Effect of Intervention with a Low-Calorie Mediterranean Diet, Intermittent Fasting, and Natural Senolytics on Aging Markers in Subjects with Low and High Vascular Risk

1. Introduction

Cardiovascular diseases (CVD) remain the leading cause of morbidity and mortality worldwide, particularly in Western countries[1]. Since the mid-20th century, numerous epidemiological studies, such as the *Framigham Heart Study*, have identified the main vascular risk factors to guide preventive strategies. However, the association between the burden of traditional cardiovascular risk factors and the occurrence of cardiovascular events is not always strong. A substantial proportion of individuals with few or no conventional cardiovascular risk factors still experienced cardiovascular events, whereas others with multiple risk factors remain event-free[2]. This apparent limitation has prompted efforts to identify additional markers that, when considered alongside classical risk factors, may improve CVD risk prediction. Such markers include systemic inflammatory biomarkers, lipoprotein (a)[3], and advanced imaging techniques for the detection of subclinical arteriosclerosis[4], all of which have shown promise in refining risk stratification and improving the precision of preventive interventions.

In recent years, aging itself, traditionally regraded as a non-modifiable vascular risk factor, has been increasingly conceptualized as a potential "treatable process." Accumulative scientific evidence suggests that appropriate interventions, it may slow biological agings, thereby reducing the incidence of aging-related diseases, such as CVD, cancer and neurodegenerative disorders. A growing body of research indicates that key cellular and molecular mechanisms underlying aging can be delayed, potentially postponing the onset of chronic diseases in older adults.

Aging is closely associated with the accumulation of "senescent cells" within tissues. Although these cells remain metabolically active, they are resistant to apoptosis, leading to their persistence and impaired tissue regeneration. Experimental studies have shown that senescent cells develop a senescence-associated secretory phenotype (SASP), characterized by the release of pro-inflammatory cytokines, chemokines, serpins, and bioactive lipids that promote fibrosis, mitochondrial dysfunction, oxidative stress and DNA damage. In parallel, advanced glycation end products (AGEs), generated through non-enzymatically reactions between sugars and proteins or lipids, further contribute to cellular dysfunction. Collectively, these processes drive chronic low-grade inflammation, cellular aging and the development of aging-related diseases, including CVD.

Evidence from experimental animals indicates that calorie restriction, physical activity, specific dietary patterns, and certain pharmacological interventions can delay cellular aging and, in some species, extend lifespan. Large epidemiological studies consistently show that predominantly plant-based dietary patterns are associated with lower incidence of CVD, reduced all-cause mortality, and improved metabolic health. Among these, the Mediterranean diet has emerged as one of the most effective dietary patern for promoting longevity and preventing chronic disease. Its anti-inflammatory and antioxidant effects, largely attributed to extra-virgin olive oil, fruits, vegetables, nuts, and legumes, are thought to play a key role in reducing systemic inflammation, a

central mechanism underlying both chronic disease and aging.[7] In addition, adherence to Mediterranean diet has been associated with improvements in cognitive function, quality of life, and longevity. [8] In parallel, intermittent fasting or time-restricted feeding has gained increasingly scientific attention as feasible and effective strategy to improve metabolic and cardiovascular health. Studies in both animals[9] and humans indicate that intermittent fasting enhances metabolic flexibility, stimulated autophagy, and improved glucose tolerance, and may also confer benefits for cognitive function and quality of life.[10-13]

More recently, senolytic agents, compounds capable of selectively eliminating senescent cells, have been identified as promising modulators of biological aging. Pharmacological senolytics such as dasatinib, navitoclax, and rapamycin, as well as natural occurring compounds including fisetin, quercetin, and piperlongumine, have shown the ability to reduce senescent cell burden and disrupt the self-perpetuating cycle of inflammation and tissue damage that accelerates aging.[14] Among natural senolytics, fisetin, a polyphenol found in apples, strawberries, grapes, onions, and kiwis, has shown particularly strong senolytic activity, potentially mediated through interactions with the mTOR signaling pathway.[14-15] In animal models, fisetin supplementation has been associated with improvements in glucose metabolism, reductions in pro-inflammatory cytokines, and preservation of renal function, without significant adverse effects.[16] Moreover, fisetin has also been shown to reduce rove glomerulosclerosis by approximately 10% compared with untreated controls, contributing to longer-term maintenance of kidney function.

Despite these encouraging preclinical findings, human data on modulation of senescence though nutritional or lifestyle interventions remain limited. Consequently, further research is needed to determine whether non-pharmacological strategies, such as dietary modification, intermittent fasting, and natural senolytics, can effectively delay biological aging and reduce the burden of age-related disease, particularly CVD.

This randomized clinical trial was designed to evaluate the effects of a low-calorie Mediterranean diet combined with intermittent fasting and the natural senolytic fisetin on biomarkers of aging, compared with the traditional MedDiet in 4,000 adults without established CVD, but at either high or very high vs. low or moderate CVD risk, stratified into two age groups (50 $\pm$ 5 and 80 $\pm$ 5 years), with 1,000 participants per intervention group. Primary aging-related outcomes will include senescent cell burden, inflammatory markers, DNA methylation patterns, and indices of mitochondrial dysfunction. In addition, the progression of subclinical arteriosclerosis will be assessed using carotid ultrasound, pulse wave velocity, endothelial function testing, and traditional cardiovascular risk factors.[17-20]

The primary objective of the study is to determine whether optimization of healthy lifestyle behaviors, including diet, physical activity, and emotional well-being, when combined with a natural senolytic intervention can delay biological aging and reduce the incidence of age-related conditions, such as CVD, dementia, sarcopenia, and frailty.

