FLADEX Project

Understanding cerebral blood Flow dynamics for Alzheimer's Disease prevention through EXercise

Statistical Analysis Plan

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1. General information of the FLADEX project

1.1 Background and rationale

Dementia encompasses a range of neurodegenerative disorders characterized by progressive cognitive decline and impaired functional abilities. Alzheimer's Disease (AD) is the most prevalent form of dementia, accounting for 60-70% of cases, and is marked by a gradual deterioration in memory, thinking, and behaviour (3,4). The cognitive impairment associated with aging presents a spectrum, ranging from mild symptoms to more severe cases, with pathophysiological signs of AD occurring at least 10-20 years before the onset of cognitive losses (5). AD progresses from preclinical stages to dementia, involving gradual memory loss and cognitive impairment.

AD progresses from preclinical stages to dementia, involving gradual memory loss and cognitive impairment. Notably, physical exercise is one of the most promising non-pharmacological interventions to delay the onset of dementia and slow down the progression of cognitive decline. Studies have explored the effects of physical exercise on CBF, highlighting improvements associated with both aerobic and strength exercise (11,12). While most research on interventions to improve CBF focuses on chronic exercise programs, there is increasing interest in understanding the acute benefits of physical exercise (13). Therefore, flADex aims to examine the acute effects of different types of exercise on CBF, AD blood-based biomarkers, and its cognitive implications in older adults.

1.2 Objective

The main objectives are:

1. The primary objective of the flADex trial is to examine the acute effect of a single bout of resistance exercise and aerobic exercise compared to resting condition on global and regional CBF (using cutting-edge MRI) in older adults.
2. The secondary objectives are to examine: (i) the acute effect of a single bout of resistance exercise and aerobic exercise compared to resting condition on blood-based biomarkers for AD and neurodegeneration in older adults; (ii) the acute effect of a single bout of resistance exercise and aerobic exercise compared to resting condition on cognitive and mood outcomes in older adults; (iii) whether exercise-induced changes in CBF mediate changes in blood-based biomarkers (or vice versa) in older adults; and, (iv) whether exercise-induced changes in CBF or blood-based biomarkers mediate changes in cognitive and mood outcomes in older adults.

2. Study methods

2.1 Trial design

The flADex (Understanding cerebral blood Flow dynamics for Alzheimer's Disease prevention through Exercise) trial is a within-subject randomized crossover design, in which 20 individuals (balanced sex distribution) aged 68-83 will be included. The study is registered in the clinicaltrial.gov database (NCT06584656; approval date: 04-09-24). The study has been designed following the Standard Protocol Items for Randomized Interventional Trials (SPIRIT) (14,15), the SPIRIT-Outcomes 2022 Extension (16) and is reported following the Consolidated

Standards of Reporting Trials (CONSORT) 2020 statement for extension to randomized crossover trials (17). FlADex has been approved Research Ethics Board of the Andalusian Health Service (CEIM/CEI Provincial de Granada; #SICEIA-2024-000602) in April 30th 2024.

2.2 Randomization

Following a counterbalanced crossover design, each participant will be included in all study conditions. The three study conditions are: (A) bout of aerobic exercise, (B) bout of resistance exercise, and (C) control resting condition. The order of each condition will be randomized for each participant to achieve a counterbalanced design.

2.3 Sample size

Previous acute studies in adults have shown that a single session of exercise changed CBF by 15% (18) and 9% (19). Specifically, the sample size is based on the mean changes (M) in CBF of 6 ml/100g/min (M1=40.5 and M2=34.2) with a standard deviation (SD) of 6.46 (20). Considering the calculation of Cohen's d based on previous studies (d=M1-M2/combined SD), we expect a large effect size (Cohen's d = 0.9) (20). Therefore, using an alpha of 0.05 and a standard power of 80%, a sample size of 20 is needed. Participants who withdraw from the study will be replaced to ensure sufficient power to assess our primary outcome. Thus, the experiment will be completed when 20 participants have completed all three experimental conditions. If one participant misses one visit, they will be rescheduled within 2-weeks of the last completed visit. This sample size is feasible based on our previous experiences involving MRI and exercise interventions.

