2D BioPAD

Supple Graphene Bio-Platform for point-of-care early detection and monitoring of Alzheimer's Disease

D5.1 Clinical Pilot Studies Initiation Package

UEF

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Executive Summary

This report, developed within the 2D-BioPAD project funded by the European Union's Horizon Europe Framework Programme for Research and Innovation 2021-2027, outlines the activities and results related to Task 5.1: Pilot Studies Deployment and Evaluation Design (M1-M12, and M22-M24) under Work Package 5 (WP5): Clinical Pilot Studies Design, Deployment, Evaluation and Validation running from M1 (October 2023) to M48 (September 2027). WP5 also includes Task 5.2: Retrospective pilot study deployment and technical validation (M25-M36); Task 5.3: Prospective pilot study deployment and clinical validation (M30-M48); and Task 5.4: Cross-regional pilot studies evaluation and validation (M40-M48).

The main aim of Task 5.1 is to prepare the clinical pilot studies initiation package, including ethics-compliant study protocol, related key documents required for ethical approval, and to prepare the study centres for deployment of the clinical pilot study at M25 (October 2025).

This report describes:

- Preparation activities at the clinical study centres: coordination activities, harmonisation of assessment procedures, benchmarking equipment for the Alzheimer's disease plasma biomarkers, and ethical and regulatory aspects
- Details on the design of the clinical pilot studies, outcome measures, data analysis, and timeline
- List of documents in the clinical pilot studies initiation package, and online link where they are publicly available
- Next steps

The clinical pilot study protocol (version 1) has received ethical approval at two study centres, and approval process is ongoing at the third study centre. Preparation of an updated regulatory-compliant clinical study protocol is ongoing, considering the device classification and differences in regulatory requirements and procedures between EU member states for clinical studies involving different types of medical devices. Final approved versions of the clinical pilot studies initiation package documents will be publicly available at the provided online link at M24 before the start of the pilot study. Following any changes in these documents throughout the duration of the clinical pilot study, updated versions will be provided online with a list of versions and dates.

Final preparations for study deployment at all clinical centres, including internal documents with 2D-BioPAD diagnostic aid details and other internal operational aspects at the study centres will also be completed at M24, and all clinical centres will initiate the pilot study at M25.

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List of Terms and Definitions

Table 1: Terms and Definitions

Abbreviation	Definition
AD	Alzheimer's disease
ADCS-ADL	Alzheimer's Disease Cooperative Study Activities of Daily Living inventory
AE	Adverse Event
AUC	The area under the receiver operating characteristic curve
Aβ	β-amyloid
BRU	Brain Research Unit at UEF
CERAD	Consortium to Establish a Registry for Alzheimer's Disease
CRF	Case Report Form
CSF	Cerebrospinal fluid
FDG	Fludeoxyglucose F18
FIMEA	Finnish Medicines Agency
FINAS	Finnish Accreditation Service
GAADRD	The Greek Association of Alzheimer's Disease and Related Disorders, Alzheimer Hellas
GCP	Good Clinical Practice
GDPR	General Data Protection Regulation
GFAP	Glial fibrillary acidic protein
HCPs	Healthcare professionals
ICD	International Classification of Diseases
ICFs	Informed Consent Form
ICH	International Conference on Harmonisation
IVD	In vitro diagnostic medical device
MADRS	Montgomery-Asberg Depression Rating Scale
MCI	Mild Cognitive Impairment
MD	Medical device
MMSE	Mini Mental State Examination
MRI	Magnetic Resonance Imaging
NfL	Neurofilament light chain
PET	Positron emission tomography
PoC	Point-of-care
p-tau217	phosphorylated-tau217
REDCap	Research Electronic Data Capture
ROC	Receiver operating characteristic curve
SAE	Serious Adverse Event
UEF	University of Eastern Finland
WP	Work Package
ZI	Central Institute of Mental Health, Mannheim, Germany

1. Introduction

Alzheimer's Disease (AD) is the most common cause of dementia[1]. With more than 1 in 9 people aged 65 and older having AD, the disease is one of the main factors affecting brain health in older people. It is expected to affect roughly 18.8 million people by 2050 in Europe alone[2], with enormous financial burden for healthcare, long-term care, and hospice (over $355b just in the US in 2021, without including additional ~$257b in unpaid caregiving) at a global scale (Figure 1). This situation will worsen due to population aging; life expectancy in Europe is expected to increase by ~10% in 2065 reaching 92.8 years for women and 90.5 years for men[3]. With no widely available effective disease-modifying drugs, and with current practices targeting mainly the symptoms rather than the cause[4], the need for a preventive approach including early diagnosis and better insight in the progression of AD is of utmost importance, especially at earlier stages such as Mild Cognitive Impairment (MCI). An early and accurate AD diagnosis could offer significant benefits such as (i) a better chance of benefiting from disease-modifying treatments, (ii) mitigating emotional and social burden for both individuals and their families, (iii) allowing more time and better quality of life, and (iv) significant savings in terms of overall costs[5]. This is particularly important given current emerging promising novel drugs (e.g., targeting amyloid accumulation), which require extensive screening to identify people with AD who would be eligible for disease-modifying treatment, while also aiming to limit adverse effects[6]. Such screening procedures are at the moment too expensive, and their accessibility is limited to highly specialized tertiary (memory) clinics.

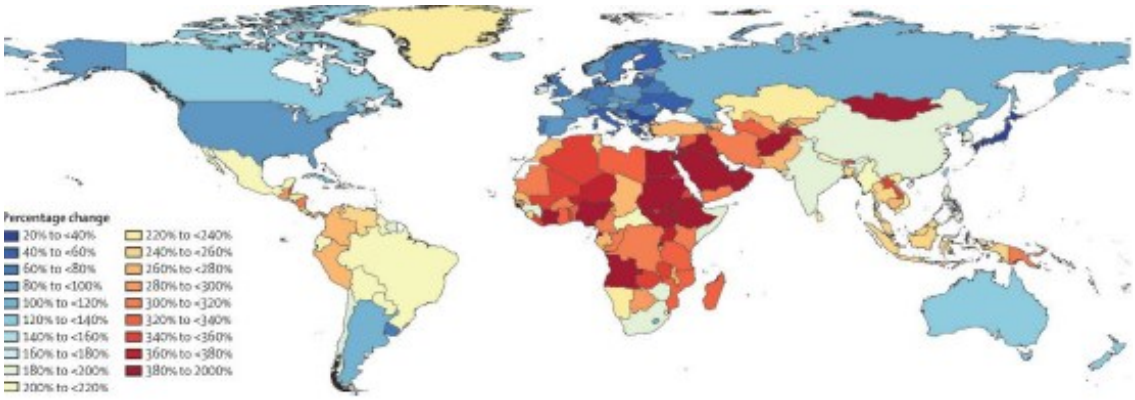

Figure 1. 2019-2050 Percentage change in global prevalence in all-age number of individuals with dementia

Currently available diagnostic techniques include magnetic resonance imaging (brain MRI), lumbar puncture (biomarkers in cerebrospinal fluid, CSF), amyloid and tau PET, FDG-PET, and neuropsychological assessment. The most reliable methods are either expensive or invasive, and most are used to confirm the AD diagnosis only in the presence of substantial symptoms. While neuropsychological tests can detect subtle cognitive impairment in early disease stages, they are inadequate for distinguishing between different

[1] https://www.alz.org/media/documents/alzheimers-facts-and-figures.pdf
[2] https://www.alzheimer-europe.org/dementia/prevalence-dementia-europe
[3] Janssen F, Bardoutsos A, El Gewily S, De Beer J. Future life expectancy in Europe taking into account the impact of smoking, obesity, and alcohol. Elife. 2021;10:e66590. doi: 10.7554/eLife.66590
[4] Kumar A, Singh A, Ekavali. A review on Alzheimer's disease pathophysiology and its management: an update. Pharmacol Rep. 2015;67(2):195-203. doi: 10.1016/j.pharep.2014.09.004
[5] https://www.alz.org/alzheimers-dementia/diagnosis/why-get-checked
[6] Gustavsson E, Raaschou P, Lärfars G, Sandman L, Juth N. Novel drug candidates targeting Alzheimer's disease: ethical challenges with identifying the relevant patient population. J Med Ethics. 2021;47(9):608-614. doi: 10.1136/medethics-2021-107304

neuropathologies. Additional challenges[7] have also been identified, due to diagnostic uncertainty and associated risks for the patient, accompanied by significant delays and costs for both patients and their families, but also for the health systems[8].

To date, clinically approved fluid biomarkers exist only for Alzheimer's disease (AD) [9,10] and until recently they were only tested in CSF. Recent major breakthroughs in accurate blood-based AD biomarkers, particularly β-amyloid (Aβ) 42/40 ratio, phosphorylated (p)-tau217, neurofilament light chain (NfL, neurodegeneration marker), and glial fibrillary acidic protein (GFAP, early marker of Aβ-related reactive astrogliosis), have the potential to truly revolutionize AD diagnostics [11,12]. The first AD blood biomarkers were validated in 2022 in clinical trials[13]. However, the problems of accessibility and costs have not yet been fully addressed, and implementation in clinical practice is still limited.

The COVID-19 pandemic and extensive use of point-of-care (PoC) in vitro diagnostic (IVD) devices has showcased the importance of massively accessible and cost-efficient solutions for a wider range of healthcare challenges. Low-cost, reliable, and easily accessible blood-based diagnostic aids for AD are urgently needed, as diagnostic accuracy of a work-up (without support of AD biomarkers) is limited with ~25-30% misdiagnosis of AD in specialist memory clinics and >50% in primary care[11]. Biomarker acceptance in clinical practice is also heavily dependent on healthcare professionals (HCPs) receiving the necessary support to understand what purposes biomarkers are validated for and interpret multi-marker data together with clinical data.