2. Study Hypothesis

Intervention with a low-calorie MedDiet combined with intermittent fasting, and fisetin supplementation will have a superior effect, compared with low-calorie MedDiet alone, in delaying the molecular and cellular mechanisms of senescence. This combined intervention is also hypothesized to slow the progression of subclinical atherosclerosis and CVD , as assessed by imaging techniques, pulse wave velocity, and endothelial function testing.

3. Objective and Purpose of the Study

The general objective is to evaluate whether a nutritional intervention combining a hypocaloric MedDiet, intermittent fasting, and supplementation with a natural senolytic compound (fisetin) can delay biological aging, slow the progression of subclinical CVD, and reduce the onset of age-related diseases. In addition, the study aims to determine whether this combined intervention produces superior effects compared with a hypocaloric MedDiet alone. The study will include 4,000 participants without established CVD, equally distributed across high and very high vs. low and moderate cardiovascular risk groups and two age groups (50 ± 5 and 80 ± 5 years).

3.1. Specific Objectives

a. Biological aging and senescence

To evaluate the effect of the combined intervention (hypocaloric MedDiet, intermittent fasting, and fisetin supplementation) on markers of cellular senescence and biological aging, including senescent cell burden, inflammatory markers, mitochondrial dysfunction, progenitor cell depletion, telomere length, and DNA methylation, compared with a hypocaloric MedDiet alone, in participants with high or very high and low or moderate cardiovascular risk across both age groups.

b. Subclinical cardiovascular disease

To assess the effect of the combined intervention on subclinical CVD, evaluated using imaging and functional vascular techniques, compared with a hypocaloric MedDiet alone, in participants with high or very high vs. low or moderate cardiovascular risk across both age groups.

c. Physical function and frailty

To evaluate the effect of the combined intervention on physical function, including sarcopenia and frailty status, compared with a hypocaloric MedDiet alone, in participants with high or very high and low or moderate cardiovascular risk across both age groups.

d. Advanced glycation end-products.

To assess the effect of the combined intervention on skin levels of advanced glycation end-products (AGEs), measured by skin autofluorescence, compared with a hypocaloric MedDiet alone, in participants with high or very high and low or moderate cardiovascular risk across both age groups.

3.2. Primary Endpoint

- Biological markers of aging, including senescent cell burden, inflammatory markers, mitochondrial function, endothelial progenitor cell levels, epigenetic DNA modifications, telomere length, DNA methylation patterns, and levels of AGEs.

3.2. Secondary Endpoints

- Changes in body composition assessed by dual-energy X-ray absorptiometry (DEXA).
- Changes in blood pressure (24-hour ambulatory blood pressure monitoring, ABPM).
- Changes in lipid profile and glucose metabolism.
- Carotid artery ultrasound (carotid intima-media thickness and number and characteristic of the atheroma plaques).
- Electrocardiogram and color Doppler echocardiographic findings.
- Clinical indicators of aging, including cognitive performance, grip strength, gait speed, and muscle mass.
- Assessment of dietary intake, physical activity, and laboratory parameters (lipid profile, carbohydrate metabolism, and iron metabolism).
- Skin levels of AGEs assessed by autofluorescence.
- Biomarkers of intervention adherence and parameters reflecting aging and treatment efficacy.
- Incidence of newly diagnosed age-related conditions, during follow-up, including CVD events, neurodegenerative disorders, bone fractures, and cancer.

4. Study Design

This study is a prospective, randomized clinical trial designed to evaluate the effects of a long-term nutritional intervention with scheduled follow-up assessments. Participants will be randomly assigned to one of the intervention arms and followed longitudinally according to a prespecified protocol. The primary outcomes are intermediate markers of biological aging and subclinical CVD. Secondary and exploratory outcomes include clinically relevant outcomes, such as all-cause mortality, incident clinical CVD events, cancer incidence, and neurodegenerative diseases. Given the long latency of these outcomes, extended duration of intervention and follow-up is required to adequately capture their occurrence.

An average minimum follow-up of five years is planned. Additional funding will be sought to allow extension of follow-up beyond this period, enabling comprehensive evaluation of long-term clinically outcomes in accordance with CONSORT recommendations for randomized trials with extended follow-up.

5. Participants

5.1. Study population and recruitment

A total of 4,000 participants will be recruited, with balanced representation of men and women, from two age groups: 50 ± 5 years and 80 ± 5 years. Participants will be recruited from the following sources:

1. Hospital Clinic Outpatient clinics.
2. Primary Care Centers affiliated with Hospital Clinic.
3. Participants from previously completed studies conducted by the research group, including PREDIMED and PREDIMED-Plus.

Eligible participants will be identified among individuals receiving care within the network of Primary Care Centers affiliated with Hospital Clínic, which collectively serve an estimated population of approximately 500,000 residents in Barcelona.

5.2. Sample Size

Based on the sample size calculation, a minimum of 3,500 participants is required to detect statistically significant differences between intervention groups. To account for an anticipated attrition rate of approximately 10%, a total of 4,000 individuals will be recruited to ensure adequate statistical power at study competition.

5.3. Stratification and Allocation Framework

Participants will be evenly distributed across two age strata, with 2,000 individuals in each age group (50 ± 5 years and 80 ± 5 years). Within each age stratum, participants will be further stratified according to cardiovascular risk, resulting in 1,000 participants with low or moderate vascular risk (L-MVR) and 1,000 participants with high or very high vascular risk (H-VHVR) per age group.

This stratification framework will be incorporated into the randomization scheme to ensure balanced allocation across intervention arms.

