CLINICAL STUDY PROTOCOL

Observational, correlational study aimed to identify healthy elderly subjects with Alzheimer pathology more efficiently.

Short Title:	Observational, correlational study to identify Alzheimer pathology in healthy elderly.
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Ethics Committee number.	P17.148
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I acknowledge accountability for this protocol in accordance with CHDR's current procedures.

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Observational, correlational study aimed to identify healthy elderly subjects with Alzheimer pathology more efficiently.

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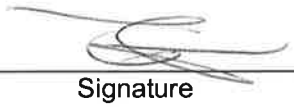

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LIST OF ABBREVIATIONS

ABR	ABR form, General Assessment and Registration form, is the application form that is required for submission to the accredited Ethics Committee; in Dutch, ABR = Algemene Beoordeling en Registratie
AD	Alzheimer's Disease
AE	Adverse Event
ANCOVA	Analysis of Covariance
BMI	Body Mass Index
BP	Blood Pressure
bpm	beats per minute
CCMO	Central Committee on Research Involving Human Subjects; in Dutch: Centrale Commissie Mensgebonden Onderzoek
CHDR	Centre for Human Drug Research
CK	creatine kinase
CRF	Case Report Form
CSF	Cerebrospinal Fluid
DSMB	Data Safety and Monitoring Board
EC	Ethics Committee (also Medical Research Ethics Committee (MREC); in Dutch: Medisch Ethische Toetsing Commissie (METC).
ECG	Electrocardiogram
EDTA	Ethylene diamine tetra-acetic acid
EU	European Union
FDA	Food and Drug Administration
GCP	Good Clinical Practice
ICH	International Conference on Harmonization
i.v.	Intravenous(ly)
IRB	Institutional Review Board
LDH	Lactate dehydrogenase
LP	Lumbar Puncture
MCI	Mild Cognitive Impairment
MedDRA	Medical Dictionary for Regulatory Activities
NfL	Neurofilament Light
SAE	Serious Adverse Event
SAP	Statistical Analysis Plan
SD	Standard Deviation
SEM	Standard Error of the Mean
SOC	System Organ Class
SOP	Standard Operating Procedure
SST	Serum Separator Tube
SUSAR	Suspected Unexpected Serious Adverse Reaction
VUmc	Vrije Universiteit medisch centrum
WHO	World Health Organization
WBP	Personal Data Protection Act; in Dutch: Wet Bescherming Persoonsgevens
WMO	Medical Research Involving Human Subjects Act; in Dutch: Wet Medisch-wetenschappelijk Onderzoek met Mensen.

PROTOCOL SYNOPSIS

Title

Observational, correlational study aimed to identify healthy elderly subjects with Alzheimer pathology more efficiently.

Short Title

Observational, correlational study to identify Alzheimer pathology in healthy elderly.

Background & Rationale

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]. New empirical criteria aim at defining early clinical, biochemical, and metabolic markers of AD before the criteria of dementia are clinically 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.

While the definite diagnosis for AD can only be made post-mortem by investigating the presence of amyloid beta (Aβ) plaques and neurofibrillary tangles, recent advances in neuroimaging, cerebrospinal fluid (CSF) assays, and other fluid biomarkers now provide the ability to detect evidence of the AD pathophysiological process in vivo [3]. Furthermore, 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 AD prior to the clinical expression of symptoms [4]. These observations are confirmed by clinical cohort studies which suggest that there may be very subtle cognitive alterations that are detectable years before meeting clinical criteria for MCI, and which predict progression to AD [5].
Based on extensive longitudinal biomarker studies [6, 7] 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 have been shown to occur in CSF up to 15 years prior to the clinical onset of AD [8]. As the disease process progresses, cognitive functions start to decline; at first only noticeable through sophisticated neuropsychological testing, but eventually also clinically evident.

In the clinical setting, the diagnosis of (probable) AD is made based on the clinical picture, 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 could result in discomfort with a chance of side-effects (e.g. post-puncture headache) while PET scans are time consuming, not available for all patients, and expensive [9, 10].

The leading hypothesis regarding the pathophysiology of AD is centered around misfolding and aggregation of toxic Aβ species such as Aβ 1-42 and drug research is focused on inhibiting formation or accelerating removal of Aβ oligomers by using antibody-based immunotherapy. Until recently, phase II and III studies with anti- Aβ antibodies all failed to improve cognition or functional ability [11-13], presumably because they were performed in patients with already manifest dementia related to AD [14]. A recent study, however, has shown promising results of anti-Aβ antibody aducanumab in patients with prodromal and mild AD by decreasing Aβ plaques in the brain [15]. Clinically, the patient group which received active compound showed slower cognitive decline than the placebo group. Future use of Aβ immunotherapy might be implemented to prevent AD in healthy elderly who show evidence of amyloid pathology as it may be able to prevent aggregation of neurotoxic forms of Aβ, thereby preventing downstream effects as synaptic dysfunction, neuronal damage and cognitive impairment [16]. With the increasing number of clinical trials with Aβ targeting compounds and consequently the search for suitable trial subjects, there is a need for a less burdensome and effective method to assess the presence of Aβ pathology in (healthy) subjects. Where clinical trials started with inclusion of AD patients and subsequently moved to earlier stage subjects (i.e. mild cognitive impairment) [17], recent early phase studies also aim to include

otherwise healthy elderly subjects with evidence of AD pathology. This means that potential subjects have to undergo CSF sampling, amyloid PET scanning, or both, to check for study eligibility which is burdensome and expensive as amongst this cognitive healthy elderly population only 19% will prove to have abnormal CSF Aβ concentration or amyloid plaques on the scan or in CSF, increasing with age (age 65+) [9].

In the current study we aim to develop an algorithm based on less-invasive 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. This algorithm could be used to identify potential trial subjects resulting in a smaller group of subjects where confirmation of actual decreased CSF Aβ levels by means of a LP is needed. In this way, subjecting a large group of subjects to LPs and/or PET scans may be avoided. The main objective of the current study is thus to develop a method to select healthy elderly with Aβ pathology for future studies in a less burdensome and more effective way.

A recent study by Lutz et al., (2016) describes a genetic-based biomarker algorithm for risk of AD development. The authors used genetic and biomarker (CSF) data from cognitively normally, MCI and AD subjects who were followed longitudinally. They included age, APOE and TOMM400'523 genotypes [18]. This study, however, was aimed at predicting conversion to clinically over dementia in subjects at risk for AD, while we intend to develop an algorithm that aims at identifying subjects at risk of having a biomarker profile consistent with AD among healthy elderly subjects. A number of variables will serve as a pre-selected pool of candidate variables and will be used in this study to develop the algorithm; plasma-based biomarkers (i.e. Aβ40, Aβ42, t-Tau, p-Tau and Neurofilament Light [NfL]), apolipoprotein ε (APOE ε) genotyping, age, educational level, grip strength, and neuropsychological and neurophysiological testing by means of a computerized test battery (i.e. NeuroCart) (for further details, see methods).
Subjects will be asked to undergo a LP to quantify AD-specific biomarkers in CSF. Based on comparable studies, the expectation is that approximately 20% of subjects who are approached will be willing to undergo a LP. In this study we aim to enroll a study population of 200 subjects and therefore think we will need to approach approximately 1000 subjects.

Plasma levels of Aβ40, Aβ42 and NfL, APOE ε status and NeuroCart tests of the 200 subjects willing to undergo a lumbar puncture will be used to define an algorithm that will be able to improve the efficiency of finding subjects who have an Aβ positive (CSF) profile. The subjects will be divided in a group yielding subjects who are suspected of AD pathology (ADpath+) by the algorithm and subjects who are suspected to be without AD pathology (ADpath-). To confirm the correct classification potential of the algorithm, the outcome will be compared to the CSF Aβ outcome of the subjects. CSF ADpath+ and a CSF ADpath- defined by an Aβ 1-42 CSF level ≤450 pg/mL for ADpath+ subjects and an Aβ 1-42 CSF level ≥451 pg/mL for ADpath- subjects, will be used to differentiate between the two groups (using the Single Molecule Array assay; Simoa assay [19]).

If the ability of the algorithm to discriminate between subjects who are likely to have a biomarker profile indicative of AD pathology and subjects with a normal biomarker profile is confirmed by evidence of pathology in CSF, it could be decided to not perform lumbar punctures in subjects who are very likely to have a *normal* CSF biomarker profile based on the algorithm in future studies. This will allow for a noninvasive method to pre-select healthy subjects likely to have Aβ pathology in the CSF and therefore to fewer healthy elderly subjects who will have to undergo a lumbar puncture. The results of this study can also be used for subject selection for research that specifically targets a certain biomarker (e.g NfL).

As an exploratory objective, plasma samples will be analyzed for diurnal fluctuation in this current study. Diurnal fluctuation in the concentration of CSF biomarkers has been reported and may represent a pre-analytical confounding factor in the laboratory diagnosis of AD [20]. Plasma biomarkers for AD have however not been extensively studied for possible fluctuating levels during the day. The results will also provide more information about the effect of the clearance systems of the brain which removes proteins (e.g. Aβ) from the brain via various overlapping clearance systems [21]. These clearance systems may be the cause of the diurnal fluctuation reported in CSF. This knowledge can be used in future clinical trials to perform certain assessments (e.g. LP and blood samples) at the most useful time points.

Objective(s)

Main objectives:

To define an algorithm based on the plasma biomarkers: Aβ40, Aβ42, t-Tau, p-Tau, NfL and APOE ε status and NeuroCart tests, age, grip strength and level of education that distinguishes between positive and negative Aβ CSF in healthy elderly.

Exploratory objective:

To determine the correlation between AD-specific biomarkers in CSF (Aβ 1-40 and 1-42, Tau proteins, NfL) and the NeuroCart scores in a cohort of 200 healthy elderly subjects.

To determine the correlation between AD-specific biomarkers in plasma (Aβ 1-40 and 1-42, Tau proteins, NfL) the NeuroCart scores in a cohort of 200 healthy elderly subjects.

To determine the correlation between AD-specific biomarkers in CSF (Aβ 1-40 and 1-42, Tau proteins, NfL) and in plasma in a cohort of 200 healthy elderly subjects.

To determine the correlation between APOE ε genotype and the Aβ /Tau/NfL concentrations in CSF in a cohort of 200 healthy elderly subjects.

To determine the correlation between APOE ε genotype and the Aβ /Tau/NfL concentrations in plasma in a cohort of 200 healthy elderly subjects.

To characterize the cohort of healthy elderly subjects in order to yield norm scores for specific NeuroCart tests in all subjects.