The 2D-BioPAD project is developing a fast, cost-effective, non-invasive, and reliable diagnostic aid for measuring blood biomarkers (Figure 2) to support the early diagnosis and progression monitoring of AD, with potential for future use in primary healthcare settings. The 2D-BioPAD diagnostic aid leverages the unique properties of 2D materials such as graphene and its derivatives. This innovative graphene-based diagnostic aid (i) introduces a versatile surface chemistry that combines nano and DNA technologies towards improved biocompatibility, stability, as well as high sensitivity and specificity for enhanced (bio-)sensing; (ii) is designed to identify and quantify in real-time and simultaneously up to 5 AD blood biomarkers; and (iii) provides an easy to use and understand digital interface with key metrics and insights regarding the measured results.

2D-BioPAD, the first multianalyte bioelectronic diagnostic aid for screening up to 5 AD-related biomarkers, is also one of the very few digitalised diagnostic aids connected with an external mobile app to guide the end-user for proper sample handling and provide user-friendly and easy to understand visualisation of the extracted results beyond a simple "biomarker positive versus negative" dichotomisation. By connecting the tests to a digital tool (e.g., smartphone or tablet), the diagnosis is brought as close as possible to the patient. This proximity combined with speed is expected to have benefits for both HCPs and patients, including higher end-user engagement. The interoperable, secure, and privacy enabling connectivity will also enable unobtrusive cloud data transfer and facilitate data-driven clinical research on AD and dementia-related diseases to improve diagnosis, treatment, and care.

[7] Porsteinsson AP, Isaacson RS, Knox S, Sabbagh MN, Rubino I. Diagnosis of Early Alzheimer's Disease: Clinical Practice in 2021. J Prev Alzheimers Dis. 2021;8(3):371-386. doi: 10.14283/jpad.2021.23

[8] Nichols E, Vos T. The estimation of the global prevalence of dementia from 1990-2019 and forecasted prevalence through 2050: An analysis for the Global Burden of Disease (GBD) study 2019. Alzheimer's & Dementia, 17, e051496, 2021. https://doi.org/10.1002/alz.051496

[9] https://www.alz.org/media/documents/inbrief-differentiating-dementias.pdf

[10] https://www.alz.org/professionals/health-systems-medical-professionals/dementia-diagnosis/differential-diagnosis

[11] Hansson et al. The Alzheimer's Association appropriate use recommendations for blood biomarkers in Alzheimer's disease. Alzheimers Dement. 2022;18(12):2669-2686

[12] Hansson O. Biomarkers for neurodegenerative diseases. Nat Med. 2021;27(6):954-963

[13] https://www.nia.nih.gov/2021-2022-alzheimers-disease-related-dementias-scientific-advances/biomarker-research

Figure 2. The 2D-BioPAD diagnostic aid concept

The remaining document consists of the following sections:

- **Section 2** articulates the overall rationale of the 2D-BioPAD clinical pilot studies.
- **Section 3** provides information on the objectives of the 2D-BioPAD clinical pilot studies.
- **Section 4** describes preparatory activities and details of the clinical pilot studies including timeline.
- **Section 5** provides concluding remarks and guides the next steps for the clinical pilot studies.

The following English-language documents are available via the 2D-BioPAD project website.

These documents will be updated as needed according to regulatory and/or ethics requirements, including a list of document versions with dates. The final versions will be available at M24 before the start of the clinical pilot studies.

- *Clinical Pilot Study Protocol, version 1.0 [link]*

This version of the protocol document was prepared using a standard template for clinical observational studies covering key information required for ethical approval. Regulatory-compliant protocols may need to use different standard document templates depending on whether the device to be investigated is a medical device (MD) or in vitro diagnostic medical device (IVD), and whether the relevant national authorities require specific notification or approval pathways for clinical investigations of devices.

- *Consent to participate in the study (HCPs) [link]*
- *Information Sheet for study participants (patients) [link]*
- *Informed Consent Form for study participants (patients) [link]*

These English-language documents have been translated into Finnish, Greek and German, and adapted to the specific format, length etc requirements of the local Ethics Committees.

- *2D-BioPAD Questionnaire Survey for patients [link]*
- *2D-BioPAD Questionnaire Survey for HCPs [link]*

The questionnaire surveys concern the participants' overall experiences with the 2D-BioPAD diagnostic aid.

- *Lifestyle questionnaire for patients [link]*

The lifestyle questionnaire was added to collect information that is not routinely and/or systematically collected as part of the standard procedures at the clinical study centres, and to ensure that lifestyle-related factors are recorded in the same way across study centres. Collecting data on lifestyle factors is important for investigating their potential impact on AD blood biomarkers. Other relevant data and questionnaires are already collected as part of standard clinical study centre procedures (see also section 4.3 Prospective study).

2. Rationale for the 2D-BioPAD clinical pilot study

An innovative device design requires a clinical pilot study as a crucial first step towards subsequent full validation. Pilot studies are conducted in the early stages of development, often before the design of a device has been finalized. They usually aim to collect a broad range of information for several purposes, including e.g. identifying modifications that may be needed for the device or use procedures, refine the intended target population, evaluate safety and preliminary clinical performance, and develop further protocols for full-scale confirmatory clinical studies. Full-scale confirmatory clinical studies are generally larger than pilot studies, are designed to provide definitive evidence for clinical performance, and their results are used to obtain regulatory approval for the tested device. While devices can be tested directly in full-scale confirmatory clinical studies, conducting a pilot study first is extremely helpful because preliminary (pilot) data can be used to improve device design and optimize the time and resources spent in confirmatory clinical studies.

Because the 2D-BioPAD diagnostic aid is currently in an early stage of development, initial clinical testing will be conducted in a pilot study covering both testing in a laboratory environment (already existing plasma samples) and in a clinical environment similar to the intended conditions of use (prospective clinical study). The clinical pilot study is also designed to provide preliminary information about the clinical performance of the diagnostic aid. All information from the 2D-BioPAD clinical pilot study will be used to plan the next steps concerning future full-scale confirmatory clinical studies, regulatory aspects of the diagnostic aid, and market-related aspects.

3. Objectives of the 2D-BioPAD clinical pilot study

The overall aim of the 2D-BioPAD clinical pilot study is to test, evaluate and validate the developed blood biomarker diagnostic aid in three clinical study centres specialised in the diagnosis and treatment of AD and dementia diseases. The study will be conducted in two stages, representing a retrospective and a prospective study.

3.1. Retrospective study

The retrospective stage focuses on technical validation of the 2D-BioPAD diagnostic aid in a laboratory environment based on existing plasma samples. The main objectives are to:

- Analyze a set of up to five plasma biomarkers that can effectively support accurate early diagnosis of AD e.g., amyloid accumulation (Aβ40, Aβ42, and the Aβ40/42 ratio), tau phosphorylation (e.g., p-tau217), neurodegeneration (NfL), or inflammation (e.g., GFAP).
- Evaluate and validate the performance of the 2D-BioPAD diagnostic aid against benchmarking equipment for the same plasma biomarkers (available at the UEF Brain Research Unit biomarker laboratory) and/or state-of-the-art equipment for CSF biomarkers (as available from routine assessments conducted at the clinical study centres), considering also other patient-related (e.g., demographics, AD disease stage, comorbidities) and sample-related parameters (e.g., temperature, thaw-freeze cycles, other processing conditions).

Results from the retrospective stage will be used to improve the diagnostic aid design and offered functionalities as appropriate.

3.2. Prospective study

The prospective stage will take place in a clinical environment, where HCPs will use the 2D-BioPAD diagnostic aid to measure AD blood biomarkers in individuals referred to the three study centres for assessment of suspected AD/dementia disease.

In *Step 1*, a small-scale feasibility test run will be performed in the three clinics. The main objective is to refine the appropriate procedure for using the diagnostic aid by identifying constraints (e.g., at which time of the day the measurement should take place), limitations (e.g., how easy it is to use), and potential improvements.

In *Step 2*, the clinical evaluation and validation will be conducted in (i) up to 300 individuals with subjective cognitive complaints, MCI, or dementia referred to the three clinical study centres for assessment, and (ii) the HCPs using the 2D-BioPAD diagnostic aid. Up to one year follow-up will be included for patients with inconclusive diagnosis following the initial assessment. The primary objectives are to (i) evaluate and validate the performance of the 2D-BioPAD diagnostic aid under real-world clinical conditions and use cases, and (ii) evaluate feedback from HCPs and patients including e.g. acceptability, trust, satisfaction, usability, and experience of using the 2D-BioPAD diagnostic aid compared with study centre-specific standard procedures for acquiring blood measurements in clinical practice. Performance of the 2D-BioPAD diagnostic aid will be compared with the same benchmarking equipment for plasma biomarkers (measured for all prospective study samples) and state-of-the-art equipment for CSF biomarkers (measurements as available from routine assessments conducted at the clinical study centres) as described above for the retrospective study.