5.4. Screening and Eligibility Assessment

Demographic characteristics and eligibility criteria will be obtained from fully computerized electronic health records during a pre-screening phase, prior to direct contact with potential participants. Eligible individuals will be identified through outpatient clinic visits, primary care encounters, or prior participation in PREDIMED and PREDIMED-Plus studies.

5.5. Informed Consent and Enrollment

At screening visit, all eligible individuals will receive a detailed explanation of the study objectives, procedures, potential risks, and expected benefits. Written informed consent will be obtained from all participants before initiation of any study-related procedures, in accordance with Good Practice and CONSORT Guidelines.

Given that the physicians involved in the trial also provide routine clinical care to participants, no conflicts are anticipated regarding confidentiality, participant identification, or access to medical records.

6. Inclusion and Exclusion Criteria

6.1. Subject Inclusion Criteria

Participants will be eligible for inclusion if they meet the following criteria:

- Age: 50 ± 5 years or 80 ± 5 years.
- Absence of established CVD, as define below.
- Ability and willingness to comply with the study procedures and provide written informed consent

Participants will be categorized at baseline into **"high or very high vascular risk** (H-VHVR) or **"low-moderate vascular risk"** (L-MVR) groups in accordance to accordance with the European Society of Cardiology (ESC) prevention guidelines and the SCORE 2 frame work[21].

*** High and Very High Vascular Risk (H/VHVR) Group**

Participants will be classified as H-VHVR if they meet at least one of the following criteria:

1. Diabetes mellitus for ≥ 10 years, with or without of target organ damage (TOD), or Diabetes with ≥ 1 additional major cardiovascular risk factor.
2. Severe hypertension: Blood pressure ≥ 180/110 mm Hg, or requirement for ≥ 3 antihypertensive drugs to achieve blood pressure control.
3. Severe Dyslipidemia: Untreated LDL cholesterol ≥ 190 mg/dL (≥ 4.9 mmol/L), or current treatment with high-intensity statins with ≥ 1 additional lipid-lowering therapy.
4. Moderate-to-severe renal disease Estimated glomerular filtration rate (eGFR) <60 mL/min/1.73 m^2 (CKD stage ≥ 3)
5. Current cigarette smoking.
6. Family history of premature CVD. First-degree relative with CVD onset <55 years in men or <65 years in women.

*** Low and Moderate Vascular Risk (L-MVR) Group[21]**

Participants aged 50 ± 5 years or 80 ± 5 years who do not meet any of the above vascular risk criteria will be classified as having low-to-moderate vascular risk, corresponding to SCORE 2 estimates below the high-risk threshold for their age and geographic region.

6.2 Subject Exclusion Criteria

Participants will be excluded from the study if they present with any chronic condition associated with a senescent phenotype or other condition that could interfere with the intervention or outcome assessment, including:

1. Established cardiovascular disease (CVD): History of ischemic heart disease, stroke, or peripheral arterial disease.
2. Chronic kidney disease: Estimated glomerular filtration rate (GFR) < 60 mL/min/1.73 m^2.
3. Chronic obstructive pulmonary disease (COPD): GOLD stage >2B.
4. Acute or chronic heart failure within the previous 12 months: Diagnosed according to Framingham criteria.
5. Idiopathic pulmonary fibrosis.
6. Hematologic or solid organ cancer diagnosed within the past five years.

7. History of osteoporotic fracture, including femoral, radial, or vertebral compression fractures.
8. Liver cirrhosis.
9. Cognitive impairment of any etiology, defined as a Global Deterioration Scale (GDS) > 4.
10. Human immunodeficiency virus (HIV) infection.
11. Frailty, defined as a FRAIL score > 3 points and/or a VIG-Frail index > 0.2.
12. Systemic autoimmune disease.
13. Current treatment with anticoagulant therapy, due to potential interactions with fisetin.
14. Food allergies or intolerances that preclude adherence to the Mediterranean diet.

7. Treatment Groups and Randomization

Participants within each of the four predefined study strata (age group and cardiovascular risk category) will be randomized in a 1:1 allocation ratio using a computerized randomization system developed by the Statistics Department of Hospital Clínic de Barcelona. The randomization sequence will be generated prior to study initiation and implemented to ensure balanced allocation across interventions arms within each stratum.

Participants will be assigned to one of the following two treatment groups:

- **Intervention Group:** Low-calorie Mediterranean diet combined with intermittent fasting and fisetin supplementation.

- **Control Group:** Traditional Mediterranean diet with placebo supplementation.

Each treatment arm will include a total of 2,000 participants, comprising 1,000 individuals with high or very high vascular risk (H-VHVR) and 1,000 participants with low-to-moderate vascular risk (L-MVR). Within each vascular risk category, participants will be equally distributed across the two age groups (50 ± 5 years and 80 ± 5 years).

8. Treatment and Study Schedule

The active intervention period will last at least 24-months. During this time, participants will attend scheduled clinical visits, according to predefined protocol.

Visit 0 – Patient Selection

Potential participants who meet the initial eligibility criteria will attend a screening and enrollment visit. During this visit, a study physician will provide information regarding the study objectives, design, intervention, and procedures. Written informed consent will be obtained prior to the initiation of any study-related assessment or procedures. This visit is expected to last approximately 30 minutes.

Visit 1 – Baseline Assessment (In-person)

Personnel: Study physician and study nurse.
Estimated duration: Approximately 60 minutes.

The baseline visit will include a comprehensive clinical, functional, and biological assessment to establish participant's initial health status and characterize aging-related conditions. The following evaluations will be performed:

- **Clinical and Physical Examination**

  A standardized, multidimensional medical and physical evaluation will be conducted to identify conditions commonly associated with aging, including CVD, neurodegenerative disorders, osteoporotic fractures, and cancer.