To characterize the plasma biomarkers across multiple times of day to gain understanding of intra- and inter-subject variability, and thus diurnal fluctuation.

Design

Single-centre, observational, correlational study.

Principal Investigator & Trial Site

Geert Jan Groeneveld / MD, PhD, Centre for Human Drug Research, Zernikedreef 8, 2333 CL Leiden, The Netherlands.

Subjects / Groups

Elderly male and female subjects of 65 years and older will be invited to participate in this study. Two hundred eligible subjects will be enrolled of which at least a hundred will be above the age of 70. Subjects will be randomised to participate in the morning or afternoon.

Sample Size Justification

This is an exploratory study, therefore the samples size is not based on statistical considerations. The aim for this study is to enroll 200 subjects. The assays to determine Aβ from CSF are well established methods and the sample size justification for this current study is therefore based on previous research on amyloid pathology in CSF. In this study we select elderly at the age of 65 and higher of which at least a hundred will be above the age of 70. According to Jansen et al., (2015) we can expect at least 19% amyloid pathology in a 65+ population and 23% amyloid pathology among cognitively healthy 70+ elderly subjects. As we expect more responsiveness for study participation from elderly between the age of 65 till 70, based on our experience with previous studies in this age range, at least 100 subjects of >70 years old will be included in this study to be able to differentiate between age, which results in approximately 23 Aβ positive subjects versus approximately 77 Aβ negative subjects in this elderly age group. Along with approximately 19 Aβ positive subjects versus approximately 81 Aβ negative subjects in the age group 65-70 we expect to find at least 42 Aβ positive healthy elderly subjects. Based on previous comparable studies, these numbers are considered appropriate for a correlational study aimed at defining an algorithm [22, 23].

Inclusion criteria

1. Males and females, aged 65 and older (inclusive);

2. Willing and able to perform the cognitive tests, as evidenced by performance on the training session of the cognitive tests;
3. Willing and able to give written informed consent and to comply with the study procedures.

Exclusion criteria

1. Legal incapacity or inability to understand or comply with the requirements of the study;
2. Evidence of cognitive deterioration, as indicated by a diagnosis of a cognitive disorder (including but not limited to MCI, Alzheimer's disease, Lewy Body Dementia, Fronto-temporal Dementia);
3. History or symptoms of significant psychiatric disease in the past 3 years (including but not limited to clinical depression, schizophrenia);
4. A Mini Mental State Examination (MMSE) score of ≤24;
5. A Geriatric Depression Scale – 15 (GDS) score of ≥6;
6. Presence of drug abuse, or positive urine drug screen (UDS) at screening or occasion;
7. Presence of severe alcohol abuse (daily alcohol consumption exceeding 2 standard drinks per day on average for females or exceeding 3 standard drinks per day on average for males (1 standard drink = 10 grams of alcohol)), or a positive breath alcohol test at screening or occasion;
8. Any contradictions for a lumbar puncture as judged by the principal investigator.
9. Any other reason that it is not safe or ethical to allow a subject to participate in the study in the opinion of the investigator.

Concomitant medications

Any medication which affects the central nervous system is not allowed as judged by the principal investigator. All prescription and over-the-counter (OTC) medications, vitamins, herbal and dietary supplements used at screening and occasion will be recorded.

Study periods

Screening to determine eligibility will take place within 21 days before the occasion. This study involves a single occasion, which will take place during the day and will not involve overnight stay.

Investigational drug

Not applicable.

Comparative drug

Not applicable.

Tolerability / safety endpoints

Not applicable.

CNS outcome variables

NeuroCart assessments

- Adaptive tracking test;
- Visual Verbal Learning Test (VVLT);
- Milner Maze test;
- Face encoding and recognition test;
- N-back test;
- Sustained Attention to Response test (SART);

- Finger tapping.

Neurophysiological NeuroCart assessments

- 21 leads electroencephalogram (EEG);
- Smooth and saccadic eye movement.

Neuropsychological tests

- Clinical Dementia Rating scale (CDR);
- Instrumental Activities of Daily Living scale (IADL).

Handgrip strength

- JAMAR hydraulic hand dynamometer

Biochemical outcome variables

CSF biomarkers

- Aβ concentration (1-40, 1-42 and 1-42/1-40 ratio);
- T-Tau and p-Tau concentrations;
- NfL concentration.

Plasma biomarkers

- Aβ concentration (1-40, 1-42);
- T-Tau and p-Tau concentrations;
- NfL concentration.

Exploratory biomarkers including but not limited to:

- Synaptic loss; Neurogranin [24],
- Glial inflammation; YKL-40 [25],
- Levels of p-Tau181 in extracts of neutrally-derives blood exosomes [26],
- MicroRNAs [MiR-155, MiR-107 and MiR-29 [27]]).

Genetics

- APOE ε genotype;

Statistical methodology

Descriptive statistics will be used to summarize the data. Data will be graphically processed for visual inspection.

In order to develop an algorithm that can classify the subjects between ADpath+ and ADpath- there will be a two-step procedure. In the first step 500 bootstrap samples will be generated out of the original data and logistic regression with LASSO penalization (in order to account for the expected small event per variable ratio) in each of the bootstrap samples with all the candidate variables (CNS and biochemical outcome variables, age and educational level) will be performed. Out of all the candidate variables the ones that consistently and most frequently appear in the bootstrap models will be selected. Hence, in this first step the variable selection will take place. In the second step another 500 bootstrap samples will be generated and logistic regression will be performed (with penalization if necessary) in each of the bootstrap samples. This time only the variables that were selected in step 1 will be entered in the model. The estimates out of all the bootstrap models will be averaged and used in order to obtain predictions in the original data. Step two will result in the bootstrap-corrected predictions that will be assessed in terms of calibration (calibration slope), discrimination (c-index) and squared mean prediction error.

To investigate the relationship between biomarkers and gene carriers and NeuroCart scores, correlations and regression analyses will be used.

NeuroCart scores will be analysed with an analysis of variance (ANOVA) and/or multivariate analysis of variance (MANCOVA) to estimate differences between the groups of elderly with different Aβ status.

The intra- and inter-individual diurnal fluctuation will be investigated with mixed-model analysis.

Table 1 Visit and Assessment Schedule

	SCR	Study day 1									
Time point / Assessment	Up to -21 d	-0.25h	0h	0.5h	1h	1.5h	2h	2.5h	3h	4h	48h
Informed consent	X										
Demography	X										
Inclusion and exclusion criteria	X										
Medical history	X										
Physical examination	X										
Fundoscopy	X										
Temperature	X										
Height and weight	X										
Concomitant medication	X	X									
Virology	X										
BsHaem	X										
UrDrug, BrAlc	X	X									
General symptoms	X										X[1]
Vital Signs (HR, BP)	X										
Meal/snack	X		X								
Geriatric Depression Scale	X										
Mini Mental State Examination	X										
Adaptive tracking test	X[2]			X							
Visual Verbal Learning Test (VVLT) immediate	X[2]				X						
VVLT delayed	X[2]					X					
VVLT recognition	X[2]					X					
Milner Maze Test (MMT) immediate	X[2]			X							
MMT reversed	X[2]			X							
MMT delayed	X[2]				X						
Face encoding and recognition test	X[2]				X						
N-Back	X[2]						X				
Sustained Attention to Response Test	X[2]				X						
Finger tapping	X[2]							X			
Electroencephalography (EEG)					X						
Smooth & saccadic eye movements	X[2]			X							
Clinical Dementia Rating scale			X								
Instrumental Activities of Daily Living scale			X								

[1] At the end of the study day, the first 20 subjects will rate the burden of the study by answering a Visual Analogue Scale question. These 20 subjects will also be contacted by telephone 48 hours after LP to check for AEs.

[2] PD tests training session.

JAMAR dynamometry							X				
Plasma sample biomarker research			X[3]				X			X[4]	
Blood sample genotyping			X								
CSF sampling									X		
Discharge	X									X	
Con-meds		<----- continuous ----->									

[3] Subjects will be unfed for first plasma biomarker sample due to the influence of fat on the adequate isolation of exosomes from plasma. Subjects will be offered breakfast/lunch thereafter.

[4] Plasma sample closest to CSF sample (4h sample) will be used to compare CSF and plasma biomarkers.

1 BACKGROUND AND RATIONALE

Title

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Background & Rationale

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]. New empirical criteria aim at defining early clinical, biochemical, and metabolic markers of AD before the criteria of dementia are clinically 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.

While the definite diagnosis for AD can only be made post-mortem by investigating the presence of amyloid beta (Aβ) plaques and neurofibrillary tangles, recent advances in neuroimaging, cerebrospinal fluid (CSF) assays, and other fluid biomarkers now provide the ability to detect evidence of the AD pathophysiological process in vivo [3]. Furthermore, 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 AD prior to the clinical expression of symptoms [4]. These observations are confirmed by clinical cohort studies which suggest that there may be very subtle cognitive alterations that are detectable years before meeting clinical criteria for MCI, and which predict progression to AD [5].
Based on extensive longitudinal biomarker studies [6;7] 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 have been shown to occur in CSF up to 15 years prior to the clinical onset of AD [8]. As the disease process progresses, cognitive functions start to decline; at first only noticeable through sophisticated neuropsychological testing, but eventually also clinically evident.

In the clinical setting, the diagnosis of (probable) AD is made based on the clinical picture, 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 could result in discomfort with a chance of side-effects (e.g. post-puncture headache) while PET scans are time consuming, not available for all patients, and expensive [9] [10].

