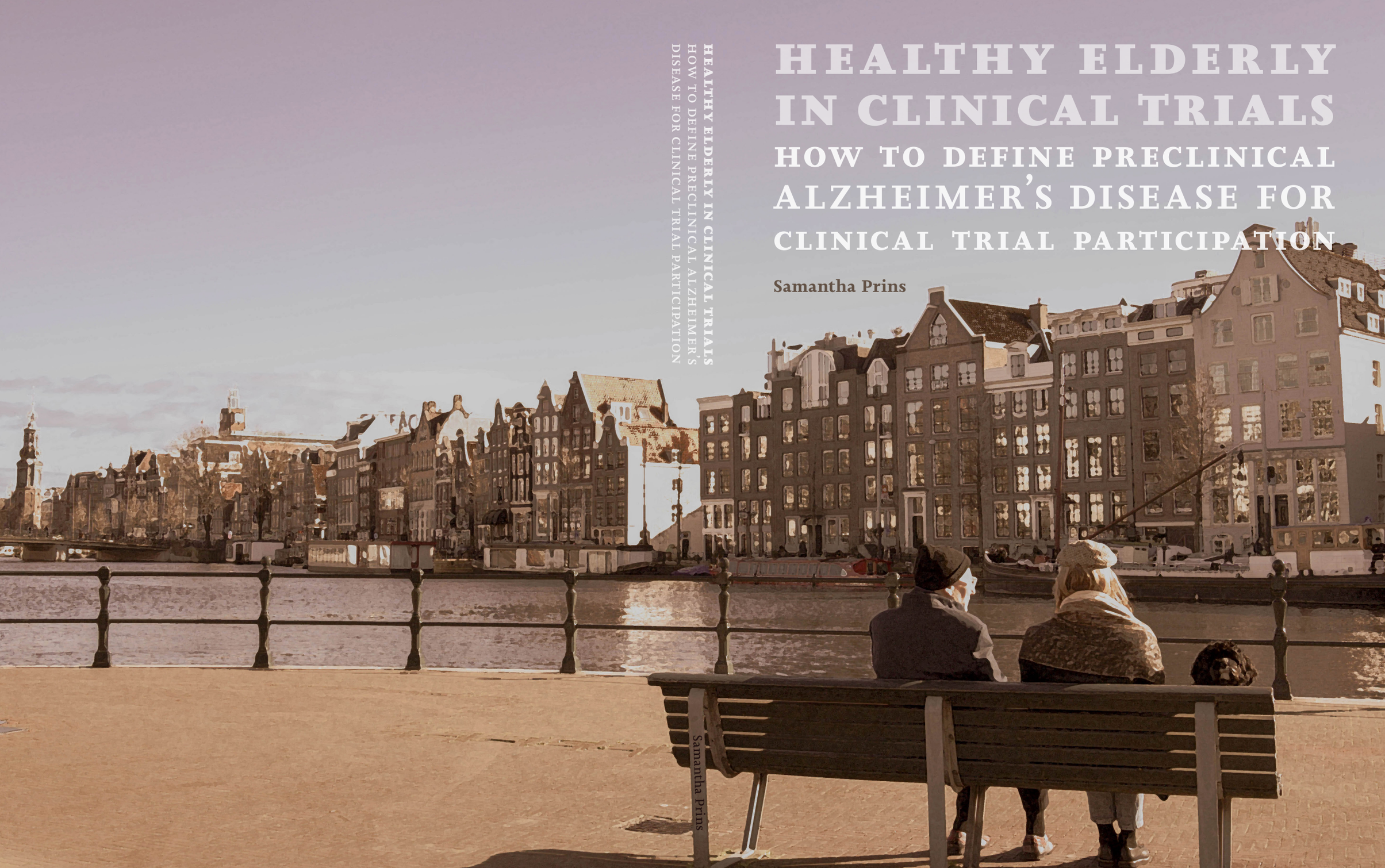

HEALTHY ELDERLY IN CLINICAL TRIALS
HOW TO DEFINE PRECLINICAL ALZHEIMER'S
DISEASE FOR CLINICAL TRIAL PARTICIPATION
HEALTHY ELDERLY
IN CLINICAL TRIALS
HOW TO DEFINE PRECLINICAL
ALZHEIMER'S DISEASE FOR
CLINICAL TRIAL PARTICIPATION
Samantha Prins
Samantha Prins

HEALTHY ELDERLY IN CLINICAL TRIALS

HOW TO DEFINE PRECLINICAL ALZHEIMER'S DISEASE
FOR CLINICAL TRIAL PARTICIPATION

VOOR MAMA

Photography
Samantha Prins

Design
Caroline de Lint, Den Haag (caro@delint.nl)

The publication of this thesis was financially supported by the foundation Centre for Human Drug Research (CHDR) in Leiden, the Netherlands

HEALTHY ELDERLY IN CLINICAL TRIALS

HOW TO DEFINE PRECLINICAL ALZHEIMER'S DISEASE FOR CLINICAL TRIAL PARTICIPATION

Proefschrift

ter verkrijging van
de graad van doctor aan de Universiteit Leiden,
op gezag van rector magnficus prof.dr.ir. H. Bijl,
volgens besluit van het college voor promoties
te verdedigen op woensdag 5 april 2023
klokke 10:00 uur
door
Samantha Prins
geboren te Hillegom

Promotores:
Prof.dr. G.J. Groeneveld
Prof.dr. J.M.A. van Gerven
Co-promotor:
dr. Laura Borghans

Leden promotiecommisie:
Prof.dr. Huub Middelkoop
Prof.dr. Charlotte Teunissen (Amsterdam Universitair Medisch Centrum)
Prof.dr. Gerard Jan Blauw
dr Niels Prins (Brain Research Center)

CHAPTER 1

Introduction

AGEING

Ageing is the process of becoming older and is an inevitable process that entails a wide variety of molecular and cellular damage over time.[1] The World Health Organization (WHO) expects 1 in 6 people in the world to be over the age of 60 by 2030. In industrialized countries, the elderly people represent the fastest growing group in the age pyramid but low- and middle income countries are following quickly.[2] Therefore, clinical research related to ageing in the elderly is important. Not only for elderly to age in a comfortable way, but also to reduce the burden on our healthcare systems. During ageing, many changes take place in the brain. Apart from neuronal cell death and brain atrophy, there are functional changes in the brain due to changes in neurotransmitter and hormone levels.[3] The ability of humans to acquire knowledge, understanding through, experience and senses is what makes us humans superior to animals. This ability is called cognitive functioning or in short, cognition.[4] Cognition depends on different brain areas to work together and combine external input (e.g., sounds, visual stimuli, touch) which may all be affected during normal aging. The cholinergic system, which is involved in memory function, consisting of cholinergic neurons in the nucleus basalis of Meynert, frontal cortex, anterior cingulate cortex, and posterior cingulate cortex, has been assumed to moderately degenerate during normal ageing of the brain.[5,6] However, cholinergic dysfunction has also been associated with several other neurodegenerative disease e.g., Alzheimer Disease (AD), Parkinson's Disease (PD) and Huntington's Disease (HD).[7] The process of ageing can be difficult to differentiate from a neurological condition as some of the processes of normal brain ageing can also be the preliminary stage of a neurological disease.[8] Usually, difficulties in performing normal daily life tasks are the first signs of change in functioning of the brain and a reason for someone to further examine if this decrease in cognitive performance is due to normal age-related decline in brain function or due to a neurological disorder.

NEURODEGENERATION

When a person ages, cognitive abilities decline, however, there is a difference in normal decline of functioning due to age and decline due to progressive loss of neurons in the context of a neurodegenerative disorder. Neurodegeneration is the progressive process of loss of structure or function of neurons, which may ultimately lead to cell death or apoptosis.[9] Accumulation or misfolding of proteins in the brain, gliosis, synaptic dysfunction, microglial activation, and inflammation are common pathologies in neurodegenerative disorders leading to apoptosis and necrosis. These processes are involved in many neurodegenerative diseases such as AD, PD and HD, which all have a different typical age of disease onset and course of the disease.[9–11] In Alzheimer's disease, neurofibrillary tangles containing phosphorylated tau and plaques consisting of amyloid peptides are observed. In Parkinson's disease, the synaptic peptide α-synuclein aggregates as Lewy bodies in the dopaminergic neurons within the substantia nigra. In Huntington's disease, the polyglutamine protein huntingtin is present in intranuclear inclusions.[12] In most neurodegenerative diseases, the first symptoms typically appear after middle age (65 years old) and increase over time. This is also the case in AD and PD. Patients with HD have a younger average age of disease onset, between 35-45 years old.[13] Where AD is characterized by episodic memory loss in the early stages of the disease, PD is characterized by movement disorders e.g., slower movements, rather than cognitive problems, which develop in the majority of patients later in the disease process.[14] HD, which is always a hereditary genetic neurodegenerative disorder, presents with mood swings and depressive feelings at the early stage of the disease followed by movements disorders and cognitive complaints.[15] These are some examples of neurodegenerative diseases with a different pathophysiology and age of disease onset but all with cognitive decline in common. The profile of cognitive symptoms, however, differs between these diseases. Patients with AD are usually more impaired in memory functioning, while patients with PD experience more difficulties with initiation of cognitive processes.[16] In HD, cognitive decline can appear before any motor symptoms but can also be mild in advanced stages of the disease.[15] Measuring cognitive functions over time can help to determine if an elderly person is experiencing normal age-related cognitive decline or cognitive decline due to a neurodegenerative disease. As the cognitive symptoms in neurodegenerative diseases can vary in time of onset, affected cognitive function, and severity of the cognitive deficit, measuring cognitive function over time is important. This is especially so in the preclinical stage of disease where no formal diagnosis has yet been established

There are many ways of measuring cognitive functions. Overall, cognition can be divided in domains of cognitive functioning, which include sensation, perception, motor skills and construction, attention and concentration, memory, executive functioning, processing speed and language or verbal skills.[17] Different brain areas are involved in these different functions. The brain regions with major involvement in neurodegeneration are the (pre)frontal lobe for attention and behavior inhibition, the temporal and parietal lobe for e.g., language, speech and memory, the cerebellum for regulation of movement and

the occipital lobe for processing of visual stimuli.[4] Measuring cognitive brain functions can be done by neuropsychological assessments, which are traditionally performed as paper-and-pencil tasks but increasingly in computerized form since the digitalization of tests in the past decades.[18] Neuropsychological testing can be used to assess the presence or absence of cognitive dysfunction, help establish a diagnosis or to help clarify the cognitive effects of neurodegenerative diseases.[19] For most of the neuropsychological tests, normative data are available to make a distinction between normal cognitive performance of a subject and when cognitive functioning can be considered abnormal. Also, confounding factors such as age, sex and education level are taken into account to further differentiate if a subject has for instance normal age-related cognitive decline or abnormal cognitive functioning.[4] At the Centre for Human Drug Research (CHDR), a computerized neuropsychological and neurophysiological test battery was developed, the NeuroCart, for the purpose of systematically studying the effects of drugs on central nervous system (CNS) functioning in the context of early phase clinical drug studies.[20] This test battery makes neuropsychological and neurophysiological test performance less time-consuming, less prone to interrater variability, and most importantly sensitive to detect pharmacological effects with minimal learning effects. Also, it is electronically available for standardized test performance in subjects.[21]

ALZHEIMER'S DISEASE

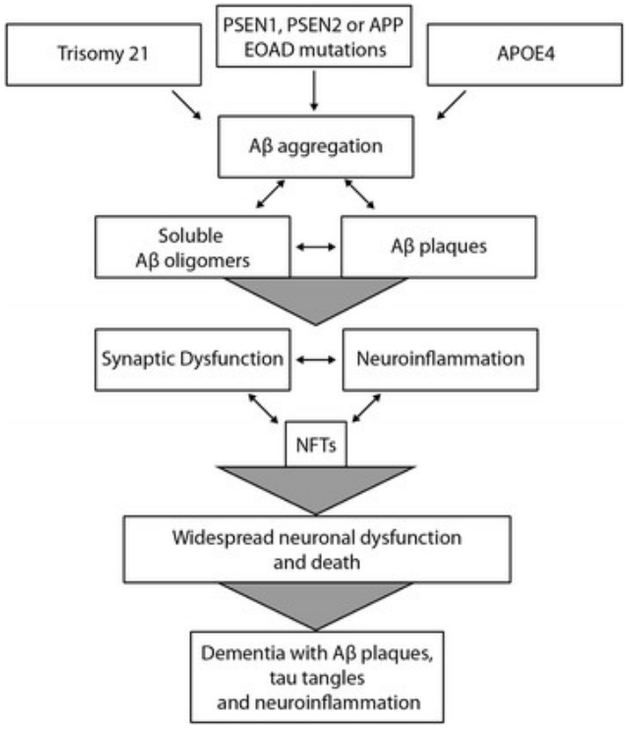
The World Health Organization (WHO) estimates the worldwide prevalence of dementia to be approximately 50 million people (in 2020).[22] Dementia is a general term for the loss of memory, language, problem solving abilities, in other words decline in cognitive functioning that is severe enough to interfere with daily life. The most common form of dementia, which represents approximately 70% of the dementia cases, is Alzheimer's Disease (AD).[23] In 1906, the German psychiatrist and neuropathologist Alois Alzheimer first discovered the typical pathological brain alterations after studying the brain of a psychiatric patient postmortem. He found amyloid plaques surrounding cells in the brain and neurofibrillary tangles inside the cells.[24] Since then, much research has been performed focused on the role of amyloid in AD. The amyloid cascade hypothesis states that AD is caused by abnormal accumulation of the amyloid beta (Aβ) protein in the brain causing plaques of this protein in various areas of the brain.[25] The Aβ peptides 39-43 are formed through sequential enzymatic cleavage by β-secretase and γ-secretase of the amyloid precursor protein (APP).[26] $A\beta_{1-40}$ is the most prevalent amyloid peptide in AD followed by $A\beta_{1-42}$, which aggregates faster in AD and is therefore intensively studied.[27,28] The Aβ peptides have the tendency to polymerize into toxic oligomers, which have been correlated with severity of dementia.[29] The Aβ plaque formation, in turn, leads to local microglia activation, cytokine release and astrocyte activation.[30] This toxic and inflammatory response leads to synaptic loss, neuronal loss, and gross cerebral atrophy, which in the end leads to clinical AD and to plaque and tangle pathology, as summarized in figure 1.[31]

Neurofibrillary tangles, which were similarly already described by Alois Alzheimer, also play a role in the development of AD and consist of misfolded hyperphosphorylated tau proteins. Normal, non-hyperphosphorylated tau proteins are mainly found inside neurons and play an important role in stabilization of the neuronal microtubules network.[32] Six different isoforms of tau have been identified in the adult brain. The process of hyperphosphorylation of tau causes disturbance in structural and regulatory function of the cytoskeleton, which is usually protected by the tau protein. The hyperphosphorylated tau proteins turn into neurofibrillary tangles inside the neurons and affect the normal cellular function and can lead to synaptic dysfunction and neurodegeneration.[33]

Genetics play a role in the chance of a person's development of AD and influence the progression rate. Autosomal dominant mutations of APP, presenilin1 (PSEN1) and presenilin1 (PSEN2) genes, both subunits of the γ-secretase causing an increase in $A\beta_{1-42}$ protein, cause early-onset familial AD (age <65 years old). The apolipoprotein E (ApoE) gene, specifically the ApoE ε4 allele, is a genetic risk factor for the typical late-onset (age >65 years old) variant of AD.[34,35]

AD is characterized by cognitive decline, specifically memory deficits which are not explained by age related decline. Up to 20 years before clinical onset of AD, the biological changes in Aβ plaques and tau tangles have already been observed in cerebrospinal fluid (CSF) of otherwise healthy elderly.[37] These changes may predict if a person will develop AD later in life. Especially when already experiencing subjective cognitive symptoms, 40-60% of these subjects are expected to convert to AD.[38-40] When a healthy subject with no cognitive complaints has a lowered CSF protein Aβ42 level, comparable with AD, this subject is considered to have preclinical AD according to the NIA-AA standards from 2011.[41] As having lowered CSF levels of Aβ42 does not per definition lead to developing AD, Dubois et al., (2016) recommend to use the term preclinical AD when an otherwise healthy subject has both Aβ and tau markers (CSF or PET) beyond pathological thresholds.[42] As PET and CSF are not commonly available for the qualification of a subject in the preclinical phase, the NIA-AA standards are used throughout this thesis.

Figure 1 The amyloid cascade by Morris et al., (2014).[36]



BIOMARKERS

A 'biological marker', or the portmanteau 'biomarker', is a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacological responses to a therapeutic intervention.[43] A biomarker can be fluid (blood, CSF), imaging (magnetic resonance imaging; MRI, positron emission tomography; PET), or can be a functional measurement such as a neuropsychological test. Biomarkers can be used for various purposes. A diagnostic biomarker detects or confirms the presence of a disease but can sometimes also be used to monitor a disease. A pharmacodynamic biomarker responds to exposure to a drug. Biomarkers may also be predictive in the sense that they may predict a subject's response to a drug. Prognostic biomarkers are used to, as the name implies, give a prognosis on the likelihood that a certain response (or disease) will occur and are useful in longitudinal clinical trials.[44] To create consensus on the use of biomarkers and the terminology, a joint task force was formed by the U.S. Food and Drug Administration (FDA) and National Institutes of Health (NIH) and created the Biomarkers EndpointS and other Tools (BEST) resource which is publicly available.[44,45]

Many different biomarkers have been associated with AD. Aβ42, the misfolded protein mainly involved in developing AD, can be measured in CSF where *lower* concentration levels are counter-intuitively associated with a higher amount of Aβ42 plaques in the brain.[46,47] The CSF Aβ42/Aβ40 ratio and CSF (p)tau/Aβ42 ratio are also considered reliable markers for either research-related diagnostics or as prognostic biomarkers.[48,49] CSF Aβ42/Aβ40 ratio has a high concordance with evidence of amyloid accumulation in the brain visualized using amyloid PET imaging.[50] In PET imaging three amyloid tracers have been approved by both the FDA and the EMA for Aβ plaque imaging; ([18F]florbetapir, [18F]flutemetamol, and [18F]florbetaben).[51] Given that CSF sampling is an invasive procedure and PET is costly and not commonly available, many have worked on finding blood-based biomarkers that can be used in the diagnosis or staging of AD. Aβ42 can be measured in blood plasma and several relatively recent studies have shown high agreement with CSF Aβ42/Aβ40 ratio and amyloid PET measurements.[52,53] Further research is needed to confirm these results and also to evaluate the course over time of Aβ42 plasma concentrations in subjects developing AD.

Neurofibrillary tangles are not specific for AD.[51] Tau protein is measurable in CSF with higher concentrations in AD patients compared to healthy controls. Higher concentrations of CSF tau have been correlated with greater cognitive impairment in preclinical and clinical AD.[54] In patients with AD, several hyperphosphorylated tau (P-TAU) isoforms can be measured in CSF, likely as a neuronal response to Aβ exposure. Therefore, an elevated CSF total tau and P-TAU concentration may be regarded as related to AD-type neurodegeneration. The only tau PET tracer approved by the FDA is 18F-Flortaucipir, which can measure the cortical tau burden in the brain.[55] Multiple other tracers are in development for measuring tau in the brain using PET. The possibility to measure P-TAU isoforms in plasma also appears to yield promising biomarkers. P-TAU181 has been reported to correlate with Aβ and tau PET and in longitudinal studies plasma P-TAU181 changed significantly before plasma Aβ, which could mean that plasma P-TAU may be used for diagnostic purposes as well as for early disease staging.[56,57]
Several biomarkers related to inflammatory and astroglial activation have been

found to be associated with AD. YKL-40 (also known as chitinase-3-like protein-1 [CHI3L1]) is a glycoprotein, which is mainly expressed in astrocytes. YKL-40 is a marker for inflammatory processes and activated astrocytes, which can be measured in CSF and in plasma and has been reported to be increased in AD compared to healthy controls.[58] Another protein that plays a role in the inflammatory response to Aβ, the soluble variant of triggering receptor expressed on myeloid cell 2 (STREM2), can be detected in CSF and can function as a biomarker for inflammation in AD. STREMS2 has been reported to increase at different stages of AD and is associated with tau pathology.[59] Glial fibrillary acidic protein (GFAP) is a marker for astrogliosis and has been reported to be increased in CSF of patients with AD and post mortem in brains of patients with AD. GFAP can also be measured in plasma and was found to be associated with brain Aβ pathology but not tau aggregation in patients with AD and in cognitively normal subjects.[60] Promising CSF biomarkers for AD related to synaptic dysfunction are neurogranin (NG), which is a post-synaptic protein and marker for synaptic loss, and neurofilament light (NFL) a marker for axonal damage.[61] None of these inflammatory or neuronal damage markers (YKL-40, STREM32, GFAP, NG, NFL) are specific for AD and elevated concentrations should be evaluated in combination with CSF and/or plasma Aβ and tau concentrations.

CSF concentrations of Aβ42, Aβ42/ Aβ40 ratio, P-TAU and total tau are considered reliable diagnostic biomarkers for AD, as are amyloid and tau PET and T1-weighted imaging MRI for overall atrophy of the brain, although all of these biomarkers are primarily used for research purposes. Other biomarkers should, at this stage, still be seen as experimental.[48] As most disease modifying therapies target the accumulation of misfolded Aβ and P-TAU proteins, or the inflammatory response to them, the classic AD biomarkers can also be regarded as potential pharmacodynamic biomarkers. CSF P-TAU may also be a prognostic biomarker as it is measurable before onset of accumulation of Aβ plaques and can predict future disease progression.[62]

Before the introduction of the above mentioned diagnostic biomarkers, a definite diagnosis of AD was only possible through autopsy of the brain to determine brain atrophy and presence of Aβ plaques and neurofibrillary tangles.[63] Fortunately, it is becoming clear that a clinical diagnosis based on neuropsychological testing combined with positive AD biomarkers corresponds highly with a diagnosis made through brain autopsy.[64] Use of CSF Aβ42 and PET Aβ as diagnostic biomarkers are now encouraged as supportive tool for the diagnosis of AD but are not yet formally approved by regulatory authorities.[62] PET and CSF sampling might not be available to all, and the NIA-Alzheimer's Association (NIA-AA) workgroups

therefore decided not to include these biomarkers as official diagnostic criteria in the last revision of the AD diagnostic criteria in 2011.[41,65] As the NIA-AA guidelines of 2011, and in particular the updated guidelines from NIA-AA in 2018, point out, biomarkers are important in biologically defining the presence of AD in humans and the use of CSF and PET biomarkers are accepted in the research framework for AD and therefore widely used in clinical trials.[66] Also, the definition of preclinical AD has been adjusted to include the use of biomarkers.[66] When a subject has no cognitive complaints but does have CSF Aβ42 levels consistent with AD, this subject is in the preclinical phase of AD, as mentioned previously.

CLINICAL TRIALS

Since the discovery of amyloid beta plaques and neurofibrillary tau tangles in the brain approximately 100 years ago, many clinical drug trials have been performed. The development of new drugs can be divided into different phases before a new drug is approved and enters the market. The first step is drug discovery, in which new technologies, new molecular compounds or existing treatments are evaluated for their potential as a medical treatment. Preclinical research follows where new compounds are being tested in vitro (in a test tube) and in vivo, in animal models to determine the pharmacological characteristics, and to determine the safety and toxicity. After extensive testing in preclinical stage, the successful new compound reaches the clinical stage of drug development, in which a compound is first administered to humans. Clinical drug research follows three phases. Phase 1 is the phase in which a compound is administered for the first time to humans and is mostly aimed at exploring the safety, tolerability, pharmacokinetics and pharmacodynamic effects of the compound. These studies are mostly performed in healthy, usually younger adult, people. Approximately 70% of all drugs move to phase 2 where the efficacy and side effects are studied in the target population. Phase 3 follows for approximately 33% of the drugs tested in phase 2. Phase 3 studies are performed in the target patient population and usually take several years, which is considerably longer than the several months needed for a typical phase 1 study. Studies in phase 2 often take several months to years to complete. Phase 3 studies are confirmatory, after phase 2 studies have already shown positive results in the target population. Phase 3 investigates the clinical efficacy of a compound in patients and monitors for adverse events. Studies in phase 3 include hundreds or thousands of patients and target to demonstrate clinical efficacy. Randomized (placebo-)controlled trials (RCT) are the preferred way to perform clinical drug studies. An RCT reduces bias and provides a rigorous tool to

examine cause-effect relationships between an intervention and outcome. This is because randomization balances participant characteristics between groups allowing attribution of any differences in outcome to the study intervention.[67] Clinical drug trials are importantly influenced by the choice of the study population as this can greatly influence the study outcome.

As the number of AD patients worldwide continues to grow, there is a huge need for disease modifying drugs. A disease modifying drug is a treatment that affects the underlying pathophysiology of the disease, in this case AD, and slows the progression of disease.[68]Despite more than a hundred phase 3 clinical trials, only one (possibly) disease modifying compound has been approved by the FDA.[69]

There may be several reasons why most clinical trials with potential disease modifying compounds in AD have so far yielded negative results. Many different pathophysiological changes play a role in the development of AD, from Aβ plaques in the brain to inflammation, glia activation and phosphorylation of tau. Targeting just one of those biomarkers may not influence the development or progression of AD sufficiently to slow disease progression. Also, not all patients with AD have the same alterations in AD related biomarkers. Better understanding of these AD related biomarkers and the complexity of the interaction between these biomarkers are needed. Combined therapy which does not focus on one single pathophysiological mechanism and therefore not one biomarker might lead to a better clinical trial outcome. As patients with AD are usually an older population with significant comorbidities, the interaction of these comorbidities with AD should be better understood. Dose selection may lead to different clinical results in patients with interacting comorbidities. The timing of the initiation of treatment in patients with AD is also of great importance. Starting treatment in AD patients with irreversible neurological damage may lead to a negative clinical trial even though the compound demonstrated positive results in an earlier phase of the disease at which less structural damage is observed.[70,71] The correct selection of trial participants is of great importance, incorporating all the above-mentioned reasons for trail failure. Pathophysiological changes such as Aβ formation and tau aggregation are already present and measurable up to 20 years before clinical disease onset and early intervention might prevent or delay a subject to become clinical.[37] Drug development in AD is shifting its attention from performing trials in patients with clinically overt AD to subjects in the preclinical phase, prior to widespread brain damage and clinical symptoms of the disease have occurred, in the hope that this will lead to positive results.[72,73]
143 agents are currently in development for AD (2022) according to the clintrials.

gov website. Mechanisms of action range from anti-inflammatory agents to the classic acetylcholinesterase inhibitors, which lead to symptomatic improvement of cognitive dysfunction, but most of them are disease modifying treatments (83.2%). The disease modifying treatments (DMT) can be divided into biologicals and small molecules. Biologicals are generally derived from living organisms and include antibodies, vaccines, antisense oligonucleotides (ASOs), and therapeutic proteins. The term small molecules refer to drugs typically taken orally that are <500 Daltons in size and can regulate a biological process.[74] In 2022, 14 trials include subjects with preclinical AD which is slightly more than in 2021 (which in turn was more than in 2020 [8 versus 4 trials]-2021). Among the phase 2 trials 7 studies recruited subjects with preclinical AD and among phase 3 trials, another 7 studies recruited subjects with preclinical AD. Table 1 gives an overview of the DMTs in development in the three clinical phases with their mechanism of action as described by Cummings et al., (2022).[74]

In June 2021 the first drug aimed at disease modification was approved for the treatment of AD. This may change the Alzheimer's Disease research field completely. Aducanumab, or Aduhelm®, promises to remove amyloid plaques from the brain that have accumulated due to AD disease process. Aducanumab is a human monoclonal antibody that selectively binds to Aβ aggregates, including soluble oligomers and insoluble fibrils and removes Aβ plaques in the brain, which has been demonstrated using florbetapir PET.[75] Two large phase 3 clinical trials, however, resulted in inconclusive results: after 18 months of treatment with aducanumab there were no reproducible clinical benefits and there was no correlation between the degree of amyloid lowering and the main clinical outcome measures.[76] The advisory board to the FDA consisting of experts in the field advised against approval of aducanumab as the studies did not prove clinical efficacy of aducanumab in the treatment of AD. Nevertheless, the FDA approved the drug in the United States of America through the 'acceleration pathway' which allows approval of drugs that do not (yet) show clinical benefit but do show effects at a biomarker level, in this case lowering of amyloid in the brain measured by PET scan. When a disease is deemed 'serious or life-threatening and a drug may provide meaningful therapeutic benefit over existing treatments by having demonstrated to influence a surrogate endpoint that is reasonably likely to predict a clinical benefit to patients the acceleration pathway can be used to approve a drug, awaiting long term clinical trial results, even when there remains some uncertainty about the drug's clinical benefit.[77] The decision of the FDA to approve the drug is considered highly debatable, as the advice of the advisory board was in this case not followed. Several members of the advisory board resigned

after this decision. The actual clinical benefit of aducanumab clearly must still be proven. If aducanumab proves not only to reduce amyloid plaques in the brain but also that long term use and removal of these plaques results in clinical benefit for AD patients, this may be considered as proof of the 'amyloid hypothesis', which states that misfolding and aggregation of beta amyloid is the primary cause of AD. Future research will then more likely target amyloid in an earlier stage to prevent the development of AD. As no drug has proven that targeting amyloid in the brain leads to clinical improvement, the amyloid hypothesis has been under discussion and it is debated if removing amyloid is the best way to move forward in AD research, causing disunity in the AD research field.

Very important for further development of aducanumab but also for other new disease modifying compounds for the treatment of AD, is correct study subject selection. For aducanumab, the discussion related to which study subjects best to enroll in further clinical trials is already ongoing. Initially the FDA approved aducanumab for all patients with AD, but they now adjusted the approval by restricting the label to patients with mild cognitive impairment or mild AD, in which the drug was also tested in the phase 3 clinical trials. The advice of the FDA to start treatment in the MCI phase or prodromal phase of AD in which only mild symptoms are present and with less severe disease pathology, is based on the expectation that treatment at these stages will lead to greater clinical benefit. Again, the importance of proper characterization of clinical trial subjects (and patients receiving newly approved treatments) is emphasized by the FDA.

OUTLINE OF THIS THESIS

This thesis comprises publications based on a several studies in healthy elderly subjects, subjects with preclinical AD and subjects with neurodegenerative diseases that were all aimed at gaining a better understanding of the difference between these subject groups and a better characterization of potential candidates for clinical trial participation in (preclinical) AD. In these studies, different biomarkers were investigated to gain more insight into healthy elderly, elderly with preclinical AD and patients with AD in order to better understand the course over time of AD biomarkers as the disease progresses and to better select the optimal potential clinical trial participants for new disease modifying treatments being developed for AD. **Chapter II** describes a large dataset analysis in which the NeuroCart, a computerized test battery to measure neuropsychological and neurophysiological performance, was used to assess age-related decline in test performance and whether test outcomes differentiate between healthy subjects and patients with Alzheimer's Disease, Parkinson's Disease, Huntington's Disease and Vascular dementia.

Chapter III reviews animal models of AD and the translation to human AD based on diagnostic and prognostic biomarkers. Using animal models to understand AD may help to fill the knowledge gap of the pathophysiological systems involved in AD and to better define the preclinical stage of AD.

In **Chapter IV** and **Chapter V** of this thesis we discuss a study in the context of which we aimed to characterize subjects with preclinical AD and how these subjects can be selected for clinical trial participation using an algorithm including multiple neuropsychological tests and blood-based diagnostic biomarkers. The aim of this study was to reduce the need for invasive procedures (e.g., lumbar puncture) in otherwise healthy elderly trial subjects.

Chapter VI and **Chapter VII** discuss how new diagnostic biomarkers for AD behave in preclinical AD. Inflammatory responses that are expected to be involved in AD pathology were measured in plasma samples to investigate if these biomarkers are already different in subjects with preclinical AD compared to healthy elderly. Phosphorylated tau, which recently has shown to be a good predictive biomarker for the development of AD, was measured in CSF and plasma with the goal to replicate previous research.

Finally, **Chapter VIII** aims to integrate all previous chapters and discusses the issue of the selection of study participants for clinical trials with disease modifying therapies being developed for AD, putting it into in a broader perspective considering the ethical point of view of selecting preclinical subjects for trials.

Table 1 Alzheimer's Disease Drug development pipeline: Disease modifying treatment in development for Alzheimer's Disease in phase 1, 2 and 3.[74]

Phase	Mechanism class	Mechanism of action
Phase 1, 27 DMT / (90% of phase 1 compounds)/ 9 biologicals / 18 small molecules	inflammation	CSF-1R antagonist; attenuates microglial proliferation and neurodegeneration
		NRTI; reduce neuroinflammation
		Non-steroidal anti-inflammatory to reduce inflammation
		Regulatory T cells
		TNF inhibitor; reduce neuroinflammation
	epigenetic regulators	Extending telomeres may benefit AD; reduce Aβ-induced neurotoxicity; effects on multiple cellular pathways
		NNRTI; promote cholesterol removal; enhance amyloid reduction.
		10hApoE2, serotype rh. Ten AAV gene transfer vector expressing the cDNA coding for human ApoE ε2, directly to the CNS/CSF of ApoE ε4 homozygotes with AD
		Histone deacetylase (HDAC) inhibitor; enhanced synaptic plasticity
	amyloid	Monoclonal antibody targeting soluble Aβ
		Monoclonal antibody to reduce Aβ
		Anti-amyloid monoclonal antibody
	tau	O-GlycNAcase Inhibitor
		Monoclonal antibody to reduce tau
		Anti-tau monoclonal antibody
	proteostasis	Aβ and tau aggregation inhibitor; inhibits neuronal death
		Prevents Aβ and tau aggregation
		Aggregation inhibitor
	synaptic plasticity/ neuroprotection	mGluR5 allosteric modulator
		Lysine-gingipain inhibitor
		Regulates calcium dyshomeostasis; tau and Aβ reduction
	neurogenesis	GABA-A receptor modulator; promote neurogenesis and reduce inflammation
		Enhance neurogenesis; activates progenitor cells
	vasculature	Direct thrombin inhibitor; reduce neurovascular damage
		Angiotensin II receptor blocker
	autophagy	Induces autophagy and promotes clearance of aggregated proteins
	metabolism and bioenergetics	Caprylic triglyceride

(continuation Table 1)

Phase	Mechanism class	Mechanism of action
Phase 2, 71 DMT (86.6% of phase 2 compounds) / 26 biologicals / 45 small molecules / 7 studies in preclinical AD	inflammation	Monoclonal antibody targeting TREM2 receptors to promotemicroglial clearance of Aβ
		Janus kinase inhibitor; reduces neuroinflammation
		Immunomodulator
		Anti-IL-1β monoclonal antibody
		Herb with antioxidant and anti-inflammatory properties
		Monoclonal antibody targeting CD38; regulates microglial activity
		Tyrosine kinase inhibitor (dasatinib) and flavonoid (quercetin); senolytic therapy approach to reduce senescent cells and tau aggregation
		Regulatory T cells; reduce neuroinflammation
		Reduce inflammatory cytokines; modulate innate and adaptive immune responses
		Dietary amino acid; reduce brain inflammation and preserve nerve cells
		Cysteinyl leukotriene type 1 (cysLT-1) receptor antagonist; effects on inflammatory processes, neuronal injury, blood-brain-barrier integrity, and Aβ protein accumulation
		Monoclonal antibody directed at semaphoring 4D to reduce inflammation
		Granulocyte macrophage colony stimulating factor
		Calcium-activated potassium channel blocker
		Monoclonal antibody targeting galactin 3
		Immune reaction to diphtheria, pertussis, tetanus vaccine
		Antiviral against HSV-1 and -2 infection; to prevent Aβ aggregation and plaque deposition
	synaptic plasticity/ neuroprotection	PDE-4 inhibitor; prolongs cAMP activity and improves neuronal plasticity
		Protein Kinase C inhibitor; facilitates synaptogenesis
		Guanylate cyclase positive allosteric modulator
		Neurotrophic agent; activates sigma receptors to preserve synaptic plasticity; protect against Aβ toxicity
		Sigma-2 receptor antagonist; competes with oligomeric Aβ binding; protect against Aβ-induced synaptic toxicity
		Plasma transfusion from exercise-trained donors
		Activates signaling via the hepatocyte growth factor system to regenerate neurons and enhance synaptic plasticity

(continuation Table 1)

Phase	Mechanism class	Mechanism of action
Phase 2, 71 DMT (86.6% of phase 2 compounds) / 26 biologicals / 45 small molecules / 7 studies in preclinical AD		SV2A modulator; improve synaptic function; reduce Aβ-induced neuronal hyperactivity
		p38MAPK-α inhibitor
		p38MAPK-α inhibitor; enhance endolysosomal function to reduce synaptic dysfunction
		Filamin A protein inhibitor; stabilizes the interaction of soluble Aβ and the alpha7 nicotinic acetylcholine receptor, reducing Aβ and synaptic dysfunction
	amyloid	Glutamatemodulator; prodrug of riluzole; improve synaptic function
		Active immunotherapy to remove Aβ
		Alpha-secretase modulator to reduce Aβ production
		Monoclonal antibody targeting soluble Aβ oligomers
		Monoclonal antibody specific for pyroglutamate Aβ
		Monoclonal antibody directed at Aβ plaques and oligomers
		Anti-Aβ monoclonal antibody (gantenerumab) with enhanced blood-brain barrier penetration
		Monoclonal antibody directed at protofibrils
		Sirtuin-nicotinamide adenine dinucleotide stimulator to enhance alpha-secretase
		Activates transport protein ABCC1 to remove Aβ
		Prodrug of tramiprosate; inhibits Aβ aggregation into toxic oligomers
		Glutaminyl cyclase (QC) enzyme inhibitor to reduce pyroglutamate Aβ production
	tau	Active immunotherapy targeting tau
		Anti-tau monoclonal antibody
		Anti-tau monoclonal antibody
		Antisense oligonucleotide targeting tau expression; MAPT RNA inhibitor
		Monoclonal antibody targeting soluble tau
		O-GlycNAcase inhibitor; promote tau glycosylation, prevent tau aggregation
		HDAC inhibitor; to reduce tau-induced microtubule depolymerization and tau phosphorylation
		Heat shock protein 90 inhibitor; to prevent aggregation and hyperphosphorylation of tau
		Monoclonal antibody to remove extracellular tau

(continuation Table 1)

Phase	Mechanism class	Mechanism of action
Phase 2, 71 DMT (86.6% of phase 2 compounds) / 26 biologicals / 45 small molecules / 7 studies in preclinical AD	metabolism and bioenergetics	SGLT2 inhibitor; to improve insulin sensitivity and CNS glucose metabolism
		Decrease glucose resistance and increase insulin signaling in the brain
		SGLT2 inhibitor (empagliflozin) and insulin combination therapy; decrease glucose resistance and increase insulin signaling in the brain
		Dual agonist of PPARδ/γ; reduce glucose and lipid metabolism
	proteostasis	Polyphenolic compound; antioxidant; prevent aggregation of Aβ and tau
		Inhibitor of APP and α-synuclein
		mTOR inhibitor; ameliorate metabolic and vascular effects of aging
	vasculature	Polyunsaturated fatty acid; reduce damage to small blood vessels
		Angiotensin II receptor blocker (telmisartan); angiotensin converting enzyme inhibitor (perindopril)
		Cerebral blood flow enhancer
	neurotransmitter receptors	Dopamine agonist with anti-Aβ effects
		NMDA receptor antagonist
		Dual Orexin receptor antagonist; improved sleep with effects on CSF Aβ
	epigenetic regulators	hTERT peptide vaccine; mimics extra-telomeric functions to inhibit neurotoxicity, apoptosis, and reactive oxygen species
		Nucleoside reverse transcriptase inhibitor; reduces genetic rearrangements
	growth factors and hormones	GnRH receptor agonist; reduce effects of elevated GnRH and gonadotropins on the brain
		11-beta-hydroxysteroid dehydrogenase type 1 inhibitor
	neurogenesis	Allosteric modulator of GABA-A receptors
		Endothelin B receptor agonist; augments activity of neuronal progenitor cells
	cell death	Iron chelating agent; reduce damaging reactive oxygen species
	ApoE, lipids and lipoprotein receptors	Cholesteryl ester transfer protein (CETP) inhibitor
	oxidative stress	Omega 3 fatty acid; improve synaptic function; antioxidant

(continuation Table 1)

Phase	Mechanism class	Mechanism of action
Phase 3, 21 DMT (67.8% of phase 3 compounds)/ 5 biologicals / 16 small molecules / 7 trials in preclinical AD	amyloid	Monoclonal antibody directed at Aβ plaques and oligomers
		Monoclonal antibody specific for pyroglutamate form of Aβ
		Monoclonal antibody directed at Aβ plaques and oligomers
		Monoclonal antibody directed at Aβ protofibrils
		Monoclonal antibody directed at Aβ monomers
		Prodrug of tramiprosate; inhibits Aβ aggregation into toxic oligomers
	Combination of amyloid DMTs	Monoclonal antibody specific for pyroglutamate form of Aβ (donanemab); monoclonal antibody directed at plaques and oligomers (aducanumab); given in separate arms of the trial
	Combination of amyloid DMTs	Monoclonal antibody directed at Aβ plaques and oligomers (gantenerumab); Monoclonal antibody directed at Aβ monomers (solanezumab); given in separate arms of the trial
	synaptic 4 plasticity/ neuroprotection	SV2A modulator; to reduce Aβ-induced neuronal hyperactivity
		Bacterial protease inhibitor targeting gingipain produced by P. gingivalis to reduce neuroinflammation and hippocampal degeneration
		Sigma-1 receptor agonist, M2 autoreceptor antagonist; to ameliorate oxidative stress, protein misfolding, mitochondrial dysfunction, and inflammation
		Filamin A protein inhibitor; stabilizes amyloid-alpha-7 nicotinic receptor interaction
	oxidative stress	Free radical scavenger
		Purified form of the omega-3 fatty acid EPA; to improve synaptic function and reduce inflammation
		Antioxidant
	metabolism and bioenergetics	Insulin sensitizer to improve CNS glucose metabolism
		GLP-1 agonist; reduces neuroinflammation and improves insulin signaling in the brain
		Caprylic triglyceride; induces ketosis and improves mitochondrial and neuronal function
	tau	Tau protein aggregation inhibitor
	inflammation	MAPK-1/3 inhibitor; reduces proinflammatory NFκB activation
	proteostasis	Tyrosine kinase inhibitor; autophagy enhancer; promotes clearance of Aβ and tau
	vasculature	Angiotensin II receptor blocker (losartan), calcium channel blocker (amlodipine), cholesterol agent (atorvastatin)
	gut-brain axis	Algae-derived acidic oligosaccharides; changes microbiome to reduce peripheral and central inflammation

REFERENCES

1 López-Otín, C., et al., *The hallmarks of aging.* Cell, 2013. **153**(6): p. 1194-1217.
2 Sousa, N., *Clinical studies in the elderly – Why and how should we care?* Aging Brain, 2021. **1**: p. 100001.
3 Peters, R., *Ageing and the brain.* Postgraduate medical journal, 2006. **82**(964): p. 84-88.
4 Lezak, M.D., et al., *Neuropsychological assessment, 5th ed.* Neuropsychological assessment, 5th ed. 2012, New York, NY, US: Oxford University Press. xxv, 1161-xxv, 1161.
5 Schliebs, R. and T. Arendt, *The cholinergic system in aging and neuronal degeneration.* Behav Brain Res, 2011. **221**(2): p. 555-63.
6 Huang, L.-K., S.-P. Chao, and C.-J. Hu, *Clinical trials of new drugs for Alzheimer disease.* Journal of Biomedical Science, 2020. **27**(1): p. 18.
7 Tata, A.M., et al., *Cholinergic system dysfunction and neurodegenerative diseases: cause or effect?* CNS Neurol Disord Drug Targets, 2014. **13**(7): p. 1294-303.
8 Denver, P. and P.L. McClean, *Distinguishing normal brain aging from the development of Alzheimer's disease: inflammation, insulin signaling and cognition.* Neural regeneration research, 2018. **13**(10): p. 1719-1730.
9 Przedborski, S., M. Vila, and V. Jackson-Lewis, *Neurodegeneration: what is it and where are we?* The Journal of clinical investigation, 2003. **111**(1): p. 3-10.
10 Seeley, W.W., *Mapping Neurodegenerative Disease Onset and Progression.* Cold Spring Harbor perspectives in biology, 2017. **9**(8): p. a023622.
11 Katsuno, M., et al., *Preclinical progression of neurodegenerative diseases.* Nagoya journal of medical science, 2018. **80**(3): p. 289-298.
12 Benilova, I., E. Karran, and B. De Strooper, *The toxic clothes.* Nature Neuroscience, 2012. **15**(3): p. 349-357.
13 Ahmad, K., et al., *Commonalities in Biological Pathways, Genetics, and Cellular Mechanism between Alzheimer Disease and Other Neurodegenerative Diseases: An In Silico-Updated Overview.* Current Alzheimer research, 2017. **14**(11): p. 1190-1197.
14 Nussbaum, R.L. and C.E. Ellis, *Alzheimer's Disease and Parkinson's Disease.* New England Journal of Medicine, 2003. **348**(14): p. 1356-1364.
15 Roos, R.A.C., *Huntington's disease: a clinical review.* Orphanet Journal of Rare Diseases, 2010. **5**(1): p. 40.
16 Salmon, D.P., et al., *Differentiation of Alzheimer's Disease and Huntington's Disease With the Dementia Rating Scale.* Archives of Neurology, 1989. **46**(11): p. 1204-1208.
17 Harvey, P.D., *Domains of cognition and their assessment* Dialogues in clinical neuroscience, 2019. **21**(3): p. 227-237.
18 Kane, R.L. and G.G. Kay, *Computerized assessment in neuropsychology: a review of tests and test batteries.* Neuropsychol Rev, 1992. **3**(1): p. 1-117.
19 Zucchella, C., et al., *Neuropsychological testing.* Pract Neurol, 2018. **18**(3): p. 227-237.
20 Groeneveld, G.J., J.L. Hay, and J.M. Van Gerven, *Measuring blood-brain barrier penetration using the NeuroCart, a CNS test battery.* Drug Discov Today Technol, 2016. **20**: p. 27-34.
21 Schatz, P. and J. Browndyke, *Applications of computer-based neuropsychological assessment.* J Head Trauma Rehabil, 2002. **17**(5): p. 395-410.
22 WHO. https://www.who.int/news-room/fact-sheets/detail/dementia. https://www.who.int/news-room/fact-sheets/detail/dementia 2021 02 September 2021 [cited 2021 07Oct2021].
23 *2021 Alzheimer's disease facts and figures.* Alzheimers Dement, 2021. **17**(3): p. 327-406.
24 Alzheimer, A., *Uber eine eigenartige Erkrankung der Hirnrinde.* 1907(64): p. 146-8.
25 Barage, S.H. and K.D. Sonawane, *Amyloid cascade hypothesis: Pathogenesis and therapeutic strategies in Alzheimer's disease.* Neuropeptides, 2015. **52**: p. 1-18.
26 Chow, V.W., et al., *An overview of APP processing enzymes and products.* Neuromolecular medicine, 2010. **12**(1): p. 1-12.
27 Hardy, J., *Has the amyloid cascade hypothesis for Alzheimer's disease been proved?* Curr Alzheimer Res, 2006. **3**(1): p. 71-3.
28 Michno, W., et al., *Pyroglutamation of amyloid-βx-42 (Aβx-42) followed by Aβ1–40 deposition underlies plaque polymorphism in progressing Alzheimer's disease pathology.* Journal of Biological Chemistry, 2019. **294**(17): p. 6719-6732.
29 Tomic, J.L., et al., *Soluble fibrillar oligomer levels are elevated in Alzheimer's disease brain and correlate with cognitive dysfunction.* Neurobiology of Disease, 2009. **35**(3): p. 352-358.
30 Wang, W.-Y., et al., *Role of pro-inflammatory cytokines released from microglia in Alzheimer's disease.* Annals of translational medicine, 2015. **3**(10): p. 136-136.

31 Serrano-Pozo, A., et al., *Neuropathological alterations in Alzheimer disease*. Cold Spring Harbor perspectives in medicine, 2011. **1**(1): p. a006189-a006189.

32 Barbier, P., et al., *Role of Tau as a Microtubule-Associated Protein: Structural and Functional Aspects*. Front Aging Neurosci, 2019. **11**: p. 204.

33 Tracy, T.E. and L. Gan, *Tau-mediated synaptic and neuronal dysfunction in neurodegenerative disease*. Current opinion in neurobiology, 2018. **51**: p. 134-138.

34 Xiao, X., et al., APP, PSEN1, and PSEN2 *Variants in Alzheimer's Disease: Systematic Re-evaluation According to* ACMG *Guidelines*. Front Aging Neurosci, 2021. **13**: p. 695808.

35 Lanfranco, M.F., C.A. Ng, and G.W. Rebeck, *ApoE Lipidation as a Therapeutic Target in Alzheimer's Disease*. Int J Mol Sci, 2020. **21**(17).

36 Morris, G.P., I.A. Clark, and B. Vissel, *Inconsistencies and Controversies Surrounding the Amyloid Hypothesis of Alzheimer's Disease*. Acta Neuropathologica Communications, 2014. **2**(1): p. 135.

37 Jack, C.R., et al., *Hypothetical model of dynamic biomarkers of the Alzheimer's pathological cascade*. The Lancet Neurology, 2010. **9**(1): p. 119-128.

38 Hansson, O., et al., *Prediction of Alzheimer's Disease Using the* CSF *Aβ42/Aβ40 Ratio in Patients with Mild Cognitive Impairment*. Dementia and Geriatric Cognitive Disorders, 2007. **23**(5): p. 316-320.

39 DeCarli, C., *Mild cognitive impairment: prevalence, prognosis, aetiology, and treatment*. Lancet Neurol, 2003. **2**(1): p. 15-21.

40 Hansson, O., et al., *Association between* CSF *biomarkers and incipient Alzheimer's disease in patients with mild cognitive impairment: a follow-up study*. The Lancet Neurology, 2006. **5**(3): p. 228-234.

41 Jack, C.R., Jr., et al., *Introduction to the recommendations from the National Institute on Aging-Alzheimer's Association workgroups on diagnostic guidelines for Alzheimer's disease*. Alzheimers Dement, 2011. **7**(3): p. 257-62.

42 Dubois, B., et al., *Preclinical Alzheimer's disease: Definition, natural history, and diagnostic criteria*. Alzheimer's & dementia: the journal of the Alzheimer's Association, 2016. **12**(3): p. 292-323.

43 Cohen, A.F., et al., *The Use of Biomarkers in Human Pharmacology (Phase I) Studies*. Annual Review of Pharmacology and Toxicology, 2015. **55**(1): p. 55-74.

44 Califf, R.M., *Biomarker definitions and their applications*. Experimental biology and medicine (Maywood, N.J.), 2018. **243**(3): p. 213-221.

45 FDA-NIH *Biomarker Working Group*. BEST *(Biomarkers, EndpointS, and other Tools) Resource. Silver Spring* (MD): *Food and Drug Administration* (US); *Bethesda* (MD): *National Institutes of Health* (US), *www.ncbi.nlm.nih.gov/books/NBK326791/*. 2016.

46 Tapiola, T., et al., *Cerebrospinal fluid {beta}-amyloid 42 and tau proteins as biomarkers of Alzheimer-type pathologic changes in the brain*. Arch Neurol, 2009. **66**(3): p. 382-9.

47 Palmqvist, S., et al., *Accuracy of Brain Amyloid Detection in Clinical Practice Using Cerebrospinal Fluid β-Amyloid 42: A Cross-Validation Study Against Amyloid Positron Emission Tomography*. JAMA Neurology, 2014. **71**(10): p. 1282-1289.

48 Cummings, J., *The National Institute on Aging – Alzheimer's Association Framework on Alzheimer's disease: Application to clinical trials*. Alzheimer's & Dementia, 2019. **15**(1): p. 172-178.

49 Fagan, A.M., et al., *Cerebrospinal Fluid tau/β-Amyloid42 Ratio as a Prediction of Cognitive Decline in Nondemented Older Adults*. Archives of Neurology, 2007. **64**(3): p. 343-349.

50 Hansson, O., et al., *Advantages and disadvantages of the use of the* CSF *Amyloid β (Aβ) 42/40 ratio in the diagnosis of Alzheimer's Disease*. Alzheimers Res Ther, 2019. **11**(1): p. 34.

