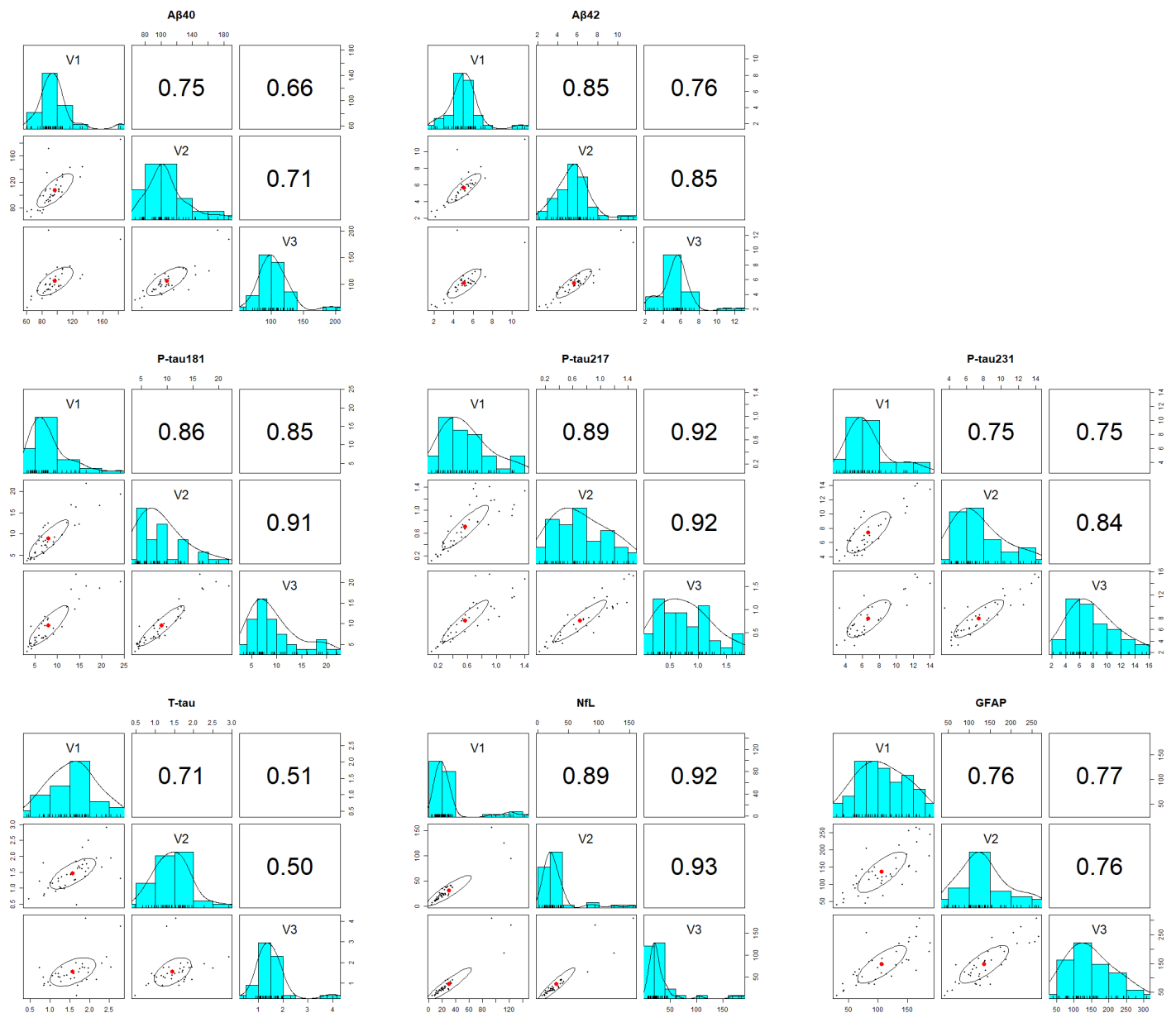




Spagettiplots of all BBM concentrations pr. visit stratified by patient and coloured by diagnosis.

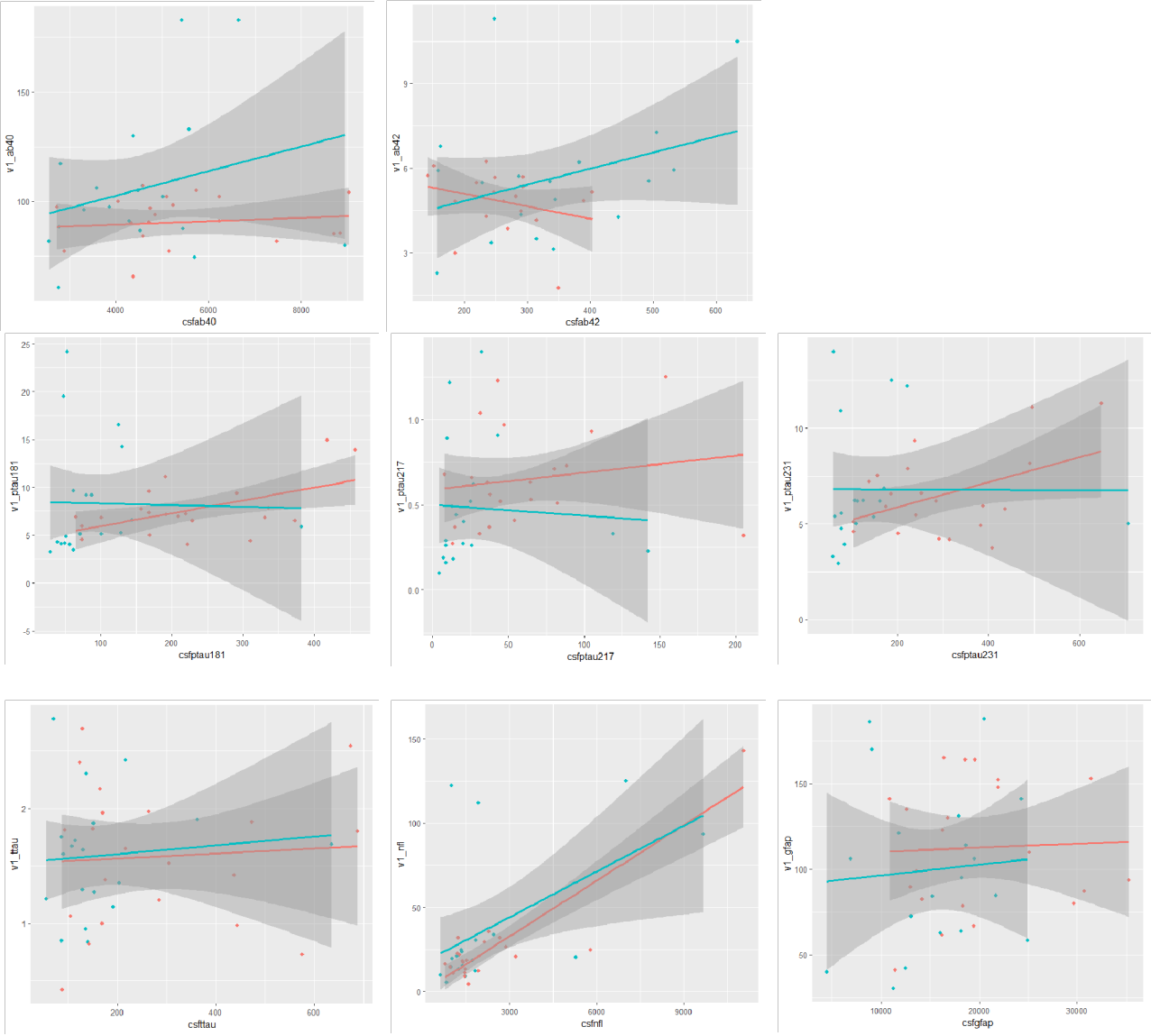
Additional file 9

Figure 7. Correlations in BBMs between visits



Histograms and scatterplot matrices based on Spearman's correlations coefficient for each biomarker between visits. V1, visit 1; V2, visit 2; V3, visit 3; Aβ40, amyloid beta 40; Aβ 20, amyloid beta 42; P-tau, phoshorylated tau; T-tau, total-tau; NfL, neurofilament light; GFAP, glial fibrillary acidic protein.

Figure 8. Scatterplots plasma and CSF correlations



Scatterplots with baseline plasma and CSF markers divided in AD and non-AD group. ■ AD ■ nonAD

8.2 Study II

The plasma p-tau₂₁₇/BD-tau ratio improves biomarker short-term variability in memory clinic patients

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RESEARCH ARTICLE

The plasma p-tau217/BD-tau ratio improves biomarker short-term variability in memory clinic patients

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Abstract

INTRODUCTION: Assessment of short-term intra- and inter-individual variability for Alzheimer's disease (AD) plasma biomarkers is essential for clinically relevant interpretation of biomarker levels. We hypothesized that the variability of plasma tau phosphorylated at threonine 217 (p-tau217) could be reduced by combining it with a tau marker, plasma brain-derived tau (BD-tau), as the p-tau217/BD-tau ratio.

METHODS: Three consecutive blood samples were collected from memory clinic patients within 36 days. Patients were dichotomized by cerebrospinal fluid (CSF) amyloidosis ($A\beta^+ = 29$, $A\beta^- = 18$). We compared intra- and inter-individual variability (coefficient of variation [CV]) in the plasma p-tau217/BD-tau ratio with p-tau217 alone and tested if kidney function, glycated hemoglobin, and body mass index (BMI) affected the variability. Finally, we compared the p-tau217/BD-tau ratio with CSF p-tau217.

RESULTS: We found that for $A\beta^+$ individuals, the intra-individual variability of the plasma p-tau217/BD-tau ratio (CV 7.1% [95% confidence interval {CI} 5.6;8.4]) was lower than for p-tau217 alone (CV 9.4% [95% CI 7.4;11.5]). At the group level, the

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variability in the p-tau217/BD-tau ratio was reduced in both A β + (CV 15.1% [95% CI 11.7;18.7]) and A β - (CV 18.4% [95% CI 13.0;23.8]) individuals compared to p-tau217 alone (A β + CV 19.1 [15.0;23.4], A β - 27.1 [18.4;36.0]). Adjusting for estimated glomerular filtration rate, hemoglobin A1C, and BMI further reduced the inter-individual variability of p-tau217/BD-tau in the A β + group. CSF p-tau217 showed higher correlation with plasma p-tau217/BD-tau ($\rho = 0.53$, $p = 0.0005$) than with p-tau217 alone ($\rho = 0.37$, $p = 0.02$).

DISCUSSION: Our findings suggest that using the ratio of plasma p-tau217 to plasma BD-tau and accounting for the influence of peripheral confounders improves biomarker stability, which is important for the interpretation of longitudinal biomarker changes and to prevent misclassification.

KEYWORDS

p-tau217, brain-derived tau, within-subject variability, p-tau217/BD-tau ratio, short-term variability, between-subject variability, plasma biomarker, Alzheimer's disease

HIGHLIGHTS

- The plasma p-tau217/BD-tau ratio lowered short-term intra- and, especially, inter-individual variability compared to the variability in plasma p-tau217 alone.
- Plasma BD-tau did not correlate with eGFR, HbA1c, or BMI, while plasma p-tau217 was significantly negatively associated with BMI.
- Adjusting for eGFR, HbA1c, and BMI further reduced the inter-individual variability of p-tau217/BD-tau.
- Additionally, CSF p-tau217 correlated better with plasma p-tau217/BD-tau than with p-tau217 alone.

1 | BACKGROUND

Blood-based biomarkers (BBMs) have proven useful in research settings in identifying amyloid and tau pathology in the brain. Current research has indicated plasma phosphorylated tau at threonine 217 (p-tau217) as a valid biomarker associated with brain amyloidosis in Alzheimer's disease (AD).^{1,2} With a view to the clinical implementation of AD BBMs, knowledge of the markers' variability, within- and between-subject (i.e., intra- and inter-individual variability) is essential for the correct interpretation of biomarker changes as clinically meaningful.³ Biomarker variability can be caused by biological variation, assay variability, comorbidities, disease-related factors (e.g., altered biomarker production and blood-brain barrier changes), and peripheral influences and comorbidities like glycated hemoglobin, body mass index (BMI), and kidney function, which affect clearance mechanisms.³⁻⁹

Brain-derived tau is, as the name implies, tau that originates exclusively from neurons in the central nervous system, targeting the six tau isoforms in the brain. The assay excludes tau produced peripherally, for example in the liver, kidneys, or heart, that contains amino acids from an extra exon compared with tau originating from the brain.¹⁰ This is

opposed to plasma T-tau, which is influenced by peripherally produced tau and has been shown to be weakly correlated with CSF T-tau.¹¹⁻¹³ In this study, we examine whether the intra- and inter-variability of p-tau217 could be reduced by combining it with a brain-derived tau marker, BD-tau.

Plasma BD-tau is an analytically robust marker¹⁴ that may ensure more accurate assessment of brain tau, as it has shown a strong correlation with CSF T-tau¹⁰ and with plasma p-tau217.¹⁵ Studies have investigated blood BD-tau as a promising marker of neurodegenerative intensity in AD and rapidly progressive dementias, such as Creutzfeldt-Jakob disease (CJD)¹⁶⁻¹⁸ and as a severity marker in stroke^{19,20} and severe traumatic brain injury.²¹

Combining markers in ratios may reduce biomarker variability, which may confound biomarker performance and interpretation. A key example is the CSF amyloid beta (A β) 42/40 ratio compared to A β 42 and A β 40 alone in detecting brain amyloid pathology.²²⁻²⁴ Further, plasma %P-tau217 (the ratio of phosphorylated tau217 to non-phosphorylated tau) has recently been described as superior in the classification of tau-positron emission tomography (PET) compared to CSF A β 42/40²⁵ and to be less affected by kidney dysfunction than p-tau isoforms alone.²⁶ Thus, biomarker ratios may

enhance the ability to distinguish pathological changes from normal variation.

Thus, we hypothesized that the plasma p-tau217/BD-tau ratio reduces the intra- and inter-individual variability compared to p-tau217 alone. We used data from a previously published AD BBM variability study³ and performed secondary analyses measuring plasma BD-tau. We compared intra- and inter-individual variability in plasma p-tau217 with the p-tau217/BD-tau and p-tau217/T-tau ratio in memory clinic patients with and without CSF brain amyloidosis. We further tested whether kidney function, glycated hemoglobin, and BMI affected the variability and examined correlations between plasma and CSF markers and peripheral confounders.

2 | METHODS

2.1 | Study design

This is a test-retest variability study conducted between January 2022 and May 2023, which included patients undergoing CSF analysis for AD biomarkers as part of the routine diagnostic evaluation for suspected neurodegenerative disease in the Memory Clinic at Rigshospitalet, Copenhagen, Denmark. Inclusion criteria were as follows: (1) had undergone lumbar puncture as part of the diagnostic assessment and (2) Mini-Mental State Examination (MMSE) $\geq$ 20. Exclusion criteria were (1) stroke within the past 3 months, (2) previous or existing major psychiatric conditions, (3) alcohol or substance abuse within the last 2 years, or (4) participation in intervention studies. Demographic data, medical history, and cognitive test results (MMSE²⁷ and Addenbrooke's Cognitive Examination [ACE]²⁸) were extracted from medical files from the patients' diagnostic work-up program in the clinic. A full description of the study method was published previously.³

Each participant gave written informed consent prior to enrollment. The study was approved by the Danish Research Ethics Committee (H-21044863), followed the tenets of the 1975 Helsinki Declaration, and was registered at clinicaltrials.gov (NCT05175664).

2.2 | Participants

As part of the diagnostic evaluation, CSF from all 47 participants was analyzed at the Clinical Biochemistry Laboratory at Rigshospitalet. CSF from patients enrolled before June 30, 2022, was analyzed by the Innotech enzyme-linked immunosorbent assays (ELISA) by Fujirebio, Ghent ($n = 8$), and CSF collected after July 1, 2022, was analyzed by the Elecsys sandwich electrochemiluminescence immunoassay by Roche, Cobas 8000. For this study, we dichotomized participants according to A β status, positive (A β +) and negative (A β -), based on CSF p-tau181/A β 42 ratio cutoff for Innotech: (0.077 [locally established] and for Elecsys [0.023]).^{29,30} Clinical diagnoses were obtained at a multidisciplinary consensus conference and based on the criteria for AD (mild cognitive impairment [MCI] or dementia), vascular dementia,³¹⁻³³ mixed AD and vascular,³³ cerebral amy-

RESEARCH IN CONTEXT

- 1. Systematic review:** The authors reviewed the literature using PubMed and found very few studies investigating the intra- and inter-individual variability of AD plasma biomarkers and how this variability may impact clinical biomarker interpretation.
- 2. Interpretation:** We found that the short-term intra- and especially inter-individual variability was lower in the plasma p-tau217/BD-tau ratio compared to the variability in plasma p-tau217 alone.
- 3. Future directions:** Reduced variability of plasma AD markers is important to ensure relevant clinical interpretation of longitudinal biomarker changes and to prevent misclassification.

loid angiopathy,³⁴ dementia with Lewy bodies,³⁵ and frontotemporal dementia.³⁶

2.3 | Blood sample collection, processing, and biomarker measurement

A baseline blood sample (V1) was drawn immediately following the lumbar puncture (LP) procedure, via venipuncture in ethylenediaminetetraacetic acid (EDTA)-plasma tubes. The second and third blood samples were collected 4 to 16 days (visit 2 [V2]) and 19 to 36 days (visit 3 [V3]) after V1, respectively. All blood samples were collected between 9:00 a.m. and 3:00 p.m. Fasting status was unknown. CSF from V1 and plasma from V1-V3 were centrifuged at 2000 g, 4°C for 10 min within 30 to 120 min after collection, redistributed into 250- μ L aliquots, and stored at -80°C and shipped to Gothenburg. Analyses of CSF and plasma T-tau and p-tau217 were performed from November 2023 to February 2024, and the analyses of BD-tau were carried out in December 2024 at the Clinical Neurochemistry Laboratory, Sahlgrenska University Hospital, Mölndal, University of Gothenburg, Sweden. Separate aliquots of the same blood draw were used for each round of analyses to minimize pre-analytical variability and avoid additional freeze-thaw cycles. Thus, measurements for all markers were obtained from simultaneously collected specimens. Single-molecule array (Simoa) technology by Quanterix HD-X³⁷ was used to measure T-tau (Quanterix single plex TAU kit) and p-tau217 (Quanterix single plex ALZpath p-tau217 assay). See the detailed description of T-tau and p-tau217 measurements elsewhere.³ Plasma BD-tau was measured with the previously validated Gothenburg University method.¹⁰ The lower limit of detection (LOD) was 0.044 pg/mL and the dynamic range was 0 to 600pg/mL (for serum/EDTA-plasma). Both assays demonstrated robust analytical performance with low mean intra-assay CV (p-tau217 assay: 5.5%, BD-tau assay: 9.1%) and mean inter-assay CV (p-tau217 assay: 10.5%, BD-tau assay: 11.7%).

2.4 | Statistical analysis

Demographics and baseline characteristics are presented as means with standard deviations. Differences in baseline characteristic variables between $A\beta_{\pm}$ were examined with two-sample *t*-tests for continuous variables and with Pearson's chi-squared tests for categorical variables. To accommodate skewed plasma and CSF distribution, $\log_{10}$ -transformation was applied and assessed by visual inspection of histograms and QQ-plot data. Correlations between plasma and CSF biomarkers, visits, and possible confounders (kidney function, HbA1c, BMI) were examined with Spearman's correlation coefficients.

CVs for intra- and inter-individual variability were examined by fitting linear mixed models (LMMs) with sampling time point as fixed effect and patients as random effects, using the "lmer" function in the lme4 package in R and the "confint" function with the bootstrap method to generate 95% confidence intervals (95% CIs). Intra-individual CVs were based on the residual's standard deviation, whereas the inter-individual CVs were calculated from the square root of the total variances, incorporating intra-individual variability. The method was previously described in detail elsewhere.³ Assumptions for the linear mixed-effects models were assessed through diagnostic plots and were adequately met (Figure S1). Absolute biomarker variability was visualized by spaghetti plots, and to account for differing intervals between visits, we calculated standardized per-day changes in both p-tau217 and the p-tau217/BD-tau ratio (Figure S2).

Models were adjusted separately for estimated glomerular filtration rate (eGFR), hemoglobin A1C (HbA1c), and BMI, added as fixed effects to the model. Further, we tested the combined effect of the covariates, including sex, in a fully adjusted multivariable analysis. Concentrations under the limit of quantification (<LOQ) (T-tau $n = 3$, BD-tau $n = 1$) were labelled as missing data (NA). Observations labelled NA were removed from the LMMs.

To assess the discriminatory performance of p-tau217 and the p-tau217/BD-tau and p-tau217/T-tau ratio at each visit (i.e., their ability to distinguish between $A\beta_{+}$ and $A\beta_{-}$ individuals), we calculated the area under the receiver operating characteristic curve (AUC) using the pROC package in R. AUCs and corresponding 95% CIs were computed for the full dataset and for complete cases. No train/test data splitting was performed; that is, AUCs were calculated using the entire available dataset at each time point.

All statistical analyses were performed using the R Statistical Software, version 4.4.1, and statistical significance was set to $\alpha = 0.05$ using only two-tailed tests.

3 | RESULTS

3.1 | Cohort characteristics

No differences in age, level of cognitive impairment, and distribution of disease severity were found between the $A\beta_{+}$ and $A\beta_{-}$ groups. The distribution of sex differed between groups, with only two females in the

$A\beta_{-}$ group. The $A\beta_{+}$ group had significantly higher baseline CSF measures of T-tau and BD-tau (Table 1). BMI and HbA1c were significantly higher in $A\beta_{-}$ individuals, of which seven patients had type 2 diabetes compared to two patients with type I and II diabetes in the $A\beta_{+}$ group.

Visual inspection of individual trajectories showed less variation for some patients in p-tau217/BD-tau compared to p-tau217 (Figure 1A,B). We observed two patients with high BD-tau and T-tau values (Figure S3) and noted that these patients had reduced kidney function ($eGFR \leq 36/\text{min}/1.73 \text{ m}^2$). The p-tau217/T-tau ratio still fluctuated in some $A\beta_{+}$ patients (Figure S3E). Plasma p-tau217 and the p-tau217/BD-tau group median and interquartile range across visits are visualized in Figure 1C,D.

3.2 | Biomarker short-term variability

3.2.1 | Intra-individual variability

The results for intra-individual variability, adjusted for sampling time point, are presented in Table 2. The lowest intra-individual variability of the single markers in $A\beta_{+}$ individuals was observed for BD-tau, while for $A\beta_{-}$ individuals, the lowest intra-individual variability was observed in p-tau217.

The p-tau217/BD-tau ratio showed lower intra-individual variability in $A\beta_{+}$ individuals (7.1% [95% CI: 5.6;9.1]) than the intra-individual variability of p-tau217 alone (CV 9.4% [95% CI 7.4;11.5]); however, the 95% CIs overlapped.

The p-tau217/T-tau ratio showed higher intra-individual variability (CV 13.9% [95% CI 10.9;16.8]) than p-tau217 in isolation, and this difference was most pronounced in $A\beta_{+}$ individuals.

Adjusting for HbA1c, eGFR, BMI, and sampling time point, by addressing the impact of each variable in separate models and by combining all covariates into one model, we observed that adjusting for sampling time point lowered the intra-individual variability for p-tau217 and the p-tau217/T-tau ratio, in $A\beta_{+}$ individuals. No other marker's variability was impacted by adjustment. For a full overview of the covariates' individual impact on the variability (Table S1).

3.2.2 | Inter-individual variability

The lowest inter-individual variability in the single markers was observed for BD-tau in the $A\beta_{+}$ group, showing lower variability compared to p-tau217 alone (Table 2).

The inter-individual variability for p-tau217/BD-tau was lower in both $A\beta_{\pm}$ compared with the variability of p-tau217 alone. p-tau217/T-tau showed the highest inter-individual variability in both $A\beta_{+}$ and $A\beta_{-}$ and showed higher variability compared with p-tau217.

Adjusting for all covariates, we observed a lower variability in p-tau217 (from CV 19.1% [95% CI 15.0;23.4] to CV 13.1% [95% CI 10.5;15.5]) in the $A\beta_{+}$ group, partially driven by adjustment for BMI;

TABLE 1 Demographics

	A β + (n = 29)	A β - (n = 18)**	p-value
Age, years mean (SD)	71.51 (5.22)	71.98 (6.52)	0.79 ⁽¹⁾
Sex (men) n (%)	15 (51.7%)	16 (88.9%)	0.01 ⁽²⁾
MMSE Mean (SD)	26.9 (2.3)	26.8 (2.4)	0.89 ⁽¹⁾
ACE Mean (SD)	79.5 (8.0)	76.7 (11.6)	0.34 ⁽¹⁾
Disease severity (MCI/Mild dementia, n (%))	16 (55%)/13 (45%)	9/9 (50%)	0.73 ⁽²⁾
eGFR	81.7 (17.9)	81.6 (23.4)	0.99 ⁽²⁾
HbA1c	38.9 (4.8)	45.1 (13.2)	0.03* ⁽²⁾
BMI	24.5 (4.3)	27.5 (5.1)	0.02* ⁽¹⁾
CSF			
p-tau181/A β 1-42 mean (SD)***	0.05 (0.03)	0.01 (0.004)	< 0.0001* ⁽³⁾
T-tau mean (SD)****	286.7 (191.2)	134.44 (71.2)	0.001* ⁽³⁾
BD-tau mean (SD) pg/mL****	134.9 (61.5)	73.26 (18.1)	0.00007* ⁽³⁾

N-missing: AD: CSF n = 4, non-AD: CSF n = 1. (1) two-way sample t-test, (2) Chi-squared test, (3) T-test on log-transformed variables.

*significant <0.05.

**In the A β - group, participants were diagnosed with dementia with Lewy bodies n = 4, frontotemporal dementia n = 2, other tauopathy = 1, cerebral amyloid angiopathy = 1, vascular dementia = 1, normal pressure hydrocephalus = 1, severe kidney disease = 1, head trauma = 1, and unclassified = 6.

***CSF analyses by Elecsys n = 39 (n = 6 with CSF analysis by Innotech, excluded from this comparison).

****CSF analyses by Simoa.

Abbreviations: ACE, Addenbrooke's Cognitive Examination; BD-tau, brain-derived tau; CSF, cerebrospinal fluid; eGFR, estimated glomerular filtration rate; HbA1c, glycated hemoglobin A1c; MCI, mild cognitive impairment; MMSE, Mini-Mental Status Examination; P-tau, phosphorylated tau; T-tau, total tau; A β , amyloid beta.

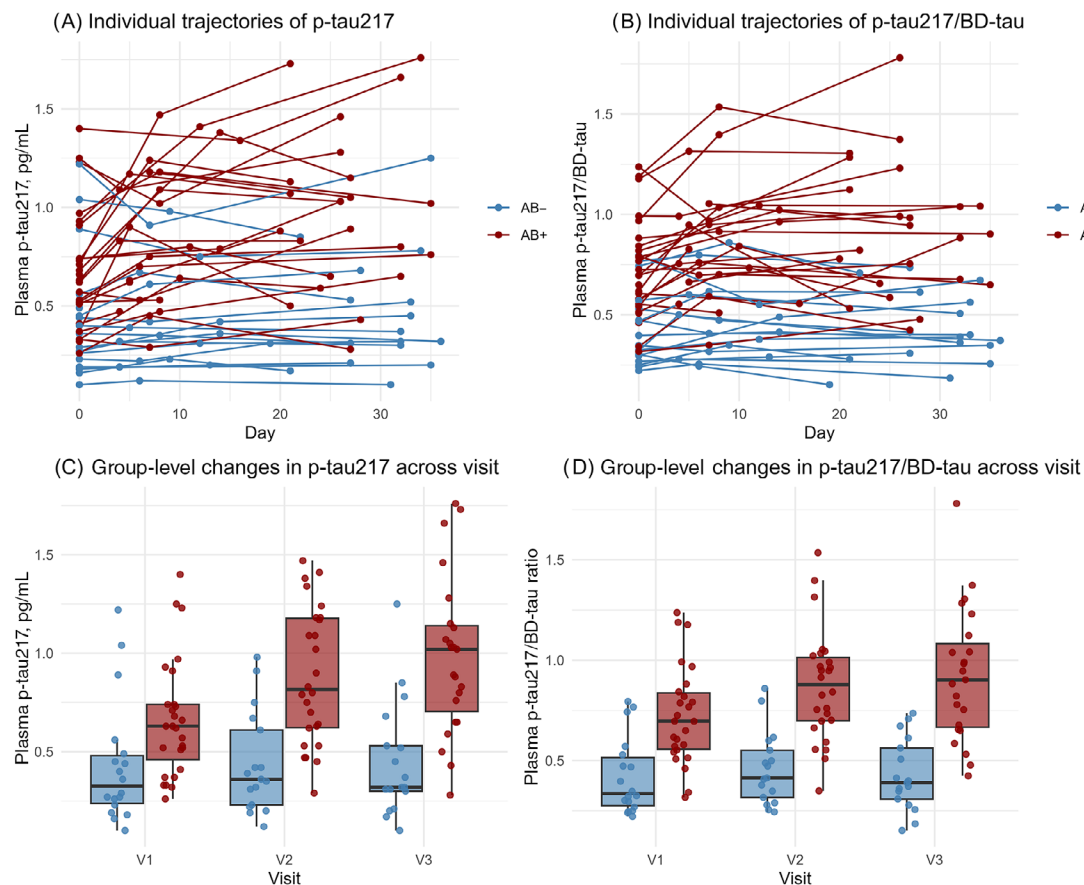


FIGURE 1 Plasma levels at each time point: Plasma samples: visit 1 = 45, visit 2 = 43, visit 3 = 40. (A and B) Spaghetti plots with subject-level p-tau217 and p-tau217/BD-tau changes across three visits. (C and D) Box plots with group median and interquartile range. For better visual inspection, two observations with high plasma values were removed in (B) BD-tau > 1.75pg/mL. See Figure S2 for all observations.