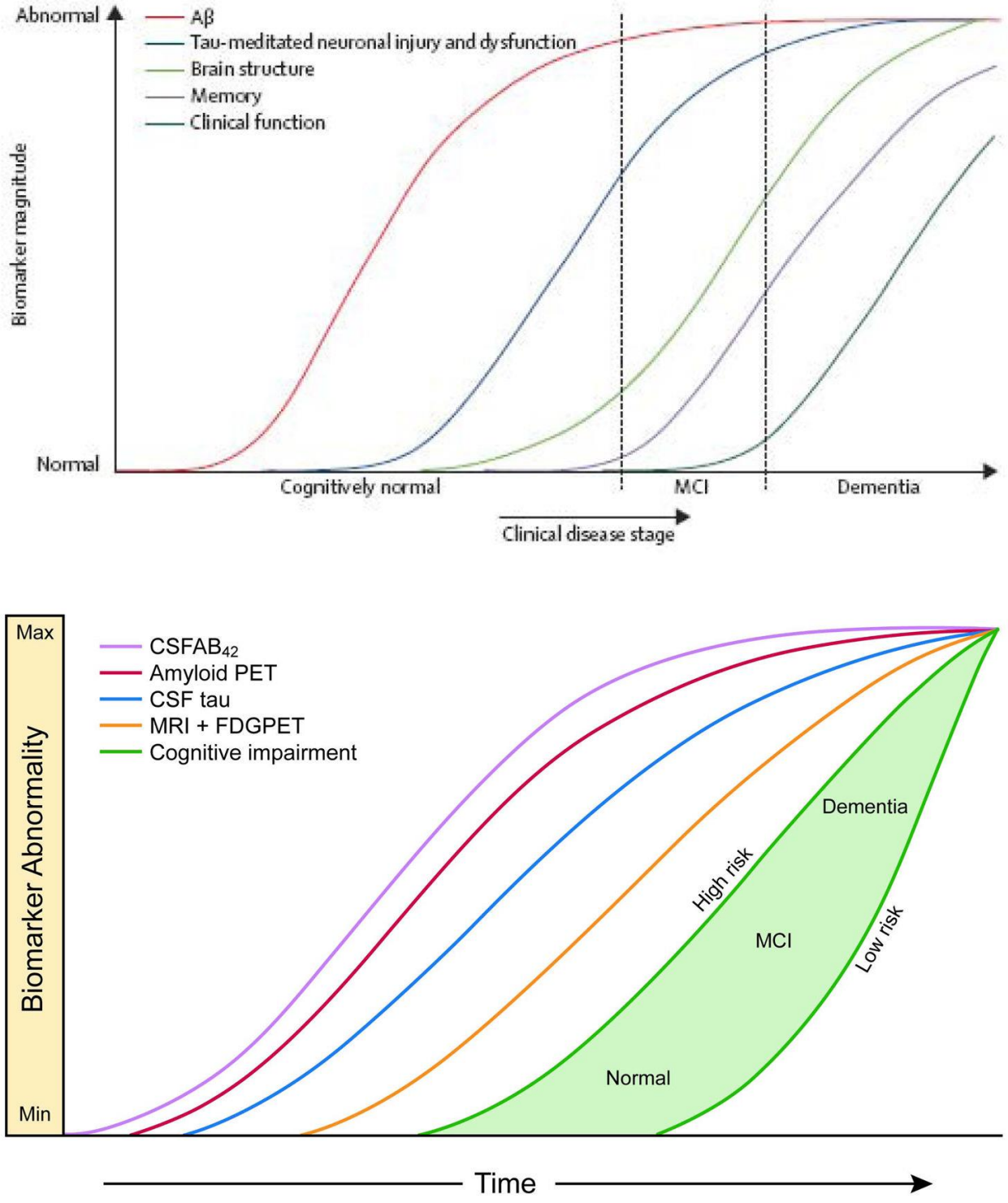

Figure 1a and 1b. Temporal ordering of AD biomarkers (reproduced from Jack et al., 2013)

1a. Original dynamic biomarkers of the AD pathological cascade model – 2010. Aβ amyloid is identified by CSF Aβ 42 or PET amyloid imaging. Neuronal injury and dysfunction is identified by CSF tau or FDG-PET. Neurodegenerative atrophy is measured by structural MRI.
1b. Revised dynamic biomarkers of the AD pathological cascade model – 2012. Aβ amyloid is identified by CSF Aβ 42 (purple) or PET amyloid imaging (red). Elevated CSF tau (blue). Neurodegeneration is measured by FDG PET and structural MRI respectively which are drawn concordantly (orange). By definition, all curves converge at the top right-hand corner of the plot, the point of maximum abnormality. The horizontal axis of disease progression is expressed as time. Cognitive response is illustrated as a zone (green filled area) with low and high risk boarders.

The leading hypothesis regarding the pathophysiology of AD is centered around misfolding and aggregation of toxic Aβ species such as Aβ 1-42 and drug research is focused on inhibiting formation or accelerating removal of Aβ oligomers by using antibody-based immunotherapy. Until

recently, phase II and III studies with anti- Aβ antibodies all failed to improve cognition or functional ability [11] [12] [13], presumably because they were performed in patients with already manifest dementia related to AD [14]. A recent study, however, has shown promising results of anti-Aβ antibody aducanumab in patients with prodromal and mild AD by decreasing Aβ plaques in the brain [15]. Clinically, the patient group which received active compound showed slower cognitive decline than the placebo group. Future use of Aβ immunotherapy might be implemented to prevent AD in healthy elderly who show evidence of amyloid pathology as it may be able to prevent aggregation of neurotoxic forms of Aβ, thereby preventing downstream effects as synaptic dysfunction, neuronal damage and cognitive impairment [16]. With the increasing number of clinical trials with Aβ targeting compounds and consequently the search for suitable trial subjects, there is a need for a less burdensome and effective method to assess the presence of Aβ pathology in (healthy) subjects. Where clinical trials started with inclusion of AD patients and subsequently moved to earlier stage subjects (i.e. mild cognitive impairment) [17], recent early phase studies also aim to include otherwise healthy elderly subjects with evidence of AD pathology. This means that potential subjects have to undergo CSF sampling, amyloid PET scanning, or both, to check for study eligibility which is burdensome and expensive as amongst this cognitive healthy elderly population only 19% will prove to have abnormal CSF Aβ concentration or amyloid plaques on the scan or in CSF, increasing with age (age 65+) [9].

In the current study we aim to develop an algorithm based on less-invasive 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. This algorithm could be used to identify potential trial subjects resulting in a smaller group of subjects where confirmation of actual decreased CSF Aβ levels by means of a LP is needed. In this way, subjecting a large group of subjects to LPs and/or PET scans may be avoided. The main objective of the current study is thus to develop a method to select healthy elderly with Aβ pathology for future studies in a less burdensome and more effective way.

A recent study by Lutz et al., (2016) describes a genetic-based biomarker algorithm for risk of AD development. The authors used genetic and biomarker (CSF) data from cognitively normally, MCI and AD subjects who were followed longitudinally. They included age, APOE and TOMM400’523 genotypes [18]. This study, however, was aimed at predicting conversion to clinically over dementia in subjects at risk for AD, while we intend to develop an algorithm that aims at identifying subjects at risk of having a biomarker profile consistent with AD among healthy elderly subjects. A number of variables will serve as a pre-selected pool of candidate variables and will be used in this study to develop the algorithm; plasma-based biomarkers (i.e. Aβ40, Aβ42, t-Tau, p-Tau and Neurofilament Light [NfL]), apolipoprotein ε (APOE ε) genotyping, age, educational level, grip strength and neuropsychological and neurophysiological testing by means of a computerized test battery (i.e. NeuroCart) (for further details, see methods).
Subjects will be asked to undergo a LP to quantify AD-specific biomarkers in CSF. Based on comparable studies, the expectation is that approximately 20% of subjects who are approached will be willing to undergo a LP. In this study we aim to enroll a study population of 200 subjects and therefore think we will need to approach approximately 1000 subjects.

Plasma levels of Aβ40, Aβ42 and NfL, APOE ε status and NeuroCart tests of the 200 subjects will be used to define an algorithm that will be able to improve the efficiency of finding subjects who have an Aβ positive (CSF) profile. The subjects will be divided in a group yielding subjects who are suspected of AD pathology (ADpath+) by the algorithm and subjects who are suspected to be without AD pathology (ADpath-). To confirm the correct classification potential of the algorithm, the outcome will be compared to the CSF Aβ outcome of the subjects. CSF ADpath+ and a CSF ADpath- defined by an Aβ 1-42 CSF level ≤450 pg/mL for ADpath+ subjects and an Aβ 1-42 CSF level ≥451 pg/mL for ADpath- subjects, will be used to differentiate between the two groups (using the Single Molecule Array assay; Simoa assay [19]).

If the ability of the algorithm to discriminate between subjects who are likely to have a biomarker profile indicative of AD pathology and subjects with a normal biomarker profile is confirmed by evidence of pathology in CSF, it could be decided to not perform lumbar punctures in subjects who are very likely to have a *normal* CSF biomarker profile based on the algorithm in future studies. This will allow for a noninvasive method to pre-select healthy subjects likely to have Aβ pathology in the

CSF and therefore to fewer healthy elderly subjects who will have to undergo a lumbar puncture. The results of this study can also be used for subject selection for research that specifically targets a certain biomarker (e.g NfL).

As an exploratory objective, plasma samples will be analyzed for diurnal fluctuation in this current study. Diurnal fluctuation in the concentration of CSF biomarkers has been reported and may represent a pre-analytical confounding factor in the laboratory diagnosis of AD [20]. Plasma biomarkers for AD have however not been extensively studied for possible fluctuating levels during the day. The results will also provide more information about the effect of the clearance systems of the brain which removes proteins (e.g. Aβ) from the brain via various overlapping clearance systems [21]. These clearance systems may be the cause of the diurnal fluctuation reported in CSF. This knowledge can be used in future clinical trials to perform certain assessments (e.g. LP and blood samples) at the most useful time points.

1.1 Study rationale

1.1.1 Benefit and risk assessment

This is a study without an intervention (e.g. novel drug administration) which makes the risk of injuries or side effect from drug administration none. Taking blood samples can be an unpleasant procedure and can lead to bruises. The CSF sampling could be an uncomfortable procedure and may lead to side effects, for instance post-dural puncture headache or bruising.

There is no benefit for the subjects who participate in the study. This study increases the knowledge about possible relationships between different types of blood plasma biomarkers in this specific cohort of elderly. The study may yield an algorithm (base on e.g. the NeuroCart results, CSF information and plasma based biomarkers from the subjects) that can be used to predict the likelihood that an otherwise healthy subject has AD pathology before clinical disease onset. This may lead to fewer lumbar punctures in future studies aimed at finding subjects to participate in clinical trials with drugs aiming at slowing AD disease onset. Subjects of this current study may be asked to participate in future studies.

1.1.2 Medical and regulatory background

Not applicable.

1.1.3 Study population

For this study a population of healthy elderly subjects aged 65 years and over has been chosen as the prevalence of geriatric cognitive disorders such as AD is increasing from approximately this age onwards.

1.1.4 Sample Size Justification

This is an exploratory study, therefore the samples size is not based on statistical considerations. The aim for this study is to enroll 200 subjects. The assays to determine Aβ from CSF are well established methods and the sample size justification for this current study is therefore based on previous research on amyloid pathology in CSF. In this study we select elderly at the age of 65 and higher of which at least a hundred will be above the age of 70. According to Jansen et al., (2015) we can expect at least 19% amyloid pathology in a 65+ population and 23% amyloid pathology among cognitively healthy 70+ elderly subjects. As we expect more responsiveness for study participation from elderly between the age of 65 till 70, based on our experience with previous studies in this age range, at least 100 subjects of >70 years old will be included in this study to be able to differentiate between age, which results in approximately 23 Aβ positive subjects versus approximately 77 Aβ negative subjects in this elderly age group. Along with approximately 19 Aβ positive subjects versus approximately 81 Aβ negative subjects in the age group 65-70 we expect to find at least 42 Aβ positive healthy elderly subjects. Based on previous comparable studies, these numbers are considered appropriate for a correlational study aimed at defining an algorithm [22, 23].