51 Zetterberg, H. and B.B. Bendlin, *Biomarkers for Alzheimer's disease – preparing for a new era of disease-modifying therapies*. Molecular Psychiatry, 2021. **26**(1): p. 296-308.

52 Nakamura, A., et al., *High performance plasma amyloid-β biomarkers for Alzheimer's disease*. Nature, 2018. **554**(7691): p. 249-254.

53 Schindler, S.E., et al., *High-precision plasma β-amyloid 42/40 predicts current and future brain amyloidosis*. Neurology, 2019. **93**(17): p. e1647-e1659.

54 Shaw, L.M., et al., *Cerebrospinal fluid biomarker signature in Alzheimer's disease neuroimaging initiative subjects*. Ann Neurol, 2009. **65**(4): p. 403-13.

55 Pontecorvo, M.J., et al., *A multicentre longitudinal study of flortaucipir (18F) in normal ageing, mild cognitive impairment and Alzheimer's disease dementia*. Brain: a journal of neurology, 2019. **142**(6): p. 1723-1735.

56 Palmqvist, S., et al., *Cerebrospinal fluid and plasma biomarker trajectories with increasing amyloid deposition in Alzheimer's disease*. EMBO Mol Med, 2019. **11**(12): p. e11170.

57 Karikari, T.K., et al., *Blood phosphorylated tau 181 as a biomarker for Alzheimer's disease: a diagnostic performance and prediction modelling study using data from four prospective cohorts*. Lancet Neurol, 2020. **19**(5): p. 422-433.

58 Villar-Piqué, A., et al., *Plasma* YKL-40 *in the spectrum of neurodegenerative dementia*. Journal of Neuroinflammation, 2019. **16**(1): p. 145.

59 Suárez-Calvet, M., et al., *Early increase of* CSF *sTREM2 in Alzheimer's disease is associated with tau related-neurodegeneration but not with amyloid-β pathology*. Molecular Neurodegeneration, 2019. **14**(1): p. 1.

60 Pereira, J.B., et al., *Plasma* GFAP *is an early marker of amyloid-β but not tau pathology in Alzheimer's disease*. Brain, 2021.

61 Mattsson, N., et al., *Cerebrospinal fluid tau, neurogranin, and neurofilament light in Alzheimer's disease*. EMBO molecular medicine, 2016. **8**(10): p. 1184-1196.

62 Zetterberg, H. and K. Blennow, *Moving fluid biomarkers for Alzheimer's disease from research tools to routine clinical diagnostics*. Molecular Neurodegeneration, 2021. **16**(1): p. 10.

63 McKhann, G., et al., *Clinical diagnosis of Alzheimer's disease: report of the* NINCDS-ADRDA *Work Group under the auspices of Department of Health and Human Services Task Force on Alzheimer's Disease*. Neurology, 1984. **34**(7): p. 939-44.

64 Fearing, M.A., et al., *Autopsy-confirmed Alzheimer's disease versus clinically diagnosed Alzheimer's disease in the Cache County Study on Memory and Aging: a comparison of quantitative* MRI *and neuropsychological findings*. J Clin Exp Neuropsychol, 2007. **29**(5): p. 553-60.

65 McKhann, G.M., et al., *The diagnosis of dementia due to Alzheimer's disease: recommendations from the National Institute on Aging-Alzheimer's Association workgroups on diagnostic guidelines for Alzheimer's disease*. Alzheimers Dement, 2011. **7**(3): p. 263-9.

66 Jack, C.R., Jr., et al., NIA-AA *Research Framework: Toward a biological definition of Alzheimer's disease*. Alzheimer's & dementia: the journal of the Alzheimer's Association, 2018. **14**(4): p. 535-562.

67 Hariton, E. and J.J. Locascio, *Randomised controlled trials - the gold standard for effectiveness research: Study design: randomised controlled trials*. BJOG: an international journal of obstetrics and gynaecology, 2018. **125**(13): p. 1716-1716.

68 Cummings, J.L., *Defining and labeling disease-modifying treatments for Alzheimer's disease*. Alzheimers Dement, 2009. **5**(5): p. 406-18.

69 FDA. *https://www.fda.gov/news-events/press-announcements/fda-grants-accelerated-approval-alzheimers-drug*. https://www.fda.gov/news-events/press-announcements/fda-grants-accelerated-approval-alzheimers-drug 2021 content current as of 06Jul2021 [cited 2021 23Nov2021].

70 Yiannopoulou, K.G., et al., *Reasons for Failed Trials of Disease-Modifying Treatments for Alzheimer Disease and Their Contribution in Recent Research*. Biomedicines, 2019. **7**(4): p. 97.

71 Anderson, R.M., et al., *Why do so many clinical trials of therapies for Alzheimer's disease fail?* The Lancet, 2017. **390**(10110): p. 2327-2329.

72 Wang, J., L. Tan, and J.-t. Yu, *Prevention Trials in Alzheimer's Disease: Current Status and Future Perspectives*. Journal of Alzheimer's Disease, 2016. **50**: p. 927-945.

73 Rafii, M.S. and P.S. Aisen, *Alzheimer's Disease Clinical Trials: Moving Toward Successful Prevention*. CNS drugs, 2019. **33**(2): p. 99-106.

74 Cummings, J., et al., *Alzheimer's disease drug development pipeline: 2022*. Alzheimers Dement (N Y), 2022. **8**(1): p. e12295.

75 Sevigny, J., et al., *The antibody aducanumab reduces Aβ plaques in Alzheimer's disease*. Nature, 2016. **537**(7618): p. 50-56.

76 Gandy, S., D.S. Knopman, and M. Sano, *Talking points for physicians, patients and caregivers considering Aduhelm® infusion and the accelerated pathway for its approval by the* FDA. Molecular Neurodegeneration, 2021. **16**(1): p. 74.

77 FDA. *https://www.fda.gov/patients/fast-track-breakthrough-therapy-accelerated-approval-priority-review/accelerated-approval*. https://www.fda.gov/patients/fast-track-breakthrough-therapy-accelerated-approval-priority-review/accelerated-approval 2018 Content current as of 01Apr2018 [cited 2021 23Nov2021].

CHAPTER II

Cognitive performance in healthy clinical trial participants and patients with the NeuroCart, a neurodegenerative disease measured with an automated neuropsychological and neurophysiological test battery

Samantha Prins,[1,2] Laura Borghans,[1,2] Marieke L. de Kam,[1] Geert Jan Groeneveld,[1,2]
Joop van Gerven[1,2]

SUBMITTED TO NEUROBIOLOGY OF AGING 2022

1. Centre for Human drug Research, Leiden, NL | 2. Leiden University Medical Center, Leiden, NL

ABSTRACT

BACKGROUND The prevalence of neurodegenerative diseases increases significantly with increasing age. Neurodegeneration is the progressive loss of function of neurons that eventually leads to cell death, which in turn leads to cognitive disfunction. Cognitive performance can therefore also be considered age dependent. The current study investigated if the NeuroCart can detect age related decline on drug-sensitive CNS-tests in healthy volunteers (HV), and whether there are interactions between the rates of decline and sex. This study also investigated if the NeuroCart was able to differentiate disease profiles of neurodegenerative diseases, compared to age-matched HV and if there is age related decline in patient groups.

METHODS This retrospective study encompassed 93 studies, performed at CHDR between 2005 and 2020 that included NeuroCart measurements, which resulted in data from 2729 subjects. Five NeuroCart tests were included in this analysis: smooth and saccadic eye movements, body sway, adaptive tracking, VVLT and N-back. Data from 84 healthy male and female volunteer studies, aged 16-90, were included. Nine studies were performed in patients with Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD) or vascular dementia (VaD). The data were analyzed with regression analyses on age by group, sex, sex by age, group by sex and group by sex by age. Least square means (LSMs) and 95% confidence intervals (CIs) were calculated for each group at the average age of the group, and at the average age of each of the other groups, and per sex.

RESULTS Mean age and standard deviation (SD) for all groups was: HV 36.2 years (19.3), AD 68.3 years (8), PD 62.7 years (8.5), HD 51.4 years (9.8) and VaD 66.9 years (8.1). Performance on all NeuroCart tests decreased significantly each year in HV. Saccadic peak velocity (SPV) was increased in AD compared to age-matched HV (+26.28 degrees/s, p =0.007), while SPV was decreased for PD and HD compared to age-matched HV (PD: -15.87 degrees/s, p=0.038, HD: -22.52 degrees/s, p=0.018). In HD patients SPV decreased faster with age compared to HV. On saccadic peak velocity the slopes between HD vs HV were significantly different, indicating a faster decline in performance on this task for HD patients compared to HV per age year. Smooth pursuit showed an overall significant difference between subject groups (p=0.037. Significantly worse performance was found for AD (-12.87%, p=<0.001), PD (-4.45%, p=<0.001) and VaD (-5.69%, p=0.005) compared to age-matched HV. Body sway significantly increased with age (p=0.021). Postural stability was decreased for both PD and HD compared to age-matched HV (PD: +38.8%, p=<0.001, HD: 154.9%, p=<0.001). The adaptive tracking was significantly decreased with age (p=<0.001). Adaptive tracking performance by AD (-7.54%, p=<0.001), PD (-8.09%, p=<0.001), HD (-5.19%, p=<0.001) and VaD (-5.80%, p=<0.001) was decreased compared to age-matched HV. Adaptive tracking in PD patients vs HV and in PD vs HD patients was significantly different, indicating a faster decline on this task per age year for PD patients compared to HV and HD. The VVLT delayed word recall showed an overall significant effect of subject group (p=0.006. Correct delayed word recall was decreased for AD (-5.83 words, p=<0.001), HD (-3.40 words, p=<0.001) and VaD (-5.51 words, p=<0.001) compared to age-matched HV.

CONCLUSION This study showed that the NeuroCart can detect age-related decreases in performance in HV, which were not affected by sex. The NeuroCart was able to detect significant differences in performance between AD, PD, HD, VAD and age-matched HV. Disease durations were unknown, therefore this cross-sectional study was not able to show age-related decline after disease onset. This article shows the importance of investigating age-related decline on digitalized neurocognitive test batteries. Performance declines with age, which emphasizes the need to correct for age when including HV in clinical trials. Patients with different neurogenerative diseases have distinct performance patterns on the NeuroCart , which this should be considered when performing NeuroCart tasks in patients with AD, PD, HD and VAD.

BACKGROUND

The prevalence of neurodegenerative diseases increases significantly with increasing age.[1] Neurodegeneration is the progressive loss of function of neurons, eventually leading to cell death which in turn leads to cognitive disfunction.[2] Cognitive performance can therefore also be considered age dependent. A subtle but consistent decline in cognitive performance is noticeable when a person ages, not only in case of neurodegenerative diseases but also with normal aging.[3–5] At a certain point, cognitive decline is not considered as age-related cognitive decline but decline due to neurodegeneration, which can have many causes e.g., dementia.

Cognition is defined by the ability of humans to acquire knowledge, understanding through thought, experience and senses and can be classified by different domains (e.g., memory, attention, executive functioning) in which there can be overlap of functions; for instance attention that is needed when performing a task involving memory.[6] Cognitive change is quantified by measuring performance on different domains with standardized neuropsychological tests, that can, most of the time, be corrected for education level.[7] Education can influence cognitive performance, as cognitive reserve makes a subject more resilient to deterioration of cognitive function.[8] Traditionally, neuropsychological tests are 'pen and paper' tasks, performed (as the name reveals) with pencils and paper and administered by trained neuropsychologists. However, human error and inter-rater variability are not uncommon.[9,10] The past decades multiple pen and paper tasks have been digitalized with great advantages such as standardized test administration, reduced inter-rater variability and less time-consuming procedures.[11] The NeuroCart is an example of a digital neuropsychological and neurophysiological test battery, developed and used by the Centre of Human Drug Research (CHDR).[12] The advantage of the NeuroCart is that this test battery can easily be implemented in (early phase) drug development.

The NeuroCart has been used for over two decades in clinical studies both in healthy volunteers (HV) as well as in studies with patients suffering from neurodegenerative diseases. NeuroCart assessments are used to identify subtle cognitive changes when administering new (pro) cognitive compounds.[12] After extensive use of the NeuroCart, enough data has been gathered to make valid assumptions about age related decline measured with the NeuroCart.

Different neurodegenerative diseases have distinct profiles in cognitive decline, although overlap in decline in cognitive functions is not uncommon.[13,14] For instance, memory deficits occur in Alzheimer's Disease (AD), Parkinson's Disease (PD) and Huntington's Disease (HD) although in different forms and with different symptomatic features.[15] These neurodegenerative diseases do not have the same progression in cognitive decline and different cognitive domains are affected in different stages of the disease.[16,17]

The current study investigated if the NeuroCart is able to detect age related decline on tests in healthy volunteers, and whether there is an interaction between the rate of decline and sex. This study also investigated if the NeuroCart is able to differentiate disease profiles of neurodegenerative diseases, compared to healthy volunteers in the same age group and if there is age related decline in patient groups. Implementing the results of this analysis in future research may lead to better subject selection for clinical research. If, for instance, a compound is developed to improve working memory function, normal age-related deterioration could be used as a model of cognitive impairment. Moreover, early development studies in healthy subjects that are age-matched to the target population, will provide more relevant outcomes for subsequent clinical trials in patients. Age-linked biomarkers may also be more sensitive to cognitive enhancers or other compounds for age-related diseases, than tests which are not affected by aging. Determination of NeuroCart-test related to ageing or neurodegenerative diseases can also generate benchmarks for 'clinical' relevance of drug effects. This could be relevant for cognitive challenge models, aiming to induce cognitive decline in healthy volunteers (e.g., mecamylamine, biperiden, scopolamine challenge models[18–20]), which can be interpreted better by comparing results to normal aging and disease profiles. Similarly, age- or disease-related changes can provide a frame of reference for effects of cognitive enhancers and disease modifying pro-cognitive drugs. All these reasons warranted an analysis of the age-relatedness of NeuroCart tests in healthy volunteers and patients with different neurodegenerative conditions that have been collected at CHDR in the past fifteen years.

METHODS

This retrospective study encompassed 93 studies, performed at CHDR between 2005 and 2020 that included NeuroCart measurements, which resulted in 2729 subjects with data from at least one of five NeuroCart tests. From the 93 studies, 9 studies were performed in patients with AD, PD, HD or vascular dementia (VAD). Data from 84 healthy male and female volunteer studies, aged 16-90, were included. The following five NeuroCart tests covering different functional domains were selected that have been used in a substantial number of studies.

EYE MOVEMENTS – SMOOTH AND SACCADIC MOVEMENTS Analysis of smooth pursuit and saccadic eye movements are frequently used for the assessment of (side) effects of drugs involving the central nervous system. The use of a computer for measurement of saccadic eye movements was originally described by Baloh et al.[21] and for smooth pursuit by Bittencourt et al.,[22] and has been extensively validated at the CHDR, e.g., by Van Steveninck et al.[23] The subjects were required to follow a light source with the eyes, which moved horizontally on a screen at 58 cm distance. The light source moved continuously with increasing speed for measurement of smooth pursuit and jumped from side to side with slightly varying intervals for saccadic eye movements. The duration of each of the tests was approximately 1 minute. The test parameter for smooth pursuit eye movements was the percentage of time the subject's eyes were in smooth pursuit of the target. For saccadic eye movement, the parameter peak velocity (deg/s) was extracted. Eye movements were recorded in a quiet room with dimmed lightning and with only one study subject in the room.

BODY MOVEMENT – BODY SWAY The body sway meter allows measurement of body movements in a single plane, providing a simple measure of postural stability. Body sway is measured with a pot string meter based on the Wright ataxia meter.[24] At CHDR, the method has been frequently used to demonstrate effects of sleep deprivation,[25] alcohol,[26] benzodiazepines.[26,27] among many others. With a string attached to the waist, all body movements over a period of 2 minutes were integrated and expressed as millimetre (mm) sway. Subjects were instructed to wear comfortable, low-heeled shoes, asked to stand still and comfortably, with their feet approximately 10 centimetres (cm) apart and their hands in a relaxed position alongside the body and eyes closed. Subjects were not allowed to talk during the measurement. The total period of body-sway measurement was two minutes.

ATTENTION AND EYE-HAND COORDINATION – ADAPTIVE TRACKING The adaptive tracking test was performed as originally described by Borland and Nicholson,[28,29] using customised equipment and software (based on TrackerUSB hard-/software (Hobbs, 2004, Hertfordshire, UK)). Adaptive tracking is a pursuit-tracking task that measures (sustained) attention and executive functioning. A circle moved randomly on a screen, and the subject had to try and keep a dot inside the moving circle by operating a joystick. As long as this effort was successful, the speed of the moving circle increased. Conversely, the velocity was reduced if the test subject was unable to maintain the dot inside the circle. The percentage

of correct performance (dot in circle) was used for analysis. The tests took 3.5 minutes, including a run-in time of 0.5 minute, in which data are not recorded.

MEMORY CONSOLIDATION – VISUAL VERBAL LEARNING TASK, DELAYED RECOGNITION Visual verbal learning.[30,31] contains three different subtests that cover basic aspects of learning behaviour: acquisition, consolidation, storage, and retrieval. Subjects that performed the Visual Verbal Learning Test (VVLT) were presented 30 words (or 15 words for subjects with dementia) in three consecutive word trials, i.e., word learning test (VVLT30 or VVLT15). Approximately thirty minutes after start of the first trial, the subjects were asked to recall as many words as possible (delayed recall – this test measures active retrieval from long term memory). Subjects were not allowed to write down words at any time during the test. Correct words were recorded (correct response), as well as words that were mentioned more than once (double response) and words that were mentioned but not presented (incorrect response). For this study, the number of correct recalls during the delayed recall condition were used in the analyses. CHDR created a computerized VVLT script based on a script from the University of Maastricht. Since the VVLT aims to avoid ceiling effects while also preventing overtaxing of subjects, patients with Alzheimer's disease performed the VVLT15 version with 15 words, as memory performance is strongly affected in this group. All other studies included the VVLT30 words version.

WORKING MEMORY – N-BACK, ONE-BACK The N-Back test measures working memory. Different versions of the N-Back test were employed in studies investigating the neural basis of working memory.[32] The test has also been widely used for measuring working memory deficits.[32–34] Performing the N-Back test requires buffering and updating consonants, matching, encoding and responding.[35] The version of the N-Back used at CHDR is a shorter version compared to the original version of Rombouts et al.[34] The maximal duration for this test was 10 minutes. Following Rombouts et al. (2002),[34] the N-Back test consisted of three conditions, with increased working memory load. In condition 0 ('X' condition), subjects were required to indicate whether the presented letter is a 'X' (=target) or another letter. In Condition 1 and 2, letters were presented sequentially (1.5 seconds for a letter [consonant, except for the letter 'z'], followed by a black screen for 0.5 seconds). Key 'z' was pressed for a target and '/' was pressed for a non-target. Condition 1, '1-back' condition, in which subjects were required to indicate whether the letter presented earlier, was a repetition without any other letter intervening (e.g., B … B); In condition 2, '2-back' condition, subjects were required to indicate

whether a letter was repeated with one other letter in between (e.g., B … C … B). The 3 conditions were presented in 3 blocks with increasing working memory load. Each condition started with a training (7 consonants; target:non-target 3:4), followed by the test (24 consonants; target:non-target 1:3). For the current analysis, the 1-back condition was used in the analyses.

Only the baseline values (before possible drug intervention) of these tests were used in this analysis, except for the VVLT. The VVLT was measured once during the intervention, and so only the values measured under placebo were used. When more baseline values per subject were available, the average of the baseline values was analysed. All tests except body movement, were performed in all five groups: HV, AD, HD, PD and VaD. To prevent falls in the most fragile subjects with dementia, body sway was measured in only three groups: HV, HD and PD.

STATISTICAL ANALYSIS

The data of selected NeuroCart tests were analyzed with regression analyses on age by group, sex, sex by age, group by sex and group by sex by age. The regression results are presented as the age, group, sex and interaction effects; the intercept and slope per group; the contrasts of the slopes of the groups; and the 'age-matched' contrasts of each disease group and HV at the mean age of the disease group. Least square means (LSMS) and 95% confidence intervals (CIS) are given for each group at the average age of the group, and at the average age of each of the other groups, and per sex and average ages.

When a subject participated in multiple studies of this batch analyses, the average age of this subject was used to calculate the mean age of the total group. For calculating age effect per NeuroCart test, the exact age at the time of test performance was calculated, but floor age (e.g., age 30.5 = age 30) was used for graphs and in the regression for all subjects.

All calculations were performed using SAS (version 9.4, SAS, Cary, NC).

RESULTS

In Table 1 the basic characteristics of the subjects included in this study are presented. Subjects were categorized into HV or patient (AD, PD, HD, VAD) as a total group. This table also demonstrates the average scores on the NeuroCart tests for the groups.

Table 1 Basic characteristics and average test scores on NeuroCart tests for healthy volunteers, Alzheimer's Disease patients, Parkinson's Disease patients, Huntington's Disease patients and Vascular dementia patients.

	Healthy volunteers	Alzheimer's Disease patients	Parkinson's Disease patients	Huntington's Disease patients	Vascular dementia
Mean age (median, total range)	N=2511 36.2 (26, 15-89)	N=63 68.3 (69, 49-90)	N=74 62.7 (64, 40-80)	N=51 51.4 (53, 21-69)	N=30 66.9 (68, 46-82)
Sex, female, mean age (median, total range)	N=711 40.7 (31, 16-83)	N=30 67.9 (70, 49-90)	N=27 60.6 (61, 40-75)	N=22 47.8 (51, 21-69)	N=9 65.3 (66, 55-73)
Sex, male, mean age (median, total range)	N=1800 34.5 (25, 15-89)	N=33 68.6 (69, 57-82)	N=47 63.9 (65, 46-80)	N=29 54 (54, 39-67)	N=21 67.6 (71, 46-82)
Saccadic peak velocity (degrees/s), mean (SD)	N=2232 490.1 (59.31)	N=39 498.1 (58.05)	N=71 453.8 (59.30)	N=44 459.1 (66.13)	N=30 479.0 (79.08)
Smooth pursuit (%), mean (SD)	N=1835 43.65 (10.700)	N=50 23.77 (12.500)	N=74 33.19 (8.891)	N=48 37.30 (7.090)	N=30 30.85 (7.930)
Body sway (mm), geometric mean (SD)	N=1994 250.2 (52.0)	Not available	N=72 363.0 (64.7)	N=49 649.3 (96.9)	Not available
Adaptive Tracking (%), mean (SD)	N=2185 26.86 (6.245)	N=62 15.01 (7.531)	N=74 15.05 (5.942)	N=48 19.43 (7.588)	N=30 17.15 (5.590)
VVLT-delayed recall (number correct), Mean (SD)	N=912 10.630/30 (6.403)	N=62 1.048/15 (1.750)	N=14 5.571/30 (2.827)	N=40 6.400/30 (4.112)	N=27 2.111/30 (1.928)
N-back (one back ratio), mean (SD)	N=853 0.9134 (0.1709)	N=10 0.3710 (0.6088)	N=25 0.8804 (0.1508)	Not available	Not available

Table 2 presents the decrease in performance per age year compared to no (0) decrease, for each of the tests on the NeuroCart for HV and patients in the different neurogenerative disease groups. Performance on all NeuroCart tests decreased significantly each year in HV, compared to no decrease. Performance on the adaptive tracking task decreased significantly for both HV as AD and PD patients.

Table 2 Change in performance per age year (=slope) per group, Healthy volunteers, Alzheimer's disease, Parkinson's disease, Huntington's disease and Vascular dementia patients.

	Healthy volunteers	Alzheimer's Disease patients	Parkinson's Disease patients	Huntington's Disease patients	Vascular dementia
Saccadic peak velocity (degrees/s)	-0.557*	-1.230	-0.619	1.486	0.131
Smooth pursuit (% point)	-0.202*	0.127	-0.181	-0.003	-0.372
Body sway (%)	0.328*	NA	1.18	0.961	NA
Adaptive tracking (% point)	-0.130*	-0.281*	-0.295*	-0.026	-0.232
VVLT delayed word recall (number correct)	-0.166*	-0.001	-0.135	-0.013	-0.087

Significant: p=<0.05, NA = Not Available

Figure 1-5 visually plot the data per NeuroCart test per age year and per subject group. Regression lines were added to the figures to visually represent the decrease in performance. The body sway data was log transformed as the data was not normally distributed. Since the performance on the 1-back task is expressed as a ratio score no regression analyses could be performed, hence no graphical representation is provided for the N-back test. Figure 6 represents all individual scores on the N-back of HV, AD and PD. A pattern of decrease after the age of 50 can be assumed based on this data, which also suggested worse performance in AD compared to HV.

Figure 1 Overall plots of estimated regression lines per subject population (Healthy volunteers, Alzheimer's disease, Parkinson's disease, Huntington's disease and Vascular dementia patients) for Saccadic peak velocity (degrees/s).

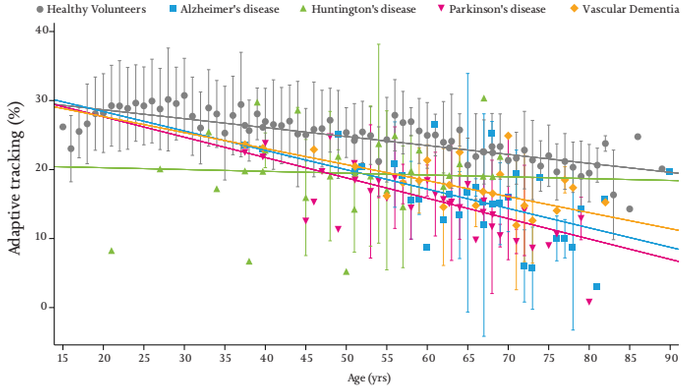


Figure 2 Overall plots of estimated regression lines per subject population (Healthy volunteers, Alzheimer's disease, Parkinson's disease, Huntington's disease and Vascular dementia patients) for Smooth pursuit (%) eye movements.

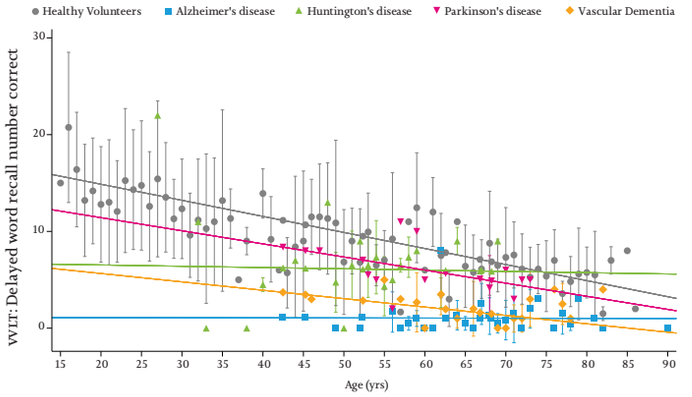


Figure 3 Overall plots of estimated regression lines per subject population (Healthy volunteers, Parkinson's disease and Huntington's disease) for Body sway (log mm).

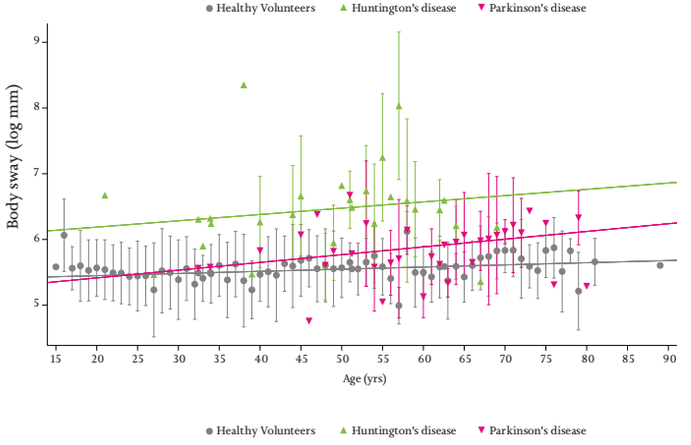


Figure 4 Overall plots of estimated regression lines per subject population (Healthy volunteers, Alzheimer's disease, Parkinson's disease, Huntington's disease and Vascular dementia patients) for Adaptive tracker (%).

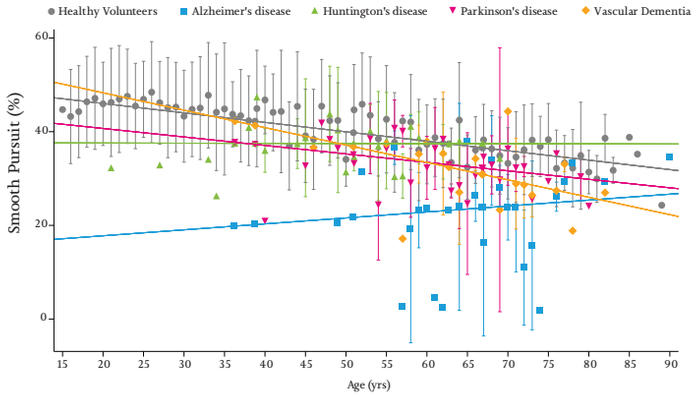


Figure 5 Overall plots of estimated regression lines per subject population (Healthy volunteers, Alzheimer's disease, Parkinson's disease, Huntington's disease and Vascular dementia patients) for VVLT delayed word recall (number correct).

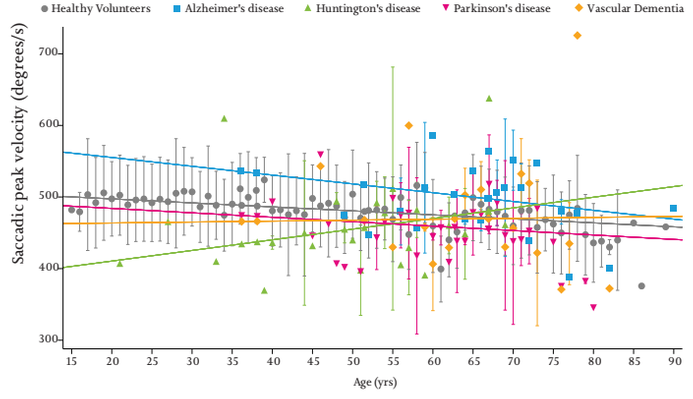
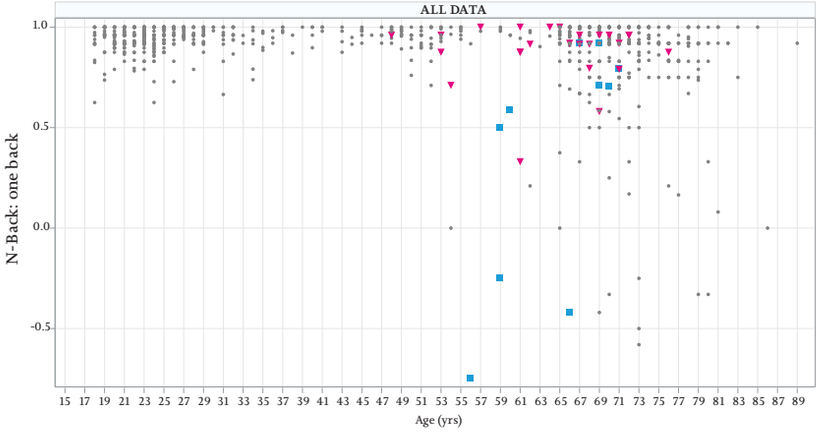


Figure 6 Individual plot of N-Back: one-back condition in healthy volunteers, Alzheimer's disease and Parkinson's disease.



To investigate the overall effect of age on the NeuroCart tests, linear regression analyses were performed. In addition to this, least square means (LSMs) and 95% confidence intervals (CIs) were calculated for each patient group, comparing performance between patient and HV at the average age of the respective patient group.

Saccadic peak velocity (SPV) was increased in AD compared to age-matched HV (+26.28 degrees/s, p =0.007). In PD, SPV was decreased compared to age-matched HV (-15.87 degrees/s, p=0.038. This was also the case in HD-patients (-22.52 degrees/s) who showed an age-related decrease in SPV compared with HV, as demonstrated by the significant difference in slope (Figure 1).

Smooth pursuit eye movements showed an overall significant difference between subject groups (p=0.037). Significantly worse performance was found for AD (-12.87%, p=<0.001), PD (-4.45%, p=<0.001) and VaD (-5.69%, p=0.005) compared to age-matched HV.

Body sway significantly increased with age (p=0.021). Furthermore, both PD and HD show decreased postural stability compared to age-matched HV (PD: +38.8%, p=<0.001, HD: 154.9%, p=<0.001).

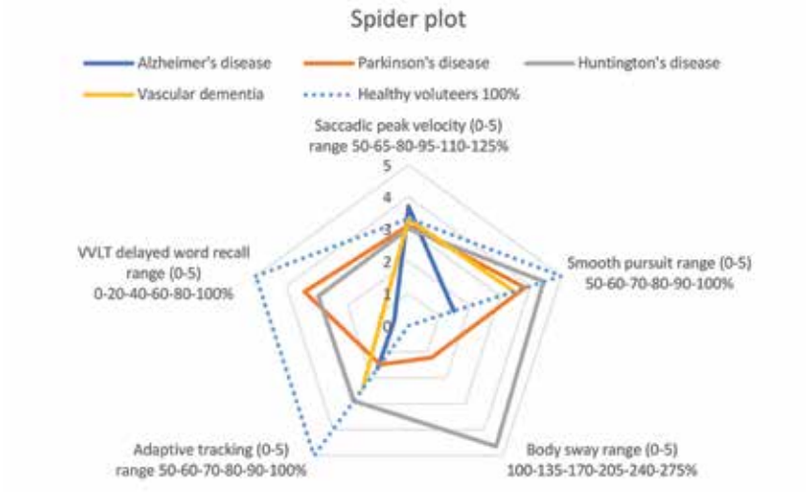
Adaptive tracking decreased significantly with age (p=<0.001). Adaptive tracking performance by subjects with AD (-7.54%, p=<0.001), PD (-8.09%, p=<0.001), HD (-5.19%, p=<0.001) and VaD (-5.80%, p=<0.001) was decreased compared to

age-matched HV. The differences in slopes between PD vs HV and PD vs HD were significant, indicating a faster decline on this task per age year for PD patients compared to HV and HD.

The VVLT delayed word recall showed an overall significant effect of subject group (p=0.006), indicating worse memory performance in patients. Correct delayed recall was decreased for AD (-5.83 words, p=<0.001), HD (-3.40 words, p=<0.001) and VaD (-5.51 words, p=<0.001) compared to age-matched HV.

A spider plot was created to visualize the NeuroCart disease profiles for AD, PD, HD and VAD compared to HV. The spider plot summarizes the performance on the NeuroCart per group and per test, see figure 7.

Figure 7 Spider plot summarizing the NeuroCart performance of patients with Alzheimer's Disease, Parkinson's Disease, Huntington's Disease and Vascular Dementia, compared to healthy volunteers at 100%.



DISCUSSION

This study investigated whether the NeuroCart can detect age-related decline in NeuroCart performance in close to 3000 healthy volunteers and specific patients, and whether there is an interaction between group, age and sex. Based on these results the NeuroCart showed age-related decreases in performance in HV, which were not affected by sex. The NeuroCart was able to detect significant differences in performance between AD, PD, HD, VAD and age-matched HV. Because disease durations were unknown, this cross-sectional study was not able to show age-related decline after disease onset. Therefore, the rate of deterioration as a consequence of neurodegenerative disease independent of age could not be quantified reliably.

The NeuroCart is a digitalized neuropsychological- and neurophysiological test battery, used in early phase drug development to detect (subtle) changes in performance of healthy volunteers and patients after the administration of a CNS-active (including pro-cognitive) compounds, and (thereby) to detect penetration of the blood brain barrier and target engagement.[12] Age-related decreases in performance in healthy volunteers were demonstrated on five different NeuroCart tests: smooth and saccadic eye movements, adaptive tracking, body sway, VVLT and N-Back. Age-related decline on cognitive tests corresponds to previous literature on cognitive decline at older age,[36] but this was not yet reported for most digitalized tests within the NeuroCart.

Patients with PD and VAD performed comparable to HV on the smooth and saccadic eye movement task. AD patients performed worse on the smooth pursuit eye movement task but better on the saccadic eye movement task compared to the other patient groups and HV. In AD, abnormalities of both smooth pursuit eye movements and saccadic eye movements have been previously reported.[37] A study found decreased saccadic peak velocity in a small number of AD patients compared to age-matched HV, which contrasts with our findings. However, this was only the case when visual stimuli were 'unpredictable', which may have been different from our test setup.[38] These authors also detected more abnormal or delayed saccades in AD, which was not analyzed in the current study. In another study, smooth pursuit eye movements differed significantly between AD and HV, similar to what was found in this current study with a significant difference between AD and age-matched HV.[39] As Moser et al., (1995) suggest, these somewhat discrepant results could be due to the different phases of the disease in the AD patients. In the current dataset the AD group was mostly in the early phase of the disease, considering the relatively low mean age of 68.3 years old, which was confounded by the requirement for legal competence in the studies in which they participated. Partly for safety reasons, body sway was not performed in AD and VaD patients, but this test resulted in worse postural stability for HD and PD compared to HV. Both PD and HD are movement disorders and previous literature confirm these findings using similar tests as the body sway.[40,41]

Most of the NeuroCart tests (smooth and saccadic eye movements, body sway, VVLT and N-Back) did not show age-related decline within any of the patient

groups. Only adaptive tracking test demonstrated age-related decline not only in HV but also in patients with AD and PD, whereas a non-significant decline was seen in HD and VAD patients. Adaptive tracking is affected by different CNS-functions, particularly sustained attention, eye-hand coordination and vigilance, which may render this test more sensitive to worsening not only during normal aging, but also to different forms and sites of neurodegeneration.

Attention is controlled by the prefrontal cortex, which is one of the first brain areas that deteriorates in both normal aging and most age-related neurodegenerative diseases.[42,43] The memory test VVLT was specifically worse in AD and VAD patients compared to HV, HD and PD. AD patients did not show a significant additional decline in word recall with age, but an overall poorer performance compared to the other groups.[44] It must be noted that in the current dataset, AD patients took an adjusted version of the test with 15 words instead of 30, to avoid overstraining, but this test was still performed worse than the more difficult 30-word version in all other subject groups. Looi et al., (1999) compared neuropsychological test performance between AD and VAD and found VAD to perform better on memory tasks than AD patients,[44] which is in line with the current data set. Although no quantitative regression analyses could be performed on the percentage scores of the N-back test results, the results do suggest decreased performance with age. A pattern of decrease after the age of 50 can be surmised based on the data from the individual scores of HV, AD and PD on the one-back task. Furthermore, the AD population seems to score lower on accuracy on the one-back paradigm of the N-Back task than HV. Fraga et al., (2018) measured event-related desynchronization with EEG in AD patients while performing the N-Back task and found a clear difference between the performance of HV and AD, which was already present in the mild cognitive impairment stage.[45]

No apparent age-related decline could be detected in the patient groups, other than on the adaptive tracking test for AD and PD. This might be explained by the decrease in cognitive performance in patients after disease onset, which could have obscured detection of additional effects of aging. Linear analyses were appropriate to investigate the decline in performance in HV with a large age range of 16 to 90 years old. In the patients' groups however, linear regression analysis may not be appropriate in patients as age ranges were smaller. Moreover, in neurodegenerative diseases performance does not decrease in a linear fashion.[46] No conclusion can be made about the rate of decline in performance on the NeuroCart of patients compared to HV, as our data did not comprise longitudinal data. Patients were generally younger (~62 years) than in comparable studies, in which the disease may have progressed for a longer period. In AD patients, memory decline was worse than expected for their age, as indicated by their particularly poor performance on a simpler VVLT version. As using a linear model did not suit the patient data, the average age per patient group was compared to the performance of healthy volunteers at that same age. All patients with neurodegenerative disease show worse performance compared to age-matched HV. Overall, the NeuroCart seems to differentiate patient groups from HVs, which is of relevance when administering NeuroCart tests in clinical research, as this can be expected to affect study outcome.

Several studies tried to mimic cognitive neurodegenerative disorders by inducing cognitive deficits in otherwise healthy subjects, and furthermore to reverse these deficits by administering a pro-cognitive compound; the so-called pharmacological challenge models of cognitive impairment.[18–20] Bakker et al., (2021) investigated the effect of 4mg biperiden p.o. in healthy elderly subjects and found a decrease in performance on several NeuroCart tests (adaptive tracking -3.04% to -1.15%; VVLT delayed recall -5.9 to -0.2 words; body sway 79.7mm increase; and smooth pursuit eye movements -5.58% to -1.53%).[19] The effect of this challenge test on cognitive test performance is less than the decreased performance of AD patients found in this study (adaptive tracking -7.5%; VVLT delayed recall -5.9 words; smooth pursuit eye movements -12.9%). Baakman et al., (2017).[20] used another challenge model, where they administered 0.5 mg scopolamine in healthy male subjects. Their findings seem to agree better with our results in patients (adaptive tracking -10.4% accuracy; VVLT delayed recall -7.1 words), but the sedative effect of scopolamine is known to negatively influence results of cognitive performance.[47]

This study shows the importance of investigating age-related decline on digitalized cognitive test batteries. The fact that performance declines with age emphasizes the need to correct or match for age when including HV in clinical trials. Patients with neurogenerative diseases have different performance patterns on the NeuroCart and this should be considered when performing digitalized neurocognitive tasks in patients with AD, PD, HD and VAD. In addition, the current dataset provides a frame of reference for impairment models and (adverse or pro-cognitive) effects of CNS-active drugs.

REFERENCES

1 Mayeux, R., *Epidemiology of neurodegeneration*. Annu Rev Neurosci, 2003. **26**: p. 81-104.
2 Katsnelson, A., B.D. Strooper, and H.Y. Zoghbi, *Neurodegeneration: From cellular concepts to clinical applications*. Science Translational Medicine, 2016. **8**(364): p. 364ps18-364ps18.
3 Harada, C.N., M.C. Natelson Love, and K.L. Triebel, *Normal Cognitive Aging*. Clinics in Geriatric Medicine, 2013. **29**(4): p. 737-752.
4 Juan, S.M.A. and P.A. Adlard, *Ageing and Cognition*. Subcell Biochem, 2019. **91**: p. 107-122.
5 Salthouse, T.A., *Trajectories of normal cognitive aging*. Psychol Aging, 2019. **34**(1): p. 17-24.
6 Harvey, P.D., *Domains of cognition and their assessment* Dialogues Clin Neurosci, 2019. **21**(3): p. 227-237.
7 Lezak, M.D., et al., *Neuropsychological assessment, 5th ed*. Neuropsychological assessment, 5th ed. 2012, New York, NY, US: Oxford University Press. xxv, 1161-xxv, 1161.
8 Stern, Y., *Cognitive reserve in ageing and Alzheimer's disease*. The Lancet Neurology, 2012. **11**(11): p. 1006-1012.
9 White, R.F., et al., *Interrater reliability of neuropsychological diagnoses: a Department of Veterans Affairs cooperative study*. J Int Neuropsychol Soc, 2002. **8**(4): p. 555-65.
10 Kozora, E., et al., *Effects of examiner error on neuropsychological test results in a multi-site study*. Clin Neuropsychol, 2008. **22**(6): p. 977-88.
11 Schatz, P. and J. Browndyke, *Applications of computer-based neuropsychological assessment*. J Head Trauma Rehabil, 2002. **17**(5): p. 395-410.
12 Groeneveld, G.J., J.L. Hay, and J.M. Van Gerven, *Measuring blood-brain barrier penetration using the NeuroCart, a CNS test battery*. Drug Discov Today Technol, 2016. **20**: p. 27-34.
13 Levy, J.A. and G.J. Chelune, *Cognitive-Behavioral Profiles of Neurodegenerative Dementias: Beyond Alzheimer's Disease*. Journal of Geriatric Psychiatry and Neurology, 2007. **20**(4): p. 227-238.
14 Ophey, A., et al., *Cognitive profiles of patients with mild cognitive impairment due to Alzheimer's versus Parkinson's disease defined using a base rate approach: Implications for neuropsychological assessments*. Alzheimers Dement (Amst), 2021. **13**(1): p. e12223.
15 Heindel, W., et al., *Neuropsychological evidence for multiple implicit memory systems: a comparison of Alzheimer's, Huntington's, and Parkinson's disease patients*. The Journal of Neuroscience, 1989. **9**(2): p. 582-587.
16 Pillon, B., et al., *Severity and specificity of cognitive impairment in Alzheimer's, Huntington's, and Parkinson's diseases and progressive supranuclear palsy*. Neurology, 1991. **41**(5): p. 634-43.
17 Pillon, B., et al., *Explicit Memory in Alzheimer's, Huntington's, and Parkinson's Diseases*. Archives of Neurology, 1993. **50**(4): p. 374-379.
18 Alvarez-Jimenez, R., et al., *Reversal of mecamylamine-induced effects in healthy subjects by nicotine receptor agonists: Cognitive and (electro) physiological responses*. Br J Clin Pharmacol, 2018. **84**(5): p. 888-899.
19 Bakker, C., et al., *Biperiden Challenge Model in Healthy Elderly as Proof-of-Pharmacology Tool: A Randomized, Placebo-Controlled Trial*. J Clin Pharmacol, 2021.
20 Baakman, A.C., et al., *An anti-nicotinic cognitive challenge model using mecamylamine in comparison with the anti-muscarinic cognitive challenge using scopolamine*. Br J Clin Pharmacol, 2017. **83**(8): p. 1676-1687.
21 Baloh, R.W., et al., *Quantitative measurement of saccade amplitude, duration, and velocity*. Neurology, 1975. **25**(11): p. 1065-70.
22 Bittencourt, P.R., et al., *Benzodiazepines impair smooth pursuit eye movements*. Br J Clin Pharmacol, 1983. **15**(2): p. 259-62.
23 van Steveninck, A.L., et al., *A comparison of the sensitivities of adaptive tracking, eye movement analysis and visual analog lines to the effects of incremental doses of temazepam in healthy volunteers*. Clin Pharmacol Ther, 1991. **50**(2): p. 172-80.
24 Wright, B.M., *A simple mechanical ataxia-meter*. J Physiol, 1971. **218 Suppl**: p. 27p-28p.
25 van Steveninck, A.L., et al., *The sensitivity of pharmacodynamic tests for the central nervous system effects of drugs on the effects of sleep deprivation*. J Psychopharmacol, 1999. **13**(1): p. 10-7.
26 van Steveninck, A.L., et al., *Pharmacodynamic interactions of diazepam and intravenous alcohol at pseudo steady state*. Psychopharmacology, 1993. **110**(4): p. 471-478.
27 van Steveninck, A.L., et al., *A study of the effects of long-term use on individual sensitivity to temazepam and lorazepam in a clinical population*. Br J Clin Pharmacol, 1997. **44**(3): p. 267-75.
28 Borland, R.G. and A.N. Nicholson, *Visual motor co-ordination and dynamic visual acuity*. Br J Clin Pharmacol, 1984. **18 Suppl 1**(Suppl 1): p. 69s-72s.
29 Borland, R.G. and A.N. Nicholson, *Comparison of the residual effects of two benzodiazepines (nitrazepam and flurazepam hydrochloride) and pentobarbitone sodium on human performance*. Br J Clin Pharmacol, 1975. **2**(1): p. 9-17.
30 de Haas, S.L., et al., *The pharmacokinetic and pharmacodynamic effects of SL65.1498, a GABA-A alpha2,3 selective agonist, in comparison with lorazepam in healthy volunteers*. J Psychopharmacol, 2009. **23**(6): p. 625-32.
31 Van der Elst, W., *The neuropsychometrics of aging [electronic resource]: normative studies in the Maastricht Aging Study*. 2006.
32 Owen, A.M., et al., *N-back working memory paradigm: a meta-analysis of normative functional neuroimaging studies*. Hum Brain Mapp, 2005. **25**(1): p. 46-59.
33 Lim, H.K., et al., *Altered verbal working memory process in patients with Alzheimer's disease: an fMRI investigation*. Neuropsychobiology, 2008. **57**(4): p. 181-7.
34 Rombouts, S.A., et al., *Alterations in brain activation during cholinergic enhancement with rivastigmine in Alzheimer's disease*. J Neurol Neurosurg Psychiatry, 2002. **73**(6): p. 665-71.
35 Sweet, L.H., et al., *Functional magnetic resonance imaging response to increased verbal working memory demands among patients with multiple sclerosis*. Hum Brain Mapp, 2006. **27**(1): p. 28-36.
36 Murman, D.L., *The Impact of Age on Cognition*. Seminars in hearing, 2015. **36**(3): p. 111-121.
37 Molitor, R.J., P.C. Ko, and B.A. Ally, *Eye movements in Alzheimer's disease*. Journal of Alzheimer's disease: JAD, 2015. **44**(1): p. 1-12.
38 Fletcher, W.A. and J.A. Sharpe, *Saccadic eye movement dysfunction in Alzheimer's disease*. Ann Neurol, 1986. **20**(4): p. 464-71.
39 Moser, A., D. Kömpf, and J. Olschinka, *Eye Movement Dysfunction in Dementia of the Alzheimer Type*. Dementia and Geriatric Cognitive Disorders, 1995. **6**(5): p. 264-268.
40 Talman, L.S. and A.L. Hiller, *Approach to Posture and Gait in Huntington's Disease*. Frontiers in Bioengineering and Biotechnology, 2021. **9**(632).
41 Apthorp, D., et al., *Postural sway correlates with cognition and quality of life in Parkinson's disease*. BMJ Neurol Open, 2020. **2**(2): p. e000086.
42 Zanto, T.P. and A. Gazzaley, *Chapter 20 – Aging of the frontal lobe*, in *Handbook of Clinical Neurology*, M. D'Esposito and J.H. Grafman, Editors. 2019, Elsevier. p. 369-389.
43 Cabeza, R. and N. Dennis, *Frontal lobes and aging: Deterioration and compensation*. Principles of Frontal Lobe Function, 2013: p. 628-652.
44 Looi, J.C. and P.S. Sachdev, *Differentiation of vascular dementia from AD on neuropsychological tests*. Neurology, 1999. **53**(4): p. 670-8.
45 Fraga, F.J., et al., *Early diagnosis of mild cognitive impairment and Alzheimer's with event-related potentials and event-related desynchronization in N-back working memory tasks*. Comput Methods Programs Biomed, 2018. **164**: p. 1-13.
46 Katsuno, M., et al., *Preclinical progression of neurodegenerative diseases*. Nagoya journal of medical science, 2018. **80**(3): p. 289-298.
47 Pergolizzi, J.V., et al., *Perspectives on transdermal scopolamine for the treatment of postoperative nausea and vomiting*. Journal of Clinical Anesthesia, 2012. **24**(4): p. 334-345.

CHAPTER III

Utility of animal models to understand human Alzheimer's disease, using the mastermind research approach to avoid unnecessary further sacrifices of animals

Tian Qin[1], Samantha Prins [2], Geert Jan Groeneveld[2], Gerard Van Westen[3], Helga E. de Vries[4], Yin Cheong Wong[5], Luc J.M. Bischoff[1] and Elizabeth C.M. de Lange [1]

INT. J. MOL. SCI. 2020, 21, 3158; DOI:10.3390/IJMS21093158, EPUB 30 APRIL 2020

1. Predictive Pharmacology, Division of Systems Biomedicine and Pharmacology, Leiden Academic Centre of Drug Research, Leiden University, Leiden, NL | 2. Centre for Human Drug Research (CHDR), Leiden, NL | 3. Computational Drug Discovery, Division of Drug Discovery and Safety, Leiden Academic Centre of Drug Research, Leiden University, Leiden, NL | 4. Neuro-immunology research group, Department of Molecular Cell Biology and Immunology, Amsterdam Neuroscience, Amsterdam UMC, Amsterdam, NL | 5. Advanced Modelling and Simulation, UCB Celltech, Slough, UK

ABSTRACT

To diagnose and treat early-stage (preclinical) Alzheimer's disease (AD) patients, we need body-fluid-based biomarkers that reflect the processes that occur in this stage, but current knowledge on associated processes is lacking. As human studies on (possible) onset and early-stage AD would be extremely expensive and time-consuming, we investigate the potential value of animal AD models to help to fill this knowledge gap. We provide a comprehensive overview of processes associated with AD pathogenesis and biomarkers, current knowledge on AD-related biomarkers derived from on human and animal brains and body fluids, comparisons of biomarkers obtained in human AD and frequently used animal AD models, and emerging body-fluid-based biomarkers. In human studies, amyloid beta (Aβ), hyperphosphorylated tau (P-TAU), total tau (T-TAU), neurogranin, SNAP-25, glial fibrillary acidic protein (GFAP), YKL-40, and especially neurofilament light (NFL) are frequently measured. In animal studies, the emphasis has been mostly on Aβ. Although a direct comparison between human (familial and sporadic) AD and (mostly genetic) animal AD models cannot be made, still, in brain, cerebrospinal fluid (CSF), and blood, a majority of similar trends are observed for human AD stage and animal AD model life stage. This indicates the potential value of animal AD models in understanding of the onset and early stage of AD. Moreover, animal studies can be smartly designed to provide mechanistic information on the interrelationships between the different AD processes in a longitudinal fashion and may also include the combinations of different conditions that may reflect comorbidities in human AD, according to the Mastermind Research approach.