TABLE 2 Intra- and inter-individual variability

	Amyloid status	Intra-individual variability CV (%; 95% CI)		Inter-individual variability CV (%; 95% CI)	
		Model 1: Time-adjusted	Model 2: Fully adjusted	Model 1: Time-adjusted	Model 2: Fully adjusted
p-tau217	A β +	9.4 (7.4;11.5)*	9.5 (7.6;11.3)	19.1 (15.0;23.4)	13.1** (10.5;15.5)*
	A β -	5.9 (4.5;7.3)*	6.2 (4.5;7.9)	27.1 (18.4;36.0)	27.7 (16.1;39.5)*
BD-tau	A β +	7.5 (5.97;9.1)	7.5 (6.0;9.1)	12.1 (9.8;14.7)	10.7 (8.4;12.7)
	A β -	6.6 (5.0;8.2)	6.5 (5.0;8.6)	20.1 (14.1;26.2)	16.8 (10.5;23.3)
T-tau	A β +	10.4 (8.2; 12.5)	10.4 (8.3;12.6)	15.0 (12.1;18.3)	12.7 (10.4;15.1)
	A β -	11.1 (8.3;14.0)	10.9 (7.8;14.0)	20.6 (14.8;26.7)	19.4 (13.2;26.4)
p-tau217/ BD-tau	A β +	7.1 (5.6;8.4)	7.1 (5.6;8.5)	15.1 (11.7;18.7)	11.1 (8.8;13.7)
	A β -	7.3 (5.6;9.1)	7.4 (4.5;9.6)	18.4 (13.0;23.8)	18.8 (12.0;26.1)
p-tau217/ T-tau	A β +	13.9 (10.9;16.8)	13.9 (11.2;16.8)	19.3 (15.6;23.1)	18.2 (14.9;21.7)
	A β -	9.6 (6.9;12.0)	9.5 (6.8;12.2)	30.2 (21.1;39.2)	32.5 (19.8;45.1)

Note: Intra- and inter-individual variability for each biomarker is presented together with the corresponding confidence interval. Bold highlights how the variability of p-tau217 is reduced in the ratio and at the group level when adjusted for confounders.

Model 1 is adjusted for sampling time point. Model 2 is adjusted for eGFR, HbA1c, BMI, and sampling time point. Excluding patients with eGFR < 36/min/1.73m² did not alter the results. Adjusting for sex in model 2 did not affect the variability.

*Non-overlapping 95% CI between A β +

**Non-overlapping 95% CI between raw (see supplementary Table S1) and Model 2 adjusted for all covariates.

Abbreviations: BD-tau, brain-derived tau; CV, coefficient of variation; CI, confidence interval; p-tau, phosphorylated tau; T-tau, total tau.

TABLE 3 Comparison of AUC between visits

	V1, n = 45 AUC (95% CI)	V2, n = 43 AUC (95% CI)	V3, n = 40 AUC (95% CI)
p-tau217	0.76 (0.61;0.92)	0.86 (0.74;1)	0.86 (0.74;1)
p-tau217/BD-tau	0.84 (0.72;0.97)	0.91 (0.81;1)	0.93 (0.86;1)
p-tau217/T-tau	0.59 (0.40;0.79)	0.75 (0.58;0.9)	0.87 (0.73;1)

Classification performance for each sampling time point, visit 1 (V1), visit 2 (V2), visit 3 (V3).

Abbreviations: AUC, area under the curve; BD-tau, brain-derived tau; CI, confidence interval; p-tau, phosphorylated tau; T-tau, total tau.

however, the 95% CIs overlapped. This change was not observed in the A β - group. Furthermore, we observed a significantly lower variability in the p-tau217/BD-tau ratio when adjusting for all covariates (CV 11.1% [95% CI 8.8;13.7]) compared to the crude p-tau217 variability (CV 19.1% [95% CI 15.0;23.4]). For a full overview of the covariates' individual impact on the variability, see Table S1.

Results from complete-case sensitivity analyses were consistent with these findings (Table S2).

3.3 | Comparison of AUC performance between visits

Table 3 presents the AUC values for classifying A β -positive and A β -negative individuals (defined by the CSF p-tau181/A β 42 ratio) using p-tau217, p-tau217/BD-tau, and p-tau217/T-tau. Across V1 and V3, classification performance remained relatively stable for the p-tau217 and p-tau217/BD-tau ratio, while more variability was observed for the p-tau217/T-tau ratio. The p-tau217/BD-tau ratio showed a numerical improvement in discriminative ability com-

pared to p-tau217 alone; however, this was not statistically significant. AUC analysis on complete cases did not alter the results (Table S3).

3.4 | Correlations: plasma biomarkers, CSF, and confounders

Plasma p-tau217 and BD-tau showed significant and high correlations between each other at all three visits, whereas plasma p-tau217 and T-tau showed a moderately significant correlation at V1 and V2, but not at V3 (Figure S3).

There were no significant correlations between plasma BD-tau and age, MMSE, ACE, BMI, eGFR, or HbA1c. Plasma T-tau correlated significantly with both eGFR ($\rho = -0.39$, $p = 0.008$) and HbA1c ($\rho = -0.38$, $p = 0.012$), but not with MMSE, ACE, or BMI. p-tau217 was significantly negatively associated with BMI ($\rho = -0.55$, $p = 0.0001$) (Figure S4).

CSF p-tau217 correlated significantly with plasma p-tau217 ($\rho = 0.37$, $p = 0.02$) and with p-tau217/BD-tau ($\rho = 0.53$, $p = 0.0005$). No

correlation was found between CSF p-tau217 and plasma p-tau217/T-tau ratio ($\rho = 0.28, p = 0.08$) (Figure S4).

4 | DISCUSSION

In this study, we examined whether the short-term variability of plasma p-tau217 could be reduced when combined with the novel plasma marker BD-tau and whether the variability of the ratio was less affected by biological variables. We found lower intra- and inter-individual variability of p-tau217/BD-tau compared to p-tau217. However, the confidence intervals overlapped, suggesting that the reduction in variability may be modest. For p-tau217/T-tau the variability was higher than the variability of p-tau217, especially the intra-individual variability in A β + individuals. Adjusting for eGFR, HbA1c, and sampling time point did not change the intra-individual variability, but we observed reduced inter-individual variability, especially in the p-tau217/BD-tau ratio, in the A β + group.

Assessing single biomarkers, the intra-individual variability reflects the variation (biological and disease-related differences in production, release, and clearance) in the biomarker within the individual. Combining biomarkers in ratios, the variability may decrease – if the biomarkers correlate and exhibit the same patterns of variability within the individual (both numerator and denominator fluctuate in the same directions). Hence, the ratio will capture a stable relationship, as we observe in the p-tau217/BD-tau ratio. The high variability for p-tau217/T-tau ratio in the A β + group compared with p-tau217 alone could reflect a non-stable relationship between these markers. Reducing the intra-individual variability of plasma AD BBMs is important when tracking longitudinal changes of biomarkers levels, that is, in intervention studies or clinical trials.

Inter-individual variability reflects the variation of a biomarker between individuals within a given group, caused by, e.g. disease heterogeneity, comorbidities, and disease severity. We found that the p-tau217/BD-tau ratio exhibited a lower inter-individual variability compared to p-tau217 alone, indicating a stable relationship. Adjusting for eGFR, HbA1c, BMI, and sampling time point markedly reduced the variability of p-tau217 and p-tau217/BD-tau in the A β + group, resulting in a halving of the unadjusted variability for p-tau217 (CV 19.1% [95% CI 15.0;23.4]) compared to the adjusted variability of the p-tau217/BD-tau ratio (CV 11.1% [95% CI 8.8;13.7]). Given the importance of correct clinical interpretation of plasma p-tau217 changes, halving the variability is deemed relevant. We noted that BMI in particular had an impact on p-tau217 intra-individual variability, supporting previous findings that BMI influences levels of plasma p-tau217.^{38,39} The effect of BMI adjustment on biomarker variability in A β + was not seen in the A β – group. While the higher BMI observed in the A β – group may suggest an interaction between BMI and A β status on plasma p-tau217, this study design and small cohort size limit causal inference. Alternatively, lower p-tau217 levels in A β – may reduce the observable impact of BMI. We found limited effect of eGFR adjustment, a well-described peripheral confounder of plasma p-tau217 levels.^{5,6,26,38} This finding could be explained by an overall normal eGFR in this study

cohort. A full overview of the individual covariate impact can be found in Table S1. For p-tau217/T-tau the inter-individual variability was higher than the variability of p-tau217 alone, especially in A β + individuals, which could reflect biomarkers fluctuating in different directions. We did not observe a relevant reduction of variability in T-tau or the p-tau217/T-tau ratio when adjusting for covariates. Low inter-individual variability, meaning patients within the same disease group are likely to exhibit similar biomarker levels, is preferable as it allows for more consistent interpretations across individuals. This should be distinguished from a preferred wide dynamic range, which captures a variety of values, enabling differentiation across, that is, disease stages or conditions.

CSF p-tau217 correlated significantly with plasma p-tau217, but a higher correlation was observed with the p-tau217/BD-tau ratio. The significant correlations found between plasma T-tau and HbA1c and eGFR indicate that plasma T-tau levels are affected by peripheral factors. The finding that these confounders were not correlated with plasma BD-tau shows that plasma BD-tau is less likely to be affected by these peripheral factors. We observed a few patients with moderate to severe reduced kidney function $\text{eGFR} < 36/\text{min}/1.73\text{m}^2$ and noted high plasma markers in these patients. This is in line with a recently published paper concluding that $\text{eGFR} > 60$ has a limited effect on AD BBM, but caution should be taken when $\text{eGFR} < 60/\text{min}/1.73\text{m}^2$.²⁵ However, excluding these patients or adjusting for eGFR did not alter our results in this study. Future research should study the impact of moderate to severe reduced kidney function on AD blood biomarkers in clinical perspectives.

Classification performance remained relatively stable for the p-tau217 and p-tau217/BD-tau ratio, while more variability was observed for the p-tau217/T-tau ratio. It is important to note that the classification analyses aimed to explore potential changes in performance between visits, rather than to provide estimates of diagnostic accuracy for clinical application. The study was not powered for formal diagnostic validation, and our modest sample size in this regard limited the possibility of performing internal validation procedures, such as cross-validation. However, sensitivity analyses based on complete-case data yielded similar AUC results, suggesting that missing data did not substantially impact our findings. We acknowledge that assessing the variability of the p-tau217/BD-tau ratio may be premature until its clinical relevance is more clearly established.

This study has some limitations, such as the modest sample size. There was a notable sex imbalance between the A β + and A β – groups, which may impact the generalizability of the findings. Sex was included in the fully adjusted multivariable model to mitigate confounding, and though we did not observe significant changes in the variability, the uneven distribution limited interpretation of sex-specific effects. Nevertheless, the two groups were well characterized, and the 1-month timeframe for sample collection was considered appropriate to minimize the potential influence of disease progression on biomarker levels, given the chronic nature of neurodegenerative diseases. Blood collection was performed without information on last-food intake, and though studies have suggested significant fluctuations due to food intake,⁴⁰ we consider potential postprandial differences as part of

the observed short-term variability this study was designed to investigate. While circadian rhythms may influence plasma levels of AD biomarkers,⁴¹ evidence suggests that plasma p-tau217 may not exhibit substantial diurnal variability.⁴² To limit potential circadian effects and variability from sampling intervals, blood sampling was restricted to daytime hours, consistent with clinical practice, and sampling time points were included in all models. However, exact sampling times were not recorded, and circadian influence cannot be entirely ruled out. Our findings should be replicated in larger, more demographically balanced cohorts. Future studies should focus on demonstrating the clinical utility of the p-tau217/BD-tau ratio and evaluating the performance of the p-tau217/BD-tau ratio at predicting or tracking disease progression in AD patients.

In conclusion, the reduced variability of the plasma p-tau217/BD-tau ratio, adjusted for various confounders, is promising to ensure relevant clinical interpretation of longitudinal biomarker changes and to prevent misclassification. We suggest that the p-tau217/BD-tau ratio should be considered in instances where low variability is important and, further, to consider the influence of peripheral confounders, especially BMI.

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CONFLICT OF INTEREST STATEMENT

A.H.S. has served as a consultant for Eisai/BioArctic (remuneration paid to the institution).

K.B. has served as a consultant and on advisory boards for AbbVie, AC Immune, ALZPath, AriBio, BioArctic, Biogen, Eisai, Lilly, Moleac Pte. Ltd, Neurimmune, Novartis, Ono Pharma, Prothena, Roche Diagnostics, and Siemens Healthineers; has served on data monitoring committees for Julius Clinical and Novartis; has given lectures, produced educational materials, and participated in educational programs for AC Immune, Biogen, Celdara Medical, Eisai, and Roche Diagnostics; and is a co-founder of Brain Biomarker Solutions in Gothenburg AB (BBS), which is a part of the GU Ventures Incubator Program, outside the work presented in this paper.

H.Z. has served at scientific advisory boards and/or as a consultant for AbbVie, Acumen, Alektor, Alzinova, ALZPath, Amylyx, Annexon, Apellis, Artery Therapeutics, AZTherapies, Cognito Therapeutics, CogRx, Denali, Eisai, LabCorp, Merry Life, Nervgen, Novo Nordisk, Optoceutics, Passage Bio, Pinteon Therapeutics, Prothena, Red Abbey Labs, reMYND, Roche, Samumed, Siemens Healthineers, Triplet Therapeutics, and Wave; has given lectures at symposia sponsored by Alzecure, Biogen, Cellectricron, Fujirebio, Lilly, Novo Nordisk, and Roche; and is a co-founder of Brain Biomarker Solutions in Gothenburg AB (BBS), which is a part of the GU Ventures Incubator Program (outside submitted work).

K.F. serves or has served on a scientific advisory board and as scientific consultant for Novo Nordisk, Roche Diagnostics, and Eisai; has given lectures at symposia for Novo Nordisk, Eli Lilly, and Eisai/BioArctic (remuneration paid to institution); and has served or serves as principal investigator in trials for Roche, Biogen, AbbVie, Novo Nordisk, and Roche Diagnostics (remuneration paid to institution). K.F. also serves as editor-in-chief for Alzheimer's Research and Therapy (Springer) for which personal remuneration is paid.

The other authors have no conflict of interest to report.

CONSENT STATEMENT

All participants gave written informed consent prior to enrollment.

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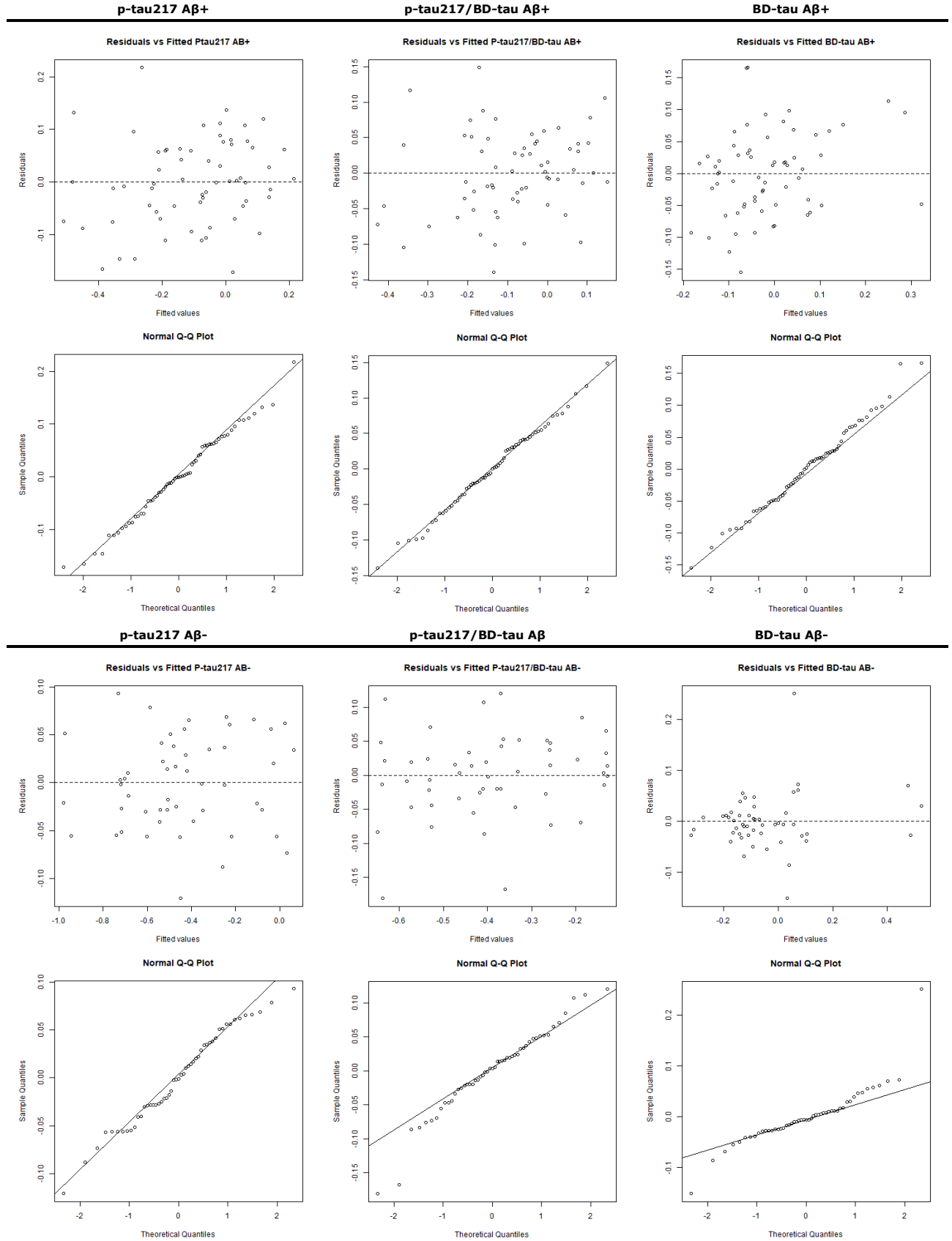
SUPPORTING INFORMATION

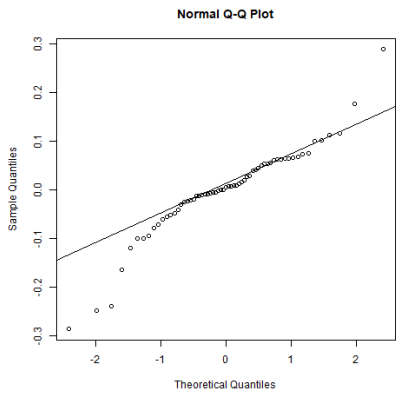
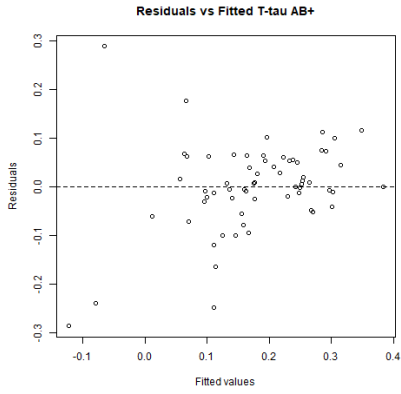
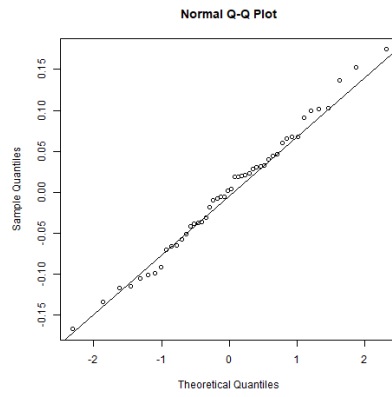
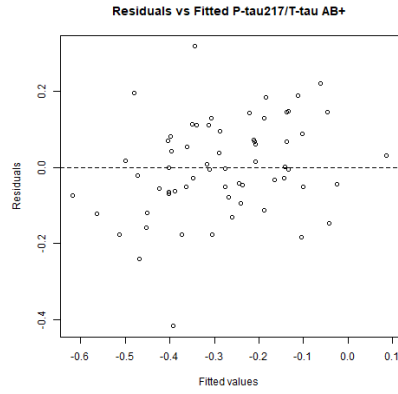
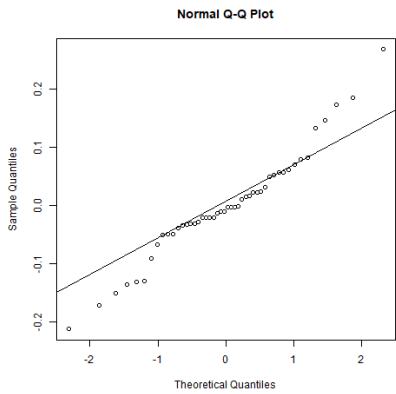
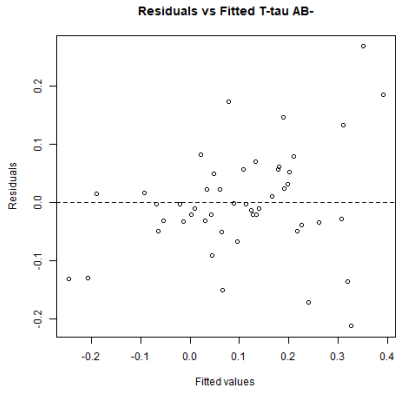
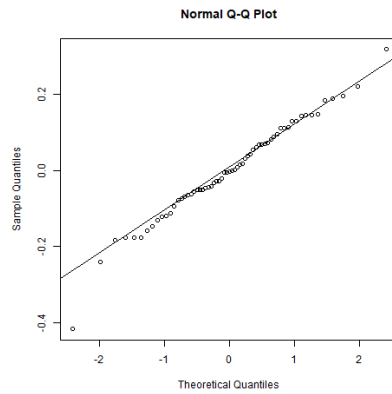
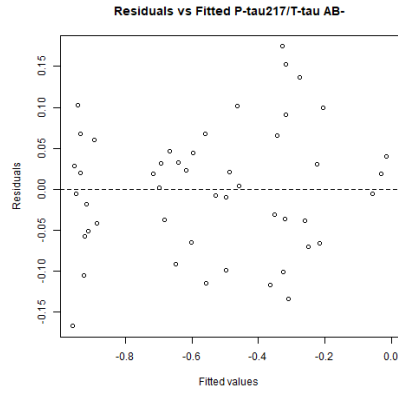
Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Clemmensen FK, Gonzalez-Ortiz F, Gramkow MH, et al. The plasma p-tau217/BD-tau ratio improves biomarker short-term variability in memory clinic patients. *Alzheimer's Dement*. 2025;11:e70143. <https://doi.org/10.1002/trc2.70143>

Supplementary material

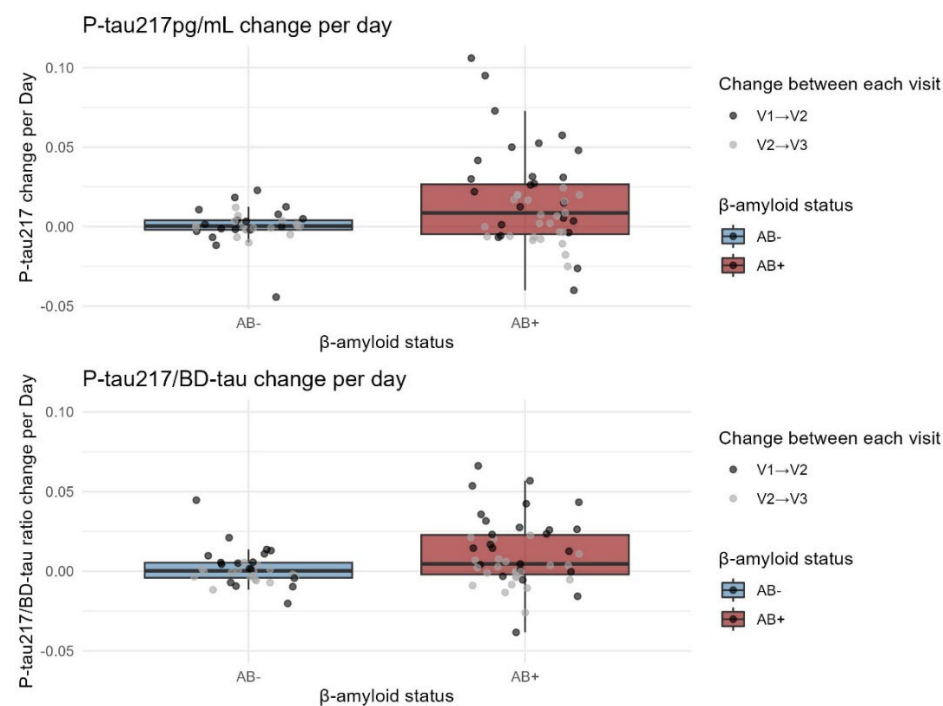
Supplementary Figure 1. Residual & corresponding Q-Q plots



T-tau A β +**p-tau217/T-tau A β +****T-tau A β -****p-tau217/T-tau A β -**

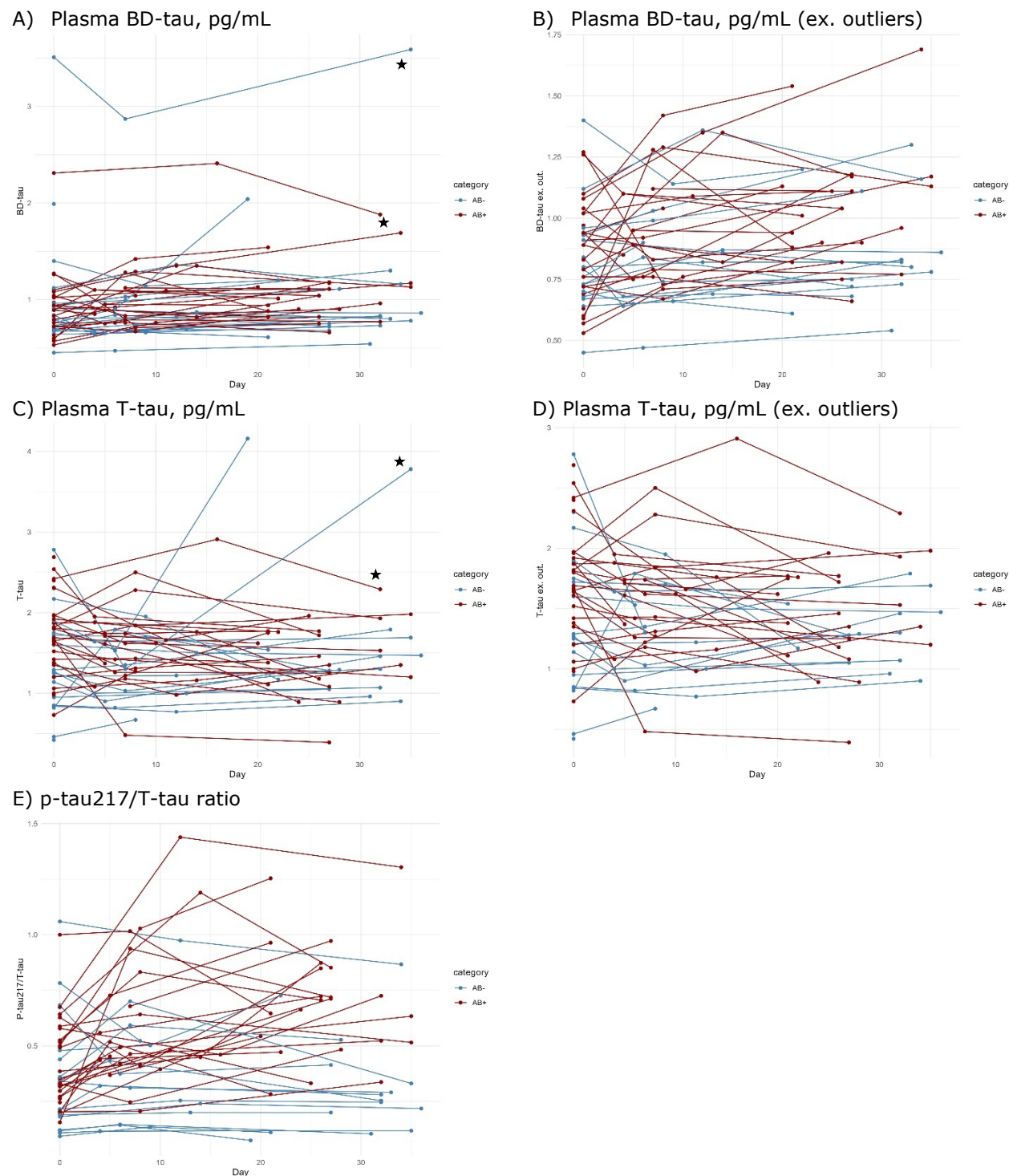
Residual and Q-Q plots from linear mixed models with logtransformed biomarker as outcome, day of sampling as fixed effect and patient as random effect.