- **Functional and Cognitive Assessments**

Validated instruments will be used to evaluate assess functional capacity, cognitive function, frailty, and sarcopenia:

- Functional status**:** *Barthel* and *Lawton-Brody* Instrumental Activities of Daily Living scale.
- Cognitive function**:** *Pfeiffer Short Portable Mental Status Questionnaire.*
- Frailty: *VIG-Frail* index and *Clinical Frailty Scale (CFS).*
- Sarcopenia: Evaluation of muscle strength and physical performance using a handgrip dynamometer and gait speed testing.

- **Cardiovascular Evaluation**

- Resting 12-lead electrocardiogram (ECG).
- Transthoracic echocardiography to assess cardiac structure and function.
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- **Biological Sample Collection**

- Venous blood samples for hematological and routine biochemical analyses.
- Spot and 24-hour urine samples for metabolic assessment.
- Collection of cells, plasma, and serum for project-specific analyses, including biomarkers of cellular senescence, progenitor cell populations, inflammatory pathways, and mitochondrial dysfunction.

- **Nutritional and Physical Activity Assessment**

- Administration of a validated 151-item food frequency questionnaires to evaluate habitual dietary intake.
- Assessment of physical activity using the Minnesota Leisure-Time Physical Activity Questionnaire.
- Application of a GeneActive accelerometer to objectively measure physical activity and sleep patterns.

- **Body Composition Assessment**

- Body mass index (BMI) will be calculated from weight and height measurements
- Dual-energy X-ray absorptiometry (DEXA) to quantify total and regional body composition, including lean mass, fat mass, and bone mineral density.

- **Vascular and Hemodynamic Assessment**

- Carotid artery ultrasound to measure carotid intima-media thickness (CIMT) as an index of subclinical atherosclerosis and to assess the number and characteristic of the atherosclerotic plaques in supra-aortic trunks.
- Twenty-four-hour ambulatory blood pressure monitoring (ABPM) using a validated Holter device to assess continuous blood pressure measurements and circadian variability.
- Blood pressure and pulse rate will be measured using a validated electronic device (Omron HEM-705CP, Netherlands). Blood pressure will be recorded after a 10-minute rest period, with three consecutive measurements on each arm, taken 3 minutes apart. The average value of all readings will be used for analysis.

- **Assessment of Skin Advanced Glycation End-Products (AGEs):**
- Measurements of skin AGEs using an AGE Reader at baseline and at 12 months.
- This validated, noninvasive technique quantifies tissue AGE accumulation through skin autofluorescence and correlates with histologically assessed tissue glycation levels.

Dietary Intervention

Estimated duration per visit: Approximately 30 minutes.

At baseline, all participants will undergo a standardized medical and lifestyle assessment, including detailed information on alcohol consumption, smoking status, and dietary patterns.

- **Control Group**

Participants randomized to the control group will follow a hypocaloric Mediterranean diet. They will receive standardized dietary counselling and general exercise recommendations aimed at promoting a healthy lifestyle. Follow-up assessments will be conducted at 3 months and subsequently every 6 months to monitor adherence and reinforce lifestyle guidance.

- **Intervention Group**

Participants randomized to the intervention group will follow a hypocaloric Mediterranean diet combined with a 14/10 intermittent fasting regimen, under the supervision of a trained study dietitian. Follow-up visits will be conducted at 3 months and subsequently every 6 months to promote adherence to dietary intervention and to provide standardized exercise recommendations aimed at increasing physical activity.

In addition, participants in the intervention group will receive a nutritional supplement containing the natural senolytic compound fisetin, administered at a dose of 20 mg/kg/day for two consecutive days each month, supplied as 150 mg tablets. Participants assigned to the intervention group will comprise approximately 50% of the total study population and will be selected though the randomization procedure described above.

- **Concomitant medication**

All participants will continue their usual pharmacological treatments as prescribed by their primary care or specialist physicians. Study investigators will not modify, discontinue, or interfere with ongoing medical therapies.

Follow-up Visits and Monitoring Schedule

Participants will be followed for a minimum of 24 months, with a combination of in-person and virtual visits conducted by the study team to monitor adherence, collect study data, and evaluate clinical and functional outcomes.

Visit 2 – 3 Months
Dietitian (in-person visit)
Duration: Approximately 30 minutes.
Procedures: Collection of anthropometric parameters, assessment of dietary intake and physical activity, using validated questionnaires.
Purpose: Reinforcement of adherence to the dietary and lifestyle recommendations.

Physician (telephone contact)
Purpose: Monitoring clinical status and identification of any new or emerging age-related conditions.

Visit 3 – 6 Months
In-person visit with dietitian, physician, and nurse

- Comprehensive evaluation including anthropometric measurements, clinical assessment, and review of dietary adherence.
- Reinforcement of dietary and physical activity recommendations.

Visit 4 – 12 Months
In-person visit with the dietitian, physician, and nurse

- Repetition of all baseline assessments, as applicable, including laboratory analyses, imaging studies, and functional evaluations.
- Measurement of skin advanced glycation end-products using the Age Reader.
- Review of clinical outcomes, intervention adherence, and adverse events.

Visit 5 – 18 Months
Hybrid visit: In-person visit with the dietitian and virtual consultation with the physician and nurse

- Focus on dietary and physical activity adherence, anthropometric monitoring, and update of clinical status.

Visit 6 – 24 Months

Final in-person visit with dietitian, physician, and nurse

- Comprehensive clinical, functional, and biochemical reassessment mirroring baseline and 12-month evaluations.
- Final documentation of study outcomes, adherence, and adverse events.

