WORK PLAN (5 Pages)

The outline presented here is extending to 2 years and structured around 3 milestones aligning with study objectives (see Figure 1).

MILESTONE 1 (month 3): PROTOCOL DEVELOPMENT AND ETHICS PREPARATION

Until month 3, we will write the protocol to be submitted to the ethics committee to ensure the highest standards as well as the protection of participants' rights, safety, and well-being throughout the study. It will provide us with the opportunity to address any ethical considerations, conflicts, or concerns that may arise.

In parallel to obtaining ethics committee approval, the protocol for conducting a pilot study will be solidified, based on the proposed framework of this project. This milestone completely aligns with **objective 1**. The protocol will be documented comprehensively outlining the following sections: (i) cognitive and demographic data collection (ii) sample collection procedure: type of blood collection, tubes to be used and any specific instructions, (iii) storage requirements: specific temperature conditions , (iv) sample handling: guidelines for labeling the samples, centrifugation, aliquoting and storage (v) packaging and shipping: description of packaging materials and procedures for shipping the blood samples that allow maintaining the required temperature during transit. ***Deliverable 1:*** Study protocol to be used throughout the study duration. ***Deliverable 2:*** Writing of the scientific paper detailing the protocol. ***Tasks lead:*** The tasks will be overseen by Dr. Arenaza-Urquijo with involvement of the full research team including a key role of Dr. Juan Fortea as advisor and Drs. Pomat, Laman and Sozinho at FMICISM and PNG-IMR.

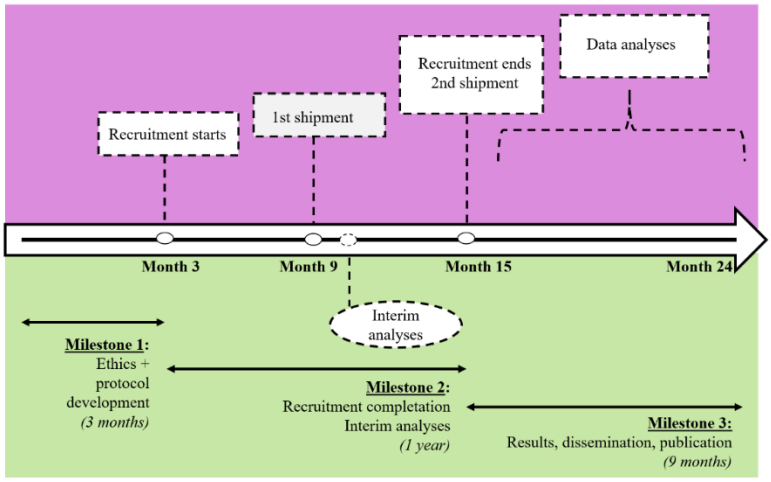

Figure 1. Illustration depicting a basic overview of the study timeline structured around the main logistic tasks and milestones. A full description can be found in the text.

MILESTONE 2 (Month 15) RECRUITMENT COMPLETION AND INTERIM ANALYSES

Recruitment will start at month 3 after ethics approval and protocol development. There is budget allocated to both sites to contract personnel during 1 year (part time) to carry out the study. Moreover, an ISGlobal researcher will oversee and support this task (refer to *Budget justification* section).

We have implemented two shipments of biological samples to minimize the risk of sample loss throughout the study. Furthermore, acknowledging the limitations of existing data for sample calculation, we intend to conduct an interim analysis. This will enable us to potentially refine our recruitment strategy in response to emerging insights. This milestone is a key milestone supporting **objectives 2 and 3.**

Data collection: We envision a **concise study visit** following participant engagement through **home visits.** The outlined data collection methods provided here, will be solidified during the first 3 months of the study (see *Deliverable 1*). At month 10, following interim analyses, the feasibility of the protocol will be evaluated by the three centers, and adjustments will be made as needed. The visit will encompass the following:

1.Informed Consent: Participants (or their families if participant is unable to provide consent) will be invited to formally provide their consent by signing the informed consent form, ensuring their full understanding and voluntary participation in the study. **2.Demographic and Comorbidity Assessment:** Detailed information regarding demographics and medical history, including any prevalent comorbidities, will be gathered. To assess the impact of vascular risk factors and comorbidities, we will collect information about the presence of high blood pressure, dyslipidemia, diabetes, and history of stroke or other co-morbidities of interest.