Supplementary Figure 2. Biomarker changes per day



To account for differing intervals between visits, we calculated standardized per-day changes in both p-tau217 and the p-tau217/BD-tau ratio. These rates, expressed in pg/mL/day and ratio units/day respectively, plotted to assess temporal stability.

Supplementary Figure 3. Individual plasma biomarker trajectories



Individual trajectories of brain-derived tau (BD-tau), total-tau (T-tau) and the phosphorylated tau 217 to t-tau ratio (p-tau217/BD-tau). ★ = two patients with eGFR between 28-36/min/1,73m².

Supplementary Table 1. Intra- & Inter-individual Variability, CV – covariate adjustment

		Intra-individual Variability CV (%; 95% CI)						Inter-individual Variability CV (%; 95% CI)					
		Unadjusted	Adjusted for eGFR	Adjusted for HbA1c	Adjusted for BMI	Adjusted for Time	All covariates	Unadjusted	Adjusted for eGFR	Adjusted for HbA1c	Adjusted for BMI	Adjusted for Time	All covariates
p-tau217	A β +	11.1* (8.8;13.4)	11.1* (8.8;13.6)	11.1* (9.1;13.3)	11.3 (9.0;13.7)	9.4* (7.4;11.5)	9.5 (7.6;11.3)	20.4** (16.2;25.0)	20.3 (15.8;25.0)	20.7 (16.1;25.7)	14.6 (12.0;17.5)	19.1 (15.0;23.4)	13.1 ** (10.5;15.5)
	A β -	6.1* (4.6;7.4)	6.1* (4.6;7.6)	6.2* (4.6;7.8)	6.2 (4.7;7.7)	5.9* (4.5;7.3)	6.2 (4.5;7.9)	27.1 (18.5;35.7)	26.0 (17.1;34.9)	27.3 (17.1;36.6)	28.0 (18.8;37.8)	27.1 (18.4;36.0)	27.7 (16.1;39.5)
BD-tau	A β +	7.97 (6.3;9.5)	7.98 (6.4;9.6)	7.98 (6.4;9.6)	7.97 (6.3;9.5)	7.5 (5.97;9.1)	7.5 (6.0;9.1)	12.6 (10.2;15.3)	11.8 (9.4;14.4)	12.6 (9.9;15.3)	11.97 (9.5;14.4)	12.1 (9.8;14.7)	10.7 (8.4;12.7)
	A β -	6.8 (5.2;8.5)	6.9 (5.4;8.6)	7.2 (5.4;9.0)	6.8 (5.1;8.5)	6.6 (5.0;8.2)	6.5 (5.0;8.6)	20.0 (13.6;25.8)	17.8 (11.9;24.0)	20.3 (13.7;27.2)	19.7 (13.1;26.0)	20.1 (14.1;26.2)	16.8 (10.5;23.3)
T-tau	A β +	10.9 (8.7;13.1)	10.9 (8.7;12.9)	10.8 (8.6;13.1)	11.0 (8.8;13.1)	10.4 (8.2;12.5)	10.4 (8.3;12.6)	15.2 (12.4;18.0)	14.6 (11.9;17.5)	14.9 (12.1;17.9)	13.8 (11.1;16.3)	15.0 (12.1;18.3)	12.7 (10.4;15.1)
	A β -	11.3 (8.6;14.0)	10.9 (8.2;13.8)	11.4 (8.4;14.4)	11.6 (8.5;14.5)	11.1 (8.3;14.0)	10.9 (7.8;14.0)	20.9 (15.4;27.3)	17.9 (13.3;23.0)	22.3 (15.6;29.8)	22.1 (15.7;29.1)	20.6 (14.8;26.7)	19.4 (13.2;26.4)
p-tau217 / BD-tau	A β +	7.7 (6.0;9.3)	7.7 (6.2;9.3)	7.7 (6.2;9.2)	7.7 (6.2;9.2)	7.1 (5.6;8.4)	7.1 (5.6;8.5)	15.5 (12.1;18.9)	15.8 (12.2;19.4)	15.3 (11.9;19.1)	12.1 (9.5;14.5)	15.1 (11.7;18.7)	11.1 (8.8;13.7)
	A β -	7.2 (5.5;8.9)	7.4 (5.6;9.3)	7.5 (5.6;9.6)	7.0 (5.3;8.8)	7.3 (5.6;9.1)	7.4 (4.5;9.6)	18.3 (12.5;23.8)	17.1 (11.9;22.4)	17.8 (12.1;23.5)	19.3 (12.7;25.1)	18.4 (13.0;23.8)	18.8 (12.0;26.1)
p-tau217 / T-tau	A β +	16.8* (13.4;20.2)	16.8* (13.7;20.2)	16.8* (13.3;20.2)	16.9 (13.6;20.2)	13.9 (10.9;16.8)	13.9 (11.2;16.8)	21.6 (17.8;25.6)	21.8 (17.7;25.8)	21.7 (17.8;25.9)	20.2 (16.8;23.7)	19.3 (15.6;23.1)	18.2 (14.9;21.7)
	A β -	9.6* (7.1;11.9)	9.5* (7.1;12.1)	9.8* (7.3;12.6)	9.6 (7.1;12.1)	9.6 (6.9;12.0)	9.5 (6.8;12.2)	30.2 (20.6;39.8)	30.6 (20.2;41.0)	32.3 (20.9;43.7)	31.4 (22.2;40.4)	30.2 (21.1;39.2)	32.5 (19.8;45.1)

Intra- and inter-individual variability for each biomarker is presented together with the corresponding confidence interval.

*Non-overlapping 95% CI between A β + and A β - group. **Non-overlapping 95% CI between raw and model adjusted for all covariates.

CV: Coefficient Variant; CI: Confidence Interval; p-tau: phosphorylated tau; T-tau: Total tau; BD-tau: brain-derived tau.

Adjusting for sex in the multivariable model did not affect the variability.

Supplementary Table 2. Intra- & Inter-individual Variability, CV – COMPLETE CASES

		INTRA-INDIVIDUAL VARIABILITY CV (%, 95% CI) - TIME ADJUSTED	INTER-INDIVIDUAL VARIABILITY CV (%, 95% CI) - TIME ADJUSTED
p-tau217	Aβ+	9.8 (7.8;11.8)	20.2 (15.2;25.6)
	Aβ-	5.9 (4.4;7.1)	27.6 (18.1; 35.8)
p-tau217/ BD-tau	Aβ+	7.1 (5.5; 8.6)	15.7 (11.8;19.8)
	Aβ-	7.3 (5.7;9.1)	18.2 (13.0;23.6)
BD-tau	Aβ+	7.9 (6.2;9.5)	13.2 (9.95;16.4)
	Aβ-	6.6 (5.1;7.96)	18.9 (13.2;24.7)
T-tau	Aβ+	10.7 (8.2;12.9)	15.5 (12.1;19.2)
	Aβ-	10.7 (8.1;13.9)	15.5 (14.7;25.6)
p-tau217/ T-tau	Aβ+	14.6 (11.5;17.7)	20.4 (16.2;25.1)
	Aβ-	9.6 (7.0;11.98)	30.9 (20.8;40.98)

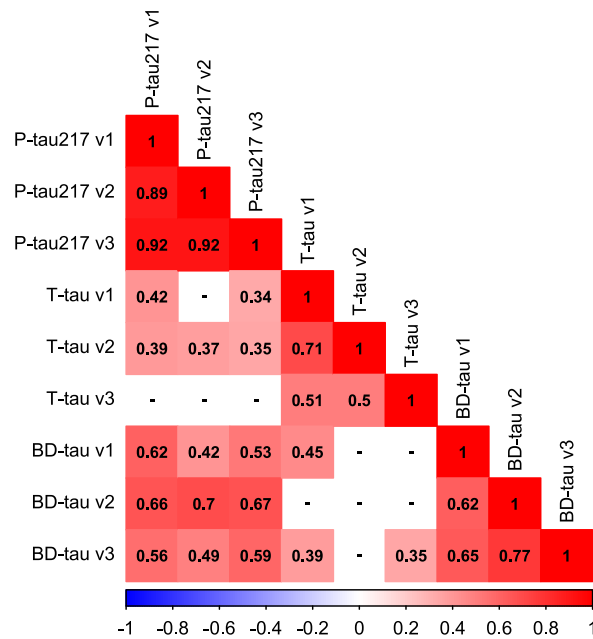
Intra- and inter-individual variability based on only complete cases (n=38) are presented together with the corresponding confidence interval. CV: Coefficient Variant; CI: Confidence Interval; p-tau: phosphorylated tau; T-tau: Total tau; BD-tau: brain-derived tau. Adjusting for sex in the multivariable model did not affect the variability.

Supplementary table 3. AUC across visits – COMPLETE CASES

	V1, n=38 AUC (95% CI)	V2, n=38 AUC (95% CI)	V3, n=38 AUC (95% CI)
p-tau217	0.77 (0.62;0.94)	0.88 (0.76;0.99)	0.85(0.73;0.98)
p-tau217/BD-tau	0.86 (0.74;0.98)	0.92 (0.83;1)	0.92 (0.84;1)
p-tau217/T-tau	0.61 (0.41;0.81)	0.76 (0.59;0.92)	0.86 (0.72;1)

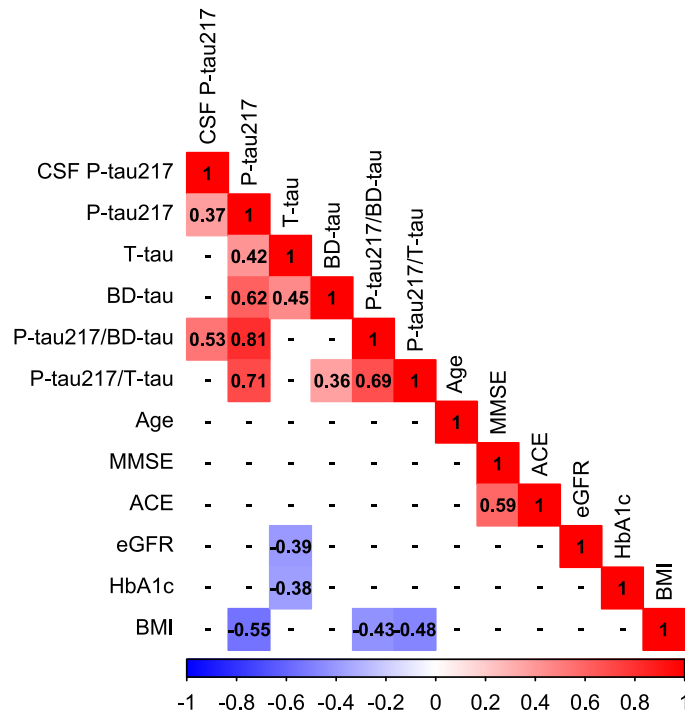
Classification performance for each sampling time point, visit 1 (V1), visit 2 (V2), visit 3 (V3). AUC, Area Under the Curve; CI, confidence intervals. p-tau: phosphorylated tau; T-tau: Total tau; BD-tau: brain-derived tau.

Supplementary Figure 3. Correlations between biomarkers & visits



Spearman's correlations. Significant correlations coefficients, p-value >0.05, are shown.

Supplementary Figure 4. Correlations between biomarkers & confounders



Spearman's correlations. Significant correlations coefficients, p-value >0.05, are shown.

8.3 Study III

Prognostic value of plasma biomarkers in early Alzheimer's disease: a longitudinal clinical and neuroimaging study

Frederikke Kragh Clemmensen, Mathias Holsey Gramkow, Fernando Gonzalez-Ortiz, Andrea Lessa Benedet, Kubra Tan, Wiebke Traichel, Ulrich Lindberg, Otto Mølby Henriksen, Ian Law, Henrik Zetterberg, Kaj Blennow, Anja Hviid Simonsen, Kristian Steen Frederiksen & Steen Gregers Hasselbalch.

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RESEARCH

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Prognostic value of plasma biomarkers in early Alzheimer's disease: a longitudinal clinical and neuroimaging study

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Abstract

Background While blood biomarkers for AD have proven useful in identifying AD pathology from other neurodegenerative conditions, their prognostic value in real-world clinical settings, such as memory clinics, remains unexplored. We aimed to examine the prognostic value of AD plasma biomarkers and their short-term changes within 4 months, for predicting clinical and neuroradiological progression over two years.

Method We performed a prospective observational longitudinal cohort study of patients with early stages of AD and followed them for up to 2 years. Plasma was analysed for neurofilament light (NfL), brain-derived tau (BD-tau), phosphorylated tau 217 (p-tau217), and glial fibrillary acidic protein (GFAP) on single molecule array. The outcomes were annual changes in clinical dementia rating scale, sum of boxes (CDR-SB) and changes in [¹⁸F]-fluorodeoxyglucose positron emission tomography ([¹⁸F]FDG-PET) and magnetic resonance imaging (MRI) brain scans. We performed linear regression models with baseline and short-term plasma biomarker changes as predictors, adjusted for age, sex, and creatinine.

Results In total, 94 patients with MCI or dementia due to AD had baseline and one-year follow-up, with 85 patients completing two-year follow-up. We found that baseline plasma NfL was significantly associated with an increase in CDR-SB at one- and two-year follow-up. Neither plasma BD-tau, p-tau217 or GFAP were associated with future clinical progression. Short-term changes of plasma NfL were associated with reduced glucose metabolism in the hippocampus and temporal cortex on [¹⁸F]FDG-PET. Short-term changes in plasma p-tau217 were significantly associated with reduced hippocampal glucose metabolism, and further, short-term changes in the p-tau217/BD-tau ratio were significantly associated with reduced metabolism in hippocampal and isthmus cingulate cortex on [¹⁸F]FDG-PET. We found no association between plasma biomarkers and MRI volumetric changes after multiple testing correction.

Conclusion Our findings indicate that plasma NfL can serve as a predictor for subsequent clinical progression in early AD. Short-term changes in AD-related plasma biomarkers may reflect underlying disease processes, however, several

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limitations affect their interpretability. Therefore, short-term changes should be evaluated carefully and always in the context of baseline levels and clinical characteristics.

Trial registration Clinicaltrials.gov (NCT05175664), date of registration 2021–12-01.

Keywords Alzheimer's disease, Dementia, Disease progression, Prognosis, Blood biomarkers, Memory clinic, Neurodegeneration, Variability

Background

Alzheimer's disease (AD) is a progressive neurodegenerative disease characterized by the accumulation of beta-amyloid plaques, neurofibrillary tangles, and brain atrophy, causing a decline in cognitive functions consequentially leading to death. These pathological alterations start decades before symptom onset [1, 2]. Progression of AD varies considerably across individuals making disease prognosis a significant challenge. Development of prognostic tools is important to help guide clinical decisions, particular in patients in the early stages of AD when disease modifying therapies, i.e., anti-amyloid drugs, are presumed to be most beneficial. Blood biomarkers for AD have emerged as promising in reflecting various aspects of AD pathology including neurodegeneration and neuroinflammation, and should ideally work to identify individuals at risk of disease, detect disease pathology, monitor effects of interventions, and predict disease progression [3, 4].

Blood biomarkers reflecting neurodegeneration, such as neurofilament light (NfL), have been proposed as a potential prognostic neurodegenerative marker in AD [5–8]. Brain-derived tau (BD-tau) has recently been suggested as a neurodegenerative marker in plasma, selectively measuring tau proteins derived from the brain [9, 10] and has been shown to predict AD progression [11]. Phosphorylated tau at amino acid 217 (p-tau217) has been associated with risk of cognitive decline in cognitively unimpaired individual [12] and different p-tau isoforms have been found to be elevated depending on disease stage [13, 14]. Glial fibrillary acidic protein (GFAP), released by activated astrocytes, has been suggested as a marker of neuroinflammation and elevated in the early stages of AD [15]. Further NfL, GFAP, and p-tau217 have been used as outcome measures in clinical trials with anti-amyloid drugs [16, 17]. These four markers each reflect different aspects of AD neuropathology and, thus, may contribute differently to our understanding of the AD continuum.

The prognostic value of AD biomarkers has been studied in preclinical cohorts with a high predictive value for progression in amyloid-positive vs. -negative individuals [7, 18]. Most of these studies are retrospective and aim to predict the risk of progression from cognitively unimpaired to MCI or dementia [14, 19–23]. In contrast, relatively few studies have explored the ability of plasma

biomarkers to predict progression *within* the symptomatic AD spectrum—from early stages to more advanced dementia stages. These studies have reported that baseline plasma NfL was associated with cognitive decline [5, 6, 24], and further predicted MRI-detected brain volume loss in AD patients [6]. A recent review of blood biomarkers and MR modalities further supports these findings, reporting associations between plasma NfL, GFAP, p-tau181, and p-tau217 with various AD-specific regional brain atrophy patterns [25].

Biomarker levels can be affected by protein production, release and clearance, as well as pre-analytical and analytical procedures, causing variability in biomarker measures [26]. Factors such as sex, age, kidney function [27], BMI and fasting status [28], along with comorbidities (e.g., hypertension, diabetes, peripheral nervous systems diseases, stroke history), significantly influence biomarker levels and must be considered when interpreting biomarker levels [29]. Using the plasma p-tau217 to non-phosphorylated tau ratio (%p-tau217) has been shown to mitigate the influence of peripheral confounders [30, 31] while the p-tau217 to BD-tau ratio has shown reduced variability [32], which reduces the possible risk of misclassification and misinterpretation of blood biomarkers levels and changes. However, whether changes in blood biomarkers over a few months only reflect intra-individual or assay variability, or whether it may reflect disease activity that entails prognostic value is not well-understood.

In this prospective longitudinal observational cohort study, we aimed to examine the predictive value of plasma biomarker levels and their short-term changes in memory clinic patients with early AD. Specifically, we examined if plasma NfL, p-tau217, BD-tau or GFAP levels could predict clinical progression in up to two years. Further, we assessed the prognostic value of the same markers when predicting imaging progression as assessed by volumetric MRI and semi-quantitative 18F-fluorodeoxyglucose positron emission tomography ([¹⁸F]FDG-PET).

Methods

Study design

Prospective longitudinal cohort study.

Participants

From January 2022 to June 2023, we included patients with MCI or mild to moderate dementia due to AD from a university memory clinic at Rigshospitalet, Copenhagen, Denmark. Patients were included at the time of their diagnosis and followed for up to two years, see study flow chart in Fig. 1. Follow-up ended in November 2024, where the study had to be terminated due to logistic factors and therefore not all patients were followed for two years.

Participants were referred to the Memory clinic (a tertiary centre) due to cognitive complaints. The diagnostic standard work-up program includes assessment of medical history and physical examination by an experienced physician, cognitive testing by Mini-Mental State Examination (MMSE) [33] and Addenbrooke's Cognitive Examination (ACE) [34], a structural brain scan (magnetic resonance imaging (MRI) or computed tomography (CT), and routine blood examination (kidney function, D-vitamin, and B12-vitamin status). If necessary, additional examinations such as cerebrospinal fluid examination, [¹⁸F]FDG-PET, amyloid PET, or dopamine active transporter scans, are carried out. At a multidisciplinary consensus conference, the diagnosis is given based on the criteria for MCI or dementia due to AD [35, 36]. For further information on the diagnostic evaluation in the memory clinic Rigshospitalet, see [37].

Inclusion criteria for this study were a diagnosis of MCI or mild to moderate dementia due to AD, a MMSE > 19, a baseline diagnostic [¹⁸F]FDG-PET/MRI or [¹⁸F]FDG-PET/CT scan, presence of a caregiver to serve as an

informant, and the patient's ability to provide written informed consent. Exclusion criteria were current alcohol or substance abuse, other major neurologic or psychiatric disorder, or participation in intervention trials. The study was approved by the Regional Scientific Ethics Committee (H-21040317) and was registered at clinicaltrials.gov (NCT05175664). The study followed the tenets of the 1975 and 2024 Declaration of Helsinki.

Baseline clinical, demographic and biochemical data

Baseline cognitive test results, which included MMSE, ACE, and the Clinical Dementia Rating (CDR) were stored in an electronic database (REDCap, Vancouver). Demographic and medical data were extracted from the medical records and included age, sex, weight, height, body mass index (BMI), years of education, medication status and comorbidities. Biochemical data of relevance in this study included glycated haemoglobin A1c (HbA1c), creatinine and estimated glomerular filtration rate (eGFR). For patients with an eGFR > 90, the numeric value was calculated as Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) mL/min/1.73m² based on creatinine level, age and sex. Cerebrospinal fluid (CSF) from patients who underwent cerebrospinal fluid examination as part of their diagnostic evaluation was analysed at the Clinical Biochemistry Laboratory at Rigshospitalet. CSF from patients enrolled before June 30th, 2022, was analysed with the Innotech enzyme-linked immunosorbent assays (ELISA) (Fujirebio, Ghent) (n = 8) and CSF collected after July 1st, 2022, was analysed with

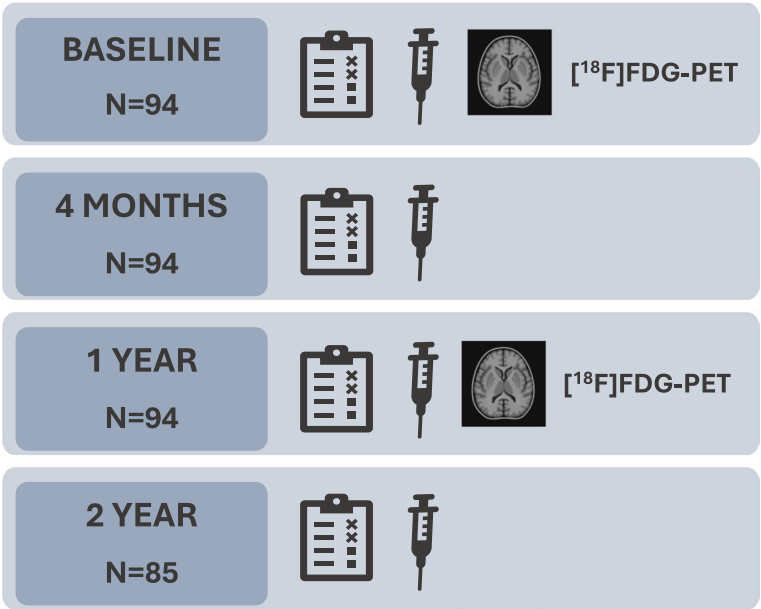


Fig. 1 Study flow. Outline of study flow. The median time interval between baseline and second blood collection was 4.4 months (1st IQR 3.2, 3rd IQR 5.2). Median time for 1Y = 1.1 years (1st IQR: 1.05, 3rd IQR: 1.2), and for 2Y = 1.9 years (1st IQR: 1.7, 3rd IQR: 2.1). One baseline blood sample was lost in collection. In total, 70 MRI and 68 [¹⁸F]FDG-PET scans were eligible for analyses of changes between baseline and 1Y FU

the Elecsys sandwich electrochemiluminescence-immunoassay (Roche, Cobas 8000) [38, 39].

Longitudinal follow up visits

At each study visit patients were asked to bring a primary caregiver. Study visits were scheduled 3–5 months, 12 months (denoted 1Y), and 18–24 (denoted 2Y) months after baseline visit. At each study visit a blood sample was collected, and a MMSE was administered. At 1 and 2Y, additionally cognitive testing included ACE and the semi-structured interview, the Clinical Dementia Rating (CDR) was carried out with the caregiver and patient [40]. CDR rates cognition and function by assessing six domains (Memory, Orientation, Judgement and Problem solving, Community affairs, Home and Hobbies, and Personal care) on a scale ranging from 0 to 18 (higher the score indicating greater impairment), termed the Sum-of-Boxes (SB).

A follow-up hybrid [^{18}F]FDG-PET/MRI brain scan was carried out at the Department of Clinical Physiology and Nuclear Medicine, Rigshospitalet, approximately two weeks prior to the 1Y FU.

Finally, the study investigator carried out a routine medical assessment at each study visit, including evaluation of disease severity stage, blood pressure, weight, and medicine, including antidementia medication drugs, which were modified when clinically indicated.

Blood sampling and analyses

Baseline plasma was collected as part of the patient's diagnostic work up program in the clinic. If, at baseline, a blood sample was collected more than 3 months earlier, a new blood sample was collected. At each study visit a blood sample was collected in ethylenediaminetetraacetic acid (EDTA)–plasma tubes. Within 30 to 120 min after collection, the tubes were centrifuged at 2,000 g, 4°C for 10 min, then redistributed into 250 µl aliquots and, stored at – 80°C until further analysis.

Plasma was analyzed at the Department of Psychiatry and Neurochemistry, the Sahlgrenska Academy, University of Gothenburg, in December 2024 and January 2025. Using the single molecule array (Simoa) technology by Quanterix HD-X [29]. Plasma NfL and GFAP were measured by the Simoa Neurology 2-*PLEX B* assay, p-tau217 by the Simoa singleplex assay *ALZpath p-tau217*, and plasma BD-tau was measured with the Gothenburg University previously validated method [9]. Analyses were carried out according to Simoa instructions [29] with samples run in singlicates, and in one round of experiment. Samples from each participant were placed next to each other on the same plate. NfL and GFAP analysis were diluted fourfold, and p-tau217 threefold—results were compensated for the dilution. Concentrations lower

than the calibration curve were labelled under limits of quantification (<LOQ).

Biomarker changes between baseline and 4M were calculated as the relative change (%),

$(\frac{4M - \text{baseline}}{\text{baseline}} \bullet 100)$, denoted *short-term changes*. We also included the p-tau217/BD-tau ratio as this has previously been found to reduce biomarker variability [32].

Clinical progression

To assess clinical progression, our primary outcome was changes in score on the CDR-SB. We defined changes as a continuous variable, hence the differences in points between baseline and 1- or 2-year FU, normalized to represent annual changes ($\frac{\text{follow up} - \text{baseline}}{\text{time between baseline and follow up}}$).

As secondary clinical outcomes, we used changes in disease severity stages, which were based on the full clinical assessment at each visit (CDR-SB, MMSE, ACE, patient and caregiver information) and assessed by the trained study investigator at each study visit in accordance with the NIA-AA [36] and the WHO ICD-11 classification [41]. We categorized patients into four groups based on: *MCI stable* (MCI patients who remained MCI), *MCI converter* (MCI patients who progressed to dementia), *dementia stable* (patients with dementia who remained at the same severity stage (mild, moderate, severe), and *dementia progression* (dementia patients who advanced to more severe stages), assessed at one- and two-year FU. To improve statistical power given the limited sample size, these four groups were further merged into two categories: *stable* and *progression*.

Imaging progression—[^{18}F]FDG-PET/MRI

All patients who had an [^{18}F]FDG-PET ($n=67$) and MRI ($n=70$) both at baseline and at one year FU were included for analysis. The scans were performed on a hybrid 3T PET/MRI scanner (Biograph mMR, Siemens, Erlangen, Germany), except for 14 individuals who received separate 1.5T MRI and PET scan at baseline. PET imaging was performed according to current guidelines [42]. To assess potential impact of scanner differences, we conducted a sensitivity analysis excluding these 14 participants. Simultaneous PET and MRI was commenced 40 min after i.v. administration of approx. 200 MBq [^{18}F]FDG. The MRI protocol included 3D-T1-weighted (T1), T2-weighted (T2), T2 fluid attenuated inversion recovery (FLAIR), and T2*-weighted imaging. The T1-weighted MRI images together with FDG-PET data were analysed using the volumetric longitudinal pipeline and PETSURFER (FreeSurfer, ver. 7.4.1) [43]. Volumetric quantifications were extracted for predefined AD specific regions of interest (ROIs) that included total grey matter, cortex, hippocampal, and lateral ventricle volumes,

normalised to the intra-cranial volume (ICV). Selection of regions was based on previous findings of changes in MRI regions in AD [7, 44]. From [^{18}F]FDG-PET scans, regional Standardised Uptake Value ratios (SUVr) was calculated by normalizing ROI SUV to uptake in the reference region (cerebellum). Based on previous findings, predefined AD specific regional SUVr measures of interest were hippocampal, isthmus cingulate, temporal, and parietal cortex [45].

Annual changes in [^{18}F]FDG-PET and MRI measures were defined as the difference between baseline and FU volume/SUVr, adjusted for baseline volume/SUVr and time between baseline and

$$1Y \left(\frac{1Y - \text{baseline}}{\text{time between baseline and 1Y}} * 100 \right).$$

Statistical methods

Descriptive statistics summarized baseline cohort characteristics. Normality of distribution was assessed by visual inspection of histograms and quantile–quantile plots. Continuous variables are presented as mean (+ standard deviation (SD)) and median and interquartile range (IQR) depending on data distribution. Categorical variables are summarised as frequency and percentage. Outliers were assessed for each biomarker by the interquartile range (IQR) method. One observation of baseline plasma NfL above the 95% percentile, was identified. The patient had a baseline NfL value above >100mg/mL with levels that decreased by –50% at follow-up visits. The abnormal level was, based on the medical record, determined to be caused by a mild head trauma prior to the baseline blood sampling, and the patient was therefore excluded from analyses with NfL. Group differences were assessed by the two-sample t-test or chi-square test. To assess group differences in baseline plasma levels, these were log-transformed for normal distribution and examined by linear regression models, adjusted for age, sex, and creatinine.