4. Implementation and evaluation

4.1. Preparation of clinical study centres

4.1.1. Overview of clinical study centres

4.1.1.1. University of Eastern Finland (UEF), Kuopio, Finland

The Brain Research Unit (BRU) at the UEF School of Medicine has experience in conducting dementia-related clinical trials since 1992[14]. Clinical studies include e.g. Finnish and multi-national randomized controlled pharmacological and non-pharmacological intervention studies, epidemiological population-based studies, and memory clinic-based studies, both industry- and academia-funded. The UEF BRU works together with the memory clinic at Kuopio University Hospital. The UEF BRU biomarker laboratory has over 20 years' experience in clinical development and testing of biomarkers for dementia diseases. Since 2005 it provides a national biomarker laboratory service available for physicians and hospitals. The quality management system is based on international standards for quality management and for clinical laboratories.

4.1.1.2. The Greek Association of Alzheimer's Disease and Related Disorders (Alzheimer Hellas, GAADRD), Thessaloniki, Greece

GAADRD is a non-profit organisation, founded in 1995 by relatives of patients with Alzheimer's Disease and other forms of dementia and doctors of all specialties[15]. It operates three (3) Day Care Centres in Greece, a Day centre for caregivers, a Home-care unit, and an inpatient facility for End-stage Dementia patients. Since 2000, Alzheimer Hellas has organised fourteen (14) Pan-Hellenic Interdisciplinary Conferences, along with informative lectures and seminars. With over 5,000 members, it focuses on fighting stigma associated with neurodegenerative diseases, controlling behavioural and affective disorders, and providing non-pharmacological interventions and appropriate medical care from specialised health care professionals. Also, GAADRD participates in many clinical studies under the umbrella of Horizon Europe projects or the Erasmus+ programs

4.1.1.3. Central Institute of Mental Health (ZI), Mannheim, Germany

The Department of Geriatric Psychiatry focuses on clinical dementia research[16]. All projects are conducted on patients either from the memory clinic or on in-patients from the three gerontopsychiatric wards. One thematic research priority is on the evaluation of biomarkers for Alzheimer's disease and other dementias. The Department has extensive experience with large multinational EU-funded and other projects involving clinical studies in dementia diseases.

4.1.2. Clinical study coordination activities

At the start of the 2D-BioPAD project, a clinical pilot study coordination team was established, including the Principal Investigators and one core team member from each clinical study centre. Coordination and preparation activities included:

- Joint online and face-to-face meetings for overall planning and decision-making related to specific clinical study aspects

[14] https://uefconnect.uef.fi/en/group/brain-research-unit-bru-clinical-trial-unit /
[15] https://www.alzheimer-hellas.gr/index.php/en /
[16] https://www.zi-mannheim.de/en/research/departments-research-groups-institutes/gerontopsychiatry.html

- Mapping of existing clinical and research protocols at all clinical study centres, including clinical, cognitive, and other biomarker assessments, to facilitate decisions on specific study design aspects and harmonization of a core set of procedures to be used at all study centres during the clinical pilot study
- At each clinical study centre, ensuring availability of healthcare professionals (HCPs) for the study; availability of samples with the appropriate storage conditions and consent for re-use and shipping for the retrospective study; pathways for patient recruitment with adequate capacity for the prospective study sample size; and any other centre-specific preparatory activities as needed
- Preparation of documents included in the main English-language clinical pilot study initiation package
- Online meetings and communication with EVNIA regarding ethical and regulatory aspects of the clinical pilot study
- Online meetings and communication with technical partners (UP-CATRIN, ICN2, AUTH, GRAPHEAL, NOVAPTECH and CeADAR) to ensure alignment of the clinical pilot study protocol with technical requirements of the 2D-BioPAD project
- Providing clinical pilot study initiation package documents to the other partners for feedback, and making the necessary modifications
- Translation of relevant documents from the clinical pilot study initiation package into Finnish, Greek or German, preparation of documents according to templates required by the relevant local Ethics Committees, and submission of documents for ethical approval
- Communication with the Scientific Advisory Board regarding the clinical pilot study initiation package

Table 2: Contribution of 2D-BioPAD consortium partners to the development of the clinical pilot study initiation package

Expertise area	Partners' main role
Clinical pilot study coordination (UEF, GAADRD, ZI)	Activities as describe above. As the Task 5.1 leader, UEF was responsible for the coordination of the work.
Ethics & regulatory (EVNIA)	Read the clinical pilot study initiation package and provided key input on ethical aspects and overall content input for all documents. Developed the 2D-BioPAD Questionnaire Surveys for patients and HCPs. Led the work on regulatory aspects of the clinical pilot study (see also section 4.1.5 Ethical and regulatory aspects).
Technical (ICN2, NOVA, AUTH, UP-CATRIN, GRAPHEAL, CeADAR)	Read documents related to the clinical pilot study circulated by the Task leader and provided input where applicable. Provided continuous information about work on technical aspects with relevance for the clinical pilot study design.
Management (QPLAN)	Reviewed all documents circulated by the Task leader. Interaction in terms of timeline and expectations as per the GA. Alignments with T1.1 findings.

4.1.3. Harmonisation of assessment procedures across clinical study centres

To ensure harmonisation of clinical pilot study procedures across the three study centres, a detailed mapping of centre-specific standard procedures was first conducted, covering the following aspects:

A) Standard diagnostic procedures

General

- Diagnostic criteria
- Estimated changes in diagnostic criteria in the near future
- Use of consensus diagnosis and details on the diagnostic process

All clinical study centres use International Classification of Diseases (ICD)-10 based diagnostic coding in medical records. This is not expected to change for the duration of the 2D-BioPAD clinical pilot study. Diagnostic criteria are overall well aligned. While there are differences in referral pathways based on healthcare system organisation and specialty (e.g. Neurology in Finland and Greece, Psychiatry in Germany), there are significant similarities in the overall diagnostic process and the use of multidisciplinary teams. Consensus diagnosis is consistently used for cases that are less clear-cut or require additional discussion of diagnostics and treatment.

Follow-up schedule

- Which patient groups are followed-up over time
- Follow-up intervals
- Assessments included in follow-up visits

Follow-up schedules and procedures are sufficiently similar up to one year after the first visit. After one year, there are significant differences due to healthcare system organisation, e.g. in Finland specialized Neurology clinics usually transfer care back to primary care after one year (a small number of patients may be followed up beyond one year in specific circumstances). Overall, follow-up procedures for unclear/special cases are similar across clinical study centres. Thus, there is adequate alignment between the clinical study centres to include up to one year follow-up in the prospective study design and rely on diagnosis data from routine assessments.

Cognitive and daily life functioning tests

- Screening tests
- In-depth neuropsychological test batteries
- Patient groups referred for in-depth testing, criteria used to decide on referral, and estimated percentage of patients undergoing in-depth testing

The clinical study centres use a variety of cognitive and daily functioning tests. Some of the in-depth testing may also be tailored to a patient's specific situation. Thus, to ensure alignment, a core set of tests (based on those used in at least two centres) was chosen to be conducted prospectively at all study centres in the 2D-BioPAD clinical pilot study (details in Table 6). Screening tests are common across centres, i.e. data can be used in connection to the already existing samples for the retrospective study.

Cerebrospinal fluid (CSF) biomarkers

- Lumbar puncture procedures, i.e. routine in all patients versus selected patient groups, including selection criteria and overall percentage of patients undergoing lumbar puncture
- Assays used for CSF analysis and laboratory cut-offs for AD-relevant biomarkers

Lumbar puncture is not done in all patients, due to contraindications, refusal of some patients to consent to an invasive and burdensome procedure, or guidelines not recommending CSF biomarkers in specific cases (e.g. no objective cognitive impairment and no other dementia risk indicators). State-of-the-art CSF biomarkers are estimated to be available via routine assessments in about 100-150 of the total 300 patients in the prospective study. Plasma biomarkers using benchmarking equipment (section 4.1.4 Benchmarking equipment for AD plasma biomarkers) will thus be measured in all 300 patients. For the retrospective study, existing plasma samples will be selected if CSF biomarker data is also available from routine assessments. Aspects related to

CSF biomarker assays used at each study centre are detailed below in section 4.1.4.1 State-of-the-art CSF biomarkers.

Blood

- Blood sampling procedures, i.e. routine sampling from all patients versus selected groups, sample storage for research purposes
- Current availability of AD blood biomarkers for routine clinical use
- Availability of kidney and liver function measurements, including list of relevant biomarkers

AD blood biomarkers are not currently available for routine clinical use at the study centres, except for plasma NfL in Finland provided by the UEF biomarker laboratory. Blood sampling is common at all study centres, especially as part of research studies. Kidney and liver function measurements are available either measured at the study centre or at primary care; creatinine and ASAT are common across all study centres.
A set of conditions for storage of existing samples applicable across study centres was identified (Table 4).

Neuroimaging

- Structural MRI procedures, i.e. routine for all patients versus selected groups
- Possibility to retrieve MRI-related data for research
- Availability and type of PET scans, and procedures (routine for all patients versus selected groups)

Structural brain MRI is done at all study centres, except in patients with MRI contraindications. Quantitative image analysis is not done as part of routine procedures but validated semi-quantitative visual rating scales (e.g. for hippocampus atrophy and white matter lesions) are used at all centres. PET scans (e.g. FDG-PET, amyloid-PET) are only done in selected cases due to higher cost.

B) Other assessments done as part of research

- Cognitive tests
- Daily life functioning level
- Other

These included additional scales/tests available at each study centre but not part of routine assessments.

After completion of the mapping, the clinical pilot study coordination team met to discuss and decide on a core set of assessments to be conducted at all study centres (details in Section 4.3 Prospective study). The decision was based on assessments providing key data for the 2D-BioPAD clinical study, and already used in at least two study centres, in order to minimize additional burden on the patients participating in the study.