1.1.5 Study design

Single-centre, observational, correlational study.

1.1.6 Study duration

This study involves a single occasion, which will take place during the day and will not involve overnight stay.

1.1.7 CNS outcome variables

<u>NeuroCart assessments</u>

- Adaptive tracking test;
- Visual Verbal Learning Test (VVLT);
- Milner Maze test;
- Face encoding and recognition test;
- N-back test;

- Sustained Attention to Response test (SART);
- Finger tapping.

Neurophysiological NeuroCart assessments

- 21 leads electroencephalogram (EEG);
- Smooth and saccadic eye movement.

Neuropsychological tests

- Clinical Dementia Rating scale (CDR);
- Instrumental Activities of Daily Living scale (IADL).

Handgrip strength

- JAMAR hydraulic hand dynamometer

1.1.8 Biochemical outcome variables

CSF biomarkers

- Aβ concentration (1-40, 1-42 and 1-42/1-40 ratio);
- T-Tau and p-Tau concentrations;
- NfL concentration.

Plasma biomarkers

- Aβ concentration (1-40, 1-42);
- T-Tau and p-Tau concentrations;
- NfL concentration.

Exploratory biomarkers including but not limited to:

- Synaptic loss; Neurogranin [24],
- Glial inflammation; YKL-40 [25],
- Levels of p-Tau181 in extracts of neutrally-derives blood exosomes [26],
- MicroRNAs [MiR-155, MiR-107 and MiR-29 [27]]).

Genetics

- APOE ε genotype;

1.1.9 Statistical methodology

Descriptive statistics will be used to summarize the data. Data will be graphically processed for visual inspection.

In order to develop an algorithm that can classify the subjects between ADpath+ and ADpath- there will be a two-step procedure. In the first step 500 bootstrap samples will be generated out of the original data and logistic regression with LASSO penalization (in order to account for the expected small event per variable ratio) in each of the bootstrap samples with all the candidate variables (CNS and biochemical outcome variables, age and educational level) will be performed. Out of all the candidate variables the ones that consistently and most frequently appear in the bootstrap models will be selected. Hence, in this first step the variable selection will take place. In the second step another 500 bootstrap samples will be generated and logistic regression will be performed (with penalization if necessary) in each of the bootstrap samples. This time only the variables that were selected in step 1 will be entered in the model. The estimates out of all the bootstrap models will be averaged and used in order to obtain predictions in the original data. Step two will result in the bootstrap-corrected predictions that will be assessed in terms of calibration (calibration slope), discrimination (c-index) and squared mean prediction error.

To investigate the relationship between biomarkers and gene carriers and NeuroCart scores, correlations and regression analyses will be used.

NeuroCart scores will be analysed with an analysis of variance (ANOVA) and/or multivariate analysis of variance (MANCOVA) to estimate differences between the groups of elderly with different Aβ status.

The intra- and inter-individual diurnal fluctuation will be investigated with mixed-model analysis.

2 STUDY OBJECTIVES

Main objectives:

To define an algorithm based on the plasma biomarkers: Aβ40, Aβ42, t-Tau, p-Tau, NfL and APOE ε status and NeuroCart tests, age, grip strength and level of education that distinguishes between positive and negative Aβ CSF in healthy elderly.

Exploratory objective:

To determine the correlation between AD-specific biomarkers in CSF (Aβ 1-40 and 1-42, Tau proteins, NfL) and the NeuroCart scores in a cohort of 200 healthy elderly subjects.

To determine the correlation between AD-specific biomarkers in plasma (Aβ 1-40 and 1-42, Tau proteins, NfL) the NeuroCart scores in a cohort of 200 healthy elderly subjects.

To determine the correlation between AD-specific biomarkers in CSF (Aβ 1-40 and 1-42, Tau proteins, NfL) and in plasma in a cohort of 200 healthy elderly subjects.

To determine the correlation between APOE ε genotype and the Aβ /Tau/NfL concentrations in CSF in a cohort of 200 healthy elderly subjects.

To determine the correlation between APOE ε genotype and the Aβ /Tau/NfL concentrations in plasma in a cohort of 200 healthy elderly subjects.

To characterize the cohort of healthy elderly subjects in order to yield norm scores for specific NeuroCart tests in all subjects.

To characterize the plasma biomarkers across multiple times of day to gain understanding of intra- and inter-subject variability, and thus diurnal fluctuation.

3 STUDY DESIGN

3.1 Overall study design and plan

The total duration of the study for each subject will be a minimal of 2 days divided as follows:

- Screening: Up to 21 days before study day;
- Study assessments: Day 1.

3.1.1 Screening

Before deciding to participate in this study, subjects will be invited to take part in an information meeting during which all study related procedures will be explained and possible risks will be extensively addressed.
The screening phase will only be started after full written, verbal and signed informed consent has been obtained, according to CHDR standard operating procedures. A medical screening (medical history, physical examination, routine haematology and urinalysis) will be performed to assess a subject's eligibility for this study. This to exclude any comorbid disorders which might influence the study results. Next to the medical screening there will also be a cognitive screening part. Subjects will be screened for significant symptoms of dementia and depression using the MMSE and GDS-15, respectively. The NeuroCart assessments will be practiced following the medical and cognitive screening, to study the subject's capability to perform the NeuroCart tests during the study visit.

3.1.2 Neuropsychological testing period

The time schedule of the study day is provided in the study flow charts Table 1. Subjects will visit CHDR for approximately 4 hours on the study day and will not have to be admitted or stay overnight.

3.1.3 Follow-up

There will be no follow-up visit as no clinical/safety follow-up measurements are needed in this non-interventional, correlational study. The first 20 subjects who have participated in the study will be asked to fill in a visual analogue scale question rating the burden of the study. Also, these 20 subjects will be contacted by telephone 48 hours after the LP was performed to check for adverse events.

4 STUDY POPULATION

4.1 Subject population

A minimum of 200 subjects will be enrolled in the study following satisfactory completion of a screening visit where eligibility for the study will be checked. Males and females will be included from the age of 65 and over. At least a hundred subjects will be above the age of 70. Subjects will be randomised to participate in the morning or afternoon to have an equal time of day distribution.

Subjects will be recruited via media advertisement or from the subjects' database of the Centre for Human Drug Research, Leiden, the Netherlands.

4.2 Inclusion criteria

1. Males and females, aged 65 and older (inclusive);
2. Willing and able to perform the cognitive tests, as evidenced by performance on the training session of the cognitive tests;
3. Willing and able to give written informed consent.

4.3 Exclusion criteria

1. Legal incapacity or inability to understand or comply with the requirements of the study;
2. Evidence of cognitive deterioration, as indicated by a diagnosis of a cognitive disorder (including but not limited to MCI, Alzheimer's disease, Lewy Body Dementia, Fronto-temporal Dementia);
3. History or symptoms of significant psychiatric disease in the past 3 years (including but not limited to clinical depression, schizophrenia);
4. A Mini Mental State Examination (MMSE) score of ≤24;
5. A Geriatric Depression Scale - 15 (GDS) score of ≥6;
6. Presence of drug abuse, or positive urine drug screen (UDS) at screening or occasion;
7. Presence of severe alcohol abuse (daily alcohol consumption exceeding 2 standard drinks per day on average for females or exceeding 3 standard drinks per day on average for males (1 standard drink = 10 grams of alcohol)), or a positive breath alcohol test at screening or occasion;
8. Any contradictions for a lumbar puncture as judged by the principal investigator;
9. Any other reason that it is not safe or ethical to allow a subject to participate in the study in the opinion of the investigator.

4.4 Study population subgroups

General cognitive functioning, dementia status and depressive status will be assessed at pre-study screening. All subjects eligible according to the in- and exclusion criteria will be entered in the study. Subjects will be asked to give consent for a LP.

Plasma levels of Aβ40, Aβ42 and NfL, APOE ε status and NeuroCart tests of the 200 subjects will be used to define an algorithm that will be able to improve the efficiency of finding healthy subjects who have an Aβ positive (CSF) profile. The subjects will be divided in a group yielding subjects who are suspected of AD pathology (ADpath+) by the algorithm and subjects who are suspected to be without AD pathology (ADpath-)

To confirm the correct classification potential of the algorithm, the outcome will be compared to the CSF Aβ outcome of the subjects. CSF ADpath+ and a CSF ADpath- defined by an Aβ 1-42 CSF

level ≤450 pg/mL for ADpath+ subjects and an Aβ 1-42 CSF level ≥451 pg/mL for ADpath- subjects, will be used to differentiate between the two groups.

4.5 Referral to specialized memory clinic

Before participation in this study, subjects will be tested for eligibility. To check if a subject is able to give consent, the subjects will be screened on cognitive and psychiatric functioning at screening. The general practitioner of each subject will be informed about possible study participation, following written consent from the subject.

When a previously presumed cognitively healthy elderly subject is showing signs of dementia, as defined by a MMSE score of ≤18, or when a subject fulfills the criteria of prodromal AD (MCI and a *positive* CSF biomarker profile (Aβ 1-42 level ≤450 ng/L)), a referral to the specialized memory clinic of the Vrije Universiteit University Medical Center (VUmc) will be discussed. The diagnosis of MCI is met according to the following criteria: (1) memory complaints, (2) normal activities of daily living, (3) normal general cognitive function, (4) abnormal memory for age, and (5) not demented [28]. At the memory clinic of the VUmc, further diagnostics (e.g. MRI) and counselling will take place.

4.6 Concomitant medications

Any medication which affects the central nervous system is not allowed as judged by the principal investigator. All prescription and over-the-counter (OTC) medications, vitamins, herbal and dietary supplements used at screening and occasion will be recorded.

4.7 Lifestyle restrictions

Approximate meal times will be according to the study schedule. Poppy seed or foods containing poppy seeds are not permitted from 3 days before screening.

Alcohol will not be allowed from at least 24 hours before screening and before the study day. Between screening and the study visit, daily alcohol consumption exceeding 2 standard drinks per day on average for females or exceeding 3 standard drinks per day on average for males will not be permitted (one unit is 10 grams of alcohol). Subjects will undergo an alcohol breath test the morning of the study day.