To ensure consistency and minimize inter-observer variability, baseline assessment procedures will be repeated at follow-up visits by the same team multidisciplinary team (physician, dietitian, and nurse) whenever possible.

9. Measurement Procedures

9.1. Dietary and Physical Activity Assessment

- Dietary assessment:
  - Habitual dietary intake and adherence to the Mediterranean diet will be evaluated using a validated Food Frequency Questionnaire (151-item FFQ).
- Physical activity (self reported)
  - The Minnesota Leisure-Time Physical Activity Questionnaire will be administered to assess physical activity patterns.
- Objective pjysical activity monitoring
  - Participants will wear a GeneActive accelerometer to continuously record physical activity levels and sleep parameters over a predefined monitoring period.

9.2. Biological Sampling and Laboratory Analyses

Blood and Urine samples will be collected for biochemical, hematological, and molecular analyses related to biological aging, senescence and intervention efficacy.

9.2.1. Markers of Cellular Aging:

- *Senescent Cell Burden*:
  - Senescence cells will be quantified by measuring β-galactosidase expression in peripheral blood mononuclear cells (PBMCs) using flow cytometry. The commercial SPIDER β-Gal kit (Cayman Chemical, Ann Arbor, MI, USA) will be employed and analyzed on a BD LSR Fortessa SORP cytometer.
- *Additional age-related markers*:
  - Complementary analyses will include inflammatory biomarkers, mitochondrial function indices, and circulating progenitor cell populations, as specified in the analytical protocol.

9.2.2. Mitochondrial dysfunction and Cellular Bioenergetics

Mitochondrial function will be evaluated using complementary biochemical and molecular markers, as outlined below:

a. *Metabolic Profile and Aerobic–Anaerobic Balance*

- The balance between aerobic and anaerobic metabolism will be assessed by analyzing plasma glucose and lactate concentrations.

- Oxidative Stress and Redox Balance will be evaluated by analyzing both pro-oxidant and antioxidant capacities in serum:
  * Pro-oxidant capacity: Lipid peroxidation products measured as thiobarbituric acid reactive substances (TBARS) by spectrophotometry.
  * Antioxidant capacity: Total Antioxidant Capacity (TAC) assessed using commercial kits (Sigma-Aldrich) by spectrophotometric analysis.

b. *Circulating Mitochondrial DNA (mtDNA)*

Cell-free mitochondrial DNA will be isolated from plasma using standard extraction kits (Qiagen Midi Kit)**.** Quantification will be performed by quantitative real-time PCR (qRT-PCR) targeting the mitochondrial 12S rRNA gene**.** Circulating mtDNA will be analyzed as a marker of immunogenicity and systemic inflammation related to damage-associated molecular patterns**.**

c. *Endothelial Progenitor Cells (EPCs)*

EPCs will be quantified by flow cytometry using whole blood will be collected after a 12-hour fast. Samples will be processed within 1 hour of collection. Peripheral blood mononuclear cells (PBMCs) will be isolated by Ficoll density gradient centrifugation and stained monoclonal antibodies against CD34, CD133, and KDR (Miltenyi Biotec, MACS, Spain). Non-viable cells will be excluded using 7-aminoactinomycin D (7-AAD) (Sigma-Aldrich, Spain). A minimum of 500,000 PBMCs per sample will be acquired using a FACSCalibur cytometer (Becton Dickinson, CA, USA) and analyzed with CellQuest Pro software.

d. *Senescent Cell-Associated Inflammatory Phenotype (SASP)*

Circulating inflammatory markers associated with the SAP will be quantified, including high-sensitivity C-reactive protein (hsCRP) and a panel of cytokines: TNF-α, MCP-1, IL-1α, IL-6, IL-10, and IL-18. Measurements will be performed using the Bio-Plex Pro Human Cytokine 27-Plex Assay Kit® (Bio-Rad Laboratories, CA, USA), based on xMAP multiplex technology (Luminex Corporation, Austin, TX, USA). All samples will be analyzed in duplicate by a single blinded investigator to minimize inter-operator variability.

e. *Telomere Length and DNA Methylation (Epigenetic Aging).*

Telomere length and DNA methylation patterns will be assessed as robust biomarkers of biological aging. DNA methylation will be analyzed using the Illumina Infinium Methylation EPIC BeadChip array (EPIC array) allowing quantification of > 850,000 CpG sites. Epigenetic age will be estimated using validated clocks, including Horvath**,** GrimAge**,** PhenoAge**,** and Skin & Blood clocks. Raw IDAT files will be processed through the Clock Foundation or the Horvath Lab bioinformatics pipelines, including quality control and derivation of surrogate aging-related traits.

9.3. Body Composition Assessment by Dual-Energy X-ray Absorptiometry (DEXA)

Body composition will be assessed using Dual-Energy X-ray Absorptiometry (DEXA) with a GE Lunar iDXA Whole-Body Scanner (GE Healthcare, WI, USA). The measured parameters will be include:

a. Lean (fat-free) mass (LM)
b. Fat mass (FM)
c. Bone mass
d. Total mass (LM + FM + bone mass)

Regional analyses will include trunk**,** android, gynoid regions, and the android-to-gynoid fat ratio (A/G ratio). Measurements will be performed at baseline and study completion.

9. 4. 24-Hour Ambulatory Blood Pressure Monitoring (ABPM)

Twenty-four-hour ABPM will be conducted at baseline and at study end using Spacelabs 90207/90217 devices (Spacelabs Inc., Richmond, WA, USA). BP will be recorded every 20 minutes during daytime and every 30 minutes during nighttime. Valid recordings must meet ESH/ESC quality criteria: $\geq$ 24 hours duration and $\geq$ 70 valid readings[22].