Linear regression models were used to assess the relationship between annual clinical and imaging changes by the explanatory variables, baseline and short-term changes in plasma biomarker levels. Outcome measures were annual changes in CDR-SB, annual changes in MRI region of interest (ROI) volumes, and annual changes in [^{18}F]FDG-PET, regional SUVr. We evaluated two models 1) baseline biomarker, adjusted for age, sex, and creatinine (see correlation coefficients in Additional file 10, Fig. 3), and 2) short-term biomarker changes, additionally adjusted for baseline biomarker values. As there was a significant negative association between baseline biomarker levels and short-term changes, with a tendency of lower baseline levels leading to greater short-term changes (see Additional file 9, Fig. 2), all linear regression models were examined with an interaction term

(baseline*short-term changes), to adjust for regression toward the mean. If a significant effect, the interaction term was included in the model. If not, the models were adjusted for baseline biomarker level.

As a secondary clinical outcome, we assessed the biomarkers performance to discriminate between *stable* and *progression*, by fitting two logistic regression models: 1) baseline biomarker, adjusted for age, sex, and creatinine and 2) short-term biomarker changes, additionally adjusted for baseline biomarker values. The dataset was split into two: 80% training and a 20% hold out test set. Model performance was evaluated using the area under the receiver operating characteristic curve (AUC). To quantify uncertainty, 95% confidence intervals for AUCs were computed using bootstrap resampling with 2,000 replicates.

To better address how both intra-individual and assay-specific variability influence short-term changes of biomarkers, we included biomarker specific reference change values (RCV, %) from previously published data on RCVs of plasma NfL, GFAP, and p-tau217 in AD patients [26]. RCV represents the minimum change between two measurements needed to exceed intra-individual variability and assay variation, and thus is important to ensure clinically relevant interpretation of changes in biomarker levels. For plasma BD-tau, we included data from a previously published study and calculated the RCV [32]. We created a three-level categorical variable with short-term changes grouped as *within*, *above* or *below* the markers' RCV, and used it in model 2.

Model assumptions were checked and met. To account for multiple comparisons across imaging regions, *p*-values were adjusted using the Benjamini–Hochberg method with a false discovery rate (FDR) threshold of 0.05. β -estimates, 95% confidence intervals and *p*-values (both raw and multivariable correction) are reported. All statistical analyses were performed using the R Statistical Software, version 4.5.0 and statistical significance was set to $\alpha = 0.05$.

Results

Participants

A total of 94 patients had baseline and 1 year FU. Out of these, 85 also completed 2-year follow-up. Nine patients discontinued participation after the one-year follow-up, either because they changed their mind or felt that the testing burden was too high. Baseline characteristics are shown in Table 1, grouped as *stable* ($n=54$) vs. *progression* ($n=40$). In general, the two groups were comparable, however the progression group was significantly older, had higher levels of plasma creatinine, and showed a higher proportion of patients treated with anti-thrombotic medication (45% vs. 17%; Table 1). We found no differences in various comorbidities (hypertension,

Table 1 Baseline demographics & clinical measures

	Stable (N = 54)	Progression (N = 40)	p-value
Age, mean (SD)	73.78 (7.1)	78.1 (6.1)	0.003
Sex, female	28 (51.9%)	21 (52.5%)	0.95
Education, years, mean (SD)	11.4 (3.3)	12.3 (4.2)	0.3
Dementia severity (MCI/dementia)	14/4** (26/94%)	12/28 (30/70%)	0.6
MMSE, mean (SD)	25.7 (2.5)	25.50 (2.4)	0.7
CDR-SB, mean (SD)	2.6 (1.0)	2.9 (1.7)	0.3
CSF β -amyloid (pg/mL) (n = 42), median (IQR)	703 (586, 848)	649 (579, 857)	0.5
CSF t-tau (pg/mL) (n = 42), median (IQR)	337 (263, 466)	324 (231, 422)	0.4
CSF p-tau (pg/mL) (n = 42), median (IQR)	40 (27, 51)	38 (23, 60)	0.7
Plasma NfL (pg/mL), median (IQR)*	17 (14, 24)	24 (19, 32)	0.035
Plasma GFAP (pg/mL), median (IQR)*	253 (189, 349)	286 (242, 415)	0.5
Plasma BD-tau (pg/mL), median (IQR)*	1.1 (0.9, 1.3)	1.2 (1.0, 1.5)	0.7
Plasma p-tau217 (pg/mL), median (IQR)*	0.8 (0.6, 1.1)	0.8 (0.7, 1.3)	0.7
BMI, mean (SD)	24.6 (3.8)	25.5 (4.1)	0.3
EGFR mL/min/1.73m ² , mean (SD)	79.4 (13.8)	71.1 (18.4)	0.014
Creatinine, mean (SD)	72.7 (17.4)	83.3 (23.0)	0.012
Hba1c, mean (SD)	39.3 (4.7)	41.7 (11.5)	0.2

Baseline demographic and clinical measures of patients who had baseline and 1-year FU data. P-values from two-sample t-test or chi-square test

*p-values based on log-transformed values, linear regression adjusted for age, sex, and creatinine. Plasma GFAP was higher in the progression group, however not significant higher after age, sex, and creatinine adjustment

**One patient presented with moderate dementia at baseline. One outlier, plasma NfL > 100pg/mL was excluded

hypercholesterolemia, diabetes, or depression) or treatment with cholinesterase inhibitors (data not shown) between groups. Plasmatic levels of NfL were higher in the progression group at baseline, but no other differences in biomarker concentrations were found. Patients in the *progression* group progressed more on the CDR-SB compared with *stable*, see Additional file 1, Table 1. Baseline measures of SUVR and MRI regional volumes did not differ between *stable* and *progression*, see Additional file 2 & 5, Tables 2 and 5.

Prognostic value of plasma biomarkers to detect annual clinical progression

Baseline plasma NfL was significantly associated with annual changes in CDR-SB at 1- and 2-year, see Table 2 (age, sex, and creatinine adjusted). The models showed that for each 10pg/mL baseline plasma NfL, the annual change in CDR-SB was 0.5 points at 1Y (p-value 0.01, 95% CI 0.1;0.8) and 0.6 points at 2Y (p-value = <0.01, CI 0.2;1.00). None of the other biomarker levels were associated with progression on the CDR-SB. The p-tau217/BD-tau ratio showed a significant association with a one-year decrease in CDR-SB. Also, we found no association between changes in CDR-SB and short-term changes in plasma biomarkers.

Plasma NfL could, with intermediate accuracy, distinguish between *stable* and *progression* groups, see Fig. 2. No differences were observed between baseline NfL or short-term changes. The other markers had lower accuracy than plasma NfL indicating a limited clinical utility.

Plasma biomarkers and their association with annual changes in [¹⁸F]FDG-PET

No association was found between [¹⁸F]FDG-PET SUVR annual changes and baseline biomarker levels. Short-term changes of plasma NfL were significantly associated with an annual decrease in bilateral hippocampal (β : -0.1, p-value=0.008, 95% CI: -0.2; -0.03) and temporal cortex SUVR (β : -0.07, p-value=0.02, 95%CI: -0.1.4;-0.01), also after multiple testing correction (see Fig. 3 and Additional file 3, Table 3).

Short-term changes of plasma p-tau217, were significantly associated with decrease in bilateral hippocampal SUVR (β : -0.03, p-value=0.03, 95% CI: -0.05; -0.003), however not significant after multiple testing correction. Furthermore, we explored p-tau217 combined with BD-tau and found that an increase in short-term changes of the p-tau217/BD-tau ratio was associated with an annual decrease in bilateral hippocampal SUVR (β : -0.03, p-value=0.006, 95% CI: -0.1; -0.01) and bilateral isthmus cingulate cortex SUVR (β : -0.03, p-value=0.01, 95% CI: -0.05; -0.01), see Fig. 3. These associations remained significant after multiple testing correction. Although not statistically significant, we observed a trend suggesting a potential association between short-term changes of p-tau217/BD-tau and temporal and parietal cortex, see Additional file 2, Table 2.

Neither plasma BD-tau nor GFAP were associated with annual changes in glucose metabolism, see Additional file 3, Table 3.

Table 2 Plasma markers as predictors of annual changes in CDR-SB

	Predictor	Estimate per 10 units increase	p-value	95% CI
CDR-SB 1Y	Baseline NfL, pg/mL	0.5	0.01	0.1;0.9
	RC NfL, %	−0.001	0.26	−0.002;0.0004
	Baseline BD-tau, pg/mL	−3.5	0.54	−14.9;7.8
	RC BD-tau, %	−0.0002	0.49	−0.001;0.0004
	Baseline p-tau217, pg/mL	−3.4	0.41	−11.3;4.6
	RC p-tau217, %	0.001	0.43	−0.001;0.002
	Baseline GFAP, pg/mL	−0.005	0.73	−0.03;0.02
	RC GFAP, %	−0.001	0.15	−0.001;0.0002
	Baseline p-tau217/BD-tau, pg/mL	−0.9	0.13	−1.8;0.3
	RC p-tau217/BD-tau, %	−0.01	0.01	−0.02;−0.001
CDR-SB 2Y	Baseline NfL, pg/mL	0.6	< 0.01	0.2;1.0
	RC NfL, %	0.0001	0.84	−0.001;0.001
	Baseline BD-tau, pg/mL	5.0	0.33	−5.2;15.1
	RC BD-tau, %	0.0002	0.52	−0.0004;0.001
	Baseline p-tau217, pg/mL	3.1	0.39	−4.1;10.3
	RC p-tau217, %	−0.0001	0.90	−0.001;0.001
	Baseline GFAP, pg/mL	−0.01	0.65	−0.03;0.02
	RC GFAP, %	−0.0003	0.46	−0.001;0.0004
	Baseline p-tau217/BD-tau, pg/mL	0.1	0.8	−0.9;1.1
	RC p-tau217/BD-tau, %	0.002	0.6	−0.01;0.01

Table showing results from linear regression models with CDR-SB as outcome and predictors: 1) baseline biomarker, 2) short-term biomarker changes (adjusted for baseline), denoted relative change (RC). Both models were adjusted for age, sex, and creatinine. Age had a significant effect in all models. Sex and creatinine adjustment altered the models but were not significant associated in any of the models. All models were additionally adjusted for baseline CDR-SB, which did not alter the results (data not shown). Finally, models with p-tau217 were further adjusted for BMI, which did not alter the results (data not shown). Baseline and short-term changes of NfL, GFAP, p-tau217, and BD-tau estimates are for 10 units of increase

Plasma biomarkers and their association with annual changes in MRI

We found no association between annual changes in MRI volumes and baseline biomarker levels. Short-term changes of plasma BD-tau were significantly associated with an annual decrease in cortex- and total grey matter volume, see Fig. 4. However, after correction for multiple testing, these associations were no longer significant. None of the other plasma markers were associated with changes in MRI volumes, see Additional file 6, Table 6. Excluding patients, $n = 14$, with baseline MRI performed on a 1.5T MRI scan, we observed the same trends between short-term BD-tau changes and MRI regions, but no associations were significant after multiple testing correction, see Additional file 7 & 8, Table 7 & Fig. 1.

Evaluating short-term biomarker changes in the context of reference change values

Given the impact of intra-individual and assay variability on biomarkers levels, we evaluated short-term changes by applying reference change values (RCV) to evaluate those exceeding expected variability thresholds. We observed that most short-term changes were within these references, see Fig. 5. Changes exceeding RCVs were especially observed in plasma NfL and p-tau217, in the dementia groups.

In the group that exhibited NfL changes above the RCV, we found a significant association with SUVr changes in

the bilateral hippocampus (β : -8.0 , p -value = 0.02 , 95%CI: -14.8 ; -1.3) and bilateral temporal cortex (β : -5.7 , p -value = 0.02 , 95%CI: -10.4 ; -1.0) compared to the group of patients that stayed were *within* the RCV. These associations remained significant after multiple testing correction, see Additional file 4, Table 4. No other significant associations were found.

Discussion

In this prospective longitudinal study of patients with early stages of AD, we investigated whether baseline and short-term changes in plasma NfL, BD-tau, p-tau217, and GFAP could predict clinical progression. We further investigated whether these were supported by changes in brain metabolism and structural changes measured by [^{18}F]FDG-PET and MRI, respectively.

Our findings demonstrate that plasma NfL was significantly associated with annual clinical progression up to two years after blood sampling. None of the other biomarkers were associated with clinical progression. We found associations between annual decreased relative regional brain glucose metabolism and short-term changes in plasma NfL, p-tau217, and the p-tau217/BD-tau ratio. Regarding MRI, the association with short-term changes in BD-tau and cortical and grey matter atrophy, was not significant after multiple testing correction.

Our finding that elevated plasma NfL levels were associated with clinical progression is consistent with

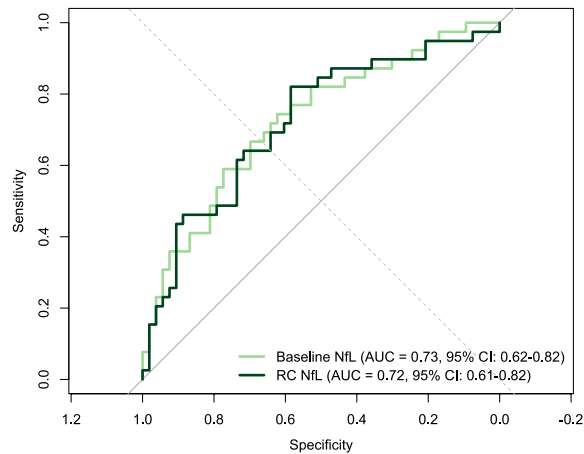
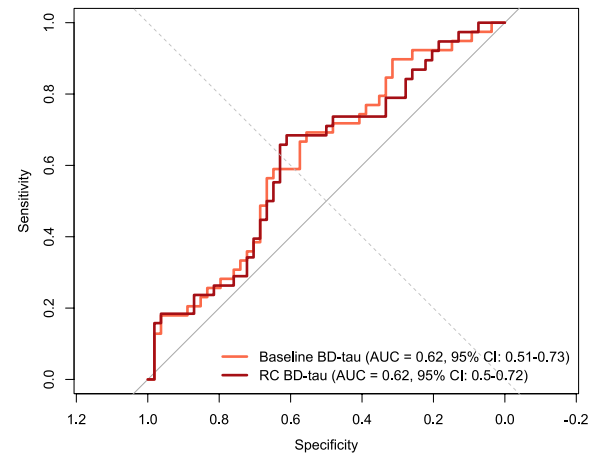
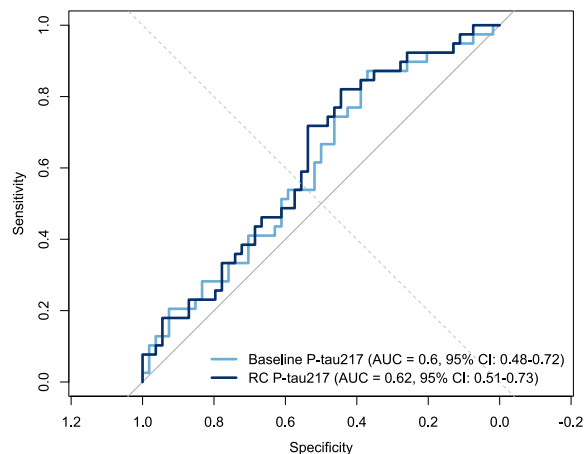
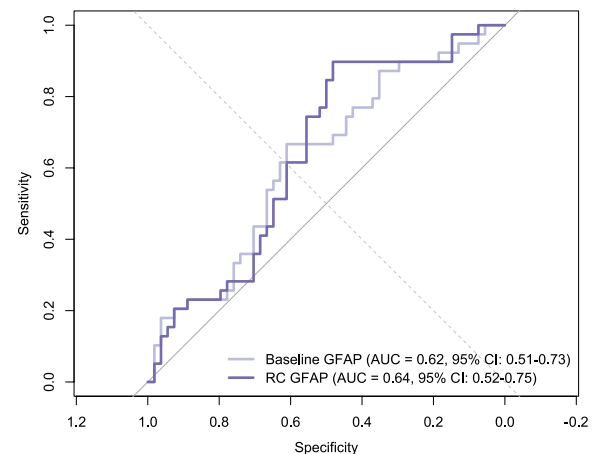
A) NfL AUC**B) BD-tau AUC****C) p-tau217 AUC****D) GFAP AUC**

Fig. 2 Plasma biomarker as predictors of *stable* vs. *progression*. Plasma **A)** NfL, **B)** BD-tau, **C)** p-tau217, and **D)** GFAP as predictors of stable vs. progression. ROC AUCs discriminating between stable and progression, based on changes in the classification of MCI and Dementia from baseline to 1-year FU. Short-term biomarker change denoted relative change (RC)

previous studies linking plasma NfL levels to cognitive progression [5–7, 24, 46]. The association between plasma NfL and reduced relative regional glucose uptake on [^{18}F]FDG-PET, is also consistent with previous studies [45, 47–49]. In contrast, we found no association between plasma NfL and MRI brain volume loss, which has been suggested previously in several studies [7, 6, 44, 49–51], and recently summarised in a comprehensive review on AD blood biomarkers and MRI measures in AD [25]. Many of these studies on NfL and MRI had longer follow up time which may explain the discrepancies [7, 44, 49, 50]. Our finding of a relationship between short-term NfL changes and glucose metabolism, but not with MRI atrophy, may reflect more subtle underlying

neuropathological changes, that are not yet associated with structural changes detectable by MRI.

In contrast, we found that short-term BD-tau changes were associated with MRI atrophy, including cortical and total grey matter volume, consistent with previous findings [11]. This implies that plasma BD-tau could be sensitive to widespread MRI-detected atrophy and may be more robust in reflecting structural brain changes than NfL.

P-tau217 is suggested as a core blood biomarker for detection of amyloid pathology [52, 53] and has been proposed to also hold prognostic abilities across the AD continuum [54]. In this study we did not find any association with clinical progression, in contrast to previous studies reporting that baseline plasma p-tau217 could

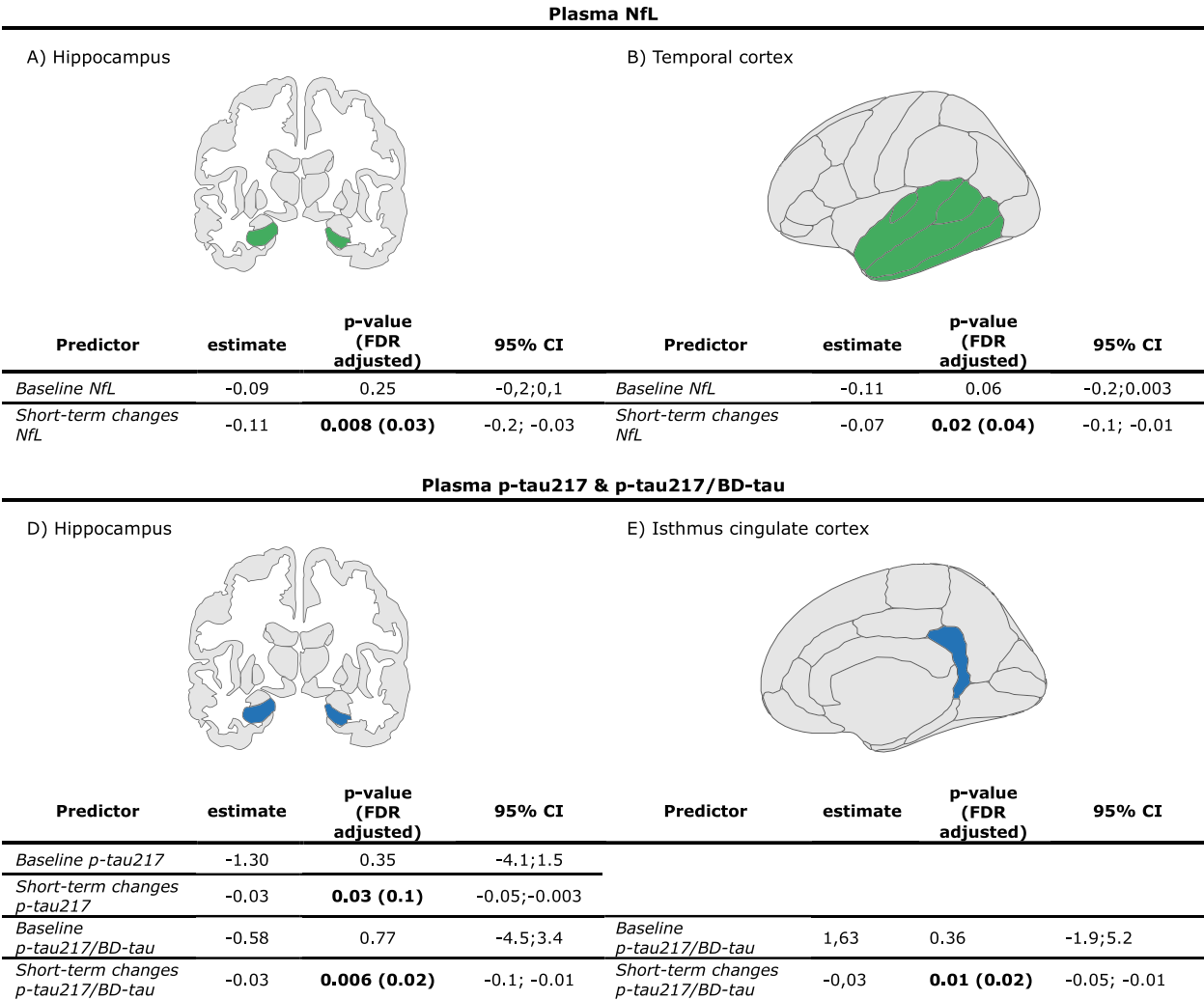


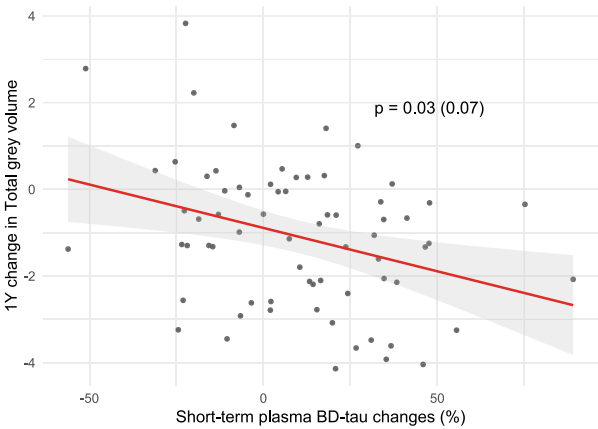
Fig. 3 Plasma biomarkers and their association with annual [¹⁸F]FDG-PET SUVR changes. Linear regression models, *p*-value adjusted for age, sex and creatinine. The NfL models contain an interaction term. Plasma short-term changes in p-tau217 and the p-tau217/BD-tau ratio were adjusted for baseline values. *P*-values in () are after multiple testing correction

predict cognitive decline in cognitively unimpaired older individuals [12, 55]. This supports plasma p-tau217 as a valuable marker for predicting conversion from cognitively unimpaired to MCI or dementia, while suggesting that p-tau217 may provide different information within the clinical AD continuum. We also considered if the p-tau217 to BD-tau ratio, could be a more sensitive prognostic marker as the ratio may be less influenced by peripheral confounders [31] and lead to less intra-individual variability [32]. Though we found a negative association with one-year CDR-SB, the estimate was considered of limited clinical importance. We did find an association with relative brain glucose metabolism across several AD specific regions (hippocampus and isthmus cingulate cortex). Thus, we cannot exclude that the ratio may offer better insight to the underlying neuropathological changes by reducing the effect of peripheral confounders.

Lastly, we found no association between plasma GFAP and either clinical or imaging progression. Plasma GFAP has been suggested as a marker that could predict clinical AD incidence several years prior to diagnosis [56] but may also entail considerable concerns regarding its discrepancy with CSF GFAP and expression in other non-brain tissue complicating its disease specificity as a brain biomarker [57].

In general, we observed that most short-term changes were within the expected range defined by the marker's RCV, suggesting that these fluctuations may be largely attributed to intra-individual and assay variability rather than true pathological change. While Simoa is highly sensitive, alternative platforms such as the Fujirebio Lumipulse platform or the NUCleic acid-Linked Immuno-Sorbent Assay developed by Alamar Biosciences could offer improved precision, which is important for detecting subtle short-term pathological changes. Furthermore,

Short-term BD-tau & Cortex volume changes



Short-term BD-tau & Total grey matter volume changes

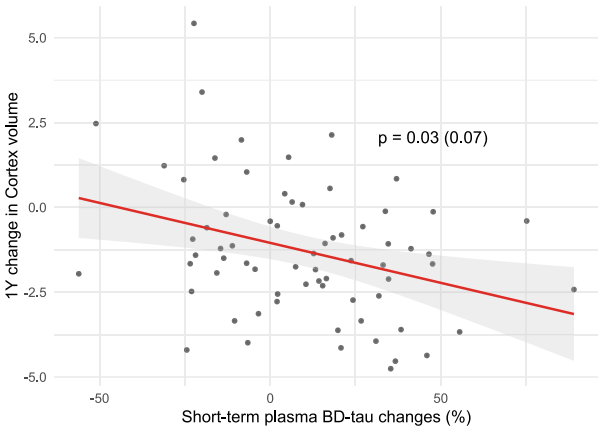
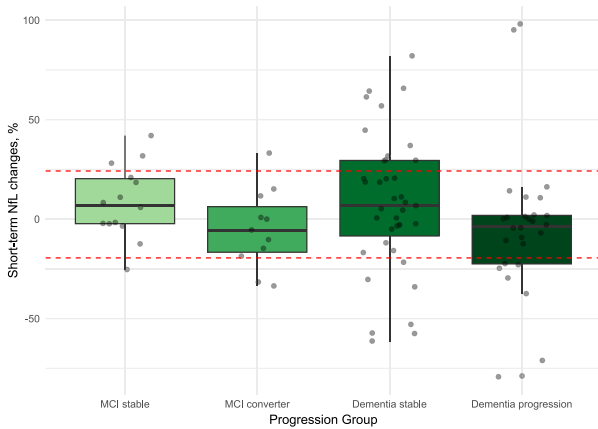
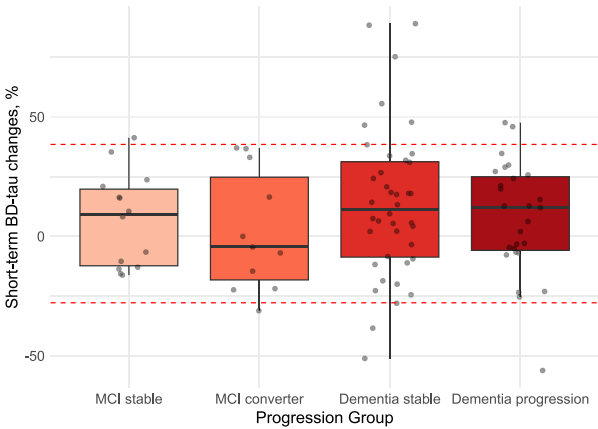


Fig. 4 Plasma biomarkers and annual MRI changes. Plots of linear regression models of the relationship between short-term BD-tau changes and MRI changes, plots not adjusted for age, sex, and creatinine. *P*-values are adjusted for age, sex and creatinine. *P*-values in () are after multiple testing correction. MRI brain scans were performed on a 1.5-3T scan

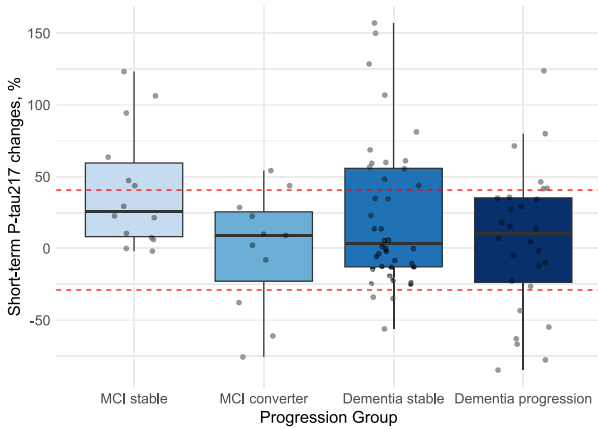
B1) NfL short-term changes w. reference change values, %



B2) BD-tau short-term changes w. reference change values, %



B3) p-tau217 short-term changes w. reference change values, %



B4) GFAP short-term changes w. reference change values, %

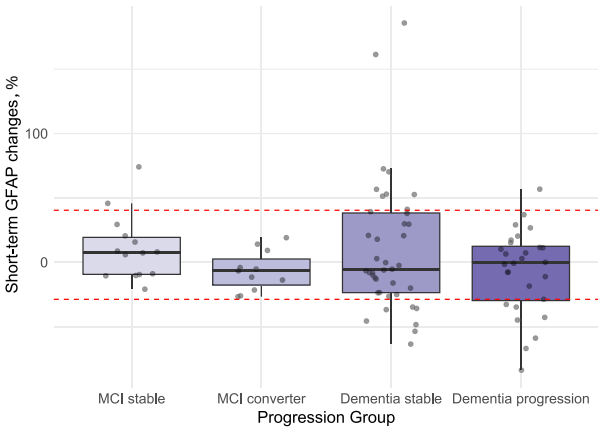


Fig. 5 Short-term biomarkers changes across disease severity. Short-term biomarker changes across 1-year disease severity progression. The red dotted lines represent the markers corresponding reference change values (RCV), %

we found that short-term changes in biomarker levels were negatively associated with baseline, likely reflecting the statistical phenomenon of regression toward the mean. Thus, we had to adjust for baseline levels to ensure unbiased models. Therefore, we suggest that for clinical interpretation of biomarker changes, one should be careful not to oversimplify biomarker trajectories without considering their interaction with baseline status. However, we acknowledge that the relationship between short-term biomarker changes and imaging findings is exploratory and that these results should be interpreted with the caveat of the above-mentioned. Future work on biomarker changes should continue to consider statistical controls for this phenomenon and, where possible, take RCVs into account. We suggest that future studies should investigate short-term biomarker changes in diverse cohorts as indicators of disease susceptibility and risk of progression, while concurrently monitoring external factors such as infection or inflammation (e.g., C-reactive protein, leukocytes, neutrophils), physical activity, and fasting status, which could further improve the interpretation of these biomarkers' variabilities.