2.4 Framework

A superiority hypothesis testing framework is used, emphasizing the evaluation of an exercise condition's positive impact compared to a resting condition. In the main analysis, we will compare a resistance exercise condition or aerobic exercise condition versus resting (no exercise) condition at four time points (30 minutes before the experimental condition; and 20, 27 and 34 minutes after the experimental condition).

2.5 Statistical interim analyses and stopping guidance

Given the acute nature of flADex, no pre-specified interim analyses will be performed, and, therefore, a stopping guidance is not applicable.

2.5 Timing of the final analyses

The main final analyses will be performed once all the data processing of the primary outcome has been completed.

2.6 Timing of outcome assessment

The primary outcome (CBF) will be assessed 30 minutes before the experimental condition; and 20, 27 and 34 minutes after the experimental condition. Specific details for timing on secondary outcomes are reported in table 1.

3. Statistical principles

3.1 Confidence intervals and P values

All statistical tests will be two-tailed. *P* for significance will be set at 0.05 and 95% confidence intervals will be estimated. No adjustment for multiplicity will be made as multiplicity adjustments may be of lesser importance in the case of distinct treatment arms (21). Moreover, only one primary outcome was defined and other outcomes (secondary/tertiary outcomes, exploratory analyses) don't required adjustment for multiple testing.

3.2 Protocol deviations

All protocol deviations made to the protocol (e.g., change in pre-defined inclusion/exclusion criteria, data cleaning/processing) will be reported and described in the analysis. Compliance will be measured based on the exercise prescription.

3.3 Analysis populations

A dataset will be created for statistical analyses. The primary and secondary outcomes will be analyzed following a per-protocol approach which will include only participants who strictly adhered to the intervention protocol and completed all three conditions.

4. Trial population

4.1 Recruitment

FlADex trial proposes a cost and time-effective strategy that builds ecologically from our previous trial (AGUEDA project (22)) for the recruitment of the participants (23,24). All participants from the AGUEDA trial—aged 65 to 80 years at enrolment and are now 3 years older, making their age range 68 to 83 years for flADex– who meet the negative brain amyloid criteria and ApoE4 non-carrier status will receive a phone call invitation to participate in the flADex project. Of the 90 participants randomized, 80 completed the AGUEDA trial. Among those, 26 participants were excluded due to amyloid beta positivity (n=17) or ApoE carriers (n=26). Thus, 54 participants (n=32 females) meet the initial eligibility criteria for the flADex trial. During this call, there is a phone screening with eligibility questions regarding health, medications, and MRI safety. After verbal consent, participants received a detailed explanation of the flADex project, benefits and potential risk, as well as the complete schedule and organization of assessments. Recruitment began in September 2024.

4.2 Eligibility criteria

Eligibility criteria will be checked in two phases, pre-screening phase and phone-screening phase. In the pre-screening phase, participants must meet the following inclusion criteria: (i) non-pathological cerebral beta-amyloid status (based on Centiloid cut-point <12 measured by PET), and (ii) ApoE4 non-carrier status; the pre-screening phase will be based on the AGUEDA trial information. In the phone-screening phase, participants will be excluded if they meet any of the following: (i) ambulatory with pain or regular use of an assisted walking device; (ii) not living in community settings during the study; (iii) pathological diagnosis related to physical or mental condition that would preclude participants from performing the cognitive tests or exercise condition; or, (iv) MRI incompatibility.

4.3 Withdrawal/follow-up

The number, timing, and reasons (e.g. adverse events, withdraw consent, lost to follow-up) for withdrawal will be noted and described. The number of replaced subjects will be recorded and the reason for withdrawal will be carefully documented.

4.4 Baseline patient characteristics

A baseline table will be created to describe the characteristics of the study population. The main characteristics will include age, sex, education, body mass index, medications and the outcomes of interest. The characteristics of the total study population and each study arm will be summarized using mean (SD) or median (interquartile range) for normally and not normally distributed continuous variables, respectively, and as number (percentage) for categorical variables.