3.Dementia Screening Tool: A specialized dementia screening tool will be administered to assess cognitive function. This step is crucial for identifying potential cognitive impairment or dementia among participants. The

Community Screening Interview for Dementia (CSI 'D') will be used. It is a simple screening technique, that can be used by non-specialists, adapted to a range of health and social service settings for dementia detection in cross-cultural studies, population with diverse socioeconomic backgrounds and minoritized populations. The instrument was validated in Portuguese and English. We will provide translation to local languages as necessary (Bantu languages in Mozambique and Tok Pisin or Papuan languages in PNG). It consists of two components, a cognitive test for non-literate and literate populations and an informant interview regarding performance in everyday living. Previous studies have demonstrated the adaptability and utility of the CSI 'D' in LMICs. The CSI'D" will be evaluated during the study visit. **4. Plasma Biomarker Collection:** Blood samples will be collected in EDTA-K2 tubes and subsequently centrifuged (2000rpm × 10 mins, 4°C) within 2 hours after extraction. Plasma will be aliquoted and stored at −80°C until shipping in specialized packaging with dry ice or liquid nitrogen to maintain such ultra-low temperature (month 9 and month 15).
Deliverable 2 (month 9): 1st ~200 blood samples shipment to Barcelona (N~100 from each site). ***Deliverable 3 (month 10):*** Interim analyses and assessment of protocol feasibility. ***Deliverable 4 (month 15)*** 2nd ~200 blood samples shipment to Barcelona (N~100 from each site). ***Deliverable 5 (month 15):*** Clinical data sharing (cognitive evaluations, demographics) (see *Data Management* section). ***Task lead:*** A designated individual will be employed at ISGlobal to oversee this task. For Deliverables 1, 4, and 5, designated employees from FMICISM and The PNG-IMR will be involved. Regarding Deliverable 3 including interim analyses, after biomarker determinations are performed, Dr. Arenaza-Urquijo's team will take the lead on data analysis.

STUDY POPULATION AND RECRUITMENT METHODS

We propose a **2- stage recruitment strategy** that combines **random sampling of older adults** ≥ 60 years old **and risk enrichment/oversampling of adults** >70 years, stratified by country (Mozambique, PNG, see Figure 2). This approach addresses both **feasibility considering the time and budget constraints of the study and mitigates the risk of underreporting** by targeting older individuals in whom AD prevalence is expected to be higher. Through **demographic surveillance systems and census lists** (The Pacific Public Health Surveillance Network [PPHSN] in PNG and the CISM census list in Mozambique) participants would initially be recruited randomly ensuring representation across the demographic group we are interested in (>60 yrs) in Manhiça (Mozambique) and Port Moresby (PNG). Thus, we will filter by age range, to then select individuals randomly. Then, we will conduct a risk stratification strategy based on older age (>70 years). The reason to target individuals over 60 years instead of 70 years at first place stems from 2 key factors: first, life expectancy is 57.7 and 59 in Mozambique and PNG, respectively. Additionally, research suggest that amyloid positivity can be manifest even two decades before noticeable symptoms.

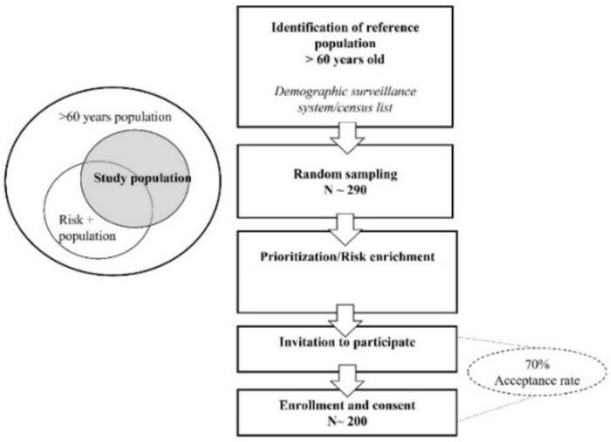

Figure 2. Recruitment chart. Risk + population: refers to the older stata of the targeted population.