Recorded variables will include:

- Mean 24-h daytime, and nighttime systolic blood pressure (SBP) and diastolic blood pressure (DBP).

9.5. Noninvasive Cardiovascular Assessment: Carotid Vascular Wall and Cardiac Function

a. Carotid Ultrasound

Carotid ultrasound will be performed to assess the arterial wall using 2D and 3D B-mode imaging. The following parameters will be eavluated:

- Carotid intima-media thickness (IMT)
- Presence, number, and morphological characteristics of atherosclerotic plaques

This technique is noninvasive, rapid, and safe, with no procedure-related adverse effects, and it is routinely employed in clinical practice to assess vascular risk.

b. Transthoracic Cardiac Echocardiography

A 2D and 3D B-mode transthoracic echocardiogram will be performed using an Acuson X300 device (Siemens, Germany). The following cardiac parameters will be obtained:

- Dimensions and volumes of the four cardiac chambers
- Left ventricular shortening fraction
- Left ventricular ejection fraction
- Left ventricular mass

c. Standardization and Quality Control

All imaging procedures will follow a standardized, semi-automated acquisistion and interpretation protocol, previously validated in another randomized clinical trial conducted by our group (WAHA trial, ClinicalTrials.gov Identifier: NCT01634841).

This standardized workflow enhances acquisition efficiency and reproducibility while minimizing operator dependency. Image evaluation will be performed by a blinded external evaluator using dedicated software on anonymized datasets, thereby ensuring objectivity and reducing the risk of assessment bias.

10. Safety Questionnaires

Safety assessments will be conducted at each study visit using a standardized questionnaire to monitor the occurrence of adverse events and potential supplement-related side effects.

General Symptoms

Participants will be asked the following questions:

1. Have you experienced any general malaise since the last visit?
2. Have you experienced nausea or vomiting?
3. Have you had episodes of unusual diarrhea or constipation?

Allergic or Skin Reactions

4. Have you experienced any skin rash, itching, or redness?
5. Have you had swelling of the face, lips, tongue, or extremities?
6. Have you had experienced difficulty breathing or any severe allergic reaction?

Investigator Assessment

Based on participant responses and clinical judgment, the investigator will assess:

- Whether any reported symptoms are possibly, probably, or definite related to the study supplement
- Whether dosage adjustment or discontinuation of the supplement is required
- Whether additional medical evaluation or intervention is indicated?

All adverse events will be documented and managed in accordance with Good Clinical Practice guidelines and applicable regulatory requirements.

11. Clinical Event Monitoring

Participnats in both treatments will be followed for a long-term period with a mean duration of approximately five years to evaluate the sustained effects of the intervention on major clinical outcomes. Clinical event monitoring will focus on the occurrence of prespecified hard endpoints, including:

1. All-cause mortality.

2. Cause-specific mortality, including cardiovascular, cancer-related, and other causes.
3. Incident cardiovascular events, such as acute myocardial infarction, stroke, and peripheral arterial disease.
4. Incident diabetes mellitus, among participants without diabetes at baseline.
5. Incident cancer, excluding non-melanoma skin cancer.
6. Incident cognitive impairment and dementia

Clinical events will be prospectively identified and systematically adjudicated using information from electronic medical records, hospital discharge reports, and death certificates, as applicable. This comprehensive event ascertainment will enable robust assessment of the long-term impact of the intervention on morbidity and mortality.

12. Statistics

12.1 Sample Size Calculation

Based on the sample size calculation, a minimum of 3,500 participants is required to detect statistically significant differences between the intervention groups for the primary outcomes. To account for an anticipated attrition rate of approximately 10%, a total of 4,000 participants will be recruited to ensure adequate statistical power at study completion. Eligible participants will be recruited from individuals receiving care at affiliated Primary Care Centers**.**

12.2. Data Management and Statistical Software

All study data will be entered into a secure, permanent, interactive database. Data entry will include verification procedures, including double data checks, to minimize transcription and input errors. Statistical analyses will be performed using SPSS software for Windows (IBM Corp., Armonk, NY, USA).

12.3. Study Variables

a. *Primary Variables*

The primary variables to be analyzed will include biological markers of aging, including:

- Mitochondrial function.
- Quantification of senescent cell burden.
- Inflammatory markers.
- Circulating endothelial progenitor cell counts.
- Epigenetic markers of biological aging, including DNA methylation clocks, and telomere length.
- Whole-genome sequencing-derived variables.
- Gene–diet interaction measures.

b. *Secondary Variables*

The secondary variables will include:

- Changes in body composition assessed by DEXA.

- Blood pressure changes measured by 24-hour ABPM.
- Lipid profile and glucose metabolism parameters.
- Carotid artery ultrasound (intima-media thickness and vascular compliance).
- Electrocardiographic and color Doppler echocardiographic parameters.
- Clinical indicators of aging**,** including cognitive performance**,** grip strength**,** gait speed, and muscle mass.
- Dietary intake and physical activity measures.
- Laboratory biomarkers, including lipid, carbohydrate, and iron metabolism indices.
- Advanced glycation end products (AGEs).
- Biomarkers of treatment adherence, biological aging, and intervention effectiveness.
- Incidence of new diagnosed age-related diseases during follow-up**,** including cardiovascular disease**,** neurodegenerative disorders**,** bone fractures, and cancer.