4.1.4. Benchmarking equipment for AD plasma biomarkers

2D-BioPAD plasma biomarker values measured in both the retrospective and prospective studies will be compared with the same plasma biomarkers (e.g., Quanterix Simoa-based) measured at the UEF Brain Research Unit biomarker laboratory[17]. The UEF Brain Research Unit laboratory offers diagnostic services of AD biomarkers available for physicians and hospitals since 2005 and has extensive experience with large-scale international studies on AD fluid biomarkers. Quality management system is based on international standards for quality management and for clinical laboratories[18]. The laboratory has ISO 15189 accreditation (FINAS

[17] https://sites.uef.fi/aivobiomarkkeritutkimukset/?lang=en
[18] https://sites.uef.fi/aivobiomarkkeritutkimukset/

Finnish Accreditation Service) for CSF β-amyloid 42, t-tau and p-tau analyses, which are run using automated immunoassays (Roche Elecsys).

In the EU, AD plasma biomarkers are not currently available in clinical practice on a wide scale. Finland is one of the few EU countries with available diagnostic services of AD plasma biomarkers for clinical practice, i.e., Quanterix Simoa-based plasma NfL service offered by the UEF Brain Research Unit biomarker laboratory (other Simoa-based AD plasma markers will soon become available). These in vitro diagnostic medical devices of the UEF laboratory are registered with the Finnish Medicines Agency (FIMEA, e.g.[19]). Other devices, reagents etc used for diagnostic services by the laboratory are CE-marked.

Depending on availability / validation status, Fujirebio Lumipulse-based plasma AD biomarkers may be considered as an alternative for benchmarking. Simoa- and Lumipulse-based plasma markers have been shown to have similar diagnostic accuracy in research studies, e.g.[20] [21].

4.1.4.1. State-of-the-art CSF biomarkers

Standard assessment procedures at all three clinical study centres include CSF biomarkers measured using state-of-the-art equipment: Roche Elecsys (β-amyloid 42, t-tau and p-tau181) at UEF and GAADRD, or Fujirebio Lumipulse (β-amyloid 42/40, t-tau and p-tau181) at GAADRD and ZI. As lumbar puncture is an invasive procedure, CSF biomarkers are measured based on clinical indication and/or patient consent, provided that no contraindications are present. CSF biomarker information is used together with other clinical, cognitive, neuroimaging etc information to establish the diagnosis in standard clinical practice.

To reduce patient burden, the 2D-BioPAD clinical pilot study will use CSF biomarker values measured during standard assessment procedures at the clinical study centres. For the retrospective study, existing plasma samples will be selected from individuals who also have available CSF biomarker data. Each assay has established cut-offs for AD pathology which are used in clinical practice. The Lumipulse and Elecsys CSF AD assays have been shown to have high analytical and clinical performances[22], with strong correlation and high concordance in identifying AD pathology[23].

4.1.5. Ethical and regulatory aspects

4.1.5.1. Ethical aspects

The investigators at all study centres will ensure that this study is conducted in conformance with the principles of the "World Medical Association Declaration of Helsinki" (52nd WMA General Assembly, Edinburgh, Scotland, October 2000; including the Notes of Clarification as added in 2002, Washington; 2004, Tokyo; 2008, Seoul; and 2013, Fortaleza), International Conference on Harmonisation (ICH) guidelines for Good Clinical Practice (GCP), and relevant legislation in each participating country.

Ethics Committee review

[19]https://sites.uef.fi/aivobiomarkkeritutkimukset/wp-content/uploads/sites/112/2023/08/Vakuutus-Biomarkkerilaboratorio-140823.pdf

[20] Pilotto A., et al. Plasma p-tau217 in Alzheimer's disease: Lumipulse and ALZpath SIMOA head-to-head comparison. medRxiv [Preprint]. 2024 May 3:2024.05.02.24306780. doi: 10.1101/2024.05.02.24306780

[21] Dakterzada, F., et al. Assessment of the Correlation and Diagnostic Accuracy between Cerebrospinal Fluid and Plasma Alzheimer's Disease Biomarkers: A Comparison of the Lumipulse and Simoa Platforms. Int. J. Mol. Sci. 2024, 25, 4594. https://doi.org/10.3390/ijms25094594

[22] Dakterzada F, et al. Assessment of the Concordance and Diagnostic Accuracy Between Elecsys and Lumipulse Fully Automated Platforms and Innotest. Front Aging Neurosci. 2021 Mar 4;13:604119. doi: 10.3389/fnagi.2021.604119

[23] Campbell MR, et al. P-tau/Aβ42 and Aβ42/40 ratios in CSF are equally predictive of amyloid PET status. Alzheimers Dement (Amst). 2021 May 18;13(1):e12190. doi: 10.1002/dad2.12190

The relevant clinical pilot study documents have been submitted to the following Ethics Committees as per standard local procedures:

- Regional Medical Research Ethics Committee of the Wellbeing Services County of North Savo (UEF study center, Finland)
  Status: approved September 17, 2024
- Bioethics Committee of Alzheimer Hellas (GAADRD study centre, Greece)
  Status: approved September 11, 2024
- Ethics Committee (ZI study center, Germany)
  Status: in processing

Approval by the relevant Ethics Committee must be obtained before starting the study at each centre and will be documented in a letter to the study centre investigator, specifying the date of approval, the composition of the ethics committee, and version and date of submitted documents.

Any substantial change and/or amendments to the study protocol will be submitted to the relevant Ethics Committees in accordance with current requirements. Approval by the Ethics Committee must be obtained before the implementation of any substantial change or amendment, except for changes necessary to remove any apparent immediate hazard to the participant.

Informed consent

Prior to study enrolment, written informed consent will be obtained from each participant (patients and HCPs). It is the responsibility of the study centre investigator to make sure written informed consent is obtained after adequate explanation of the aims, methods, source of funding, the anticipated benefits and potential risks of the study and the discomfort it may entail. The nature, significance and scope of the study will be explained in writing and verbally in an appropriate and comprehensible manner. Care should be taken to ensure that sufficient time is allowed for the decision-making process. It will be made clear that participants may withdraw their consent to participate in the study at any time without giving reasons. The original informed consent forms (ICFs) will be kept in a study folder at each study centre and stored separately from the study data. A copy of the written study information and the ICF will be handed out to the participant.

In the case of patients who are diagnosed with AD or other dementia, ability to provide written informed consent for the study will be established according to the already existing standard procedures at each study centre. Individuals with impaired capacity to consent will not be eligible for the study. Each clinical study centre will adhere to the relevant local/national regulations and ethical guidelines to determine when an informant/legally acceptable representative is necessary and to ensure that the consent process is conducted appropriately.

Disclosure of AD blood biomarker results

Given that the overall aim of this clinical pilot study is to evaluate and validate AD blood biomarkers measured using the 2D-BioPAD diagnostic aid, these biomarker results will not be disclosed to patients and will not be used by HCPs for making diagnosis and treatment/care decisions. AD blood biomarker results measured using state-of-the-art equipment may be disclosed to patients and may be used by HCPs for making diagnosis and treatment/care decisions only if they are already included in the centre-specific standard assessment procedures. In such cases the disclosure and related procedures will be conducted by the treating physician at the relevant study centre, i.e., not as part of the 2D-BioPAD clinical pilot study.

Data protection and confidentiality of study data

All investigators, study staff and other parties involved in the study must comply with the requirements of the General Data Protection Regulation (GDPR) and relevant national data protection legislation as applicable regarding the collection, storage, processing, and disclosure of personal information.

All team members, partners, and collaborators who have access to the data and are involved in the study are responsible for treating subject and study information as confidential. The investigators will ensure that the subject information will not be made publicly available. To maintain subjects' confidentiality, study records and samples will be de-identified. All participants will be given a random subject ID and all data and samples will be processed, analysed, saved, and stored as de-identified. Identification code keys linking the subjects' names and other personal information to the subjects' identification number must be stored in the Investigator Site File, in a locked or password protected location at each centre separately from all other data. Access to the code keys and subjects' personal information will be given only to the main investigators or those research-team members for whom access is necessary to conduct the study.

Sharing of de-identified data and samples between Consortium partners will be done using secure encrypted methods after a material transfer agreement has been signed by all relevant partners.

Data and samples storage and archiving

All clinical pilot study data in paper format, as well as samples collected, will be stored as de-identified in a locked location at each study centre. De-identified data will be also saved for the study duration in an electronic format in the centre institution disk/server space which will be protected with username and password. Access right will be limited, and granted, only to the members of the research group at the respective centre. All participating institutions have information protection and processing policies and the relevant IT infrastructure for their implementation.

Participant's personal information, such as name, social security number, and contact information, will be stored and kept separately from all other data and samples, in a password-protected disk/server space and/or locked location with limited access rights at each study centre. Access to the code keys and subjects' personal information will be granted only to those researchers at the respective study centre for whom access is absolutely necessary in order to conduct the study.

De-identified data and samples will be shared between 2D-BioPAD Consortium partners for analyses as specified in the protocol. Prior to the sharing of data and samples, a material transfer agreement will be prepared, approved, and signed by all partners involved. Any excess of samples shipped outside of the study centre where samples were collected will be destroyed after completion of the analysis.

After the end of the study, research data in paper format and electronic de-identified data from each study centre will be archived at the respective study centre according to the relevant local/national regulations.

Insurance/Compensation

The study participants will be insured according to the relevant legislation at each study centre. No financial compensation will be provided to research participants for participating in the study.