5. Analysis

5.1 Outcome definitions

The primary and secondary outcomes will be assessed before and after the experimental condition, while some pre-specific outcomes are assessed only during each condition. See table 1 for Measurement timepoints of the flADex outcomes

Table 1. Measurement timepoints of the outcomes of the flADex project.

Primary outcome	Before the condition	During the condition	After the condition	Specific measurement
Cerebral Blood Flow	-30’		20’, 27’ and 34’	Pseudo continuous Arterial Spin Labeling (pCASL) and Magnetization Prepared Rapid Gradient Echo (MPRAGE)
Secondary outcome	**Before the condition**	**During the condition**	**After the condition**	**Specific measurement**
Blood biomarkers of AD patahology and neurodegeneration	-5’		3’, 50`and 70’	Aβ42/40 ratio, p-tau217, p-tau181, NfL BD-tau and GFAP
Growth factor	-5’		3’, 50`and 70’	BDNF, IGF-1
Episodic memory	-15’		60’	Picture Sequence Memory Test (Raw score)
Cognitive inhibition	-15’		60’	Flanker test (Incongruent trials inverse efficiency score)
Mood status	-60’		70’	Scale Profile of Mood States (POMS)
Feeling scale	-1’		1’	Feeling scale (FS)
Exercise related-variable	**Before the condition**	**During the condition**	**After the condition**	**Specific measurement**
Rate of perceived exertion (Aerobic and resistance condition)		12’, 20’ and 30’		OMNI-Resistance Exercise Scale (OMNI-RES)
Repetitions in reserve (Resistance condition)		After each exercise		The Repetitions in Reserve (RIR)
Heart Rate		12’, 20’ and 30’		Heart Rate per second (Polar H10)
Cognitive engagement		12’,20’ and 30’		Cognitive Load Measurement scale
Enjoyment			5’	Physical activity Enjoyment scale (PACES)
Blood pressure	-60’		1’	Omron M3, Intellisense, OMRON Healthcare Europe, Spain

AD, Alzheimer’s disease; Aβ42/40, Amyloid-beta 42; p-tau217, p-tau181, phosphorylated tau protein at positions 217 and 181; NfL, Neurofilament Light Chain; BD-tau, brain-derived tau, GFAP, Glial Fibrillary Acidic Protein; BDNF, Brain-Derived Neurotrophic Factor; IGF-1, Insulin-Like Growth Factor 1.

Primary outcome

The primary outcome is the *change in Cerebral Blood Flow (CBF)* (Time Frame: 30 minutes before; and 20, 27 and 34 minutes after the experimental condition). CBF is assessed by MRI, using a Siemens Magnetom PRISMA Fit 3T scanner with a 64-channel head coil located at Mind, Brain and Behavior Research Centre (CIMCYC) from the University of Granada. Specific acquisition parameters for Pseudo continuous Arterial Spin Labeling (pCASL) sequence are used to determinate global and regional CBF in resting supine position condition. Structural T1 sequence (only pre-condition) is used to co-register the pCASL and delineate regions of interest for CBF. Time-of-flight angiography (TOF) (before pCASL pre-condition and before first pCASL post-condition) sequence is used to identify the carotid arteries. The unit of measure of CBF is expressed as milliliters per 100 grams of brain tissue per minute (mL/100 g/min).

Secondary outcomes

*Change in AD blood-based biomarkers (Aβ42/40 ratio)(*Time Frame: 5 minutes before; and 0, 50 and 70 minutes after the experimental condition). The following biomarkers are assessed: Amyloid-beta 42 (Aβ42), Amyloid-beta 40 (Aβ40). Aβ42 and Aβ40 will be combined as the Aβ42/40 ratio to represent the relative concentration of Aβ42 to Aβ40. All blood samples will be collected in fasting conditions at CIMCYC, processed and stored at -80°C by trained staff.

Change in neurodegenerative blood-based biomarkers (p-tau217, p-tau181, NfL, BD-tau and GFAP) (Time Frame: 5 minutes before the experimental condition; and 0 minutes, 50 minutes and 70 minutes after the experimental condition). The following biomarkers are assessed: phosphorylated tau protein at positions 217 and 181 (p-tau217, p-tau181), Neurofilament Light Chain (NfL), brain-derived tau (BD-tau) and Glial Fibrillary Acidic Protein (GFAP) Unit of measurement: p-tau217, p-tau181, NfL, BD-tau and GFAP are commonly measured in picograms per milliliter (pg/mL).