INTRODUCTION

Alzheimer's disease (AD) is a complex progressive neurodegenerative disorder and is the most common cause of dementia. AD can roughly be divided into two types: familial AD (~5% of total AD patients) and late-onset (LOAD) or sporadic AD (~95% of total AD patients). Familial AD is caused by mutations in either the APP gene or in the genes encoding presenilin 1 (PSEN1) or presenilin 2 (PSEN2), which are essential components of the γ-secretase complex.[1] These mutations lead to the elevation of total amyloid beta (Aβ), a higher $A\beta_{1\text{-}42}/A\beta_{1\text{-}40}$ ratio, and Aβ aggregation.[2] In sporadic AD, *t*he disturbance of Aβ clearance mechanisms is thought to be the major contribution to Aβ accumulation in the brain, but a (causal) relationship is not fully understood.[3,4] In contrast, it is well-established that an increased frequency of the *ApoE ε4* allele indicates increased risk to develop AD.[5,6] The *ApoE ε4* allele plays an important role in several AD-related processes, such as the oxidative stress response,[7] synaptic loss,[8] Aβ accumulation.,[9] and ApoE/LRPI-mediated Aβ clearance.[4] Studies with transgenic ApoE−/− mice showed that these mice develop blood-brain barrier (BBB) breakdown. *ApoE ε4* drives the matrix metalloproteinase 9 (MPP-9)-mediated BBB dysfunction that finally contributes to disturbed influx/efflux of Aβ across the BBB.[10]

Different stages in AD progression have been defined.[11,12]: the first is the preclinical stage or asymptomatic stage. It occurs between the earliest pathogenic events of AD and the first appearance of specific cognitive changes, which are different from the changes observed in normal ageing. This asymptomatic stage might take many years to develop.[13,14] The second stage is the prodromal stage and is defined by mild cognitive impairment (MCI). In this stage, cognitive changes and amnestic symptoms are present. Importantly, MCI is not selective for AD as not all individuals with MCI develop AD, but individuals with MCI have an increased risk of developing AD or other forms of dementia.[15] In the third and final stage of AD, brain Aβ plaques and neurofibrillary tau tangles (NFTs) may appear on imaging tests of the brain. Individuals at this stage lose control of physical functions and depend on others for care. They sleep more often and are unable to communicate or even recognize their loved ones.

Currently there is no treatment for AD other than some symptomatic treatments that do not slow down or halt AD progression. It is thought that treatment options for AD modification will be more effective during the preclinical stage.[11,16-19] Postmortem autopsy of the AD brain, which then shows atrophy, neuronal loss, Aβ plaques, and NFTs, is the only certain AD diagnosis.[20,21] During life, clinical evaluation of AD considers cognitive deficits by neuropsychological assessments

and measurements of $A\beta_{1-42}$ and total tau (T-TAU) in cerebrospinal fluid (CSF).[22,23] The CSF $A\beta_{1-42}$ level and $A\beta_{1-42}/A\beta_{1-40}$ ratio have now been widely accepted as valid indicators of brain accumulation of Aβ.[24] Furthermore, imaging techniques like magnetic resonance imaging (MRI) and positron emission tomography (PET) are used to obtain information on Aβ plaques and the size of the brain and to rule out possible other causes of dementia.

The diagnosis of early AD is currently not yet possible, and there is a great need for information regarding and understanding of the processes that are involved in the onset and early stages of AD. Currently, subjective cognitive decline (SCD) epidemiological data provide evidence that the risk for mild cognitive impairment and dementia is increased in individuals with SCD,[25] but we do not yet know what mechanism drives the body toward developing AD. Thus, we have a gap in our understanding of onset and early development of AD.

The problem challenge facing this field of research is that of obtaining more mechanistic information on the time course and interrelationships of the rate and extent of processes that drive the onset and early development of human AD. In humans, there is the possibility for monitoring blood levels of multiple body compounds (potential biomarkers) in cohort. Many such cohort measurements are currently ongoing. Although we might learn a lot from such studies, there are crucial limitations. First, for detecting early changes in body processes that may lead to AD, plasma information is not sufficient, as the levels of body compounds may result in many disturbances not necessarily connected to AD onset. Information on the brain might be provided by what can be detected using imaging techniques. However, imaging techniques are very costly and will not be used in all subjects, let alone in each human subject at each year of follow-up. Thus, human subjects will have developed (significant indicators of) AD before the information of the human subject in relation to AD progression can be obtained. As AD progresses slowly, this will take years at least. In other words, such studies at best would be very expensive and time-consuming. Therefore, we look to additional, alternative approaches to help solve the problem.

Animal models of AD do not really reflect AD in humans. Human AD is familial for only about 5% of cases, while most animal AD models are based on mutations in APP, PSEN1, and/or PSEN2 genes. However, the 'artificial' AD in animal models of AD might still provide us with information that can be helpful to unravel processes associated with development of AD. This could be helpful in guiding research in humans, focusing on what can be learned from body fluids that can readily be obtained from humans. In animal AD models we can also investigate the influence of combinations of conditions in a well-defined setting.[26] As an example, in mice, it was shown that a combination of ApoE deficiency and high-fat diet, but not these conditions on their own, leads to BBB disruption and neuropathology,[27] as would be useful in research on comorbidities in AD.

In this review, we first provide an overview of pathophysiological hypotheses/mechanisms and underlying processes that are thought to play a role in the onset and progression of AD. Then, we summarize published data on the frequently used compounds (biomarkers) in AD research, namely $A\beta_{1-40}$, $A\beta_{1-42}$, hyperphosphorylated tau (P-TAU), T-TAU, neurogranin, SNAP-25, glial fibrillary acidic protein (GFAP), YKL-40, and neurofilament light (NFL), and their presence/concentrations in brain and body fluids (blood/serum/plasma and CSF) in human AD subjects and controls and in frequently used animal models of AD. Next, we compare the human and animal data to identify similarities. Furthermore, we include emerging new biomarkers that can be measured in body fluids, as these could extend our knowledge on changes in body fluids in AD, to help in identifying composite biomarker panels that indicate the stage of AD and could be used in AD stage diagnosis.

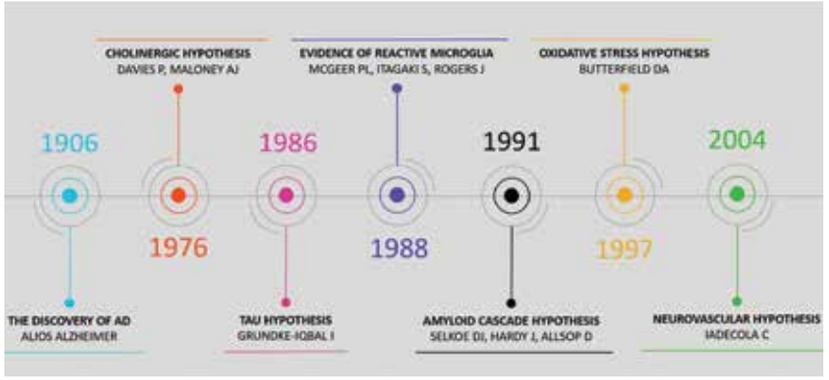
Finally, we discuss the potential value of animal AD models in helping to generate better insights in processes involved in onset and progression of AD, their interactions, and the possibility of designing experiments with well-defined conditions (e.g., to include comorbidities) to understand the influence of these conditions and combinations thereof.

Pathophysiological hypotheses and associated biomarkers

To effectively identify and validate biomarkers of AD, knowledge about the underlying molecular pathogenesis of AD is critical. A comprehensive overview of all pathological processes is needed to understand the disease. As AD is a complex disease, in which multiple processes are known to play a role, different hypotheses exists focusing on the different processes which (might) play a role in AD.[28–30] Overall, these are briefly described in Supplementary Material 1, and the timeline is displayed in Figure 1.

Some recent findings seem to provide new hypotheses. These include changes in the functioning of the endocrine pathway and the vagus nerve.[38] as well as in the gut-microbiome-derived metabolites.[29] In addition, not so long ago, glucose hypo-metabolism was found as an early pathogenic event in the prodromal phase of AD and was associated with cognitive and functional decline,[39] which makes glucose metabolism brain-imaging 2-deoxy-2-fluorine-18-fluoro-d-glucose positron emission tomography (18FDG-PET) a valuable indicator for the diagnosis of neurodegenerative diseases that cause dementia, including AD.[40]

Figure 1 Timeline showing the pathophysiological hypotheses of Alzheimer's disease: the cholinergic hypothesis,[31] tau hypothesis,[32] evidence of reactive microglia,[33] amyloid cascade hypothesis,[34,35] oxidative stress hypothesis,[36] and neurovascular hypothesis.[37]



Cerebral insulin resistance has been accepted as contributing to the neurodegenerative process in AD by activating oxidative stress, cytokine production, and apoptotic process.[41] It is also the link between sporadic AD and its risk factor of diabetes.[42] Furthermore, age-related decline in the ability of glucose to cross the BBB might lead to the production of Aβ plaques and tau-containing neurofibrillary tangles (neuro-energetic hypothesis).[43] Moreover, insulin resistance in type 2 diabetes mellitus and obesity is a risk factor for AD, as insulin resistance might contribute to neurodegeneration.[44]

Interestingly, type 2 diabetes mellitus animals develop accumulation of AD pathologies like Aβ plaques and tau phosphorylation.[45] Furthermore, insulin has effects on Aβ production and clearance via the MEK-ERK pathway. Aβ, in turn, is found to induce the removal of cell surface insulin receptors.[46] The contribution of mitochondria in the pathogenesis of AD has also been studied (which relates to oxidative stress); however, their exact role and place in the disease cascade is still not fully known.[47] Furthermore, the disruption of neural circuits has also been studied in relation to AD. Again, the question arises as to whether the disruption of neuronal circuits is a cause or consequence in AD pathology; however, impairment of neuronal circuits is already found in early stages of the disease. Interestingly, some aspects of AD-related neuronal dysfunction reported in mice are quite similar to the human situation.[48]

Overall, there are multiple mechanisms that possibly contribute to AD, and these have led to the investigation of a broad range of different biomarkers (Table 1) that are related to these different processes, with neuronal degeneration as a final outcome.[49–60] As indicated, biomarkers are urgently needed to provide information regarding the pathobiology of AD in order to find a cure and to diagnose AD, preferably in the preclinical stage, with minimal burden for the patient and minimal costs. Biomarkers obtained from body fluids like CSF and blood are therefore needed. Preferably, these markers will provide information in the preclinical stage.

Most frequently studied brain-, CSF-, and blood-derived biomarkers of AD in humans and in most frequently used rat and mouse AD models

First, we selected the most related disease processes in AD (as described in Table 1), and within these processes, we selected the biomarkers that currently have been identified with alteration in body fluids such as CSF and blood in human AD patients. Thus, we ended up with a panel of nine biomarkers, namely, $A\beta_{1-40}$, $A\beta_{1-42}$, P-TAU, T-TAU, neurofilament light (NFL), neurogranin, SNAP-25, GFAP, and YKL-40. It should be noted that some of these compounds are not specific for AD but may be of value by having a role in AD pathology. Second, we summarized the most frequently used rat and mouse models in AD, to compare the information of these biomarkers in humans and in animals. A detailed literature-searching strategy is provided in Supplementary Material3.

Although all these biomarkers are frequently measured in AD patients, $A\beta_{1-40}$ and $A\beta_{1-42}$ were the most frequently studied ones in AD animal models (as there has been much focus on the amyloid cascade hypothesis). We wondered whether the changes in biomarkers in human and animal body fluids would reflect those in the brain and also whether the biomarker changes in animal models would reflect those found in AD patients. Below, we have provided information on human and animal brain, CSF, and plasma as available for each biomarker. The information has shown to be fragmented such that no direct comparison between animals and humans can be made, but rather a comparison of trends could be made as a heatmap (Figure 2). Our detailed findings on actual data are summarized in Supplementary Material 2.

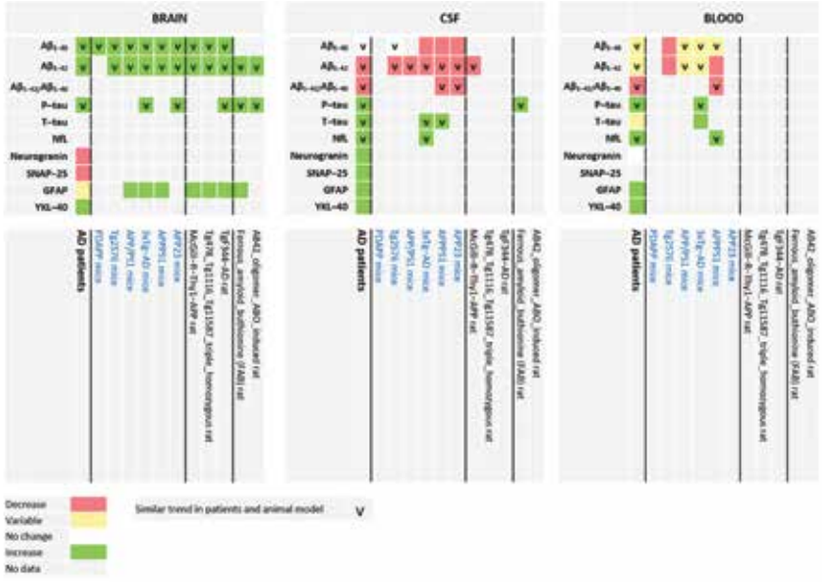
Table 1 Alzheimer's disease (AD)-related processes and the corresponding potential body-fluid-based biomarkers. *It should be noted that some of these compounds are not specific for AD but may be of value by having a role in AD pathology.*

Process	Remarks	Related (Potential) Body-Fluid-Based Biomarkers	Reference
Decreased cholinergic transmission	Not a definitive causation of the disease, but merely a consequence	Acetyltransferase (ChAT)	[54]
		Acetylcholinesterase (AChE)	[54]
		SNAP-25	[61]
Dysfunction in phosphorylation process of tau protein resulting in hyperphosphorylation of the molecule	Secondary pathogenic event that subsequently causes neurodegeneration in AD	Total tau (T-TAU) Hyperphosphorylated tau (P-TAU)	[51,60, 62–64]
Reactive gliosis and neuroinflammation	Reactive microglia and astrocytes surround amyloid plaques and secrete proinflammatory cytokines, which are an early, prime movers in AD evolution	Glial fibrillary acidic protein (GFAP)	[49,58]
		S-100B	[49,58]
		YKL-40	[50,55]
Inequality between production and clearance leads to amyloid β (Aβ) accumulation in brain	The triggering event and the most important factor with highest acceptance but still not exclusively the cause of the disease	$A\beta_{1-42}$ $A\beta_{1-40}$	[24,52]
Characteristic presence of oxidative stress in AD brains	Reactive oxygen species (ROS) and neuronal apoptosis are involved not only in AD but also other neurodegenerative diseases. Below, we propose the oxidative stress pathways specific to AD and involved kinases as potential biomarkers for these processes	-	-
	N-Methyl-D-aspartate receptor (NMDR)-mediated oxidative stress inducing abnormal hyperphosphorylation of tau	Mitogen-activated protein kinase (MAPK) and extracellular receptor kinase (ERK)	[65,66]
		Calmodulin-dependent protein kinase (CaMKII)	[67,68]

(continuation Table 1)

Process	Remarks	Related (Potential) Body-Fluid-Based Biomarkers	Reference
	Aβ activates GSK-3β, which induces oxidative stress, resulting in hyperphosphorylation of tau, NFT formation, neuronal death, and synaptic loss	Glycogen synthase-3β (GSK-3β)	[69,70]
	NMDR-mediated oxidative stress leads to activation and phosphorylation of CREB	cAMP response element-binding protein (CREB)	[71–73]
	Calcineurin activation leads to release of intracellular Ca2+ and reduced NMDR function. Aβ reduces NMDR function whichimpairs LTP through enhanced calcineurin activity	Calcineurin	[74,75]
Cerebrovascular dysfunction, alterations in cerebral blood flow, and impairment of low-density lipoprotein receptor-related protein-1 (LRP-1).	Morphological alterations in cerebral capillaries and increased use of CBF and glucose utilization have been reported in AD patients	LRP-1	[76,77]
Neurodegeneration	Endpoint of different processes	Neurofilament light (NFL)	[78]
		Neurogranin	[79]
		SNAP-25	[61]

Figure 2 Heat map of the changes of the selected biomarkers measured in the brain, CSF, and blood in humans and different animal models of AD. *In humans, the change in biomarker level is compared between AD patients and healthy volunteers. In AD animal models, the change in biomarker level is compared between 'old' and 'young' AD animals (different age groups) and therefore the change during life in which AD features are assumed to progress. Literature used: [50,52,55,78-144] (for details on values of individual studies please see also Supplementary Material 2).*



$A\beta_{1-40}$ and $A\beta_{1-42}$

Aβ, especially $A\beta_{1-42}$, is the most frequently studied biomarker of AD. $A\beta_{1-42}$ and $A\beta_{1-40}$ are increased in the brains of AD patients compared to healthy volunteers, as well as in brains of 'young' versus 'old' animals of different AD rat and mouse models.

In human CSF, $A\beta_{1-42}$ levels are also found to be significantly decreased in the preclinical stage of AD,[52,57] while $A\beta_{1-40}$ levels in CSF do not seem to change. In Tg2576 transgenic mice, also no change in $A\beta_{1-40}$ CSF level is found between different age groups (3-23 months); however, there is a significantly decrease in $A\beta_{1-42}$ in the CSF in these rats. Furthermore, like in human AD, a decrease of $A\beta_{1-42}$ in CSF has been reported in older mice compared to young mice for 3xTg-AD, APPPS1, and APP23 AD transgenic mouse models.[80–82] Following this, a decreased human CSF $A\beta_{1-42}/A\beta_{1-40}$ ratio (as widely accepted as a biomarker with better diagnostic accuracy than CSF $A\beta_{1-42}$ alone for the significantly better association with brain Aβ deposition).[83,145–147]was found. Similarly, Maia et al., 2015 found that the CSF $A\beta_{1-42}/A\beta_{1-40}$ ratio in the APP23, APP24, and APP51 mouse models decreases with increasing age (i.e., AD progression).[82] However, this is not a perfect comparison, as in the animal studies, the decrease of $A\beta_{1-42}/A\beta_{1-40}$ was with increasing age of the transgenic mice, and this is not perfectly comparable with the cross-sectional comparisons in human studies.

Until recently, Aβ peptides measured in human plasma were considered not, or only slightly, correlated with cerebral β-amyloidosis,[57,83] and plasma $A\beta_{1-40}$ concentrations were considered to be influenced by production of platelets with $A\beta_{1-40}$-loaded α-granules.[147] However, Verberk et al., 2018 concluded that the plasma $A\beta_{1-42}/A\beta_{1-40}$ ratio has great potential as a prescreening tool to identify AD pathology.[148] Recently, using advanced immune-precipitation coupled with mass spectrometry analytical platforms, Nakamura et.al., 2018 could indeed indicate that the decreased plasma $A\beta_{1-42}/A\beta_{1-40}$ ratio shares the trend in CSF and shows high performance when predicting brain amyloid-β burden.[149] This result is validated by using two independent cohorts of datasets, and both datasets contain cognitively normal individuals, individuals with MCI, and individuals with AD dementia. Furthermore, the plasma APP669-711/$A\beta_{1-42}$ ratio may differentiate between individuals (i.e., cognitively normal individuals, those with MCI, and those with AD) with high and low Aβ load in the brain, with a significant correlation between brain Aβ burden (as measured by amyloid-PET) and CSF $A\beta_{1-42}$ level.[149] Interestingly, the plasma levels of both $A\beta_{1-40}$ and $A\beta_{1-42}$ of Tg2576 transgenic mice also significantly decrease with age (comparing different age groups between 3- and 23-month-old mice). This decrease with age was not observed in the non-transgenic control groups, which had about 100 times lower levels of both $A\beta_{1-40}$ and $A\beta_{1-42}$; these levels did not change with age (6-23 months).[84]

The ratio between P-TAU and $A\beta_{1-42}$ is also used. The CSF P-TAU/$A\beta_{1-42}$ ratio has been shown to be a sensitive predictive diagnostic biomarker of AD and is recommended by the IWG-2 diagnostic criteria for prodromal AD stage.[11,12,150] Moreover, the human plasma P-TAU/$A\beta_{1-42}$ ratio, characterized with tau-PET, has recently been indicated to be significantly correlated with cerebral tauopathy.[85] Furthermore, a decrease in plasma $A\beta_{1-42}$ has recently been indicated as a biomarker of prodromal AD progression in patients with amnestic MCI positive

subjects (based on $A\beta_{1-42}$/P-TAU in the CSF as well as *ApoE ε4*genotype) versus amnesic MCI AD negative subjects.[151]

However, compared to the changes found in $A\beta_{1-40}$ and $A\beta_{1-42}$ levels measured in the CSF, we found less consistency in the reported changes in $A\beta_{1-40}$ and $A\beta_{1-42}$ plasma levels in AD patients compared to healthy controls in the different studies. Additionally, no consistency was found in changes in plasma $A\beta_{1-40}$ levels between studies using APP/PSI, 3xTg-AD, or APPPSI mice, and no consistency was found in plasma $A\beta_{1-42}$ levels between studies using APP/PSI and 3xTg-AD transgenic mice. However, a decrease of plasma-derived $A\beta_{1-42}$ was reported in the APPPSI mice, and a decrease was found in both $A\beta_{1-40}$ and $A\beta_{1-42}$ plasma levels in aging TG2576 mice. No studies were found measuring $A\beta_{1-40}$ and $A\beta_{1-42}$ plasma levels in rat models over time.

T-TAU and P-TAU

Two different forms of the microtubule-associated protein tau are measured in body fluids: total tau (T-TAU) and hyperphosphorylated tau (P-TAU). P-TAU has decreased capacity to stabilize microtubules. Furthermore, in the brain, P-TAU is the primary component of the NFTs.[64] In humans, the deposition of fibrillar tau that aggregates in brain can be accessed with tau PET,[152] though with limited level of accuracy for the determination of P-TAU versus T-TAU. In animal studies, T-TAU and P-TAU levels in brain tissue homogenate can directly be quantified, and an increase of both P-TAU and T-TAU levels in brains of older animals compared to young animals was found.

In human CSF samples, P-TAU and T-TAU were found to be increased in sporadic AD patients compared to healthy controls. In two animal studies, T-TAU was measured in CSF. Maia et al., 2013 found an increase in T-TAU in APP23 and APPPS1 mice with age.[81] Lecanu et al., 2006 reported an increase of P-TAU in CSF in aging FAB rats.[86] Furthermore, P-TAU and T-TAU were also found to be mostly increased in plasma samples of 3xTg-AD compared to wild-type mice, although this was dependent on the antibody types used.[87] This directly indicates an important point: P-TAU has many phosphorylation sites and multiple fragments in body fluids, and the results of measurements depend on the antibody pairs used, which may hamper proper comparison of P-TAU data. While the same P-TAU form (P-TAU181) was measured in most of the human studies,[79,85,88–91,153,154] not all studies specify the P-TAU form. In the selected animal studies, the Ser202/Thr205 form was most often used [86,92]; furthermore, less consistency in the use of the same antibody was found in animal studies compared to human studies.

In plasma, P-TAU data are variable. Different studies measuring T-TAU plasma levels reported an increase.[85,93,94]or no significant change in T-TAU plasma levels in AD patients compared to healthy controls.[94–96] This might be related to the stage of AD or the severity of AD of the AD subpopulation, as a T-TAU increase was also not detectable in patients at MCI AD stage.[93,94]

NFL

Neurofilament light (NFL) has been shown to be a dynamic cross-disease biomarker for neurodegeneration. Although the increase of NFL in CSF is not specific for AD, it is an important predictor of neurodegeneration in AD as well as cognitive deterioration and structural brain changes over time.[155] In contrast to CSF T-TAU and neurogranin alterations, NFL reflects neurodegeneration independent of Aβ pathology.[153] NFL was reported to be increased in human CSF.[96–98] and plasma.[78,96,99–101] of AD patients compared to healthy controls. Recently, a study has been conducted, involving patients in a presymptomatic stage of familial AD, in which NFL levels in the CSF (n = 187) and serum (n = 405) were found to be correlated. Interestingly, the rate of change of serum NFL over time (i.e., log10(serum NFL) per year) could distinguish mutation carriers (i.e., highly penetrant autosomal-dominant mutations in APP, PSEN1, or PSEN2) from nonmutation carriers, almost a decade earlier than cross-sectional absolute NFL levels.[156] These promising results indicate the promise of that blood-based NFL as a valuable biomarker for AD diagnosis in the preclinical stage.
NFL was detected in two transgenic mice models of AD by Bacioglu et al., 2016.[102] In APPPS1 mice, CSF NFL increased with age: from 3 months to 12 months and to 18 months. Plasma NFL increased accordingly. In the tau-overexpressed P301S-tau mice, CSF NFL increased with age from 2-4 months to 6-8 months and to 10-12 months until 14-16 months, as did plasma NFL.[102]

Neurogranin

Neurogranin is a neural-enriched dendritic protein involved in long-term potentiation of synapses, particularly in the hippocampus and basal forebrain. CSF neurogranin is used as a biomarker for synapse loss and synaptic dysfunction in neurodegenerative diseases, including AD. While Kvartsberg et al., 2019 reported a decrease of neurogranin in the brain of AD patients,[103] several other studies have reported an increase of neurogranin in CSF of AD patients.[79,88,95,104–106,157] CSF neurogranin levels did not significantly differ between AD patients and patients

with Lewy body dementia (LBD).[158] In contrast, Mavroudis et al., 2019 found that CSF levels of neurogranin were significantly higher in AD patients compared with cognitively normal participants, as well as between AD patients and patients with mild cognitive impairment (MCI), So, AD patients have higher neurogranin levels compared to MCI , and MCI has higher neurogranin levels compared to controls, which indicates that neurogranin levels might be used to differentiate MCI patients from AD patients.[158] In blood, no significant change in neurogranin concentration has been detected in AD compared to controls.[95,105]

No studies have reported on measuring neurogranin in the brain, CSF, or blood in the selected animal models in a longitudinal fashion. One study using CamKII-TetOp25 mice (inducible transgenic mice overexpressing p25, or Cdk5, which is required for normal development of the mammalian brain) reported a significant increase of neurogranin in the CSF after 3 weeks of p25 induction.[159]

SNAP-25

Massive synapse loss is another critical pathological process that occurs in the AD brain and correlates with cognitive decline.[160] Synaptic dysfunction can occur and eventually progresses into massive synaptic loss.[161] This process can be indicated by the decline of synaptic protein levels. Various synaptic proteins, such as SNAP-25, synaptophysin, rab 3A (presynaptic protein), PSD-95, synaptopodin (postsynaptic protein), synapsin I, and chromogranin B (synaptic vesicle proteins), have been reported to be significantly reduced in the brains of patients with AD.[91,107,162–164] Neuronal death alone is not believed to be sufficient to explain the magnitude of synapse loss, suggesting that synapses are selectively damaged or degenerated prior to brain cell death.[165] Reduced gene expression patterns of genes related to synaptic vesicle trafficking have been found, which indicate that synapses in AD may not function effectively, even prior to visible structural alteration of neurons.[166] In contrast to the decrease of SNAP-25 found in human brains, SNAP-25 is found to be increased in the CSF of AD patients compared to age-matched controls.[91,108–110] No studies were found measuring the difference of SNAP-25 in the blood of AD patients compared to healthy controls.

Additionally, no studies were found measuring SNAP-25 in brain, CSF, or blood samples in the selected animal models. Reports about synaptic loss in transgenic mice models of AD are mainly based on histological studies. For instance, dendritic spine loss was reported in 8-month-old PDAPP mice and 4.5-month-old Tg2576 mice.[167] Early synaptic loss was identified in hippocampus

of 3-4-month-old J20 mice bearing APPswe and APPind mutations. In the brain of these mice, the levels of synaptophysin, PSD95, synaptotagmin, and homer significantly decreased, preceding the deposition of senile plaques.[168] Dendritic spine loss around Aβ plaques began approximately at 3 months of age in APP/PS1 mice.[169] Age-dependent loss of synaptophysin, synaptotagmin, PSD-95, and homer immunoreactivity was reported in the hippocampus of 4-month-old APP/PS1 mice.[168] Although the immunoactivity of certain synaptic proteins has been used to evaluate synaptic loss in these histological studies, the corresponding body-fluid-based changes of these proteins have not been measured.

GFAP

Gliosis is a nonspecific phenomenon that occurs in response to injuries to the brain and involves the activation and proliferation of glial cells. In AD, gliosis is marked by an increase in activated microglia and reactive astrocytes near the sites of Aβ plaques.[58] It is widely accepted that the interaction of microglia with fibrillary Aβ leads to microglia and astrocyte activation, which results in the production of chemokines, neurotoxic cytokines, and reactive oxygen and nitrogen species that are deleterious to the brain.[170] Moreover, it has been shown that reactive astrocytes play an additional role in AD by their contribution to an overall amyloid burden in the brain, given the wide expression of APP, BACE1, and γ-secretase in astrocytes.[58] Glial fibrillary acidic protein (GFAP) is an established indicator for astrocyte activation. GFAP is found to be increased in postmortem human brain tissue samples of AD patients,[111] but no significant change is found in postmortem cerebellar brain tissue compared to age-matched controls.[112] An increase in GFAP with age was found in brains from APP/PS1,[113] 3xTg-AD,[114] and APPPS1.[115] transgenic mouse models. The same was reported for the McGill-R-Thy1-APP.[116] and TgF344-AD.[117] transgenic rat model, as well as the FAB rat.[86]

Furthermore, GFAP is increased in the CSF of AD patients.[98,118] GFAP levels in serum have also been reported to be increased in serum from AD patients compared to non-neurodegenerative controls, and the increase of serum GFAP correlated with the Mini-Mental State Examination score. Moreover, serum GFAP might be used to discriminate between AD and behavioral variant of frontotemporal dementia.[119]

Body-fluid-based markers for gliosis in AD mouse and rat models are not reported.

YKL-40

YKL-40, also called chitinase-3-like 1 (CHI3L1), is a glycoprotein expressed by different cells (such as astrocytes and macrophages) and, although its function is not yet completely understood, is linked to inflammation.[120,171] YKL-40 was found to be increased in the brains of AD patients compared to healthy controls.[120] Furthermore, increased levels of YKL-40 are found in CSF.[50,55,89,98,120]] and in plasma.[50,121]of AD patients compared to healthy controls. Interestingly, Wenström et al., 2015 found increased levels of YKL40 in the CSF of AD patients, compared to the nondemented control group, but not in patients with Lewy body disease or Parkinson's disease.[55] Moreover, the increase of CSF YKL-40 has also been observed in preclinical (based on clinical dementia rating (CDR) or Mini-Mental State Examination (MMSE) score) AD patients and AD subjects with MCI.[50,89] No studies were found measuring YKL-40 in brain, CSF, or blood samples in the selected animal models.

Overall, for animal studies, researchers tend to seek for 'direct answers' of the AD-like pathology in the brain; however, we believe that there is an underestimation of the value in also including body-fluid-based biomarkers of the animals to understand how AD pathology in brain is reflected in body fluids.

Emerging Techniques and Body-Fluid-Based Biomarkers

In the near future, emerging biomarkers, measured in different body fluids, might provide additional valuable information on processes occurring in the early onset and progression of AD. These include, but are not limited to, extracellular vesicles (EVs); microRNAs; and proteomic-, metabolomic-, and lipidomic-based body fluid biomarkers.

MicroRNAs as Body-Fluid-Derived Biomarkers for AD

MicroRNAs (miRNAs) are a group of biomarkers that can be found in different body fluids, like CSF, blood, and saliva. These small (about 20 nucleotides), noncoding RNAs play a role in many different biological processes. Importantly, miRNAs are known to be conserved across species. Accumulating evidence suggests that alterations in the miRNA networks could contribute to the pathology of AD, or can at least be used as an early indication of the development of AD. Results from recent studies in humans suggest that a number of specific miRNAs are differently expressed in disease conditions, of which some are thought to be involved in the regulation of key genes in AD, including APP and BACE-1.

A subset of miRNAs seems to be specifically altered in the AD brain, including miR-29, miR-15, miR-107, miR-181, miR-146, miR-9, miR-101, miR-106, miR-125b, and miR-132. All were independently validated in two or more studies.[60,172] Furthermore, several large-scale genome-wide profiling studies have been performed, demonstrating that, beside the brain, miRNA levels in blood and CSF are also differently expressed in AD patients, compared to age-matched healthy volunteers.[173] Several miRNAs are indicated to be putatively proinflammatory, including miR-9, miR-125b, miR-146a, and miR-155. The expression levels of these miRNAs are increased in both postmortem brain extracellular fluid (brain ECF) and the CSF of postmortem AD patients.[173] The levels of miRNAs, including miR-137, miR-9, miR-29a, and miR-29b, were found to be significantly reduced in plasma of probable AD patients with a Mini-Mental State Examination score of 23–28.[174]

Recent studies on miRNA expression changes in AD patients suggest the potential of body-fluid-based miRNAs to assist the early diagnosis of AD. However, many of these studies were cross-sectional, with one measurement per patient only, limiting the usage of miRNAs as biomarkers when assessing AD progression. Longitudinal observations of miRNA alterations in AD animal models might provide complementary information of pathology-associated differences in miRNA levels during the progression of AD, particularly in an early stage. Genome-wide analysis of the brain miRNA signature in an APP/PS1dE9 mouse model was investigated by Luo et al., 2014.[175] In this study, nine miRNAs, namely miR-99b-5p, miR-7b-5p, miR-7a-5p, miR-501-3p, miR-434-3p, miR-409-5p, miR-331-3p, miR-138-5p, and miR-100-5p, showed consistent changes at 2, 4, 6 and 12 months of age in the APPswe/PS1dE9 mouse model. Another study showed 37 miRNAs that are consistently changed in the cerebral cortex of APP/PS1dE9 mice, among which 17 miRNAs are downregulated. These include miR-20a, miR-29a, miR-125b, miR-128a, and miR-106b,[176] which are linked to AD based on information of human AD studies. miR-106b is increased in the cerebral cortex of 3- and 6-month-old APP/PS1dE9 mice but decreased in 9-month-old mice (although still remaining slightly higher compared to the level of miR-106b in 3-month-old mice).[177] This indicates that miRNA expression patterns may change over time.

Furthermore, the plasma miRNA profile was investigated at different timepoints during the AD-like pathology progression in 3xTg-AD mice.[178] Plasma samples were obtained from 2-3-month-old and 14-15-month-old 3xTg-AD and wild-type (WT) mice. No significant differences in miRNA levels were detected

between WT and transgenic mice at the young (2-3 months) age, while age-related significant changes in miRNAs were observed in both WT and transgenic mice, with some of these changes being specific for 3xTg-AD mice. Nineteen miRNAs show similar change over time of both WT and transgenic mice. These include family members of let-7, miR-30, and the miR-17-92 cluster and its paralogs. A group of miRNAs, including miR-132, miR-138, miR-146a, miR-146b, miR-22, miR-24, miR-29a, miR-29c, and miR-34a, show significant changes in plasma levels only in the transgenic group. These age-dependent changes are of interest as they could consequently derive from AD pathology progression in this mouse line. The plasma miRNA profile has also been studied over time in the APP/PS1dE9 mice model. At 4 months, when these mice are in the prepathological stage of AD, a significant decrease in expression of miR-200b-3p, miR-139-5p, and miR-27b-3p was observed, together with a significant increase of miR-205-3p and miR-320-3p expression.[179] At 8 months, when amyloidosis is apparent in these mice, the expression of a different set of miRNAs is altered, with an increase in 4 miRNAs (miR-140-3p, miR-486-3p, miR-339-5p and miR-744-5p) and a decrease in miR-143-3p and miR-34a-5p. At 15 months, expression of miR-339-5p and miR-140-3p remained significantly increased, suggesting a sustained increase in expression of these two miRNAs over time.

Proteomic Body-Fluid-Based Biomarkers

Proteomics is a multidisciplinary, technology-driven science that focuses on the analysis of proteomes, i.e., the proteins of a biological system, their structures, interactions, post-translational modifications, and, in particular, the changes in their levels and their modifications as the result of specific diseases or external factors.[180]

Although untargeted proteomic analysis can provide us with unbiased body fluid protein panels that have potential diagnostic value in AD, issues around quantification and reproducibility should be considered. Thus, an alternative approach in the proteomic study was advocated where the subjects involved in the studies are grouped to a continuous variable such as brain atrophy, rate of cognitive decline, Aβ burden, and CSF biomarker level (so-called 'endophenotype discovery').[181] Based on this, Shi et al., 2018 summarized the 'endophenotype discovery' for plasma proteomic biomarkers in AD and eight most replicated protein biomarkers were selected.[181] However, it should be noted that for the discovery of robust (i.e., reliable and quantifiable) proteomic biomarkers for AD, the comparability of data from multiple large studies across heterogeneous populations is needed; this remains challenging, as has been addressed by Carlyle et al., 2018.[182]

In case of AD, several CSF- and blood-based proteomics analyses have been conducted by comparing the proteome profiles of these body fluids of AD patients to the control groups to collect information about gene products involved in AD, i.e., alterations in protein levels and post-translational modifications. Several proteomic targets have been discovered that displayed significant alteration in the CSF of AD subjects compared to the control group.[183–185] In these studies, a certain consistency was observed in the alteration of apolipoproteins in the CSF of AD patients, suggesting that a pronounced reduction of pro-apolipoproteins might be a potential CSF-based biomarker of AD. In blood, however, only few putative blood-based protein biomarkers could be replicated in independent studies. This was concluded by a large-scale replication check for 94 of the 163 candidate biomarkers from 21 published studies in plasma samples from 677 subjects.[186]

In AD animal models, proteomic biomarker profiles from the brain hippocampus homogenate of ADLPAPT mice,[187] APP/PS1 mice, and *ApoE4* knock-in mice models.[188] have been identified with age-dependent alterations; many of the differentially expressed proteins were identical at presymptomatic stage of the mice, earlier than the formation of Aβ plaque. However, body-fluid-based proteomic biomarkers in AD animal models are still missing. It would be interesting to also investigate these markers in CSF and plasma of the animal AD models to relate these body-fluid-based to brain-tissue-based proteomic markers and the pathological changes of the brain. Furthermore, these biomarkers can be traced throughout the lifespan of animals as well as the disease progression, and this could provide insights in refinement of biomarkers in human body fluids.

Metabolomic and Lipidomic Body-Fluid-Based Biomarkers

Metabolomics is one of the latest systems biology approaches where multiple platforms are utilized to measure levels of small-molecule metabolites in biological samples. Metabolic signatures are unique to an individual wherein perturbations in metabolite levels may inform on the disease state and underlying mechanisms of the disorder.[189] Given their close association with the host's phenotypes, the profile of metabolites demonstrates the current physiological state of a cell and is the end result of the upstream biological information that flows from genome over to transcriptome and proteome to metabolome.[190]

In 2018, Hurtado et al. reported the state of the art in AD-related metabolomic biomarker evidence based on studies on metabolomics and lipidomics in AD.[191]; in 2019, several novel targets were reported as potential body-fluid-based biomarkers in AD, where it was found that kynurenine pathway metabolites and primary fatty amides showed great significance in their alterations in AD subjects

compared to the controls.[192,193] Multiplatform metabolomics has emerged as an essential tool for the identification of potential AD biomarkers in different human body fluids, and many potential biomarkers have been discovered in the last decade. However, only a few of these have been validated. Moreover, fellow researchers in this area expressed concerns about the consistency of the proteomics, metabolomics, and lipidomics studies and called for interlaboratory validations.[190] So, while this is an emerging field of high interest, it suffers from interlaboratory differences and reproducibility issues. However, different laboratories have extensively validated their metabolic and lipidomic platforms, and series of studies all using the same platform will definitely provide relevant data on changes in AD onset and progression.

Extracellular-Vesicle-Based Biomarkers

EVs include, from small to large size, exosomes, microvesicles, and apoptotic bodies; are secreted from different cells in the body; and contain unique molecular information regarding their cell of origin. They are released into the extracellular environment and are known to play a role in intercellular communication between cells in close proximity, as well as between distant cells. This, together with the fact that EVs have been found in many different body fluids including blood, urine, and CSF, has raised interest in the use of EVs as a source for the discovery of novel biomarkers. It should be noted that the methodologies to isolate EVs, the techniques used for quantification of EVs, and the techniques to quantify their characteristics and content are all crucial for the data obtained, and good comparisons of data currently published are not yet possible.[194] (unpublished data of our group). So, what is described below should be interpreted given these drawbacks

Several studies indicate that AD patients could be distinguished from cognitive normal controls based on the (synaptic) protein cargo from neural-derived EVs extracted from plasma. These biomarkers would reflect AD pathology up to 10 years before the clinical onset of AD.[195] Different types of AD biomarkers were investigated by Goetzl et al., showing that Aβ and tau proteins increased along with AD progression,[195] while synaptic markers significantly declined.[196,197] In these studies, the EV-based lysosomal proteins, brain insulin resistance factor, and cellular survival factors appeared to be useful in distinguishing between control and AD progression in multiple stages of the disease.[198,199] However, the work of the Goetzl group has not yet been reproduced by others, while their LICAM work was done in what they claimed as early stages of AD (i.e., not specified/defined), all of which means that the value of their work remains to be seen.

In addition, EVs originating from the Central Nervous System (CNS) were found in plasma, containing the AD-related biomarkers $A\beta_{1-42}$, P-TAU, and T-TAU. These EVs were found to identify patients converting from MCI to dementia.[200] Moreover, Gui et al., 2015 reported that the miRNA profile in CSF-derived EVs was altered in AD. The MRNA transcripts of APP, Tau, NFL, DJ-1/PARK7, fractalkine and neurosin were altered, and long noncoding RNAs (RP11-462G22.1 and PCA3) were also found to be differentially expressed in CSF-derived EVs.[201] Overall, these studies demonstrate the potential of EV-based biomarkers in the early stages of AD.

Still, only a few studies investigated EV-based biomarkers in AD transgenic mouse and rat models. One study, by Eitan et al., 2016, reported higher levels of $A\beta_{1-42}$ and $A\beta_{1-40}$ in plasma-derived EVs of six APP/PS1 mice and five 3xTgAD mice compared to nine age-matched WT control mice. The absolute levels of $A\beta_{1-42}$ and $A\beta_{1-40}$ in plasma-derived EVs in these transgenic mice were lower compared to the levels of these markers found in EV-depleted plasma, while the ratio of $A\beta_{1-42}/A\beta_{1-40}$ was significantly higher in EVs.[202] This indicates that EV-based biomarker alterations can be different from body-fluid-based alterations and that plasma-derived EVs might provide biomarkers with higher sensitivity than whole-plasma-derived biomarkers.

Discussion and Conclusions

The number of currently existing and emerging pathophysiological hypothesis, mechanisms, theories, and processes related to AD is high and is still increasing. This indicates that AD is a very complex and multifactorial disease. An important problem is that we do not have a cure or treatment for AD. Another problem is that the disease cannot be diagnosed in an early stage. Currently, we lack information and understanding of processes in the onset and early stage of the disease. As such, we lack an early diagnosis and treatment option in the early phase of AD. This highlights the need to find adequate, preferably body-fluid-based biomarkers of AD. Currently, the biomarkers that are mostly measured in human studies are Aβ, P-TAU, T-TAU, neurogranin, SNAP-25, GFAP, YKL-40, and especially NFL. Additionally, there is a high volume of animal research, in which the emphasis has mostly been on Aβ.

For early diagnosis and treatment of AD, we first need to solve the problem of the gap in the knowledge and understanding of the onset and early phase of AD. miRNAs and EVs, together with proteomic, metabolomic, and lipidomic body-fluid-based biomarkers are emerging as (early) biomarkers of AD as well as other diseases. miRNAs are conserved across species, which makes it easier to extrapolate findings between humans and animal models of AD. However, as a drawback,

it is hard to assess whether a change in microRNA expression level is a result or a cause of AD. Moreover, a single microRNA can target multiple genes, and one gene can be targeted by different microRNAs. Furthermore, while omic technics show great potential in the discovery of new biomarkers in AD, a drawback here is the current lack of consistency and reproducibility, which indicates that omic markers in AD need to be further validated. Then, the use of EVs combined with the measurement of EV-associated and non-EV-associated miRNAs together with techniques like metabolomics and lipidomics show great promise for the detection of novel biomarkers in body fluids and for the collection of new information to increase our understanding of the pathogenesis of AD. Here, the drawback is in the multiple methodologies in isolating and characterizing EVs, which influence the data obtained and thereby make comparisons between the data difficult.

For the biomarkers that have been investigated in brain, CSF, and blood, the changes in disease stage are rather different (Figure 2), and so they are not directly related. This indicates that body fluids might not directly provide mechanistic information on the disease stage, and in humans it might be difficult to study the interrelationships and time-dependencies of the biomarkers. Therefore, we need different approaches for more mechanistic understanding of early AD and its progression.

We believe that the problem of the gap in knowledge and understanding of the onset and early phase of AD cannot be solved by human studies alone. This is for the simple reasons that it is too costly and too time-intensive to measure many compounds in human samples, let alone imaging studies, in well-controlled and longitudinal studies in the ageing human population, in which a small percentage will actually develop AD. Thus, there is the need for alternative approaches to obtain a useful understanding on the relation between the changes in (body fluid) biomarkers and (early) AD stage. In our view, animal studies could be helpful.

An ideal animal model that exhibits all features of human AD does not exist. Current animal AD models are at best to be regarded as reductionist tools, as the majority of the animal models represent familial AD while most AD patients have sporadic AD (although a few non-transgenic rat models of AD have been developed.[117]). However, animal models of AD may still be of value to gain understanding on the pathological processes involved in AD if enough similarities exist between animal AD models and human AD. To that end, an overview is given on associated and most frequently measured biomarkers in brain, CSF, and blood of human AD and animal models of AD. Where possible, the trend in changes of these biomarkers is assessed and summarized in a heat map (Figure 2).

Although a direct comparison between human (familial and sporadic) AD and (mostly genetic) animal AD models cannot be made, a majority of similar trends are observed in brain, CSF, and blood for human AD stage and animal AD model life stage, assuming that a later stage in life of the AD animal represents a later stage in AD. Despite the current limitation of exact knowledge on AD stage in humans and in animals, we see many similarities. This makes us believe that animal models of AD have a good potential to provide information that can be useful for also better understanding the (early) processes in AD.

While we all strive for the reduction/replacement in the use of animals, we should realize two things. (1) The problem of AD is too big not to make all efforts possible to diagnose AD in an early stage, where the disease might be halted or even reversed. (2) AD is too complex to be understood from single-biomarker and single-timepoint measurements. Therefore, we must study this complex disease in a very systematic research manner including multiple biomarker measurements at multiple sites in the body (fluids and tissue) in a longitudinal fashion under well-defined conditions, applying advanced mathematical modeling (according to the Mastermind Research approach[26]) to unravel the processes and their interactions (Figure 3). Ideally, composite biomarker panels will reflect all processes that occur in AD in a stage-dependent manner. However, it should be noted that due to the small sample size of brain ECF, CSF, and blood that can be obtained from rats and especially mice, sampling as well as detecting compounds and EVs in these body fluids is challenging.

Recently, we have shown that the strategic use of animals and the collection of smart-data have led to a mathematical model that can adequately predict drug distribution into multiple physiologically relevant compartments of the CNS, not only in animals, but also in humans. It should be noticed that CNS drug distribution is also the result of multiple processes and their interdependencies. The CNS drug distribution model has been developed using systematic research in experimental animals by varying conditions, measuring drug concentrations at multiple locations in the CNS, and making differences between drug and body properties explicit, such that the body properties of the rat could be replaced by human body properties. Our CNS drug distribution model can now replace the use of animals and directly predict CNS drug distribution in humans on the basis of plasma pharmacokinetics and drug properties.[203,204] This indicates a much better and efficient use of animals.

Thus, for AD, longitudinal early-life-studies can be performed in both transgenic and non-transgenic animal AD models and their control littermates, on a much shorter time scale than in humans, while measurements can also be taken

at multiple timepoints at multiple body sites (including body fluids) and finally also in the brain, to relate to currently known brain markers of AD. This anticipated approach is depicted in Figure 4.

Altogether, in our view, strategic and well-controlled animal studies are needed to fill the crucial knowledge gap, especially on the processes involved in the onset and early stage of AD. Thus, as much as possible in individual animals, multiple (putative) biomarkers should be measured at multiple body sites, including body fluids, to understand their interdependencies and time-dependencies in AD onset and progression (according to the Mastermind Research approach). By this approach, systematic research can also be performed on combinations of conditions, such as comorbidities.

Once we have such understanding, we will have a good basis for defining (multiple) targets to be modified by therapeutic approaches in order to halt the disease or even be able to reverse the disease in its early stage into a healthy condition again.

Figure 3 The recently developed human CNS drug distribution model is an example of application of the Master Research Approach.[26] *The model is developed on the basis of animal research, and now CNS drug distribution in humans can be predicted without the need of experimental animals.[203,204]*

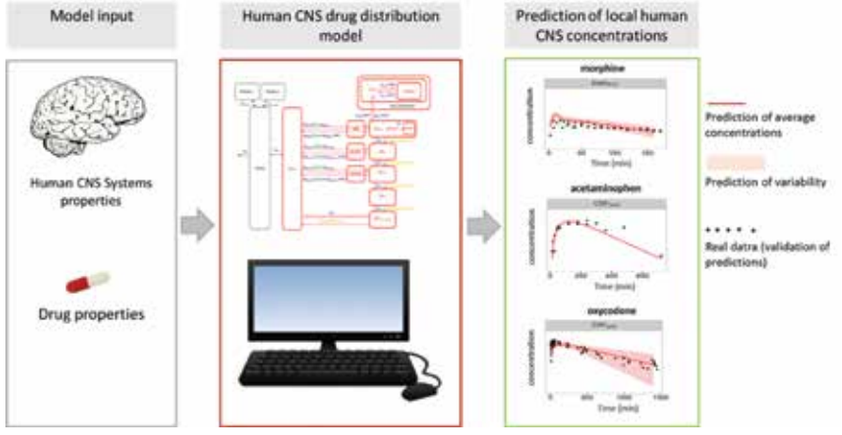
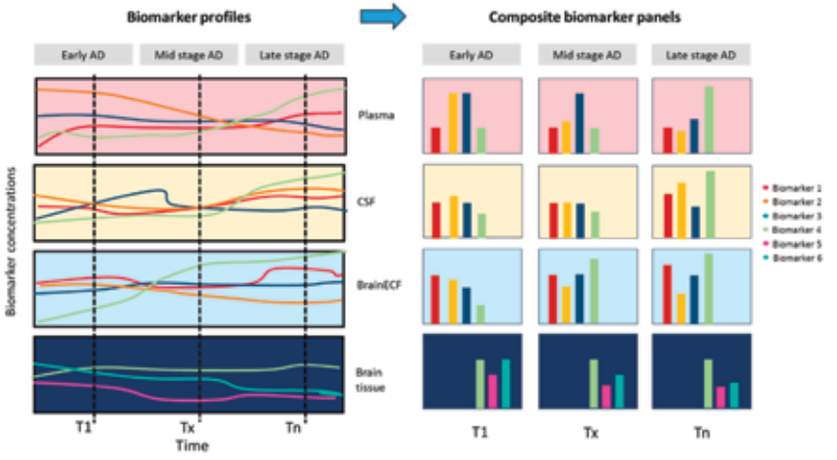


Figure 4 Anticipated approach to study and understand the processes in AD progression. *Longitudinal, multiple-biomarker, multiple-body-site measurements in AD animals (and their control littermates – not shown here) should be able to reveal processes and their interdependencies in AD and in normal ageing as stage (T1, Tx, Tn)-dependent 'composite biomarker panels', leading to insights that are AD-specific to be targeted as therapy.*



REFERENCES

1 Karran, E.; Mercken, M.; De Strooper, B. The amyloid cascade hypothesis for Alzheimer's disease: An appraisal for the development of therapeutics. Nat. Rev. Drug Discov. 2011, 10, 698–712.