Subjects will not be allowed to have excessive caffeine consumption, defined as >800 mg per day from 7 days prior to the study day. Subjects will abstain from caffeine-containing products for 24 hours prior to the start of the screening and study day. Caffeine quantities defined as: one cup of coffee contains 100 mg of caffeine; one cup of tea, or one glass of cola, or potion of chocolate (dark:100 g, milk 200 g) contains approximately 40 mg of caffeine; one bottle of Red Bull contains approximately 80 mg of caffeine.

Strenuous physical activity (e.g., heavy lifting, weight or fitness training) is not allowed from 48 hours prior to the screening. Light ambulatory activities (e.g. walking at normal pace) will be permitted.

Subjects are asked not to use NSAIDs (e.g. ibuprofen, diclofenac) in the ten days prior to the study day till one day after the study day (LP).

Subjects will be required to fast 4 hours before the start of the study occasion. Water is allowed as required. Subject will only have to fast for the first blood sample on the study day and will be offered breakfast/lunch thereafter.

4.8 Study withdrawal and replacement

4.8.1 Subject withdrawal

Subjects have the right to withdraw from the study at any time for any reason. Should a subject decide to withdraw from the study, all efforts should be made to complete and report the observations as thoroughly as possible.

4.8.2 Replacement policy

Subjects withdrawing will be replaced at the discretion of the investigator.

5 INVESTIGATIONAL MEDICINAL PRODUCT

Not applicable.

5.1.1 Blinding

Not applicable.

6 CLINICAL ENDPOINTS

6.1 Clinical endpoints

- Concomitant medication
- Clinical laboratory tests
  - Haematology
  - Urinalysis
- Vital signs
  - Pulse Rate (bpm)
  - Systolic blood pressure (mmHg)
  - Diastolic blood pressure (mmHg)

6.2 CNS outcome variables

NeuroCart tests:

- Adaptive tracking:
  - average performance (%);

- Visual Verbal Learning Test (VVLT) memory testing
  - immediate recall 1 (number correct),
  - immediate recall 2 (number correct),
  - immediate recall 3 (number correct),
  - delayed recall (number correct),
  - delayed recognition (number correct), and
  - delayed recognition (average reaction time correct) (msec).

- Milner Maze test:
  - Immediate
    - Total errors
    - Total moves
    - Time (msec)
  - Reversed
    - Total errors
    - Total moves
    - Time (msec)
  - Delayed
    - Total errors
    - Total moves
    - Time (msec);

- Face Encoding and Recognition Task:
  - Average reaction time for number correct recognition task;
  - Number correct recognition task;

- N-Back task:
  - (nr correct-nr incorrect)/total for zero-back

  - (nr correct-nr incorrect)/total for one-back
  - (nr correct-nr incorrect)/total for two-back
  - average reaction time for zero back (msec)
  - average reaction time for one back (msec)
  - average reaction time for two back (msec);

- Sustained Attention to Response Task:
  - Total errors;
  - Total number of omission errors;
  - Total number of commission errors;
  - Mean reaction time correct responses;
  - Reaction time variability;
  - Post error slowing score.
- Finger tapping:
  - Mean tapping rate;
  - Standard deviations.

Neurophysiological NeuroCart assessments:

- The following EEG endpoints will be determined at each time point and summarized:

Primary:

  - Alpha-power Fz-Cz (uV)
  - Alpha-power Pz-O1 (uV)
  - Alpha-power Pz-O2 (uV)
  - Beta-power Fz-Cz (uV)
  - Beta-power Pz-O1 (uV)
  - Beta-power Pz-O2 (uV)
  - Gamma-power Fz-Cz (uV)
  - Gamma-power Pz-O1 (uV)
  - Gamma-power Pz-O2 (uV)
  - Delta-power Fz-Cz (uV)
  - Delta-power Pz-O1 (uV)
  - Delta-power Pz-O2 (uV)
  - Theta-power Fz-Cz (uV)
  - Theta-power Pz-O1 (uV)
  - Theta-power Pz-O2 (uV)

When primary EEG endpoints show results that warrant further analysis in the opinion of the investigator, further analysis will be performed and will be described in the SAP.

- Saccadic eye movements:
  - saccadic reaction time (second),
  - saccadic peak velocity (degrees/second), and
  - saccadic inaccuracy (%);
- Smooth pursuit eye movements:

- percentage of time the eyes of the subjects are in smooth pursuit of the target (%).

Neuropsychological tests

- GDS-15 (only at screening):
  - Total score
- MMSE (only at screening):
  - Total score
- CDR:
  - Sum of boxes
- IADL:
  - Total score

Handgrip strength

- Handgrip strength dominant hand

6.3 Biochemical endpoints

CSF biomarkers

- Aβ concentration (1-40, 1-42 and 1-42/1-40 ratio);
- T-Tau and p-Tau concentrations;
- NfL concentration.

Plasma markers

- Aβ concentration (1-40, 1-42);
- T-Tau and p-Tau concentrations;
- NfL concentration.

Exploratory biomarkers including but not limited to:

- Synaptic loss; Neurogranin [24],
- Glial inflammation; YKL-40 [25],
- Levels of p-Tau181 in extracts of neutrally-derives blood exosomes [26],
- MicroRNAs [MiR-155, MiR-107 and MiR-29 [27]]).

Genetic assessments

- APOE ε genotype.

7 STUDY ASSESSMENTS

See Table 1 for the time points of the assessments.

7.1 Clinical assessments

A short physical examination is performed before study participation to check if a subject is eligible e.g. has no general health problems, no comorbidities which might influence study participation and/or the study endpoints.

7.1.1 Vital signs

Evaluations of systolic and diastolic blood pressure, pulse rate, and temperature will be performed at screening. Pulse and blood pressure will be taken after 5 minutes in the supine position, thereafter the blood pressure will be measured in a standing position. There will be a gradual transition to the erect position. Automated oscillometric blood pressures will be measured using a Dash 3000, Dash 4000, Dynamap 400 or Dynamap ProCare 400. Additionally, the pulse rate data is provided by the pulse oximeter attached to the monitor.

7.1.2 Weight and height

Weight (kg) will be recorded at screening. Height (cm) will be recorded and body mass index (BMI) calculated at screening. Also the waist/hip ratio will be measured at screening as BMI and the waist/hip ratio is associated with worse cognitive functioning at later age [29].

7.1.3 Physical examination

Physical examination (i.e., inspection, percussion, palpation and auscultation) is performed during the screening. Clinically relevant findings that are present prior to study participation must be recorded with the subject's Medical History. Clinically relevant findings found after the start of study participation and meeting the definition of an adverse event (AE, new AE or worsening of previously existing condition) must be recorded.

7.1.4 Laboratory assessments

Laboratory parameters

Blood and other biological samples will be collected during screening for the following clinical laboratory tests:

Lab	Tests	Collection & Analysis
Haematology	Haemoglobin [including Mean Corpuscular volume (MCV), Mean corpuscular haemoglobin (MCH), Mean corpuscular haemoglobin concentration (MCHC)], haematocrit, red cell count (RBC), total white cell count (WBC), leukocyte differential count and Platelet count. Differential blood count, including: basophils, eosinophils, neutrophils, lymphocytes, and monocytes.	4 mL of venous blood in a BD Vacutainer® K2EDTA tube. Samples will be analysed by the Central Clinical Hematology Laboratory (CKHL) of Leiden University Medical Center.
Serology	HIV1 and HIV2 antibodies, Hepatitis B antigen and Hepatitis C antibodies	5 mL of venous blood in a BD Vacutainer® SST Gel and Clot Activator tube. Samples will be analysed by the Central Clinical Microbiology Laboratory (CKML) of the Leiden University Medical Center.
Urinalysis	Leucocytes, blood, nitrite, protein, urobilinogen, bilirubin, pH, specific gravity, ketones, glucose. If there is a clinically	A midstream, clean-catch urine specimen will be analysed by dipstick (Multistix® 10 SG,

	significant positive result, urine will be sent to the CKCL for microscopy and/or culture.	Siemens Healthcare Diagnostics, Frimley, UK).
Alcohol	Alcohol Breath Test	The hand-held Alco-Sensor IV meter (Honac, Apeldoorn, the Netherlands) will be used to measure the breath ethanol concentrations.
Urine drug screen	Cocaine, amphetamines, opiates (morphine), benzodiazepines and cannabinoids.	A urine specimen will be analysed at CHDR by test kit (InstAlert, Innovacon, San Diego, USA).

DNA collection

Genotypes of the APOE ε gene have been shown to be relevant for amyloid metabolism and deposition. The APOE ε4 genotype is associated with the risk to develop AD, but as well with higher amyloid load in the brain in healthy elderly and patients with AD [30]. A blood sample of 4mL will be collected in a K2EDTA tube according to standard operating procedures. Samples will be stored at –80°C. Remaining blood from the samples will be used for future exploratory DNA research after which possible remaining samples will be destroyed.

CSF sampling

A CSF sample for estimation of Aβ concentration (1-40, 1-42 and 1-42/1-40 ratio), Total tau concentration, Phosphorylated tau concentration and Neurofilament Light will be collected at a pre-determined time point, see Table 1. A recent study by Janelidze et al., (2016) concluded that calculating the ratio between Aβ40/Aβ42 is significantly better in detecting amyloid deposition in prodromal AD and to differentiate AD patients from healthy elderly than (only) measuring lowered Aβ42 and will therefore also be calculated in the current study [31]. Apart from the standard assessments used for the diagnosis of Alzheimer's disease as Aβ concentrations and Tau, Neurofilament Light will also be determined. Neurofilament light (NfL) chain is a putative marker of subcortical large-caliber axonal degeneration and increased NfL in CSF has been linked to inflammatory diseases, frontotemporal lobe dementia and AD [32]. Recent studies have found that NfL is associated with AD disease progression and that NfL correlates with cognitive performance over time [33]. Also the presence of white matter changes was associated with increased levels of NfL, which probably reflects axonal degeneration [34], this in turn will cause worse cognitive performance.

A CSF sample of 4 mL will be collected in a 10 mL polypropylene tubes and transferred to the lab. Time of sampling is recorded. CSF is centrifuged within one hour, at 2000g for 10 minutes at 4°C. Prior to centrifugation, CSF samples are kept at room temperature. Immediately after centrifugation, samples are divided into 0.5 ml aliquots in clean Sarstedt polypropylene 0.5 mL tubes and stored at –80°C. All aliquots are clearly labeled. Lumbar punctures will be performed by a trained, physician with a 25 G atraumatic lumbar puncture needle (Braun, 25G), who will be supervised by an experienced neurologist. The needle will be placed at the L3-L4 or L4-L5 interspace with the subject in supine or sitting position. If a subject suffers from post-dural headaches, the subject will be treated according to our standard operating procedures.