4.1.5.2. Regulatory aspects

Regulatory Authority approvals

Different Regulatory requirements and procedures persist between EU member states for clinical studies on the safety and performance of an IVD. As of July 2024, Greece and Germany do not have a centralized national

Regulatory Authority approval process in place and require notification concerning clinical studies after ethical approval has been obtained. Finland has recently established a national process with a 2-step approach: (1) ethical approval by the relevant local Ethics Committee, and (2) Finnish Medicines Agency (FIMEA) approval or notification, depending on whether the clinical study will involve a medical device (MD) or in vitro diagnostic medical device (IVD).

Regulatory assessments of the* 2D-BioPAD device and *Regulatory pathway

EVNIA has been leading the work on assessing the device type and classification and identifying the relevant regulatory pathways to facilitate preparing a regulatory-compliant clinical study protocol based on the current ethics-compliant protocol version 1.0. A T6.4 Regulatory Acceptability Plan template was prepared in M2 and has the objective to provide guidance on the regulatory pathway, requirements, and standards applicable for the 2D-BioPAD device. A meeting was held the 9th of July 2024 to discuss the 2D-BioPAD device classification. A 1st draft was prepared of specification of the device (Table 3). Further assessment of the device type and classification will be performed by EVNIA with input from consortium partners. Based on this knowledge an update of the Regulatory Acceptability Plan will be made by EVNIA and will guide on the preparation of an updated regulatory-compliant clinical protocol (M24).

Table 3: 2D-BioPAD device specification

Intended use	**2D-BioPAD is a blood based diagnostic aid, based on AD related biomarkers detection and quantification.**
Intended population	Individuals in the AD continuum (preclinical, prodromal or dementia stage)
Intended user	Qualified healthcare professionals who, in combination with other diagnostic tools, assess individuals within the AD continuum.
Pathology	Alzheimer's Disease (AD)

4.1.6. Study registration

Study registration in a suitable public registry (e.g., clinicaltrials.gov or equivalent) will be done following final ethical approval at all centres, and regulatory approval/notification as applicable, before the start of the study (M24).

4.2. Retrospective study

The 2D-BioPAD retrospective study (M25-M36) will use existing plasma samples from the three clinical study centres in Finland, Germany, and Greece. Study design is summarised in Figure 3.

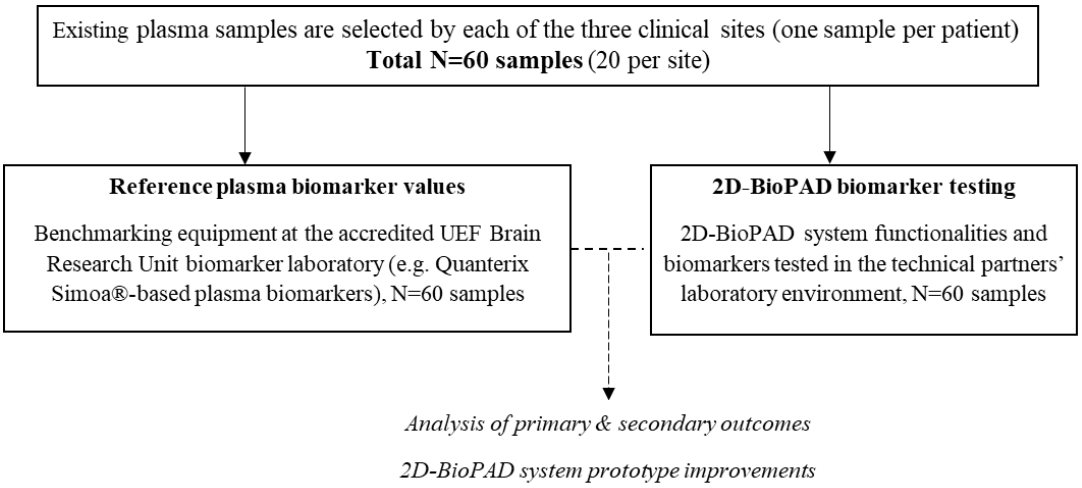

Figure 3. 2D-BioPAD clinical pilot study design for the retrospective stage

4.2.1. Blood samples and selection criteria

Existing samples will be used if they are maximum 10 years old, continuously stored in -70^{0}C freezer since collection, and there is consent for use of the samples and related clinical data, including shipping/sharing within the EU.

Table 4. Inclusion and exclusion criteria for the retrospective study

Inclusion criteria	
Plasma samples	• Maximum 10 years old • Up to 2 ml available for 2D-BioPAD pilot study • Stored in -70^{0}C freezer • Existing consent to re-use for research, including shipping within the EU
Sample-related clinical data	• Diagnosis AD dementia, MCI due to AD, or no dementia-related disease (control) in connection to the blood sample collection time • Available data on CSF biomarkers, cognitive tests (at least MMSE), comorbidities, and medications in connection to the diagnostic workup • Existing consent to re-use data for research, including sharing within the EU
Exclusion criteria	
Sample-related clinical data	• Diagnosis of dementia other than AD • Mixed dementia • MCI suspected due to non-AD causes, as per clinical judgement based on centre-specific standard assessments

4.2.2. Data collection

The following patient data already collected during the standard diagnostic workup in connection to the plasma sampling time will be used: diagnosis (AD dementia, MCI, or control/no dementia-related disease), cognitive tests, comorbidities, medications, other blood biomarkers (e.g., kidney and liver function).

Samples will be shipped on dry ice to the technical partners (i.e., ICN2 and GRAPHEAL) using established services for biological specimen shipping. Timelines are shown in Section 4.5.

4.2.3. Risk analysis

The retrospective study does not involve significant risks. It will use a total of 60 already existing plasma samples (20 per centre). Samples are already stored on-site in appropriate conditions. A smaller number of samples is considered sufficient for initial technical validation purposes in a laboratory environment. Stricter selection criteria (Table 4) are applied to ensure a well-defined case-control design, and availability of all necessary data. All clinical study centres have extensive experience and established contacts with appropriate services for biological specimen shipping.

4.3. Prospective study

The prospective study will be conducted between M30-M48. Patients and HCPs will be recruited at the three centres in Finland, Germany, and Greece. Up to 300 patients (≈100 per centre) will be recruited from individuals referred to the study centres (or related memory clinics where applicable) for assessment of cognitive status as per centre-specific standard procedures. The research participants may have subjective cognitive complaints, MCI, or dementia diagnosis.

The HCPs can be nurses or physicians (neurologists, psychiatrists, or geriatricians) depending on centre-specific organisation of standard healthcare. Per centre at least 2 HCPs will be recruited. HCPs may have different levels of experience, from limited experience (e.g., residency or <3 years in the dementia field) to extensive experience (e.g., specialist or at least 3 years in the dementia field). At least one HCP at each centre should have more extensive experience.

4.3.1. Selection criteria

Table 5. Inclusion and exclusion criteria for the prospective study

Inclusion criteria	
Patients	• Age 50 years or older • Subjective cognitive complaints, MCI or dementia diagnosis, as per centre-specific standard assessment procedures • Ongoing assessment after first referral to the clinical study centre, or diagnostic procedures completed ≤3 months before the first 2D-BioPAD study visit • Ability to provide written informed consent
HCPs	• 18 years or older • Certified HCP (e.g., nurse, physician) • Actively involved in the centre-specific assessment process for dementia-related diseases
Exclusion criteria	
Patients	• Any health conditions that substantially affect communication and/or ability to participate in study procedures, as judged by the study nurse or physician • Impaired capacity to consent as per centre-specific standard assessment

4.3.2. Overall study design

This is a **multicentre prospective observational study**. HCPs will use the 2D-BioPAD diagnostic aid to measure AD blood biomarkers in individuals referred to the three study centres for assessment of suspected AD/dementia disease. 2D-BioPAD baseline visit activities will be conducted only after the informed consent form has been signed. The overall study design is summarised in Figure 4.

The 2D-BioPAD baseline visit will be conducted after centre-specific standard assessment procedures are completed and a diagnosis and treatment/care plan have been decided according to standard clinic procedures. The 2D-BioPAD baseline visit can be a separate visit or can be conducted at the end of an already scheduled standard clinic visit, depending on patient preference and study centre logistics. The aim is to reduce burden for patients and limit interference with routine clinical activities.

As validation of the biomarker measures from the 2D-BioPAD diagnostic aid will be done as part of this study, these biomarker results will not be used for diagnosis or to guide treatment plans. HCPs participating in the 2D-BioPAD clinical pilot study who are directly involved in the standard diagnostic work-up and decision-making for patients tested with the 2D-BioPAD diagnostic aid must not have access to the biomarker results before the diagnosis and treatment/care plan are decided (See also Section 4.1.5.1 Ethical aspects).