*Change in growth factors (BDNF) (*Time Frame: 5 minutes before the experimental condition; and 0 minutes, 50 minutes and 70 minutes after the experimental condition). Brain-Derived Neurotrophic Factor (BDNF) is measured. BDNF is commonly measured in picograms per milliliter (pg/mL).

Change in growth factors (IGF-1) (Time Frame: 5 minutes before the experimental condition; and 0 minutes, 50 minutes and 70 minutes after the experimental condition). Insulin-Like Growth Factor 1 (IGF-1) will be measured. Unit of measurement: IGF-1 is commonly measured in nanograms per milliliter (ng/mL).

*Change in episodic memory (*Time Frame: 15 minutes before the experimental condition; and 60 minutes after the experimental condition). Episodic memory is assessed using the Picture Sequence Memory Test from the Cognitive NIH Toolbox, a computer-based validated battery available in Spanish. The Picture Sequence Memory Test measures the participant's score from the cumulative number of adjacent pairs of pictures remembered correctly over two

consecutive learning trials. The number of adjacent pairs placed correctly for each of trials 1 and 2 is used.
*Change in inhibition/attention (*Time Frame: 15 minutes before the experimental condition; and 60 minutes after the experimental condition). The Flanker task measures inhibitory control and attention by using the inverse efficiency score of incongruent trials. The inverse efficiency score is calculated as reaction time/accuracy (RT/ACC).

Mood status (Time Frame: 60 minutes before; and 70 minutes after the experimental condition). Mood is evaluated using a shortened version of the validated Profile of Mood States (POMS) (25) scale The 15-item POMS scale is a psychological assessment tool used to measure and evaluate a person's mood states. It consists of a questionnaire with a list of 15 adjectives or mood descriptors, where individuals rate how they have been feeling on a scale typically ranging from "Not at all" to "Extremely". The items are divided into 5 dimensions: depression, vigor, anger, tension and fatigue.

Feeling scale (Time Frame: 1 minute before; and 1 minute after the experimental condition). Emotional response is evaluated using the feeling scale (FS) (26) The FS is an 11-point scale ranging from -5 (very bad) to +5 (very good) used to measure an individual's emotional feeling in terms of pleasure or displeasure at a specific moment.

Exercise-related variables.

*Rate of perceived exertion (in aerobic condition and resistance condition) (*Time Frame: During the experimental condition at minute 12, minute 20, and minute 30). Perceived exertion of exercise (RPE) is assessed using the OMNI-Resistance Exercise Scale (OMNI-RES)(28) of perceived exertion from 0-10 points.

*Repetitions in reserve (in resistance condition) (*Time Frame: During the resistance condition after each exercise at minutes 5, 6, 7, 8, 9, 10, 11, 12 / 14, 15, 16, 17, 18, 19, 20, 21 / 23, 24, 25, 26, 27, 28, 29, 30). The Repetitions in Reserve (RIR) is assessed after each exercise in the resistance condition. RIR method is a self-regulation technique used in strength training to gauge exercise intensity. It involves estimating how many more repetitions you could perform before reaching failure after completing a set.

*Heart Rate (*Time Frame: During the experimental condition at minute 12, minute 20, and minute 30). Heart rate is assessed using H10 Polar Bands via the "Polar Team" app installed on an iPad.

*Cognitive engagement (*Time Frame: During the experimental condition at minute 12, minute 20, and minute 30)(29). Cognitive engagement is assessed by the Cognitive Load Measurement scale. The Cognitive Load Scale is a 9-item scale used to assess the mental effort or cognitive load experienced by individuals when engaging in a task, particularly in educational or learning contexts. It involves a self-reported measure where participants rate their perceived mental effort on a scale, ranging from 1 very low mental effort; to 9 very high mental effort.

*Enjoyment (*Time Frame: 5 minutes after the experimental condition) of physical activity is assessed using a shortened version of the Physical activity Enjoyment scale (PACES) (27), an 8-item validated version, which measures the extent (on a 7-point Likert scale) to which participants enjoy doing a specific activity.