7.2 Blood collection

Approximately 10 mL blood will be collected via an i.v. catheter placed in an antecubital vein in the arm in appropriate K2EDTA tubes. Immediately following collection of the required blood volume, the tubes will be slowly tilted backwards and forwards (no shaking) to bring the anti-coagulant into

solution. Blood samples will be collected over the time periods indicated in the schedule of assessments. The blood plasma samples for bioanalysis are centrifuged within one hour, at 2000g for 10 minutes at 4°C. Prior to centrifugation, plasma samples are kept at room temperature. Immediately after centrifugation, supernatant is divided into 0.5 ml aliquots in Sarstedt polypropylene 0.5mL tubes and stored at –80°C. Plasma samples should not be hemolytic.
The exact actual clock time of withdrawal of the blood sample will be recorded.
The blood plasma samples for bioanalysis will be used for the bioanalysis with the ultrasensitive Single Molecule (Simoa HD-1 analyzer) immunoassay technology. Left-over blood samples will be used for exploratory analysis for AD biomarkers including but not limited to neurogranin, YKL-40 and microRNA.

7.2.1 Labelling

Pre-printed, waterproof labels will be used to identify the tubes used during sample collection and for storage of separated plasma. Each label will contain the following information:

- CHDR Protocol number
- Subject Number
- Occasion number (date)
- Protocol (delta) time
- Activity: Sample type (blood) & purpose

7.2.2 Shipping Procedures

CHDR will arrange shipment of the samples. The samples must be packed securely together with completed shipment forms in polystyrene insulated shipping containers together with enough dry ice to last for 48 hours.

The APOE ε genotyping samples will be shipped in batches to the LUMC Humane Genetics lab. Until analysis, aliquoted samples are stored at -80°C.

7.2.3 Bioanalysis

The LUMC Humane Genetics lab will determine APOE ε genotype status for all subjects by isolate the DNA using the commercially available QIAamp DNA Blood MINI kit. After isolation of the DNA, the DNA samples will be used to determine the APOE genotype using the PCR sequence protocol followed by a sequential analysis (according the Sanger method). APOE genotype will be derived from the analysis and reported per subjects.

CSF and Blood plasma will be assessed for the presence of the biomarkers Aβ40, Aβ42, total tau and NfL, using the ultrasensitive Single Molecule (Simoa HD-1 analyzer) immunoassay technology. Phosphorylated Tau will also be assessed in the CSF samples using this method. The left-over aliquots are used for repetition of measurement, when necessary, or exploratory analysis (including but not limited to neurogranin, YKL-40 and microRNA).

For a full description of the bioanalysis of the CSF samples and plasma samples using the Simoa HD-1 analyzer please refer to the lab manual. Exploratory biomarker analysis will also be described in the lab manual.

7.2.4 Concomitant medications

Concomitant medications initiated, stopped, up-titrated or down-titrated will be recorded.

7.3 CNS assessments and questionnaires

CHDR has developed and validated a computer-based battery of non-invasive, minimally burdensome cognitive, psychomotor and neurophysiological measures that are sensitive to the functional pharmacodynamic (PD) effects of a vast array of different central nervous system (CNS) active drugs. The NeuroCart includes tests that allow standardized assessment of the principal

cognitive domains, including memory and attention. In previous studies the NeuroCart showed a decline in performance on tests related to sustained attention in healthy elderly subjects compared to healthy young, and yet lower performance in healthy elderly with below normal cognition with lowest performance in patients with mild to moderate AD (not yet published). Exploring the relationship between NeuroCart performance and Aβ status in (healthy) subjects will be an exploratory objective of this study. The NeuroCart will also be used to create norm scores on the performed measurements and create a NeuroCart phenotype in healthy elderly subjects. All measurements will be performed in a quiet room with ambient illumination. Per session, there will only be one subject in the same room.

GDS-15
The geriatric depression scale - 15 is a screening instrument used to screen for depression, specifically for elderly [35]. The elderly subjects is asked to fill in 15 questions which represent the subject's feelings during the past week. The GDS-15 will be assessed during screening.

MMSE
The mini mental state examination is the most commonly used instrument for screening cognitive function. The MMSE is a brief, quantitative measure of cognitive status in adults. It can be used to screen for cognitive impairment, to estimate the severity of cognitive impairment at a given point in time, to follow the course of cognitive changes in an individual over time, and to document an individual's response to treatment. This examination is not suitable for making a diagnosis but can be used to indicate the presence of cognitive impairment such as in a person with suspected dementia or following a head injury. The MMSE is far more sensitive in detecting cognitive impairment than the use of informal questioning or overall impression of a subjects orientation. The test takes about 10 minutes [36]. The MMSE will be assessed during the screening.

Adaptive tracking
The adaptive tracking test will be performed as originally described by Borland and Nicholson [37, 38], using customised equipment and software (based on TrackerUSB hard-/software (Hobbs, 2004, Hertfordshire, UK)). The average performance and the standard deviation of scores over a 3.5-minute period will be used for analysis. This 3.5-minute period is including a run in time of 0.5 minute, in this run in time the data is not recorded. Adaptive tracking is a pursuit-tracking task. A circle moves randomly about a screen. The subject must try to keep a dot inside the moving circle by operating a joystick. If this effort is successful, the speed of the moving circle increases. Conversely, the velocity is reduced if the test subject cannot maintain the dot inside the circle.

Each test is preceded by three training sessions and includes two baseline measurements. After 4 to 6 practice sessions, learning effects are limited. The adaptive tracking test is more sensitive to impairment of eye-hand coordination by drugs than compensatory pursuit tasks or other pursuit tracking tasks, such as the pursuit rotor. The adaptive tracking test has proved to be useful for measurement of CNS effects of alcohol [39], various other psychoactive drugs [40] and sleep deprivation [41].

The adaptive tracking test has proved very sensitive to cholinergic stimulation in both healthy young and healthy elderly in earlier studies performed at CHDR (data not published yet). Voluntary attention and psychomotor functioning, which are measured by the adaptive tracking test, was found to be enhanced after cholinergic stimulation [42, 43].

Visual verbal learning test (VVLT, 30 words)
Volunteers that perform the VVLT are presented 30 words in three consecutive word trials, i.e. word learning test (VVLT30). Each trial ends with a free recall of the presented words (Immediate Recall- a test to determine acquisition and consolidation of information). Approximately thirty minutes after start of the first trial, the volunteers are asked to recall as many words as possible (Delayed Recall-

this test measures active retrieval from long term memory). Immediately thereafter, the volunteers undergo memory recognition test, which consists of 15 presented words and 15 'distractors' (Delayed Recognition- testing memory storage). Importantly, volunteers are not allowed to write words down at any time during the whole test procedure.

Nathan and colleagues [44] have found that the International Shopping List test, a test of both immediate recall and delayed recall over multiple trials showed strong pharmacodynamics effects after administration of an M1 receptor allosteric agonist. The VVLT is similar to the test used by Nathan et al. as over several trials both immediate and delayed recall of words is tested, as well as delayed recognition.

Milner Maze test

The Milner Maze Test (MMT) is a computerised version of the Milner Maze (1965). This is a spatial working memory test which was developed Milner and based on an early hidden maze task developed by Barker (1931) and extended by Milner (1965). Volunteers have to complete a maze by using trial and error learning to locate a 28-step pathway that is hidden beneath a 10×10 grid of tiles. Individuals attempt to find the same pathway on successive trials. The test contains thirty well-matched maze versions to ensure that the volunteer never performs the same hidden path twice throughout the study. There are three types of trials in the MMT: Immediate for imprinting (five times the same path version) and Delayed (the same path once) and Reversed (the same path once in reversed direction) for memory function. Spatial working memory function can be inferred from the improved accuracy and speed of decisions across these trials [45].

The Milner maze test is similar to the Groton maze learning test. The Groton maze learning test has been shown to be very sensitive to cholinergic compounds such as donepezil and the anticholinergic effects of scopolamine [46, 47]. Furthermore, spatial learning tests in general have been able to show the negative pharmacodynamics effects of muscarinic antagonism [37].

On each study day, the volunteer pseudo-randomly receives different mazes from the thirty well-matched alternate forms for this test. This is to prevent that a volunteer completes the same hidden path form more than once throughout the study. Therefore, a randomization scheme for the path versions is created for each MMT trial in the study.

During a CNS training session, the volunteer will complete the complete MMT (Immediate, Reversed and Delayed), with a training maze version. During the occasion, volunteers are presented a MMT immediate with five consecutive trials. Immediately thereafter, the volunteers undergo the reversed Milner Maze Test, which consists of one trial of the same maze in reverse (from bottom-right to upper-left). Approximately thirty minutes after start of the Immediate-Reversed block, the volunteers are asked to perform the Milner Maze again with the same maze (Delayed MMT). The main outcomes of the MMT are the total time that is used for exploration (this excludes time involved with illegal moves) and accuracy (correct or incorrect) of the responses. Incorrect responses can be divided into exploration errors, illegal moves, and repeat errors. An exploration error is an incorrect response made in accord with the standard MMT rules whereas illegal moves are errors made as a result of breaking one of the rules. Repeat errors are registered when the subject is repeatedly trying to explore in the same direction. In total, the task takes about 4 - 8 min to complete.

Face Encoding and Recognition Task

Recognition tests involving memory have appeared in several formats. The facial encoding and recognition task measures the episodic memory. This task requires successful storage of new explicit memory and successful retrieval of episodic memory. A simplified version of the face encoding and recognition task has been used in studies with Alzheimer patients [48].
The face encoding and recognition task consists of two parts; the encoding task and the recognition task. In the encoding task, participants are presented with a series of 24 photographs (faces) at a

rate of one every 2 seconds and are asked to attend to the stimuli. In the recognition task participants are asked to indicate whether the picture (N= 48; 24 familiar, 24 unfamiliar) shown is a familiar or an unfamiliar face by button presses. Seven different (0-6) but comparable versions have been constructed, randomized and validated. Version 0 can be used for training of subjects. Reaction time will be recorded and accuracy will be determined by subtracting the false answers from the correct answers, and dividing this by the total of items (= 24), resulting in a value ranging from -1 to 1 (with 0 as chance level). This version was adapted from the WMS – III Faces (Wechsler, 1997b; as described in Lezak, et al., 2004).