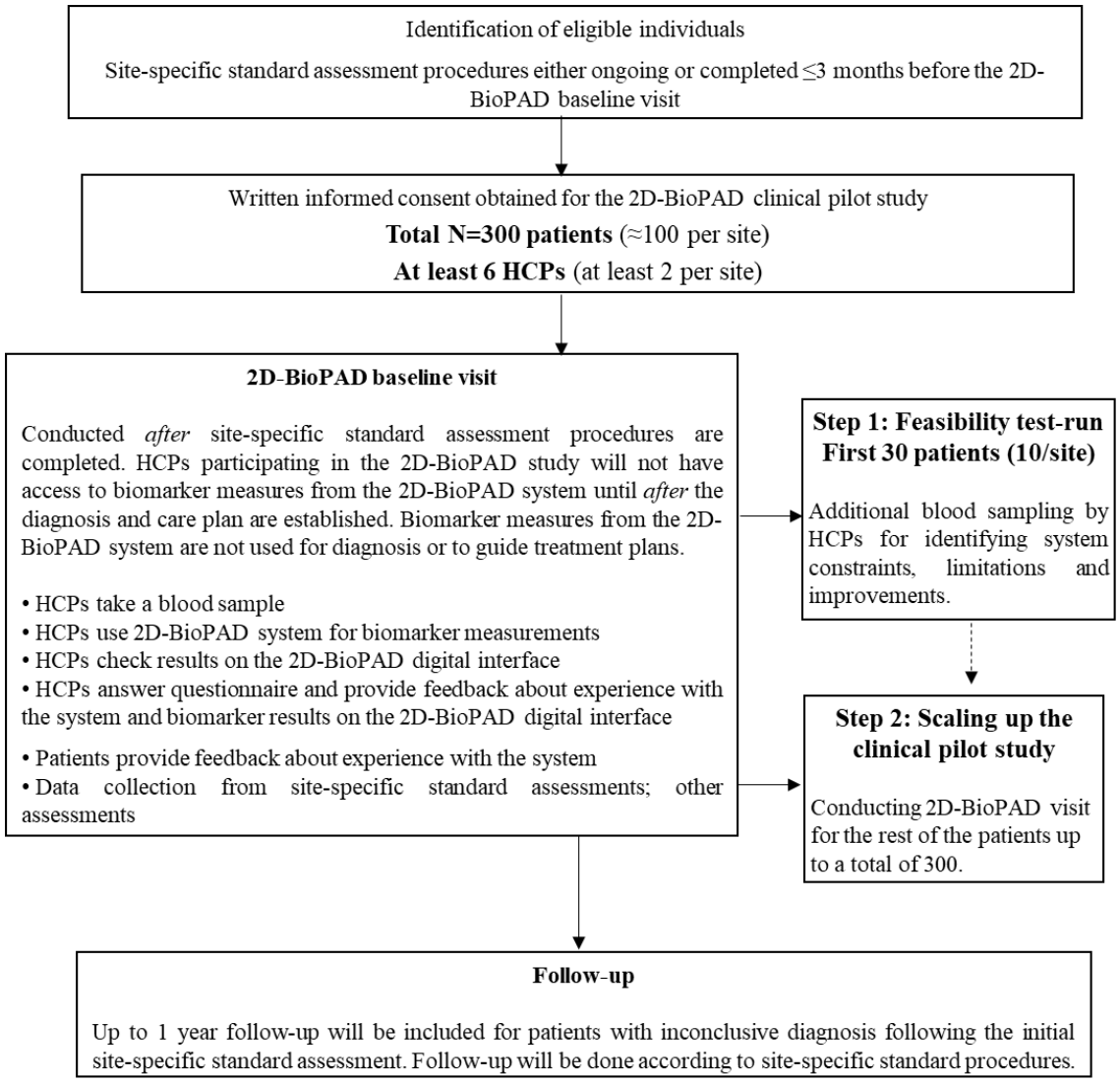

Figure 4. 2D-BioPAD clinical pilot study design for the prospective stage

4.3.2.1. Training on using the 2D-BioPAD Diagnostic Aid

Training workshops for HCPs

ICN2 will plan a training workshop for the HCPs in cooperation with the three study centres. The format will likely consist of virtual sessions, combining practical hands-on training with app-based tutorials. In-person session could be considered according to the needs of the HCPs and the availability of the trainers.

User guides and manuals

The mobile app will feature a comprehensive Digital User Guide that provides step-by-step instructions on device usage. This includes guidance on:

- Setting up the device.
- Procedure for collecting samples.
- Procedure for pre-processing samples.

- Using the device for testing.
- Accessing the results and safely disposing of consumables.

The guide will be available in both digital (within the app) and downloadable PDF formats, as well as through video tutorials and interactive elements embedded in the app for ease of use.

Handling technical issues and troubleshooting

For troubleshooting, there will be app-based support features for technical issues. The app will include common troubleshooting scenarios, and in case of more critical problems, HCPs will be able to directly contact the technical support team via phone or through the app's integrated support feature. The importance of real-time assistance will be carefully considered, particularly when samples are being processed.

4.3.3. Recruitment

All clinical study centres have well-established recruitment procedures for research studies, including both HCPs and patients. Recruitment timeline for the 2D-BioPAD clinical pilot study is shown in section 4.5. Continuous recruitment will be conducted until a total sample of ≈300 patients will be reached. Step 1 (feasibility test-run) will include the first 30 recruited patients (10 per study centre). In Step 2, recruitment will be scaled up to include 270 patients until the total N≈300 is reached.

4.3.3.1. Step 1: feasibility test-run

In Step 1, a small-scale feasibility test run will be performed with the first 30 recruited patients (10 per study centre) to refine the procedure for using the diagnostic aid by identifying constraints, limitations, and potential improvements. To assess the potential impact of fasting versus non-fasting state, and time of day, blood sampling for each patient will be done **in the morning** (one fasting sample before 09:00, and one sample about one hour after breakfast on-site) **and in the afternoon** (non-fasting after 13:00 on the same day). This is based on recent studies indicating that AD blood biomarker levels may be modified by food intake, and also showing significant changes over a 3-hour interval in the fasting control group[24].

4.3.3.2. Step 2: scaling up the clinical pilot study

In Step 2, the clinical pilot study will be scaled up with recruitment of the rest of the patients (up to a total of 300, 100 patients per centre for step 1 and step 2 together). Up to one year follow-up will be included for patients with inconclusive diagnosis following the initial assessment. The follow-up protocol will be according to centre-specific standard procedures.

4.3.4. Data collection

Data and samples will be collected as described below. The *2D-BioPAD baseline visit* refers to data and samples collection for all 300 recruited patients. The *2D-BioPAD feasibility test-run* refers to additional data and samples collection for the first 30 patients recruited during Step 1. A total of up to 30 ml blood will be drawn per patient for all samples together (including the additional Step 1 sampling).

[24] Huber H, Ashton NJ, Schieren A, Montoliu-Gaya L, Molfetta GD, Brum WS, Lantero-Rodriguez J, Grötschel L, Stoffel-Wagner B, Coenen M, Weinhold L, Schmid M, Blennow K, Stehle P, Zetterberg H, Simon MC. Levels of Alzheimer's disease blood biomarkers are altered after food intake-A pilot intervention study in healthy adults. Alzheimers Dement. 2023 Dec;19(12):5531-5540. doi: 10.1002/alz.13163

The *follow-up* is potentially applicable to all 300 recruited patients, but the primary focus is on patients whose diagnosis is unclear at baseline and have a follow-up visit scheduled as per centre-specific standard procedures.

4.3.4.1. At the 2D-BioPAD baseline visit

Data (including existing data from centre-specific standard assessments) will be collected for use in this study only after the informed consent form has been signed.

Table 6. Data collected from patients

Existing data from centre-specific standard assessments
Cognitive status • Control / no dementia disease / subjective cognitive complaints • MCI • Dementia type • Date of diagnosis
Demographics, e.g.: • Age • Gender (as recorded in medical records) • Years of formal education • Civil status • Work status
Family history of AD/other dementias in first degree relatives
#Neuropsychological test results and function in activities of daily living according to the standard diagnostic protocol of the respective centre.
Medical history (ICD-10 codes)
Current medication (ATC codes)
Markers of kidney and liver function, e.g., creatinine (with the Chronic Kidney Disease Epidemiology Collaboration CKD-EPI equation used to estimate glomerular filtrate rate, eGFR) and ASAT; other centre-specific markers may include e.g., urea, ALAT, GGT, CPK)
Systolic and diastolic blood pressure
Height and weight, BMI
Semiquantitative/visual brain MRI ratings if available (e.g., MTA, Fazekas)
*Results of CSF biomarker analysis according to the centre-specific standard protocol (e.g., Aβ40, Aβ42, total tau, ptau-181) if available
Results of genetic testing (APOE genotype) if available
Amyloid-PET visual ratings (e.g., positive/negative) if available
Visual readings of FDG-PET scans if available
New data
Blood sample for measurement of plasma AD biomarkers (Aβ40, Aβ42 and the Aβ42/40 ratio, p-tau217, NfL, GFAP) using benchmarking equipment **
Blood sample for measurement of plasma AD biomarkers using the 2D-BioPAD diagnostic aid
Questionnaire including feedback about experience with 2D-BioPAD diagnostic aid
Self-reported lifestyle questionnaire

 # A minimum set of tests conducted at all study centres should include:

- *Mini Mental State Examination (MMSE)*
- *Consortium to Establish a Registry for Alzheimer's Disease (CERAD)*
- *Alzheimer's Disease Cooperative Study Activities of Daily Living inventory (ADCS-ADL)*
- *Montgomery-Asberg Depression Rating Scale (MADRS)*

** State-of-the-art CSF biomarkers as described in section 4.1.4.1. To reduce patient burden, the 2D-BioPAD prospective study will use CSF biomarker values where available from standard assessment procedures at the clinical study centres.*

*** Benchmarking equipment for plasma AD biomarkers as described in Section 4.1.4. All plasma samples for all patients will be analysed at the UEF Brain Research Unit biomarker laboratory.*

Data collected from HCPs will include:

- Age
- Gender (self-reported)
- HCP group (e.g., nurse, physician)
- Clinical specialty
- Years and degree of experience (e.g., resident, specialist)
- Questionnaire including feedback about experience with the diagnostic aid and biomarker results on the 2D-BioPAD digital interface, acceptability, trust and satisfaction (short questions with Likert-scale answers to be answered via the digital interface after blood sampling and visualisation of plasma biomarker results for each patient; longer questionnaire to be answered e.g., 3-4 times during the study after the HCP has conducted blood sampling and visualisation of plasma biomarker results for multiple patients).