2 Pimplikar, S.W. Reassessing the amyloid cascade hypothesis of Alzheimer's disease. Int. J. Biochem. Cell Biol. 2009, 41, 1261–1268.

3 Holtzman, D.M. Role of apoE/Aβ interactions in the pathogenesis of Alzheimer's disease and cerebral amyloid angiopathy. J. Mol. Neurosci. **2001**, 17, 147–155.

4 Mawuenyega, K.G.; Sigurdson, W.; Ovod, V.; Munsell, L.; Kasten, T.; Morris, J.C.; Yarasheski, K.E.; Bateman, R.J. Decreased clearance of CNS beta-amyloid in Alzheimer's disease. Science **2010**, 330, 1774.

5 Farrer, L.A.; Cupples, L.A.; Haines, J.L.; Hyman, B.; Kukull, W.A.; Mayeux, R.; Myers, R.H.; Pericak-Vance, M.A.; Risch, N.; van Duijn, C.M. Effects of Age, Sex, and Ethnicity on the Association Between Apolipoprotein E Genotype and Alzheimer Disease: A Meta-analysis. JAMA **1997**, 278, 1349–1356.

6 Bu, G. Apolipoprotein E and its receptors in Alzheimer's disease: Pathways, pathogenesis and therapy. Nat. Rev. Neurosci. **2009**, 10, 333–344.

7 Dose, J.; Huebbe, P.; Nebel, A.; Rimbach, G. APOE genotype and stress response – A mini review. Lipids Health Dis. **2016**, 15, 121.

8 Sen, A.; Alkon, D.L.; Nelson, T.J. Apolipoprotein E3 (ApoE3) but not ApoE4 protects against synaptic loss through increased expression of protein kinase C epsilon. J. Biol. Chem. **2012**, 287, 15947–15958.

9 Hashimoto, T.; Serrano-Pozo, A.; Hori, Y.; Adams, K.W.; Takeda, S.; Banerji, A.O.; Mitani, A.; Joyner, D.; Thyssen, D.H.; Bacskai, B.J.; et al. Apolipoprotein E, especially apolipoprotein E4, increases the oligomerization of amyloid beta peptide. J. Neurosci. **2012**, 32, 15181–15192.

10 Montagne, A.; Zhao, Z.; Zlokovic, B.V. Alzheimer's disease: A matter of blood-brain barrier dysfunction? J. Exp. Med. **2017**, 214, 3151–3169.

11 Dubois, B.; Feldman, H.H.; Jacova, C.; Hampel, H.; Molinuevo, J.L.; Blennow, K.; DeKosky, S.T.; Gauthier, S.; Selkoe, D.; Bateman, R.; et al. Advancing research diagnostic criteria for Alzheimer's disease: The IWG-2 criteria. Lancet Neurol. **2014**, 13, 614–629.

12 Dubois, B. The Emergence of a New Conceptual Framework for Alzheimer's Disease. J. Alzheimers Dis. **2018**, 62, 1059–1066.

13 Caselli, R.J.; Locke, D.E.; Dueck, A.C.; Knopman, D.S.; Woodruff, B.K.; Hoffman-Snyder, C.; Rademakers, R.; Fleisher, A.S.; Reiman, E.M. The neuropsychology of normal aging and preclinical Alzheimer's disease. Alzheimers Dement. **2014**, 10, 84–92.

14 Mormino, E.C.; Papp, K.V.; Rentz, D.M.; Donohue, M.C.; Amariglio, R.; Quiroz, Y.T.; Chhatwal, J.; Marshall, G.A.; Donovan, N.; Jackson, J.; et al. Early and late change on the preclinical Alzheimer's cognitive composite in clinically normal older individuals with elevated amyloid beta. Alzheimers Dement. **2017**, 13, 1004–1012.

15 Aerts, L.; Heffernan, M.; Kochan, N.A.; Crawford, J.D.; Draper, B.; Trollor, J.N.; Sachdev, P.S.; Brodaty, H. Effects of MCI subtype and reversion on progression to dementia in a community sample. Neurology **2017**, 88, 2225.

16 Sperling, R.A.; Jack, C.R., Jr.; Aisen, P.S. Testing the right target and right drug at the right stage. Sci. Transl. Med. **2011**, 3, 111cm33.

17 Sperling, R.A.; Karlawish, J.; Johnson, K.A. Preclinical Alzheimer disease-the challenges ahead. Nat. Rev. Neurol. **2013**, 9, 54–58.

18 Ostrowitzki, S.; Lasser, R.A.; Dorflinger, E.; Scheltens, P.; Barkhof, F.; Nikolcheva, T.; Ashford, E.; Retout, S.; Hofmann, C.; Delmar, P.; et al. A phase III randomized trial of gantenerumab in prodromal Alzheimer's disease. Alzheimers Res. Ther. **2017**, 9, 95.

19 Egan, M.F.; Kost, J.; Voss, T.; Mukai, Y.; Aisen, P.S.; Cummings, J.L.; Tariot, P.N.; Vellas, B.; van Dyck, C.H.; Boada, M.; et al. Randomized Trial of Verubecestat for Prodromal Alzheimer's Disease. N. Engl. J. Med. **2019**, 380, 1408–1420.

20 Mufson, E.J.; Ikonomovic, M.D.; Counts, S.E.; Perez, S.E.; Malek-Ahmadi, M.; Scheff, S.W.; Ginsberg, S.D. Molecular and cellular pathophysiology of preclinical Alzheimer's disease. Behav. Brain Res. **2016**, 311, 54–69.

21 Bao, W.; Jia, H.; Finnema, S.; Cai, Z.; Carson, R.E.; Huang, Y.H. PET Imaging for Early Detection of Alzheimer's Disease: From Pathologic to Physiologic Biomarkers. PET Clin. **2017**, 12, 329–350.

22 Paterson, R.W.; Slattery, C.F.; Poole, T.; Nicholas, J.M.; Magdalinou, N.K.; Toombs, J.; Chapman, M.D.; Lunn, M.P.; Heslegrave, A.J.; Foiani, M.S.; et al. Cerebrospinal fluid in the differential diagnosis of Alzheimer's disease: Clinical utility of an extended panel of biomarkers in a specialist cognitive clinic. Alzheimers Res. Ther. **2018**, 10, 32.

23 Jack, C.R., Jr.; Bennett, D.A.; Blennow, K.; Carrillo, M.C.; Dunn, B.; Haeberlein, S.B.; Holtzman, D.M.; Jagust, W.; Jessen, F.; Karlawish, J.; et al. NIA-AA Research Framework: Toward a biological definition of Alzheimer's disease. Alzheimers Dement. **2018**, 14, 535–562.

24 Blennow, K.; Mattsson, N.; Scholl, M.; Hansson, O.; Zetterberg, H. Amyloid biomarkers in Alzheimer's disease. Trends Pharmacol. Sci. **2015**, 36, 297–309.

25 Jessen, F.; Amariglio, R.E.; Buckley, R.F.; van der Flier, W.M.; Han, Y.; Molinuevo, J.L.; Rabin, L.; Rentz, D.M.; Rodriguez-Gomez, O.; Saykin, A.J.; et al. The characterisation of subjective cognitive decline. Lancet Neurol. **2020**, 19, 271–278.

26 de Lange, E.C. The mastermind approach to CNS drug therapy: Translational prediction of human brain distribution, target site kinetics, and therapeutic effects. Fluids Barriers CNS **2013**, 10, 12.

27 Mulder, M.; Blokland, A.; van den Berg, D.J.; Schulten, H.; Bakker, A.H.; Terwel, D.; Honig, W.; de Kloet, E.R.; Havekes, L.M.; Steinbusch, H.W.; et al. Apolipoprotein E protects against neuropathology induced by a high-fat diet and maintains the integrity of the blood-brain barrier during aging. Lab. Invest. **2001**, 81, 953–960.

28 Sanabria-Castro, A.; Alvarado-Echeverría, I.; Monge-Bonilla, C. Molecular Pathogenesis of Alzheimer's Disease: An Update. Ann. Neurosci. **2017**, 24, 46–54.

29 Du, X.; Wang, X.; Geng, M. Alzheimer's disease hypothesis and related therapies. Transl. Neurodegener. **2018**, 7, 2.

30 Liu, P.P.; Xie, Y.; Meng, X.Y.; Kang, J.S. History and progress of hypotheses and clinical trials for Alzheimer's disease. Signal Transduct. Target. Ther. **2019**, 4, 29.

31 Davies, P.; Maloney, A.J.F. Selective Loss of Central Cholinergic Neurons in Alzheimer's Disease. Lancet **1976**, 308, 1403.

32 Grundke-Iqbal, I.; Iqbal, K.; Tung, Y.C.; Quinlan, M.; Wisniewski, H.M.; Binder, L.I. Abnormal phosphorylation of the microtubule-associated protein tau (tau) in Alzheimer cytoskeletal pathology. Proc. Natl. Acad. Sci. USA **1986**, 83, 4913–4917.

33 McGeer, P.L.; Itagaki, S.; Tago, H.; McGeer, E.G. Reactive microglia in patients with senile dementia of the Alzheimer type are positive for the histocompatibility glycoprotein HLA-DR. Neurosci. Lett. **1987**, 79, 195–200.

34 Selkoe, D.J. The molecular pathology of Alzheimer's disease. Neuron **1991**, 6, 487–498.

35 Hardy, J.; Allsop, D. Amyloid deposition as the central event in the aetiology of Alzheimer's disease. Trends Pharmacol. Sci. **1991**, 12, 383–388.

36 Butterfield, D.A. β-Amyloid-Associated Free Radical Oxidative Stress and Neurotoxicity: Implications for Alzheimer's Disease. Chem. Res. Toxicol. **1997**, 10, 495–506.

37 Iadecola, C. Neurovascular regulation in the normal brain and in Alzheimer's disease. Nat. Rev. Neurosci. **2004**, 5, 347–360.

38 Breit, S.; Kupferberg, A.; Rogler, G.; Hasler, G. Vagus Nerve as Modulator of the Brain-Gut Axis in Psychiatric and Inflammatory Disorders. Front. Psychiatry **2018**, 9, 44.

39 Caldwell, C.C.; Yao, J.; Brinton, R.D. Targeting the prodromal stage of Alzheimer's disease: Bioenergetic and mitochondrial opportunities. Neurotherapeutics **2015**, 12, 66–80.

40 Tripathi, M.; Tripathi, M.; Damle, N.; Kushwaha, S.; Jaimini, A.; D'Souza, M.M.; Sharma, R.; Saw, S.; Mondal, A. Differential diagnosis of neurodegenerative dementias using metabolic phenotypes on F-18 FDG PET/CT. Neuroradiol. J. **2014**, 27, 13–21.

41 Akhtar, A.; Sah, S.P. Insulin signaling pathway and related molecules: Role in neurodegeneration and Alzheimer's disease. Neurochem. Int. **2020**, 135, 104707.

42 Kulas, J.A.; Weigel, T.K.; Ferris, H.A. Insulin resistance and impaired lipid metabolism as a potential link between diabetes and Alzheimer's disease. Drug Dev. Res. **2020**, 81, 194–205.

43 Blonz, E.R. Alzheimer's Disease as the Product of a Progressive Energy Deficiency Syndrome in the Central Nervous System: The Neuroenergetic Hypothesis. J. Alzheimers Dis. **2017**, 60, 1223–1229.

44 Arnold, S.E.; Arvanitakis, Z.; Macauley-Rambach, S.L.; Koenig, A.M.; Wang, H.Y.; Ahima, R.S.; Craft, S.; Gandy, S.; Buettner, C.; Stoeckel, L.E.; et al. Brain insulin resistance in type 2 diabetes and Alzheimer disease: Concepts and conundrums. Nat. Rev. Neurol. **2018**, 14, 168–181.

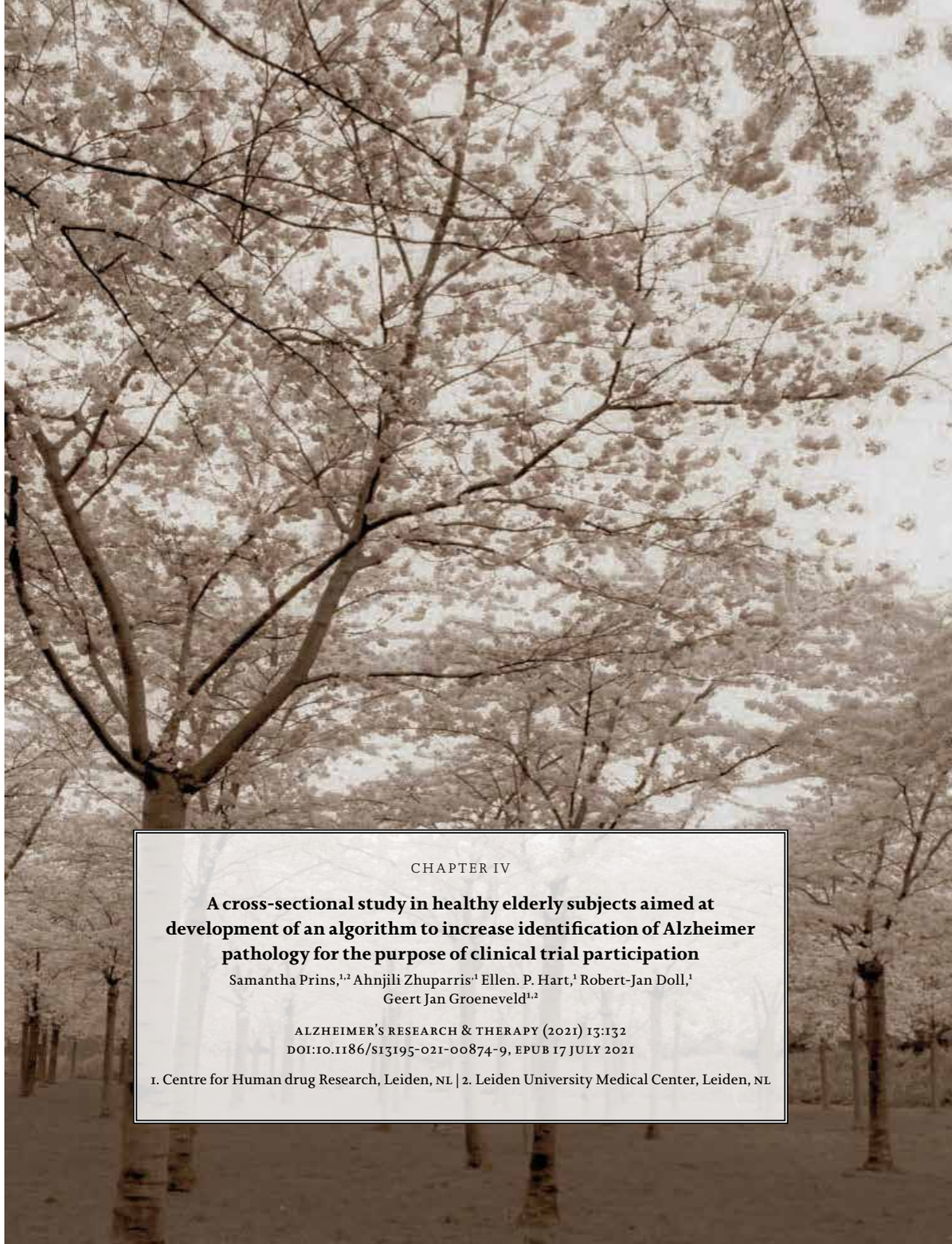
45 Kimura, N. Diabetes Mellitus Induces Alzheimer's Disease Pathology: Histopathological Evidence from Animal Models. Int. J. Mol. Sci. **2016**, 17, 503.
46 Dineley, K.T.; Jahrling, J.B.; Denner, L. Insulin resistance in Alzheimer's disease. Neurobiol. Dis. **2014**, 72 Pt A, 92–103.
47 Swerdlow, R.H. Mitochondria and Mitochondrial Cascades in Alzheimer's Disease. J. Alzheimers Dis. **2018**, 62, 1403–1416.
48 Zott, B.; Busche, M.A.; Sperling, R.A.; Konnerth, A. What Happens with the Circuit in Alzheimer's Disease in Mice and Humans? Annu. Rev. Neurosci. **2018**, 41, 277–297.
49 Jesse, S.; Steinacker, P.; Cepek, L.; von Arnim, C.A.; Tumani, H.; Lehnert, S.; Kretzschmar, H.A.; Baier, M.; Otto, M. Glial fibrillary acidic protein and protein S-100B: Different concentration pattern of glial proteins in cerebrospinal fluid of patients with Alzheimer's disease and Creutzfeldt-Jakob disease. J. Alzheimers Dis. **2009**, 17, 541–551.
50 Craig-Schapiro, R.; Perrin, R.J.; Roe, C.M.; Xiong, C.; Carter, D.; Cairns, N.J.; Mintun, M.A.; Peskind, E.R.; Li, G.; Galasko, D.R.; et al. YKL-40: A novel prognostic fluid biomarker for preclinical Alzheimer's disease. Biol. Psychiatry **2010**, 68, 903–912.
51 Wallin, A.K.; Blennow, K.; Zetterberg, H.; Londos, E.; Minthon, L.; Hansson, O. CSF biomarkers predict a more malignant outcome in Alzheimer disease. Neurology **2010**, 74, 1531–1537.
52 Bateman, R.J.; Xiong, C.; Benzinger, T.L.; Fagan, A.M.; Goate, A.; Fox, N.C.; Marcus, D.S.; Cairns, N.J.; Xie, X.; Blazey, T.M.; et al. Clinical and biomarker changes in dominantly inherited Alzheimer's disease. N. Engl. J. Med. **2012**, 367, 795–804.
53 Chen, Z.; Zhong, C. Decoding Alzheimer's disease from perturbed cerebral glucose metabolism: Implications for diagnostic and therapeutic strategies. Prog. Neurobiol. **2013**, 108, 21–43.
54 Orta-Salazar, E.; Cuellar-Lemus, C.A.; Díaz-Cintra, S.; Feria-Velasco, A.I. Cholinergic markers in the cortex and hippocampus of some animal species and their correlation to Alzheimer's disease. Neurología (English Edition) **2014**, 29, 497–503.
55 Wennstrom, M.; Surova, Y.; Hall, S.; Nilsson, C.; Minthon, L.; Hansson, O.; Nielsen, H.M. The Inflammatory Marker YKL-40 Is Elevated in Cerebrospinal Fluid from Patients with Alzheimer's but Not Parkinson's Disease or Dementia with Lewy Bodies. PLoS ONE **2015**, 10, e0135458.
56 Kamat, P.K.; Kalani, A.; Rai, S.; Swarnkar, S.; Tota, S.; Nath, C.; Tyagi, N. Mechanism of Oxidative Stress and Synapse Dysfunction in the Pathogenesis of Alzheimer's Disease: Understanding the Therapeutics Strategies. Mol. Neurobiol. **2016**, 53, 648–661.
57 Olsson, B.; Lautner, R.; Andreasson, U.; Öhrfelt, A.; Portelius, E.; Bjerke, M.; Hölttä, M.; Rosén, C.; Olsson, C.; Strobel, G.; et al. CSF and blood biomarkers for the diagnosis of Alzheimer's disease: A systematic review and meta-analysis. Lancet Neurol. **2016**, 15, 673–684.
58 Frost, G.R.; Li, Y.M. The role of astrocytes in amyloid production and Alzheimer's disease. Open Biol. **2017**, 7, 170228.
59 Huynh, R.A.; Mohan, C. Alzheimer's Disease: Biomarkers in the Genome, Blood, and Cerebrospinal Fluid. Front. Neurol. **2017**, 8, 102.
60 Li, K.; Wei, Q.; Liu, F.F.; Hu, F.; Xie, A.J.; Zhu, L.Q.; Liu, D. Synaptic Dysfunction in Alzheimer's Disease: Abeta, Tau, and Epigenetic Alterations. Mol. Neurobiol. **2018**, 55, 3021–3032.
61 Agliardi, C.; Guerini, F.R.; Zanzottera, M.; Bianchi, A.; Nemni, R.; Clerici, M. SNAP-25 in Serum Is Carried by Exosomes of Neuronal Origin and Is a Potential Biomarker of Alzheimer's Disease. Mol. Neurobiol. **2019**, 56, 5792–5798.
62 Hampel, H.; Blennow, K.; Shaw, L.M.; Hoessler, Y.C.; Zetterberg, H.; Trojanowski, J.Q. Total and phosphorylated tau protein as biological markers of Alzheimer's disease. Exp. Gerontol. **2010**, 45, 30–40.
63 Morris, M.; Maeda, S.; Vossel, K.; Mucke, L. The many faces of tau. Neuron **2011**, 70, 410–426.
64 Khan, S.S.; Bloom, G.S. Tau: The Center of a Signaling Nexus in Alzheimer's Disease. Front. Neurosci. **2016**, 10, 31.
65 Wang, J.Z.; Liu, F. Microtubule-associated protein tau in development, degeneration and protection of neurons. Prog. Neurobiol. **2008**, 85, 148–175.
66 Kim, E.K.; Choi, E.J. Pathological roles of MAPK signaling pathways in human diseases. Biochim. Biophys. Acta **2010**, 1802, 396–405.
67 Gomperts, S.N.; Carroll, R.; Malenka, R.C.; Nicoll, R.A. Distinct Roles for Ionotropic and Metabotropic Glutamate Receptors in the Maturation of Excitatory Synapses. J. Neurosci. **2000**, 20, 2229.
68 Shankar, G.M.; Bloodgood, B.L.; Townsend, M.; Walsh, D.M.; Selkoe, D.J.; Sabatini, B.L. Natural oligomers of the Alzheimer amyloid-beta protein induce reversible synapse loss by modulating an NMDA-type glutamate receptor-dependent signaling pathway. J. Neurosci. **2007**, 27, 2866–2875.
69 Phiel, C.J.; Wilson, C.A.; Lee, V.M.Y.; Klein, P.S. GSK-3α regulates production of Alzheimer's disease amyloid-β peptides. Nature **2003**, 423, 435–439.
70 Hooper, C.; Killick, R.; Lovestone, S. The GSK3 hypothesis of Alzheimer's disease. J. Neurochem. **2008**, 104, 1433–1439.
71 Vitolo, O.V.; Sant'Angelo, A.; Costanzo, V.; Battaglia, F.; Arancio, O.; Shelanski, M. Amyloid beta -peptide inhibition of the PKA/CREB pathway and long-term potentiation: Reversibility by drugs that enhance cAMP signaling. Proc. Natl. Acad. Sci. USA **2002**, 99, 13217–13221.
72 Pugazhenthi, S.; Wang, M.; Pham, S.; Sze, C.I.; Eckman, C.B. Downregulation of CREB expression in Alzheimer's brain and in Abeta-treated rat hippocampal neurons. Mol. Neurodegener. **2011**, 6, 60.
73 Souza, M.A.; Magni, D.V.; Guerra, G.P.; Oliveira, M.S.; Furian, A.F.; Pereira, L.; Marquez, S.V.; Ferreira, J.; Fighera, M.R.; Royes, L.F. Involvement of hippocampal CAMKII/CREB signaling in the spatial memory retention induced by creatine. Amino Acids **2012**, 43, 2491–2503.
74 Chen, Q.S.; Wei, W.Z.; Shimahara, T.; Xie, C.W. Alzheimer amyloid beta-peptide inhibits the late phase of long-term potentiation through calcineurin-dependent mechanisms in the hippocampal dentate gyrus. Neurobiol. Learn. Mem. **2002**, 77, 354–371.
75 Reese, L.C.; Zhang, W.; Dineley, K.T.; Kayed, R.; Taglialatela, G. Selective induction of calcineurin activity and signaling by oligomeric amyloid beta. Aging Cell **2008**, 7, 824–835.
76 Jaeger, L.B.; Dohgu, S.; Hwang, M.C.; Farr, S.A.; Murphy, M.P.; Fleegal-DeMotta, M.A.; Lynch, J.L.; Robinson, S.M.; Niehoff, M.L.; Johnson, S.N.; et al. Testing the neurovascular hypothesis of Alzheimer's disease: LRP-1 antisense reduces blood-brain barrier clearance, increases brain levels of amyloid-beta protein, and impairs cognition. J. Alzheimers Dis. **2009**, 17, 553–570.
77 Spuch, C.; Antequera, D.; Pascual, C.; Abilleira, S.; Blanco, M.; Moreno-Carretero, M.J.; Romero-Lopez, J.; Ishida, T.; Molina, J.A.; Villarejo, A.; et al. Soluble Megalin is Reduced in Cerebrospinal Fluid Samples of Alzheimer's Disease Patients. Front. Cell. Neurosci. **2015**, 9, 134.
78 Mattsson, N.; Andreasson, U.; Zetterberg, H.; Blennow, K.; Alzheimer's Disease Neuroimaging Initiative. Association of Plasma Neurofilament Light with Neurodegeneration in Patients with Alzheimer Disease. JAMA Neurol. **2017**, 74, 557–566.
79 Kester, M.I.; Teunissen, C.E.; Crimmins, D.L.; Herries, E.M.; Ladenson, J.H.; Scheltens, P.; van der Flier, W.M.; Morris, J.C.; Holtzman, D.M.; Fagan, A.M. Neurogranin as a Cerebrospinal Fluid Biomarker for Synaptic Loss in Symptomatic Alzheimer Disease. JAMA Neurol. **2015**, 72, 1275–1280.
80 Cho, S.M.; Lee, S.; Yang, S.H.; Kim, H.Y.; Lee, M.J.; Kim, H.V.; Kim, J.; Baek, S.; Yun, J.; Kim, D.; et al. Age-dependent inverse correlations in CSF and plasma amyloid-beta(1-42) concentrations prior to amyloid plaque deposition in the brain of 3xTg-AD mice. Sci. Rep. **2016**, 6, 20185.
81 Maia, L.F.; Kaeser, S.A.; Reichwald, J.; Hruscha, M.; Martus, P.; Staufenbiel, M.; Jucker, M. Changes in Amyloid-β and Tau in the Cerebrospinal Fluid of Transgenic Mice Overexpressing Amyloid Precursor Protein. Sci. Transl. Med. **2013**, 5, 194re2.
82 Maia, L.F.; Kaeser, S.A.; Reichwald, J.; Lambert, M.; Obermuller, U.; Schelle, J.; Odenthal, J.; Martus, P.; Staufenbiel, M.; Jucker, M. Increased CSF Abeta during the very early phase of cerebral Abeta deposition in mouse models. EMBO Mol. Med. **2015**, 7, 895–903.
83 Janelidze, S.; Stomrud, E.; Palmqvist, S.; Zetterberg, H.; van Westen, D.; Jeromin, A.; Song, L.; Hanlon, D.; Tan Hehir, C.A.; Baker, D.; et al. Plasma beta-amyloid in Alzheimer's disease and vascular disease. Sci. Rep. **2016**, 6, 26801.
84 Kawarabayashi, T.; Younkin, L.H.; Saido, T.C.; Shoji, M.; Ashe, K.H.; Younkin, S.G. Age-dependent changes in brain, CSF, and plasma amyloid (beta) protein in the Tg2576 transgenic mouse model of Alzheimer's disease. J. Neurosci. **2001**, 21, 372–381.
85 Park, J.C.; Han, S.H.; Yi, D.; Byun, M.S.; Lee, J.H.; Jang, S.; Ko, K.; Jeon, S.Y.; Lee, Y.S.; Kim, Y.K.; et al. Plasma tau/amyloid-beta1-42 ratio predicts brain tau deposition and neurodegeneration in Alzheimer's disease. Brain **2019**, 142, 771–786.
86 Lecanu, L.; Greeson, J.; Papadopoulos, V. Beta-amyloid and oxidative stress jointly induce neuronal death,

amyloid deposits, gliosis, and memory impairment in the rat brain. Pharmacology **2006**, 76, 19–33.
87 Li, L.; Jiang, Y.; Hu, W.; Tung, Y.C.; Dai, C.; Chu, D.; Gong, C.X.; Iqbal, K.; Liu, F. Pathological Alterations of Tau in Alzheimer's Disease and 3xTg-AD Mouse Brains. Mol. Neurobiol. **2019**, 56, 6168–6183.
88 Thorsell, A.; Bjerke, M.; Gobom, J.; Brunhage, E.; Vanmechelen, E.; Andreasen, N.; Hansson, O.; Minthon, L.; Zetterberg, H.; Blennow, K. Neurogranin in cerebrospinal fluid as a marker of synaptic degeneration in Alzheimer's disease. Brain Res. **2010**, 1362, 13–22.
89 Antonell, A.; Mansilla, A.; Rami, L.; Llado, A.; Iranzo, A.; Olives, J.; Balasa, M.; Sanchez-Valle, R.; Molinuevo, J.L. Cerebrospinal fluid level of YKL-40 protein in preclinical and prodromal Alzheimer's disease. J. Alzheimers Dis. **2014**, 42, 901–908.
90 Zhao, Q.; Liu, M.; Ha, L.; Zhou, Y.; Alzheimer's Disease Neuroimaging Initiative. Quantitative (18)F-AV1451 Brain Tau PET Imaging in Cognitively Normal Older Adults, Mild Cognitive Impairment, and Alzheimer's Disease Patients. Front. Neurol. **2019**, 10, 486.
91 Ohrfelt, A.; Brinkmalm, A.; Dumurgier, J.; Zetterberg, H.; Bouaziz-Amar, E.; Hugon, J.; Paquet, C.; Blennow, K. A Novel ELISA for the Measurement of Cerebrospinal Fluid SNAP-25 in Patients with Alzheimer's Disease. Neuroscience **2019**, 420, 136–144.
92 Belfiore, R.; Rodin, A.; Ferreira, E.; Velazquez, R.; Branca, C.; Caccamo, A.; Oddo, S. Temporal and regional progression of Alzheimer's disease-like pathology in 3xTg-AD mice. Aging Cell **2019**, 18, e12873.
93 Zetterberg, H.; Wilson, D.; Andreasson, U.; Minthon, L.; Blennow, K.; Randall, J.; Hansson, O. Plasma tau levels in Alzheimer's disease. Alzheimer's Res. Ther. **2013**, 5, 9.
94 Mattsson, N.; Zetterberg, H.; Janelidze, S.; Insel, P.S.; Andreasson, U.; Stomrud, E.; Palmqvist, S.; Baker, D.; Tan Hehir, C.A.; Jeromin, A.; et al. Plasma tau in Alzheimer disease. Neurology **2016**, 87, 1827–1835.
95 De Vos, A.; Jacobs, D.; Struyfs, H.; Fransen, E.; Andersson, K.; Portelius, E.; Andreasson, U.; De Surgeloose, D.; Hernalsteen, D.; Sleegers, K.; et al. C-terminal neurogranin is increased in cerebrospinal fluid but unchanged in plasma in Alzheimer's disease. Alzheimers Dement. **2015**, 11, 1461–1469.
96 Palmqvist, S.; Janelidze, S.; Stomrud, E.; Zetterberg, H.; Karl, J.; Zink, K.; Bittner, T.; Mattsson, N.; Eichenlaub, U.; Blennow, K.; et al. Performance of Fully Automated Plasma Assays as Screening Tests for Alzheimer Disease-Related beta-Amyloid Status. JAMA Neurol. **2019**. doi:10.1001/jamaneurol.2019.1632.
97 Rosengren, L.E.; Karlsson, J.-E.; Karlsson, J.-O.; Persson, L.I.; Wikkelsø, C. Patients with Amyotrophic Lateral Sclerosis and Other Neurodegenerative Diseases Have Increased Levels of Neurofilament Protein in CSF. J. Neurochem. **1996**, 67, 2013–2018.
98 Abu-Rumeileh, S.; Steinacker, P.; Polischi, B.; Mammana, A.; Bartoletti-Stella, A.; Oeckl, P.; Baiardi, S.; Zenesini, C.; Huss, A.; Cortelli, P.; et al. CSF biomarkers of neuroinflammation in distinct forms and subtypes of neurodegenerative dementia. Alzheimers Res. Ther. **2019**, 12, 2.
99 Zhou, W.; Zhang, J.; Ye, F.; Xu, G.; Su, H.; Su, Y.; Zhang, X.; Alzheimer's Disease Neuroimaging Initiative. Plasma neurofilament light chain levels in Alzheimer's disease. Neurosci. Lett. **2017**, 650, 60–64.
100 Lewczuk, P.; Ermann, N.; Andreasson, U.; Schultheis, C.; Podhorna, J.; Spitzer, P.; Maler, J.M.; Kornhuber, J.; Blennow, K.; Zetterberg, H. Plasma neurofilament light as a potential biomarker of neurodegeneration in Alzheimer's disease. Alzheimers Res. Ther. **2018**, 10, 71.
101 Lin, Y.S.; Lee, W.J.; Wang, S.J.; Fuh, J.L. Levels of plasma neurofilament light chain and cognitive function in patients with Alzheimer or Parkinson disease. Sci. Rep. **2018**, 8, 17368.
102 Bacioglu, M.; Maia, L.F.; Preische, O.; Schelle, J.; Apel, A.; Kaeser, S.A.; Schweighauser, M.; Eninger, T.; Lambert, M.; Pilotto, A.; et al. Neurofilament Light Chain in Blood and CSF as Marker of Disease Progression in Mouse Models and in Neurodegenerative Diseases. Neuron **2016**, 91, 56–66.
103 Kvartsberg, H.; Lashley, T.; Murray, C.E.; Brinkmalm, G.; Cullen, N.C.; Hoglund, K.; Zetterberg, H.; Blennow, K.; Portelius, E. The intact postsynaptic protein neurogranin is reduced in brain tissue from patients with familial and sporadic Alzheimer's disease. Acta Neuropathol. **2019**, 137, 89–102.
104 Hellwig, K.; Kvartsberg, H.; Portelius, E.; Andreasson, U.; Oberstein, T.J.; Lewczuk, P.; Blennow, K.; Kornhuber, J.; Maler, J.M.; Zetterberg, H.; et al. Neurogranin and YKL-40: Independent markers of synaptic degeneration and neuroinflammation in Alzheimer's disease. Alzheimers Res. Ther. **2015**, 7, 74.
105 Kvartsberg, H.; Portelius, E.; Andreasson, U.; Brinkmalm, G.; Hellwig, K.; Lelental, N.; Kornhuber, J.; Hansson, O.; Minthon, L.; Spitzer, P.; et al. Characterization of the postsynaptic protein neurogranin in paired cerebrospinal fluid and plasma samples from Alzheimer's disease patients and healthy controls. Alzheimers Res. Ther. **2015**, 7, 40.
106 Wang, L.; Alzheimer's Disease Neuroimaging Initiative. Association of cerebrospinal fluid Neurogranin with Alzheimer's disease. Aging Clin. Exp. Res. **2019**, 31, 185–191.
107 Shimohama, S.; Kamiya, S.; Taniguchi, T.; Akagawa, K.; Kimura, J. Differential Involvement of Synaptic Vesicle and Presynaptic Plasma Membrane Proteins in Alzheimer's Disease. Biochem. Biophys. Res. Commun. **1997**, 236, 239–242.
108 Brinkmalm, A.; Brinkmalm, G.; Honer, W.G.; Frölich, L.; Hausner, L.; Minthon, L.; Hansson, O.; Wallin, A.; Zetterberg, H.; Blennow, K.; et al. SNAP-25 is a promising novel cerebrospinal fluid biomarker for synapse degeneration in Alzheimer's disease. Mol. Neurodegener. **2014**, 9, 53.
109 Zhang, H.; Therriault, J.; Kang, M.S.; Ng, K.P.; Pascoal, T.A.; Rosa-Neto, P.; Gauthier, S.; Alzheimer's Disease Neuroimaging Initiative. Cerebrospinal fluid synaptosomal-associated protein 25 is a key player in synaptic degeneration in mild cognitive impairment and Alzheimer's disease. Alzheimers Res. Ther. **2018**, 10, 80.
110 Wang, S.; Zhang, J.; Pan, T.; Alzheimer's Disease Neuroimaging Initiative. APOE epsilon4 is associated with higher levels of CSF SNAP-25 in prodromal Alzheimer's disease. Neurosci. Lett. **2018**, 685, 109–113.
111 Barroeta-Espar, I.; Weinstock, L.D.; Perez-Nievas, B.G.; Meltzer, A.C.; Siao Tick Chong, M.; Amaral, A.C.; Murray, M.E.; Moulder, K.L.; Morris, J.C.; Cairns, N.J.; et al. Distinct cytokine profiles in human brains resilient to Alzheimer's pathology. Neurobiol. Dis. **2019**, 121, 327–337.
112 Singh-Bains, M.K.; Linke, V.; Austria, M.D.R.; Tan, A.Y.S.; Scotter, E.L.; Mehrabi, N.F.; Faull, R.L.M.; Dragunow, M. Altered microglia and neurovasculature in the Alzheimer's disease cerebellum. Neurobiol. Dis. **2019**, 132, 104589.
113 Minkeviciene, R.; Ihalainen, J.; Malm, T.; Matilainen, O.; Keksa-Goldsteine, V.; Goldsteins, G.; Iivonen, H.; Leguit, N.; Glennon, J.; Koistinaho, J.; et al. Age-related decrease in stimulated glutamate release and vesicular glutamate transporters in APP/PS1 transgenic and wild-type mice. J. Neurochem. **2008**, 105, 584–594.
114 Caruso, D.; Barron, A.M.; Brown, M.A.; Abbiati, F.; Carrero, P.; Pike, C.J.; Garcia-Segura, L.M.; Melcangi, R.C. Age-related changes in neuroactive steroid levels in 3xTg-AD mice. Neurobiol. Aging **2013**, 34, 1080–1089.
115 Radde, R.; Bolmont, T.; Kaeser, S.A.; Coomaraswamy, J.; Lindau, D.; Stoltze, L.; Calhoun, M.E.; Jaggi, F.; Wolburg, H.; Gengler, S.; et al. Abeta42-driven cerebral amyloidosis in transgenic mice reveals early and robust pathology. EMBO Rep. **2006**, 7, 940–946.
116 Hanzel, C.E.; Pichet-Binette, A.; Pimentel, L.S.; Iulita, M.F.; Allard, S.; Ducatenzeiler, A.; Do Carmo, S.; Cuello, A.C. Neuronal driven pre-plaque inflammation in a transgenic rat model of Alzheimer's disease. Neurobiol. Aging **2014**, 35, 2249–2262.
117 Cohen, R.M.; Rezai-Zadeh, K.; Weitz, T.M.; Rentsendorj, A.; Gate, D.; Spivak, I.; Bholat, Y.; Vasilevko, V.; Glabe, C.G.; Breunig, J.J.; et al. A transgenic Alzheimer rat with plaques, tau pathology, behavioral impairment, oligomeric abeta, and frank neuronal loss. J. Neurosci. **2013**, 33, 6245–6256.
118 Ishiki, A.; Kamada, M.; Kawamura, Y.; Terao, C.; Shimoda, F.; Tomita, N.; Arai, H.; Furukawa, K. Glial fibrillar acidic protein in the cerebrospinal fluid of Alzheimer's disease, dementia with Lewy bodies, and frontotemporal lobar degeneration. J. Neurochem. **2016**, 136, 258–261.
119 Oeckl, P.; Halbgebauer, S.; Anderl-Straub, S.; Steinacker, P.; Huss, A.M.; Neugebauer, H.; von Arnim, C.A.F.; Diehl-Schmid, J.; Grimmer, T.; Kornhuber, J.; et al. Glial Fibrillary Acidic Protein in Serum is Increased in Alzheimer's Disease and Correlates with Cognitive Impairment. J. Alzheimers Dis. **2019**, 67, 481–488.
120 Llorens,F.; Thune,K.;Tahir,W.;Kanata,E.; Diaz-Lucena, D.; Xanthopoulos, K.; Kovatsi, E.; Pleschka, C.; Garcia-Esparcia, P.; Schmitz, M.; et al. YKL-40 in the brain and cerebrospinal fluid of neurodegenerative dementias. Mol. Neurodegener. **2017**, 12, 83.
121 Villar-Pique, A.; Schmitz, M.; Hermann, P.; Goebel, S.; Bunck, T.; Varges, D.; Ferrer, I.; Riggert, J.; Llorens, F.; Zerr, I. Plasma YKL-40 in the spectrum of neurodegenerative dementia. J. Neuroinflamm. **2019**, 16, 145.
122 Roher, A.E.; Esh, C.L.; Kokjohn, T.A.; Castaño, E.M.; Van Vickle, G.D.; Kalback, W.M.; Patton, R.L.; Luehrs, D.C.; Daugs, I.D.; Kuo, Y.-M.; et al. Amyloid beta peptides

in human plasma and tissues and their significance for Alzheimer's disease. Alzheimer's Dement. **2009**, 5, 18–29.
123 Games, D.; Adams, D.; Alessandrini, R.; Barbour, R.; Borthelette, P.; Blackwell, C.; Carr, T.; Clemens, J.; Donaldson, T.; Gillespie, F.; et al. Alzheimer-type neuropathology in transgenic mice overexpressing V717F β-amyloid precursor protein. Nature **1995**, 373, 523–527.
124 Hsiao, K.; Chapman, P.; Nilsen, S.; Eckman, C.; Harigaya, Y.; Younkin, S.; Yang, F.; Cole, G. Correlative Memory Deficits, Aβ Elevation, and Amyloid Plaques in Transgenic Mice. Science **1996**, 274, 99–103.
125 Snellman, A.; Lopez-Picon, F.R.; Rokka, J.; Salmona, M.; Forloni, G.; Scheinin, M.; Solin, O.; Rinne, J.O.; Haaparanta-Solin, M. Longitudinal amyloid imaging in mouse brain with 11C-PIB: Comparison of APP23, Tg2576, and APPswe-PS1dE9 mouse models of Alzheimer disease. J. Nucl. Med. **2013**, 54, 1434–1441.
126 Jacobsen, J.S.; Wu, C.-C.; Redwine, J.M.; Comery, T.A.; Arias, R.; Bowlby, M.; Martone, R.; Morrison, J.H.; Pangalos, M.N.; Reinhart, P.H.; et al. Early-onset behavioral and synaptic deficits in a mouse model of Alzheimer's disease. Proc. Natl. Acad. Sci. USA **2006**, 103, 5161–5166.
127 Izco, M.; Martinez, P.; Corrales, A.; Fandos, N.; Garcia, S.; Insua, D.; Montanes, M.; Perez-Grijalba, V.; Rueda, N.; Vidal, V.; et al. Changes in the brain and plasma Abeta peptide levels with age and its relationship with cognitive impairment in the APPswe/PS1dE9 mouse model of Alzheimer's disease. Neuroscience **2014**, 263, 269–279.
128 Ordonez-Gutierrez, L.; Anton, M.; Wandosell, F. Peripheral amyloid levels present gender differences associated with aging in AbetaPP/PS1 mice. J. Alzheimers Dis. **2015**, 44, 1063–1068.
129 Oddo, S.; Caccamo, A.; Shepherd, J.D.; Murphy, M.P.; Golde, T.E.; Kayed, R.; Metherate, R.; Mattson, M.P.; Akbari, Y.; LaFerla, F.M. Triple-Transgenic Model of Alzheimer's Disease with Plaques and Tangles. Neuron **2003**, 39, 409–421.
130 Harach, T.; Marungruang, N.; Duthilleul, N.; Cheatham, V.; Mc Coy, K.D.; Frisoni, G.; Neher, J.J.; Fak, F.; Jucker, M.; Lasser, T.; et al. Reduction of Abeta amyloid pathology in APPPS1 transgenic mice in the absence of gut microbiota. Sci. Rep. **2017**, 7, 41802.
131 Van Dam, D.; D'Hooge, R.; Staufenbiel, M.; Van Ginneken, C.; Van Meir, F.; De Deyn, P.P. Age-dependent cognitive decline in the APP23 model precedes amyloid deposition. Eur. J. Neurosci. **2003**, 17, 388–396.
132 Sturchler-Pierrat, C.; Abramowski, D.; Duke, M.; Wiederhold, K.-H.; Mistl, C.; Rothacher, S.; Ledermann, B.; Bürki, K.; Frey, P.; Paganetti, P.A.; et al. Two amyloid precursor protein transgenic mouse models with Alzheimer disease-like pathology. Proc. Natl. Acad. Sci. USA **1997**, 94, 13287–13292.
133 Iulita, M.F.; Allard, S.; Richter, L.; Munter, L.M.; Ducatenzeiler, A.; Weise, C.; Do Carmo, S.; Klein, W.L.; Multhaup, G.; Cuello, A.C. Intracellular Abeta pathology and early cognitive impairments in a transgenic rat overexpressing human amyloid precursor protein: A multidimensional study. Acta Neuropathol. Commun. **2014**, 2, 61.
134 Rorabaugh, J.M.; Chalermpalanupap, T.; Botz-Zapp, C.A.; Fu, V.M.; Lembeck, N.A.; Cohen, R.M.; Weinshenker, D. Chemogenetic locus coeruleus activation restores reversal learning in a rat model of Alzheimer's disease. Brain **2017**, 140, 3023–3038.
135 Forny-Germano, L.; Lyra e Silva, N.M.; Batista, A.F.; Brito-Moreira, J.; Gralle, M.; Boehnke, S.E.; Coe, B.C.; Lablans, A.; Marques, S.A.; Martinez, A.M.; et al. Alzheimer's disease-like pathology induced by amyloid-beta oligomers in nonhuman primates. J. Neurosci. **2014**, 34, 13629–13643.
136 Fukuyama, R.; Mizuno, T.; Mori, S.; Nakajima, K.; Fushiki, S.; Yanagisawa, K. Age-dependent change in the levels of Abeta40 and Abeta42 in cerebrospinal fluid from control subjects, and a decrease in the ratio of Abeta42 to Abeta40 level in cerebrospinal fluid from Alzheimer's disease patients. Eur. Neurol. **2000**, 43, 155–160.
137 Giedraitis, V.; Sundelof, J.; Irizarry, M.C.; Garevik, N.; Hyman, B.T.; Wahlund, L.O.; Ingelsson, M.; Lannfelt, L. The normal equilibrium between CSF and plasma amyloid beta levels is disrupted in Alzheimer's disease. Neurosci. Lett. **2007**, 427, 127–131.
138 Motter, R.; Vigo-Pelfrey, C.; Kholodenko, D.; Barbour, R.; Johnson-Wood, K.; Galasko, D.; Chang, L.; Miller, B.; Clark, C.; Green, R.; et al. Reduction of β-amyloid peptide42 in the cerebrospinal fluid of patients with Alzheimer's disease. Ann. Neurol. **1995**, 38, 643–648.
139 Riemenschneider, M.; Schmolke, M.; Lautenschlager, N.; Guder, W.G.; Vanderstichele, H.; Vanmechelen, E.; Kurz, A. Cerebrospinal beta-amyloid (1–42) in early Alzheimer's disease: Association with apolipoprotein E genotype and cognitive decline. Neurosci. Lett. **2000**, 284, 85–88.
140 Clark, C.M.; Xie, S.; Chittams, J.; Ewbank, D.; Peskind, E.; Galasko, D.; Morris, J.C.; McKeel, D.W., Jr.; Farlow, M.; Weitlauf, S.L.; et al. Cerebrospinal Fluid Tau and β-Amyloid: How Well Do These Biomarkers Reflect Autopsy-Confirmed Dementia Diagnoses? Arch. Neurol. **2003**, 60, 1696–1702.
141 Liu, L.; Herukka, S.K.; Minkeviciene, R.; van Groen, T.; Tanila, H. Longitudinal observation on CSF Abeta42 levels in young to middle-aged amyloid precursor protein/presenilin-1 doubly transgenic mice. Neurobiol. Dis. **2004**, 17, 516–523.
142 Parent, M.J.; Zimmer, E.R.; Shin, M.; Kang, M.S.; Fonov, V.S.; Mathieu, A.; Aliaga, A.; Kostikov, A.; Do Carmo, S.; Dea, D.; et al. Multimodal Imaging in Rat Model Recapitulates Alzheimer's Disease Biomarkers Abnormalities. J. Neurosci. **2017**, 37, 12263–12271.
143 Toledo, J.B.; Vanderstichele, H.; Figurski, M.; Aisen, P.S.; Petersen, R.C.; Weiner, M.W.; Jack, C.R., Jr.; Jagust, W.; Decarli, C.; Toga, A.W.; et al. Factors affecting Abeta plasma levels and their utility as biomarkers in ADNI. Acta Neuropathol. **2011**, 122, 401–413.
144 Rembach, A.; Faux, N.G.; Watt, A.D.; Pertile, K.K.; Rumble, R.L.; Trounson, B.O.; Fowler, C.J.; Roberts, B.R.; Perez, K.A.; Li, Q.X.; et al. Changes in plasma amyloid beta in a longitudinal study of aging and Alzheimer's disease. Alzheimers Dement. **2014**, 10, 53–61.
145 Niemantsverdriet, E.; Ottoy, J.; Somers, C.; De Roeck, E.; Struyfs, H.; Soetewey, F.; Verhaeghe, J.; Van den Bossche, T.; Van Mossevelde, S.; Goeman, J.; et al. The Cerebrospinal Fluid Abeta1-42/Abeta1-40 Ratio Improves Concordance with Amyloid-PET for Diagnosing Alzheimer's Disease in a Clinical Setting. J. Alzheimers Dis. **2017**, 60, 561–576.
146 Lehmann, S.; Delaby, C.; Boursier, G.; Catteau, C.; Ginestet, N.; Tiers, L.; Maceski, A.; Navucet, S.; Paquet, C.; Dumurgier, J.; et al. Relevance of Abeta42/40 Ratio for Detection of Alzheimer Disease Pathology in Clinical Routine: The PLMR Scale. Front. Aging Neurosci. **2018**, 10, 138.
147 Zetterberg, H. Plasma amyloid beta-quo vadis? Neurobiol. Aging **2015**, 36, 2671–2673.
148 Verberk, I.M.W.; Slot, R.E.; Verfaillie, S.C.J.; Heijst, H.; Prins, N.D.; van Berckel, B.N.M.; Scheltens, P.; Teunissen, C.E.; van der Flier, W.M. Plasma Amyloid as Prescreener for the Earliest Alzheimer Pathological Changes. Ann. Neurol. **2018**, 84, 648–658.
149 Nakamura, A.; Kaneko, N.; Villemagne, V.L.; Kato, T.; Doecke, J.; Dore, V.; Fowler, C.; Li, Q.X.; Martins, R.; Rowe, C.; et al. High performance plasma amyloid-beta biomarkers for Alzheimer's disease. Nature **2018**, 554, 249–254.
150 Dubois, B.; Feldman, H.H.; Jacova, C.; Cummings, J.L.; DeKosky, S.T.; Barberger-Gateau, P.; Delacourte, A.; Frisoni, G.; Fox, N.C.; Galasko, D.; et al. Revising the definition of Alzheimer's disease: A new lexicon. Lancet Neurol. **2010**, 9, 1118–1127.
151 Albani, D.; Marizzoni, M.; Ferrari, C.; Fusco, F.; Boeri, L.; Raimondi, I.; Jovicich, J.; Babiloni, C.; Soricelli, A.; Lizio, R.; et al. Plasma Abeta42 as a Biomarker of Prodromal Alzheimer's Disease Progression in Patients with Amnestic Mild Cognitive Impairment: Evidence from the PharmaCog/E-ADNI Study. J. Alzheimers Dis. **2019**, 69, 37–48.
152 Leuzy, A.; Chiotis, K.; Lemoine, L.; Gillberg, P.G.; Almkvist, O.; Rodriguez-Vieitez, E.; Nordberg, A. Tau PET imaging in neurodegenerative tauopathies-still a challenge. Mol. Psychiatry **2019**, 24, 1112–1134.
153 Mattsson, N.; Insel, P.S.; Palmqvist, S.; Portelius, E.; Zetterberg, H.; Weiner, M.; Blennow, K.; Hansson, O.; Alzheimer's Disease Neuroimaging Initiative. Cerebrospinal fluid tau, neurogranin, and neurofilament light in Alzheimer's disease. EMBO Mol. Med. **2016**, 8, 1184–1196.
154 Mattsson, N.; Scholl, M.; Strandberg, O.; Smith, R.; Palmqvist, S.; Insel, P.S.; Hagerstrom, D.; Ohlsson, T.; Zetterberg, H.; Jogi, J.; et al. (18)F-AV-1451 and CSF T-tau and P-tau as biomarkers in Alzheimer's disease. EMBO Mol. Med. **2017**, 9, 1212–1223.
155 Zetterberg, H.; Skillback, T.; Mattsson, N.; Trojanowski, J.Q.; Portelius, E.; Shaw, L.M.; Weiner, M.W.; Blennow, K.; Alzheimer's Disease Neuroimaging Initiative. Association of Cerebrospinal Fluid Neurofilament Light Concentration With Alzheimer Disease Progression. JAMA Neurol. **2016**, 73, 60–67.
156 Preische, O.; Schultz, S.A.; Apel, A.; Kuhle, J.; Kaeser, S.A.; Barro, C.; Graber, S.; Kuder-Buletta, E.; LaFougere, C.; Laske, C.; et al. Serum neurofilament dynamics