Sample size: The sample size has been adjusted to **align with feasibility criteria** considering the duration and budget constraints of the current project, which is **designed as a pilot study** (refer to Risk and contingency for limitations). We anticipate a recruitment of approximately 400 participants, 200 participants by site (N=200 Manhiça, N=200 Port Moresby). We will target at N=290 participants considering an acceptance rate of 70%. Based on Jansen et al., 2022 (CSF), A+ prevalence in cognitively unimpaired older adults varies by APOE4 allele status, 18ranging from 13% to 35% at age 60 and increasing with age. Estimates peak at 25% to 66% by age 80. Using Manhiça's population data as a reference (average expected A+ prevalence of 20% in individuals

over 65 without APOE4), a sample size of at least 251 individuals is needed from a population of 2280 to estimate a proportion of 20% with a 10% margin of error and 95% confidence level. Though slightly higher than our target sample size, we used as reference cognitively unimpaired older adults without APOE4, while we expect a high prevalence of APOE4 carriers and feasibility considerations necessitate this compromise for the pilot study (refer to risk and contingency)

Household visits will be conducted to reach selected individuals. Those expressing interest in participating will be invited to visit the designated research center or clinic for enrollment and data collection. Inclusion and exclusion criteria will be applied during this visit, prior to obtaining informed consent. **Inclusion criteria** entail individuals aged 60 years or older, residing in either Manhiça or Port Moresby, and willingly consenting to participate in the study. **Exclusion criteria** involve individuals who are deemed by their healthcare provider to have contraindications for undergoing a blood extraction. ***Task lead:*** Dr. Arenaza-Urquijo will oversee this task with the support of Prof. Garcia-Aymerich who has extensive experience with epidemiological research.

DATA COLLECTION, BIOMARKER DETEMINATION AND STATISTICAL ANALYSES

We aim to measure **Ptau-217** to gain initial insights into the **prevalence of AD pathophysiology** in the study population. Plasma p-tau species, including p-tau217, have shown high performance to identify underlying AD.[17] Plasma p-tau217 (tau phosphorylated at Thr217) shows the highest fold-changes in Aβ-positive patients with cognitive impairment, thus being less susceptible to analytical variation.[18] Plasma pTau217 has consistently shown exceptionally high accuracy across different platforms and has demonstrated strong correlations with other markers of AD (CSF biomarkers, amyloid PET and Tau PET) and notably with neuropathology.[19] Further previous evidence supports its implementation as a single biomarker.

Moreover, Neurofilament light (**Nfl), a non-AD specific marker of neurodegeneration** will be determined to have a more comprehensive understanding of neurodegenerative processes in the population. Nfl is a blood protein released from damaged neurons, indicating neurodegeneration. While not specific to any one pathology like Alzheimer's, its presence in plasma serves as a general biomarker for neurological damage and disease progression. Due to its specificity to neuronal damage and its ability to reflect ongoing pathological processes NFL in plasma is associated with or disease progression and severity. Given that such a marker appears to be heavily influenced by HIV-derived immunosuppression, and given the high rate of background HIV infection in Mozambique, serological status will be determined on site for all Mozambican participants, and adequate counseling provided according to test's results.