4.3.4.2. At the 2D-BioPAD feasibility test-run

This applies only to the first 30 recruited patients, and the additional plasma sampling in the feasibility test run.

Data collected from patients:

- Additional blood samples for measurement of plasma AD biomarkers using the 2D-BioPAD diagnostic aid, and using benchmarking equipment, as per schedule described in section 4.3.3.1

Data collected from HCPs:

- Questionnaire including feedback about experience with the diagnostic aid and biomarker results on the 2D-BioPAD digital interface (short questions with Likert-scale answers to be answered via the digital interface after blood sampling and visualisation of plasma biomarker results for each patient; optional free form written notes/comments).

4.3.4.3. At follow-up

For all 300 patients, up to 1 year follow-up after baseline will be included if the diagnosis is unclear at baseline and follow-up is scheduled as per centre-specific standard procedures. The following patient-related data will be collected:

- Cognitive status
  - Control / no dementia disease / subjective cognitive complaints
  - MCI
  - Dementia type
- Date of diagnosis

4.3.5. Risk analysis

4.3.5.1. Development of the 2D-BioPAD diagnostic aid

Potential clinical pilot study risks related to the 2D-BioPAD diagnostic aid development and mitigation strategies are listed below.

- **Prototype functionality limitations requiring more extensive pre-processing of samples at the clinical study centres** (risk: moderate | impact: low)

Impact: A potential need for more extensive pre-processing of samples would not affect the clinical pilot study at UEF, i.e. the UEF biomarker laboratory can handle additional pre-processing on-site. Benchmarking at UEF for samples from all study centres would also not be affected. However, if the 2D-BioPAD prototype requires more extensive sample pre-processing, the GAADRD and ZI study centres may need to rely on the local laboratories that they already use for routine blood sampling and testing. This would change the prototype testing setup from *direct* HCP-patient testing with immediate results on-site to the currently used *indirect* routine setup, i.e. laboratory-based sampling/measurement with results delivered separately to HCPs, who then inform patients as needed.

Mitigation: Because this is a clinical *pilot* study, the design has sufficient adjustment room for covering two different prototype testing setups (direct and indirect). In the indirect setup, the HCPs feedback questions would be separated into two parts, one for the laboratory (sampling/testing) and one for the HCPs (results/digital interface). The patient feedback questionnaire can be easily adapted because patients are not informed about not yet validated biomarker results. Additional feedback would allow comparisons between direct and indirect setups. Comparison with benchmarking equipment would be the same. GAADRD and ZI are currently assessing the details of a potential shift from direct to indirect prototype testing setup at these study centres.

- **Number of 2D-BioPAD diagnostic aids ready-to-use by the clinical study centres lower than expected** (risk: low | impact: moderate)

Impact: With a lower number of diagnostic aids, recruitment would be expected to take longer time.

Mitigation: The total number of 300 patients can still be recruited because the current timeline (section 4.5) accounts for 1-year follow-up in cases where the diagnosis is initially not fully clear. The recruitment duration could thus be extended, and selection criteria adjusted to limit or exclude unclear cases, i.e., shift towards a more typical case-control study design, which is fully appropriate in a pilot study.

- **Significant delay in delivery of a prototype ready-to-use by the clinical study centres** (risk: low | impact: moderate)

Impact: Significantly delayed start of recruitment, and/or potential shift from prototype testing in real-life clinical environments to laboratory-only testing.

Mitigation: As above for delays in recruitment. In case of major delays estimated to significantly affect the total sample size that is feasible to recruit before the end of the project, the clinical pilot study design will be adapted, with more emphasis on plasma samples analysis in a laboratory/technical environment. I.e. recruitment can start as planned, including collection of new plasma samples, but prototype-based biomarkers will be measured by the technical partners in their laboratory environment (benchmarking at UEF laboratory would not change). The HCPs and patient feedback questionnaires would be replaced with e.g. usability and user experience assessments, focus groups, and/or workshops based on prototype-related scenarios identified by technical partners as most useful for further prototype development. A smaller real-life environment testing could still be included when at least one ready-to-use prototype becomes available.

4.3.5.2. Clinical pilot study sample size considerations

This is a clinical pilot study, i.e., the first study to evaluate and validate AD blood biomarkers measured using the newly developed 2D-BioPAD diagnostic aid in human plasma samples and in clinical environments in three countries. Sample size considerations for the prospective study are summarized in Table 6.

Table 7. Sample size for the prospective study

Study	Sample size	Rationale
Prospective, feasibility	30 patients 3 plasma samples/patient	10 patients per study centre appropriate for an initial feasibility assessment when shifting from a laboratory to a clinical environment. Repeated blood sampling allows initial assessment of use conditions in a clinical environment.
Prospective, full study	300 patients 1 plasma sample/patient	Sample size is in line with recent pilot studies of AD blood biomarkers validating new assessment methods for AD blood biomarkers[25] or studies comparing different assessment methods[26,27,28].
Prospective, feasibility & full study	At least 6 HCPs (2/study centre)	Sample size allows detailed initial feedback from several medical specialties and HCP categories with various degrees of experience.

4.3.5.3. Missing data

Missing data in the 2D-BioPAD prospective study is defined as:

- Missing blood samples and/or related AD biomarkers measured using the 2D-BioPAD diagnostic aid and/or benchmarking equipment. The three study centres have extensive experience with blood sampling for clinical research studies, which reduces the risk of sampling failures. A sufficient amount of blood will be collected to allow completion of AD blood biomarker measurements in case of technical issues with some of the samples.
- Missing answers from patients in self-reported questionnaires. Completion will be ensured by HCPs checking the questionnaires during the 2D-BioPAD baseline visit.
- Missing answers from HCPs in questionnaires. Technical solutions will be applied in the 2D-BioPAD digital interface to design the questionnaires in a way that reduces the potential to skip or miss questions.

Most data collected from patients in the 2D-BioPAD clinical pilot study will be data generated during the standard assessment procedures at each study centre. Since these data reflect real-world clinical routines, missing assessments will not be treated as missing data. A pre-defined core set of assessments will be conducted in the same way across all centres (section 4.3.4).

4.3.5.4. Data handling and monitoring

The three clinical study centres will jointly design the case report forms (CRF) and other data collection forms (e.g., information related to study visits, such as contact details, bookings etc) for the 2D-BioPAD clinical pilot

[25] Huber H., et al. Biomarkers of Alzheimer's disease and neurodegeneration in dried blood spots-A new collection method for remote settings. Alzheimers Dement. 2024 Apr;20(4):2340-2352. doi: 10.1002/alz.13697

[26] Therriault, J., et al. Comparison of Two Plasma P-Tau217 Assays to Detect and Monitor Alzheimer's Pathology. Available at SSRN: https://ssrn.com/abstract=4572850 or http://dx.doi.org/10.2139/ssrn.4572850

[27] Hirtz, C., et al. Comparison of ultrasensitive and mass spectrometry quantification of blood-based amyloid biomarkers for Alzheimer's disease diagnosis in a memory clinic cohort. Alz Res Therapy 15, 34 (2023). https://doi.org/10.1186/s13195-023-01188-8

[28] Janelidze S., et al. Head-to-Head Comparison of 8 Plasma Amyloid-β 42/40 Assays in Alzheimer Disease. JAMA Neurol. 2021;78(11):1375–1382. doi:10.1001/jamaneurol.2021.3180

study. Investigators at all clinical study centres will file all data per subject. An explanation for the omission of any required data should appear on the appropriate CRF section. All data recorded in the CRF will be signed off by the study centre PI or their appropriate designee.

Data from CRFs will be entered in a web-based database, e.g., via REDCap (Research Electronic Data Capture), a secure web application for building and managing online surveys and databases (already available at UEF), by trained personnel at each clinical study centre. All changes made following initial submission of data will have an electronic audit trail with a date. Specific instructions and further details will be outlined in the study manual of operations. The PI at each centre will monitor the study at their own centre to ensure quality and integrity of data collected. They will review study files, ethical/regulatory documents, consent forms and allocate adequate time for other study monitoring activities. Each PI must permit study-related monitoring visits, audits, review by the relevant authorities, and allow direct access to source data and source documents provided that subject confidentiality is protected.

Source documents (paper or electronic) are those in which research participant data are recorded and documented for the first time. Source data will be collected in Finnish (UEF), Greek (GAADRD), or German (ZI). Source documents in paper format will be kept in a locked location at each study centre, to which only authorized people involved in the study will have access. Each clinical study centre must maintain adequate source documentation to allow reliable verification and validation of the study data.

CRFs, other data collection forms, manual of operations, and the web-based database will be ready before the start of the study (M24).

4.3.5.5. Safety evaluations

Due to the design of this study, HCPs and patients will not have access to AD plasma biomarker results from the 2D-BioPAD diagnostic aid during the diagnostic workup of a patient, i.e., there is no risk of interference with diagnostic, treatment, and care decisions, which will be made according to established standard procedures at each study centre.

Blood sampling is overall safe and well tolerated, and participation in this study is expected to have a low risk of adverse events. Blood sample collection may cause discomfort, mild bruising, and/or bleeding where the needle is inserted. Some people may become dizzy, lightheaded, or feel faint. Infections rarely occur. For efficient collection of safety information relevant to the 2D-BioPAD study participation, we will collect and evaluate data regarding (serious) adverse events that occur according to the following pre-specified conditions:

- During blood sampling for the 2D-BioPAD clinical pilot study and/or afterwards for the duration of the 2D-BioPAD study visit
- Involving the puncture site used for blood sampling.