This study has some limitations. The modest sample size and dropouts after one-year follow-up may compromise statistical power and generalisability. The study was not designed to account for pre-analytical factors such as fasting status that may influence plasma biomarker levels [28]. Further, in this study we did not include markers of common AD co-pathology, such as vascular changes or alpha-synuclein, that may contribute to clinical symptoms and neurodegeneration, potentially masking associations between AD specific biomarkers and disease progression [58]. The restriction of imaging analyses to a predefined set of regions warrants some limitations. While our selection was based on methodological considerations, including the risk of type I error, other regions may also be highly informative in early disease stages. Future studies with larger cohorts should extend the regional analyses to capture a more complete picture of disease progression. Finally, previous studies reporting associations between baseline plasma markers and MRI changes used follow-up periods of 2–4 years [7, 44], suggesting that longer follow-up may be needed to detect structural changes that biomarkers could potentially predict. Because some patients had their baseline scan performed on a 1.5T MRI scanner, we re-evaluated the MRI analysis after excluding these individuals. This exclusion, however, did not alter our results after correction for multiple testing, see Additional file 8, Fig. 1. Finally, clinically observed progression in AD is a complex interplay between neuropathological changes, comorbidities and psychosocial factors and remains a challenge to measure objectively. Although this study included well-validated objective measures of clinical progression, the authors

acknowledge that establishing a relationship between biological markers and clinical disease progression is a complex task. Future studies should further validate plasma biomarkers in larger and more diverse populations to support their clinical implementation as AD specific prognostic biomarkers.

We consider that our use of a prospective study design in a real-life memory clinic cohort of patients with early AD provides an important addition to studies investigating retrospective cohorts measuring disease progression from cognitively unimpaired to MCI or AD dementia [14, 19–23]. Despite our limited sample size, this cohort is a unique collection of well-characterised patients including information on several potential confounders and comorbidities. We found a significant effect of several confounding factors, including age, sex, and creatinine, suggesting that these may confound the relationship between biomarker levels and progression risk. Finally, the study design allowed novel investigations of short-term changes in AD plasma biomarkers.

Conclusion

In conclusion, our findings indicate that plasma NfL may serve as a predictor of disease progression in early stages of AD. Baseline plasma NfL was associated with subsequent clinical progression, while short-term changes in NfL were linked to changes in AD-specific regional brain hypometabolism. However, none of the other markers were associated with clinical progression. Although some of the biomarkers' short-term changes may reflect underlying disease processes, there are several caveats to this interpretation, such as intra-individual variability and comorbidities. Therefore, short-term biomarker changes should be carefully evaluated and always in the context of baseline values.

Abbreviations

AD	Alzheimer's disease
p-tau	Phosphorylated tau
NfL	Neurofilament light
GFAP	Glial fibrillary acidic protein
BD-tau	Brain-derived tau
RCV	Reference change value
EDTA	Ethylenediaminetetraacetic acid
CDR-SB	Clinical Dementia Rating Scale - Sum of Boxes
MMSE	Mini-mental state examination
ACE	Addenbrookes cognitive examination
HbA1c	Hemoglobin A1c
eGFR	Estimated glomerular filtration rate
RC	Relative change

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s13195-025-01892-7>.

Supplementary Material 1: Table 1. Progression in CDR-SB at 1- & 2- year FU.

Supplementary Material 2: Table 2. Baseline FU [18 F]FDG-PET.

Supplementary Material 3: Table 3. Plasma biomarkers and FU [18 F]FDG-PET changes.

Supplementary Material 4: Table 4. Short-term changes above or below the markers reference change value, %.

Supplementary Material 5: Table 5. MRI baseline.

Supplementary Material 6: Table 6. Plasma biomarkers & MR changes.

Supplementary Material 7: Table 7. Plasma biomarkers & MR – excluding patients with baseline 1.5T MRI.

Supplementary Material 8: Figure 1. MRI plots – excluding patients with baseline 1.5T MRI.

Supplementary Material 9: Figure 2. Plasma biomarkers baseline levels and short-term changes - Regression toward the mean.

Supplementary Material 10: Figure 3. Plasma markers and correlations with peripheral factors.

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Authors' contributions

Conceptualization: FKC, MHG, AHS, KSF, SGH Methodology: FKC, MHG, UL, OMH, IL, AHS, KSF, SGH Data Collection: FKC, MHG, UL, ALB, KT, WT Formal Analysis: FKC, AHS, SGH, KSF, UL, OMH, IL Writing—Original Draft: FKC Writing—Review & Editing: FKC, MHG, FG, ALB, KT, WT, UL, OMH, IL, AHS, KB, HZ, KSF, SGH Supervision: AHS, KSF, SGH Funding Acquisition: FKC, KSF, SGH Resources: KB, HZ, IL, OMH, AHS, KSF, SGH All authors read and approved the final manuscript.

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Data availability

The data analysed in this study is not publicly available due to the sensitivity of participant information and to protect privacy. Data may be made available from the corresponding author upon reasonable request and pending approval from the relevant ethics committee.

Declarations

Ethics approval and consent to participate

This study was approved by the Danish Research Ethics Committee (H-21040317) and followed the tenets of the 1975 Helsinki Declaration. The study was registered at clinicaltrials.gov (NCT05175664). Written informed consent was obtained from each participant prior to enrolment.

Consent for publication

Not applicable.

Competing interests

AHS has served at scientific advisory board for Eisai/BioArctic (remuneration paid to institution). KF serves or has served on a scientific advisory board and as scientific consultant for Novo Nordisk, Roche Diagnostics and Eisai, has given lectures in symposia for Novo Nordisk, Eli Lilly and Eisai/BioArctic, (remuneration paid to institution) and has served or serves as principal investigator in trials for Roche, Biogen, AbbVie, Novo Nordisk and Roche Diagnostics (remuneration paid to institution). KF also serves as Editor-in-Chief for Alzheimer's Research and Therapy (Springer) for which personal remuneration is paid. HZ has served at scientific advisory boards and/or as a consultant for Abbvie, Acumen, Alektor, Alzinova, ALZpath, Amylyx, Annexon, Apellis, Artery Therapeutics, AZTherapies, Cognito Therapeutics, CogRx, Denali, Eisai, Enigma, LabCorp, Merck Sharp & Dohme, Merry Life, Nervgen, Novo Nordisk, Optoceutics, Passage Bio, Pinteon Therapeutics, Prothena, Quanterix, Red Abbey Labs, reMYND, Roche, Samumed, ScandiBio Therapeutics AB, Siemens Healthineers, Triplet Therapeutics, and Wave, has given lectures sponsored by Alzecure, BioArctic, Biogen, Cellectricon, Fujirebio, LabCorp, Lilly, Novo Nordisk, Oy Medix Biochemica AB, Roche, and WebMD, is a co-founder of Brain Biomarker Solutions in Gothenburg AB (BBS), which is a part of the GU Ventures Incubator Program, and is a shareholder of MicThera (outside submitted work). KB has served as a consultant and at advisory boards for Abbvie, AC Immune, ALZPath, AriBio, Beckman-Coulter, BioArctic, Biogen, Eisai, Lilly, Moleac Pte. Ltd, Neurimmune, Novartis, Ono Pharma, Prothena, Quanterix, Roche Diagnostics, Sunbird Bio, Sanofi and Siemens Healthineers; has served at data monitoring committees for Julius Clinical and Novartis; has given lectures, produced educational materials and participated in educational programs for AC Immune, Biogen, Celdara Medical, Eisai and Roche Diagnostics; and is a co-founder of Brain Biomarker Solutions in Gothenburg AB (BBS), which is a part of the GU Ventures Incubator Program, outside the work presented in this paper. The other authors have no conflict of interest to report.

KF serves or has served on a scientific advisory board and as scientific consultant for Novo Nordisk, Roche Diagnostics and Eisai, has given lectures in symposia for Novo Nordisk, Eli Lilly and Eisai/BioArctic, (remuneration paid to institution) and has served or serves as principal investigator in trials for Roche, Biogen, AbbVie, Novo Nordisk and Roche Diagnostics (remuneration paid to institution). KF also serves as Editor-in-Chief for Alzheimer's Research and Therapy (Springer) for which personal remuneration is paid. HZ has served at scientific advisory boards and/or as a consultant for Abbvie, Acumen, Alektor, Alzinova, ALZpath, Amylyx, Annexon, Apellis, Artery Therapeutics, AZTherapies, Cognito Therapeutics, CogRx, Denali, Eisai, Enigma, LabCorp, Merck Sharp & Dohme, Merry Life, Nervgen, Novo Nordisk, Optoceutics, Passage Bio, Pinteon Therapeutics, Prothena, Quanterix, Red Abbey Labs, reMYND, Roche, Samumed, ScandiBio Therapeutics AB, Siemens Healthineers, Triplet Therapeutics, and Wave, has given lectures sponsored by Alzecure, BioArctic, Biogen, Cellectricon, Fujirebio, LabCorp, Lilly, Novo Nordisk, Oy Medix Biochemica AB, Roche, and WebMD, is a co-founder of Brain Biomarker Solutions in Gothenburg AB (BBS), which is a part of the

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Supplementary material

Additional file 1. Table 1. Progression in CDR-SB at 1- & 2- year FU

	Stable (N=54)	Progression (N=40)	p-value*
CDR-SB 1y change	0.76 (0.95)	2.82 (1.67)	< 0.001
CDR-SB 2y change	0.90 (0.93)	2.54 (1.52)	< 0.001
MMSE 1y change	-0.99 (2.75)	-3.42 (3.24)	< 0.001
MMSE 2y change	-1.13 (1.70)	-2.92 (2.39)	< 0.001

*age and sex adjusted p-values.

Additional file 2. Table 2. Baseline FU [18F]FDG-PET

<i>Mean (SD)</i>	Stable (n=42)	Progression (n=25)	p value*
Hippocampus bilat. SUVr	0.77 (0.07)	0.75 (0.06)	0.8
Isthmus cing. Cortex bilat. SUVr	1.08 (0.13)	1.02 (0.10)	0.3
Temporal cortex bilat. SUVr	0.9 (0.06)	0.9 (0.06)	0.3
Parietal cortex bilat SUVr	1.03 (0.09)	0.99 (0.07)	0.3

*age and sex adjusted p-values.

Additional file 3. Table 3. Plasma biomarkers and FU [18F]FDG-PET changes

<i>Change pr. year</i>	<i>Predictor</i>	<i>estimate</i>	<i>p-value (FDR adjusted p-value)</i>	<i>95% CI</i>	
<i>Hippocampal bilat. SUVr</i>	<i>Baseline NfL</i>	-0.09	0.25	-0.2;	0.1
	<i>Short-term changes NfL*</i>	-0.11	0.008 (0.03)	-0.2;	-0.03
	<i>Baseline BD-tau</i>	-2.87	0.13	-6.6;	0.8
	<i>Short-term changes BD-tau</i>	0.01	0.78	-0.03;	0.05
	<i>Baseline p-tau217</i>	-1.30	0.35	-4.1;	1.5
	<i>Short-term changes p-tau217</i>	-0.03	0.03 (0.1)	-0.05;	-0.003
	<i>Baseline p-tau217/BD-tau</i>	-0.58	0.77	-4.5;	3.4
	<i>Short-term changes p-tau217/BD-tau</i>	-0.03	0.006 (0.02)	-0.1;	-0.01
<i>Isthmus cingulate cortex bilat. SUVr</i>	<i>Baseline GFAP</i>	-0.01	0.28	-0.01;	0.004
	<i>Short-term changes GFAP</i>	-0.02	0.18	-0.04;	0.01
	<i>Baseline NfL</i>	-0.03	0.68	-0.2;	0.1
	<i>Short-term changes NfL*</i>	-0.05	0.17	-0.1;	0.02
	<i>Baseline BD-tau</i>	-0.51	0.76	-3.9;	2.9
	<i>Short-term changes BD-tau</i>	0.03	0.11	-0.01;	0.1
	<i>Baseline p-tau217</i>	0.62	0.62	-1.9;	3.1
	<i>Short-term changes p-tau217</i>	-0.02	0.11	-0.04;	0.004
<i>Temporal cortex SUVr</i>	<i>Baseline p-tau217/BD-tau</i>	1.63	0.36	-1.9;	5.2
	<i>Short-term changes p-tau217/BD-tau</i>	-0.03	0.01 (0.02)	-0.05;	-0.01
	<i>Baseline GFAP</i>	-0.002	0.62	-0.01;	0.01
	<i>Short-term changes GFAP</i>	0.004	0.75	-0.02;	0.03
	<i>Baseline NfL</i>	-0.11	0.06	-0.2;	0.003
	<i>Short-term changes NfL*</i>	-0.07	0.02 (0.04)	-0.1;	-0.01
	<i>Baseline BD-tau</i>	-1.45	0.28	-4.1;	1.2
	<i>Short-term changes BD-tau</i>	0.01	0.45	-0.02;	0.04
<i>Parietal cortex SUVr</i>	<i>Baseline p-tau217</i>	-1.1	0.27	-3.0;	0.85
	<i>Short-term changes p-tau217</i>	-0.01	0.33	-0.03;	0.01
	<i>Baseline p-tau217/BD-tau</i>	-1.01	0.47	-3.8;	1.8
	<i>Short-term changes p-tau217/BD-tau</i>	-0.02	0.05	-0.03;	0.0002
	<i>Baseline GFAP</i>	-0.0004	0.90	-0.01;	0.01
	<i>Short-term changes GFAP</i>	-0.003	0.75	-0.02;	0.01
	<i>Baseline NfL</i>	-0.07	0.35	-0.2;	0.1
	<i>Short-term changes NfL</i>	-0.06	0.11	-0.1;	0.02
<i>Parietal cortex SUVr</i>	<i>Baseline BD-tau</i>	-0.75	0.66	-4.2;	2.7
	<i>Short-term changes BD-tau</i>	0.03	0.17	-0.01;	0.1
	<i>Baseline p-tau217</i>	0.65	0.61	-1.9;	3.2
	<i>Short-term changes p-tau217</i>	-0.01	0.35	-0.03;	0.01
	<i>Baseline p-tau217/BD-tau</i>	1.84	0.31	-1.7;	5.4
	<i>Short-term changes p-tau217/BD-tau</i>	-0.02	0.07	-0.04;	0.002
	<i>Baseline GFAP</i>	-0.002	0.65	-0.01;	0.01
	<i>Short-term changes GFAP</i>	-0.01	0.58	-0.03;	0.02

Linear regression models, all adjusted for age, sex and creatinine (no sign. association with the individual covariates).

*significant interaction term between baseline and short-term NfL changes.

Additional file 4. Table 4. Short-term changes above or below the markers reference change value, %

	Predictor	estimate	p-value (FDR adjusted p-value)	95% CI	
<i>Hippocampal bilat. SUVR</i>	Above NfL RCV%	-8.0	0.02 (0.04)	-14.8;	-1.3
	Below NfL RCV%	4.5	0.2	-3.2;	12.2
	Above BD-tau RCV%	0.4	0.7	-1.8;	2.7
	Below BD-tau RCV%	-2.5	0.1	-5.5;	0.5
	Above p-tau217 RCV%	-1.4	0.2	-3.6;	0.8
	Below p-tau217 RCV%	3.2	0.1	-0.4;	6.8
	Above GFAP RCV%	-2.7	0.1	-5.7;	0.3
	Below GFAP RCV%	-1.1	0.5	-4.1;	1.9
<i>Isthmus cingulate cortex bilat. SUVR</i>	Above NfL RCV%	-5.4	0.1	-11.7;	1.0
	Below NfL RCV%	-0.6	0.9	-7.8;	6.7
	Above BD-tau RCV%	1.6	0.1	-0.5;	3.7
	Below BD-tau RCV%	-1.1	0.4	-3.8;	1.7
	Above p-tau217 RCV%	-1.5	0.1	-3.6;	0.5
	Below p-tau217 RCV%	0.9	0.6	-2.4;	4.2
	Above GFAP RCV%	0.0	1.0	-2.7;	2.8
	Below GFAP RCV%	-2.1	0.1	-4.9;	0.6
<i>Temporal cortex SUVR</i>	Above NfL RCV%	-5.7	0.02 (0.04)	-10.4;	-1.0
	Below NfL RCV%	3.7	0.2	-1.7;	9.0
	Above BD-tau RCV%	0.5	0.6	-1.1;	2.1
	Below BD-tau RCV%	-1.6	0.1	-3.8;	0.5
	Above p-tau217 RCV%	-1.1	0.2	-2.7;	0.5
	Below p-tau217 RCV%	0.0	1.0	-2.7;	2.6
	Above GFAP RCV%	-0.8	0.5	-3.0;	1.4
	Below GFAP RCV%	-0.8	0.5	-3.0;	1.5
<i>Parietal cortex SUVR</i>	Above NfL RCV%	-4.6	0.2	-10.9;	1.7
	Below NfL RCV%	4.9	0.2	-2.3;	12.1
	Above BD-tau RCV%	2.0	0.1	-0.1;	4.1
	Below BD-tau RCV%	-0.7	0.6	-3.4;	2.1
	Above p-tau217 RCV%	-0.8	0.4	-2.9;	1.3
	Below p-tau217 RCV%	0.7	0.7	-2.7;	4.1
	Above GFAP RCV%	-0.6	0.7	-3.4;	2.2
	Below GFAP RCV%	-1.1	0.4	-4.0;	1.7

Linear regression models evaluating the relationship between short-term changes in the context of reference change values (RCV) with [¹⁸F]FDG-PET changes. Reference levels were participant with short-term changes *within* RCV. All models were adjusted for baseline biomarker, age, sex and creatinine.

Additional file 5. Table 5. MRI baseline

	Mean (SD)	Stable (N=44)	Decline (N=26)	p-value*
Total grey matter volume		0.38 (0.02)	0.37 (0.02)	0.3
Cortex volume		0.28 (0.02)	0.27 (0.02)	0.3
Hippocampus bilat.		0.004 (0.001)	0.004 (0.001)	0.4
Lateral ventricle volume		0.03 (0.01)	0.04 (0.01)	0.2

Baseline volume measures. *Linear regression models adjusted for age and sex. Based on MRI volumes, normalised to the intra-cranial volume. MRI baseline data was not available for analyses for n=4 in stable and n=7 in progression group.

Additional file 6. Table 6. Plasma biomarkers & MR changes

Change pr. year	Predictor	estimate	p-value (FDR adjusted p-value)	95% CI
Total Grey Volume	Baseline NfL	0.005	0.9	-0.05; 0.06
	Short-term changes NfL	-0.003	0.6	-0.02; 0.01
	Baseline BD-tau	-0.09	0.9	-1.48; 1.30
	Short-term changes BD-tau	-0.02	0.03 (0.07)	-0.03; -0.001
	Baseline p-tau217	0.16	0.8	-0.87; 1.19
	Short-term changes p-tau217	0.003	0.5	-0.01; 0.01
	Baseline p-tau217/BD-tau	-0.11	0.9	-1.58; 1.36
	Short-term changes p-tau217/BD-tau	0.01	0.05	0.0002; 0.02
Cortex volume	Baseline GFAP	0.0004	0.8	-0.003; 0.004
	Short-term changes GFAP	-0.001	0.9	-0.01; 0.01
	Baseline NfL	-0.02	0.7	-0.08; 0.05
	Short-term changes NfL	-0.01	0.5	-0.02; 0.01
	Baseline BD-tau	-0.56	0.5	-2.17; 1.06
	Short-term changes BD-tau	-0.02	0.03 (0.07)	-0.03; -0.002
	Baseline p-tau217	-0.01	1.0	-1.22; 1.20
	Short-term changes p-tau217	0.0003	1.0	-0.01; 0.01
Hippocampus bilat. volume	Baseline p-tau217/BD-tau	-0.35	0.7	-2.06; 1.37
	Short-term changes p-tau217/BD-tau	0.01	0.2	-0.004; 0.02
	Baseline GFAP	0.0002	0.9	-0.004; 0.004
	Short-term changes GFAP	-0.002	0.7	-0.01; 0.01
	Baseline NfL	0.05	0.5	-0.09; 0.20
	Short-term changes NfL	0.01	0.6	-0.02; 0.04
	Baseline BD-tau	-2.99	0.1	-6.42; 0.43
	Short-term changes BD-tau	-0.02	0.2	-0.06; 0.01
Lateral ventricle bilat. volume	Baseline p-tau217	-1.08	0.4	-3.67; 1.52
	Short-term changes p-tau217	-0.001	0.9	-0.02; 0.02
	Baseline p-tau217/BD-tau	0.98	0.6	-2.71; 4.67
	Short-term changes p-tau217/BD-tau	0.01	0.4	-0.01; 0.03
	Baseline GFAP	-0.003	0.4	-0.01; 0.005
	Short-term changes GFAP	0.01	0.2	-0.01; 0.04
	Baseline NfL	0.05	0.5	-0.10; 0.19
	Short-term changes NfL	-0.003	0.8	-0.04; 0.03
Lateral ventricle bilat. volume	Baseline BD-tau	1.75	0.3	-1.59; 5.10
	Short-term changes BD-tau	0.01	0.7	-0.03; 0.04
	Baseline p-tau217	-0.47	0.7	-2.98; 2.04
	Short-term changes p-tau217	0.004	0.7	-0.02; 0.03
	Baseline p-tau217/BD-tau	-1.95	0.3	-5.48; 1.58
	Short-term changes p-tau217/BD-tau	-0.001	1.0	-0.02; 0.02
	Baseline GFAP	0.005	0.2	-0.003; 0.01
	Short-term changes GFAP	-0.01	0.5	-0.03; 0.01

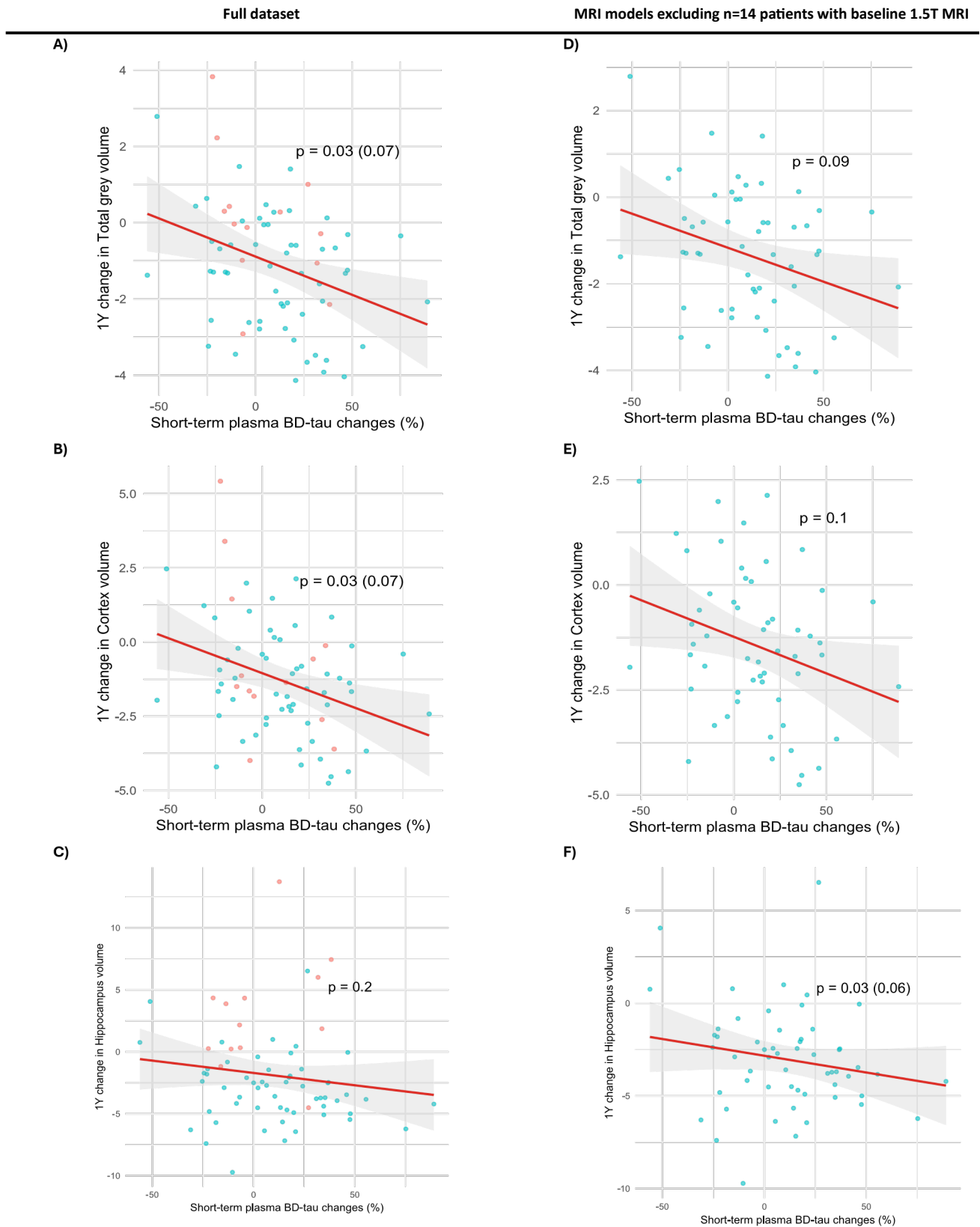
Linear regression models, adjusted for age, sex and creatinine. Models with short-term changes were additionally adjusted for baseline biomarker level. Age was significant associated with bilat. Hippocampus volume. Creatinine and sex were significant associated with cortex & grey matter volume.

Additional file 7. Table 7. Plasma biomarkers & MR – excluding patients with baseline 1.5T MRI

<i>Change pr. year</i>	<i>Predictor</i>	<i>estimate</i>	<i>p-value (FDR adjusted p- value)</i>	<i>95% CI</i>	
<i>Total Grey Volume</i>	<i>Baseline NfL</i>	-0.01	0.7	-0.08	0.05
	<i>Short-term changes NfL</i>	0.0002	1.0	-0.01	0.02
	<i>Baseline BD-tau</i>	0.4	0.6	-1.2	2.0
	<i>Short-term changes BD-tau</i>	-0.01	0.1	-0.03	0.002
	<i>Baseline p-tau217</i>	0.31	0.6	-0.8	1.4
	<i>Short-term changes p-tau217</i>	0.003	0.5	-0.01	0.01
	<i>Baseline p-tau217/BD-tau</i>	-0.18	0.8	-1.8	1.4
	<i>Short-term changes p-tau217/BD-tau</i>	0.01	0.1	-0.001	0.02
	<i>Baseline GFAP</i>	-0.001	0.4	-0.01	0.002
	<i>Short-term changes GFAP</i>	0.001	0.8	-0.01	0.01
<i>Cortex volume</i>	<i>Baseline NfL</i>	-0.03	0.5	-0.1	0.05
	<i>Short-term changes NfL</i>	-0.01	0.5	-0.02	0.01
	<i>Baseline BD-tau</i>	-0.02	1.0	-1.9	1.8
	<i>Short-term changes BD-tau</i>	-0.01	0.1	-0.03	0.003
	<i>Baseline p-tau217</i>	0.13	0.8	-1.1	1.4
	<i>Short-term changes p-tau217</i>	0.003	0.6	-0.01	0.01
	<i>Baseline p-tau217/BD-tau</i>	-0.4	0.6	-2.24	1.4
	<i>Short-term changes p-tau217/BD-tau</i>	0.01	0.1	-0.003	0.02
	<i>Baseline GFAP</i>	-0.002	0.4	-0.01	0.003
	<i>Short-term changes GFAP</i>	-0.002	0.8	-0.02	0.01
<i>Hippocampus bilat. volume</i>	<i>Baseline NfL</i>	0.005	0.9	-0.1	0.1
	<i>Short-term changes NfL</i>	0.002	0.9	-0.03	0.03
	<i>Baseline BD-tau</i>	-2.7	0.06	-5.6	0.1
	<i>Short-term changes BD-tau</i>	-0.03	0.03 (0.1)	-0.05	-0.002
	<i>Baseline p-tau217</i>	-0.6	0.6	-2.6	1.5
	<i>Short-term changes p-tau217</i>	-0.01	0.3	-0.03	0.01
	<i>Baseline p-tau217/BD-tau</i>	1.05	0.5	-1.9	4.0
	<i>Short-term changes p-tau217/BD-tau</i>	0.004	0.7	-0.01	0.02
	<i>Baseline GFAP</i>	-0.01	0.02 (0.06)	-0.01	-0.002
	<i>Short-term changes GFAP</i>	0.01	0.4	-0.01	0.03
<i>Lateral ventricle bilat. volume</i>	<i>Baseline NfL</i>	0.04	0.6	-0.1	0.2
	<i>Short-term changes NfL</i>	-0.01	0.4	-0.04	0.02
	<i>Baseline BD-tau</i>	-0.46	0.8	-4.0	3.1
	<i>Short-term changes BD-tau</i>	0.003	0.8	-0.03	0.04
	<i>Baseline p-tau217</i>	-1.5	0.2	-3.9	0.9
	<i>Short-term changes p-tau217</i>	0.01	0.2	-0.01	0.03
	<i>Baseline p-tau217/BD-tau</i>	-1.9	0.3	-5.4	1.6
	<i>Short-term changes p-tau217/BD-tau</i>	0.01	0.5	-0.01	0.03
	<i>Baseline GFAP</i>	0.01	0.2	-0.002	0.01
	<i>Short-term changes GFAP</i>	-0.004	0.8	-0.03	0.03

Linear regression models, adjusted for age, sex and creatinine. Models with short-term changes were additionally adjusted for baseline biomarker level.