Biomarker determination are outsourced to a third-party service provider with whom we will collaborate for this purpose. In brief, plasma samples will be measured in the Lumipulse fully-automated platform G600II using commercially available kits (Fujirebio Europe, Ghent, Belgium). On the day of the analysis, plasma samples will be brought to room temperature, mixed thoroughly, centrifuged for 5 minutes at 2000g, and subsequently transferred to specific cuvettes for analysis in the Lumipulse platform.[20]

In a previous study, amyloid positive participants had 3.2 times higher concentrations of plasma pTau217. [20] This magnitude of effect, combined with the advantages of automation, makes this assay particularly promising for integration into standard clinical practices. **This automation aspect makes it particularly promising for use in LMICs in future studie**s, where streamlined and efficient diagnostic tools are essential for widespread implementation and effective Alzheimer's disease screening program

Apolipoprotein 4 status: DNA will be extracted from full blood using standard procedures, and *APOE* was genotyped. Briefly, direct DNA sequencing of exon 4 was performed routinely for all participants, followed by visual analysis of the resulting electropherogram to identify the two coding polymorphisms that encode the three possible *APOE* isoforms. Participants will be classified as APOE4+ (2/4, 3/4, 4/4) or APOE4- (2/2, 2/3, 3/3)

Kidney function: Participants will be classified according to the estimated glomerular filtrate rate (eGFR)- rate at which the kidneys filter waste from the blood- in different stages (1–5) of chronic kidney disease (CKD) using CKD-EPI formula based on serum creatinine levels. For this purpose, serum Creatinine levels will be determined (on site) alongside APOE4 determinations.

STATISTICAL ANALISES

Prevalence of AD pathophysiology and neurodegeneration (Objective 2) We will follow the methodology delineated by Brum et *al.* [21] stratifying the risk of having AD pathophysiology. By implementing this strategy, it is feasible to define threshold values that are highly sensitive and specific to either detect or rule out amyloid pathology minimizing the probability of misclassification. Then, **weighted prevalence rates** will be calculated to account for the unequal probabilities of selection for different groups, ensuring that the prevalence estimates are representative of the entire population. Weighted prevalence rates are calculated by assigning weights to individuals in a sample based on their representation in the population. These weights are then used to adjust prevalence rates observed in the sample, yielding weighted counts for each subgroup. The weighted counts are aggregated across all subgroups to calculate the overall weighted prevalence rate, providing a more accurate representation of prevalence in the entire population. In the absence of validated threshold for NfL, the prevalence of NfL will be described with percentile ranges, for example, the percentage of the population falling within the 25th to 75th percentile range, indicating the middle 50% of values.

Association with cognition and comorbidities (objective 3): Multiple and/or logistic regression analyses will be carried out to assess the association of continuous and ptau217 levels and Nfl levels with: (1) cognitive outcomes: CSI 'D' (scores ranging from 0-30), (2) presence/absence of main comorbidities (high blood pressure, dyslipidemia, diabetes, and history of stroke, HIV) (3) APOE4 status and (4) kidney function. Depending on the variable range and distribution log transformation, non-parametric statistical approaches and robust regressions methods will be considered. ***Task lead:*** Statistical analyses will be carried out by our PI group, in collaboration with a skilled statistician employed for these tasks. The process of scientific writing is a collaborative effort among all researchers involved in the study. Through iterative revisions and feedback sessions, we aim to produce comprehensive scientific manuscripts that accurately depict our findings and their significance.

MILESTONE 3 (Month 15-24): RESULTS' DISEMINATION AND PUBLICATIONS

In our project, several key milestones are dedicated to ensuring the effective dissemination of our findings to both scientific communities and the general public.

1. Presentation at meetings: We will present findings at national and international meetings, including the Alzheimer's Association International Conference (AAIC), offering insights into our research design and early findings. Final results will be shared at AAIC and through webinars organized by ISTAART PIAs. **2. Publication in journals**: Results will be published in high-impact open-access journals due to their wide academic interest and impact. **3. Lectures at scientific meetings and institutions**: Researchers will communicate results through plenary, keynote, and invited lectures at major scientific meetings and academic institutions. **4. Webinars**: A webinar focused on Alzheimer's and LMICs will be organized, attracting international researchers to share project framework and preliminary results. **5. Social media and media impact**: Results will be publicized on the ISGlobal webpage and social networks, engaging media outlets through interviews and press releases. Informational materials will be developed for wider accessibility. **6. Dissemination articles and presentations**: Non-technical articles and presentations tailored for general audiences will promote result dissemination. Public engagement lectures and forums will educate stakeholders about aging and Alzheimer's disease. **7. Participation in open days**: We'll engage in public awareness initiatives such as yearly open doors events and the Science Festival in Barcelona, hosting community workshops and seminars to educate the public about aging and AD. We will proactively engage, with dissemination purposes of study results and AD awareness, with communities both in Mozambique and PNG.