12.4. Statistical Methods

Continuous variables will be summarized as means ± standard deviations or medians with interquartile ranges, as appropriate. Categorical variables will be described using frequencies and percentages. Between-group comparisons will be performed using Student's *t*-test**,** Mann–Whitney *U* test**,** χ^2 test, or Fisher's exact test, depending on data distribution and variable type. Longitudinal changes over time will be evaluated using repeated-measures ANOVA or mixed-effects regression models, with adjustment for baseline values and relevant covariates, including age, sex, BMI, medication use. Multivariate regression analyses will be conducted to explore associations between biological aging markers and clinical outcomes, as well as to assess gene-diet interaction were appropriate. All statistical tests will be two-sided, and P value < 0.05 will be considered statistically significant.

Descriptive statistics will be summarized as means ± standard deviations (SD) for continuous variables and as appropriate measures for categorical variables. Because most study variables will be assessed at two or more time points, participant-level values will be summarized using the mean of all available measurements for each participant in the primary analyses. Variables exhibiting non-normal distributions will be log-transformed (natural logarithm) prior to analysis to approximate normality. Within-group changes from baseline to follow-up differences will be evaluated using paired Student's *t*-test, whereas between-group differences will be assessed using analysis of covariance (ANCOVA), with baseline values included as covariates. Multivariate models will be adjusted for age, sex, and any clinical parameters showing significant baseline imbalances between to control for potential confounding. *Post-hoc* comparisons will be corrected for multiple testing using Bonferroni method. Effect estimates will be expressed as mean differences with 95% confidence intervals (CIs).

13. Ethics and Legal Aspects

The study will be conducted in strict accordance with international accepted ethical principles governing research involving human participants and clinical trials. The protocol compiles with the principles of the Declaration of Helsinki and with Spanish Law 14/2007 of 3 July on Biomedical Research.

Prior to study enrollment, all participants will be required to provide written informed consent. A study investigator will explain to each potential participant, or to their legally authorized representative when applicable, the nature and objectives of the study, procedures, expected duration, potential risks and benefits, and any foreseeable inconveniences associated with participation. Participants will be informed that their participation is entirely voluntary and that they may withdraw from the study at any time without penalty or any effect on their ongoing their medical care or relationship with their treating physician. Participants will be given sufficient time to review and understand the Participant Information Sheet before dating and signing the informed consent form. A copy of the signed document will be retained, and no participant will be enrolled in the study without prior written informed.

Throughout the study, participants will complete a standardized safety questionnaire before and after each intervention to identify potential adverse effects, or intervention-related side effects. Based on previous experience with similar dietary and lifestyle intervention studies, no significant adverse events are anticipated.

The study will be conducted in accordance with the approved protocol and follow Standard Operating Procedures (SOPs) to ensure compliance with Good Clinical Practice (GCP) standards, as defined in the International Council for Harmonization (ICH) Harmonized Tripartite Guideline for Good Clinical Practice (1996)**.**

14. Data Management

All health data collected in this study will be encrypted to ensure participant privacy and data protection. No direct identifiable personal information, including name, surname, initials, identification number, medical record number, CIP, postal address, or email address, will be included in the analytical dataset. Each participant will instead be assigned a unique study identification code.

A separate, secure file linking study identification codes to corresponding medical record numbers will be maintained exclusively by the principal investigator and will be accessible to other members of research team.

The primary study database will be password-protected and access will be restricted to the Principal Investigators and authorized study personnel only. Data will be stored on a secure institutional serve (Drive F) within the research center's protected network environment. Dr. Rosa M. Casas will be responsible for the custody, maintenance, and security of this database.

Under no circumstances will study data be uploaded to or stored on public and commercial cloud-based platforms (e.g., Google Drive). When data sharing is required for collaborative analyses, all files will be fully encrypted and transferred exclusively through the institution's secure sharing platform: https://compartir.clinic.cat/storagecloud/index.php/login.

All data handling procedures will comply with applicable data protection regulations, including the General Data Protection Regulation (GDPR) and relevant national legislation.

15. Data Processing and Record Keeping: Data Confidentiality

The objectives of data collection and processing in this study are as follows:

1. To identify the stage of disease progression at which initiation of the intervention provides the greatest benefits on clinical aging outcomes and associated biological parameters.
2. To develop novel diagnostic tools for the assessment of biological aging prior to the onset of age-related diseases.
3. To characterize the interaction between calorie restriction, intermittent fasting, and adherence to the traditional Mediterranean diet in relation to biological aging processes.

The processing, communication, and transfer of participants' personal data will comply with Regulation (EU) 2016/679 of the European Parliament and Council of 27 April 2016 on the protection of natural persons with regard to the processing of personal data and on the free movement of such data (General Data Protection Regulation, GDPR), as well as Organic Law 3/2018 of 5 December on the Protection of Personal Data and the Guarantee of Digital Rights (LOPDGDD).

All study data will be identified exclusively by a unique participant code, ensuring that no directly identifiable personal information is included in the research database. Only the Principal Investigators and authorized collaborators with legitimate access to source data (medical records) will be able to link coded data to individual participants. The identity of participants will not be disclosed to any other person or entity, except in the cases of medical emergency or when disclosure is required by law.

Authorized representatives of health authorities**,** the Research Ethics Committee, and the study sponsor may access personally identifiable data when necessary to verify study conduct or data integrity. In all such circumstances, confidentiality will be strictly maintained in accordance with applicable legislation.

Only encrypted data will be transferred to third parties or collaborative entities, and such datasets will never include information capable of directly identifying participants (e.g., name, initials, address, or social security number). Data transfers will be performed solely for research purposes consistent with the objectives of this study and will always ensure participant confidentiality.

If encrypted data are transferred outside the European Union, whether to hospital-affiliated entities, external service providers, or collaborating research institutions, such transfers will be governed by appropriate contractual safeguards or other mechanisms approved by data protection authorities, in accordance with GDPR and LOPDGDD requirements.