predicts neurodegeneration and clinical progression in presymptomatic Alzheimer's disease. Nat. Med. **2019**, 25, 277–283.
157 Wellington, H.; Paterson, R.W.; Portelius, E.; Törnqvist, U.; Magdalinou, N.; Fox, N.C.; Blennow, K.; Schott, J.M.; Zetterberg, H. Increased CSF neurogranin concentration is specific to Alzheimer disease. Neurology **2016**, 86, 829.
158 Mavroudis, I.A.; Petridis, F.; Chatzikonstantinou, S.; Kazis, D. A meta-analysis on CSF neurogranin levels for the diagnosis of Alzheimer's disease and mild cognitive impairment. Aging Clin. Exp. Res. **2019**. doi:10.1007/s40520-019-01326-z.
159 Hoglund, K.; Schussler, N.; Kvartsberg, H.; Smailovic, U.; Brinkmalm, G.; Liman, V.; Becker, B.; Zetterberg, H.; Cedazo-Minguez, A.; Janelidze, S.; et al. Cerebrospinal fluid neurogranin in an inducible mouse model of neurodegeneration: A translatable marker of synaptic degeneration. Neurobiol. Dis. **2020**, 134, 104645.
160 Terry, R.D.; Masliah, E.; Salmon, D.P.; Butters, N.; DeTeresa, R.; Hill, R.; Hansen, L.A.; Katzman, R. Physical basis of cognitive alterations in alzheimer's disease: Synapse loss is the major correlate of cognitive impairment. Ann. Neurol. **1991**, 30, 572–580.
161 Taoufik, E.; Kouroupi, G.; Zygogianni, O.; Matsas, R. Synaptic dysfunction in neurodegenerative and neurodevelopmental diseases: An overview of induced pluripotent stem-cell-based disease models. Open Biol. **2018**, 8, 180138.
162 Masliah, E.; Mallory, M.; Alford, M.; DeTeresa, R.; Hansen, L.A.; McKeel, D.W.; Morris, J.C. Altered expression of synaptic proteins occurs early during progression of Alzheimer's disease. Neurology **2001**, 56, 127–129.
163 Marksteiner, J.; Kaufmann, W.A.; Gurka, P.; Humpel, C. Synaptic proteins in Alzheimer's disease. J. Mol. Neurosci. **2002**, 18, 53–63.
164 Reddy, P.H.; Mani, G.; Park, B.S.; Jacques, J.; Murdoch, G.; Whetsell, W., Jr.; Kaye, J.; Manczak, M. Differential loss of synaptic proteins in Alzheimer's disease: Implications for synaptic dysfunction. J. Alzheimers Dis. **2005**, 7, 103–117; discussion 173–180.
165 Masliah, E. Mechanisms of synaptic dysfunction in Alzheimer's disease. Histol. Histopathol. **1995**, 10, 509–519.
166 Yao, P.J.; Zhu, M.; Pyun, E.I.; Brooks, A.I.; Therianos, S.; Meyers, V.E.; Coleman, P.D. Defects in expression of genes related to synaptic vesicle traffickingin frontal cortex of Alzheimer's disease. Neurobiol. Dis. **2003**, 12, 97–109.
167 Lanz, T.A.; Carter, D.B.; Merchant, K.M. Dendritic spine loss in the hippocampus of young PDAPP and Tg2576 mice and its prevention by the ApoE2 genotype. Neurobiol. Dis. **2003**, 13, 246–253.
168 Hong, S.; Beja-Glasser, V.F.; Nfonoyim, B.M.; Frouin, A.; Li, S.; Ramakrishnan, S.; Merry, K.M.; Shi, Q.; Rosenthal, A.; Barres, B.A.; et al. Complement and microglia mediate early synapse loss in Alzheimer mouse models. Science **2016**, 352, 712–716.
169 Bittner, T.; Burgold, S.; Dorostkar, M.M.; Fuhrmann, M.; Wegenast-Braun, B.M.; Schmidt, B.; Kretzschmar, H.; Herms, J. Amyloid plaque formation precedes dendritic spine loss. Acta Neuropathol. **2012**, 124, 797–807.
170 Mandrekar-Colucci, S.; Landreth, G.E. Microglia and inflammation in Alzheimer's disease. CNS Neurol. Disord. Drug Targets **2010**, 9, 156–167.
171 Muszynski, P.; Groblewska, M.; Kulczynska-Przybik, A.; Kulakowska, A.; Mroczko, B. YKL-40 as a Potential Biomarker and a Possible Target in Therapeutic Strategies of Alzheimer's Disease. Curr. Neuropharmacol. **2017**, 15, 906–917.
172 Delay, C.; Mandemakers, W.; Hebert, S.S. MicroRNAs in Alzheimer's disease. Neurobiol. Dis. **2012**, 46, 285–290.
173 Alexandrov, P.N.; Dua, P.; Hill, J.M.; Bhattacharjee, S.; Zhao, Y.; Lukiw, W.J. microRNA (miRNA) speciation in Alzheimer's disease (AD) cerebrospinal fluid (CSF) and extracellular fluid (ECF). Int. J. Biochem. Mol. Biol. **2012**, 3, 365–373.
174 Geekiyanage, H.; Jicha, G.A.; Nelson, P.T.; Chan, C. Blood serum miRNA: Non-invasive biomarkers for Alzheimer's disease. Exp. Neurol. **2012**, 235, 491–496.
175 Luo, H.; Wu, Q.; Ye, X.; Xiong, Y.; Zhu, J.; Xu, J.; Diao, Y.; Zhang, D.; Wang, M.; Qiu, J.; et al. Genome-wide analysis of miRNA signature in the APPswe/PS1DeltaE9 mouse model of alzheimer's disease. PLoS ONE **2014**, 9, e101725.
176 Wang, X.; Liu, P.; Zhu, H.; Xu, Y.; Ma, C.; Dai, X.; Huang, L.; Liu, Y.; Zhang, L.; Qin, C. miR-34a, a microRNA up-regulated in a double transgenic mouse model of Alzheimer's disease, inhibits bcl2 translation. Brain Res. Bull. **2009**, 80, 268–273.
177 Wang, H.; Liu, J.; Zong, Y.; Xu, Y.; Deng, W.; Zhu, H.; Liu, Y.; Ma, C.; Huang, L.; Zhang, L.; et al. miR-106b aberrantly expressed in a double transgenic mouse model for Alzheimer's disease targets TGF-beta type II receptor. Brain Res. **2010**, 1357, 166–174.
178 Garza-Manero, S.; Arias, C.; Bermudez-Rattoni, F.; Vaca, L.; Zepeda, A. Identification of age- and disease-related alterations in circulating miRNAs in a mouse model of Alzheimer's disease. Front. Cell. Neurosci. **2015**, 9, 53.
179 Ryan, M.M.; Guevremont, D.; Mockett, B.G.; Abraham, W.C.; Williams, J.M. Circulating Plasma microRNAs are Altered with Amyloidosis in a Mouse Model of Alzheimer's Disease. J. Alzheimers Dis. **2018**, 66, 835–852.
180 Papassotiropoulos, A.; Fountoulakis, M.; Dunckley, T.; Stephan, D.A.; Reiman, E.M. Genetics, transcriptomics, and proteomics of Alzheimer's disease. J. Clin. Psychiatry **2006**, 67, 652–670.
181 Shi, L.; Baird, A.L.; Westwood, S.; Hye, A.; Dobson, R.; Thambisetty, M.; Lovestone, S. A Decade of Blood Biomarkers for Alzheimer's Disease Research: An Evolving Field, Improving Study Designs, and the Challenge of Replication. J. Alzheimers Dis. **2018**, 62, 1181–1198.
182 Carlyle, B.C.; Trombetta, B.A.; Arnold, S.E. Proteomic Approaches for the Discovery of Biofluid Biomarkers of Neurodegenerative Dementias. Proteomes **2018**, 6, 32.
183 Davidsson, P.; Westman-Brinkmalm, A.; Nilsson, C.L.; Lindbjer, M.; Paulson, L.; Andreasen, N.; Sjogren, M.; Blennow, K. Proteome analysis of cerebrospinal fluid proteins in Alzheimer patients. Neuroreport **2002**, 13, 611–615.
184 Puchades, M.; Hansson, S.F.; Nilsson, C.L.; Andreasen, N.; Blennow, K.; Davidsson, P. Proteomic studies of potential cerebrospinal fluid protein markers for Alzheimer's disease. Brain Res. Mol. Brain Res. **2003**, 118, 140–146.
185 Olah, Z.; Kalman, J.; Toth, M.E.; Zvara, A.; Santha, M.; Ivitz, E.; Janka, Z.; Pakaski, M. Proteomic analysis of cerebrospinal fluid in Alzheimer's disease: Wanted dead or alive. J. Alzheimers Dis. **2015**, 44, 1303–1312.
186 Kiddle, S.J.; Sattlecker, M.; Proitsi, P.; Simmons, A.; Westman, E.; Bazenet, C.; Nelson, S.K.; Williams, S.; Hodges, A.; Johnston, C.; et al. Candidate blood proteome markers of Alzheimer's disease onset and progression: A systematic review and replication study. J. Alzheimers Dis. **2014**, 38, 515–531.
187 Kim, D.K.; Park, J.; Han, D.; Yang, J.; Kim, A.; Woo, J.; Kim, Y.; Mook-Jung, I. Molecular and functional signatures in a novel Alzheimer's disease mouse model assessed by quantitative proteomics. Mol. Neurodegener. **2018**, 13, 2.
188 He, K.; Nie, L.; Zhou, Q.; Rahman, S.U.; Liu, J.; Yang, X.; Li, S. Proteomic Profiles of the Early Mitochondrial Changes in APP/PS1 and ApoE4 Transgenic Mice Models of Alzheimer's Disease. J. Proteome Res. **2019**, 18, 2632–2642.
189 Wilkins, J.M.; Trushina, E. Application of Metabolomics in Alzheimer's Disease. Front. Neurol. **2017**, 8, 719.
190 Enche Ady, C.N.A.; Lim, S.M.; Teh, L.K.; Salleh, M.Z.; Chin, A.V.; Tan, M.P.; Poi, P.J.H.; Kamaruzzaman, S.B.; Abdul Majeed, A.B.; Ramasamy, K. Metabolomic-guided discovery of Alzheimer's disease biomarkers from body fluid. J. Neurosci. Res. **2017**, 95, 2005–2024.
191 Hurtado, M.O.; Kohler, I.; Lange, E.C.d. Next-generation biomarker discovery in Alzheimer's disease using metabolomics – From animal to human studies. Bioanalysis **2018**, 10, 1525–1546.
192 Jacobs, K.R.; Lim, C.K.; Blennow, K.; Zetterberg, H.; Chatterjee, P.; Martins, R.N.; Brew, B.J.; Guillemin, G.J.; Lovejoy, D.B. Correlation between plasma and CSF concentrations of kynurenine pathway metabolites in Alzheimer's disease and relationship to amyloid-beta and tau. Neurobiol. Aging **2019**, 80, 11–20.
193 Kim, M.; Snowden, S.; Suvitaival, T.; Ali, A.; Merkler, D.J.; Ahmad, T.; Westwood, S.; Baird, A.; Proitsi, P.; Nevado-Holgado, A.; et al. Primary fatty amides in plasma associated with brain amyloid burden, hippocampal volume, and memory in the European Medical Information Framework for Alzheimer's Disease biomarker discovery cohort. Alzheimers Dement. **2019**, 15, 817–827.
194 Brennan, K.; Martin, K.; FitzGerald, S.P.; O'Sullivan, J.; Wu, Y.; Blanco, A.; Richardson, C.; Mc Gee, M.M. A comparison of methods for the isolation and separation of extracellular vesicles from protein and lipid particles in human serum. Sci. Rep. **2020**, 10, 1039.
195 Fiandaca, M.S.; Kapogiannis, D.; Mapstone, M.; Boxer, A.; Eitan, E.; Schwartz, J.B.; Abner, E.L.; Petersen, R.C.; Federoff, H.J.; Miller, B.L.; et al. Identification of preclinical Alzheimer's disease by a profile of pathogenic proteins in neurally derived blood exosomes: A case-control study. Alzheimers Dement. **2015**, 11, 600–607 e1.

196 Goetzl, E.J.; Kapogiannis, D.; Schwartz, J.B.; Lobach, I.V.; Goetzl, L.; Abner, E.L.; Jicha, G.A.; Karydas, A.M.; Boxer, A.; Miller, B.L. Decreased synaptic proteins in neuronal exosomes of frontotemporal dementia and Alzheimer's disease. FASEB J. **2016**, 30, 4141–4148.
197 Goetzl, E.J.; Abner, E.L.; Jicha, G.A.; Kapogiannis, D.; Schwartz, J.B. Declining levels of functionally specialized synaptic proteins in plasma neuronal exosomes with progression of Alzheimer's disease. FASEB J. **2018**, 32, 888–893.
198 Kapogiannis, D.; Boxer, A.; Schwartz, J.B.; Abner, E.L.; Biragyn, A.; Masharani, U.; Frassetto, L.; Petersen, R.C.; Miller, B.L.; Goetzl, E.J. Dysfunctionally phosphorylated type 1 insulin receptor substrate in neural-derived blood exosomes of preclinical Alzheimer's disease. FASEB J. **2015**, 29, 589–596.
199 Goetzl, E.J.; Boxer, A.; Schwartz, J.B.; Abner, E.L.; Petersen, R.C.; Miller, B.L.; Carlson, O.D.; Mustapic, M.; Kapogiannis, D. Low neural exosomal levels of cellular survival factors in Alzheimer's disease. Ann. Clin. Transl. Neurol. **2015**, 2, 769–773.
200 Winston, C.N.; Goetzl, E.J.; Akers, J.C.; Carter, B.S.; Rockenstein, E.M.; Galasko, D.; Masliah, E.; Rissman, R.A. Prediction of conversion from mild cognitive impairment to dementia with neuronally derived blood exosome protein profile. Alzheimers Dement. (Amst) **2016**, 3, 63–72.
201 Gui, Y.; Liu, H.; Zhang, L.; Lv, W.; Hu, X. Altered microRNA profiles in cerebrospinal fluid exosome in Parkinson disease and Alzheimer disease. Oncotarget **2015**, 6, 37043–37053.
202 Eitan, E.; Hutchison, E.R.; Marosi, K.; Comotto, J.; Mustapic, M.; Nigam, S.M.; Suire, C.; Maharana, C.; Jicha, G.A.; Liu, D.; et al. Extracellular Vesicle-Associated Abeta Mediates Trans-Neuronal Bioenergetic and Ca(2+)-Handling Deficits in Alzheimer's Disease Models. NPJ Aging Mech. Dis. **2016**, 2, 16019.
203 Yamamoto, Y.; Valitalo, P.A.; Huntjens, D.R.; Proost, J.H.; Vermeulen, A.; Krauwinkel, W.; Beukers, M.W.; van den Berg, D.J.; Hartman, R.; Wong, Y.C.; et al. Predicting Drug Concentration-Time Profiles in Multiple CNS Compartments Using a Comprehensive Physiologically-Based Pharmacokinetic Model. CPT Pharmacomet. Syst. Pharmacol. **2017**, 6, 765–777.
204 Yamamoto, Y.; Valitalo, P.A.; Wong, Y.C.; Huntjens, D.R.; Proost, J.H.; Vermeulen, A.; Krauwinkel, W.; Beukers, M.W.; Kokki, H.; Kokki, M.; et al. Prediction of human CNS pharmacokinetics using a physiologically-based pharmacokinetic modeling approach. Eur. J. Pharm. Sci. **2018**, 112, 168–179.



CHAPTER IV

A cross-sectional study in healthy elderly subjects aimed at development of an algorithm to increase identification of Alzheimer pathology for the purpose of clinical trial participation

Samantha Prins,[1,2] Ahnjili Zhuparris[1] Ellen. P. Hart,[1] Robert-Jan Doll,[1] Geert Jan Groeneveld[1,2]

ALZHEIMER'S RESEARCH & THERAPY (2021) 13:132
DOI:10.1186/S13195-021-00874-9, EPUB 17 JULY 2021

1. Centre for Human drug Research, Leiden, NL | 2. Leiden University Medical Center, Leiden, NL

ABSTRACT

BACKGROUND In the current study we aimed to develop an algorithm based on biomarkers obtained through non- or minimally invasive procedures to identify healthy elderly subjects who have an increased risk of abnormal cerebrospinal fluid (CSF) Amyloid beta42 (Aβ) levels consistent with the presence of Alzheimer's Disease (AD) pathology. Use of the algorithm may help to identify subjects with preclinical AD who are eligible for potential participation in trials with disease modifying compounds being developed for AD. Due to this pre-selection, fewer lumbar punctures will be needed, decreasing overall burden for study subjects and costs.

METHODS Healthy elderly subjects (n=200; age: 65-70 (N=100) and age >70 (N=100) with an MMSE >24 were recruited. An automated central nervous system test battery was used for cognitive profiling. CSF $A\beta_{1-42}$ concentrations, plasma $A\beta_{1-40}$, $A\beta_{1-42}$, neurofilament Light and total Tau concentrations were measured. $A\beta_{1-42}$/1-40 ratio was calculated for plasma. The neuroinflammation biomarker YKL-40 and ApoE ε4 status were determined in plasma. Different mathematical models were evaluated on their sensitivity, specificity and positive predictive value. A logistic regression algorithm described the data best. Data were analyzed using a 5-fold cross validation logistic regression classifier.

RESULTS Two hundred healthy elderly subjects were enrolled in this study. Data of 154 subjects were used for the per protocol analysis. The average age of the 154 subjects was 72.1 (65-86) years. Twenty-four (27.3%) were Aβ positive for AD (age 65-83). Results of the logistic regression classifier showed that predictive features for Aβ positivity/negativity in CSF consists of sex, 7 CNS tests and 1 plasma-based assay. The model achieved a sensitivity of 70.82% (±4.35) and a specificity of 89.25% (± 4.35) with respect to identifying abnormal CSF in healthy elderly subjects. The receiver operating characteristic curve showed an AUC of 65% (±0.10).

CONCLUSION This algorithm would allow for a 70% reduction of lumbar punctures needed to identify subjects with abnormal CSF Aβ levels consistent with AD. Use of this algorithm can be expected to lower overall subject burden and costs of identifying subjects with preclinical AD and therefore of total study costs.

BACKGROUND

As new disease-modifying therapies for Alzheimer's disease (AD) enter clinical trials, identifying the disease at a clinical stage where the pathological injury is not too severe to allow functionally meaningful recovery, or at least stabilization, is a major issue of current research.[1] Classification criteria aim at defining early clinical, biochemical, and metabolic markers of AD before the clinical criteria of dementia are fulfilled.[2] Identification of the pre-dementia phase of AD is crucial to allow progress of new treatments designed to intervene in the disease process at the earliest possible stage.

The current leading hypothesis regarding the pathophysiology of AD is centered on the misfolding and aggregation of toxic amyloid beta (Aβ) species such as $A\beta_{1-42}$, and drug research has therefore so far focused most on this therapeutic target. Emerging data in otherwise healthy elderly individuals suggest that biomarker evidence of Aβ accumulation and neurofibrillary tangles are associated with functional and structural brain alterations, consistent with the patterns of abnormality seen in patients with mild cognitive impairment (MCI) and even AD, prior to the clinical expression of symptoms.[3] A phase one study in 2016 showed promising results of the anti-Aβ antibody aducanumab in patients with prodromal and mild AD by decreasing Aβ plaques in the brain.[4] Following this phase one study, the compound was further studied in two identically designed phase 3 trials. In March 2019, the trial was halted due to ineffectiveness. Further analyses showed that in one of the two phase 3 trials the patient group that received the highest dose of the active compound showed slower cognitive decline than the placebo group. Based on these results, the FDA recently approved aducanumab for the treatment of AD in the USA under the 'accelerated approval pathway' which provides patients access to drugs when there is an expectation of clinical benefit despite some uncertainty about the clinical benefit of the drug.[12] Aβ immunotherapy could prevent (progression to) AD in healthy elderly who show evidence of amyloid pathology and could prevent (further) aggregation of neurotoxic forms of Aβ and would thereby prevent downstream effects as synaptic dysfunction, neuronal damage and cognitive impairment.[6] However, many phase 3 anti-amyloid trials have failed to demonstrate effects on progression of cognitive decline in patients with (mild to moderate) AD, despite clear Aβ lowering effects in cerebrospinal fluid (CSF) or PET.[7-11]

Based on extensive longitudinal biomarker studies.[13,14] a specific pattern of deterioration of AD specific biomarkers has been proposed, which reflects the

underlying progressive neuropathology of the disease. In this model, described by Jack et al., 2013, concentrations of Aβ in CSF start decreasing decades before clinical symptoms appear. Changes in total and phosphorylated tau (t-Tau, p-Tau) concentrations in CSF have been shown to occur up to 15 years prior to the clinical onset of AD.[15,16] Studies with Aβ lowering compounds are increasingly performed in cognitively healthy subjects with a CSF profile consistent with AD or 'preclinical AD', due to this early decrease of Aβ in CSF and the hypothesis that cognitive deterioration can still be prevented at this stage.[17,18] Over the age of 65, approximately 20% of cognitively healthy subjects can be expected to have a CSF profile with lowered Aβ levels consistent with AD as this is shown to be an age-related process.[19] This means that to identify a single healthy elderly subject with CSF values consistent with AD, four subjects will have to undergo a lumbar puncture unnecessarily. This leads to unnecessary overall burden for study subjects and to higher study costs.

In the clinical setting, the diagnosis of (probable) AD is made based on clinical symptoms (e.g., self-reported memory loss, partner reports, difficulties in daily functioning), combined with neuropsychological testing, and confirmed by evidence of amyloid pathology in CSF (abnormal Aβ and/or Tau levels) or on amyloid PET scans, when available. The collection of CSF is however, an invasive technique, which is burdensome in itself but also carries a risk of adverse effects (e.g. post-puncture headache) while PET scans are time consuming, not available for all patients, and expensive.[19,20]

As a result of the aforementioned, many studies have attempted to identify blood assays which can reliably measure AD related biomarkers.[21,22] Some seem to be successful in making a distinction between blood Aβ levels in subjects with (subjective) cognitive impairment, MCI or AD.[23,24] Also, the biomarkers T-TAU and Neurofilament Light (NFL) have been able to make this distinction.[25,26] Limitations of the current blood-based biomarkers are that outcomes are not consistent between studies and the methods used are highly diverse.[27]

In the current study we aimed to develop an algorithm based on minimally invasive biomarkers (plasma and cognitive tests), to be used for pre-selection of subjects with an increased risk of lowered, abnormal, CSF Aβ levels ('Aβ positive subjects') consistent with the presence of AD pathology. This algorithm can be used to preselect cognitively healthy Aβ positive people for drug studies in preclinical AD, thereby resulting in fewer subjects needing to undergo a lumbar puncture.

METHODS

This was a single-center, cross-sectional, observational, correlational study. All study participants visited the research unit twice, once for a medical screening and once for the study measurements.

PARTICIPANTS We aimed to enroll 200 healthy male and female participants, with an age of 65 years and older. Of these 200 subjects, at least 100 participants were to be above the age of 70. All the subjects visited Centre for Human Drug Research (CHDR) between October 2017 and November 2018 where all study assessments took place. CHDR is a clinical pharmacology research facility where early phase clinical drug studies and methodology and biomarker research are performed. For this study, a population of healthy elderly subjects aged 65 years and over was chosen as the prevalence of neurodegenerative disorders with an important cognitive component such as AD increases significantly from this age onwards.[19] Main exclusion criteria were a diagnosis of a cognitive disorder (including but not limited to MCI, AD, Lewy Body dementia, Frontotemporal dementia), history of psychiatric disease in the past 3 years, Mini Mental State Examination (MMSE) ≤ 24, Geriatric Depression Scale (GDS) ≥ 6, presence of drug or alcohol abuse (<2 standard drinks per day for female and <3 standard drinks per day for male), any medication which influences the central nervous system or is contraindicative for the performance of a lumbar puncture.

All subjects underwent medical screening, including medical history, physical examination, vital signs measurements in supine and standing position, routine hematology, urinalysis and urine drug screen.

All subjects visited the clinical research unit once for the study day and underwent blood sampling at predefined time points (0, 2 and 4 hour[s]). A single lumbar puncture was performed for the collection of CSF (at 4 hours). Furthermore, a CNS test battery was performed to collect data on different CNS domains.

BLOOD SAMPLING Approximately 10mL blood was collected via an i.v. catheter placed in an antecubital vein in the arm in appropriate K2EDTA tubes at the predefined time points mentioned above. Immediately following collection if the required blood volume, the tubes were slowly tilted backwards and forwards (no shaking) to bring the anticoagulant into solution. The blood plasma samples for bioanalysis were centrifuged within one hour, at 2000g for 10 minutes at 4°C. Prior to centrifugation, plasma samples were kept at room temperature. Immediately after centrifugation, supernatant was divided into 0.5 ml aliquots in Sarstedt polypropylene 0.5mL tubes and stored at -80°C.

LUMBAR PUNCTURE A CSF sample of 4 mL was collected in a 10 mL polypropylene tube. CSF was centrifuged within one hour, at 2000g for 10 minutes at 4°C. Prior to centrifugation, CSF samples were kept at room temperature. Immediately after centrifugation, samples were divided into 0.5 ml aliquots in Sarstedt polypropylene 0.5 mL tubes and stored at -80°C. Lumbar punctures were performed by a trained, physician with a 25G atraumatic lumbar puncture needle (Braun, 25G) under supervision of an experienced neurologist. The needle was placed at the L3-L4 or L4-L5 interspace with the subject in supine or sitting position. If a subject suffered from post-dural headaches, the subject was treated according to our standard operating procedures.

AMYLOID STATUS Amyloid beta1-42 was measured in the CSF using the fully automated Elecsys platform as this is widely used for diagnostics.[28] Lowered Aβ levels classified as amyloid abnormal and consistent with the presence of Alzheimer pathology were dichotomized by creating a group of 'Aβ positive subjects' (Aβ < 1000 PG/mL) and 'Aβ negative subjects' (Aβ ≥ 1000 PG/mL).

PLASMA ANALYSIS Several plasma analyses were performed in plasma samples that were taken within one hour from the CSF sample. Plasma biomarkers have been selected based on promising previous research of the use of plasma biomarkers to predict AD pathology. Although analytical methods vary, previous research has been able to measure Aβ, T-TAU and NFL in plasma and have therefore been included to this study and the algorithm.[23–26] Plasma concentration of Aβ 1-40, Aβ 1-42, T-TAU and NFL were measured using the fully automated, highly sensitive single molecule array Simoa technology.[29] The Aβ scores have been used as single variables as well as in a ratio score $A\beta_{1-42}$/ $A\beta_{1-40}$.

Chitinase 3-like 1 (CHI3L1), or more commonly called YKL-40, is a glycoprotein which is mainly expressed in astrocytes. Insoluble Aβ aggregates in the brain can induce the activation of microglia, resulting in the synthesis of proinflammatory mediators, which further can stimulate astrocytic expression of YKL-40.[30] Higher concentrations of YKL-40 were found in patients with prodromal AD, MCI and full-blown AD.[31,32] when measured in CSF. Measuring YKL-40 in plasma can lead to a less invasive method of measuring inflammation related to AD in healthy subjects. YKL-40 was measured in plasma samples using the CHI3L1 Human ELISA Kit (Thermo Fisher).

APOLIPOPROTEIN E GENOTYPING Apolipoprotein E (ApoE) genotyping was performed after isolating DNA from EDTA blood. DNA was isolated using QIAamp DNA Blood MINI kit after which a polymerase chain reaction (PCR) technique was applied on the clean DNA. A sequential analysis (according to the Sanger method) than determined the ApoE genotype. One or 2 ApoE ε4 alleles classified subjects as ApoE ε4 carriers, when no ApoE ε4 alleles were present a subject was classified as noncarrier.

COGNITIVE ASSESSMENTS AND QUESTIONNAIRES The NeuroCart is a battery of CNS tests used to assess a wide range of CNS domains.[33] All measurements were performed in a quiet room with ambient illumination. Per session there was only one participant in the room. The following tests were performed using the NeuroCart: the Adaptive tracking test to measure attention and eye-hand coordination,[34] the Face encoding and Recognition task (FACE) to measure visual memory,[35] the Visual Verbal Learning Test (VVLT, 30 words) to measure the whole scope of learning behavior (i.e. acquisition, consolidation, storage and retrieval),[36] the Milner Maze test (MMT) evaluated visuospatial working memory,[37] the N-Back test was assessed to evaluate working memory,[38] the Sustained Attention to Response Task (SART) as a vigilance task,[39] finger tapping for motor fluency,[40] saccadic and smooth eye movement.[41] were also measured.

21-Leads electro encephalography (EEG).[42] recordings were made for all subjects to monitor (abnormal) brain activity. An 8-minute resting EEG was performed while the subjects alternated 4 minutes with their eyes closed and 4 minutes with their eyes opened while resting in a chair. Subjects face a featureless wall and are instructed not to stare, not to move their head and eyes, and to suppress eye blinks. The Refa-40 (TMSi B.V., the Netherlands) recording system and 32-lead cap (TMSi B.V.) have been used. The five standard EEG band have been analyzed Delta (1.5 < 6.0), Theta (6.0 < 8.5), Alpha (8.5 < 12.5), Beta (12.5 < 30.) and Gamma (30.0 < 40.0).

The clinical dementia rating scale (CDR).[43] was assessed via a semi-structured interview with the participating subject only, to rate impairment in six different cognitive categories (memory, orientation, judgement and problem solving, community affairs, home and hobbies and personal care). To rate impairment in more complex daily activities the Instrumental Activities of Daily Living Scale (IADL).[44] was assessed. Both questionnaires were administered by trained neuropsychologists.

SAMPLE SIZE JUSTIFICATION In this study we selected elderly at the age of 65 years old and higher of which at least a hundred above the age of 70. According to Jansen et al., (2015).[19] we expected at least 19% amyloid pathology in a 65+

population and 23% amyloid pathology among cognitively healthy 70+ elderly subjects. We expected more responsiveness for study participation from elderly between the age of 65 till 70, based on our experience with previous studies with participants in this age range. Participants in this age range have participated in studies at CHDR before and are therefore registered in our local database and have received emails about this study. A higher number of participants within the age range 65-70 are present in the database compared to older elderly. Therefore, we aimed to enroll at least 100 subjects of >70 years old in this study as prevalence of amyloid pathology is expected to be higher in this age group. This would result in an estimated 23 Aβ positive subjects versus approximately 77 Aβ negative subjects in the >70 years old age group. Along with approximately 19 Aβ positive subjects versus 81 Aβ negative subjects in the age group 65-70, we expected to identify at least 42 Aβ positive healthy elderly subjects among the total group of 200. Based on previous comparable studies, these numbers were considered appropriate for a correlational study aimed at defining an algorithm.[45,46]

STATISTICAL ANALYSIS Statistical analyses were performed using Python (version 3.7.3) and the sklearn package (version 0.21.3). To build a classification model that could differentiate between Aβ positive subjects and Aβ negative subjects, all parameters such as plasma data, genetic information, cognitive assessments, level of education, age and gender were included as features.

When a classifier contains more features than can be justified by the observed data, there is a risk of the model overfitting. Overfitting occurs when a classifier corresponds too closely to a particular subpopulation and cannot be generalized to the wider population. Two methods were used to reduce the feature space, Variance Inflation Factor (VIF) and Penalized Regression. VIF identified the pairs of highly correlated features and subsequently removes one of the features from the classifier. Penalized Regression was applied to the logistic regression classifier to shrink the coefficients of features that were less predictive of the outcome.

For this study, we reviewed the performance of four classifiers – Ridge-penalized Logistic Regression, Random Forest Classifier, Support Vector Machine Classifier, and k-Nearest Neighbours Classifier — on four datasets – a dataset with all features, only the VIF-selected features, all features except the EEG features and all features except the genotyping feature. To ensure that the models were not under- or overfitting, we performed 5-fold stratified cross-validation. This data partitioning approach ensures that we built a more generalized model that can perform well when presented with unseen data. The 5-fold stratified cross-validation randomly samples the data into 5 folds of approximately equal proportions. In this case, there were 30 or 31 subjects per fold. Each fold contained the same ratio of Aβ positive and Aβ negative subjects. The model was trained on 4 folds of data and validated on the 5th fold. The cross-validation process was repeated 5 times, with each of the subsamples used exactly once as the validation data. The validation results were averaged over each iteration to estimate the model's predictive performance. We selected the optimal classifier by selecting the classifier with the highest sensitivity and specificity. If the sensitivity and specificity scores were identical between classifier, we then choose the classifier with the highest F1 score.

RESULTS

DEMOGRAPHIC AND CLINICAL CHARACTERISTICS Two hundred healthy elderly subjects were enrolled in this study of which 189 were included in the CSF and plasma analyses due to CSF availability. The 11 missing CSF samples were due to absent CSF flow during lumbar puncture. The 189 CSF samples were analyzed on $A\beta_{42}$ using the Elecsys method and 55 healthy elderly had CSF $A\beta_{42}$ levels consistent with AD (Aβ < 1000 PG/mL). Of the 189 subjects with CSF availability, 154 subjects were included in the per protocol analyses. Plasma analyses were missing for 27 subjects due to analytic errors. NeuroCart data was incomplete for 8 subjects. Forty-nine subject were female (68.2% were male and 31.8% female). Their mean age was 72.1 years (range: 65-86), with a median MMSE score of 29 (range 25-30), and GDS score of 0 (median, range 0-5). Self-reported memory performance and daily functioning were assessed using CDR and IADL scores with averaged scores of 0 in all subjects. Of the 154 elderly, 42 (27.3%) were Aβ positive for AD (average age 73.7.[65–83] See Table 1).

DATA ANALYSIS For each dataset and classifier, we calculated the sensitivity, specificity, precision and F1 score. The VIF-selected features dataset and logistic regression classifier achieved a sensitivity and specificity of 70.8% and 89.2%. The receiver operating characteristic (ROC) curve showed an AUC of 65% (±0.10) see Figure 11. However, the mean performance for all four classifiers (for the VIF-selected features dataset) was 68.0% sensitivity and 76.4% specificity. The lowest sensitivity and specificity for the worse performing model (the random forest) was 63.6% and 70.6% respectively. While we found similar performance when applying different classifiers, the logistic regression showed the highest sensitivity and specificity for the classification task.

The best performing classifier, logistic regression, included 32 of the 90 parameters measured in this study. Results of the logistic regression algorithm analyses conclude that the best prediction of Aβ positivity/negativity in CSF in an elderly subject is made by combining the 32 parameters measured with the NeuroCart (table 2). The algorithm included the following 7 CNS tests and 1 plasma analysis: MMT, VVLT, finger tapping, N-Back, SART, Face, EEG, and the plasma biomarker YKL-40. Sex was also included. We can use the logistic regression equation to calculate the probability (between 0 to 1) of a new subject being classified as amyloid positive or negative. If the subject is given a probability greater than 0.5, they will be classified as amyloid positive.

Table 1 Demographics, clinical characteristics and biomarker information of the study population.

Characteristics		Amyloid status CSF	
	Total group, n=154	Aβ positive, n=42 (27.3%)	Aβ negative, n=112 (72.7%)
Age, yr	72.1 [65;86]	73.7 [65;85]	71.4 [65;86]
Female gender	49 (31.8 %)	13 (30.6%)	36 (32.1%)
MMSE	29 (25-30)	29 (25-30)	29 (25-30)
GDS	0 (0-5)	1 (0-5)	0 (0-5)
CDR	0.0 (0-0.5)	0.0 (0)	0.0 (0-0.5)
IADL	0.0 (0)	0.0 (0)	0.0 (0)
Education*	6 (1-7)	6 (1-7)	6 (1-7)
ApoE ε4/e4 (n=150)	5 (3.3 %)	5 (100%)	0 (0%)
ApoE at least one e4 allele (n=150)	39 (26 %)	18 (42.9%)	21 (18.8%)

*Continuous data are presented as mean [min; max] and dichotomous data as n (%). MMSE: Mini Mental State Examination; GDS: Geriatric Depression Scale; CDR: Clinical Dementia rating Scale; IADL: Instrumental Activity of Daily Living scale; ApoE ε4: apolipoprotein E 4. *: Level of education defined as 1) lower than primary school, 2) primary school, 3) less than lower professional education, 4) Lower professional education, 5) Mid-level professional education, 6) High school/college, 7) university.*

As EEG- and genotyping-based assessments are time and resource consuming tasks, we built two additional classification models excluding these features. By excluding the EEG features, the highest sensitivity and specificity achieved was 70.6% and 73.5%, respectively using ridge-penalized logistic regression classifier. Hence the exclusion of the EEG features had little to no effect on the sensitivity of the classifier but lead to a 15 percentage points drop in specificity compared to the best performing logistic regression model. When omitting the genotyping features (the ApoE ε4 status), the best performing model was the k-Nearest Neighbour. This model achieved a sensitivity and specificity of 70.4% and 72.3% respectively. Like the classifier with no EEG features, the exclusion of the genotyping data had little to no effect on the classifier's sensitivity, while the specificity did drop by 16 percentage points compared to the best performing logistic regression model.

Table 2 NeuroCart activities and parameters included in the algorithm*

Activity	Cognitive domain	Parameter
Visual Verbal Learning Test (VVLT, 30 words)	Memory	Delayed word recall number correct Immediate word recall number doubles, 3e trial Immediate word recall number incorrect 1st trial Delayed word recall number doubles Immediate word recall number doubles, 2e trial Immediate word recall number doubles, 1st trial Immediate word recall number incorrect 3e trial Delayed word recognition number incorrect Immediate word recall number incorrect 2e trial
Electro-encephalography (EEG)	Electrical brain activity	Delta-power Fz-Cz (eyes open) Theta-power Fz-Cz (eyes closed) Beta-power Fz-Cz (eyes open) Gamma-power Pz-O2 (eyes open) Delta-power Pz-O2 (eyes open) Gamma-power Pz-O1 (eyes closed) Alpha-power Fz-Cz (eyes open) Theta-power Pz-O1 (eyes open) Gamma-power Fz-Cz (eyes open) Alpha-power Pz-O1 (eyes closed)
Finger Tapping	Motor activation and fluency	Standard deviation of the mean (dominant hand)
Sustained Attention to Response Task (SART)	Vigilance	Total omission errors Post error slowing
N-Back	Working memory	Number correct – number incorrect/total for one back
Milner Maze test (MMT)	Spatial working memory	Reversed total illegal moves Immediate total repeat errors Immediate total illegal moves Delayed total illegal moves Reversed total repeat errors Delayed total repeat errors
Face encoding and recognition task (Face)	Episodic memory	Number incorrect

** Top activities/parameters have more impact on the algorithm than the bottom activities in this table.*

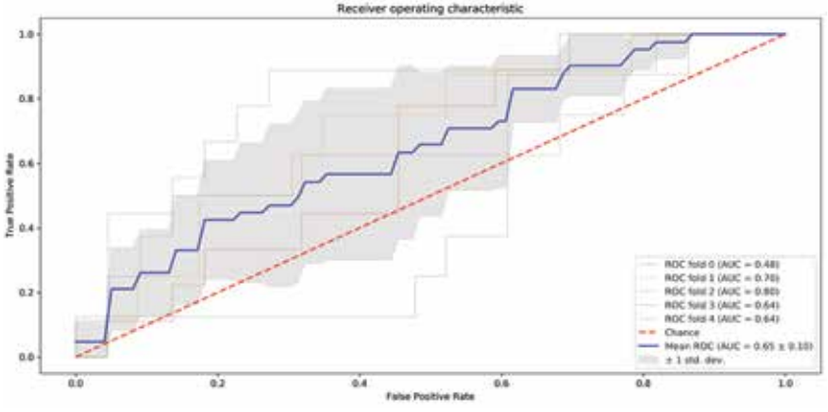
When aiming for 50 healthy elderly with Aβ CSF levels consistent with AD, 220 elderly must undergo the (non-invasive) tests included in the algorithm. Of these 220 subjects, the algorithm will predict 66 elderly with Aβ positive levels in CSF, 50 of which will be true positives (Aβ CSF levels consistent with AD), the remaining 16 will be false positive (Aβ negative). However, 21 Aβ positive subjects will be mislabeled as Aβ negative (see Table 3). This algorithm would allow for a 70% reduction of lumbar punctures needed to identify subjects with abnormal CSF Aβ levels consistent with AD, meaning 66 lumbar punctures instead of 220 (see Figure 2).

Table 3 Sensitivity/specificity table of the logistic regression algorithm

	Predicted Aβ +	Predicted Aβ -	Total
Actual Aβ +	50	21	71
Actual Aβ -	16	133	149
Total	66	154	220

Sensitivity & specificity table calculated with a sensitivity of 70.82% and specificity of 89.25%. When aiming for 50 positively predicted Aβ positive subjects, 66 will be predicted as such. Therefore 16 subjects will falsely be predicted as being Aβ positive and 21 will falsely be predicted as being Aβ negative.

Figure 1 Receiver Operating Characteristic (ROC) metric to evaluate the logistic regression output quality using 5-fold cross-validation.



DISCUSSION

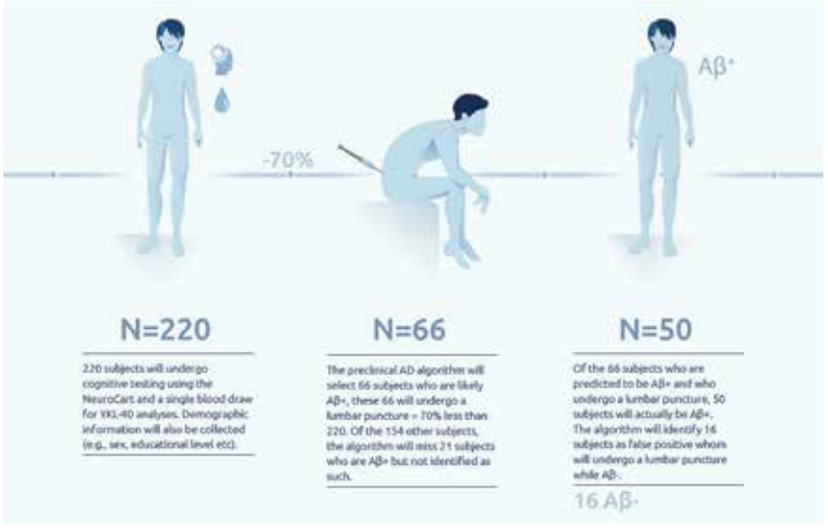
This study aimed to develop an algorithm based on less-invasive (plasma) biomarkers for AD pathology, to be used for pre-selection of subjects who are suspected of lowered, abnormal, CSF Aβ levels ('Aβ positive subjects') consistent with the presence of AD pathology. The algorithm includes sex, 7 cognitive tests measured with the NeuroCart (MMT, VVLT, finger tapping, N-Back, SART, Face and EEG) and one plasma biomarker (YKL-40) and was successful in predicting CSF Aβ+ in healthy elderly with a sensitivity of 70.82% and specificity of 89.25%. When using this algorithm, 70% fewer lumbar punctures will have to be performed to enroll subjects based on lowered Abeta CSF. The overall subject burden and costs of trials will reduce as fewer lumbar punctures will need to be performed. This may also increase subject's willingness to participate.

Four classification algorithms (Random Forest, Logistic Regression, Support Vector Machine Classifier and a K-nearest neighbors classifier) were used to classify Aβ positivity. A comparison of classification models is necessary to identify a model that best fits the data. Logistic regression outperformed the other algorithms in terms of accuracy, precision and recall. The logistic regression model is ideal for Aβ positivity classification as it provides an estimation of the association between the predictor and the outcome. Palmqvist et al., (2019).[47] and Jang et al., (2019).[48] have also demonstrated the use of logistic regression to reliably dichotomize amyloid status using plasma. This further supports the notion that logistic regression can use multimodal non-invasive cognitive and blood-based biomarkers to stratified enrollment of subjects with preclinical AD into clinical trials. In this study, 200 healthy elderly were included of which 154 were eventually included in the model. This is a satisfactory amount of subjects to support the conclusion of this study. For the logistic regression classifier, we have selected 0.5 to be the probabilistic threshold for classifying a patient as Aβ+ or Aβ-. Using the ROC curve (Figure 1), a researcher may choose a different threshold depending on what they choose to prioritize, the true positive rate (sensitivity) or the false positive rate (1-specificity)).

Approximately 50 subjects is an acceptable number for a Proof-Of-Concept study of a novel compound, 20-80 subjects is common in phase one trials according to the FDA.[49] Based on the 27.3% Aβ positivity in our study we estimate that in a new group of 220 healthy elderly, 71 subjects will be Aβ+. The algorithm will identify 66 subjects as having Aβ+CSF. Due to the sensitivity of 70.82%, 21 Aβ+ subjects would not be identified as such. Also, 16 Aβ-subjects would wrongfully be identified as Aβ+ which results in 50 truly Aβ+ subjects. Using the algorithm

would reduce the number of lumbar punctures in healthy elderly by 70%, i.e., 66 lumbar punctures instead of 220. As this algorithm is designed to select healthy elderly with Aβ CSF concentrations consistent with AD, having a 100% accuracy is of no importance, contrary to when using a test or an algorithm for diagnostic purposes. We would not perform unnecessary lumbar punctures in 89.25% patients with an increased chance of being Aβ-. In our opinion, this decrease in overall burden justifies the use of such an algorithm for subject selection for trial.

Figure 2 Visualization of reduction of lumbar punctures using the algorithm.



Other studies developed algorithms focused on predicting the progression to dementia due to AD,[50,51] the classification of different stages of AD,[52,53] and for the diagnosis of AD in the early stages.[54] These algorithms were developed for diagnostic purpose rather than for clinical trial participation, such as the one described in our study. Also, the data used in these algorithms were collected in clinical settings such as behavioral observation, clinical presentation and MRI data. When selecting healthy elderly for clinical trial participation, this information is commonly not available. Others have tried to identify healthy subjects with amyloid pathology using considerably burdensome and costly MRI data.[55,56] Khan et al., (2018) suggests an algorithm for preclinical diagnosis of AD based

on a combination of three AD biomarkers: neuroimaging, genetic markers and abnormalities in CSF $A\beta_{1-42}$, T-TAU and P-TAU (the gold standard for the diagnosis of AD). However, as mentioned before, data from neuroimaging is not commonly available and far more costly and time consuming than the tests used in our algorithm. Reduction of the number of lumbar punctures performed in healthy subjects is of great value to increase participation willingness in healthy elderly and to lower overall subject burden. A comparable study to this current study showed that Aβ positivity (confirmed by either CSF or PET-MRI) can be predicted by a combination of demographic variables, ApoE status, baseline cognition and 24-month follow up rates.[57] A 24-month follow up is usually not available and gathering follow up information on healthy subjects before the start of a clinical trial is too time consuming.

Accumulation of Aβ plaques in the brain associated with lowered levels of Aβ in CSF is still seen as the main pathological cause of AD. Various clinical trials have therefore focused on reducing Aβ plaques in the brain. Where reducing Aβ has been successful, lowering the prevalence of dementia due to AD has not been a result. Huang et al., (2020) reported 9 failed phase 3 anti-amyloid trails since 2016 with 6 different compounds.[58] Two of these trails were performed in subjects with preclinical AD, both with BACE inhibitors.[17,18] and both were discontinued due to either toxicity or lack of efficacy. Researchers claim that interfering early in the disease process will probably result in higher efficacy than when the clinical disease process has already started, evidenced by a diagnosis of preclinical AD or MCI. Looking at the inclusion criteria of the aforementioned studies shows that healthy elderly with CSF Aβ levels consistent with AD have been selected for participation. Healthy elderly are defined as having a clinical interview, namely the clinical dementia rating scale (CDR) of 0. Using the CDR total score is well accepted in clinical research and is widely used for clinical diagnosis of AD.[59] Still, very subtle cognitive changes are not detected using this crude screening tool. Using the algorithm proposed in this article will help to better select trial participants by including diverse cognitive assessments instead of the more general cognitive score of the CDR.

Shifting focus from invasive measurements (CSF, PET-MRI) to blood-based biomarkers for AD has been a major topic in research as new technics have been developed claiming to be ultrasensitive to detecting AD related proteins.[24] Using a blood test would make it more accessible to diagnose patients but also to identify possible trial participants. Challenges in the use of blood-based AD biomarkers are the different biological system compared to the CSF system, use of different analytical methods (ELISA, Simoa, etc.), and the specificity for AD of

these biomarkers.[27] Specifying pre-AD stages with the use of blood-based biomarkers has yet to be standardized. The preclinical AD algorithm created in this study includes only one blood-based biomarker (YKL-40) and the limitations of using blood-based biomarkers are therefore minor. Use of a different analytical method may alter the outcome of the analysis slightly and therefore could have led to a different composition of the algorithm. This should be kept in mind when comparing the outcome of this study to those of other studies. The combination of blood-based biomarkers with genetic information and cognitive assessments appears to be a powerful tool in preselection of preclinical AD subjects in clinical trials.

Four out of the seven NeuroCart tasks that are included in the algorithm are memory tasks. Loss of memory early on in the disease process is common for (amnestic) MCI and often lead to the AD diagnosis.[60] Especially the visual verbal learning task is important for the algorithm to differentiate between preclinical AD and healthy elderly. Visual and verbal memory problems are common in AD.[61] and have also been reported in preclinical AD.[62,63]

Reducing the number of lumbar punctures in healthy subjects and the additional benefits for clinical research must be weighed against the ethical consequences of identifying healthy subjects with an elevated risk of developing AD, which at this moment is an untreatable disease. Approximately 53% of subjects fulfilling the criteria of preclinical AD will actually develop MCI or AD.[64] When selecting trial subjects based on specific biomarkers, these subjects will become aware that they have CSF Aβ levels consistent with AD. The development of Aβ plaques in the brain and eventually developing AD can be a 20- to 30-year long process.[65] This is a substantial amount of time to be concerned about a disease that one might develop. Knowledge about predispositions to develop a disease can even have financial consequences and reduce health benefits as people might not be hired for certain jobs and health insurances may increase insurance premium. Nevertheless, studying cognitively healthy elderly is important as treatment in a pre-disease phase might prevent or retard the process of developing clinically overt Alzheimer's dementia. With the ultimate goal of preventing AD, the need to include preclinical subjects in clinical studies is vital.

Limitations

Among the limitations is that a logistic algorithm was used which cannot incorporate incomplete datasets.[66] Hence, the model will fail to predict a class if a subject is missing a single feature. Missing data is not uncommon in research, especially when cognitive tests are performed. Benefits of using this model however proceed this limitation. Inconclusiveness about the validity of blood based biomarkers can also be regarded as a limitation of this study. This study only includes one plasma biomarker which reduces the inconvenience. The ethical consequences of using an algorithm like ours in healthy elderly should always be taken into account and could be regarded as a limitation. The study population is a relatively highly educated group. This might not be completely representative with regards to the cognitive performance of an average population.

Conclusion

This algorithm would allow for a 70% reduction of lumbar punctures needed to identify subjects with abnormal CSF Aβ levels consistent with AD. We have identified an algorithm that is able to preselect healthy elderly who are more likely to have Aβ CSF levels consistent with AD. Using this algorithm, fewer lumbar punctures will have to be performed when selecting subjects for clinical trials. Use of this algorithm can be expected to lower overall subject burden and costs of identifying subjects with preclinical AD and therefore of total study costs.