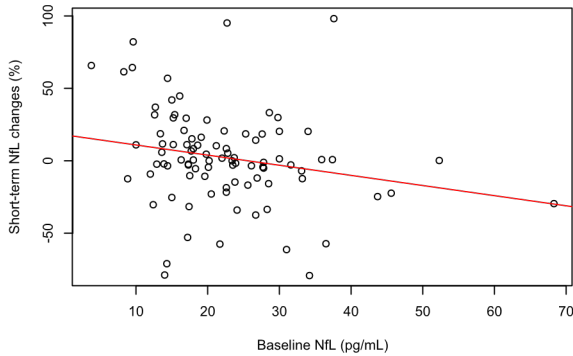
Additional file 8: Figure 1. MRI plots – excluding patients with baseline 1.5T MRI



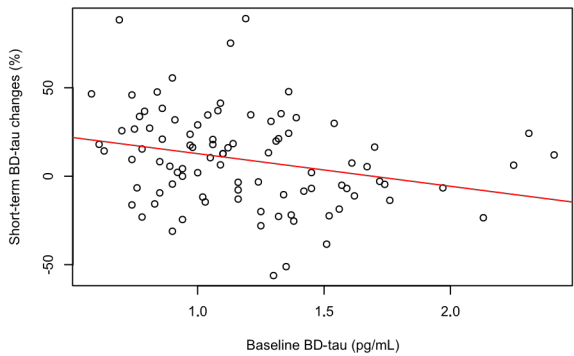
A, B, C: plots including all observations, color-coded by scanner **blue** = same scanner, **red** = different baseline scanner. D, E, F: plots with 14 patients are excluded. In A & B the “original” association becomes non-significant on D & E, but the trends remain clearly visible. C, the “original” non-significant association between BD-tau changes and hippocampal volume, which in F) becomes significant after excluding the 14 patients (12 of whom show an increase in volume). After multiple testing correction, none of the associations remain significant.

Additional file 9. Figure 2. Plasma biomarkers baseline levels and short-term changes - Regression toward the mean

A) NfL



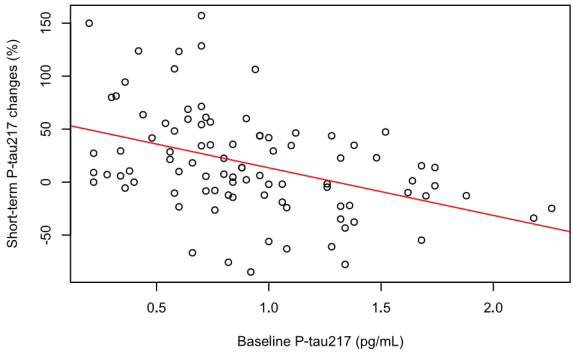
B) BD-tau



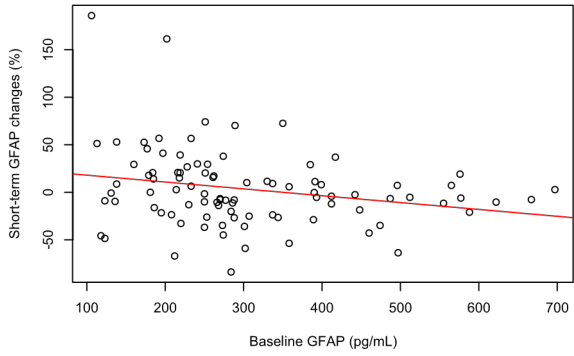
p=0.01

p=0.01

C) p-tau217



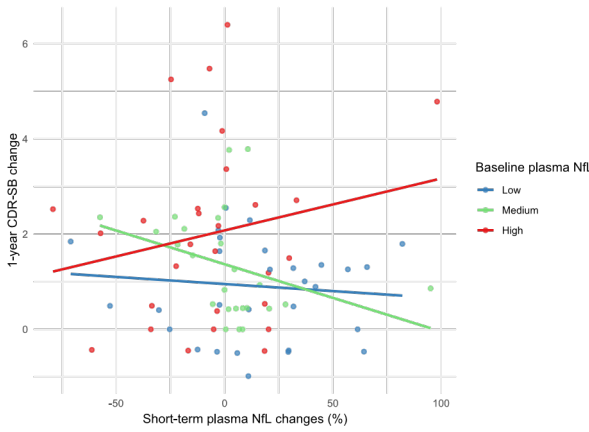
D) GFAP



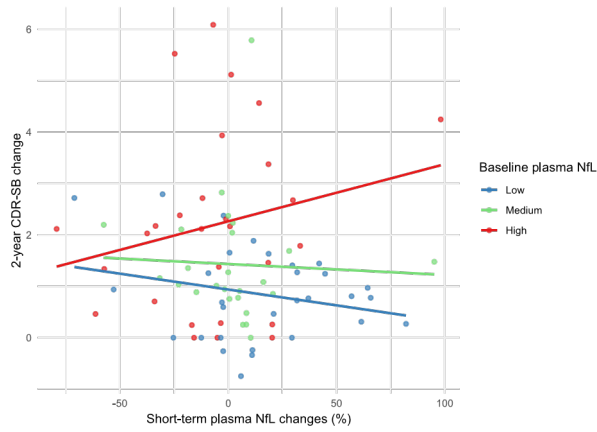
p=6.17e-06

p=0.02

E) 1y CDR-SB changes & Short-term NfL changes dependent on baseline NfL

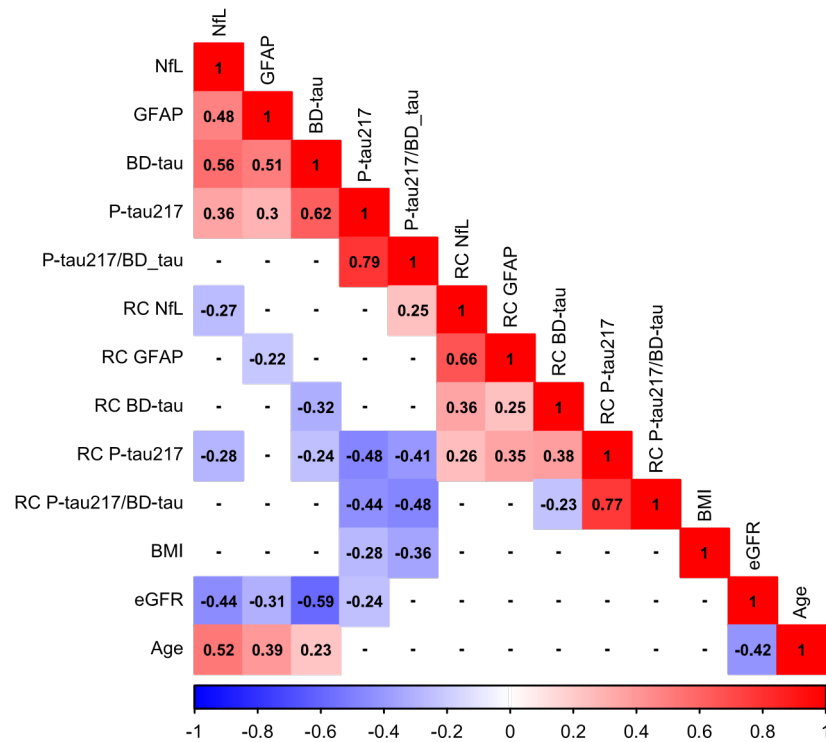


D) 2y CDR-SB changes & Short-term NfL changes dependent on baseline NfL



A-D) plots of linear regression models plotting the associations between baseline and short-term biomarker changes. E-F) shows how the interaction between changes in CDR-SB and short-term NfL levels is dependent on the baseline level.

Additional file 10: Figure 3. Plasma markers and correlations with peripheral factors



To identify covariates to include in the linear regression models, correlations between biomarkers and age, eGFR, BMI were assessed by Spearman's rank correlations coefficient. Baseline NfL, p-tau217, and GFAP was significantly negatively associated with baseline eGFR. Baseline NfL, BD-tau, and GFAP was significantly positively associated with age. Further baseline p-tau217 was significantly negatively associated with BMI. We found that using the p-tau217/BDtau ratio there was no correlation with eGFR. We found a negative significant correlation between all biomarkers baseline level and corresponding short-term changes.

8.4 Study IV

Tracking clinical and neuroimaging progression of Alzheimer's disease by longitudinal trajectories of plasma biomarkers

Frederikke Kragh Clemmensen, Mathias Holsey Gramkow, Andrea Lessa Benedet, Fernando Gonzalez-Ortiz, Kubra Tan, Wiebke Traichel, Ulrich Lindberg, Otto Mølby Henriksen, Henrik Zetterberg, Kaj Blennow, Ian Law, Anja Hviid Simonsen, Kristian Steen Frederiksen & Steen Gregers Hasselbalch.

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Title: Tracking clinical and neuroimaging progression of Alzheimer's disease by longitudinal trajectories of plasma biomarkers

Frederikke Kragh Clemmensen¹, Mathias Holsey Gramkow¹, Andrea L. Benedet², Fernando Gonzalez-Ortiz², Kubra Tan², Wiebke Traichel², Ulrich Lindberg³, Otto Mølby Henriksen³, Henrik Zetterberg^{2,4,5,6,7,8}, Kaj Blennow^{2,4}, Ian Law^{3,9}, Anja Hviid Simonsen¹, Kristian Steen Frederiksen^{1,9}, Steen Gregers Hasselbalch^{1,9}.

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Running title

Plasma biomarkers tracking progression of Alzheimer's disease.

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Abstract

Introduction: Blood biomarkers reflecting clinical and neuroimaging progression are essential to monitor Alzheimer's disease (AD).

Methods: In a two-year prospective study of 94 early AD patients, four consecutive blood samples were collected and analysed for p-tau217, NfL, BD-tau and GFAP. Linear mixed models were used to compare plasma biomarker trajectories between clinically stable and progressors, and to assess correlations with individual changes in CDR-SB, magnetic resonance imaging volumes, and [^{18}F]-fluorodeoxyglucose positron emission tomography glucose metabolism.

Results: Plasma p-tau217 and the p-tau217/BD-tau ratio showed steeper trajectories in progressors. No plasma biomarkers correlated with individual clinical or neuroimaging changes, except for the p-tau217/BD-tau ratio, which weakly correlated with CDR-SB changes. Most plasma biomarker changes from baseline to follow-up remained within reference change values.

Discussion: Plasma p-tau217 increases more rapidly in clinical progressors, reflecting tau-related neurodegeneration underlying cognitive decline. The absence of a clear relationship with individual clinical measures suggests biomarker variability may mask disease-specific changes.

Highlights

- Plasma p-tau217 showed significantly steeper increase over two years in clinically AD progressors compared with stable AD patients.
- No differences between stable and progressors were observed in plasma NfL, BD-tau or GFAP trajectories.
- Neither plasma p-tau217, NfL, BD-tau, nor GFAP correlated with individual changes in CDR-SB or changes in MRI regional volumes or [^{18}F]FDG-PET regional metabolism.
- Most changes in plasma biomarker levels from baseline to follow-up remained within their reference change values.

Research in context

Systematic review: We conducted a PubMed literature review on plasma biomarkers for monitoring Alzheimer's disease (AD) progression and found few longitudinal studies with repeated blood sampling, most of which focused on patients with preclinical AD. Thus, longitudinal studies with repeated blood sampling in clinically manifest AD are essential to determine whether plasma biomarkers can reliably monitor disease progression.

Interpretation: Our findings show that plasma p-tau217 exhibits a steeper longitudinal trajectory in clinically progressors versus stable within the clinically AD continuum. Neither plasma p-tau217, NfL, BD-tau, nor GFAP correlated with individual changes in CDR-SB, MMSE or changes in MRI regional volumes or [¹⁸F]FDG-PET regional metabolism. As this is in contrast with previous studies on preclinical AD, it may suggest that plasma p-tau217 entail different information within the clinical AD continuum. Plasma biomarker changes from baseline to follow-up were mostly within their specific reference change values, suggesting that only few changes may reflect actual disease-related changes.

Future directions: Future studies should explore plasma biomarker trajectories within the clinically AD continuum compared with trajectories in preclinical cohorts and focus on how biomarker variability may mask disease-specific changes.

Background

As disease-modifying treatments for Alzheimer's disease (AD) develop, there is a pressing need for tools that monitor and track disease progression, as this may help to better evaluate treatment response. Here, specifically blood biomarkers have shown not only diagnostic ¹ but also prognostic ^{2,3} potential. However, whether AD blood biomarkers can effectively monitor and track the natural disease course, remains to be determined. This is important considering blood biomarkers as surrogate endpoints in clinical trials, as their alignment with disease progression should reflect clinical status.

Currently, blood biomarkers included in the revised criteria include biomarkers for detection of various aspects of AD pathology ⁴. Plasma phosphorylated tau at threonine acid 217 (p-tau217) has shown promise in detection of early AD-related changes and in predicting disease progression ^{1,2,5-8}. Elevated plasma levels of

neurofilament light (NfL), a marker of neurodegeneration, and glial fibrillary acidic protein (GFAP), a marker of astrocytic reactivity, can be detected up to a decade before diagnosis ⁹. Further baseline plasma NfL has been linked to progression of cognitive decline ^{10–14}. Plasma brain-derived tau (BD-tau) has been associated with neurodegeneration in AD ¹⁵. These prognostic studies are based on baseline measures of plasma markers predicting future cognitive decline ^{2,6,8,11}. However few studies have evaluated the longitudinal trajectories of AD blood biomarkers and have primarily focused on cognitively unimpaired individuals at risk of developing dementia ^{16–18}. We are aware of one study that has investigated patients after the onset of amyloid PET positivity, focusing on sex differences in biomarker trajectories ¹⁹. Thus, there is a lack of studies evaluating the trajectories of AD blood biomarkers during the natural disease course in the clinically relevant population, i.e., memory clinic patients with a clinical diagnosis of mild cognitive impairment (MCI) or dementia due to AD.

Furthermore, AD blood biomarkers have already been used as surrogate endpoints for target engagement in anti-amyloid clinical trials ^{20–22}. However, clinical trial cohorts often differ from memory clinic populations, with, e.g., comorbidities and concurrent co-pathologies, so that findings from clinical trials cannot easily be translated to real-world memory clinic patients. Finally, research has previously shown considerable within-subject variability in AD plasma biomarker levels ^{23–25}. Within-subject variability reflects the extent to which an individual exhibit changes in biomarker levels due to age, physiology, genetics, comorbidities, etc., i.e., changes which may not be related to specific disease pathology. Previous studies have suggested that using the plasma p-tau217 to non-phosphorylated tau217 ratio can reduce variability in plasma p-tau217 levels caused by kidney dysfunction ²⁶ and that the p-tau217 to BD-tau ratio can reduce within-and between subject variability in plasma p-tau217 levels²⁵. High within-subject variability may mask true disease progression or treatment effects, posing a significant challenge when assessing biomarkers as surrogate endpoints in clinical trials. Together, this knowledge gap calls for research that simultaneously measures biological markers and clinical measures over time to clarify their relationship and validate biomarkers as indicators of disease status or treatment response.

In this longitudinal study, we aimed to investigate the disease tracking properties of AD plasma biomarkers in memory clinic patients with MCI or mild-moderate dementia due to AD. We aimed to examine whether biomarker trajectories of plasma p-tau217, NfL, BD-tau, GFAP, and the p-tau217/BD-tau ratio differed between clinical progressors and stable individuals and if individual plasma biomarker trajectories correlated with concurrent changes in cognitive tests and neuroimaging measures. Finally, we aimed to explore the longitudinal biomarker variability in context with previously established reference changes values for each specific biomarker.

Methods

Study design

Prospective longitudinal cohort study.

Participants

This study was carried out at the Memory clinic at Rigshospitalet, Copenhagen. We included patients with MCI or mild to moderate dementia due to AD. Inclusion was carried out from January 2022 to June 2023. Participants were eligible for inclusion if they met the following criteria: 1) a diagnosis of MCI or mild to moderate dementia due to AD; 2) MMSE >19; 3) an available diagnostic brain [¹⁸F]FDG-PET/magnetic resonance scan (MRI) or [¹⁸F]FDG-PET /computed tomography (CT) scan to be used as baseline scan; 4) and the availability of a caregiver informant. Exclusion criteria were substance abuse, major neurological or psychiatric disorders, or participation in intervention trials.

The diagnosis of MCI or dementia due to AD^{27,28} was given at a multidisciplinary consensus conference in the memory clinic, following a comprehensive assessment consisting of: cognitive evaluation using the Mini-Mental State Examination (MMSE)²⁹ and Addenbrooke's Cognitive Examination (ACE)³⁰, medical history and physical examination, a brain [¹⁸F]FDG-PET scan combined with structural neuroimaging, either MRI or CT routine blood tests, and additional examinations, such as cerebrospinal fluid examination, neuropsychological evaluation, or amyloid PET when clinically indicated. The standard diagnostic evaluation at the memory clinic Rigshospitalet is described in detail elsewhere³¹.

Patients were included at the time of their diagnosis (baseline) and followed for up to two years, with scheduled visits at 3-5 months, 12 months (1Y), and 18-24 (2Y) after baseline. All visits were carried out by a study investigator. Follow-up ended in November 2024. A detailed description of the study design has been previously published ³².

The study was approved by the Regional Scientific Ethics Committee (H-21040317) and was registered at clinicaltrials.gov (NCT05175664). The study followed the tenets of the 1975 and 2024 Declaration of Helsinki.

Baseline data:

We collected baseline data from the patients' medical record including age, sex, weight, height, body mass index (BMI), years of education, medication status and comorbidities, cognitive tests results of MMSE, ACE, and the Clinical Dementia Rating (CDR), neuroimaging data, and biochemical data (glycated haemoglobin A1c (HbA1c), creatinine and estimated glomerular filtration rate (eGFR)). eGFR values greater than 90 mL/min/1.73m² were calculated using the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation. If available, we included baseline data on AD cerebrospinal fluid (CSF) biomarkers including amyloid beta 1-42, phosphorylated tau 181 and total tau. The Innotech enzyme-linked immunosorbent assays (ELISA) (Fujirebio, Ghent) (n=8) were used on CSF collected before June 30th 2022, and the Elecsys sandwich electrochemiluminescence-immunoassay (Roche, Cobas 8000) was used on CSF collected after July 1st, 2022 ^{33,34}.

Longitudinal study visits:

Cognitive assessments included an MMSE (all visits), and the Clinical Dementia Rating (CDR) (carried out at 1Y and 2Y). The CDR is a semi-structured interview, rating six cognitive and functional domains, yielding a Sum-of-Boxes (SB) score from 0 to 18, where higher scores indicate greater impairment ³⁵. All study visits included a routine medical assessment with blood pressure, weight, and medication review, including adjustment of antidementia drugs, when clinically indicated. Based on all available clinical information, including CDR-SB, disease stage (MCI or mild, moderate, or severe dementia) was determined at each study visit in accordance with NIA-AA ²⁸ and the WHO ICD-10 classification ³⁶. Participants were classified as

either *stable* or *progressors* based on clinical change from baseline to the final follow-up. *Stable* was defined as no change in disease severity, while *progressors* was defined as conversion from MCI to dementia or advancement to a more severe dementia stage.

Baseline and 1 year FU [¹⁸F]FDG-PET and MRI scans were performed at the Department of Nuclear Medicine, Rigshospitalet according to guidelines³⁷. All scans were performed on a hybrid PET/3T MRI scanner (Siemens mMR, Siemens Healthineers, Erlangen, Germany), except for 14 baseline scans performed on a PET scanner and 1.5T MRI scanner, separately. Patients had approx. 200MBq [¹⁸F]FDG administered and after 40 minutes the MRI and PET images were conducted simultaneously, including 3D-T1-weighted (T1), T2-weighted (T2), T2 fluid attenuated inversion recovery (FLAIR), and T2*-weighted imaging. For the FDG-PET analysis, regional standardized uptake value ratios (SUVr) were derived by normalizing PET uptake in regions of interest to cerebellar uptake. We used the volumetric longitudinal pipeline and PETSurfer (FreeSurfer, ver. 7.4.1) to process T1-weighted MRI and PET data³⁸. Guided by previous studies, the analysis focused on predefined MRI regions including total grey matter, cortex, hippocampus, and lateral ventricles^{39,40} (all normalized to intracranial volume (ICV)) and predefined SUVr measures from the hippocampus, isthmus cingulate, temporal, and parietal cortices⁴¹.

Plasma analysis:

At each study visit, blood was collected in ethylenediaminetetraacetic acid (EDTA)–plasma tubes and within 30 to 120 minutes after collection, centrifuged at 2,000 g, 4°C for 10 minutes, then distributed in 250µl aliquots and stored at –80°C.

Plasma biomarker quantification was performed at the Department of Psychiatry and Neurochemistry, Sahlgrenska Academy, University of Gothenburg, using ultra-sensitive Single Molecule Array (Simoa) immunoassays on the Quanterix HD-X Analyzer [30]. GFAP and NfL were measured with the Neurology 2-Plex B assay, p-tau217 with the ALZpath pTau217 Plus assay, and BD-tau with a University of Gothenburg in-house assay⁴². All analyses were conducted in a single experimental round with one batch of reagents, following manufacturer instructions. Analysis was carried out in one round of experiments, with samples from each participant placed next to each other on the same plate. Within and between analytical variability

were assessed by three internal quality control samples at the start and end of each run, with two quality control levels run in duplicate at both the beginning and end of each run. Concentrations below the quantifiable range on the calibration curve were labelled as < LOQ).

Statistical analysis:

Baseline differences of continuous variables between *stable* individuals and *progressors* were assessed by two-sample t-test or linear regression (when adjusting for age, sex, and creatinine), and for categorical variables with χ^2 tests. Log-transformed, natural logarithm, plasma variables were used to ensure normal distribution. To assess biomarker baseline differences between stable and progressors, we conducted an analysis of covariances, adjusted for age, sex, and creatinine. Individual trajectories of biomarker levels were plotted using *ggplot2* package in R.

To examine differences in biomarker trajectories between groups (*stable* and *progressors*), we conducted linear mixed effect models (LMMs) using the *lmer* function from the lme4 R package. We added progression status as fixed effect, adjusted for time, age, sex and baseline creatinine, and to allow each participant to have individual baseline levels and different rate of change over time, we added time and patient, as random intercept and random slope, respectively. Time and progression status were included as an interaction term to test if the effect of time on the outcome (biomarker) differed between the progression and stable groups. To determine whether including an interaction between time and progression status improved model fit, we compared two nested linear mixed-effects models (with and without the interaction term) using the Akaike Information Criterion (AIC).

We then investigated the correlations between individual biomarker trajectories with CDR-SB and MMSE. First, we performed LMMs with plasma biomarkers, CDR-SB, and MMSE as outcome, separately. We added time as a fixed effect, age, sex (and baseline creatinine in models with plasma biomarkers) as covariates, and time and patient, as random intercept and random slope, respectively. Finally, to allow comparison of rate of change across variables with different units, we extracted the random slopes from the LMMs and computed Pearson's correlations.

LMMs handle missing data by using available data under the assumption that data is missing at random, no imputation is performed. In this data set we had limited missing data, counting one baseline blood sample lost after collection and the nine patients who did not participate in the two-year follow-up.

We further tested the correlations between baseline plasma biomarker levels and baseline levels of creatinine and eGFR, using spearman's correlations.

As the availability of only two time points limited our ability to estimate individual slopes using longitudinal models, a different analysis method was chosen for the imaging data. We calculated the annual relative change, independent of baseline value in imaging measures and blood biomarkers between baseline and one year FU($((FU-baseline)/baseline)/\Delta time$). Pearson's correlations were then calculated between these changes. We only included cases with both baseline and follow-up scan available for analysis (MRI n=70, PET n=67). To assess the potential impact of scanner differences, we conducted a sensitivity analysis excluding the 14 participants with a 1.5T baseline MRI scan. Multiple testing correction was applied to all imaging analyses using the FDR approach.

Finally, to address the impact of within subject and assay variability on longitudinal biomarker changes we included biomarker specific reference change values (RCV). RCV represents an interval in which changes in biomarker levels are caused by within-subject variability and assay variability. To ensure changes between two measurement points are likely to reflect underlying pathological changes, the difference must exceed that of the given marker's RCV. In previous studies^{24,25}, we estimated RCVs in AD patients for plasma NfL, p-tau217, BD-tau, and GFAP, measured by SIMOA^{24,25}. Estimations of RCVs were performed in accordance with Fraser et. al⁴³ as

$$RCV = 100 * (\pm \exp \left(z * \sqrt{2} * \sqrt{\log (CV_{within-subject}^2 + CV_{assay}^2) - 1} \right)),$$

based on the within-subject variability and the mean intermediate assay variation for each plasma biomarker. Please see supplementary table 1, reporting these metrics and listing the RCV's used in this study. Statistical significance was assessed at a two-tailed $\alpha = 0.05$ level. All statistical analyses were performed by R version 4.5.0 with RStudio.

Results:

Cohort characteristics

Baseline and 1-year follow-up data were available for 94 patients, with 85 also completing the 2-year follow-up. Patients in the progression group were significantly older and had significantly higher baseline levels of plasma NfL, compared with the stable group, see Table 1. The presence of various comorbidities was similar between groups and no other baseline group differences were observed. Figure 1A shows the distribution of individuals with MCI or dementia at baseline and their change in disease stage during study period. Longitudinal data confirmed that the progression group declined significantly faster on cognitive tests than those in the stable group (CDR-SB: $\beta = 0.33$, $e\beta = 1.39$, $p\text{-value} < 0.001$, and MMSE: $\beta = -1.6$, $p\text{-value} < 0.001$), see Figure 1B and supplementary Table 2.

Table 1. Baseline demographics

	Stable (N=41)	Progressors (N=53)	<i>p</i> -value
Age, years	73.5 (7.3)	77.3 (6.3)	< 0.001
Sex, F/M	19/22 (46/54%)	30/23 (57/43%)	0.3
Education, years	11.1 (3.1)	12.3 (4.1)	0.2
Disease severity MCI/dementia, n (%)	7/34 (17/83%)	19/34 (36/64%)*	0.044
FAQ IADL	7.8 (4.3)	7.9 (5.8)	0.9
MMSE**	25.6 (2.4)	25.6 (2.5)	0.8
CDR-SB **	3.0 (2.0, 3.5)	2.5 (1.5, 4.0)	0.3
<i>Biochemical data</i>			
CSF β -amyloid (pg/mL) (n= 42)**	704 (634, 883)	649 (465, 760)	0.2
CSF <i>t</i> -tau (pg/mL) (n= 42)**	333(249, 480)	392 (248, 434)	0.8
CSF <i>p</i> -tau (pg/mL) (n= 42)**	36 (26, 52)	41 (30, 48)	0.9
plasma <i>p</i> -tau217 (pg/mL) *	0.8 (0.6, 1.3)	0.8 (0.7, 1.2)	0.8
plasma NfL (pg/mL) *	17.3 (13.6, 24.1)	23.0 (17.9, 28.9)	0.03
plasma BD-tau (pg/mL) *	1.1 (0.9, 1.3)	1.1 (0.9, 1.4)	0.5
plasma GFAP (pg/mL) *	241 (179, 307)	285 (246, 413)	0.07
plasma <i>P</i> -tau217/BD-tau *	0.8 (0.6, 1.0)	0.7 (0.6, 0.9)	0.3
eGFR mL/min/1.73m ²	77.7 (14.8)	74.4 (17.4)	0.3
HbA1c mmol/mol	39.9 (5.0)	40.6 (10.3)	0.7
Creatinine μ mol/L	75.4 (18.4)	78.5 (22.2)	0.5
<i>Comorbidities</i>			
BMI	24.7 (3.7)	25.2 (4.1)	0.6
Hypertension, yes (%)	27 (66%)	25 (47%)	0.1
Hyper cholesterol, yes (%)	21 (51%)	22 (42%)	0.4
DM I/II, yes (%)	6 (15%)	6 (13%)	0.4
Atrial fibrillation, yes (%)	2 (5%)	6 (11%)	0.3
Previous stroke, yes (%)	3 (7%)	1 (2%)	0.2
Previous depression, yes (%)	5 (12%)	3 (6%)	0.3
MRI Fazekas, mean (SD)	0.9 (0.8)	1.0 (0.7)	0.4

p*-value from linear regressions adjusted for age, sex and baseline creatinine. *p*-value from linear regressions adjusted for age and sex). ***one patient presented with moderate dementia at baseline. CDR-SB, CSF and plasma *p*-tau217, NfL, BD-tau, and GFAP are showed as median and inter quartile range (log transformed for normal distribution). Age, education, FAQ IADL, MMSE, eGFR, HbA1c, and creatinine are displayed as mean and standard deviations.