N-back test

Following Rombouts et al. (2002) [38], the N-Back test consists of three conditions, with increased working memory load: (Condition 0) "X" condition, in which participants are required to indicate whether the presented letter is a "X" (=target) or another letter; In Condition 1 and 2, letters will be presented sequentially (1.5 seconds for a letter [consonant, except for the letter "z"] presentation, followed by a black screen for 0.5 seconds). "z" is pressed on the keyboard for a target and "/" is pressed for a non-target. Condition 1,"1-back" condition, in which participants are required to indicate whether the letter presented is a repetition without any other letter intervening (e.g., B … B); Condition 2, "2-back" condition, in which participants are required to indicate whether a letter is repeated with one other letter in between (e.g., B … C … B).The 3 conditions are presented in 3 blocks with increasing working memory load. Each condition starts with a training (7 consonants; target:non-target 3:4), followed by the test (24 consonants; target:non-target 1:3).

Sustained Attention to Response Task

The SART is programmed in E-prime 2.0 and administered using a desktop computer. In the SART, single white digits (1-9) are randomly presented in the centre of a computer screen against a black background. The digits appeared in one of five font sizes (48, 72, 94, 100, 120 point), randomly selected at each trial. The digits are presented for 250 ms and followed by a mask slide (cross in circle) for a duration of 900 ms. Subjects are asked to respond by pressing a single response key (space bar) as quickly and accurately as possible after the appearance of each digit. The exception is the nominated no-go target; digit '3'. Formal testing is preceded by a practice session of 18 digits (2 no-go targets). The practice session is not included in the analysis. Each SART contains 225 trials (200 go targets and 25 no-go targets).

The main outcome measure of the SART is the total error score, containing 'commision errors' (i.e. key presses when no key should have been pressed; on no-go trials) and 'omission errors' (i.e. no key presses when the key should have been pressed; on go trials). Thus, the SART total error score equals the sum of the commission and the omission errors. In addition, the mean reaction time of all correct response trials (MeanRTcorrect) is calculated. The RT variability is measured by dividing the standard deviation by the mean RT. Post error slowing is a phenomenon where, choice-response tasks, reaction time is significantly increased after the occurrence of an error [49]. The post error slowing score is quantified as follows: the difference between the post error RT and the pre-error RT are divided by the mean RT of that session. In the case of the SART, the post error slowing score is based solely on the commission errors.

Finger tapping

The test has been adapted from the Halstead Reitan Test Battery [50], and evaluates motor activation and fluency. Speed of finger tapping is measured for the index finger for the dominant hand; a session contains five performances of 10 seconds (eg. 5 x 10 seconds). Feedback on performance is given by a counter in the centre of the screen, while the amount of taps of each 10 second trial is shown on the screen in between the trials. The space bar is used as tapping device. The volunteer is instructed to tap as quickly as possible with the index finger of the dominant hand and to rest the wrist on the table. The mean tapping rate and the standard deviations for the dominant hand are used for statistical analysis. The test is programmed in E-prime.

21-leads EEG

Electroencephalography (EEG) will be used to monitor brain activity, which can accurately index normal and abnormal brain aging in a non-invasive method [51]. EEG recordings will be obtained at times specified in the schedule of assessments, Table 1. A 8-minute resting EEG will be performed in which the measurement will be performed when subjects are resting alternating with their eyes closed and their eyes opened for four minutes on each condition.

Saccadic eye movements

A growing body of literature has investigated changes in eye movements as a result of Alzheimer's disease (AD). When compared to healthy, age-matched controls, patients display a number of remarkable alterations to oculomotor function and viewing behavior such as increased latency to initiate smoot or saccadic eye movements [52].

Saccadic eye movements will be recorded at a training session, pre-dose and at times specified in the protocol. Recording of eye movements will be performed in a quiet room with dimmed lightning. There will be only one subject per session in the same room.
Recording and analysis of saccadic eye movements is conducted with a microcomputer-based system for sampling and analysis of eye movements. The program for signal collection and the AD-converter is from Cambridge Electronic Design (CED Ltd., Cambridge, UK), the amplification by Grass (Grass-Telefactor, An Astro-Med, Inc. Product Group, Braintree, USA) and the sampling and analysis scripts are developed at the CHDR (Leiden, the Netherlands). Disposable silver-silver chloride electrodes (Ambu Blue Sensor N) will be applied on the forehead and beside the lateral canthi of both eyes of the subject for registration of the electro-oculographic signals. Skin resistance is reduced to less than 5 kOhm before measurements. Head movements are restrained using a fixed head support. The target consists of a moving dot that is displayed on a computer screen. This screen is fixed at 58 cm in front of the head support.

Saccadic eye movements are recorded for stimulus amplitudes of approximately 15 degrees to either side. Fifteen saccades are recorded with interstimulus intervals varying randomly between 3 and 6 seconds. Average values of latency (reaction time), saccadic peak velocity of all correct saccades and inaccuracy of all saccades will be used as parameters. Saccadic inaccuracy is calculated as the absolute value of the difference between the stimulus angle and the corresponding saccade, expressed as a percentage of the stimulus angle.

Smooth eye movements

The same system as used for saccadic eye movements is also used for measurement of smooth pursuit. For smooth pursuit eye movements, the target moves at a frequency ranging from 0.3 to 1.1 Hz, by steps of 0.1 Hz. The amplitude of target displacement corresponds to 22.5 degrees eyeball rotation to both sides. Four cycles are recorded for each stimulus frequency. The time in which the eyes are in smooth pursuit of the target will be calculated for each frequency and expressed as a percentage of stimulus duration. The average percentage of smooth pursuit for all stimulus frequencies will be used as parameter.

CDR

The Washington University Clinical Dementia Rating (CDR) is used in longitudinal studies and clinical trials for staging the severity of Alzheimer's disease (AD). The CDR is derived from a semi structured interview with the patient and rates impairment in each of six cognitive categories (Memory, Orientation, Judgment and Problem Solving, Community Affairs, Home and Hobbies, and Personal Care) on a five-point scale in which none = 0, questionable = 0 5, mild = 1, moderate 2, and severe = 3. (Note Personal Care has no questionable impairment level) From the six individual category ratings, or "box scores," the global CDR is established by clinical scoring rules which CDR 0 = no dementia and CDR 0.5, 1, 2 or 3 indicates questionable, mild, moderate or severe dementia [53].

IADL

The instrumental activities of daily living scale was developed to asses complex activities necessary for functioning in community settings (e.g. shopping, cooking, managing finances). The capacity to handle these complex functions normally is lost before basic 'activities of daily living' (e.g. eating, bathing, toileting) which are measured by ADL scales. Therefore assessing IADL may identify incipient decline in older adults or otherwise capable and healthy. The IADL contains 8 items that are rated with a summary score and takes around 10 minutes [54].

JAMAR dynamometry – handgrip strength

As handgrip strength may serve as a predictor of cognitive loss with advancing age [55], the JAMAR dynamometry is used in this study. The subject sits comfortably in a standard chair with legs, back support and fixed arms. The subject will be asked to rest the forearms on the arms of the chair with the wrist just over the end of the arm of the chair—wrist in a neutral position, thumb facing upwards. The subject must be demonstrated how to use the Jamar handgrip dynamometer to show that gripping very tightly registers the best score. The subject should perform this test with the dominant hand. The subject should position the hand so that the thumb is round one side of the handle and the four fingers are around the other side. The instrument should feel comfortable in the hand of the subject. The position of the handle can be altered if necessary. The observer should rest the base of the dynamometer on the palm of the hand of the subject as the subject holds the dynamometer. The aim of this is to support the weight of the dynamometer (to negate the effect of gravity on peak strength), but care should be taken that its movement is not restricted. The subject must be encouraged to squeeze as long and as tightly as possible. The measurement outcome (grip strength in kilograms) is digitally recorded in the data collection form and will be repeated twice to give three readings in total. The best of the three grip strength measurements is used in statistical analyses so as to encourage the subjects to get as high a score as possible. The hand dominance, i.e. right, left or ambidextrous (people who can genuinely write with both hands) of the subject must be recorded. This method is adapted from the standardised method of Roberts et al [56].

7.4 Sequence of assessments and time windows

Assessments will be performed where possible according to the schedule of assessments in a top down order. All assessments are given an indicative expected time.

7.5 Total blood volume

Blood samples are taken at screening and/or the study day with a total amount of 43 mL.

Sample	Samples taken		Sample Volume*			Volume	
Haematology	1	x	4	mL	=	4	mL
Serology	1	x	5	mL	=	5	mL
Biomarker	3	x	10	mL	=	30	mL
Genotyping	1	x	4	mL	=	4	mL
* inclusive discarded volume			**Total blood volume/subject**			**43**	**mL**

8 SAFETY REPORTING

8.1 Definitions of adverse events

An AE is any untoward medical occurrence in a subject who is participating in a (clinical) study. The AE does not necessarily have to follow the administration of a study drug, or to have a causal relationship with the study. An AE can therefore be any unfavourable and unintended sign (including an abnormal laboratory or vital sign finding), symptom, or disease temporally associated with the study participation.

8.1.1 Intensity of adverse events

The intensity of clinical AEs is graded three-point scale as defined below:

- Mild: discomfort noticed but no disruption of normal daily activity;
- Moderate: discomfort sufficient to reduce or affect normal daily activity;
- Severe: inability to work or perform daily activity.

8.1.2 Chronicity of adverse events

The chronicity of the event will be classified by the investigator on a three-item scale as defined below:

- Single occasion: single event with limited duration;
- Intermittent: several episodes of an event, each of limited duration;
- Persistent: event which remained indefinitely.

8.1.3 Action

Eventual actions taken will be recorded.

8.1.4 Serious adverse events

A Serious Adverse Event (SAE) is defined by the International Conference on Harmonization (ICH) guidelines as any AE fulfilling at least one of the following criteria:

- - results in death;
- - is life threatening (at the time of the event);
- - requires hospitalisation or prolongation of existing inpatients' hospitalisation;
- - results in persistent or significant disability or incapacity;
- - is a congenital anomaly or birth defect; or
- - any other important medical event that did not result in any of the outcomes listed above due to medical or surgical intervention but could have been based upon appropriate judgement by the investigator.

An elective hospital admission will not be considered as a serious adverse event.

8.1.5 Reporting of serious adverse events

SAEs will be reported according to the following procedure.

The investigator will report the SAEs through the web portal ToetsingOnline (see https://toetsingonline.nl/) to the accredited EC that approved the protocol, within 7 days of first knowledge for SAEs that result in death or are life threatening followed by a period of maximum of 8 days to complete the initial preliminary report. All other SAEs will be reported within a period of maximum 15 days after the sponsor has first knowledge of the serious adverse events.