REFERENCES

1 Amieva, H., et al., *Prodromal Alzheimer's Disease: Successive Emergence of the Clinical Symptoms*. Annals of Neurology, 2008. **64**(5): p. 492-498.

2 Dubois, B., et al., *Research criteria for the diagnosis of Alzheimer's disease: revising the* NINCDS-ADRDA *criteria*. Lancet Neurology, 2007. **6**(8): p. 734-746.

3 Price, J.L., et al., *Neuropathology of nondemented aging: presumptive evidence for preclinical Alzheimer disease*. Neurobiol Aging, 2009. **30**(7): p. 1026-1036.

4 Sevigny, J., et al., *The antibody aducanumab reduces Abeta plaques in Alzheimer's disease*. Nature, 2016. **537**(7618): p. 50-56.

5 Schneider, L., *A resurrection of aducanumab for Alzheimer's disease*. Lancet Neurol, 2020. **19**(2): p. 111-112.

6 Lemere, C.A. and E. Masliah, *Can Alzheimer disease be prevented by amyloid-beta immunotherapy?* Nat Rev Neurol, 2010. **6**(2): p. 108-119.

7 Rinne, J.O., et al., *11C-PiB* PET *assessment of change in fibrillar amyloid-beta load in patients with Alzheimer's disease treated with bapineuzumab: a phase 2, double-blind, placebo-controlled, ascending-dose study*. Lancet Neurol, 2010. **9**(4): p. 363-72.

8 Leyhe, T., et al., *Modulation of beta-amyloid by a single dose of GSK933776 in patients with mild Alzheimer's disease: a phase I study*. Alzheimers Res Ther, 2014. **6**(2): p. 19.

9 Salloway, S., et al., *Two phase 3 trials of bapineuzumab in mild-to-moderate Alzheimer's disease*. N Engl J Med, 2014. **370**(4): p. 322-33.

10 Selkoe, D.J., *Alzheimer disease and aducanumab: adjusting our approach*. Nat Rev Neurol, 2019. **15**(7): p. 365-366.

11 Swanson, C.J., et al., *A randomized, double-blind, phase 2b proof-of-concept clinical trial in early Alzheimer's disease with lecanemab, an anti-Aβ protofibril antibody*. Alzheimers Res Ther, 2021. **13**(1): p. 80.

12 FDA. FDA *Grants Accelerated Approval for Alzheimer's Drug*. 2021 content current as of 07Jun2018 [cited 2021 18June].

13 Chiu, M.J., et al., *Combined plasma biomarkers for diagnosing mild cognition impairment and Alzheimer's disease*. ACS Chem Neurosci, 2013. **4**(12): p. 1530-1536.

14 Jack, C.R., Jr., et al., *Tracking pathophysiological processes in Alzheimer's disease: an updated hypothetical model of dynamic biomarkers*. Lancet Neurol, 2013. **12**(2): p. 207-216.

15 Jack, C.R., Jr., et al., *Hypothetical model of dynamic biomarkers of the Alzheimer's pathological cascade*. Lancet Neurol, 2010. **9**(1): p. 119-128.

16 Jack, C.R., Jr., et al., NIA-AA *Research Framework: Toward a biological definition of Alzheimer's disease*. Alzheimers Dement, 2018. **14**(4): p. 535-562.

17 Timmers, M., et al., *Pharmacodynamics of atabecestat (JNJ-54861911), an oral BACE1 inhibitor in patients with early Alzheimer's disease: randomized, double-blind, placebo-controlled study*. Alzheimers Res Ther, 2018. **10**(1): p. 85.

18 Neumann, U., et al., *The* BACE-1 *inhibitor* CNP520 *for prevention trials in Alzheimer's disease*. EMBO Mol Med, 2018. **10**(11).

19 Jansen, W.J., et al., *Prevalence of Cerebral Amyloid Pathology in Persons Without Dementia A Meta-analysis*. Jama-Journal of the American Medical Association, 2015. **313**(19): p. 1924-1938.

20 Zwan, M., et al., *Concordance Between Cerebrospinal Fluid Biomarkers and [C-11] PIB PET in a Memory Clinic Cohort*. Journal of Alzheimers Disease, 2014. **41**(3): p. 801-807.

21 Zetterberg, H. and S.C. Burnham, *Blood-based molecular biomarkers for Alzheimer's disease*. Molecular Brain, 2019. **12**(1): p. 26.

22 Olsson, B., et al., *CSF and blood biomarkers for the diagnosis of Alzheimer's disease: a systematic review and meta-analysis*. Lancet Neurology, 2016. **15**(7): p. 673-684.

23 Verberk, I.M.W., et al., *Plasma Amyloid as Prescreener for the Earliest Alzheimer Pathological Changes*. Ann Neurol, 2018. **84**(5): p. 648-658.

24 Janelidze, S., et al., *Plasma beta-amyloid in Alzheimer's disease and vascular disease*. Sci Rep, 2016. **6**: p. 26801.

25 Mielke, M.M., et al., *Plasma phospho-tau181 increases with Alzheimer's disease clinical severity and is associated with tau- and amyloid-positron emission tomography*. Alzheimers & Dementia, 2018. **14**(8): p. 989-997.

26 Lewczuk, P., et al., *Plasma neurofilament light as a potential biomarker of neurodegeneration in Alzheimer's disease*. Alzheimers Research & Therapy, 2018. **10**.

27 Hampel, H., et al., *Blood-based biomarkers for Alzheimer disease: mapping the road to the clinic*. Nat Rev Neurol, 2018. **14**(11): p. 639-652.

28 Lewczuk, P., et al., *Biomarkers of Alzheimer's disease and mild cognitive impairment: a current perspective*. Adv Med Sci, 2015. **60**(1): p. 76-82.

29 Rissin, D.M., et al., *Single-molecule enzyme-linked immunosorbent assay detects serum proteins at subfemtomolar concentrations*. Nat Biotechnol, 2010. **28**(6): p. 595-9.

30 Muszynski, P., et al., YKL-40 *as a potential biomarker and a possible target in therapeutic strategies of Alzheimer's disease*. Curr Neuropharmacol, 2017.

31 Antonell, A., et al., *Cerebrospinal fluid level of* YKL-40 *protein in preclinical and prodromal Alzheimer's disease*. J Alzheimers Dis, 2014. **42**(3): p. 901-8.

32 Kester, M.I., et al., *Cerebrospinal fluid* VILIP-1 *and* YKL-40, *candidate biomarkers to diagnose, predict and monitor Alzheimer's disease in a memory clinic cohort*. Alzheimers Res Ther, 2015. **7**(1): p. 59.

33 Groeneveld, G.J., J.L. Hay, and J.M. Van Gerven, *Measuring blood-brain barrier penetration using the NeuroCart, a* CNS *test battery*. Drug Discov Today Technol, 2016. **20**: p. 27-34.

34 Borland, R.G. and A.N. Nicholson, *Visual motor co-ordination and dynamic visual acuity*. Br J Clin Pharmacol, 1984. **18 Suppl 1**: p. 69S-72S.

35 Lezak MD, H.D., Loring DW., *Neuropyschological assessment*. Vol. 4th. 2004: New York: Oxford University Press.

36 de Haas, S.L., et al., *The pharmacokinetic and pharmacodynamic effects of SL65.1498, a* GABA-A *alpha2,3 selective agonist, in comparison with lorazepam in healthy volunteers*. J Psychopharmacol, 2009. **23**(6): p. 625-32.

37 Milner, B., *Visually-Guided Maze-Learning in Man - Effects of Bilateral Hippocampal, Bilateral Frontal, and Unilateral Cerebral-Lesions*. Neuropsychologia, 1965. **3**(4): p. 317-338.

38 Rombouts, S.A.R.B., et al., *Alterations in brain activation during cholinergic enhancement with rivastigmine in Alzheimer's disease*. Journal of Neurology Neurosurgery and Psychiatry, 2002. **73**(6): p. 665-671.

39 Dudschig, C. and I. Jentzsch, *Speeding before and slowing after errors: is it all just strategy?* Brain Res, 2009. **1296**: p. 56-62.

40 Andrew, J.M., *Delinquents and the Tapping Test*. J Clin Psychol, 1977. **33**(3): p. 786-791.

41 Molitor, R.J., P.C. Ko, and B.A. Ally, *Eye movements in Alzheimer's disease*. J Alzheimers Dis, 2015. **44**(1): p. 1-12.

42 Rossini, P.M., et al., *Clinical neurophysiology of aging brain: from normal aging to neurodegeneration*. Prog. Neurobiol, 2007. **83**(6): p. 375-400.

43 Morris, J.C., *The Clinical Dementia Rating (CDR): current version and scoring rules*. Neurology, 1993. **43**(11): p. 2412-4.

44 Lawton, M.P. and E.M. Brody, *Assessment of older people: self-maintaining and instrumental activities of daily living*. Gerontologist, 1969. **9**(3): p. 179-86.

45 Cui, Y., et al., *Predicting the development of mild cognitive impairment: a new use of pattern recognition*. Neuroimage, 2012. **60**(2): p. 894-901.

46 Zhang, D., et al., *Multimodal classification of Alzheimer's disease and mild cognitive impairment*. Neuroimage, 2011. **55**(3): p. 856-67.

47 Palmqvist, S., et al., *Performance of Fully Automated Plasma Assays as Screening Tests for Alzheimer Disease-Related beta-Amyloid Status*. JAMA Neurol, 2019.

48 Jang, H., et al., *Prediction of fast decline in amyloid positive mild cognitive impairment patients using multimodal biomarkers*. Neuroimage Clin, 2019. **24**: p. 101941.

49 FDA. 2021 content current as of 04Jan2018 [cited 2021 14Jan].

50 Lewczuk, P., et al., *Validation of the Erlangen Score Algorithm for the Prediction of the Development of Dementia due to Alzheimer's Disease in Pre-Dementia Subjects*. Journal of Alzheimers Disease, 2015. **48**(2): p. 433-441.

51 Lutz, M.W., et al., *A Genetics-based Biomarker Risk Algorithm for Predicting Risk of Alzheimer's Disease*. Alzheimers Dement. (N Y), 2016. **2**(1): p. 30-44.

52 Ahmad, F. and W.M. Dar, *Classification of Alzheimer's Disease Stages: An Approach Using* PCA-*Based Algorithm*. American Journal of Alzheimers Disease and Other Dementias, 2018. **33**(7): p. 433-439.

53 An, N., et al., *Deep ensemble learning for Alzheimer's disease classification*. J Biomed Inform, 2020. **105**: p. 103411.

54 Zheng, W., et al., *Application of Generalized Split Linearized Bregman Iteration algorithm for Alzheimer's disease prediction*. Aging (Albany NY), 2020. **12**(7): p. 6206-6224.

55 Mielke, M.M., et al., *Indicators of amyloid burden in a population-based study of cognitively normal elderly*. Neurology, 2012. **79**(15): p. 1570-7.

56 Casamitjana, A., et al., MRI-*Based Screening of Preclinical Alzheimer's Disease for Prevention Clinical Trials*. Journal of Alzheimer's Disease, 2018. **64**: p. 1099-1112.

57 Insel, P.S., et al., *Assessing risk for preclinical beta-amyloid pathology with ApoE, cognitive, and demographic information*. Alzheimers Dement (Amst), 2016. **4**: p. 76-84.

58 Huang, L.K., S.P. Chao, and C.J. Hu, *Clinical trials of new drugs for Alzheimer disease*. Journal of Biomedical Science, 2020. **27**(1).

59 Huang, H.C., et al., *Diagnostic accuracy of the Clinical Dementia Rating Scale for detecting mild cognitive impairment and dementia: A bivariate meta-analysis*. Int J Geriatr Psychiatry, 2020.

60 Jahn, H., *Memory loss in Alzheimer's disease*. Dialogues Clin Neurosci, 2013. **15**(4): p. 445-54.

61 Tromp, D., et al., *Episodic memory in normal aging and Alzheimer disease: Insights from imaging and behavioral studies.* Ageing Res Rev, 2015. **24**(Pt B): p. 232-62.
62 Baker, J.E., et al., *Episodic Memory and Learning Dysfunction Over an 18-Month Period in Preclinical and Prodromal Alzheimer's Disease.* J Alzheimers Dis, 2018. **65**(3): p. 977-988.
63 Stricker, N.H., et al., *Diagnostic and Prognostic Accuracy of the Cogstate Brief Battery and Auditory Verbal Learning Test in Preclinical Alzheimer's Disease and Incident Mild Cognitive Impairment: Implications for Defining Subtle Objective Cognitive Impairment.* J Alzheimers Dis, 2020. **76**(1): p. 261-274.
64 Parnetti, L., et al., *Prevalence and risk of progression of preclinical Alzheimer's disease stages: a systematic review and meta-analysis.* Alzheimers Research & Therapy, 2019. **11**.
65 Jansen, W.J., et al., *Prevalence of Cerebral Amyloid Pathology in Persons Without Dementia A Meta-analysis.* Jama-Journal of the American Medical Association, 2015. **313**(19): p. 1924-1938.
66 Jiang, W., et al., *Logistic regression with missing covariates - Parameter estimation, model selection and prediction within a joint-modeling framework.* Comput. Stat. Data Anal., 2020. **145**: p. 106907.

CHAPTER V

The usefulness of plasma amyloid as a prescreener for the earliest Alzheimer pathological changes depends on the study population

S. Prins,[1,2] A. Zhuparris,[1] G.J. Groeneveld[1,2]

PUBLISHED IN ANNALS OF NEUROLOGY. LETTER TO THE EDITOR REGARDING: VERBERK, INGE MW, ET AL. 'PLASMA AMYLOID AS PRESCREENER FOR THE EARLIEST ALZHEIMER PATHOLOGICAL CHANGES.' ANNALS OF NEUROLOGY 84.5 (2018): 648-658.
DOI: 10.1002/ANA.25634, EPUB 1 NOVEMBER 2019

1. Centre for Human drug Research, Leiden, NL | 2. Leiden University Medical Center, Leiden, NL

Recently, Verberk et al. showed that plasma Aβ42/Aβ40 ratio has potential to identify Alzheimer pathological changes in subjects with subjective memory decline. Further, the inclusion of age and ApoEε4 carriership in their multivariate model improved the likelihood of identification. Based on these results, Verberk and colleagues postulated that plasma Aβ42/Aβ40 ratio could be a potential prescreener to identify the earliest Alzheimer's Disease (AD) pathological changes in individuals with subjective memory decline.

We aimed to extend the findings of Verberk et al, using the same statistical methods, but in a different population, namely healthy elderly subjects without memory complaints (n=189). Subjects in this study were male and female, aged 72 years (mean, range: 65-86), with a mean MMSE score of 28.8 (range 25-30), and Geriatric Depression Scale score-15 of 0.7 (mean, range 0-5). Subjects were excluded if they had a cognitive or psychiatric disorder, or a history of drug- and/or alcohol abuse. If a subject used medication which affected the central nervous system, or medication with a contraindication for a lumbar puncture, they would also be excluded. Self-reported memory performance/daily functioning were assessed with use of the Clinical Dementia Rating scale-sum of boxes (CDR) and the Instrumental Activities of Daily Living scale (IADL) in participating subjects only. Average CDR and IADL scores were 0 in all subjects.

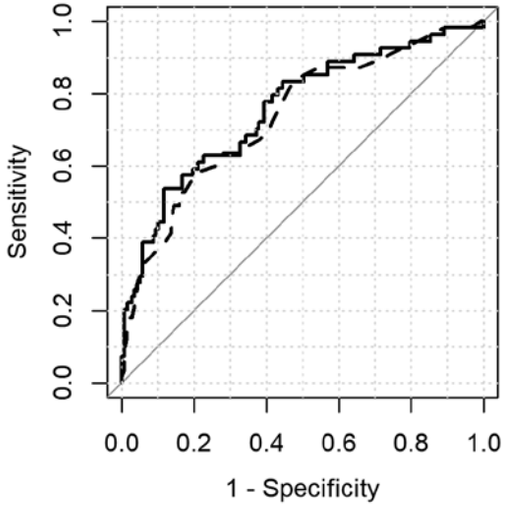
The sensitivity and specificity of the plasma Aβ42/Aβ40 ratio in our study were 30.8% and 71% respectively, compared to 76% and 75% in Verberk et al. The results of our logistic regression and receiver operating characteristic (ROC) analyses showed that the plasma Aβ42/Aβ40 ratio did not significantly affect ROC curves discriminating between cerebrospinal fluid (CSF) amyloid abnormal and amyloid normal individuals, in a multivariate model including age and ApoEε4 carriership (Fig 1).

Applying Verberk's model to subjects in our sample would theoretically identify 'preclinical' elderly, defined as elderly with biomarker evidence consistent with AD but without cognitive complaints. However, due to the low sensitivity (30.8%) of the model in our sample, we would miss a substantial number of healthy elderly with AD pathology, who we need for participation in clinical trials on the prevention of AD.

To build a model generalizable to an independent dataset, cross-validation of the regression model is crucial. Knowing that Verberk et al. did not cross-validate their model, over-fitting of the sampled data is a possible explanation for the discrepancy between Verberk's and our findings. While there are a maximum of 3 features included in Verberk's multivariate model, if the model was trained on a homogenous population, overfitting can be a likely occurrence. We therefore think that the findings from the study of Verberk et al. can only be limitedly extrapolated to a different population, and that their conclusion that plasma amyloid is a prescreener for the earliest AD pathological changes as stated in the title of their article, seems as yet too strong.

Another possible explanation for the divergent outcomes in the study by Verberk et al. and ours may simply be the difference in populations. Past research has shown that subjects with subjective memory complaints are more likely to progress to dementia than healthy elderly without. Also, these subjects tend to have a higher chance of being ApoE4 carriers4. Based on our findings, we can either conclude that Verberk's regression model was overfitted and cannot be extrapolated to new data, or that plasma Aβ42/Aβ40 ratio is not a potential prescreener to identify elderly without memory complaints.

Figure 1 Receiver operating characteristic (ROC) curves of logistic regression models that discriminate between cerebrospinal fluid (CSF) amyloid abnormal and amyloid normal (based on CSF amyloid beta 42 scores) among healthy elderly subjects. *Solid line: Variables within the logistic regression model are ABeta ratio, ApoE ε4 carriership and age. The Area under the curve [AUC] is 75.7% and 95% confidence interval [CI] is 67.8-83.6%. Dotted line: Variables within this logistic regression model only include ApoE ε4 carriership and age.* AUC: 73.8% CI: 65.8%-81.8%. *Grey line: 50% reference line.*



REFERENCES

Verberk, I.M.W., Slot, R.S., Verfaillie, S.C.J. et al. Plasma amyloid as prescreener for the earliest Alzheimer pathological changes. Ann Neurol 2018; 84:656-666.

Jack, C.R., Bennet, D.A., Blennow, K., et al. NIAA-AA Research framework: Toward a biological definition of Alzheimer's disease. Alzheimer's & Dementia 2018; 14:535-562.

Picard, R.R. and Cook, D., Cross-validation of regression models, 1984, Journal of the Amercian Statistical Association, 79:387, 575-583.

Zwan, M.D., Villemagne, V.L., Dore, V. et al. Subjective memory complaints in ApoE ε4 carriers are associated with high amyloid-β burden. Journal of Alzheimer's Disease; 49:1115-1122.

CHAPTER VI

Inflammatory plasma biomarkers in subjects with preclinical Alzheimer's disease

Samantha Prins,[1,2] Marieke L. de Kam,[1] Charlotte E. Teunissen,[3] Geert Jan Groeneveld[1,2]

ALZHEIMER'S RESEARCH & THERAPY (2022) 14:106
DOI:10.1186/S13195-022-01051-2, EPUB 3 AUGUST 2022

1. Centre for Human drug Research, Leiden, NL | 2. Leiden University Medical Center, Leiden, NL | 3. Neurochemistry Laboratory, Department of Clinical Chemistry, Neuroscience, Amsterdam UMC, Vrije Universiteit Amsterdam, Amsterdam, NL

ABSTRACT

BACKGROUND This study investigated plasma biomarkers for neuroinflammation associated with Alzheimer's Disease (AD) in subjects with preclinical AD compared to healthy elderly. How these biomarkers behave in patients with AD, compared to healthy elderly is well known, but determining these in subjects with preclinical AD is not and will add information related to the onset of AD. When found to be different in preclinical AD, these inflammatory biomarkers may be used to select preclinical AD subjects who are most likely to develop AD, to participate in clinical trials with new disease modifying drugs.

METHODS Healthy elderly (n= 50; age 71.9; MMSE >24) and subjects with preclinical AD (n=50; age 73.4; MMSE >24) defined by CSF Aβ_{1-42} levels < 1000 PG/mL were included. Four neuroinflammatory biomarkers were determined in plasma, GFAP, YKL-40, MCP-1 and Eotaxin-1. Differences in biomarker outcomes were compared using ANCOVA. Subject characteristics age, gender and ApoE ε4 status were reported per group and were covariates in the ANCOVA. Least square means were calculated for all 4 inflammatory biomarkers using both the Aβ+/ Aβ- cut off and PTAU/Aβ_{1-42} ratio.

RESULTS The mean (Standard Deviation, SD) age of the subjects (n=100) was 72.6 (4.6) years old with 62 male and 38 female subjects. Mean (SD) overall MMSE score was 28.7 (0.49) and 32 subjects were ApoE ε4 carriers. The number of subjects in the different ApoE ε4 status categories differed significantly between the Aβ+ and Aβ- group. Plasma GFAP concentration was significantly higher in the Aβ+ group compared to the Aβ- group with significant covariates age and sex, variables that also correlated significantly with GFAP.

CONCLUSION GFAP was significantly higher in subjects with preclinical AD compared to healthy elderly which agrees with previous studies. When defining preclinical AD based on the PTAU181/Aβ_{1-42} ratio, YKL-40 was also significantly different between groups. This could indicate that GFAP and YKL-40 are more sensitive markers of the inflammatory process in response to the Aβ misfolding and aggregation that is ongoing as indicated by the lowered Aβ_{1-42} levels in the CSF. Characterizing subjects with preclinical AD using neuroinflammatory biomarkers is important for subject selection in new disease modifying clinical trials.

BACKGROUND

Biomarkers for Alzheimer's Disease (AD) are primarily validated based on observed differences between cognitively healthy elderly and AD patients.[1-5]

Investigating biomarkers in subjects with preclinical AD (AD biomarker positive but cognitively normal) is important as clinical trials of new drugs shift to disease prevention in the still cognitively normal elderly.[6,7] Biomarker changes may present itself as early as 20 years prior to disease onset and therefore early intervention is important.[8] Selecting subjects with preclinical AD for clinical trials may aid in demonstrating modification of disease progression due to treatment with drugs targeting core pathophysiological processes and treatment of patients with preclinical AD may ultimately prevent conversion to AD. Characterization of individuals with preclinical AD by identifying biomarkers indicative of the earliest pathophysiological processes involved in AD is therefore of the utmost importance. Preferably minimally invasive methods are used to identify AD pathology, especially in otherwise healthy subjects.

The accumulation of amyloid plaques and intracellular neurofibrillary tangles consisting of misfolded phosphorylated tau (PTAU) protein during the development of AD eventually leads to synaptic dysfunction after which axonal damage occurs and cognitive changes can be observed. While this protein related process is ongoing, the immune system is also responsive.[9]

Misfolded and aggregated proteins can bind to pattern recognition receptors on microglia and astroglia, and trigger an innate immune response characterized by release of inflammatory mediators, which contribute to disease progression and severity.[10] Differences in immune CSF biomarkers, such as YKL-40, MCP-1 and eotaxin-1 have been well established between healthy elderly and AD patients.[11-15] An observable neuroinflammatory response of the immune system to protein aggregation could mean that the process of neurodegeneration leading to AD has already started.[9] Measurement of these innate neuroimmune response related biomarkers in the preclinical AD stages may help to predict which cognitively healthy elderly are more likely to develop AD.

YKL-40 (also known as chitinase-3-like protein-1 [CHI3L1]) is a glycoprotein, which is mainly expressed in astrocytes. AD patients have significantly higher YKL-40 levels in the CSF compared with healthy controls however it is not a specific biomarker for AD, because it merely reflects the inflammatory progress. YKL-40 is suitable as a marker for clinical drug trials to give information about neurodegeneration and glial activation independently of tau and Aβ.[12] Plasma YKL-40 levels have been investigated in patients with AD and in healthy elderly controls.[15] but not yet in subjects with preclinical AD.

Glial fibrillary acidic protein (GFAP) is a marker for astrogliosis and has been reported to be increased postmortem in brains of patients with AD and in CSF of patients with AD.[16,17] Verberk et al., (2021) found GFAP to be associated with in increased risk of dementia and a sleeper rate of cognitive decline and they conclude that GFAP has the potential to be a prognostic blood-based biomarker for AD in their cohort of cognitively normal older people.[18]Another recent study showed elevated plasma GFAP levels in subjects with preclinical AD which could mean that astrocytic damage or activation starts in the preclinical phase of AD.[19]

Chemokines are a family of chemoattractant, which play a vital role in cell migration from blood into tissue and vice versa, and in the induction of cell movement in response to a chemical (chemokine) gradient by a process known as chemotaxis.[20] In addition, chemokines have recently been shown to have a function in the nervous system as neuromodulators. Two chemokines (monocyte chemoattractant protein-1[MCP-1] and eotaxin-1) have previously been reported to be correlated with greater memory impairment in MCI and AD.[11] In a recent study these chemokines were demonstrated to be able to discriminate between healthy subjects and subjects with MCI and AD.[13]

In the current study we aimed to investigate plasma biomarkers related to neuroinflammation associated with AD in a cohort of subjects with preclinical AD and to compare these to healthy elderly. Using a preclinical subject population will add valuable information to the body of literature on the onset of AD.

METHODS

This was an exploratory sub-study of a previously performed study registered in the international trial register with ID number: ISRCTN79036545. All study participants provided written consent for exploratory analyses of material obtained during study execution.

The main study was approved by the ethics committee of the Leiden University Medical Center (LUMC), the Netherlands. The study was conducted according to the Dutch act on Medical Research Involving Human Subjects (WMO) and in compliance with Good Clinical Practice (ICH-GCP) and the Declaration of Helsinki.

PARTICIPANTS Samples of 100 healthy male and female participants of 65 years of age and older were selected from the main study in health elderly.[21] All subjects were healthy volunteers without cognitive complaints who registered for participation voluntarily. Of these 100 subjects, 50 subjects were selected with a CSF $A\beta_{1-42}$ profile consistent with Alzheimer's disease and were classified as subjects with probable brain amyloidosis, referred to as preclinical AD. A healthy control group of 50 subjects was selected based on subjects having high levels of CSF $A\beta_{1-42}$. $A\beta_{1-42}$ was measured in CSF using the fully automated Elecsys platform.[22] at the Neurochemistry Lab Amsterdam UMC, using in-house confirmed cut-offs.[23] Lowered Aβ levels classified as amyloid abnormal and consistent with the presence of Alzheimer pathology were dichotomized by creating a group of 'Aβ positive subjects' (Aβ+ = < 1000 PG/mL) and 'Aβ negative subjects' (Aβ- = > 1000 PG/mL). All the subjects visited Centre for Human Drug Research (CHDR) between October 2017 and November 2018. Main exclusion criteria were a diagnosis of a cognitive disorder (including but not limited to MCI, AD, Lewy Body dementia [LBD], Frontotemporal dementia [FTD]), history of psychiatric disease in the past 3 years, Mini Mental State Examination (MMSE) ≤ 24, Geriatric Depression Scale (GDS) ≥ 6, presence of drug or alcohol abuse (<2 standard drinks per day for female and <3 standard drinks per day for male), use of any medication that was expected to influence central nervous system function or is contraindicative of the performance of a lumbar puncture.

All subjects visited the clinical research unit once and underwent blood sampling at predefined time points (0, 2 and 4 hour[S]). A single lumbar puncture was performed for the collection of CSF (at 4 hours, either in the morning or afternoon). Furthermore, an automated CNS test battery was performed to collect data related to different domains of CNS functioning. The Clinical Dementia Rating scale (CDR) was assessed during the study day.

In the context of a post hoc analysis, subjects were also dichotomized based on the PTAU/$A\beta_{1-42}$ ratio. Previous studies have shown that the use of ratio scores may be superior to the use of a single biomarker.[24,25] PTAU information was known from the main study and determined by measuring PTAU in CSF using the fully automated Elecsys platform.[22] at the Neurochemistry Lab Amsterdam UMC, using the PTAU/ $A\beta_{1-42}$ ratio >0.02 cut-off for preclinical AD definition. Subjects with a score <0.02 were classified as healthy subjects.

BLOOD SAMPLING Approximately 10mL blood was collected via an i.v. catheter placed in an antecubital vein in the arm in appropriate K2EDTA tubes (BD, USA) at the predefined time points mentioned above. Following blood centrifugation within one hour at 2000g for 10 min at 4°C, the plasma aliquots were divided into 0.5mL aliquots in Sarstedt polypropylene tubes and stored at -80°C. All blood samples for analyses of YKL-40, GFAP, MCP-1 and Eotaxin-1 are collected in a non-fasted state within one hour of collection of the CSF sample

LUMBAR PUNCTURE Lumbar punctures were performed by a trained, physician with a 25G atraumatic lumbar puncture needle (Braun, 25G). The needle was placed at the L3-L4 or L4-L5 interspace with the subject in supine or sitting position. 4 ml CSF was collected in a 15 mL polypropylene tube (Corning, USA). CSF was centrifuged within one hour, at 2000g for 10 minutes at 4°C and stored at -80°C.[26]

APOLIPOPROTEIN E GENOTYPING Apolipoprotein E (ApoE) genotyping was performed after isolating DNA from EDTA blood by the laboratory of human genetics (department of human genetics and endocrinology, Leiden University Medical Center LUMC). DNA was isolated using QIAamp DNA Blood MINI kit after which a polymerase chain reaction (PCR) technique was applied on the clean DNA. A sequential analysis (according to the Sanger method) than determined the ApoE genotype. One or 2 ApoE ε4 alleles classified subjects as ApoE ε4 carriers, when no ApoE ε4 alleles were present a subject was classified as noncarrier.

MEASUREMENT OF YKL-40, GFAP, MCP-1 AND EOTAXIN-1 YKL-40 (Chitinase 3-like 1 [CHI3L1]) was measured in the plasma samples using the CHI3L1 Human ELISA Kit (Thermo Fisher) according to the manufacturer's instructions. YKL-40 was measured previously in a larger sample and not for the sole purpose of this study.[21] Results of the 100 subjects selected for this study, have been used in the analyses.Plasma GFAP concentrations were measured at Amsterdam University Medical Centers (Amsterdam UMC) using the Simoa GFAP Discovery kit on the Single molecule array (Simoa) platform (Quanterix, Billerica, USA). MCP-1 and Eotaxin-1 were also measured at the Amsterdam UMC using Meso scale discovery (MSD, Rockville, MD, USA) assays according to the kit instructions.

STATISTICAL METHODOLOGY Visual checks on the ranges of biomarker and clinical characteristic test scores for each group based on CSF amyloid beta status, were done using scatter plots, as well as Tukey boxplots. Independent T-Test, Pearson Chi-Square test and Mann-Whitney tests were applied as appropriate.

To establish differences between subject groups in biomarkers, data is analysed using an ANCOVA, where age, sex and E4 status are added to the model as covariate. After including all covariates, the analysis was repeated with only the significant covariates added to the model. Variables were Log transformed where applicable. Least square means were calculated for all 4 inflammatory biomarkers using both the Aβ+/ Aβ- cut off and PTAU/$A\beta_{1-42}$ ratio. All analyses were carried out using SAS for Windows V9.4 (SAS Institute, Inc., Cary, NC, USA). A p-value of <0.05 was considered significant.

RESULTS

DEMOGRAPHIC AND CLINICAL CHARACTERISTICS The mean age of the total group of study participants (n=100) was 72.6 (4.6) years old with 62 male and 38 female subjects. Mean overall MMSE score was 28.7 (0.49) and 32 subjects were ApoE ε4 carriers. All subjects had a CDR score of 0.

COMPARISON OF PLASMA YKL-40, GFAP, MCP-1 AND EOTAXIN-1 BETWEEN Aβ+ AND Aβ- SUBJECTS Table 1 presents the cross-sectional demographics and clinical characteristics of the studied population based on Aβ+/ Aβ- groups. The ApoE ε4 status were significantly different between Aβ+ and Aβ- subjects. All other clinical characteristics do not differ significantly between the Aβ+ and Aβ- group. Plasma GFAP concentration was significantly higher in the Aβ+ group compared to the Aβ- group before and after adjusting for covariates age and sex, variables that also correlated significantly with GFAP, see figure 2. YKL-40, MCP-1 and Eotaxin-1 were not significantly different between the Aβ+ and Aβ- group None of the biomarkers correlated with the MMSE score.

Table 1 Cross-sectional demographics and clinical characteristics of the studied population based on Aβ+/ Aβ- groups.

	Aβ+ (n=50)	Aβ- (n=50)	p
Aβ level (mean, SD)	706.0 (174.36)	>1700	
Sex (male/female)	33/17	29/21	0.41
BMI (mean, SD)	26.07 (3.95)	25.17 (3.44)	0.225
Age (years, mean, SD)	73.40 (4.72)	71.88 (4.45)	0.101
ApoE ε4 carrier (n, %)	25 (50%)	7 (14.6%)	0.003
MMSE (mean, SD)	28.60 (1.41)	28.82 (1.37)	0.431
CDR (mean, SD)	0 (0)	0 (0)	
GFAP PG/mL (mean, SD)	N=50 195.1 ± 87.13	N=50 134.0 ± 50.71	<0.001
YKL-40 PG/mL (mean, SD)	N=49 54662.3 ± 39697.31	N=49 82947.1 ± 83418.38	0.397
MCP-1 PG/mL (mean, SD)	N=50 91.74 ± 16.72	N=50 97.98 ± 34.01	0.358
Eotaxin-1 PG/mL (mean, SD)	N=50 195.0 ± 57.87	N=50 204.0 ± 94.80	0.783

P values in bold font were considered significant (p<0.05). Independent T-Test and Pearson Chi-Square test were applied as appropriate.

COMPARISON OF PLASMA YKL-40, GFAP, MCP-1 AND EOTAXIN-1 BETWEEN SUBJECTS DIVIDED BASED ON PTAU/Aβ42 RATIO Table 2 presents the cross-sectional demographics and clinical characteristics of the studied population based on the PTAU/$A\beta_{1-42}$ ratio score. The ApoE ε4 status were significantly different between two groups divided by PTAU/$A\beta_{1-42}$ ratio score. All other clinical characteristics do not differ significantly groups. Plasma GFAP and plasma YKL-40 concentration were significantly higher in the preclinical AD group based on the PTAU/$A\beta_{1-42}$ ratio before and after adjusting for covariates age, sex and ApoE ε4 status as these variables also correlated with GFAP, see figure 3. YKL-40 was significantly different between ApoE ε4 carriers versus non-carriers. Eotaxin-1 was significantly different between sexes. MCP-1 did not show any difference.

Table 2 Cross-sectional demographics and clinical characteristics of the studied population based on the PTAU/$A\beta_{1-42}$ ratio score.

	PTAU/Aβ + (n=36)	PTAU/Aβ – (n=64)	p
PTAU/Abeta42 ratio	0.04 (0.012)	0.01 (0.003)	
Aβ level (mean, SD)	685.2 (163.7)	1494.2 (401.9)	
Sex (male/female)	26/10	36/28	0.166
BMI (mean, SD)	26.2 (3.8)	25.3 (3.7)	0.338
Age (years, mean, SD	73.8 (4.9)	72.0 (4.4)	0.039
ApoE ε4 carrier (n, %)	18 (50%)	14 (22.6%)	0.001
MMSE (mean, SD)	28.5 (1.5)	28.8 (1.4)	0.314
CDR (mean, SD)	0 (0)	0 (0)	
GFAP PG/mL (mean, SD)	211.8 ± 97.6	138.8 ± 49.9	<0.001
YKL-40 PG/mL (mean, SD)	N=38, 87038.7 ± 74252.3	N=145, 60583.7 ± 54067.1	0.012
MCP-1 PG/mL (mean, SD)	N=34, 92.6 ± 18.4	N=64, 96.4 ± 30.7	0.602
Eotaxin-1 PG/mL (mean, SD)	N= 34 193.6 ± 62.9	N=64 202.5 ± 86.7	0.630

P values in bold font were considered significant (p<0.05). Independent T-Test, Pearson Chi-Square test and Mann-Whitney tests were applied as appropriate.

CORRELATION BETWEEN BIOMARKERS Figure 1 represents a heatmap with p-values calculated for all inflammatory biomarkers plus $A\beta_{42}$, PTAU/$A\beta_{42}$ ratio and age. Plasma YKL-40, GFAP, $A\beta_{42}$ and PTAU/$A\beta_{42}$ ratio correlated with age. YKL-40 also correlated with GFAP and PTAU/Aβ42 ratio. GFAP correlated with PTAU/$A\beta_{42}$ ratio. MCP-1 is positively correlated with Eotaxin-1 and $A\beta_{42}$. $A\beta_{42}$ and PTAU/$A\beta_{42}$ ratio are strongly correlated. N=121 for Aβ, which are the samples of all original subjects included in the main study except the subjects with a CSF $A\beta_{42}$ concentration of >1700 as no exact concentrations are available.

Figure 1 Heatmap p-values for biomarkers correlations YKL-40, GFAP, eotaxin-1, MCP-1, Aβ42, and Ptau/Aβ42.

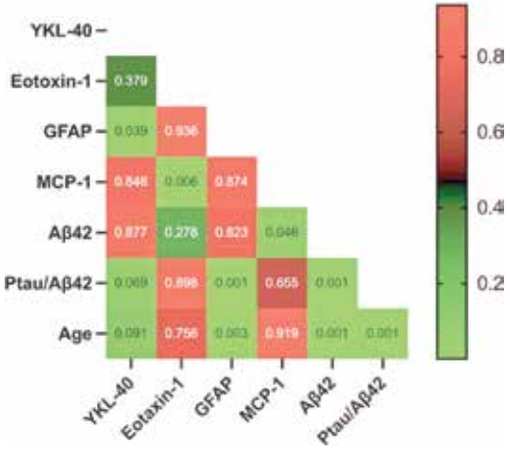


Figure 2 Significant violin plot for GFAP among healthy elderly subjects with a CSF profile consistent with Alzheimer's Disease, n=50 (Aβ+ [CSF Aβ42 <1000] versus healthy elderly subjects with normal CSF Aβ-, n=50 [CSF Aβ42 >1000]).

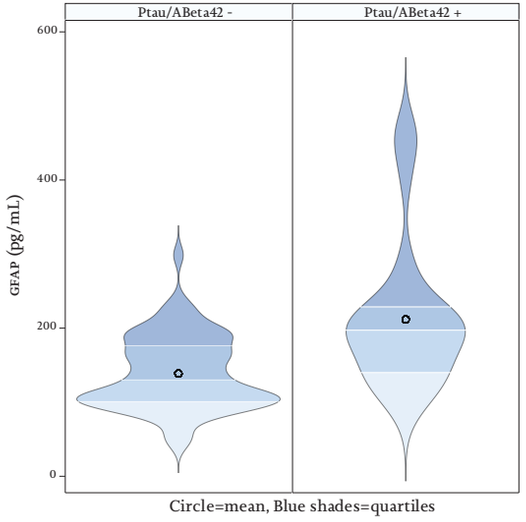
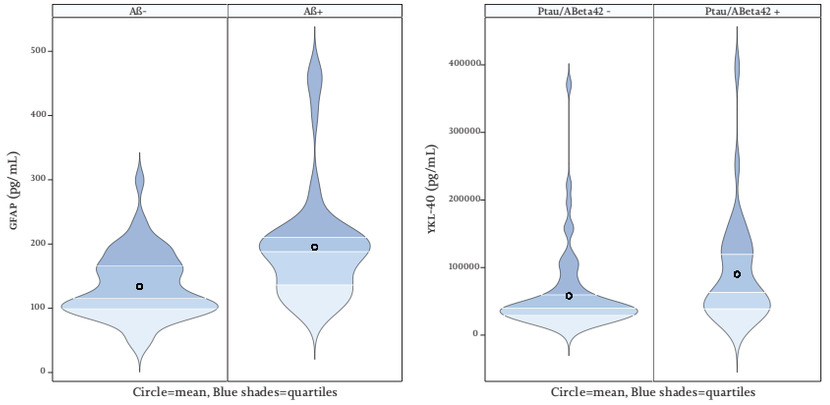


Figure 3 Significant violin plots for GFAP and YKL-40 compared to PTAU/ Aβ42 ratio.



DISCUSSION

In the current exploratory study we aimed to investigate plasma biomarkers related to neuroinflammation associated with AD in a cohort of subjects with preclinical AD, and to compare these to healthy elderly, both defined by $A\beta_{1-42}$ CSF status. Of the four inflammatory plasma biomarkers investigated in this study, only GFAP was significantly higher in subjects with preclinical AD compared to healthy elderly. When defining preclinical AD based on the PTAU181/$A\beta_{1-42}$ ratio, GFAP and YKL-40 were significantly different between groups. This could indicate that GFAP and YKL-40 are more sensitive markers of the incipient inflammatory process that occurs in response to the beta amyloid misfolding and aggregation that is ongoing as indicated by the lowered $A\beta_{1-42}$ protein levels in the CSF.

With increasing prevalence of AD.[27] it would be interesting to look at 'biomarker-positive' subjects, 50% of whom will develop AD,[24] and further investigate the course over time of the inflammatory biomarkers described here. As we found in the current study, evidence of astrogliosis as demonstrated by elevated GFAP was already increased in healthy subjects positive for CSF $A\beta_{1-42}$. If we can further characterize these subjects, we may be able to define a group of healthy subjects more likely to develop AD and treat these subjects in early (neuroinflammatory or CSF $A\beta_{1-42}$ lowering) clinical trials. Measurement of GFAP and YKL-40 in plasma is useful in healthy subjects with preclinical AD as it allows to determine the level of neuroinflammation in subjects possibly developing AD and can provide more information on the relationship between neuroinflammation and the development of AD. Disease modifying treatments targeting neuro-inflammation early in the preclinical disease process of AD may delay disease progression and prevent or delay cognitive decline as inflammation can be expected to influence cognitive performance independently from Aβ pathology.[28]

Our results showing an increase in GFAP in the preclinical stage are in line with Verberk et al., (2021) who studied a similar population of cognitively healthy elderly and found GFAP to be associated with increased risk of progression to dementia and steeper cognitive decline.[18] Aβ measured in plasma by Chatterjee et al (2021).[19] in cognitively normal older adults resulted in two groups, Aβ+ and Aβ- subjects comparable to our studied population. This study also found that GFAP was elevated in subjects with preclinical AD. Our study therefore reproduces these study results, demonstrating that these findings are real and independent of the specific samples used by Chatterjee or by us. Pereira er al., (2021).[28] mention that plasma GFAP might be specific to AD as it correlated with Aβ pathology in their study with comparable cognitive normal subjects, which is supported by the differences between groups found in our study but not the correlation with Aβ itself. Alternatively, this could be the result of a smaller sample size. Further research is needed to determine if GFAP can be used as a CSF-independent marker for (preclinical) AD.

When YKL-40 is measured in CSF, this could indicate that microglial activation is taking place, even though YKL-40 concentrations are already measurable is subject without lowered Aβ measured in CSF .[29] Several associations have been found between CSF YKL-40 and neurodegenerative biomarkers in CSF namely total tau protein and significant differences have been found between AD patients, healthy elderly, and subjects with preclinical AD.[30] Demonstrating differences in plasma levels of YKL-40 between healthy elderly and subjects with preclinical AD could help to identify inflammatory processes in a less invasive manner. In our study, plasma YKL-40 did not correlate with CSF $A\beta_{1-42}$ and was not different between subjects with preclinical AD and healthy controls. Thus, no conclusion about can be drawn about glial activation by YKL-40 in response to accumulation of Aβ in this particular sample of healthy subjects, perhaps because it is too early in the disease process to identify differences in YKL-40 concentrations in plasma. When redefining the subjects based on CSF PTAU181/$A\beta_{1-42}$ ratio scores, plasma YKL-40 concentration was found to differ between groups. This comparison was

performed post hoc, however. As plasma YKL-40 was not previously reported to be different between subjects with preclinical AD and healthy controls, this finding is of interest and a reason to further investigate this and confirm it in a properly powered study aimed at replication. Comparable to GFAP, YKL-40 levels increase with age, in CSF and also in plasma. When measured in plasma, higher plasma YKL-40 concentrations seem to be correlated with male sex, older age, ApoE ε4 status and cerebral accumulation of Aβ measured with PET.[31] Our sample did not found YKL-40 to be correlated with age, sex, ApoE ε4 status and Aβ measured in CSF. GFAP showed to be correlated with sex, age and Aβ status in our sample. GFAP and YKL-40 can be found in a vast range of peripheral cells expressing it and might therefore be measurable in plasma. Previous studies, however, conclude that measuring GFAP in plasma is related to CNS inflammation and severity of disease.[32,33] YKL-40 has been found to be increased in subjects with streptococcal pneumonia and could therefore have a peripheral origin and confound to the measurability in plasma which should be taken into account when interpreting YKL-40 results in plasma.[34]

The subjects investigated in the current study were part of a larger observational study, therefore information on cognitive status measured using a computerized cognitive test battery and several paper and pencil tasks was available. Our two groups, preclinical AD and healthy elderly, were specifically different regarding Abeta1-42 measured in CSF. We divided GFAP and YKL-40 scores into 'high' levels and 'low' levels of inflammation by using the median and compared these groups with the total group of subjects. None of the cognitive domains (e.g., memory, attention, overall cognitive performance measured with MMSE and CDR) differed significantly between groups and therefore there was no indication of early cognitive decline in the otherwise healthy subjects with elevated neuroinflammatory markers. This is in contrast to other, longitudinal studies, which have found that plasma (and CSF) GFAP could predict global cognitive decline.[18] even though plasma GFAP was not always measured longitudinally.[28]

Limitations

The correlation that we found between CSF Aβ42 and CSF PTAU/Aβ42 ratio is inherently based on the use of CSF Aβ42 in the latter ratio. YKL-40 being significantly different in the PTAU/Aβ42 ratio condition and not being different based on Aβ alone could be a result of this.

GFAP correlated significantly with CSF PTAU/Aβ42 ratio but not with CSF Aβ42, which can possibly be explained by differences in sample size. The calculation of GFAP in correlation with Aβ only includes the Aβ+ subjects as these are continuous values (n=50), the Aβ- subjects all had Aβ levels of >1700 (no exact value). For calculating the ratio score PTAU/Aβ42 the whole data set could be used (n=98) with Aβ- =1700. When including a larger N, also including the 50 Aβ- subjects, the correlation between GFAP and CSF Aβ42 could have been significant as we have established a difference between groups on GFAP and Aβ+ / Aβ- subjects. For the calculation of the correlation between Aβ42 and age, the original data set of 200 subjects was used, of which 121 subjects had exact Aβ42 values. The subjects with Aβ42 concentrations of >1700 PG/mL were not included as no exact concentrations were known; it was only indicated that levels were >1700 PG/mL. It is, however, unlikely that the correlation found between Aβ42, and age would be non-significant if exact values for all subjects with levels >1700 PG/mL were available.

The subjects included in this study were not referred to a memory clinic but voluntarily participated in this study. No subjects with proof of (subjective) memory complaints participated, demonstrated by a MMSE of >24 during prescreening, and during the study confirmed by a CDR of 0 and AIDL of 0. However, subjects with insecurities about their cognitive performance might be more likely to participate in observational studies.

This study was exploratory and further research is needed to confirm the results. Data in this study was not corrected for multiple comparisons.

Conclusions

Measuring GFAP and YKL-40 in plasma in subjects with preclinical AD could be of added value to further differentiate subjects with lowered CSF Aβ42 from otherwise healthy elderly to better define the preclinical AD status. However, this study was cross-sectional and subject discrimination needs further analyses. If further research shows that these inflammatory plasma biomarkers are specific for (preclinical) AD, measuring these can be an important step forward in characterizing otherwise healthy elderly with preclinical AD in a less invasive manner.

REFERENCES

1 Blennow, K. and H. Zetterberg, Biomarkers for Alzheimer's disease: current status and prospects for the future. J Intern Med, 2018. 284(6): p. 643-663.
2 Zetterberg, H. and B.B. Bendlin, Biomarkers for Alzheimer's disease – preparing for a new era of disease-modifying therapies. Molecular Psychiatry, 2021. 26(1): p. 296-308.
3 Budelier, M.M. and R.J. Bateman, Biomarkers of Alzheimer Disease. The Journal of Applied Laboratory Medicine, 2019. 5(1): p. 194-208.
4 Bjorkli, C., A. Sandvig, and I. Sandvig, Bridging the Gap Between Fluid Biomarkers for Alzheimer's Disease, Model Systems, and Patients. Frontiers in Aging Neuroscience, 2020. 12(272).
5 Olsson, B., et al., CSF and blood biomarkers for the diagnosis of Alzheimer's disease: a systematic review and meta-analysis. Lancet Neurol, 2016. 15(7): p. 673-684.
6 Huang, L.K., S.P. Chao, and C.J. Hu, Clinical trials of new drugs for Alzheimer disease. J Biomed Sci, 2020. 27(1): p. 18.
7 Dunn, B., P. Stein, and P. Cavazzoni, Approval of Aducanumab for Alzheimer Disease – The FDA's Perspective. JAMA Internal Medicine, 2021. 181(10): p. 1276-1278.
8 Jansen, W.J., et al., Prevalence of Cerebral Amyloid Pathology in Persons Without Dementia: A Meta-analysis. JAMA, 2015. 313(19): p. 1924-1938.
9 Saitgareeva, A.R., et al., The role of microglia in the development of neurodegeneration. Neurol Sci, 2020.
10 Heneka, M.T., et al., Neuroinflammation in Alzheimer's disease. Lancet Neurol, 2015. 14(4): p. 388-405.
11 Bettcher, B.M., et al., MCP-1 and eotaxin-1 selectively and negatively associate with memory in MCI and Alzheimer's disease dementia phenotypes. Alzheimers Dement (Amst), 2016. 3: p. 91-7.
12 Haas, D., Chapter 15 - Biomarker for Alzheimer's Disease, in Precision Medicine, H.-P. Deigner and M. Kohl, Editors. 2018, Academic Press. p. 333-349.
13 Morgan, A.R., et al., Inflammatory biomarkers in Alzheimer's disease plasma. Alzheimers Dement, 2019. 15(6): p. 776-787.
14 Oeckl, P., et al., Glial Fibrillary Acidic Protein in Serum is Increased in Alzheimer's Disease and Correlates with Cognitive Impairment. J Alzheimers Dis, 2019. 67(2): p. 481-488.
15 Villar-Pique, A., et al., Plasma YKL-40 in the spectrum of neurodegenerative dementia. J Neuroinflammation, 2019. 16(1): p. 145.
16 Muramori, F., K. Kobayashi, and I. Nakamura, A quantitative study of neurofibrillary tangles, senile plaques and astrocytes in the hippocampal subdivisions and entorhinal cortex in Alzheimer's disease, normal controls and non-Alzheimer neuropsychiatric diseases. Psychiatry Clin Neurosci, 1998. 52(6): p. 593-9.
17 Jesse, S., et al., Glial fibrillary acidic protein and protein S-100B: different concentration pattern of glial proteins in cerebrospinal fluid of patients with Alzheimer's disease and Creutzfeldt-Jakob disease. J Alzheimers Dis, 2009. 17(3): p. 541-51.
18 Verberk, I.M.W., et al., Serum markers glial fibrillary acidic protein and neurofilament light for prognosis and monitoring in cognitively normal older people: a prospective memory clinic-based cohort study. The Lancet Healthy Longevity, 2021. 2(2): p. e87-e95.
19 Chatterjee, P., et al., Plasma glial fibrillary acidic protein is elevated in cognitively normal older adults at risk of Alzheimer's disease. Transl Psychiatry, 2021. 11(1): p. 27.
20 Mackay, C.R., Chemokines: immunology's high impact factors. Nat Immunol, 2001. 2(2): p. 95-101.
21 Prins, S., et al., A cross-sectional study in healthy elderly subjects aimed at development of an algorithm to increase identification of Alzheimer pathology for the purpose of clinical trial participation. Alzheimers Res Ther, 2021. 13(1): p. 132.
22 Lewczuk, P., et al., Biomarkers of Alzheimer's disease and mild cognitive impairment: a current perspective. Adv Med Sci, 2015. 60(1): p. 76-82.
23 Willemse, E.A.J., et al., Comparing CSF amyloid-beta biomarker ratios for two automated immunoassays, Elecsys and Lumipulse, with amyloid PET status. Alzheimer's & dementia (Amsterdam, Netherlands), 2021. 13(1): p. e12182-e12182.
24 Blennow, K., et al., Predicting clinical decline and conversion to Alzheimer's disease or dementia using novel Elecsys Abeta(1-42), pTau and tTau CSF immunoassays. Sci Rep, 2019. 9(1): p. 19024.
25 Campbell, M.R., et al., P-TAU/Aβ42 and Aβ42/40 ratios in CSF are equally predictive of amyloid PET status. Alzheimers Dement (Amst), 2021. 13(1): p. e12190.
26 Teunissen, C.E., et al., A consensus protocol for the standardization of cerebrospinal fluid collection and biobanking. Neurology, 2009. 73(22): p. 1914-1922.
27 WHO. https://www.who.int/news-room/fact-sheets/detail/dementia. https://www.who.int/news-room/fact-sheets/detail/dementia 2020 21 September 2020 [cited 2021 18Aug2021].
28 Pereira, J.B., et al., Plasma GFAP is an early marker of amyloid-β but not tau pathology in Alzheimer's disease. Brain, 2021.
29 Hellwig, K., et al., Neurogranin and YKL-40: independent markers of synaptic degeneration and neuroinflammation in Alzheimer's disease. Alzheimer's research & therapy, 2015. 7: p. 74-74.
30 Baldacci, F., et al., Diagnostic function of the neuroinflammatory biomarker YKL-40 in Alzheimer's disease and other neurodegenerative diseases. Expert Review of Proteomics, 2017. 14(4): p. 285-299.
31 Vergallo, A., et al., Association of plasma YKL-40 with brain amyloid-β levels, memory performance, and sex in subjective memory complainers. Neurobiology of Aging, 2020. 96: p. 22-32.
32 Högel, H., et al., Serum glial fibrillary acidic protein correlates with multiple sclerosis disease severity. Mult Scler, 2020. 26(2): p. 210-219.
33 Wu, L., et al., Serum glial fibrillary acidic protein and ubiquitin C-terminal hydrolase-L1 for diagnosis of sepsis-associated encephalopathy and outcome prognostication. J Crit Care, 2019. 52: p. 172-179.
34 Kronborg, G., et al., Serum level of YKL-40 is elevated in patients with Streptococcus pneumoniae bacteremia and is associated with the outcome of the disease. Scand J Infect Dis, 2002. 34(5): p. 323-6.