Figure 1. CDR-SB & Disease stage trajectories

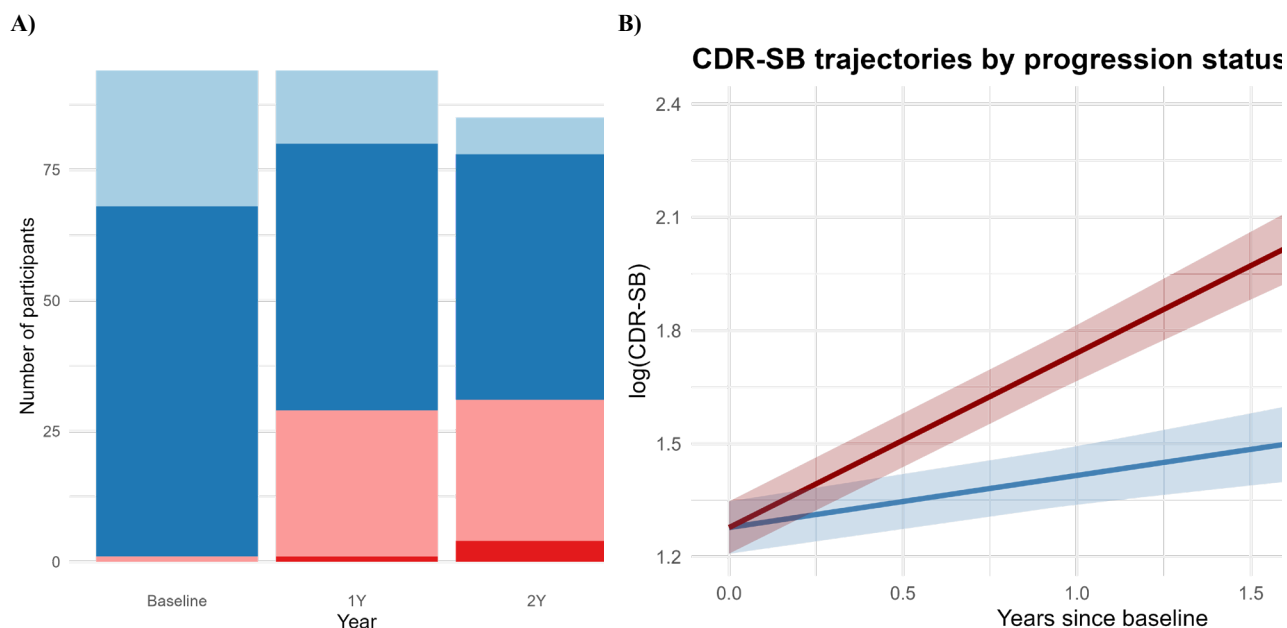


Figure 1A shows how patients change disease stage between visits, based on available clinical information and according to the ICD-10 criteria. Figure 1B shows the different CDR-SB trajectories between stable and progressors.

Biomarker trajectories

Trajectories at group level

While the stable group exhibited no significant change in any of the blood biomarkers, individuals in the progression group showed a significant increase over time in plasma NfL (slope = 0.09, $e\beta$ =9% relative annual increase), p-tau217 (slope = 0.11, $e\beta$ 12% relative annual increase), and in the p-tau217/BD-tau ratio (slope = 0.12, an absolute annual increase by 0.12 units), see Figure 2 A, B, and E. For GFAP we observed a trend of increase in both groups, whereas for BD-tau we observed a negative trend in the progression group. See supplementary Figure 1 for individual biomarker trajectories.

Differences between group trajectories

For plasma NfL there was no differences in slopes over time between stable and progressors, see Figure 2B. There was a significant difference in slopes between stable and progressors in plasma p-tau217 (β = 0.19, $e\beta$ =21% relative annual increase (p -value= .01)), and in the p-tau217/BD-tau ratio (β = 0.2, an absolute increase by 0.2 units (p -value= <0.001)), see Figure 2A and E. The p-tau217 and p-tau217/BD-tau ratio models with the interaction term had a lower AIC than the model without, indicating a better fit, thus

supporting the inclusion of the interaction term. There were no group differences in slopes in plasma GFAP and BD-tau.

In subgroup analyses for different trajectories across disease stages, we observed a numerically steeper trajectory which did not reach significance for the plasma ratio p-tau217/BD-tau in individuals who progressed from MCI to dementia compared with individuals who progressed from dementia to more severe stages. Further, we found significantly steeper trajectories in MCI progressors compared with stable MCI and dementia patients, and in the dementia progression vs. stable dementia group, see Figure 2F. In patients with MCI who converted to dementia, we found that plasma p-tau217 increased significantly faster than in stable patients with dementia (β : 0.1, p -value: 0.01), data not shown.

The relationship between biomarkers

Testing the relationship between the individual biomarkers' trajectories, we found different patterns in the stable group compared with progressors. In the progression group, the trajectory of plasma p-tau217 correlated moderately with plasma BD-tau ($\rho = 0.6$, p -value ≤ 0.000001) and NfL correlated moderately with GFAP ($\rho = 0.6$, p -value ≤ 0.00001), indicating that clinical progressors with a steeper increase in plasma p-tau217 tends to have a steeper increase in plasma BD-tau and GFAP. In contrast, in the stable group we found that plasma P-tau217 trajectories correlated negatively with plasma BD-tau and GFAP, while positively correlated with NfL. BD-tau trajectories correlated positively with BD-tan and negatively with NfL in stable individuals. See supplementary Figure 2. Finally, we evaluated plasma biomarker trajectories by sex and observed no significant differences (data not shown).

Correlations between baseline biomarker levels and kidney function

Baseline levels of creatinine correlated significantly with baseline plasma p-tau217 ($\rho = 0.2$, p -value ≤ 0.001), NfL ($\rho = 0.2$, p -value = 0.0001), BD-tau ($\rho = 0.4$, p -value ≤ 0.0001), but not with GFAP ($\rho = 0.05$, p -value = 0.3). Baseline levels of eGFR correlated significantly negatively with baseline plasma p-tau217 ($\rho = -0.3$, p -value = $p < 0.01$), NfL ($\rho = -0.4$, p -value ≤ 0.001), BD-tau ($\rho = -0.5$, p -value ≤ 0.0001), and GFAP ($\rho = -0.3$, p -value ≤ 0.01). No correlations were observed for the plasma p-tau217/BD-tau ratio with either creatinine ($\rho = 0.03$, p -value ≤ 0.8) or eGFR ($\rho = -0.1$, p -value = 0.1).

Figure 2. Plasma biomarker trajectories in stable vs. progressors

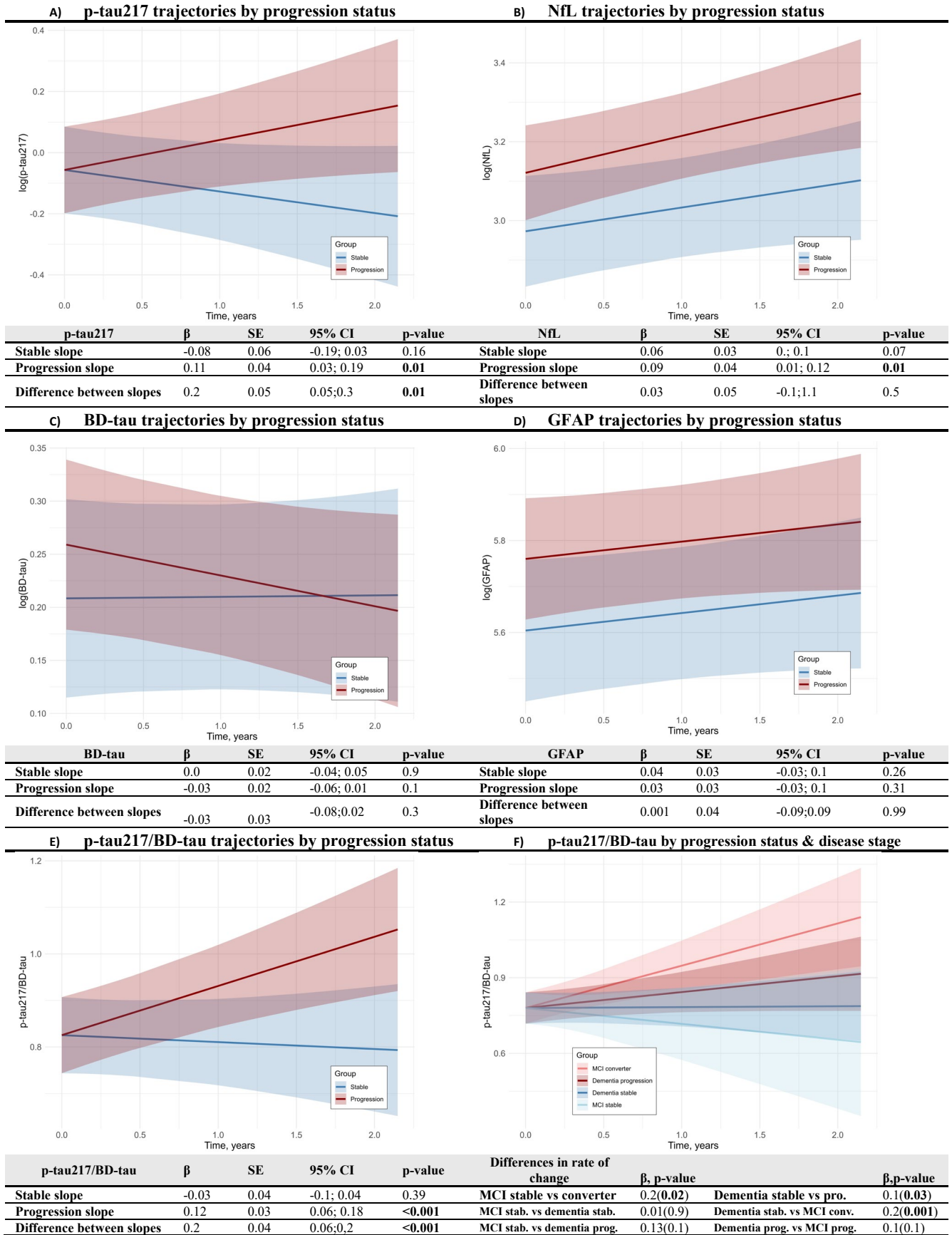


Figure 2 legend: Longitudinal plasma trajectories stratified by stable individuals vs. individuals who progressed. LMM models reflecting the change in plasma biomarker levels over time. Based on log transformed (NfL, p-tau217, BD-tau and GFAP) as outcome, time (years), age, sex and creatinine as fixed effect, and patients as random effects. *age was significant in LMM model. For p-tau217 and p-tau217/BD-tau models, there were no evidence of baseline differences, hence for visual interpretation the model was fitted without group as the main effect, but with group:time as main effect and plotted.

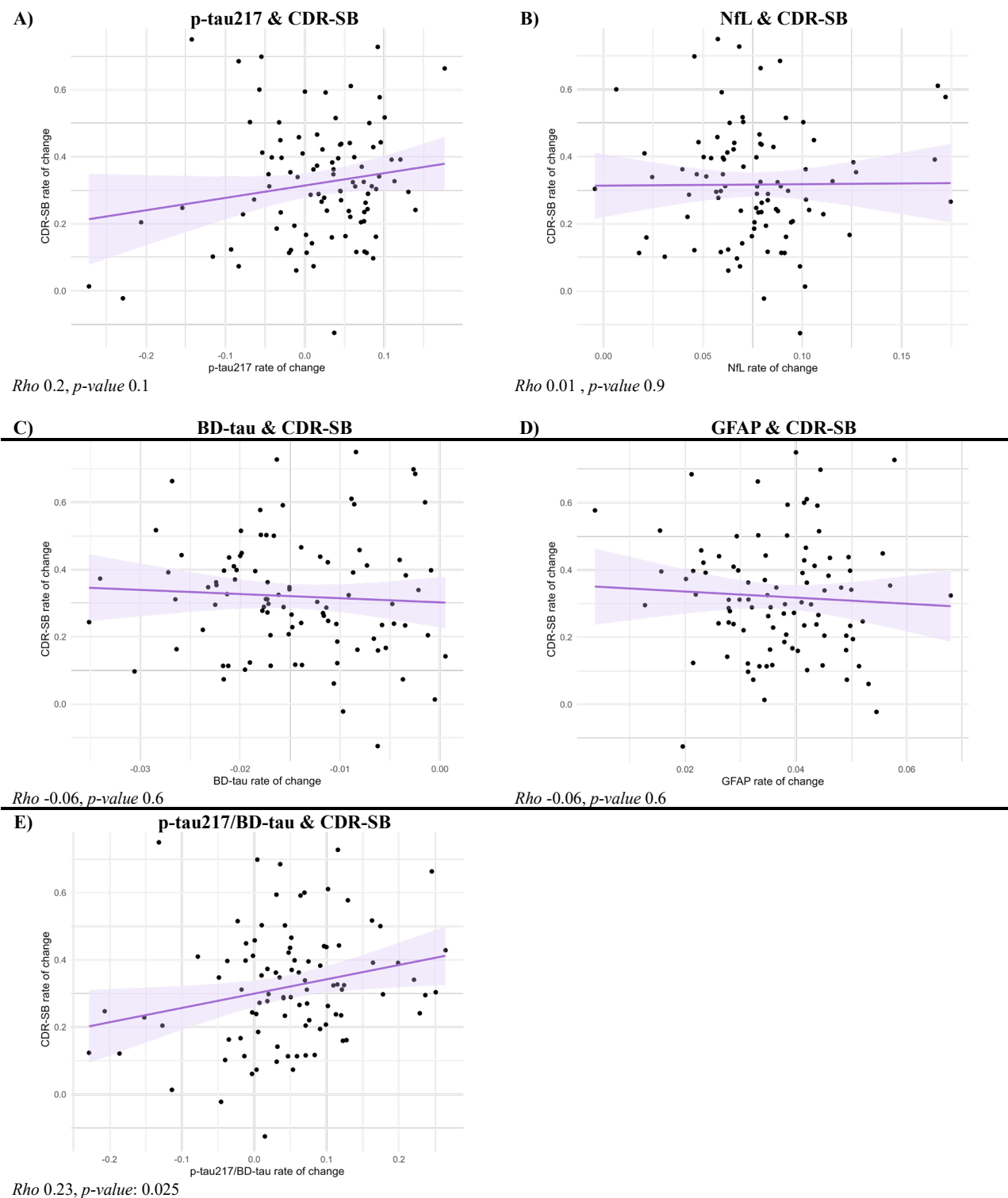
Correlations between individual rate of change of blood biomarkers & cognitive tests

No significant correlations were observed between individual rate of changes in plasma NfL, p-tau217, BD-tau or GFAP and changes in CDR-SB over time, see Figure 3A-D. However, a significant but weak correlation was observed between the p-tau217/BD-tau ratio and CDR-SB individual rate of change, see Figure 3E. No correlations were found between any biomarkers and MMSE and no correlations were found between CDR-SB and biomarkers changes tested on the stable and progression group separately (data not shown). Furthermore, subgroup analyses stratified by baseline disease stage (MCI vs. mild/moderate AD), did not reveal significant correlations between individual rates of change in blood biomarkers and cognitive decline (as measured by either MMSE or CDR-SB) within either subgroup. This is consistent with the overall findings and suggest that the absence of associations is not driven by heterogeneity in disease stage.

Plasma biomarkers & imaging progression

Linear mixed models showed a significant change in volume and metabolism in all four MRI and PET regions, respectively over one year, see supplementary Table 3. However, we found no correlation between annual changes in blood biomarkers and imaging regions, after multiple testing correction, see Figure 4A and B. Sensitivity analysis excluding the 14 patients with a 1.5T MRI baseline scan revealed a correlation between changes in plasma p-tau217 and changes in lateral ventricle volumes and between changes in plasma p-tau217/BD-tau and changes in hippocampus and lateral ventricle volumes (corrected for multiple testing), see Figure 4C.

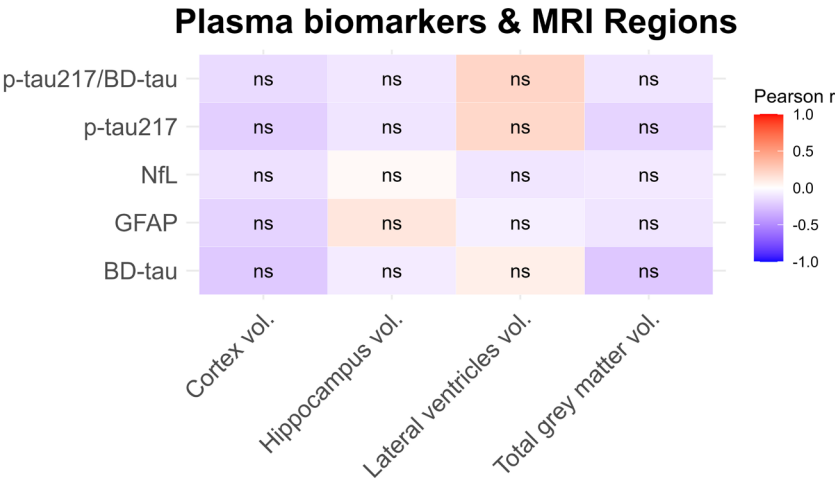
Figure 3. Plasma biomarkers & CDR-SB correlations



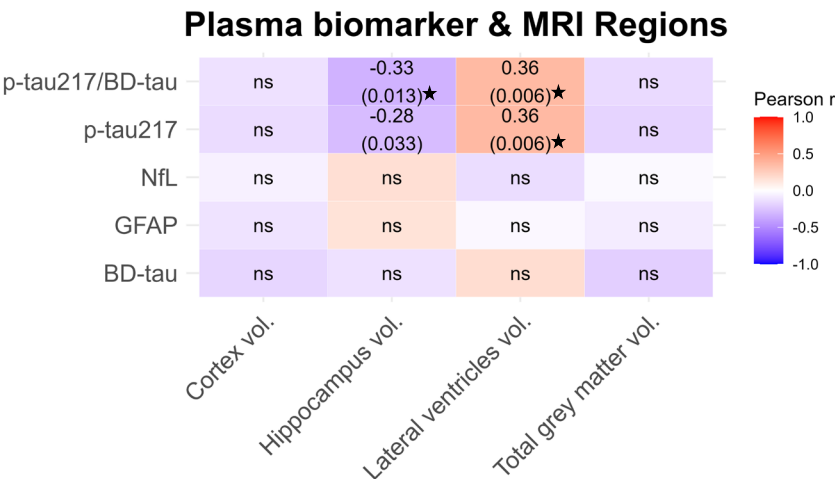
Pearsons's correlations coefficient to compare random slopes of plasma biomarkers with CDR-SB. Random slopes extracted from LMM with time (years), age, sex and creatinine as fixed effect, and patients as random effects. Each dot represents an individual and how this individual's' biomarker slope correlates with CDR-SB changes.

Figure 4. Correlations heatmaps: changes in imaging regions vs. biomarkers

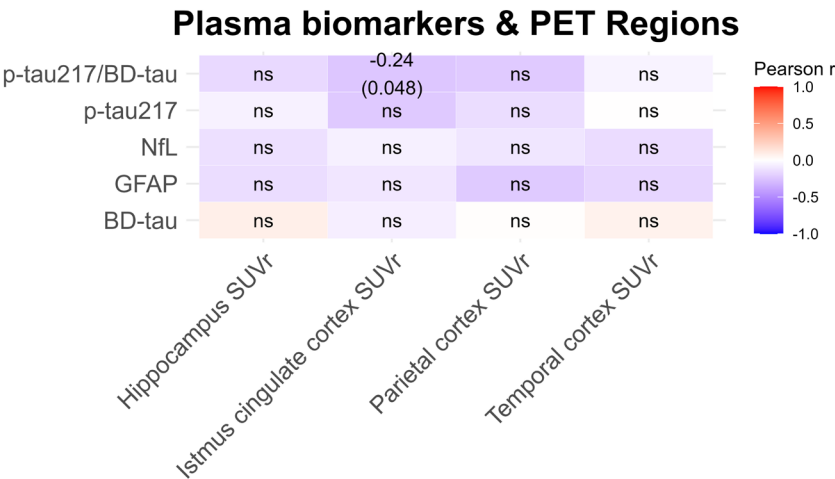
A) MRI



B) MRI
Excluding
n=14 patients
with 1.5T
baseline scan



C) FDG-PET

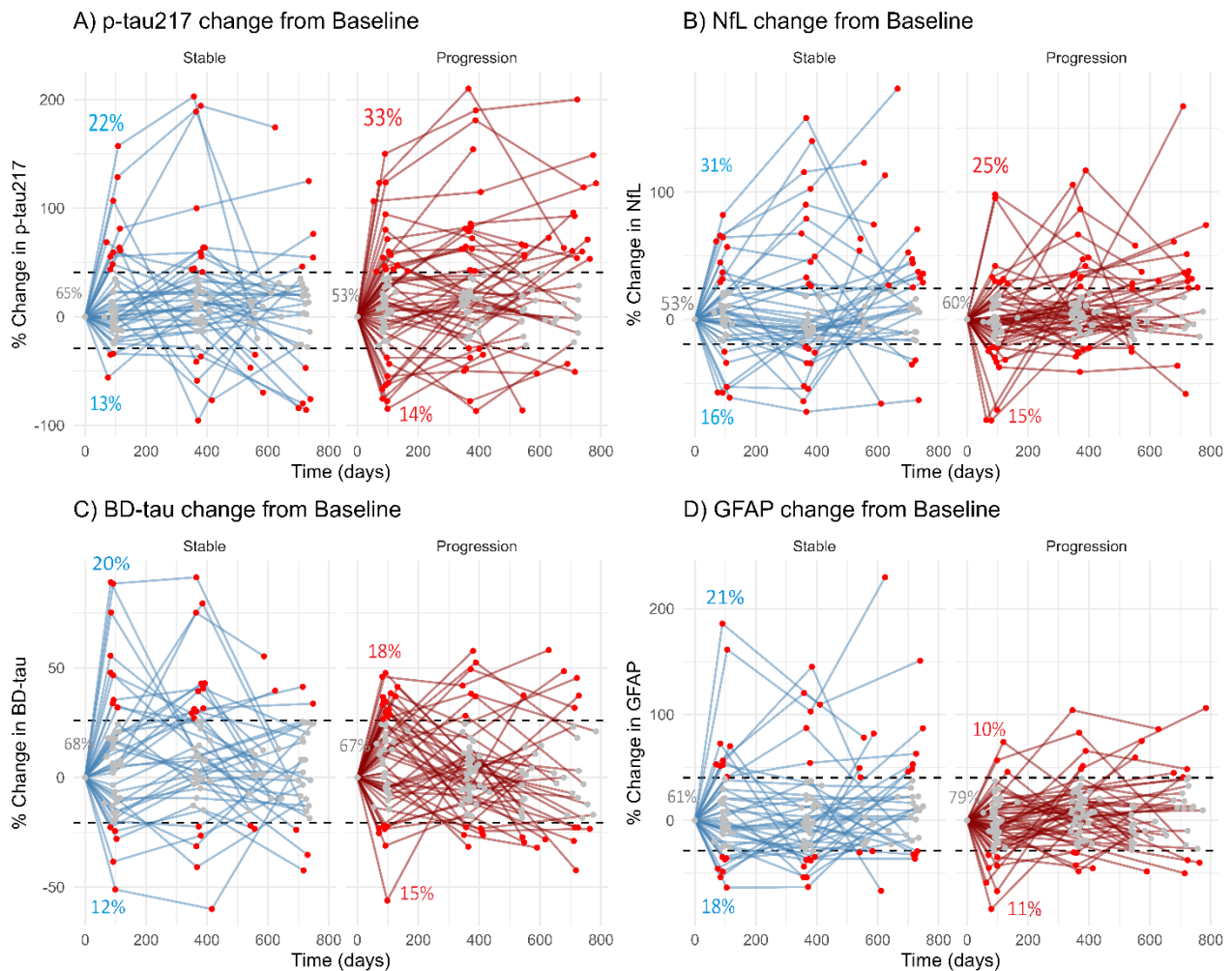


Heatmaps showing correlations between biomarker and imaging changes, rc; relative change, ns; nonsignificant. *P*-values are presented in parenthesis (), ★ are *p*-values significant after FDR adjustment.

Using reference changes values to assess disease-specific pathological changes in plasma biomarker levels

To identify those changes that lie within the RCV, we calculated the percentage change between baseline and each follow-up visit (denoted *observation*), see Figure 5. The total number of observations after baseline were 269. For plasma p-tau217, 65 and 53% observations were within the RCV, in the stable and progression group, respectively. For NfL the proportions of changes within RCV were 53 and 60% for stable and progressors. Even higher proportions were found for BD-tau and GFAP, where 68% and 67%, and 61% and 79% of the observations lay within the RCV.

Figure 5. Biomarker changes in the context of reference change value



% change from baseline above, within, and below the RCVs. The dotted lines represent the plasma biomarkers individual upper and lower reference change values^{24,25}. Red dots = exceeds RCV, Grey dots = within RCV.

Discussion

This longitudinal study evaluated the disease tracking properties of plasma NfL, p-tau217, BD-tau, and GFAP in patients with AD. Our findings demonstrate that plasma p-tau217 and the plasma p-tau217/BD-tau ratio exhibit different trajectories in stable individuals versus progressors. Moreover, for the p-tau217/BD-tau ratio trajectories also differed between those that progressed from one disease stage (MCI or more severe dementia stage). For most biomarkers we did not observe correlations with individual changes in CDR-SB or MMSE, or with concurrent neuroimaging changes. However, for p-tau217/BD-tau, we observed a significant, but weak correlation with CDR-SB. Finally, we found that most changes in biomarker levels between baseline and follow-up remained within their respective RCV, based on the biomarker specific within-subject and assay variability.

Of all the biomarkers included in the present study, only p-tau217 trajectories differed between patients who remained stable and those who progressed. Plasma p-tau217 is closely linked to tau pathology and considered an upstream marker, reflecting neurofibrillary tangle formation⁴⁴. As brain tau pathology accumulates with advancing disease, this would be expected to be reflected in longitudinal increases in plasma p-tau217, which we confirm with our findings in this study. The distinct plasma p-tau217 trajectories are consistent with previous studies that have reported similar divergent patterns across cohorts of cognitively normal (CN) individuals, subjective cognitive decline (SCD), and patients with MCI¹⁷. The p-tau217/BD-tau ratio further increased the differences in group trajectories and indicated a steeper trajectory for individuals with MCI who progressed to dementia compared with individuals with dementia who progressed to more severe stages. We acknowledge this finding is limited by the small samples size in each subgroup and should be validated in future larger cohorts. But, with the addition of our findings from patients with more advanced disease, plasma p-tau217 may represent a potential monitoring biomarker across both preclinical and clinically manifest AD continuum. Finally, p-tau217 individual trajectories did not correlate with changes in MMSE and CDR-SB. A recently published paper reported a modest correlation between changes in plasma p-tau217 and concurrent changes in cognitive measures in preclinical Alzheimer's disease⁴⁵. This discrepancy may reflect differences in plasma p-tau217 trajectories between

preclinical and clinically manifest AD. The only significant relationship between individual trajectories was between the p-tau217/BD-tau ratio and CDR-SB trajectories, indicating that the p-tau217/BD-tau ratio may be more sensitive to track longitudinal changes in individual patients. This is consistent with previous findings suggesting that plasma p-tau217/BD-tau ratio exhibits reduced variability and is less influenced by peripheral factors compared with plasma p-tau217 alone ²⁵. However, we acknowledge the correlation was weak and should be further evaluated in other comparably larger and more diverse cohorts.