Definitions

Adverse Event (AE): any untoward occurrence in a participant.

Serious Adverse Event (SAE): any untoward and unexpected occurrence or effect that:

- Results in death
- Is life-threatening – refers to an event in which the participant was at risk of death at the time of the event; it does not refer to an event which hypothetically might have caused death if it were more severe
- Requires hospitalisation
- Results in persistent or significant disability or incapacity

Medical judgement will be exercised in deciding whether an AE is serious and/or severe in all situations. Important AEs that are not immediately life-threatening or do not result in death or hospitalisation but may jeopardise the participant or may require intervention to prevent one of the other outcomes listed in the definition above, will also be considered serious.

Reporting Procedures

Following the signing the informed consent, any events that are reported by the participant or observed by study team members, and meet the abovementioned pre-specified conditions, will be reported as AE or SAE, depending on which criteria are being met. Depending on the nature of the event the reporting procedures below will be followed. Any questions concerning (S)AE reporting will be directed to the Principal Investigators in the first instance. (S)AEs will be recorded by participant study ID numbers. No identifiable information in relation to any (S)AEs reported will be disclosed outside of the relevant study centre.

A Serious Adverse Event (SAE) form will be completed and emailed to the Principal Investigators within 24 hours. Study centre investigators will report any SAEs as required by their local regulations (e.g., to their Ethics Committee or other relevant regulatory authorities).

Causality

The assignment of causality for AEs and SAEs will be made by the trial Principal Investigators as follows:

- *Unrelated*: No evidence of any causal relationship
- *Unlikely*: There is little evidence to suggest there is a causal relationship (e.g., the event did not follow a reasonable temporal sequence after administration of the study procedure or did not follow a known or expected response pattern to the suspected procedure). There is another reasonable explanation for the event (e.g., the patient's clinical condition, other concomitant procedure, or treatment).
- *Possible*: There is some evidence to suggest a causal relationship (e.g., because the event followed a reasonable temporal sequence after administration of the study procedure or followed a known or expected response pattern to the suspected procedure). However, the influence of other factors may have contributed to the event (e.g., the patient's clinical condition, other concomitant procedures, or treatments).
- *Probable*: There is evidence to suggest a causal relationship and the influence of other factors is unlikely.
- *Definite*: There is clear evidence to suggest a causal relationship and other possible contributing factors can be ruled out.

4.4. Evaluation and validation design

4.4.1. Retrospective study outcomes

Table 8. Overview of the retrospective study outcomes

Outcome type	Description
Primary	Comparison of plasma biomarker values between 2D-BioPAD prototype and benchmarking equipment. Sensitivity, specificity, positive and negative predictive values of blood biomarkers measured with the 2D-BioPAD prototype for identifying: • AD-related pathology (e.g., β-amyloid, tau), as indicated by CSF biomarkers measured during routine assessments at the clinical study centres using state-of-the-art equipment and established cut-offs. • AD diagnosis established as per centre-specific standard procedures.
Secondary	Assessing the potential impact of other parameters, e.g., demographics, comorbidities on the primary outcomes.

4.4.2. Prospective study outcomes

Table 9. Overview of the prospective study outcomes

Outcome type	Description
Primary	• Sensitivity, specificity, positive and negative predictive values of plasma biomarkers measured with the 2D-BioPAD prototype versus benchmarking equipment (same as above) for identifying AD diagnosis established as per centre-specific standard procedures. • Overall experience of HCPs with the 2D-BioPAD diagnostic aid • Overall experience of patients with the 2D-BioPAD diagnostic aid
Secondary	• Comparison of plasma biomarker values between 2D-BioPAD prototype and benchmarking equipment, considering the potential impact of other parameters, e.g., demographics, comorbidities, disease stage • Sensitivity, specificity, positive and negative predictive values of plasma biomarkers measured with the 2D-BioPAD prototype for identifying AD-related pathology (e.g., β-amyloid, tau), as indicated by either plasma biomarkers from benchmarking equipment or, where available, CSF biomarkers measured during routine assessments at the clinical study centres using state-of-the-art equipment. • Investigating plasma biomarkers measured with 2D-BioPAD prototype versus benchmarking equipment in relation to cognitive, clinical and other data • Assess if user experience (HCPs and patients) with the 2D-BioPAD diagnostic aid varies by country, clinical specialty, and/or years of experience • Assess if the information provided to HCPs via the digital interface of the 2D-BioPAD diagnostic aid is understandable and relevant • Collect feedback for diagnostic aid improvement from HCPs and patients

4.4.3. Data analysis

Analyses will include e.g.:

- Correlations (Spearman/Pearson as appropriate) between 2D-BioPAD markers and benchmarking plasma markers. Statistical tests for comparing correlation coefficients will be used where applicable.
- Linear regressions and plots of 2D-BioPAD markers and benchmarking plasma markers.
- Bland-Altman comparison of 2D-BioPAD markers against benchmarking plasma markers.

Between-group comparisons of 2D-BioPAD AD plasma biomarker levels will be conducted using e.g., Mann-Whitney U test, t-test, or ANOVA as appropriate. Groups will be defined according to both clinical and biomarker-based characteristics. Diagnostic groups will be based on diagnosis established following centre-specific standard assessments, e.g., AD dementia, other dementia, MCI, or control. Biomarker-based groups will be defined using:

- For all participants, AD blood biomarkers measured with benchmarking equipment. Cut-offs will be based on existing literature (where available) and/or data-driven, e.g., using Gaussian mixture modelling.
- For participants with available AD CSF biomarkers from standard assessments, grouping will be based on established cut-offs.
- Exploratory analyses will consider ATN-based categorizations using state-of-the-art CSF and/or benchmarking plasma biomarkers.

To assess the potential impact of other parameters, e.g., demographics, comorbidities, liver and/or kidney function, APOE genotype (where available), such parameters will be added as covariates in the analyses.

Discrimination accuracies of 2D-BioPAD AD plasma biomarkers will be determined with logistic regression models and receiver operating characteristic curve (ROC) analysis. When predicting diagnosis or CSF-based biomarker +/- status, for each AD blood biomarker the area under the receiver operating characteristic curve (AUC) of the ROCs for 2D-BioPAD and benchmarking measures will be compared with DeLong test. Sensitivity, specificity, positive and negative predictive values will be assessed for different 2D-BioPAD biomarker cut-offs. Initial analyses will consider each AD plasma biomarker individually. Further analysis will investigate combinations of plasma biomarkers to determine which combinations have the best discrimination accuracy.

AD plasma biomarkers (2D-BioPAD versus benchmarking equipment) will be investigated in relation to cognitive, clinical, and other data using appropriate regression models.

Unadjusted 2-sided p-values <0.05 will be considered statistically significant. Adjustment for multiple testing using a false discovery rate (e.g., Benjamini-Hochberg procedure) will be used where relevant.

The main hypothesis is that the accuracy of the 2D-BioPAD system measurements is noninferior to the accuracy of AD plasma biomarkers measured using benchmarking equipment.

Analyses of user experience (HCPs and patients) will include mainly descriptives, with comparisons by country, clinical specialty, and/or years of experience where relevant.

4.5. Timeline

	2025			2026												2027								
	Oct	Nov	Dec	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep
	M25	M26	M27	M28	M29	M30	M31	M32	M33	M34	M35	M36	M37	M38	M39	M40	M41	M42	M43	M44	M45	M46	M47	M48
Retrospective																								
Sample Selection																								
Shipping to tech partners																								
Technical Validation																								
Shipping to UEF & Benchmarking																								
Data analysis																								
Prospective																								
HCP Recruitment & Training																								
Patients Recruitment & Baseline visit																								
Feasibility test-run																								
Patients follow-up																								
HCP survey																								
Benchmarking																								
Data Analysis																								

5. Conclusions

The innovative 2D-BioPAD diagnostic aid is currently in an early stage of development. Thus, initial clinical testing will be conducted in a pilot study covering both testing in a laboratory environment (already existing plasma samples) and in a clinical environment similar to the intended conditions of use (prospective clinical study).

Following extensive preparatory activities at the clinical study centres in collaboration with the other 2D-BioPAD Consortium partners, the ethics-compliant version of the clinical pilot studies initiation package has been completed. The clinical pilot study protocol (version 1) has received ethical approval at two study centres, and approval process is ongoing at the third study centre.

Preparation of an updated regulatory-compliant clinical study protocol is ongoing, considering the device classification and differences in regulatory requirements and procedures between EU member states for clinical studies involving different types of medical devices. The updated version will be communicated to the members of the Ethics and Industrial Advisory Board for additional feedback. Final approved versions of the clinical pilot studies initiation package documents will be publicly available at the provided online link(s) at M24 before the start of the pilot study. Following any changes in these documents throughout the duration of the clinical pilot study, updated versions will be provided online with a list of versions and dates.

Final preparations for study deployment at all clinical centres, including internal documents with 2D-BioPAD diagnostic aid details and other internal operational aspects at the study centres will also be completed at M24, and all clinical centres will initiate the pilot study at M25.

The clinical pilot study is designed to provide preliminary information about the clinical performance of the diagnostic aid. All information from the 2D-BioPAD clinical pilot study will be used to plan the next steps concerning future full-scale confirmatory clinical studies, regulatory aspects of the diagnostic aid, and market-related aspects.

2D BioPAD

Supple Graphene Bio-Platform for point-of-care early detection and monitoring of Alzheimer's Disease

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