CHAPTER VII

Neurocognitive functions are not impaired in subjects with preclinical AD based on CSF Aβ and higher levels of CSF P-TAU217

Samantha Prins,[1,2] Thomas K. Karikari,[4] Marieke L. de Kam,[1] Przemyslaw R. Kac,[4] Henrik Zetterberg,[4] Kaj Blennow,[3,4] Geert Jan Groeneveld[1,2]

SUBMITTED TO JOURNAL OF ALZHEIMER'S DISEASE

1. Centre for Human drug Research, Leiden, NL | 2. Leiden University Medical Center, Leiden, NL | 3. Institute of Neuroscience and Physiology Department of Psychiatry and Neurochemistry, The Sahlgrenska Academy University of Gothenburg, Mölndal, SE | 4. Clinical Neurochemistry Laboratory, Sahlgrenska University Hospital, Mölndal, SE

ABSTRACT

BACKGROUND Amyloid plaques in the brain and lowered levels of amyloid beta measured in cerebrospinal fluid (CSF) are used as biomarker evidence to diagnose patients with Alzheimer's disease (AD). Along with tauopathy and hyperphosphorylated tau, which can be measured as tau deposition in the brain and increased (hyperphosphorylated) tau levels in CSF, these are the hallmark for AD. Tau is expressed predominantly in the central and peripheral nervous systems, where it is abundant in nerve cell axons. Tau binds to microtubules, providing stability and facilitating axonal transport. Tau is encoded by the microtubule-associated protein tau (MAPT) gene and is naturally unfolded. Six tau isoforms are expressed in adult human brains. The current study investigated P-TAU181, P-TAU217 and P-TAU231 isoforms in CSF and P-TAU181 and P-TAU231 in plasma in subjects with preclinical AD and healthy elderly, to investigate whether phosphor-tau CSF can differentiate healthy elderly from preclinical AD subjects and study cognitive performance of subjects with preclinical AD based on CSF Aβ in combination with higher levels of P-TAU isoforms. Results could help identify the correct study populations for clinical trials investigating disease modifying treatments (DMTs) aimed at P-TAU.

METHODS Samples of 100 healthy male and female subjects of 65 years of age and older were selected from the main study in healthy elderly based on $A\beta_{1-42}$ status. All subjects were healthy volunteer with no cognitive complaints. Of the 100 subjects, 50 subjects were selected having CSF $A\beta_{1-42}$ profiles consistent with Alzheimer's disease and were classified as preclinical AD according to the NIA-AA standards from 2011. Blood and CSF samples were taken and analyzed on CSF P-TAU181, P-TAU217 and P-TAU231 and plasma P-TAU181 and P-TAU231. The following NeuroCart tests were performed: the Adaptive tracking test to measure attention and eye-hand coordination, the Face encoding and Recognition task (FACE) to measure visual memory, the Visual Verbal Learning Test (VVLT, 30 words) to measure the whole scope of learning behavior (i.e. acquisition, consolidation, storage and retrieval), the N-Back test was assessed to evaluate working memory, finger tapping for motor fluency, saccadic and smooth eye movement were also measured. Basic characteristics such as age, gender and ApoE ε4 status were reported per group. Visual checks on the ranges of biomarker scores for each group were done using scatter plots, as well as Tukey boxplots. To explore differences between groups the biomarker outcomes were tested with an ANCOVA where age, sex and ApoE ε4 status were added in the model, or t-tests where applicable. Variables were Log transformed where applicable. Least square means were calculated for all P-TAU isoforms in both groups.

RESULTS The ApoE ε4 status was significantly different between Aβ+ and Aβ- subjects. CSF P-TAU181 and CSF P-TAU231 were significantly different on age, not on group difference between Aβ+ and Aβ-. Plasma P-TAU181 and P-TAU231 were not significantly different between Aβ+ and Aβ- subjects or any of the covariates. None of the cognitive assessments show significant difference per P-TAU concentration in CSF or plasma. Age was significantly higher in subjects with higher concentrations of CSF P-TAU181, P-TAU231 and P-TAU217. Age was also significantly higher in subjects with Aβ+ and CSF concentrations of P-TAU181 and P-TAU231. CSF P-TAU181 is strongly correlated with CSF P-TAU 217 and P-TAU231 (P=<0.0001) but also with plasma P-TAU181 (p=0.0184) and P-TAU231 (p=0.0189). CSF P-TAU217 and P-TAU231 are also strongly correlated (p=<0.0001). CSF P-TAU217 correlates with plasma P-TAU181 (P=0.0042) and P-TAU231 (p=0.0358). CSF P-TAU231 correlates with plasma P-TAU181 (P=0.0054) and P-TAU231 (p=0.0170). Plasma P-TAU181 correlates strongly with plasma P-TAU231 (p=<0.0001). None of the P-TAU biomarkers correlates with $A\beta_{1-42}$.

CONCLUSION As P-TAU seems to emerge in the preclinical phase of AD as a response to upcoming Aβ misfolding in the brain, this could be the earliest possible intervention window for treatment before neurofibrillary tangles arise. Measuring P-TAU in plasma can be used for the measurement of target engagement of these specific anti-tau DMT and early phase removal or lowering of P-TAU might lead to less subjects progressing from preclinical AD to AD. As this study does not confirm the discriminating power of P-TAU in preclinical AD, more (longitudinal) research is needed to provide more insight into the usefulness of plasma P-TAU biomarkers for distinction between preclinical AD and healthy subjects.

BACKGROUND

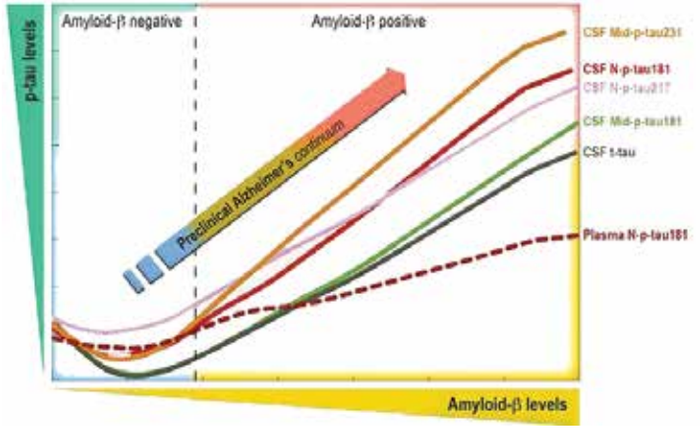
Amyloid plaques in the brain and lowered levels of amyloid beta measured in cerebrospinal fluid (CSF) are used as biomarker evidence to diagnose patients with Alzheimer's disease (AD). Along with tauopathy and hyperphosphorylated tau, which can be measured as tau deposition in the brain and increased (hyperphosphorylated) tau levels in CSF, these are the hallmark for AD.[1]

Tau is expressed predominantly in the central and peripheral nervous systems, where it is abundant in nerve cell axons.[2] Tau binds to microtubules, providing stability and facilitating axonal transport.[3]

Tau is encoded by the microtubule-associated protein tau (MAPT) gene and is naturally unfolded. Six tau isoforms are expressed in adult human brains. An imbalance in tau kinase and phosphatase activity is considered to be the reason for tau hyperphosphorylation in AD and other neurodegenerative diseases.[4] Previous research has focused on specific isoforms of phosphorylated tau to distinguish between healthy subjects and patients with AD with regard to increased CSF total and phosphorylated tau levels at threonine 181 (P-TAU181). However, P-TAU181 was shown not to be specific for AD and is increased in multiple neurodegenerative diseases.[5] Various other studies have found correlations between different phosphorylated tau isoforms and amyloid plaques in patients with Alzheimer's disease.[6–8] Especially the P-TAU isoforms P-TAU217, P-TAU231 and P-TAU 181 are found to be increased in CSF of subjects with amyloidosis related to AD. Barthelemy et al., (2020) describe that especially P-TAU217 measured in CSF is a highly specific biomarker for detecting preclinical and advanced forms of AD, more specific than P-TAU181. CSF P-TAU217 correlates strongly with presence of beta amyloid in the brain using PiB-PET imaging.[5] A publication by the same group describes significant differences in CSF and plasma P-TAU217 and P-TAU181 between amyloid beta (Aβ) positive and Aβ negative subjects, regardless of the cognitive status which indicates tauopathy in the preclinical stage of AD.[9] Preclinical AD refers to cognitively healthy subjects having lowered CSF $A\beta_{1-42}$ levels consistent with AD, so called Aβ positive subjects.[10] Palmqvist et al., (2020) tried to discriminate AD from other neurodegenerative disorders by using plasma P-TAU217 in populations ranging from healthy to AD. They found that plasma P-TAU217 performed better in discriminating AD than other plasma tau isoforms and MRI based biomarkers and was similarly effective as key CSF and PET based measures.[11] P-TAU has been suggested to correlate with cognitive impairment, better than Aβ related biomarkers.[12] Suarez-Calvet et al., (2020) published an illustration of the process of tau-phosphorylation compared to amyloidosis in CSF see, figure 1.[12]

Measuring these isoforms in CSF and plasma in healthy elderly and in people with preclinical AD can help to identify pathological disease onset and can also be used to identify early AD pathology when selecting cognitively healthy elderly for participation in clinical trials with amyloid beta targeting drugs aimed at disease modifying effects and prevention of dementia. Additionally, very few studies have shown discrimination between healthy subjects and preclinical AD based on plasma P-TAU isoforms, so confirmation of the findings of the Barthelemy papers is needed. The current study investigated P-TAU181, P-TAU217 and P-TAU231 isoforms in CSF and P-TAU181 and P-TAU231 in plasma in subjects with preclinical AD and healthy elderly, to investigate whether phosphor-tau CSF can differentiate healthy elderly from preclinical AD subjects and study cognitive performance of subjects with preclinical AD based on CSF Aβ in combination with P-TAU isoforms. Results could help identify the correct study populations for clinical trials investigating disease modifying treatments (DMTs) aimed at P-TAU.

Figure 1 The CSF continuum of different P-TAU isotope levels compared to Aβ levels.[12]



METHODS

This was an exploratory sub-study of a previously performed study registered in the international trial register with ID number: ISRCTN79036545.[13] All study participants provided written consent for exploratory analyses of material obtained during study execution.

The main study was approved by the ethics committee of the Leiden University Medical Center (LUMC), the Netherlands. The study was conducted according to the Dutch act on Medical Research Involving Human Subjects (WMO) and in compliance with Good Clinical Practice (ICH-GCP) and the Declaration of Helsinki.

PARTICIPANTS Samples of 100 healthy male and female subjects of 65 years of age and older were selected from the main study in healthy elderly.[13] based on $A\beta_{1-42}$ status. All subjects were healthy volunteer with no cognitive complaints. Subjects responded voluntarily on recruitment advertisements asking for healthy elderly trial subjects. Of the 100 subjects, 50 subjects were selected having CSF $A\beta_{1-42}$ profiles consistent with Alzheimer's disease and were classified as preclinical AD according to the NIA-AA standards from 2011.[10] The remaining 50 subjects were selected on having high levels of CSF $A\beta_{1-42}$ as healthy control group. Lowered Aβ levels classified as amyloid abnormal and consistent with the presence of Alzheimer pathology were dichotomized by creating a group of 'Aβ positive subjects' (Aβ+ = < 1000 PG/mL) and 'Aβ negative subjects' (Aβ- = > 1700 PG/mL) using confirmed cut-offs.[14] $A\beta_{1-42}$ was measured in CSF using the fully automated Elecsys platform as this is widely used for diagnostics.[15] All the subjects visited Centre for Human Drug Research (CHDR) between October 2017 and November 2018. Main exclusion criteria were a diagnosis of a cognitive disorder (including but not limited to Mild Cognitive Impairment [MCI], AD, Lewy Body dementia, Frontotemporal dementia), history of psychiatric disease in the past 3 years, Mini Mental State Examination (MMSE) ≤ 24, Geriatric Depression Scale (GDS) ≥ 6, presence of drug or alcohol abuse (<2 standard drinks per day for female and <3 standard drinks per day for male), use of any medication that was expected to influence central nervous system function or is contraindicative of the performance of a lumbar puncture.

All subjects visited the clinical research unit once and underwent blood sampling at predefined time points (0, 2 and 4 hour[s]). A single lumbar puncture was performed for the collection of CSF (at 4 hours), for measurement of $A\beta_{1-42}$ as described below.

This is an exploratory study, therefore the sample size is not based on statistical considerations. Including 50 preclinical AD subjects and an equally sized healthy elderly control group (n=50) was considered appropriate for a comparative study. Previous comparable studies have been able to show differences between groups in smaller sample sizes.[11,16]

BLOOD SAMPLING Approximately 10mL blood was collected via an i.v. catheter placed in an antecubital vein in the arm in appropriate K2EDTA tubes at the predefined time points mentioned above. Following blood sample processing, the plasma fractions were stored at -80°C.

LUMBAR PUNCTURE A CSF sample of 4 mL was collected in a 10 mL polypropylene tube. CSF was centrifuged within one hour, at 2000g for 10 minutes at 4°C and stored at -80°C. Lumbar punctures were performed by a trained, physician with a 25G atraumatic lumbar puncture needle (Braun, 25G). The needle was placed at the L3-L4 or L4-L5 interspace with the subject in supine or sitting position.

APOLIPOPROTEIN E GENOTYPING Apolipoprotein E (ApoE) genotyping was performed after isolating DNA from EDTA blood. DNA was isolated using QIAamp DNA Blood MINI kit after which a polymerase chain reaction (PCR) technique was applied on the clean DNA. A sequential analysis (according to the Sanger method) than determined the ApoE genotype. One or 2 ApoE ε4 alleles classified subjects as ApoE ε4 carriers, when no ApoE ε4 alleles were present a subject was classified as noncarrier.

MEASUREMENT OF CSF P-TAU181, P-TAU217 AND P-TAU231 AND PLASMA P-TAU181 AND P-TAU231 All blood samples for analyses of phosphorated tau were collected in a non-fasted state within one hour of collection of the CSF sample. After sample processing, the CSF and plasma fractions were stored at -80 °C until further analyses. $A\beta_{1-42}$ was measured in CSF using the fully automated Elecsys platform as this is widely used for diagnostics.[15] All P-TAU isoforms were analyses with Simoa HD-X using in-house assays at the Department of Psychiatry and Neurochemistry, University of Gothenburg, Mölndal, Sweden as described by Karikari et al., (2020).[17]

COGNITIVE ASSESSMENTS AND QUESTIONNAIRES The NeuroCart is a battery of CNS tests used to assess a wide range of CNS domains.[18] All measurements were performed in a quiet room with ambient illumination. Per session there was only one subject in the room. The following tests were performed using the NeuroCart: the Adaptive tracking test to measure attention and eye-hand coordination,[19] the Face encoding and Recognition task (FACE) to measure visual memory,[20] the Visual Verbal Learning Test (VVLT, 30 words) to measure the whole scope of learning behavior (i.e. acquisition, consolidation, storage and retrieval),[21] the N-Back test was assessed to evaluate working memory,[22] finger tapping for motor fluency,[23] saccadic and smooth eye movement were also measured.[24]

The clinical dementia rating scale (CDR).[25] was assessed via a semi-structured interview with the participating subject only, to rate impairment in six different cognitive categories (memory, orientation, judgement and problem solving, community affairs, home and hobbies and personal care).

STATISTICAL METHODOLOGY Subjects were grouped based on CSF amyloid beta status where Aβ+ equals preclinical AD and Aβ- equals healthy elderly as mentioned above. Basic characteristics such as age, gender and ApoE ε4 status were reported per group. Visual checks on the ranges of biomarker scores for each group were done using scatter plots, as well as Tukey boxplots. To explore differences between groups the biomarker outcomes were tested with an ANCOVA where age, sex and ApoE ε4 status were added in the model, or t-tests where applicable. Variables were Log transformed where applicable. Least square means were calculated for all P-TAU isoforms in both groups. All analyses were carried out using SAS for Windows V9.4 (SAS Institute, Inc., Cary, NC, USA).

RESULTS

DEMOGRAPHIC AND CLINICAL CHARACTERISTICS The mean age of the total group of study participants (n=100) was 72.6 (4.6) years old with 62 male and 38 female subjects. Mean overall MMSE score was 28.7 (0.49) and 32 subjects were ApoE ε4 carriers. All subjects had a CDR score of 0.

COMPARISON OF CSF P-TAU181, P-TAU217, P-TAU231 AND PLASMA P-TAU181 AND P-TAU231 BETWEEN Aβ+ AND Aβ- SUBJECTS Table 1 presents the cross-sectional demographics and clinical characteristics of the studied population based on Aβ+/ Aβ- groups. The ApoE ε4 status was significantly different between Aβ+ and Aβ- subjects. All other clinical characteristics do not differ significantly between the Aβ+ and Aβ- group. CSF P-TAU217 was significantly different between Aβ+ and Aβ- subjects, see Table 1 and Figure 1. CSF P-TAU181 and CSF P-TAU231 were significantly different on age, not on group difference between Aβ+ and Aβ- as the data shows more spreading, see Figure 2. Plasma P-TAU181 and P-TAU231 were not significantly different between Aβ+ and Aβ- subjects or any of the covariates.

COGNITIVE PERFORMANCE OF SUBJECTS WITH DIFFERENT P-TAU CONCENTRATIONS None of the cognitive assessments show significant difference per P-TAU concentration in CSF or plasma, see Table 2. Scatterplots of all cognitive assessments were created with subjects pooled by Aβ+ and above median P-TAU concentrations for all P-TAU isoforms. Visual check of the scatterplots resulted into no apparent differences between the groups therefore no statistical analyses were performed. Analyses on age (years) and total MMSE scores were performed for the total subject group and subjects with Aβ+ and P-TAU, see table 3. Age was significantly higher in the total subject group. Age was not correlated in subjects with Aβ+ alone. There was no difference in any of the comparisons for total MMSE score.

Table 1 Cross-sectional demographics and clinical characteristics of the studied population based on Aβ+/ Aβ- groups.

	Aβ+ (n=50)	**Aβ- (n=50)**	**p**
Aβ level (mean, SD)	706.0 (174.36)	>1700	
Sex (male/female)	33/17	29/21	0.41
BMI (mean, SD)	26.07 (3.95)	25.17 (3.44)	0.225
Age (years, mean, SD)	73.40 (4.72)	71.88 (4.45)	0.101
ApoE ε4 carrier (n, %)	25 (50%)	7 (14.6%)	0.003
MMSE (mean, SD)	28.60 (1.41)	28.82 (1.37)	0.431
CDR (mean, SD)	0 (0)	0 (0)	
P-TAU181 CSF PG/mL (mean, SD)	N=50 318.8 ± 180.6	N=50 220.2 ± 62.30	0.221
P-TAU181 Plasma PG/mL (mean, SD)	N=50 16.99 ± 6.84	N=50 15.46 ± 5.98	0.254
P-TAU231 CSF PG/mL (mean, SD)	N=50 419.46 ± 208.21	N=50 288.05 ± 81.12	0.110
P-TAU231 Plasma PG/mL (mean, SD)	N=50 14.20 ± 4.73	N=50 13.67 ± 5.05	0.897
P-TAU217 CSF PG/mL (mean, SD)	N=46 4.58 ± 3.42	N=50 1.70 ± 0.83	0.001

P values in bold font were considered significant (p<0.05). Independent T-Test and Pearson Chi-Square test were applied as appropriate.

Figure 1 Significant violin plot for CSF P-TAU217 in healthy elderly subjects (Abeta-, n=50) and subjects with preclinical AD (Abeta+, n=46).

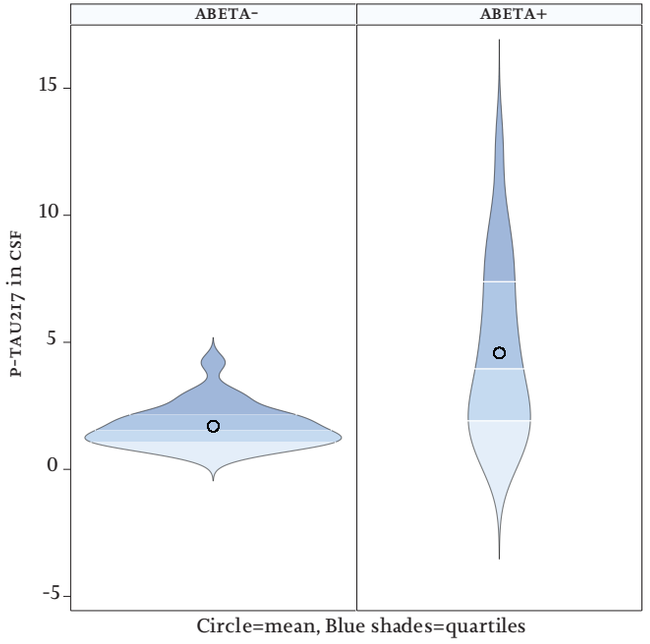


Figure 2 Boxplots of CSF P-TAU181 and CSF P-TAU231 in healthy elderly subjects (Abeta-, n=50) and subjects with preclinical AD (Abeta+, n=50).

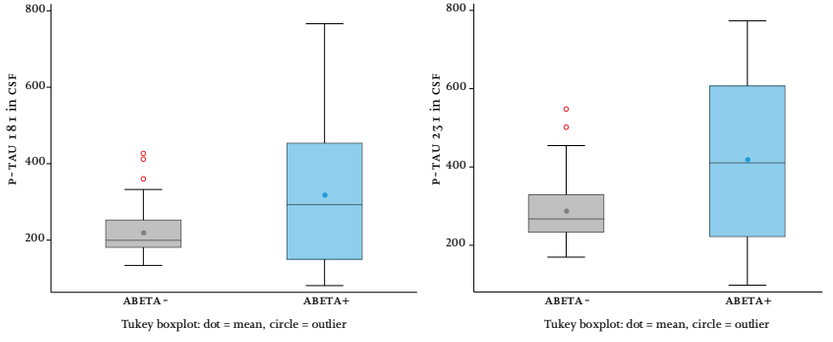


Table 2 Correlation for cognitive assessments and P-TAU concentrations in CSF and plasma calculated with Spearman r.

	P-TAU181 CSF	P-TAU181 plasma	P-TAU 231 CSF	P-TAU231 plasma	P-TAU 217 CSF
Saccadic inaccuracy (%)	r:-0.01 p=0.94 n=100	r:-0.08 p=0.43 n=100	r:-0.04 p=0.72 n=100	r:-0.12 p=0.25 n=100	r:0.01 p=0.91 n=96
Smooth pursuit (%)	r:-0.05 p=0.62 n=100	r:-0.03 p=0.81 n=100	r:-0.11 p=0.26 n=100	r:-0.01 p=0.93 n=100	r:-0.15 p=0.15 n=96
Tapping (taps/10s)	r:0.01 p=0.90 n=100	r:-0.04 p=0.68 n=100	r:-0.01 p=0.95 n=100	r:0.03 p=0.79 n=100	r:-0.04 p=0.74 n=96
Adaptive tracking (%)	r:0.06 P=0.56 n=100	r:-0.01 p=0.27 n=100	r:0.04 p=0.72 n=100	r:-0.07 p=0.47 n=100	r:0.02 p=0.83 n=96
VVLT delayed word recognition (# correct)	r:-0.08 p=0.43 n=99	r:-0.10 p=0.31 n=99	r:-0.08 p=0.46 n=99	r:-0.03 p=0.79 n=99	r:-0.14 p=0.17 n=95
VVLT delayed word recall (# correct)	r:-0.05 p=0.61 n=100	r:-0.10 p=0.30 n=100	r:-0.05 p=0.59 n=100	r:-0.11 p=0.29 n=100	r:-0.07 p=0.51 n=96
N-Back 2-back (correct)	r:-0.11 p=0.28 n=100	r:-0.05 p=0.63 n=100	r:-0.14 p=0.17 n=100	r:-0.11 p=0.28 n=100	r:-0.12 p=0.24 n=96
FACE (# correct)	r:-0.19 p=0.06 n=100	r:-0.07 p=0.48 n=100	r:-0.13 p=0.20 n=100	r:-0.04 p=0.68 n=100	R:-0.05 p=0.62 n=96

Table 3 Correlations for age (years) and MMSE (total) score per total group (n=100), per Aβ positive group (n-50) and Aβ positive group with above median P-TAU concentrations.

	Spearman r correlations total group	Spearman r correlations Aβ+ and P-TAU
Age (yrs)		
P-TAU181 CSF	r=0.21 p=0.04 n=100	r=0.22 p=0.13 n=50
P-TAU181 plasma	r=0.14 p=0.17 n=100	r=0.04 p=0.78 n=50
P-TAU231 CSF	r=0.24 p=0.02 n=100	r=0.21 p=0.14 n=50
P-TAU231 plasma	r=0.14 p=0.16 n=100	r=0.16 p=0.27 n=50
P-TAU217 CSF	r=0.22 p=0.03 n=96	r=0.14 p=0.35 n=46
MMSE (total)		
P-TAU181 CSF	r=-0.06 p=0.55 n=100	r=-0.05 p=0.71 n=50
P-TAU181 plasma	r=-0.02 p=0.81 n=100	r=0.11 p=0.47 n=50
P-TAU231 CSF	r=-0.08 p=0.42 n=100	r=-0.04 p=0.77 n=50
P-TAU231 PLASMA	r=0.03 p=0.77 n=100	r=-0.05 p=0.72 n=50
P-TAU217 CSF	r=-0.04 p=0.74 n=96	r=0.12 p=0.44 n=46

P values in bold font were considered significant (p<0.05).

Table 4 refers to the correlations between the biomarkers of the total group (n=100). CSF p-tau181 is strongly correlated with CSF p-tau 217 and p-tau231 (P=<0.0001) but also with plasma p-tau181 (p=0.0184) and p-tau231 (p=0.0189). CSF p-tau217 and p-tau231 are also strongly correlated (p=<0.0001). CSF p-tau217 correlates with plasma p-tau181 (P=0.0042) and p-tau231 (p=0.0358). CSF p-tau231 correlates with plasma p-tau181 (P=0.0054) and p-tau231 (p=0.0170). Plasma p-tau181 correlates strongly with plasma p-tau231 (p=<0.0001). None of the p-tau biomarkers correlates with $A\beta_{1-42}$.

Table 4 Correlation table with p values for correlations between CSF P-TAU181, P-TAU217, P-TAU231 and plasma P-TAU181, P-TAU231 and CSF $A\beta_{1-42}$.

	CSF P-TAU181	CSF P-TAU217	CSF P-TAU231	Plasma P-TAU181	Plasma P-TAU231	$A\beta_{1-42}$
CSF P-TAU181	-					
CSF P-TAU217	r:0.855 p=<0.0001	-				
CSF P-TAU231	r:0.959 p=<0.0001	r:0.899 p=<0.0001	-			
Plasma P-TAU181	r:0.235 p=0.0184	r:0.289 p=0.0042	r:0.276 p=0.0054	-		
Plasma P-TAU231	r:0.234 p=0.0189	r:0.215 p=0.0358	r:0.238 p=0.0170	r:0.603 p=<0.0001	-	
$A\beta_{1-42}$	r:-0.111 p=0.4425	r:-0.207 p=0.1675	r:-0.161 p=0.2638	r:-0.191 p=0.1832	r:-0.095 p=0.5128	-

P values in bold font were considered significant (p<0.05).

DISCUSSION

This exploratory study investigated P-TAU181, P-TAU217 and P-TAU231 isoforms in CSF and P-TAU181 and P-TAU231 in plasma in subjects with preclinical AD (Aβ+) and healthy elderly (Aβ-), to investigate whether phosphor-tau can differentiate healthy elderly from preclinical AD subjects. Cognitive performance was also studied in subjects with preclinical AD based on CSF Aβ in combination with higher levels of P-TAU isoforms. CSF P-TAU217 was significantly different between Aβ+ and Aβ- subjects. CSF P-TAU181 and CSF P-TAU231 were increased at higher age, there was no group difference between Aβ+ and Aβ-. Plasma P-TAU181 and P-TAU231 were not significantly different between Aβ+ and Aβ- subjects or any of the covariates. Cognitive performance did not differ in subjects with different P-TAU concentrations. A positive correlation was found between age and CSF P-TAU181, P-TAU231 and P-TAU217. All P-TAU isoforms in CSF and plasma show high correlations.

CSF phosphorylated tau and total tau together with CSF amyloid beta 42 represent the core biomarkers for AD. Research shows that even in the preclinical

stage of AD, with only slight Aβ pathology, changes in tau metabolism are already measurable.[12] When referring to P-TAU in literature, P-TAU at threonine-181 is usually meant in AD research as this isoform has been studied most and accurately distinguishes AD patients from mild cognitive impairment (MCI) and healthy subjects.[26] With new analyses methods making it possible to measure P-TAU in plasma, P-TAU181 has been studied extensively and results show that P-TAU181 in plasma has the ability to discriminate AD from other neurological diseases. Also, P-TAU181 starts to increase in preclinical AD with further increases in MCI and dementia stages.[27] In our study, P-TAU181 in CSF and plasma was not different between Aβ+ and Aβ+- subjects.

Difference in results may be due to different subject populations, where Karikari (2020).[16] included subjects from independent cohorts, this current study included 100 cognitively healthy subjects above the age of 65 with 50 subjects known to have lowered CSF $A\beta_{1-42}$ levels consistent with AD. The subjects described in the Karikari et al., study were different in an important fashion from the subjects described here. The discovery cohort which included AD patients and age-matched controls with minor neurological or psychiatric symptoms, the TRIAD and BIOFINDER-2 studies, which included cognitively healthy elderly subjects and patients with MCI, AD and frontotemporal dementia. TRIAD also included young adults (20-30 years old). No preclinical AD was determined in subjects in these trials, which could explain the differences in our data set as the analytical sensitivity of the assays may be insufficient for the detection of preclinical AD.[16]

CSF P-TAU217 has gained interest as recent studies showed this isoform to be better at detecting AD than P-TAU181.[5,28] This was also shown in subjects with preclinical AD where higher levels of P-TAU217 were observed compared to P-TAU181.[12] which is in agreement with our study. In a longitudinal study P-TAU217 was found to be able to monitor disease progression from cognitive unimpaired subjects to MCI to AD.[29] Suarez-Calvet et al., (2020) also investigated P-TAU231 in CSF finding this to be a very promising biomarker for preclinical AD as P-TAU231 was more prominently increased in preclinical AD than CSF P-TAU217 and CSF P-TAU181. This current study could not replicate these findings. The preclinical AD subjects investigated by Suarez-Calvet et al., were younger than our population, but were otherwise comparable with regards to MMSE score and ApoE ε4 disposition.

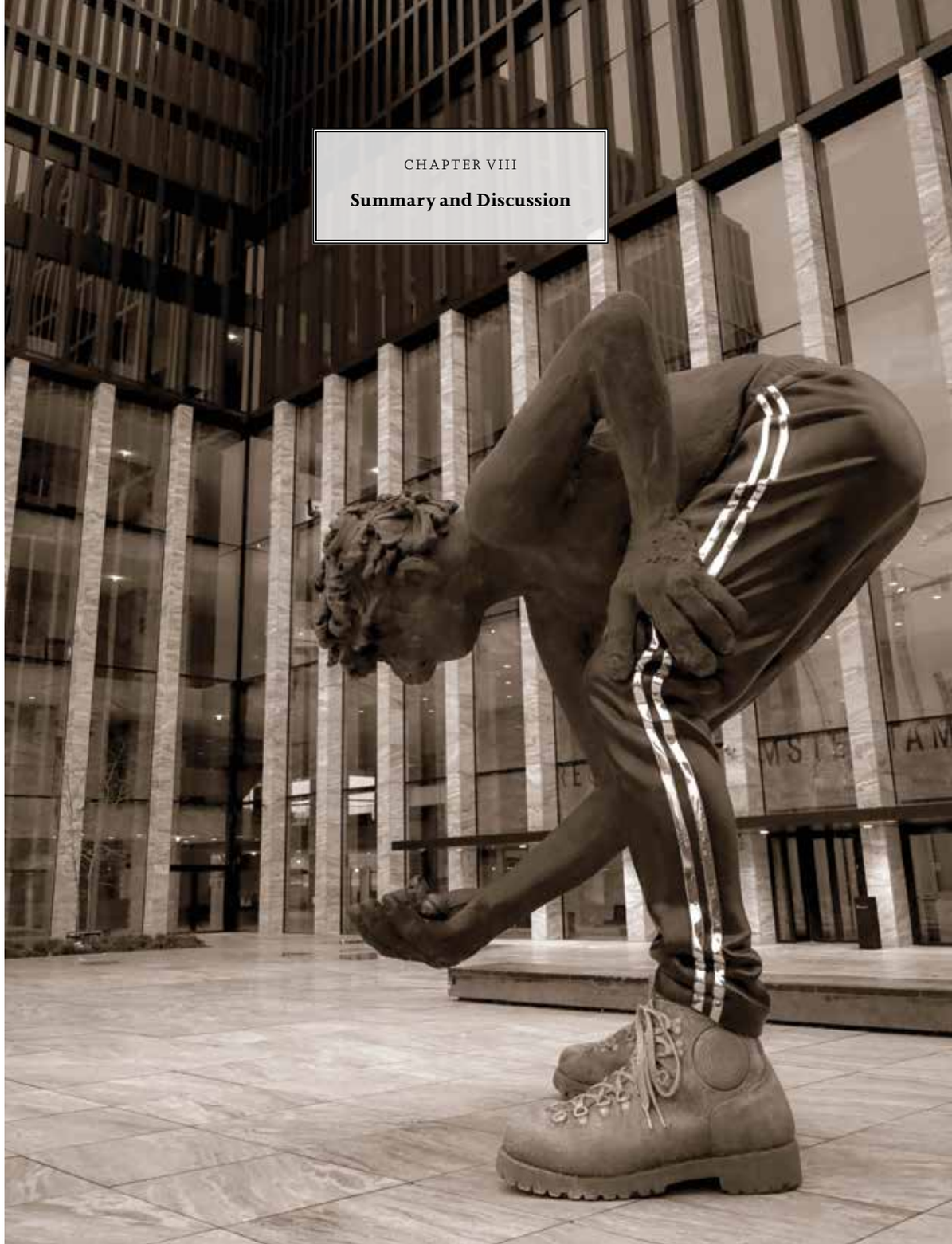
Plasma P-TAU181, P-TAU217 and P-TAU231 shows to already be increased in subjects with Aβ positive PET scans while tau-PET is still negative.[30–32] No PET was performed in the current study and based on CSF $A\beta_{1-42}$ alone, we could not replicate these findings. Having a soluble assay for the detection of tau, especially in plasma, is however far easier applicable and less costly in identifying otherwise healthy subjects with tauopathy. Apart from that, CSF P-TAU seems to precede detection with tau PET and measuring CSF or plasma P-TAU demonstrates to be indicative for early tau pathology closely related to Aβ.[32] This study measured CSF and plasma using Simoa HD-X using (in-house assays at the Department of Psychiatry and Neurochemistry, University of Gothenburg, Mölndal, Sweden as described by Karikari et al., (2020)[17]), which has been reported to be a robust analytical method.[33]

Subjects participating in this study were healthy elderly with no cognitive complaints. This was confirmed by a medical and cognitive prescreening before trial participation. This was again confirmed as data of the subjects was split into a group of subjects with CSF Aβ+ and Aβ- resulting in a group of subjects with preclinical AD. Data was further divided into subjects with Aβ+ and above median concentrations of P-TAU isoforms and cognitive performance still did not differ between these subjects, even in the preclinical stage of AD, even though lower concentration of Aβ and higher concentrations of P-TAU in CSF indicates that AD pathology is present to a greater extent. When comparing the results of some of the cognitive assessments performed in this study (Adaptive tracking, VVLT, N-Back test and saccadic and smooth eye movements) with previous literature, our population did perform below average compared to general healthy subjects. The NeuroCart scores however do not yet resemble scores of AD patients (Prins et al., 2022, submitted: Journal of the Neurological Sciences). The subjects with preclinical AD in this study might reflect a remarkably early stage of the preclinical phase in which not all P-TAU isoforms are yet increased. This studied population can therefore be referred to as cognitively healthy elderly who are likely to be enrolled in studies aimed at demonstrating disease modifying effects of a DMT in healthy elderly subjects.

Currently (2022), there are 13 DMTs in development aiming to reduce tauopathy in AD.[34] Mechanisms of action range from inhibition of tau aggregation to monoclonal antibodies promising to remove (extracellular) tau. As P-TAU seems to emerge in the preclinical phase of AD as a response to upcoming Aβ misfolding in the brain, this could be the earliest possible intervention window for treatment before neurofibrillary tangles arise. Measuring P-TAU in plasma can be used for the measurement of target engagement of these specific anti-tau DMT and early phase removal or lowering of P-TAU might lead to less subjects progressing from preclinical AD to AD. As this study does not confirm the discriminating power of P-TAU in preclinical AD, more (longitudinal) research is needed to provide more insight into the usefulness of plasma P-TAU biomarkers for distinction between preclinical AD and healthy subjects.

REFERENCES

1 Bloom, G.S., Amyloid-β and tau: the trigger and bullet in Alzheimer disease pathogenesis. JAMA Neurol, 2014. 71(4): p. 505-8.
2 Goedert, M., Tau filaments in neurodegenerative diseases. FEBS Lett, 2018. 592(14): p. 2383-2391.
3 Drummond, E., et al., Phosphorylated tau interactome in the human Alzheimer's disease brain. Brain, 2020. 143(9): p. 2803-2817.
4 Noble, W., et al., The importance of tau phosphorylation for neurodegenerative diseases. Front Neurol, 2013. 4: p. 83.
5 Barthelemy, N.R., et al., Cerebrospinal fluid phospho-tau T217 outperforms T181 as a biomarker for the differential diagnosis of Alzheimer's disease and PET amyloid-positive patient identification. Alzheimers Res Ther, 2020. 12(1): p. 26.
6 Lleó, A., et al., Phosphorylated tau181 in plasma as a potential biomarker for Alzheimer's disease in adults with Down syndrome. Nature Communications, 2021. 12(1): p. 4304.
7 Prvulovic, D. and H. Hampel, Amyloid β (Aβ) and phospho-tau (P-TAU) as diagnostic biomarkers in Alzheimer's disease. Clinical Chemistry and Laboratory Medicine, 2011. 49(3): p. 367-374.
8 Lehmann, S., et al., Cerebrospinal fluid A beta 1–40 peptides increase in Alzheimer's disease and are highly correlated with phospho-tau in control individuals. Alzheimer's Research & Therapy, 2020. 12(1): p. 123.
9 Barthelemy, N.R., et al., Blood plasma phosphorylated-tau isoforms track CNS change in Alzheimer's disease. J Exp Med, 2020. 217(11).
10 Jack, C.R., Jr., et al., Introduction to the recommendations from the National Institute on Aging-Alzheimer's Association workgroups on diagnostic guidelines for Alzheimer's disease. Alzheimers Dement, 2011. 7(3): p. 257-62.
11 Palmqvist, S., et al., Discriminative Accuracy of Plasma Phospho-tau217 for Alzheimer Disease vs Other Neurodegenerative Disorders. JAMA, 2020. 324(8): p. 772-781.
12 Suárez-Calvet, M., et al., Novel tau biomarkers phosphorylated at T181, T217 or T231 rise in the initial stages of the preclinical Alzheimer's continuum when only subtle changes in Aβ pathology are detected. EMBO Mol Med, 2020. 12(12): p. e12921.
13 Prins, S., et al., A cross-sectional study in healthy elderly subjects aimed at development of an algorithm to increase identification of Alzheimer pathology for the purpose of clinical trial participation. Alzheimer's Research & Therapy, 2021. 13(1): p. 132.
14 Willemse, E.A.J., et al., Comparing CSF amyloid-beta biomarker ratios for two automated immunoassays, Elecsys and Lumipulse, with amyloid PET status. Alzheimers Dement (Amst), 2021. 13(1): p. e12182.
15 Lewczuk, P., et al., Biomarkers of Alzheimer's disease and mild cognitive impairment: a current perspective. Adv Med Sci, 2015. 60(1): p. 76-82.
16 Karikari, T.K., et al., Blood phosphorylated tau 181 as a biomarker for Alzheimer's disease: a diagnostic performance and prediction modelling study using data from four prospective cohorts. Lancet Neurol, 2020. 19(5): p. 422-433.
17 Karikari, T.K., et al., Head-to-head comparison of clinical performance of CSF phospho-tau T181 and T217 biomarkers for Alzheimer's disease diagnosis. Alzheimers Dement, 2021. 17(5): p. 755-767.
18 Groeneveld, G.J., J.L. Hay, and J.M. Van Gerven, Measuring blood-brain barrier penetration using the NeuroCart, a CNS test battery. Drug Discov Today Technol, 2016. 20: p. 27-34.
19 Borland, R.G. and A.N. Nicholson, Visual motor co-ordination and dynamic visual acuity. Br J Clin Pharmacol, 1984. 18 Suppl 1(Suppl 1): p. 69s-72s.
20 Lezak, M.D., et al., Neuropsychological assessment, 5th ed. Neuropsychological assessment, 5th ed. 2012, New York, NY, US: Oxford University Press. xxv, 1161-xxv, 1161.
21 de Haas, S.L., et al., The pharmacokinetic and pharmacodynamic effects of SL65.1498, a GABA-A alpha2,3 selective agonist, in comparison with lorazepam in healthy volunteers. J Psychopharmacol, 2009. 23(6): p. 625-32.
22 Rombouts, S.A.R.B., et al., Alterations in brain activation during cholinergic enhancement with rivastigmine in Alzheimer's disease. Journal of Neurology Neurosurgery and Psychiatry, 2002. 73(6): p. 665-671.
23 Andrew, J.M., Delinquents and the Tapping Test. J Clin Psychol, 1977. 33(3): p. 786-91.
24 Molitor, R.J., P.C. Ko, and B.A. Ally, Eye movements in Alzheimer's disease. J Alzheimers Dis, 2015. 44(1): p. 1-12.
25 Morris, J.C., The Clinical Dementia Rating (CDR): current version and scoring rules. Neurology, 1993. 43(11): p. 2412-4.
26 Zetterberg, H. and K. Blennow, Moving fluid biomarkers for Alzheimer's disease from research tools to routine clinical diagnostics. Molecular Neurodegeneration, 2021. 16(1): p. 10.
27 Janelidze, S., et al., Plasma P-TAU181 in Alzheimer's disease: relationship to other biomarkers, differential diagnosis, neuropathology and longitudinal progression to Alzheimer's dementia. Nat Med, 2020. 26(3): p. 379-386.
28 Janelidze, S., et al., Cerebrospinal fluid P-TAU217 performs better than P-TAU181 as a biomarker of Alzheimer's disease. Nat Commun, 2020. 11(1): p. 1683.
29 Mattsson-Carlgren, N., et al., Longitudinal plasma P-TAU217 is increased in early stages of Alzheimer's disease. Brain, 2020. 143(11): p. 3234-3241.
30 Karikari, T.K., et al., Blood phosphorylated tau 181 as a biomarker for Alzheimer's disease: a diagnostic performance and prediction modelling study using data from four prospective cohorts. The Lancet Neurology, 2020. 19(5): p. 422-433.
31 Janelidze, S., et al., Associations of Plasma Phospho-Tau217 Levels With Tau Positron Emission Tomography in Early Alzheimer Disease. JAMA Neurology, 2021. 78(2): p. 149-156.
32 Ashton, N.J., et al., Plasma P-TAU231: a new biomarker for incipient Alzheimer's disease pathology. Acta Neuropathologica, 2021. 141(5): p. 709-724.
33 Bayoumy, S., et al., Clinical and analytical comparison of six Simoa assays for plasma P-TAU isoforms P-TAU181, P-TAU217, and P-TAU231. Alzheimer's Research & Therapy, 2021. 13(1): p. 198.
34 Cummings, J., et al., Alzheimer's disease drug development pipeline: 2022. Alzheimers Dement (N Y), 2022. 8(1): p. e12295.



CHAPTER VIII

Summary and Discussion

SUMMARY

Over a 100 years after Alois Alzheimer discovered amyloid plaques surrounding brain cells and neurofibrillary tangles inside the cells of a deceased patient naming it Alzheimer's Disease (AD),[1] we still have not been able to solve the mystery of this disease.

As mentioned in **Chapter I**, the growing elderly population worldwide creates a great burden on the health care systems. The WHO estimates 1 in 6 people in the world to be over the age of 60 by 2030.[2] As more people generally have access to (better) health care throughout life, people get older. With increasing age, the chance to develop a form of dementia also increases. In 2020, the prevalence of dementia was approximately 50 million people worldwide. The most common form of dementia is Alzheimer's disease which accounts for approximately 70% of the dementia cases.[3] Biomarker research has yielded many new insights in AD over the past decades. Biomarker evidence of AD pathology has shown to be measurable up to 20 years before clinical symptoms appear.[4] Apart from measuring amyloid beta and tau in brains of deceased patients, these proteins can now also be measured using cerebrospinal fluid (CSF), positron emission tomography (PET) using tracers and even in blood. New biomarkers associated with AD have also been identified related to inflammatory processes in the brain, astroglial activation and neuronal damage. The large numbers of patients emphasize the need for a disease modifying treatment. Clinical trials have been improved making use of randomized (placebo) controlled trials reducing bias in trial results. At present time (2022), 119 disease modifying compounds are in development for the treatment of AD.[5] Currently, only symptomatic treatment is available for AD patients. In June 2021, the first DMT for the treatment of AD was approved by the FDA in the United States of America.[6] Aducanumab promises to remove amyloid plaques from the brain that have accumulated due to AD disease progression. Inconclusive results from the preceding clinical trials led to this acceptance and therefore the EMA did not approve the drug in the European Union. The label of aducanumab has been adjusted since approval by the FDA. Initially the FDA approved aducanumab for all patients with AD, but they now adjusted the approval by restricting the label to patients with mild cognitive impairment or mild AD, in whom the drug was also tested in the phase 3 clinical trials. This stresses that subject selection is of great importance in AD research. Performing clinical trials in early phase of AD or even preclinical AD might prevent further disease progression as there is less disease pathology in the brain. When a healthy subject with no cognitive complaints has a lowered CSF protein Aβ42 level, comparable with AD, this subject is considered to have preclinical AD according to the NIA-AA standards from 2011.[7] This shift in subject selection is noticeable in current clinical trials with 14 DMT trials including subjects with preclinical AD.[5]

Cognitive performance is important to take into account when looking at the clinical manifestation of AD. **Chapter II** described age related decline in cognitive performance measured by the NeuroCart. The NeuroCart is a neuropsychological and neurophysiological test battery that is used to detect pharmacodynamic effects of drugs in the context of (early phase) drug development.[8] Over the years it has been used in hundreds of studies in healthy subjects and patient populations. This retrospective study encompassed 93 studies, performed at CHDR between 2005 and 2020 that included NeuroCart measurements, which resulted in 2729 subjects with data from at least one of five NeuroCart measurements. The five NeuroCart tests included in the study were: Eye Movements - Smooth and Saccadic Eye Movements, Body movement- Body sway, Attention and Eye-Hand Coordination- Adaptive Tracking, Memory Consolidation-Visual Verbal Learning Task, Delayed Recognition, Working Memory-N-Back. Results show that the NeuroCart can detect age-related decreases in performance in healthy subjects, which were not affected by sex. The NeuroCart was able to detect significant differences in performance between healthy volunteers and patients with AD, Parkinson's Disease, Huntington's Disease and Vascular dementia at the mean age of the disease group. Because disease durations were unknown, this cross-sectional study was not able to show age-related decline after disease onset. Therefore, the speed of deterioration as a consequence of neurodegenerative disease could not be quantified reliably. The healthy elderly participating in this study, declined in performance on all NeuroCart measurements on a yearly basis. After clinical onset of the studied neurogenerative diseases, this decline increases significantly.

In **Chapter III** a broad overview of biomarkers found in human AD and a comparison to biomarkers in animal studies is described. The number of currently existing and emerging pathophysiological hypotheses, mechanisms, theories, and processes related to AD is high and is still increasing. Currently, we lack sufficient information and understanding of processes in the onset and early stage of the disease. This contributes to the fact that we cannot yet diagnose or initiate treatment in the earliest phase of AD. This highlights the need to find adequate, preferably body-fluid-based biomarkers of AD. Currently, the biomarkers that are mostly measured in human studies are Aβ, P-TAU, T-TAU, neurogranin, SNAP-25, GFAP, YKL-40, and NFL.[9] Additionally, there is a high volume of animal research, in which the emphasis has mostly been on Aβ. Animal studies can be

smartly designed to provide mechanistic information on the interrelationships between the different AD processes in a longitudinal fashion and may also include the combinations of different conditions that may reflect comorbidities in human AD, according to the Mastermind Research approach.[10] The Mastermind Research approach is for strategic and systematic CNS drug research using advanced preclinical experimental designs and mathematical modeling and is able to model data extracted from animal research to predict CNS drug distribution in humans without the need of animal experiments.