Plasma NfL and BD-tau are markers of axonal damage and neuronal degeneration. NfL is considered a downstream consequence of disease processes, not closely linked to amyloid deposition, but released following various causes of neuronal damage such as cerebrovascular changes, acute brain injury, and other neurodegenerative diseases ⁴⁶. For plasma NfL, we found a statistically significant increase in the progression group. However, this increase was not markedly steeper than the near-significant increase in the stable group. This is consistent with previous findings of longitudinal increase of plasma NfL in cognitively unimpaired older adults, where no differences in NfL trajectories between amyloid- and tau PET positive and negatives were found ¹⁶. We did however notice higher baseline NfL levels in the progression group, indicating its potential as a prognostic biomarker as previously suggested ^{11–13}. Finally, we found no significant correlation between individual NfL rate of change and MMSE, CDR-SB or imaging changes. This contradicts a previous larger cohort study that established significant correlations between plasma NfL trajectories with both MRI volumes, MMSE, and CDR-SB ⁴⁷. In contrast to our study's two-year follow-up time, the maximum follow-up time in this study was 10 years. As plasma NfL levels can be influenced by comorbidities and non-AD-related pathologies ^{48,49} longitudinal changes in NfL are not exclusively explained by Alzheimer's disease-associated neurodegeneration. This may affect the strength and specificity of its correlation with clinical presentation, especially in a memory clinic cohort as ours. In contrast plasma BD-tau is believed to be less influenced by peripheral factors and linked to AD pathology ⁴², however, we did not find longitudinal increases of plasma BD-tau in this study and nor did we find any differences between stable or progressors. The progression group exhibited a near significant decrease over time, opposite to our hypothesis that BD-tau levels reflect ongoing neurodegenerative processes as the disease progress. The finding is difficult to interpret and warrants further investigation.

Finally, GFAP may reflect neuroinflammatory responses occurring throughout the disease course. In this study, we did not find any significant longitudinal change in plasma GFAP levels, neither at group level, nor did we find any relationship between individual GFAP trajectories and MMSE, CDR-SB or imaging changes. While plasma GFAP may be elevated in early disease stages of AD and correlate with cognitive impairment^{50,51} it seems to entail more diagnostic accuracy than CSF GFAP⁵². This discrepancy between plasma and CSF GFAP, together with production of GFAP in the peripheral nervous system, has raised caution regarding interpreting plasma GFAP as indicative of astrocytic reactivation^{53,54}. Our findings may support these limitations of plasma GFAP when used for monitoring AD patients.

The lack of correlations between individual patient biomarker trajectories and CDR-SB trajectories, raises further important considerations. Plasma biomarker concentrations may vary considerably within patients with AD, which may mask true disease progression²⁴. A recent study by Dodge et al.⁵⁵ demonstrated that collecting 3–5 repeated blood samples could reduce within-subject variability and enhance measurement precision of plasma p-tau217. This aligns with previous studies^{23,24} and could suggest that repeated sampling may offer more robust information than a single measurement. Such an approach could improve the clinical interpretability and utility of longitudinal changes in plasma p-tau217. To further explore this potential, future longitudinal studies should consider incorporating repeated sampling when entering a longitudinal study. This refinement may help mitigate the impact of biomarker variability, which can obscure associations between blood biomarkers and clinical progression. Additionally, factors that may influence within-subject variability, such as diurnal fluctuations, fasting status, renal function, and the presence of infection or inflammation, should be systematically accounted for in future study designs^{56–61}.

In this study we included biomarker-specific RCVs (based on within-subject and within-assay variability) and observed that most biomarker changes lie within the RCV, suggesting that only a minority of changes may reflect actual disease-related progression. Furthermore, it is notable that p-tau217 displayed the highest number of observations in the progression group exceeding the RCV, which further substantiates that plasma p-tau217 is the most promising candidate to track disease progression amongst the biomarkers investigated. While plasma p-tau217 levels may be influenced by peripheral factors such as kidney function and BMI

^{26,62,63}, potential concurrent co-pathologies should not alter plasma p-tau217 levels. In contrast, both plasma NfL, BD-tau and GFAP are more sensitive to concurrent co-pathologies. This may partly explain why p-tau217 may track AD progression more closely, especially given the larger proportion of p-tau217 changes exceeding the RCV. However, this relationship may still be confounded by concurrent co-pathologies such as alpha-synuclein, cerebrovascular pathology or TAR DNA-binding protein 43, which can influence disease severity and progression, resulting in an imbalance between clinical presentation and biological staging of AD ^{4,63,64}.

Challenges in a study of clinical progression of AD are many ⁶⁵ including the lack of valid scales that quantitatively capture the heterogeneous picture of AD progression ⁶⁶. The CDR-SB is a composite clinical rating scale that integrates cognitive and functional domains and is used as primary outcome in anti-amyloid clinical trials ⁶⁷. However, it is influenced by numerous factors such as sensitivity to demographic factors, clinical heterogeneity, and inter-rater variability, which can confound the interpretation of clinical progression ⁶⁸. Using another outcome measure for disease progression, we included longitudinal measures of [¹⁸F]FDG-PET/MRI in AD specific regions. However, we did not find that longitudinal changes on [¹⁸F]FDG-PET or MRI correlated with concurrent biomarker changes. Previous studies have suggested that baseline measures of AD pathology in blood and CSF are associated with longitudinal measures of atrophy and neuronal dysfunction assessed by MRI and FDG-PET⁶⁹. This suggests that the prognostic ability of baseline plasma markers does not necessarily translate into monitoring imaging trajectories over time. Our sensitivity analysis, excluding patients with a 1.5T baseline MRI scan, showed that changes in p-tau217 correlated significantly with increased lateral ventricle volumes and that the p-tau217/BD-tau ratio correlated significantly with decreased hippocampal volume and increased lateral ventricle volumes, after multiple testing correction. This signal may reflect a relationship between p-tau217 and neurodegeneration, which in our study could be masked by scanner differences. However, this finding remains uncertain and requires further validation.

Although our study benefits from longitudinal blood sampling in a well-characterised cohort, some limitations warrant consideration. The moderate sample size impacts the generalisability of our findings and

limits the opportunity to further subgroup our cohort to assess differences between e.g. stable, slow and fast progressors. Future work should seek to validate our findings in larger and more diverse populations. We have limited data on the influence of co-pathologies, which may impact both clinical presentation as well as biomarker levels. Finally, we only have CSF AD markers available on approximately 50% of our cohort, which limits the possibility of validating the presence of brain amyloidosis. However, the clinical diagnosis of AD, either at MCI or dementia stage, was obtained at an MDT conference by clinical experts.

Taken together, within-subject variability, comorbidities and concurrent co-pathologies all contribute to the challenging task of monitoring AD progression with a single fluid biomarker. This underscores a fundamental challenge in clinical trial design: plasma biomarkers provide biological measures of pathological processes that may not correlate with cognitive and functional decline. In the context of anti-amyloid trials, the imperfect relationship between clinical presentation and CDR-SB scores, between individual biomarkers and CDR-SB trajectories, and between clinical and biological staging necessitates cautious interpretation when using blood biomarkers as surrogate endpoints. While improvement in biomarkers may reflect a biological signal, they do not always translate into measurable clinical benefits as assessed by standard endpoints. It is essential to establish a robust link between biomarker changes and meaningful clinical outcomes, to ensure biomarkers as valid surrogate measures in clinical trials.

In conclusion, this study demonstrates that plasma p-tau217 levels increases more rapidly over time in progressors compared with stable individuals, across disease stages. We believe the examination of the relationship between individual biomarker trajectories with clinical measures and neuroimaging measures, provide novel insight into the disease tracking properties of blood biomarkers in the natural disease course of AD memory clinic patients. However, the absence of a clear relationship between individual trajectories challenges its use as reliable disease-tracking measures, which necessitates caution when using plasma biomarkers to track disease progression and eventually monitor treatment response.

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Conflicts

AHS has served at scientific advisory board for Eisai/BioArctic (remuneration paid to institution).

KF serves or has served on a scientific advisory board and as scientific consultant for Novo Nordisk, Roche Diagnostics and Eisai, has given lectures in symposia for Novo Nordisk, Eli Lilly and Eisai/BioArctic, (remuneration paid to institution) and has served or serves as principal investigator in trials for Roche, Biogen, AbbVie, Novo Nordisk and Roche Diagnostics (remuneration paid to institution). KF also serves as Editor-in-Chief for Alzheimer's Research and Therapy (Springer) for which personal remuneration is paid.

HZ has served at scientific advisory boards and/or as a consultant for Abbvie, Acumen, Alector, Alzinova, ALZpath, Amylyx, Annexon, Apellis, Artery Therapeutics, AZTherapies, Cognito Therapeutics, CogRx, Denali, Eisai, Enigma, LabCorp, Merck Sharp & Dohme, Merry Life, Nervgen, Novo Nordisk, Optoceutics, Passage Bio, Pinteon Therapeutics, Prothena, Quanterix, Red Abbey Labs, reMYND, Roche, Samumed, ScandiBio Therapeutics AB, Siemens Healthineers, Triplet Therapeutics, and Wave, has given lectures sponsored by Alzecure, BioArctic, Biogen, Cellectricon, Fujirebio, LabCorp, Lilly, Novo Nordisk, Oy Medix Biochemica AB, Roche, and WebMD, is a co-founder of Brain Biomarker Solutions in Gothenburg AB (BBS), which is a part of the GU Ventures Incubator Program, and is a shareholder of MicThera (outside submitted work).

KB has served as a consultant and at advisory boards for Abbvie, AC Immune, ALZPath, AriBio, Beckman-Coulter, BioArctic, Biogen, Eisai, Lilly, Moleac Pte. Ltd, Neurimmune, Novartis, Ono Pharma, Prothena, Quanterix, Roche Diagnostics, Sunbird Bio, Sanofi and Siemens Healthineers; has served at data monitoring committees for Julius Clinical and Novartis; has given lectures, produced educational materials and participated in educational programs for AC Immune, Biogen, Celdara Medical, Eisai and Roche

Diagnostics; and is a co-founder of Brain Biomarker Solutions in Gothenburg AB (BBS), which is a part of the GU Ventures Incubator Program, outside the work presented in this paper.

The other authors have no conflict of interest to report.

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Key words

Alzheimer's disease, progression, blood biomarker, neurodegeneration, trajectory

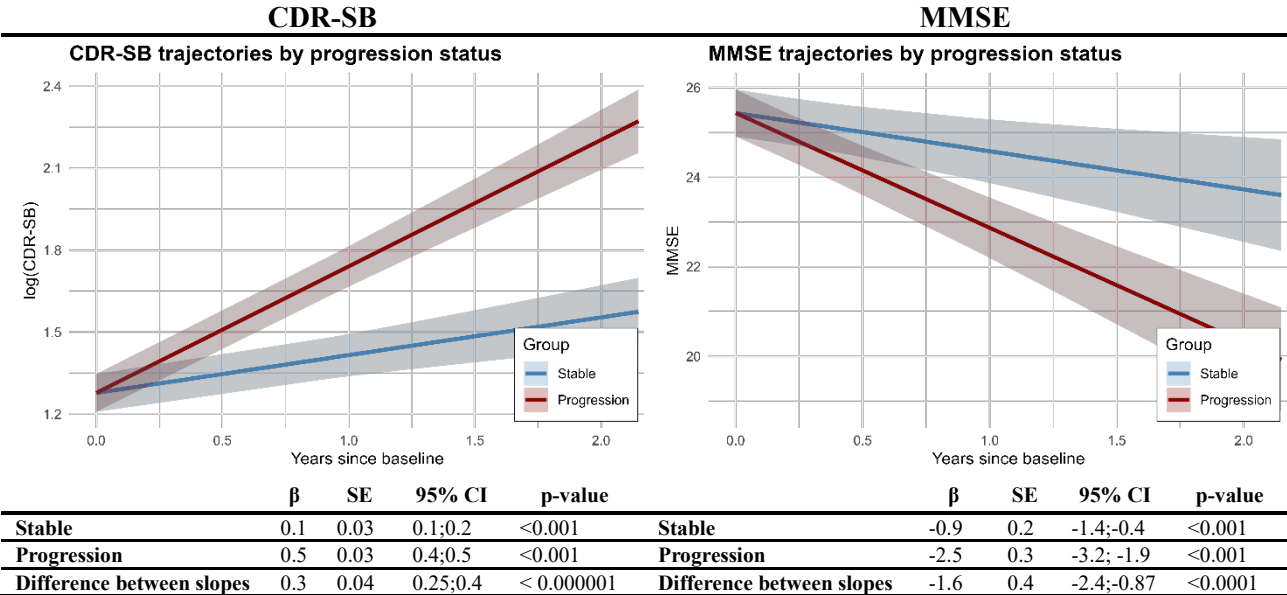
Supplementary material

Table 1. Reference change values

Biomarker	Within-subject variability (%, 95% CI)	Assay variability (%)	RCV
P-tau217	10.5 (8.3;12.8)	10.5	-28.9; +40.7
NfL	7.1 (5.5;8.6)	6.1	-19.5; +24.2
GFAP	11.4 (9.2; 13.9)	9.3	-28.8; +40.4
BD-tau	7.9 (6.3; 9.6)	6.1	-20.7; +26.1

Table outlining the within-subject and assay variability used to estimate the reference change values, RCV. For p-tau217, NfL, and GFAP data was derived from Clemmensen et. al, *Alzheimers Res. Ther.*, 2025. For BD-tau, data was derived from Clemmensen et al., *Alzheimers Dement Transl Res Clin Interv.*, 2025. Both studies are based on the same study population of AD patients. Assay variability was based on the mean intermediate precision.

Table 2. Cognitive trajectories



As the main effect of group was not significant, indicating no evidence of baseline differences, for visual interpretation the model was fitted without group as the main effect, but with group*time as main effect and plotted. LM with log transformed CDR_SB, raw MMSE with time (years) and age as fixed effect, patients as random effects. *age was significant in LMM model.

Figure 1. Individual biomarker trajectories

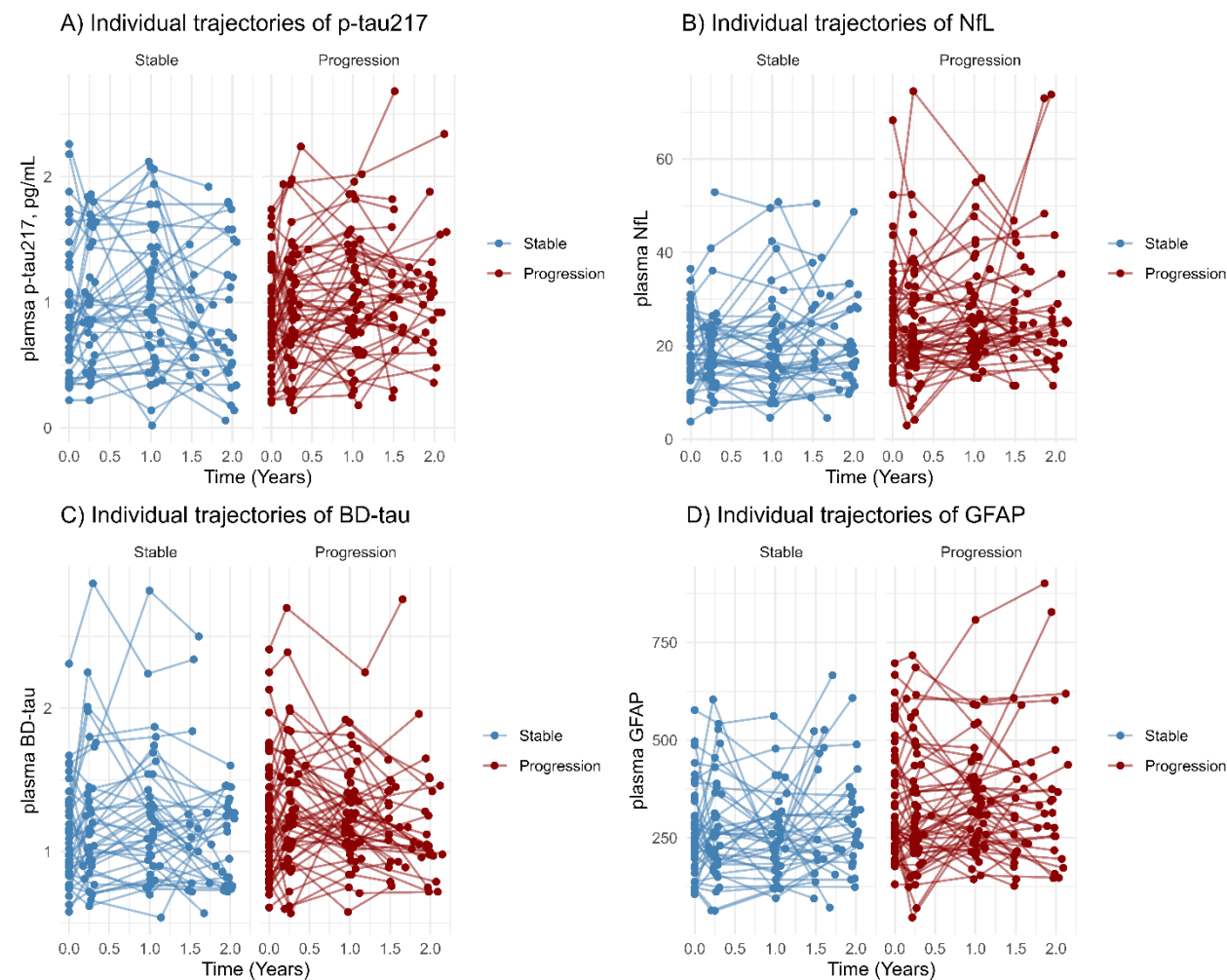
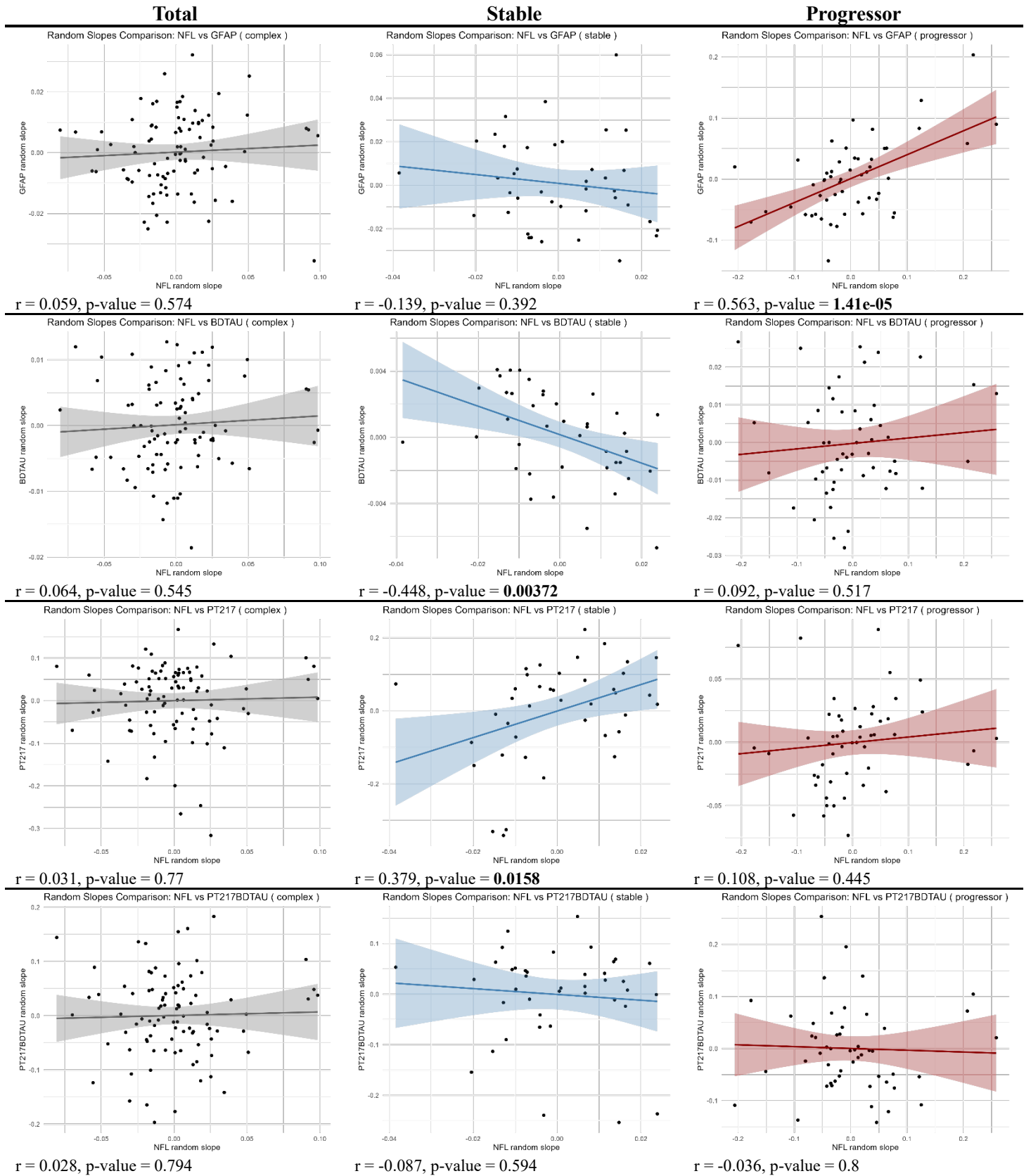
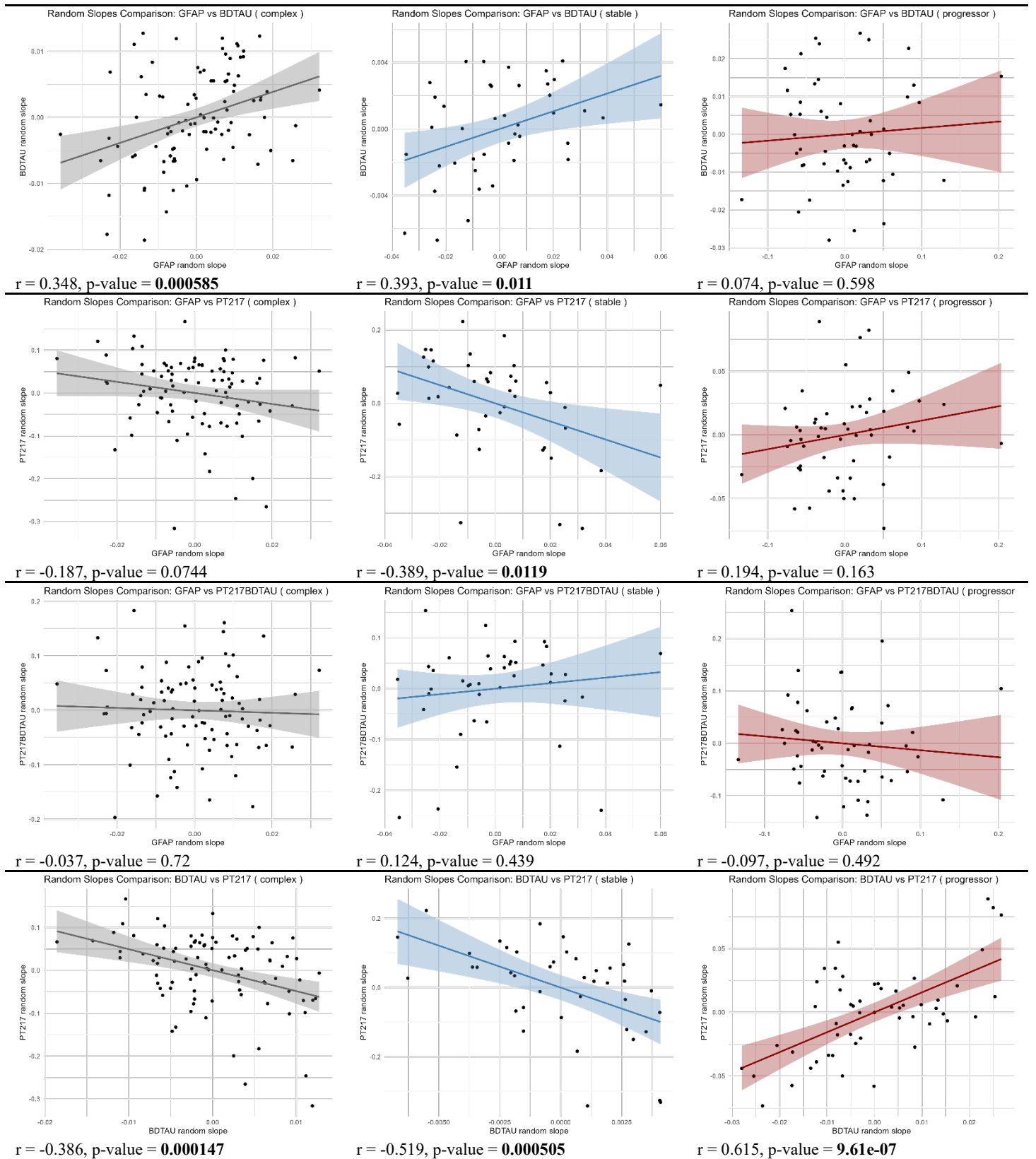
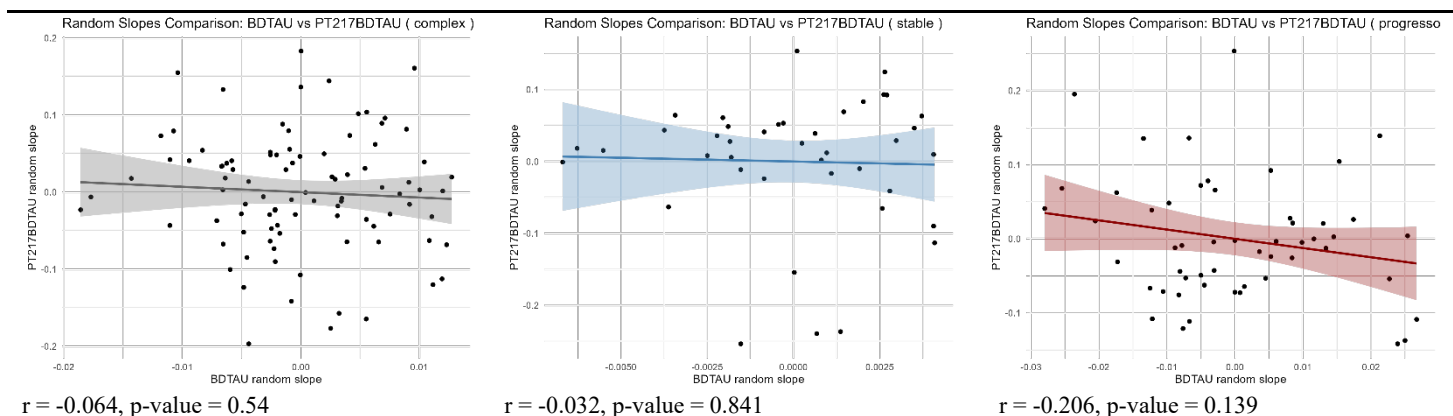


Figure 2. Correlations between rate of change in plasma biomarkers







Pearsons's correlations coefficient, r , between rate of change for each biomarker – random slopes extracted from LMMs, adjusted for age, sex and baseline creatinine.

Table 3. Change in imaging measures over time

	Region	Group	β	SE	95% CI	p-value
FDG-PET, SUVr	Temporal cortex	Total	-0.016	0.004	[-0.023, -0.008]	<0.001
		Stable	-0.017	0.005	[-0.027, -0.007]	0.002
		Progression	-0.014	0.005	[-0.025, -0.003]	0.011
	Hippocampus	Total	-0.016	0.004	[-0.024, -0.008]	<0.001
		Stable	-0.019	0.005	[-0.03, -0.009]	<0.001
		Progression	-0.013	0.006	[-0.026, 0]	0.050
	Parietal cortex	Total	-0.015	0.005	[-0.025, -0.004]	0.007
		Stable	-0.019	0.007	[-0.033, -0.005]	0.011
		Progression	-0.011	0.008	[-0.027, 0.005]	0.161
MR, vol	Isthmus cingulate cortex	Total	-0.036	0.006	[-0.047, -0.024]	<0.001
		Stable	-0.04	0.007	[-0.054, -0.025]	<0.001
		Progression	-0.032	0.008	[-0.049, -0.015]	<0.001
	Total grey matter cortex	Total	-0.004	0.001	[-0.006, -0.003]	<0.001
		Stable	-0.004	0.001	[-0.007, -0.002]	0.003
		Progression	-0.005	0.001	[-0.007, -0.002]	<0.001
	Cortex	Total	-0.004	0.001	[-0.005, -0.002]	<0.001
		Stable	-0.003	0.001	[-0.006, -0.001]	0.006
		Progression	-0.004	0.001	[-0.006, -0.003]	<0.001
	Hippocampus	Total	-0.0001	0	[0, 0]	<0.001
		Stable	-0.0001	0	[0, 0]	0.010
		Progression	-0.0001	0	[0, 0]	0.005
	Lateral ventricles	Total	0.002	0	[0.001, 0.002]	<0.001
		Stable	0.001	0	[0.001, 0.002]	<0.001
		Progression	0.002	0	[0.002, 0.003]	<0.001

Table showing longitudinal changes of imaging regions, based on linear mixed models, adjusted for time and age.