8.1.6 Follow-up of adverse events

All adverse events will be followed until they have abated or until a stable situation has been reached. Depending on the event, follow up may require additional tests or medical procedures as indicated, and/or referral to the general physician or a medical specialist.

8.2 Temporary halt for reasons of subject safety

In accordance to section 10, subsection 4, of the WMO, the investigator will inform the subjects and the EC if anything occurs, on the basis of which it appears that the disadvantages of participation may be significantly greater than was foreseen in the research proposal. The study will be suspended pending further review by the EC, except insofar as suspension would jeopardise the subjects' health. The investigator will ensure that all subjects are kept informed.

9 STATISTICAL METHODOLOGY AND ANALYSES

9.1 Statistical analysis plan

All safety and statistical programming is conducted with SAS 9.4 for Windows (SAS Institute Inc., Cary, NC, USA).

9.2 Protocol violations/deviations

Protocol deviations will be identified based on conditions related to the categories below:

- Protocol entry criteria
- Forbidden concomitant medications
- Missing evaluations for relevant endpoints
- Other protocol deviations occurring during study conduct.

Major protocol deviations will be identified before the study closure, and listed where appropriate.

9.3 Power calculation

This is an exploratory study and is therefore not based on statistical considerations.

9.4 Missing, unused and spurious data

All missing or incomplete clinical endpoints and data, including dates and times, are treated as such. Missing test results or assessments will not be imputed. Missing PD data, indicated as 'M' in the data listing, will be estimated within the statistical mixed model using SAS PROC MIXED.

For graphical and summary purposes PD and clinical endpoint values below the limit of quantification will be set to half (½) of the limit of quantification. For analysis no undetermined values will be replaced.

The handling of missing, unused and spurious data will be documented in the study report.

9.5 Analysis sets

Data of all subjects participating in the study will be included in the analyses if the data can meaningfully contribute to the objectives of the study.

9.6 Subject disposition

- Subject disposition will be listed by subject.

The following subject data will be summarized:

- Number and percentage of subjects screened,
- Number and percentage of subjects enrolled,
- Number and percentage of subjects included in the PD analysis population.
- Number and percentage of subjects completed,

A subject who completed the study is defined as a subject where the 4 hour biomarker plasma sample was assessed.

9.7 Baseline parameters and concomitant medications

9.7.1 Demographics and baseline variables

Continuous demographic variables (e.g., age, height, weight, BMI) will be summarized by descriptive statistics (n, mean, SD, median, Min, Max).

Qualitative demographic characteristics (sex, race/ethnicity) will be summarized by counts and percentages.

9.7.2 Medical history

Medical history will only be listed.

9.7.3 Concomitant Medications

All concomitant medications will be displayed in a listing.

9.7.4 Adverse events

The AE coding dictionary for this study will be Medical Dictionary for Regulatory Activities (MedDRA). It will be used to summarize both the number of events and the number of subjects with an AEs by primary system organ class (SOC) and preferred term (PT).
All adverse events will be displayed in listings.

9.8 CNS and biochemical endpoints

9.8.1 NeuroCart

All parameters will be listed by subject.

Parameters will initially be analysed without transformation, but if the data suggest otherwise, log-transformation may be applied.

Descriptive statistics will be used to summarize the data. Data will be graphically processed for visual inspection.

In order to develop an algorithm that can classify the subjects between ADpath+ and ADpath- there will be a two-step procedure. In the first step 500 bootstrap samples will be generated out of the original data and logistic regression with LASSO penalization (in order to account for the expected small event per variable ratio) in each of the bootstrap samples with all the candidate variables (CNS and biochemical outcome variables, age and educational level) will be performed. Out of all the candidate variables the ones that consistently and most frequently appear in the bootstrap models will be selected. Hence, in this first step the variable selection will take place. In the second step another 500 bootstrap samples will be generated and logistic regression will be performed (with penalization if necessary) in each of the bootstrap samples. This time only the variables that were selected in step 1 will be entered in the model. The estimates out of all the bootstrap models will be averaged and used in order to obtain predictions in the original data. Step two will result in the bootstrap-corrected predictions that will be assessed in terms of calibration (calibration slope), discrimination (c-index) and squared mean prediction error.

To investigate the relationship between biomarkers and gene carriers and NeuroCart scores, correlations and regression analyses will be used.

NeuroCart scores will be analysed with an analysis of variance (ANOVA) and/or multivariate analysis of variance (MANCOVA) to estimate differences between the groups of elderly with different Aβ status.

The intra- and inter-individual diurnal fluctuation will be investigated with mixed-model analysis.

9.8.2 Inferential methods

The study is exploratory and no formal null hypothesis is set. No adjustments for multiple comparisons will be applied.

9.9 Exploratory analyses and deviations

Exploratory data-driven analyses can be performed with the caveat that any statistical inference will not have any confirmatory value.
Deviations from the original statistical plan will be documented in the article.

10 GOOD CLINICAL PRACTICE, ETHICS AND ADMINISTRATIVE PROCEDURES

10.1 Good clinical practice

10.1.1 Ethics and good clinical practice

The investigator will ensure that this study is conducted in full compliance with the protocol, the principles of the Declaration of Helsinki, ICH GCP guidelines, and with the laws and regulations of the country in which the clinical research is conducted.

10.1.2 Ethics committee / institutional review board

The investigator will submit this protocol and any related documents to an Ethics Committee (EC) and the Competent Authority (CA). Approval from the EC and the statement of no objection from the CA must be obtained before starting the study, and should be documented in a dated letter/email to the investigator, clearly identifying the trial, the documents reviewed and the date of approval. A list of EC members must be provided, including the functions of these members. If study staff were present, it must be clear that none of these persons voted.

Modifications made to the protocol after receipt of the EC approval must also be submitted as amendments by the investigator to the EC in accordance with local procedures and regulations.

10.1.3 Informed consent

It is the responsibility of the investigator to obtain written informed consent from each individual participating in this study after adequate explanation of the aims, methods, objectives and potential hazards of the study. The investigator must also explain to the subjects that they are completely free to refuse to enter the study or to withdraw from it at any time for any reason.

The Informed Consent and Subject Information will be provided in Dutch.

10.1.4 Insurance

The investigator has a liability insurance which is in accordance with article 7, subsection 6 of the WMO.
The investigator (also) has an insurance which is in accordance with the legal requirements in the Netherlands (Article 7 WMO and the Measure regarding Compulsory Insurance for Clinical Research in Humans of 23rd June 2003). This insurance provides cover for damage to research subjects through injury or death caused by the study.

- € 650,000.- (i.e., six hundred and fifty thousand Euro) for death or injury for each subject who participates in the Research;
- € 5,000,000.- (i.e., five million Euro) for death or injury for all subjects who participate in the Research;
- € 7,500,000.- (i.e., seven million and five hundred thousand Euro) for the total damage incurred by the organisation for all damage disclosed by scientific research for the Sponsor as ‘verrichter’ in the meaning of said Act in each year of insurance coverage.

The insurance applies to the damage that becomes apparent during the study or within 4 years after the end of the study.

10.2 Study funding

CHDR is the sponsor of the study and is funding the study.

10.3 Data handling and record keeping

10.3.1 Data collection

Data will be recorded on electronic data collection forms in Promasys for subsequent tabulation and statistical analysis. The data will be handled confidentially and if possible anonymously.

A Subject Screening and Enrolment Log will be completed for all eligible or non-eligible subjects with the reasons for exclusion.

Some of the neuropsychological assessment are recorded on paper data collection forms and will be entered after quality control in a Promasys database for tabulation and statistical analysis.

10.3.2 Database management and quality control

All data from paper source will be entered into the Promasys database twice, by two different individuals. A quality control check will be done by CHDR staff on all data entered in the Promasys database, using data entry progress checks and database listings (blind data review). Errors with obvious corrections will be corrected before database lock.
Results of computer (NeuroCart) tests and electronically captured questionnaires and clinical laboratory will be sent electronically to CHDR and loaded into the database.

After the database has been declared complete and accurate, the database will be locked. Any changes to the database after that time can only be made by joint written agreement between the investigator and the statistician.

10.4 Access to source data and documents

All study data will be handled confidentially. The investigator will retain the originals of all source documents generated at CHDR for a period of 2 years after the report of the study has been finalised, after which all study-related documents will be archived (at a minimum) on micro-film which will be kept according to GCP regulations.
The investigator will permit trial-related monitoring, audits, EC review and regulatory inspections, providing direct access to source data and documents.

10.5 Quality control and quality assurance

This study will be conducted according to applicable Standard Operating Procedures (SOPs). Quality assurance will be performed under the responsibility of CHDR's Quality Assurance manager.

10.6 Protocol amendments

Any change to a protocol has to be considered as an amendment.

10.6.1 Non-substantial amendment

Administrative or logistical minor changes require a non-substantial amendment. Such changes include but are not limited to changes in study staff or contact details or minor changes in the packaging or labelling of study drug. Non-substantial amendments will be approved (signed) by the investigator(s) and will be recorded and filed by the investigator but will not be notified to the EC and the CA.

The implementation of a non-substantial amendment can be done without notification to the appropriate EC or CA. It does not require their approval.

10.6.2 Substantial amendment

Significant changes require a substantial amendment. Significant changes include but are not limited to: new data affecting the safety of subjects, change of the objectives/endpoints of the study, eligibility criteria, study assessments/procedures, study duration, with or without the need to modify the core Subject Information and Informed Consent Form.

Substantial amendments are to be approved by the appropriate EC and the CA will need to provide a 'no grounds for non-acceptance' notification prior to the implementation of the substantial amendment.

Urgent amendment

An urgent amendment might become necessary to preserve the safety of the subjects included in the study. The requirements for approval should in no way prevent any immediate action being taken by the investigators in the best interests of the subjects. Therefore, if deemed necessary, an investigator can implement an immediate change to the protocol for safety reasons. This means that, exceptionally, the implementation of urgent amendments will occur before submission to and approval by the EC(s) and CA.

10.7 End of study report

The investigator will notify the EC of the end of the study within a period of 8 weeks. The end of the study is when 200 subjects have had their study visit.
In case the study is ended prematurely, the investigator will notify the EC, including the reasons for the premature termination.

Within one year after the end of the study, the investigator will submit a final study report with the results of the study, including any publications/abstracts of the study, to the EC. The principal investigator will be the signatories for the study report.

10.8 Public disclosure and publication policy

In accordance with standard editorial and ethical practice, the results of the study will be published, if applicable. The authorship guidelines of the Vancouver Protocol[5] will be followed regarding co-authorship.

[5] http://www.icmje.org/

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