Chapter IV combined plasma-based biomarkers for AD with cognitive biomarkers measured with the NeuroCart to predict CSF amyloid beta status of healthy elderly. The study aimed to develop an algorithm based on less-invasive (plasma) biomarkers for AD pathology, to be used for pre-selection of subjects who are suspected of lowered, abnormal, CSF Aβ levels ('Aβ positive subjects') consistent with the presence of AD pathology. The algorithm that resulted from the study includes sex, 7 cognitive tests measured with the NeuroCart (MMT, VVLT, finger tapping, N-Back, SART, Face and EEG) and one plasma biomarker (YKL-40) and was successful in predicting CSF Aβ+ in healthy elderly with a sensitivity of 70.82% and specificity of 89.25%. When using this algorithm, 70% fewer lumbar punctures will have to be performed to enroll subjects based on lowered Aβ CSF. The overall subject burden and costs of trials will reduce as fewer lumbar punctures will need to be performed. This may also increase subject's willingness to participate in drug studies.[11]

Verberk et al.[12] showed that plasma Aβ42/Aβ40 ratio has the potential to identify Alzheimer pathological changes in subjects with subjective memory decline. Further, the inclusion of age and ApoEε4 carriership in their multivariate model improved the likelihood of identification. Based on these results, Verberk and colleagues postulated that plasma Aβ42/Aβ40 ratio could be a potential prescreener to identify the earliest AD pathological changes in individuals with subjective memory decline. Using plasma-based biomarkers in identifying and characterizing the preclinical AD state is a breakthrough in clinical research as taking a blood sample is less invasive than taking a CSF sample which decreases the burden for healthy subjects and patients. However, results are still preliminary and should be reviewed with caution. Results could, however, not be reproduced in a (slightly) different subject group as discussed in **Chapter V** of this dissertation. We aimed to extend the findings of Verberk et al, using the same statistical methods, but in a different population, namely healthy elderly subjects without memory complaints (n=189). The sensitivity and specificity of the plasma Aβ42/Aβ40 ratio in our study were 30.8% and 71% respectively, compared to 76% and 75% in Verberk et al. The results of our logistic regression and receiver operating characteristic (ROC) analyses showed that the plasma Aβ42/Aβ40 ratio did not significantly affect ROC curves discriminating between cerebrospinal fluid (CSF) amyloid abnormal and amyloid normal individuals, in a multivariate model including age and ApoEε4 carriership. Not cross validating a model can lead to overfitting of the sampled data. Also, different populations were used in comparing the results. Stating that plasma amyloid is a prescreener for the earliest signs of AD pathology is, in our opinion, a premature statement.[13]

What Alois Alzheimer did not know in 1906, but what we have learned since then is that AD is not simply caused by amyloid plaques and neurofibrillary tangles. As discussed in **Chapter VI**, inflammation also plays a major role. This exploratory study investigated plasma biomarkers related to neuroinflammation associated with AD in a cohort of subjects with preclinical AD, and compared them to healthy elderly, defined by $A\beta_{1-42}$ CSF status. Four inflammatory plasma biomarkers were investigated. YKL-40 (also known as chitinase-3-like protein-1 [CHI3L1]) is a glycoprotein, which is mainly expressed in astrocytes. Patients with AD have significantly higher YKL-40 levels in the CSF compared to healthy controls however it is not a specific biomarker for AD, because it merely reflects the inflammatory progress.[14] Glial fibrillary acidic protein (GFAP) is a marker for astrogliosis and was reported to be increased postmortem in brains of patients with AD and in CSF of patients with AD.[15] Two chemokines (monocyte chemoattractant protein-1[MCP-1] and eotaxin-1) have previously been reported to be correlated with greater memory impairment in MCI and AD.[16] Of the four inflammatory plasma biomarkers investigated in the study, only GFAP was significantly higher in subjects with preclinical AD compared to healthy elderly. When post hoc defining preclinical AD based on the PTAU181/$A\beta_{1-42}$ ratio, GFAP and YKL-40 were significantly different between groups. This could indicate that GFAP and YKL-40 are more sensitive markers of the incipient inflammatory process that occurs in response to the beta amyloid misfolding and aggregation that is ongoing as indicated by the lowered $A\beta_{1-42}$ protein levels in the CSF.[17]

The neurofibrillary tangles discovered by Alois Alzheimer have been studied profoundly in the past decades. **Chapter VII** described specific isotopes of tau, namely phosphorylated types and comparing results found in CSF to plasma. The study investigated P-TAU at threonine 181,217 and 231 in CSF and P-TAU181 and P-TAU231 in plasma in subjects with preclinical AD and healthy elderly defined by $A\beta_{1-42}$ CSF status, to investigate whether phosphor-tau CSF and plasma biomarkers offer a good alternative to distinct healthy elderly from preclinical AD subjects. CSF PTAU217 was significantly higher in subjects with preclinical

AD compared to healthy elderly. CSF PTAU181 and CSF PTAU231 were increased at higher age but there was no group difference between the two studied groups. All PTAU isoforms in CSF and plasma show high correlations. As PTAU seems to emerge in the preclinical phase of AD as a response to upcoming Aβ misfolding in the brain, this could be the earliest possible intervention window for treatment before neurofibrillary tangles arise. Measuring PTAU in plasma can be used for the measurement of target engagement of specific anti-tau DMT and early phase removal or lowering of ptau might lead to less subjects progressing from preclinical AD to AD. As this study does not confirm the discriminating power of PTAU in preclinical AD, more (longitudinal) research is needed to provide more insight into the usefulness of plasma PTAU biomarkers for distinction between preclinical AD and healthy subjects.

FUTURE PERSPECTIVE OF THE USE OF BIOMARKERS IN HEALTHY SUBJECTS IN THE PRECLINICAL PHASE OF ALZHEIMER'S DISEASE

In this dissertation the focus has been on preclinical AD. How we define a subject to be in the preclinical phase of AD had been a topic of discussion in the past decade. Subjects with preclinical AD included in the studies mentioned in this thesis were characterized based on the NIA-AA standards from 2011, which state that if an otherwise healthy subject without cognitive complaints has evidence of Aβ pathology in CSF, this subject is classified as being in the preclinical phase of AD.[18] Having Aβ pathology is not a guarantee that a subject will actually develop AD later in life although the odds are greatly increased. Current research states that approximately 40-60% of subjects with subjective cognitive complaints will develop AD from the preclinical phase.[19,20] New suggestions about the definition of preclinical AD have been proposed, including the use of PET to determine amyloidosis in the brain and measuring tau pathology in CSF (2014). The most recent recommendation about the classification of preclinical AD is evidence of both Aβ and tau pathology measured by either PET and/or CSF.[7] This standard is, however, still only applied in some research facilities and are not part of standard clinical care. Also, including both PET and CSF for the classification of an otherwise healthy individual is costly and invasive, which influences the willingness of a subject to undergo these procedures but also the availability of these diagnostic tools is far from common. Aβ measured with PET is concordant with measurements in CSF, which makes performing both assessments unnecessary.[21]

The development of blood-based biomarkers in the detection of (early) AD is very promising and might improve the ever-challenging field of AD research as it is a less invasive procedure. When biomarkers that are well established in CSF, such as Aβ and specific tau isotopes, can be validated properly in blood or plasma samples, this would make early diagnosis more accessible, less invasive, and far less costly. Unfortunately, we are not there yet. New high-sensitive blood-based assays have emerged with promising results on consistency between different cohorts and agreement when comparing these results with CSF and PET.[22–24] More (long term) research is needed to determine the validity of these blood-based biomarkers before these can be implemented as standard (research) practice.

And what about the cognitive aspect of preclinical AD? As described in this thesis, combining a blood-based YKL-40 test with cognitive tests using the NeuroCart can predict CSF Aβ outcome. These findings are especially useful for clinical research as, per definition, subjects in the preclinical phase of AD do not have cognitive complaints and overall do not perform worse on cognitive tests. Asking trial subjects to perform cognitive tests and a blood draw may increase willingness to participate in clinical trials and may lower costs of clinical (due to fewer PET and/or CSF measurements).

Taking the information from this thesis into account, questions arise what the perfect biomarker combination would be in a clinical trial and which trial subjects should be enrolled to improve clinical trials in preclinical AD. Based on the research performed in this thesis and recent literature, the suggested biomarkers to incorporate in a clinical trial would be a combination of CSF, blood-based- and cognitive biomarkers. Preselecting healthy subjects in an age range with higher prevalence of AD pathology results in including subjects from the age of 65 years old as approximately 20% will have Aβ pathology measured in CSF.[25] Submitting these subjects to a variety of cognitive tests (e.g., memory consolidation, verbal learning, sustained attention, motor movement and EEG) and blood-based biomarkers (GFAP, YKL-40 and ptau217) will increase the chance of finding subjects with preclinical AD likely to develop AD in the future. Taking the cognitive- and biomarker results into account, a selection of these subjects would be asked to undergo CSF sampling or a PET scan to confirm preclinical AD status based on $A\beta_{1-42}$ and ptau217. Improving the selection criteria for clinical trials to be performed in preclinical AD can be expected to lead to a less heterogenic patient population, lower primary outcome variability and greater effect size of the intervention and thereby a better powered RCT with a larger chance of a positive outcome.

It is important that subjects with preclinical AD are well characterized. Evidence of AD pathology needs to be established in order to enroll these subjects in clinical trials with DMTs aimed at prevention of progression of developing AD pathology. Recruiting patients with Alzheimer's disease in clinical trials can be a challenge due to various reasons, e.g., study burden, cognitive burden,

progression of disease and study compliance. Focusing more on enrolling subjects with preclinical AD will save time and money as trials will be completed at a faster pace due to higher compliance and lower burden for healthy subjects compared to patients with cognitive decline. On the other hand, trials may have to last longer before change on a biomarker level can be observed.[26] Finding the optimal therapeutic window for DMTs in AD will have to include subjects with preclinical AD to find the earliest window for modification. Currently ongoing longitudinal studies aimed at elucidating biomarker evolution over time will shed more light on the feasibility of inclusion of subjects with preclinical AD. Examples of these large trials are the European Prevention of Alzheimer's Disease Consortium (EPAD) and PResymptomatic EValuation of Experimental or Novel Treatments for AD (PREVENT-AD), which collect (biomarker) data of healthy elderly over several years in CSF and blood but also PET imaging when available, genetics and cognitive information.[27,28]

FUTURE CONSIDERATIONS FOR CLINICAL TRIALS IN ALZHEIMER'S DISEASE WITH DISEASE MODIFYING TREATMENTS

After selecting the ideal trial subject characterized to be in the preclinical AD phase, what would be the best design for a clinical trial with a disease modifying compound? As mentioned by Hariton and Locascio (2018), the gold standard for effectiveness research is the randomized controlled trial (RCT) design.[29] First step is to carefully select the studied population, as mentioned above. Also, the interventions that will be compared and the outcomes of interest should be determined prior to the start of the trial. A power calculation to predefine the number of subjects needed to obtain reliable results should be done beforehand. Trials should be registered to avoid selective reporting of trial outcomes. When subjects are recruited, preferably a computerized system randomizes the subjects into different trial arms to prevent selection bias. Using double-blinded conditions, meaning the trial subjects, physicians and researchers do not know which subject is in which treatment arm, further minimalizes bias. Results should be based on intention-to-treat analyses opposed to only including subjects who have completed treatment in the analyses. A problem with RCTs can be that subjects do not represent the patients for whom the results of the trial will be used in the future. Maximizing the treatment response by selecting a more limited homogeneous study population helps with demonstrating treatment effect but becomes less representative for the patient population. Including biomarker data and including subjects in the preclinical phase of a disease should minimize this generalization issue. RCT are usually more costly as more conditions are added to a trial resulting in more data, however, this should be compared to performing trials in a less optimized way resulting in having to perform more trials with debatable outcomes which will cost more in the end. Reproducibility of a trial is important. As shown in **chapter V** of this dissertation one single study does not represent certainty and multiple comparable studies should be performed before any definite conclusions can be drawn. Accordingly, RCTs in subjects with preclinical AD, phase 3 studies in patients with AD should follow the same guidelines. Patients with AD should be well characterized on biomarker level to include patients with similar pathology as to what the DMT is targeting.

ETHICAL CONSIDERATIONS IN PRECLINICAL ALZHEIMER'S RESEARCH

This dissertation focused on research in healthy elderly and subjects in the preclinical phase of AD, in whom no cognitive symptoms are (yet) measurable but in the presence of biomarkers (in this case CSF Aβ42) that are consistent with AD pathology. Ethical considerations should be taken into account when performing research in otherwise healthy elderly subjects. Since 2018, the General Data Protection Regulations (GDPR) are in place protecting all personal data of EU citizens. At the time of data collection for the studies mentioned in this thesis (**chapter IV, V, VI and VII**) the GDPR was not yet fully applicable and therefore informed consent forms were less specific about handling of personal information and the possibility of requesting personal results collected during study participation. Currently, clinical trial participants are more aware of the (personal) data collected during trial participation and requests for detailed information can be more common.

In The Netherlands, a license is needed when performing research that involves screening the population on severe diseases or abnormalities for which no treatment or prevention is available (Wet op bevolkingsonderzoek [WBO]).[30] Most biomarker research in preclinical AD related to trial participation is of course not population-based research but does investigate severe diseases or abnormalities with no treatment or prevention. Question is if large population-based studies, which would provide us with valuable information about the development of AD, would even be allowed by the Dutch government. Current so called secondary prevention trials that screen large groups of healthy elderly for presence of AD related biomarkers and genetic information in order to select subjects for trials have been approved by ethical committees, also in the Netherlands.[27,28] These trials are not by definition population-based trials as not all people above a certain age are invited but do aim to include a large number of otherwise healthy elderly.

As an example, EPAD registered over half a million people across Europe. Also, sharing information based on biomarker data indicating the possible presence of an untreatable disease to an otherwise healthy elderly will have great consequences. No disease modifying treatment is yet available (in The Netherlands) for AD and the presence of biomarkers consistent with AD is not 100% predictive of developing AD later on in life. Also, biomarkers consistent with AD can be present up to 20 years before actual disease onset, so actively diagnosing a preclinical state may lead to a long period of unnecessary worry. Enrolling subjects with preclinical AD means screening healthy subjects looking for specific AD pathology, which leads to many subjects that will have to be screened which is both time consuming and costly. Also, the treatment period for subjects with preclinical AD might have to be longer as the effect of treatment will take more time with less profound pathological damage.[26] Exposing preclinical subjects to treatment for a longer period of time must be safe and benefits of the trial results must justify the burden.

The question if the preclinical biomarker results should be shared with otherwise healthy trials subject with no cognitive complaints remains unanswered. Research shows that there might be benefits to an early diagnosis. Subjects implemented specific health behavioral changes to everyday life when learning about being an ApoEE4 carrier, according to Chao et al., (2008),[31] even when knowing that these lifestyle changes were not proven to prevent AD. Disclosing genetic information could affect trial outcome as shown by Lineweaver et al., (2014) who concluded that subjects who were familiar with their genetic disposition for AD performed worse on cognitive tests.[32] Input from the patient community and better understanding the concept of biomarkers by the general population might help researchers to understand what degree of risk is found to be acceptable in clinical trials. Knowing ones' AD biomarker status also influences the willingness to participate in clinical trials due to altruistic reasons but also to reduce personal risk of developing AD.[33] As many biomarkers in AD research are not specific for AD, extra caution is needed for the possibility of misdiagnosing subjects. The diagnostic accuracy of CSF biomarkers for AD in the MCI stage is high, with sensitivity and specificity up to 85%-90%.[34,35] These are high accuracy numbers, but still lead to many misdiagnosed subjects.[35,36] For example, even with a specificity of 90%, assuming a prevalence of preclinical AD of approximately 20% among healthy elderly above the age of 65 years, the positive predictive value of a positive test (as in a CSF profile consistent with AD) will be as low as 50%, leading to a large number of misdiagnosed subjects.

Being diagnosed with a disease that influences cognitive performance can be of great influence on certain legal rights. Caution about the consequences of having a preclinical 'diagnosis' on these rights should be taken into account. Subjects with AD can lose the right to hold a driver's license and, in the USA, lose the right to hold a gun (which might not be such a bad thing). As for legal arrangements, early diagnoses do force subjects to think about their future and for instance draw op their wills before reaching the incapacitated phase. If knowledge about biomarker status becomes common, this could also influence the health care system and in particularly could influence health care insurance policies.

Disclosing results of biomarker and genetic testing is a complex task and should only be done by trained specialists. The decision to learn about one's biomarker or genetic status should be made by the trial subject him- or herself. However, because of the importance of finding a cure for AD research related to biomarkers and genetics in the field of AD should continue. In our opinion, specific trial data (biomarker and genetic results) should only be disclosed at an individual's explicit request, after thorough (psychological) education about the possible consequences. Future research should take the ethical considerations into account, especially with longitudinal studies characterizing otherwise healthy human beings and study how biomarker disclosure impacts an otherwise healthy subject. Once DMTs are available for the preclinical stage, the ethical considerations will change drastically and will need to be reevaluated. At this point, clinical research in subjects with preclinical AD including biomarker information has a solid scientific basis and needs to be able to move forward in order to ultimately find a cure for AD.

REFERENCES

1 Alzheimer, A., Uber eine eigenartige Erkrankung der Hirnrinde. 1907(64): p. 146-8.
2 WHO. https://www.who.int/news-room/fact-sheets/detail/dementia. https://www.who.int/news-room/fact-sheets/detail/dementia 2021 02 September 2021 [cited 2021 07Oct2021].
3 2021 Alzheimer's disease facts and figures. Alzheimers Dement, 2021. 17(3): p. 327-406.
4 Jack, C.R., et al., Hypothetical model of dynamic biomarkers of the Alzheimer's pathological cascade. The Lancet Neurology, 2010. 9(1): p. 119-128.
5 Cummings, J., et al., Alzheimer's disease drug development pipeline: 2022. Alzheimers Dement (NY), 2022. 8(1): p. e12295.
6 FDA. https://www.fda.gov/news-events/press-announcements/fda-grants-accelerated-approval-alzheimers-drug. https://www.fda.gov/news-events/press-announcements/fda-grants-accelerated-approval-alzheimers-drug 2021 content current as of 06Jul2021 [cited 2021 23Nov2021].
7 Dubois, B., et al., Preclinical Alzheimer's disease: Definition, natural history, and diagnostic criteria. Alzheimer's & dementia: the journal of the Alzheimer's Association, 2016. 12(3): p. 292-323.
8 Groeneveld, G.J., J.L. Hay, and J.M. Van Gerven, Measuring blood-brain barrier penetration using the NeuroCart, a CNS test battery. Drug Discov Today Technol, 2016. 20: p. 27-34.
9 Qin, T., et al., Utility of Animal Models to Understand Human Alzheimer's Disease, Using the Mastermind Research Approach to Avoid Unnecessary Further Sacrifices of Animals. Int J Mol Sci, 2020. 21(9).
10 de Lange, E.C., The mastermind approach to CNS drug therapy: translational prediction of human brain distribution, target site kinetics, and therapeutic effects. Fluids Barriers CNS, 2013. 10(1): p. 12.
11 Prins, S., et al., A cross-sectional study in healthy elderly subjects aimed at development of an algorithm to increase identification of Alzheimer pathology for the purpose of clinical trial participation. Alzheimers Res Ther, 2021. 13(1): p. 132.
12 Verberk, I.M.W., et al., Plasma Amyloid as Prescreener for the Earliest Alzheimer Pathological Changes. Ann Neurol, 2018. 84(5): p. 648-658.
13 Prins, S., A. Zhuparris, and G.J. Groeneveld, Usefulness of Plasma Amyloid as a Prescreener for the Earliest Alzheimer Pathological Changes Depends on the Study Population. Ann Neurol, 2020. 87(1): p. 154-155.
14 Haas, D., Chapter 15 - Biomarker for Alzheimer's Disease, in Precision Medicine, H.-P. Deigner and M. Kohl, Editors. 2018, Academic Press. p. 333-349.
15 Jesse, S., et al., Glial fibrillary acidic protein and protein S-100B: different concentration pattern of glial proteins in cerebrospinal fluid of patients with Alzheimer's disease and Creutzfeldt-Jakob disease. J Alzheimers Dis, 2009. 17(3): p. 541-51.
16 Bettcher, B.M., et al., MCP-1 and eotaxin-1 selectively and negatively associate with memory in MCI and Alzheimer's disease dementia phenotypes. Alzheimers Dement (Amst), 2016. 3: p. 91-7.
17 Prins, S., et al., Inflammatory plasma biomarkers in subjects with preclinical Alzheimer's disease. Alzheimer's Research & Therapy, 2022. 14(1): p. 106.
18 Jack, C.R., Jr., et al., Introduction to the recommendations from the National Institute on Aging-Alzheimer's Association workgroups on diagnostic guidelines for Alzheimer's disease. Alzheimers Dement, 2011. 7(3): p. 257-62.
19 DeCarli, C., Mild cognitive impairment: prevalence, prognosis, aetiology, and treatment. Lancet Neurol, 2003. 2(1): p. 15-21.
20 Hansson, O., et al., Prediction of Alzheimer's Disease Using the CSF Aβ42/Aβ40 Ratio in Patients with Mild Cognitive Impairment. Dementia and Geriatric Cognitive Disorders, 2007. 23(5): p. 316-320.
21 Spallazzi, M., et al., CSF biomarkers and amyloid PET: concordance and diagnostic accuracy in a MCI cohort. Acta Neurol Belg, 2019. 119(3): p. 445-452.
22 Teunissen, C.E., et al., Blood-based biomarkers for Alzheimer's disease: towards clinical implementation. The Lancet Neurology, 2022. 21(1): p. 66-77.
23 Zetterberg, H., Blood-based biomarkers for Alzheimer's disease – An update. Journal of Neuroscience Methods, 2019. 319: p. 2-6.
24 Chong, J.R., et al., Blood-based high sensitivity measurements of beta-amyloid and phosphorylated tau as biomarkers of Alzheimer's disease: a focused review on recent advances. J Neurol Neurosurg Psychiatry, 2021. 92(11): p. 1231-1241.
25 Jansen, W.J., et al., Prevalence of Cerebral Amyloid Pathology in Persons Without Dementia A Meta-analysis. Jama-Journal of the American Medical Association, 2015. 313(19): p. 1924-1938.
26 Molinuevo, J.L., et al., Ethical challenges in preclinical Alzheimer's disease observational studies and trials: Results of the Barcelona summit. Alzheimers Dement, 2016. 12(5): p. 614-22.
27 Tremblay-Mercier, J., et al., Open science datasets from PREVENT-AD, a longitudinal cohort of pre-symptomatic Alzheimer's disease. NeuroImage: Clinical, 2021. 31: p. 102733.
28 Ritchie, C.W., et al., Development of interventions for the secondary prevention of Alzheimer's dementia: the European Prevention of Alzheimer's Dementia (EPAD) project. Lancet Psychiatry, 2016. 3(2): p. 179-86.
29 Hariton, E. and J.J. Locascio, Randomised controlled trials - the gold standard for effectiveness research: Study design: randomised controlled trials. BJOG: an international journal of obstetrics and gynaecology, 2018. 125(13): p. 1716-1716.
30 CCMO. https://www.ccmo.nl/onderzoekers/wet-en-regelgeving-voor-medisch-wetenschappelijk-onderzoek/wetten/wet-op-het-bevolkingsonderzoek-wbo. https://www.ccmo.nl/onderzoekers/wet-en-regelgeving-voor-medisch-wetenschappelijk-onderzoek/wetten/wet-op-het-bevolkingsonderzoek-wbo 2022 [cited 2022 17Aug2022].
31 Chao, S., et al., Health behavior changes after genetic risk assessment for Alzheimer disease: The REVEAL Study. Alzheimer Dis Assoc Disord, 2008. 22(1): p. 94-7.
32 Lineweaver, T.T., et al., Effect of knowledge of ApoE genotype on subjective and objective memory performance in healthy older adults. Am J Psychiatry, 2014. 171(2): p. 201-8.
33 Grill, J.D., et al., Risk disclosure and preclinical Alzheimer's disease clinical trial enrollment. Alzheimer's & Dementia, 2013. 9(3): p. 356-359.e1.
34 Hansson, O., et al., Association between CSF biomarkers and incipient Alzheimer's disease in patients with mild cognitive impairment: a follow-up study. Lancet Neurol, 2006. 5(3): p. 228-34.
35 Mattsson, N., et al., CSF Biomarkers and Incipient Alzheimer Disease in Patients With Mild Cognitive Impairment. JAMA, 2009. 302(4): p. 385-393.
36 Mattsson, N., D. Brax, and H. Zetterberg, To Know or Not to Know: Ethical Issues Related to Early Diagnosis of Alzheimer's Disease. International Journal of Alzheimer’s Disease, 2010. 2010: p. 841941

NEDERLANDSE SAMENVATTING

Meer dan 100 jaar nadat Alois Alzheimer amyloïde plaques ontdekte rondom hersencellen en neurofibrillaire knopen in de cellen van een overleden patiënt beschreef, zijn we er nog steeds niet in geslaagd het mysterie van de ziekte van Alzheimer (AD) op te lossen.

Zoals beschreven in **Hoofdstuk 1**, vormt de groeiende steeds ouder wordende bevolking wereldwijd een grote belasting voor de gezondheidszorg. De Wereldgezondheidsorganisatie (WHO) schat dat 1 op de 6 mensen ter wereld in 2030 ouder zijn dan 60 jaar. Naarmate meer mensen toegang hebben tot (betere) gezondheidszorg, worden mensen ouder. Met het ouder worden neemt ook de kans op het ontwikkelen van een vorm van dementie toe. In 2020 bedroeg de prevalentie van dementie wereldwijd ongeveer 50 miljoen mensen. De meest voorkomende vorm van dementie is de ziekte van Alzheimer, die verantwoordelijk is voor ongeveer 70% van de gevallen van dementie. Biomarker onderzoek heeft de afgelopen decennia veel nieuwe inzichten in AD opgeleverd. Biomarker bewijs van AD-pathologie is meetbaar vanaf 20 jaar voordat klinische symptomen verschijnen. Naast het meten van amyloïde bèta en tau in hersenen van overleden patiënten, kunnen deze eiwitten nu ook worden gemeten in de cerebrospinale vloeistof (CSF), met behulp van positronemissietomografie (PET) en zelfs in bloed.

Nieuwe biomarkers geassocieerd met AD zijn geïdentificeerd die verband houden met ontstekingsprocessen in de hersenen, astroglia activatie en neuronale schade. Het grote aantallen patiënten met de ziekte van Alzheimer benadrukt de noodzaak voor ontwikkeling van een behandeling die de ziekte kan remmen of stoppen (een *ziekte modificerende* behandeling). Klinisch onderzoek is verbeterd door gebruik te maken van gerandomiseerde (placebo) gecontroleerde onderzoeken. Op dit moment (2022) zijn 119 ziekte modificerende middelen in ontwikkeling voor de behandeling van AD. Momenteel is alleen symptomatische behandeling beschikbaar voor AD patiënten. In juni 2021 werd de eerste ziekte modificerende behandeling voor AD goedgekeurd door de FDA in de Verenigde Staten van Amerika. Aducanumab belooft amyloïde plaques uit de hersenen te verwijderen die zich hebben opgehoopt als gevolg van de progressie van de ziekte van Alzheimer. De resultaten van de voorgaande klinische onderzoeken waren niet overtuigend en daarom keurde de EMA het medicijn niet goed in de Europese Unie. Het label van aducanumab is sinds goedkeuring door de FDA aangepast. Aanvankelijk keurde de FDA aducanumab goed voor alle patiënten met AD, maar ze pasten de goedkeuring aan door het label te beperken tot patiënten met cognitieve stoornissen van geringe ernst (Mild Cognitive Impairement, MCI) of *mild* AD, bij wie het medicijn ook werd getest in de fase 3 klinische onderzoeken. Dit benadrukt dat de selectie van de juiste onderzoekspopulatie van groot belang

is in AD onderzoek. Wanneer een gezond persoon zonder cognitieve klachten een verlaagd gehalte van het eiwit Aβ42 in de CSF heeft, zoals ook wordt waargenomen bij patiënten met AD, wordt deze persoon volgens de NIA-AA-normen uit 2011 beschouwd preklinische AD te hebben. Het uitvoeren van klinische onderzoeken in de vroege fase van AD of zelfs gedurende de preklinische fase van AD kan verdere ziekteprogressie voorkomen omdat er minder ziektepathologie in de hersenen aanwezig is. De verschuiving in de selectie van proefpersonen is merkbaar in de huidige klinische onderzoeken met 14 studies waarin ziekte modificerende middelen worden getest op proefpersonen met preklinische AD (2022).

Cognitief functioneren is een belangrijk onderdeel van de klinische manifestatie van AD. **Hoofdstuk II** beschrijft leeftijd gerelateerde achteruitgang in cognitieve prestaties gemeten m.b.v. de NeuroCart. De NeuroCart is een neuropsychologische en neurofysiologische testbatterij die wordt gebruikt om farmacodynamische effecten van geneesmiddelen te kwantificeren in de context van (vroege fase) geneesmiddelontwikkeling. In de loop der jaren is de NeuroCart gebruikt in honderden onderzoeken bij gezonde proefpersonen en in verschillende patiëntenpopulaties. De retrospectieve studie beschreven in **Hoofdstuk II** omvatte 93 studies, uitgevoerd bij het CHDR tussen 2005 en 2020 waarbij NeuroCart metingen zijn verricht, wat resulteerde in 2729 proefpersonen met data van ten minste één van de vijf NeuroCart metingen. De vijf NeuroCart tests die in het onderzoek zijn opgenomen waren: vloeiende en saccadische oogbewegingen, body sway, adaptive tracking, VVLT en N-Back. De resultaten tonen aan dat de NeuroCart leeftijd gerelateerde achteruitgang kan detecteren bij gezonde proefpersonen, wat niet werden beïnvloed door geslacht. De NeuroCart was in staat om significante verschillen in prestatie te detecteren tussen gezonde vrijwilligers en patiënten met AD, de ziekte van Parkinson, de ziekte van Huntington en vasculaire dementie op de gemiddelde leeftijd van de ziektegroep. Omdat de duur van de ziekte niet bekend was, kon deze cross-sectionele studie geen leeftijd-gerelateerde achteruitgang aantonen over het beloop van de ziekte. Daarom kon de snelheid van verslechtering van cognitieve functies als gevolg van neurodegeneratieve ziekte zoals gemeten met NeuroCart niet betrouwbaar worden gekwantificeerd. De gezonde ouderen die deelnamen aan deze studie, presteerde elk jaar slechter op alle NeuroCart-metingen. Bij de neurodegeneratieve ziekten nemen de cognitieve prestaties significant sneller af.

In **Hoofdstuk III** wordt een overzicht van biomarkers in humane AD in vergelijking met biomarkers in dierstudies beschreven. Er bestaat een groot aantal hypothesen met betrekking tot AD. Momenteel ontbreekt het ons aan voldoende informatie en begrip van processen in het vroege stadium van de ziekte. Dit draagt ertoe bij dat we in de vroegste fase van AD nog geen diagnose kunnen stellen of behandeling kunnen instellen. Dit benadrukt de noodzaak om adequate, bij voorkeur op lichaamsvloeistof gebaseerde, biomarkers voor AD te vinden. Momenteel zijn de biomarkers die het meest worden bepaald in studies bij mensen Aβ, P-TAU, T-TAU, neurogranin, SNAP-25, GFAP, YKL-40 en NFL. Daarnaast is er veel dieronderzoek waarbij de nadruk vooral op Aβ ligt. Dierstudies kunnen slim worden ontworpen om mechanistische informatie te verschaffen over de onderlinge relaties tussen de verschillende AD processen op een longitudinale manier inclusief comorbiditeiten die veel voorkomen in mensen, volgens de Mastermind Research-benadering. De Mastermind Research aanpak is bedoeld voor strategisch en systematisch CNS-geneesmiddelenonderzoek door met behulp van geavanceerde preklinische experimentele en wiskundige modellering gegevens uit dieronderzoek te modelleren om de distributie van CNS-geneesmiddelen bij mensen te voorspellen zonder dat er dierproeven nodig zijn.

Hoofdstuk IV combineerde op plasma gebaseerde biomarkers voor AD met cognitieve biomarkers gemeten met de NeuroCart om de CSF amyloïd bèta status van gezonde ouderen te voorspellen. De studie had tot doel een algoritme te ontwikkelen op basis van minder invasieve (plasma)biomarkers voor AD-pathologie en deze te gebruiken voor de preselectie van proefpersonen met verlaagde, abnormale Aβ-spiegels in het CSF ('Aβ-positieve proefpersonen') in overeenstemming met de aanwezigheid van AD-pathologie. Het algoritme dat uit de studie voortkwam, omvat geslacht, 7 cognitieve testen gemeten met de NeuroCart (MMT, VVLT, vingertikken, N-Back, SART, FACE en EEG) en één plasma biomarker (YKL-40) en was succesvol in het voorspellen van CSF Aβ+ bij gezonde ouderen met een sensitiviteit van 70,82% en specificiteit van 89,25%. Bij gebruik van dit algoritme zouden 70% minder lumbaalpuncties verricht hoeven worden om gezonde oudere proefpersonen te detecteren met een verlaagd Aβ CSF zoals gezien wordt bij AD. De belasting van de proefpersoon en de kosten van onderzoek zullen hierdoor significant afnemen. Dit kan ook de bereidheid vergroten van proefpersonen om deel te nemen aan geneesmiddelenonderzoek.

Verberk et al., toonde aan dat de plasma Aβ42/Aβ40 ratio potentie heeft om de pathologische veranderingen van de ziekte van Alzheimer te identificeren bij personen met subjectieve geheugenklachten. Het includeren van leeftijd en ApoEε4 dragerschap verbeterde in hun multivariate model de waarschijnlijkheid van identificatie. Op basis van deze resultaten postuleerden Verberk et al., dat de plasma-Aβ42/Aβ40 ratio een potentiële *prescreener* zou kunnen zijn om de vroegste pathologische veranderingen van AD te identificeren bij personen met subjectieve geheugenklachten. Het gebruik van op plasma gebaseerde biomarkers bij het identificeren en karakteriseren van preklinische AD is een doorbraak in klinisch onderzoek, aangezien het nemen van een bloedmonster minder ingrijpend is dan

het nemen van een CSF-punctie, wat de belasting voor gezonde proefpersonen en patiënten vermindert. De resultaten zijn echter nog voorlopig en moeten met de nodige voorzichtigheid worden geïnterpreteerd. De resultaten konden niet worden gereproduceerd in een (iets) andere onderzoeksgroep zoals besproken in **Hoofdstuk v** van dit proefschrift. Wij wilden de bevindingen van Verberk et al., uitbreiden, gebruikmakend van dezelfde statistische methoden, maar in een andere populatie, namelijk gezonde ouderen zonder geheugenklachten (n=189). De sensitiviteit en specificiteit van de plasma Aβ42/Aβ40 ratio in onze studie waren respectievelijk 30,8% en 71%, vergeleken met 76% en 75% in Verberk et al. De resultaten van onze logistische regressie en Receiver Operating Characteristics-analyses (ROC) toonden aan dat de plasma Aβ42/Aβ40 ratio geen significante invloed had op de ROC-curven die onderscheid maakten tussen CSF amyloïd abnormale en amyloïd normale individuen, in een multivariaat model inclusief leeftijd en ApoEε4 status. Het niet kruis valideren van een model kan leiden tot overfitting van de data. Ook werden verschillende populaties gebruikt om de resultaten te vergelijken. Beweren dat plasma-amyloïde een *prescreener* is voor de vroegste tekenen van AD-pathologie, is naar onze mening een voorbarige uitspraak.

Wat Alois Alzheimer in 1906 niet wist, maar wat we sindsdien hebben geleerd, is dat AD niet alleen wordt veroorzaakt door amyloïd plaques en neurofibrillaire knopen. Zoals besproken in **Hoofdstuk VI** speelt ontsteking ook een grote rol. Deze verkennende studie onderzocht plasma-biomarkers gerelateerd aan neuro-inflammatie geassocieerd met AD in een cohort van proefpersonen met preklinische AD, en vergeleek deze met gezonde ouderen, gedefinieerd door CSF $A\beta_{1-42}$. Vier inflammatoire plasma-biomarkers werden onderzocht. YKL-40 (ook bekend als chitinase-3-like protein-1 [CHI3L1]) is een glycoproteïne, dat voornamelijk tot expressie komt in astrocyten. Patiënten met AD hebben significant hogere YKL-40 spiegels in de liquor in vergelijking met gezonde controles, maar het is geen specifieke biomarker voor AD, omdat het alleen ontsteking weerspiegelt. Glial fibrillary acidic protein (GFAP) is een marker voor astrogliose en is postmortaal verhoogd in de hersenen en in het liquor van patiënten met AD. Van twee chemokinen (monocyt chemoattractant proteine-1 [MCP-1] en eotaxine-1) is bekend dat deze gecorreleerd zijn met meer geheugenstoornis in MCI en AD. Van de vier inflammatoire plasma biomarkers die in het onderzoek werden onderzocht, was alleen GFAP significant hoger bij proefpersonen met preklinische AD in vergelijking met gezonde ouderen. Bij het post-hoc definiëren van preklinische AD op basis van de PTAU181/$A\beta_{1-42}$-ratio, waren GFAP en YKL-40 significant verschillend tussen groepen. Dit zou erop kunnen wijzen dat GFAP en YKL-40 gevoeligere markers zijn van het beginnende ontstekingsproces dat optreedt als reactie op de misvouwing en aggregatie van bèta-amyloïd, gemeten als verlaagde $A\beta_{1-42}$ in het CSF.

De neurofibrillaire tangles die door Alois Alzheimer zijn ontdekt, zijn de afgelopen decennia grondig bestudeerd. **Hoofdstuk VII** beschrijft specifieke isotopen van tau, namelijk gefosforyleerde typen, en vergelijkt deze in CSF met plasma. De studie onderzocht P-TAU op threonine 181, 217 en 231 in CSF en P-TAU181 en P-TAU231 in plasma bij proefpersonen met preklinische AD en gezonde ouderen gedefinieerd door CSF $A\beta_{1-42}$, om te onderzoeken of fosfor-tau CSF en plasma-biomarkers een goed alternatief zijn voor het onderscheiden van gezonde ouderen van preklinische AD-patiënten. CSF PTAU217 was significant hoger bij proefpersonen met preklinische AD in vergelijking met gezonde ouderen. CSF PTAU181 en CSF PTAU231 waren verhoogd op hogere leeftijd, maar er was geen verschil tussen de twee onderzochte groepen. Alle PTAU-isovormen in CSF en plasma vertonen onderling hoge correlaties. Aangezien PTAU in de preklinische fase van AD lijkt te ontstaan als een reactie op Aβ-misvouwing in de hersenen, zou dit het vroegst mogelijke interventievenster voor behandeling kunnen zijn voordat neurofibrillaire tangles ontstaan. Het meten van PTAU in plasma kan worden gebruikt in geneesmiddelenonderzoek waarbij specifieke anti-tau ziekte modificerende behandeling (DMT) wordt onderzocht. Verwijdering of verlaging van PTAU in een vroege fase kan ertoe leiden dat minder patiënten met preklinische AD daadwerkelijk AD ontwikkelen. Aangezien deze studie het onderscheidend vermogen van PTAU in preklinische AD niet bevestigt, is meer (longitudinaal) onderzoek nodig om meer inzicht te krijgen in het verschil tussen PTAU-biomarkers in de preklinische AD fase in vergelijking met gezonde proefpersonen.

TOEKOMST PERSPECTIEF VOOR HET GEBRUIK VAN BIOMARKERS IN GEZONDE DEELNEMERS IN DE PREKLINISCHE FASE VAN DE ZIEKTE VAN ALZHEIMER

In dit proefschrift ligt de focus op preklinische AD. De definitie van de preklinische fase van AD was het afgelopen decennium een belangrijk onderwerp van discussie. De meest recente aanbeveling (2016) voor de classificatie van preklinische AD is het hebben zowel Aβ- als tau-pathologie gemeten door PET en/of CSF. Deze standaard wordt echter slechts in enkele onderzoeksinstellingen toegepast en maakt geen deel uit van de standaard klinische zorg. In dit proefschrift is de norm uit 2011 gebruikt waarbij Aβ pathologie in CSF zonder cognitieve klachten voldoende is voor de classificatie van preklinisch AD.
De ontwikkeling van op bloed gebaseerde biomarkers voor de detectie van (vroegtijdige) AD is zeer veelbelovend en zou het uitdagende veld van AD onderzoek kunnen verbeteren omdat het een minder invasieve procedure is. Er zijn nieuwe sensitieve testen in bloed ontwikkeld met veelbelovende resultaten, er is consistentie tussen verschillende cohorten en de resultaten zijn vergelijkbaar met CSF

en PET, maar helaas zijn we er nog niet: er is meer (lange termijn) onderzoek nodig om de validiteit van deze op bloed biomarkers te onderzoeken voordat deze als standaard kunnen worden geïmplementeerd.

Zoals beschreven in dit proefschrift kan het combineren van cognitieve testen met bloedonderzoek helpen het CSF Aβ profiel te voorspellen van gezonde ouderen. Proefpersonen vragen om cognitieve onderzoeken en een bloedafname te ondergaan, kan de bereidheid om deel te nemen aan klinische onderzoeken vergroten en de klinische kosten verlagen (door minder PET- en/of CSF-metingen).

Op basis van het onderzoek dat in dit proefschrift is uitgevoerd en recente literatuur, zou een combinatie van CSF, bloed en cognitieve biomarkers de beste combinatie zijn voor de selectie van proefpersonen met preklinisch AD voor studiedeelname. Op basis van de resultaten van cognitieve testen en biomarkers, zou een selectie van deze proefpersonen worden gevraagd een lumbaalpunctie of een PET-scan te ondergaan om de preklinische AD-status te bevestigen op basis van $A\beta_{1-42}$ en PTAU. Het verbeteren van de selectiecriteria voor klinische onderzoeken in preklinische AD zal naar verwachting leiden tot een minder heterogene patientenpopulatie, lagere uitkomstvariabiliteit en een grotere effectgrootte van de interventie en daardoor is er een analyse mogelijk om het juiste aantal subjects te berekenen met een grotere kans op een positieve uitkomst.

Het werven van patiënten met de ziekte van Alzheimer in klinische onderzoeken kan om verschillende redenen een uitdaging zijn, bijvoorbeeld studiebelasting, cognitieve belasting, ziekteprogressie en het naleven van onderzoeksregels. Door ons meer te richten op deelname van proefpersonen met preklinische AD zal tijd en geld worden bespaard, aangezien onderzoeken in een sneller tempo zullen worden voltooid vanwege een hogere therapietrouw en lagere belasting voor gezonde proefpersonen in vergelijking met patiënten met cognitieve beperkingen. Aan de andere kant kunnen studies langer duren voordat verandering op biomarker niveau kan worden waargenomen. Om het optimale therapeutische venster voor DMT's bij AD te vinden, moeten proefpersonen met preklinische AD worden geïncludeerd om het vroegste stadium voor modificatie te vinden. Momenteel lopen er longitudinale studies gericht op het in kaart brengen van biomarkers voor AD en deze zullen inzicht geven over onderzoek in mensen met preklinische AD.

OVERWEGINGEN VOOR TOEKOMSTIG KLINISCH ONDERZOEK NAAR DE ZIEKTE VAN ALZHEIMER MET ZIEKTE MODIFICERENDE MIDDELEN

Zoals Hariton en Locascio (2018) vermeldden, is de gouden standaard voor klinisch onderzoek het gebruiken van gerandomiseerde gecontroleerde studies (Randomized Controlled Trials, RCT). Een probleem met RCT's kan zijn dat proefpersonen niet de patiënten vertegenwoordigen voor wie de resultaten van de studie in de toekomst zullen worden gebruikt. Het maximaliseren van de behandelrespons door het selecteren van een meer beperkte homogene onderzoekspopulatie helpt bij het aantonen van het behandeleffect, maar wordt minder representatief voor de patiëntenpopulatie. Het includeren van biomarker gegevens en het includeren van proefpersonen in de preklinische fase van een ziekte zou dit generalisatieprobleem moeten minimaliseren. RCT's zijn meestal duurder naarmate er meer voorwaarden aan een onderzoek worden toegevoegd, wat resulteert in meer data. Deze hoge kosten moeten echter worden vergeleken met het uitvoeren van onderzoeken op een minder geoptimaliseerde manier, wat resulteert in het uitvoeren van meer studies met betwistbare resultaten die uiteindelijk meer zullen kosten. Reproduceerbaarheid van een onderzoek is belangrijk Dienovereenkomstig moeten RCT's bij proefpersonen met preklinische AD en fase 3-onderzoeken bij patiënten met AD dezelfde richtlijnen volgen. Patiënten met AD moeten goed worden gekarakteriseerd op biomarker niveau om patiënten te includeren met een vergelijkbare pathologie als waarop de DMT zich richt.

ETHISCHE OVERWEGINGEN IN ONDERZOEK NAAR PREKLINISCHE ZIEKTE VAN ALZHEIMER

Bij onderzoek met gezonde (oudere) proefpersonen moet rekening worden gehouden met ethische overwegingen. Sinds 2018 is de Algemene Verordening Gegevensbescherming (AVG) van kracht die alle persoonlijke gegevens van EU-burgers beschermt. Door het invoeren van de AVG zijn deelnemers aan klinische onderzoeken zich meer bewust van de (persoonlijke) gegevens die worden verzameld tijdens deelname aan het onderzoek en kunnen verzoeken om gedetailleerde informatie vaker voorkomen.

In Nederland is een vergunning nodig voor het uitvoeren van onderzoek waarbij de bevolking wordt gescreend op ernstige ziekten of afwijkingen waarvoor geen behandeling of preventie beschikbaar is (Wet op bevolkingsonderzoek [WBO]). Huidige zogenaamde secundaire preventieonderzoeken die grote groepen gezonde ouderen screenen op de aanwezigheid van AD-gerelateerde biomarkers en genetische informatie om proefpersonen voor onderzoek te selecteren, zijn goedgekeurd door ethische commissies, ook in Nederland. Deze onderzoeken zijn niet per definitie bevolkingsonderzoeken, aangezien niet alle mensen boven een bepaalde leeftijd worden uitgenodigd, maar er wordt wel naar gestreefd een groot aantal gezonde ouderen deel te laten nemen. EPAD registreerde bijvoorbeeld meer dan een half miljoen mensen in heel Europa.

Er is (in Nederland) nog geen ziekte modificerende behandeling voor AD beschikbaar en de aanwezigheid van biomarkers die overeenkomen met AD is niet

100% voorspellend voor het ontwikkelen van AD later in het leven. Ook kunnen biomarkers die consistent zijn met AD aanwezig zijn tot 20 jaar voor het daadwerkelijke begin van de ziekte, dus het actief diagnosticeren van een preklinische fase kan leiden tot een lange periode van onnodige zorgen. Het gedurende langere tijd blootstellen van preklinische proefpersonen aan behandeling moet veilig zijn en de voordelen van het onderzoek moeten de last rechtvaardigen.

Onderzoek toont aan dat er mogelijk voordelen zijn aan een vroege diagnosen zowel positief (gezonde gedragsverandering in dagelijks leven) als negatief (slechter presenteren op cognitieve taken). Input van de patiënten gemeenschap en een beter begrip van het concept van biomarkers door de algemene bevolking zou onderzoekers kunnen helpen te begrijpen welke mate van risico acceptabel wordt bevonden in klinische onderzoeken. Omdat veel AD biomarkers niet specifiek zijn voor AD, is extra voorzichtigheid geboden voor de mogelijkheid van een verkeerde diagnose van proefpersonen. De diagnostische nauwkeurigheid van CSF-biomarkers voor AD in het MCI-stadium is hoog, met sensitiviteit en specificiteit tot 85%-90%. Dit zijn hoge nauwkeurigheidscijfers, maar leiden nog steeds tot veel verkeerd gediagnosticeerde proefpersonen.

Proefpersonen met AD kunnen het recht op het bezit van een rijbewijs verliezen en, in de VS, het recht om een wapen te bezitten (wat misschien niet eens zo erg is). Wat betreft juridische regelingen: vroege diagnoses dwingen proefpersonen om na te denken over hun toekomst en bijvoorbeeld hun testament op te stellen voordat ze de wilsonbekwame fase bereiken. Als kennis over de status van biomarkers gemeengoed wordt, kan dit ook van invloed zijn op het zorgstelsel en in het bijzonder op de zorgverzekeringen.

Het delen van resultaten van biomarker- en genetisch onderzoek is een complexe taak en mag alleen worden uitgevoerd door getrainde specialisten. Een onderzoeksdeelnemer moet zelf de beslissing kunnen nemen om op de hoogte gesteld te worden van biomaker of genetische informatie Toekomstig onderzoek moet rekening houden met ethische overwegingen, vooral met longitudinale studies die gezonde mensen karakteriseren. Zodra DMT's beschikbaar zijn voor de preklinische fase, zullen de ethische overwegingen drastisch veranderen en moeten deze opnieuw worden geëvalueerd. Op dit moment heeft klinisch onderzoek bij proefpersonen met preklinische AD, inclusief biomarkers, een solide wetenschappelijke basis en is cruciaal om uiteindelijk een remedie voor AD te vinden.

CURRICULUM VITEA

Samantha Prins (Hillegom, 1987) graduated from secondary school, Atheneum, in 2006 (Fioretti college, Lisse) and started her bachelor psychology at Leiden University in 2008. During her studies she followed courses at California State University East Bay in the USA. In 2012 she started her master Clinical Neuropsychology at Leiden University. She obtained her master's degree in 2014 after which she worked as a neuropsychologist at Leiden University Medical Center (LUMC) at the 'Discontinuation of Antihypertensive Therapy in the Elderly' (DANTE) Leiden study at the department of psychiatry and the TRACK-ON Huntington's Disease study at the neurology department. Alongside the research at LUMC and her studies, Samantha also started working as a research assistant at Centre for Human Drug Research (CHDR) (2013). In 2015 she started as a project leader in the neurology group at CHDR. She performed early phase clinical trials focused on neurodegenerative diseases under supervision of prof. dr. Geert Jan Groeneveld and prof. dr. Joop van Gerven. In 2016/2017 a PhD topic was introduced. Her main scientific focus became (preclinical) Alzheimer's Disease with multicenter studies and CHDR funded studies which resulted in this thesis. Since 2022, Samantha works at Brain Research Center as a Senior Clinical Trial Start-Up Specialist.

LIST OF PUBLICATIONS

R. Alvarez-Jimenez, E.P. Hart, **S. Prins**, M. de Kam, J.M.A. van Gerven, A.F. Cohen, G.J. Groeneveld. Reversal of mecamylamine-induced effects in healthy subjects by nicotine receptor agonists: Cognitive and (electro) physiological responses, 2018.

S. Simpraga, H.D. Mansvelder, G.J. Groeneveld, **S. Prins**, E.P. Hart, S. Poil, K. Linkenkaer-Hansen. An EEG nicotine acetylcholine index to assess the efficacy pf pro-cognitive compounds, 2018.

S. Prins, A. Zhuparris, G.J. Groeneveld. Usefulness of plasma amyloid as a prescreener for earliest Alzheimer pathological changes depends on the study population, 2019

T. Qin, **S. Prins**, G.J. Groeneveld, G. van Westen, H. E. de Vries, Y. C. Wong, L.J.M. Bischoff, E.C.M. de Lange. Utility of animal models to understand human Alzheimer's disease, using the mastermind research approach to avoid unnecessary further sacrifices of animals, 2020.

C. Bakker, T. Tasker, J. Liptrot, E.P. Hart, E.S. Klaassen, **S. Prins**, T.F. van der Doef, G.A. Brown, A. Brown, M. Congreve, M. Weir, F.H. Marshall, D.M. Cross, G.J. Groeneveld, P.J. Nathan. First-in-man study to investigate safety, pharmacokinetics and exploratory pharmacodynamics of HTL0018318, a novel M1-receptro partial agonist for the treatment of dementias, 2020.

S. Prins, A. Zhuparris, E.P. Hart, R.J. Doll, G.J. Groeneveld. A cross-sectional study in healthy elderly subjects aimed at development of an algorithm to increase identification of Alzheimer pathology for the purpose of clinical trail participation, 2021.

C. Bakker*, **S. Prins***, J. Liptrot, E.P. Hart, E.S. Klaassen, G.A. Brown, A. Brown, M. Congreve, M. Weir, F.H. Marshall, J. Stevens, D.M. Cross, T. Tasker, P.J. Nathan, G.J. Groeneveld. Safety, pharmacokinetics and pharmaco-dynamics of HTL0009936, a selective muscarinic M1-acetylcholine receptor agonist: A randomized cross-over trial, 2021.

Elzinga W, **Prins S,** Borghans L, Gal P, Vargas G, Groeneveld G, Doll R. Detection of Clenbuterol-Induced Changes in Heart Rate Using At-Home Recorded Smartwatch Data: Randomized Controlled Trial, 2021

S. Prins, M.L. de Kam, C.E. Teunissen, G. Groeneveld. Inflammatory plasma biomarkers in subjects with preclinical Alzheimer's disease, 2022.