Deliverable 6 (months 15-24): Submission of 2 scientific publications reporting study findings.

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Identified risk and contingency: (1) The effectiveness of the project hinges on its multicenter structure, demanding coordination between the partners and ISGlobal. To guarantee the efficacy of recruitment strategies and uphold consistency in data collection and quality, we will designate a dedicated individual from ISGlobal who will personally oversee operations at both sites at the outset of the study (*see budget justification).* Additionally, Deliverable 1, which encompasses a protocol for data collection, storage, and shipping developed by the 3 centers, will comprehensively address this potential risk. **(2) There is a risk associated with the feasibility of the field study and the challenge of effectively communicating with and identifying participants in dispersed locations.** However, the study benefits from the expertise of seasoned professionals with extensive experience in the field. At month 10, we will have the opportunity to assess study feasibility and make adjustments as necessary. Regardless, the insights gained will be valuable for enhancing recruitment and logistics in future studies. **(3) Low precision in prevalence estimates and low variability in study outcomes.** This could occur due to factors the small sample size. We have partially addressed this by oversampling older participants. Nevertheless, it's important to recognize that the results of this study will serve as preliminary insights for informing future, larger-scale studies rather than providing conclusive findings. Overall, despite its limitations, we believe that this pilot study will be pivotal in guiding informed decisions regarding potential modifications to the study protocol, recruitment strategies, and sampling methods for future endeavors.

Personnel engaged and fortified collaborations. This project will strengthen research collaborations between ISGlobal, the PNG Institute of Medical Research, and the Manhiça Health Research Center, establishing a **new avenue of collaboration in the research field of aging and dementia**. We envision this collaboration as a foundational study that will pave the way for securing additional funding for larger-scale epidemiological studies in the future. We anticipate that this study will foster collaboration with other initiatives, including the African Dementia Network, the Global Brain Health initiative, as well as with the Alzheimer's Association and ISTAART Professional Interest Areas, including the Diversity PIA. **Dr. Arenaza-Urquijo is Assistant Professor** at ISGlobal and Chair of the Reserve, Resilience and Protective factors PIA.She is a recognized expert in the area of resilience in aging and Alzheimer's disease and is currently leading her own research group at ISGlobal. **Prof. Quique Bassat**, Director General of the ISGlobal, is the head of the Malaria and Neglected Parasitic Diseases Program. Bassat's expertise lies in infectious disease epidemiology and public health. His extensive experience in working within low-resource settings and collaborating with LMICs through his career, which notably includes the 2 partners involved in this proposal, will be instrumental in conducting this research. **Prof. Judith Garcia-Aymerich,** Head of the Environment and Health over the Lifecourse Programme, has extensive experience in biomedical research methodology, contributes to expert committees, and collaborates with regulatory authorities. Dr. Juan Fortea, neurologist and researcher at Sant Pau Hospital, will be pivotal as an advisor of plasma biomarker protocol. **Dr. William Pomat** is a biomedical researcher specializing in immunology, particularly bacterial causes of pneumonia in children, currently directing the **PNG Institute of Medical Research** – partner on this proposal - renowned as the nation's premier academic research institute and an internationally acclaimed center of excellence. Dr. Pomat has extensive research experience in Papua New Guinea. **Dr. Moses Laman - PNG Institute of Medical Research-** has devoted his career to stem the tide of these infections and improve outcomes in those affected, especially children. Their participation in the project will ensure smooth coordination for recruitment of study individuals in PNG. **Dr. Sozinho Acácio** (MD; PhD) is a researcher working on Tuberculosis and other non-communicable chronic conditions at the **Manhiça Health Research Center** (CISM). With his expertise in coordinating studies, his contribution will be invaluable particularly in managing logistics and providing insights for future studies addressing aging and Alzheimer's disease in the area.