Blood pressure (BP) (Time Frame: 60 minutes before the experimental condition; and 1 minute after the experimental condition)*:* BP will be measured using a validated automated monitor (Omron M3, Intellisense, OMRON Healthcare Europe, Spain) with participants seated and their left arm at heart level. After 5 minutes of rest, two readings will be taken at 1-minute intervals, and the average of the readings will be used for analysis

5.2 Analysis methods

The main analyses will consist of the per-protocol analyses for the primary and secondary outcomes using linear mixed models. The model will include fixed effects for time (four or two levels depending on the outcome), condition (three levels), time*condition interaction, as well as the unique participant identifier as a random effect. The number and patterns of missing data will be explored and reported. Missing data will be assumed to be missing at random and handled within the linear mixed model analyses. Model assumptions will be assessed. If violations are detected, appropriate corrective measures will be implemented, including data transformations. Condition effects will be evaluated using a time-by-treatment interaction term and described with estimated marginal means and 95% confidence intervals. All statistical tests will be two-tailed. P for significance will be set at 0.05.

5.2.1 Primary analysis.

Effects on primary outcomes

To investigate the overall effect of the different exercise interventions (resistance, aerobic, and resting) on the change in the CBF from pre-condition to post-condition, a crossover analysis will be employed using linear mixed models with repeated measures over time. This model will account for the three conditions (resistance, aerobic, and resting) and the corresponding time points (30 minutes before; and 20, 27 and 34 minutes after the experimental condition), following a per-protocol approach. The model will include a random intercept for each participant and a fixed effect for the group-by-condition interaction to account for the crossover design. The preprocessing analysis of pCASL will be carried out using different programs (Freesurfer and ASLprep).

5.2.2. Secondary analyses

Effects on secondary outcomes

To investigate the effect of the different exercise interventions (resistance, aerobic, and resting) on the secondary outcomes (i.e., blood biomarkers for AD pathology and neurodegeneration, growth factors, episodic memory, inhibition/attention, mood status and feeling scale), the same analyses as the primary analysis using a per-protocol principle and linear mixed models' analysis will be performed.

Mediators
Mediation analysis will be performed following AGReMA (A Guideline for Reporting Mediation Analyses) recommendations (21), using CBF and blood biomarkers as mediators depending on the outcome. When blood biomarkers are the outcomes, CBF will act as the mediator, and vice versa. When cognitive and mood indicators are the outcomes, both CBF and blood biomarkers will act as mediators. In addition, bivariate correlations will be performed between changes in CBF, changes in blood-based biomarkers and changes in cognitive and mood outcomes.

Exercise condition parameters
Parameters related to the exercise intervention (i.e., enjoyment, Rate of perceived exertion, RIR and cognitive engagement*)* will be described similar to the baseline characteristics. These parameters will help, for instance, to determine whether participants reached the target intensity levels.

5.2.3. Exploratory analyses
Moderators
Moderation analysis will be performed using the experimental condition as independent variable (aerobic vs resistance vs resting), CBF as outcome, and sex as moderator. Due to the limited sample size, we will interpret these results with caution as we may not have sufficient statistical power to detect moderation effects. Nevertheless, performing this analysis remains informative for understanding exploratory trends in how sex may moderate acute responses to different exercise types.

5.3 Missing data
The number of missing data will be reported, and patterns of missing data will be explored. Based on previous experience, we expect that missing data will be assumed to be missing at random. Therefore, the linear mixed model analyses will handle our missing data. However, once the data processing is finalized, we reconsider this expectation. If this assumption does not hold, we will explore appropriate measures for the data analyses.

5.4 Harms
Any situation that may occur, such as injury, emergency, dizziness or scheduled surgery will be reported as adverse events categorized based on predefined criteria using common terminology criteria (i.e., mild, moderate, severe) (6).

5.5 Statistical software
The analyses of the primary outcomes will be performed using R. For the main analyses, we will use e.g., ‘lm4” and “nlme” packages. The use of specific packages will be reported in each manuscript. In addition, specific softwares will be used for secondary brain outcomes.

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