As study sponsors, we commit to processing data in accordance with EU Regulation 2016/679, maintaining a record of processing activities, and conducting risk assessments to ensure that appropriate technical and organizational measures are in place to protect personal data.

In accordance with current regulations, participants have the right to:

- Restrict restriction of processing of inaccurate or incomplete data.
- Request access to and obtain a copy of their personal data.
- Request data portability to another entity or third party.

To exercise these rights, participants may contact either the Principal Investigators or the Data Protection Officer of the Hospital Clínic de Barcelona at protecciodades@clinic.cat. If participants consider they their rights have not been adequately addressed, they may lodge a complaint with the Spanish Data Protection Agency (Agencia Española de Protección de Datos**)**.

In accordance with legal and regulatory requirements governing clinical research, study data will not be deleted upon participant withdraws, as retention is necessary to preserve the scientific integrity of the study and to comply with applicable obligations, including those related to regulatory oversight and authorization processes.

The investigators and sponsors will retain study data for a minimum of 20 years following, in accordance with applicable regulatory requirements. After this period, personal data may be retained by the healthcare institution or sponsor for future scientific research only if explicit participant consent has been obtained and such use fully complies with applicable legal, ethical and data protection standards.

16. Biological Sample Management

Participation in this study involves the collection and analysis of biological samples for the assessment of biomarkers related to biological aging, intervention adherence, and treatment efficacy.

16.1. *Sample Collection*

The following biological samples will be collected:

- Urine: One 24-hour urine sample and an additional 60 mL of fresh urine will be obtained for the assessment of novel biomarkers of intervention adherence and metabolic status.
- Blood (Plasma and Serum, approximately 65 mL): blood samples will be collected for the analysis of inflammatory markers, senescence-related biomarkers, circulating progenitor cells, indicators of mitochondrial dysfunction, whole-DNA sequencing, and other biochemical parameters relevant to aging and metabolic health.

16.2. *Additional Laboratory Analyses*

Subject to the availability of additional funding, further analyses may be conducted, including:

i. <u>Lipoprotein profiling and multi-omic analyses</u>

Transcriptomic, proteomic, lipidomic, metagenomic, and metabolomic approaches will be applied to improve the characterization of dietary response and interindividual phenotypic variability, supporting the development of personalized nutrition and treatment strategies.

ii. Whole-genome sequencing and gene–diet interaction studies

Genotyping will be performed using the 7900 HT Sequence Detection System with a TaqMan™ fluorescent allelic discrimination assay (Applied Biosystems).

iii. Gut microbiome analyses:

- Fecal DNA extraction will be performed using the QIAamp PowerFecal DNA Kit (Qiagen) following the manufacturer's instructions.
- DNA quantification will be conducted with the Qubit 2.0 Fluorometer using the High Sensitivity dsDNA Kit (Invitrogen).
- Microbiome profiling will be conducted through amplification and sequencing of the 16S ribosomal RNA gene.

iv. Microbiota and systemic metabolites

Short-chain fatty acids (SCFAs)**,** lipopolysaccharides (LPS)**,** trimethylamine N-oxide (TMAO), and advanced glycation end products (AGEs) will be quantified using liquid or gas chromatography coupled with mass spectrometry

v. Untargeted metabolomics

Untargeted metabolomics analyses of plasma and urine sample will be performed to identify novel biomarkers associated with intervention, aging processes and treatment effectiveness.

16.3. *Access, Storage, and Use of Biological Samples*

Access to biological samples will require written authorization from the researcher responsible for sample custody, documented in a signed agreement. Any transfer of samples or performance of analyses by third parties will require prior written approval.

All samples and derived data will be fully de-identified. If a participant withdraws consent for the use of their biological samples, the samples will be destroyed immediately.

Samples will be stored in the IDIBAPS laboratories (Floor 4B – Department 4) and at the Food and Nutrition Campus of the University of Barcelona until they are used for the purposes described in this protocol or for future studies, provided that additional funding is secured and an appropriate protocol amendment is approved. Following study completion, any remaining samples will be destroyed.

Each biological sample will be labeled exclusively by a code identifier, without any personally identifiable information. Only the study physician and authorized collaborators will be able to link samples to an individual participant. All data derived from biological samples will be processed in accordance with the same confidentiality and data protection standards applied to all other study data.

Provision of biological samples is voluntary and free of charge, and participants will not hold any rights to commercial benefits that may arise from potential discoveries or results derived from this research.

16.4. *Communication of Relevant Findings*

If clinically relevant information is identified that may affect the health of participants or their relatives, participants will be informed using the contact information contained in their medical record. Participants may decline receipt of such information by indicating their preference in the informed consent form.

With respect to genetic analyses, neither the participant nor their physician will be automatically informed of the results. Participants may, however, request access to their individual results by contacting the study physician. As these analyses are exploratory, research will not be considered clinically actionable and will not be used to guide diagnosis or treatment.

16.5. *Legal and Ethical Compliance*

This study complies with Law 14/2007 on Biomedical Research and Royal Decree 1716/2011, which regulate the collection, storage, use, and transfer of biological samples for biomedical research in Spain. By signing the informed consent form, participants authorize the use of their biological samples exclusively for the purposes described in this protocol, namely, to investigate and compare the long-term effects of a healthy calorie-restricted diet and/or fisetin on clinical aging outcomes and associated biological processes.

17. Funding

This study is supported by a research grant from the Carlos III Health Institute (Instituto de Salud Carlos III, ISCIII), project FIS 23/00921, awarded to Drs. Ramon Estruch and E. Sacanella.

Given the scope and multidisciplinary nature of the project, additional funding will be sought from complementary sources, including annual funds allocated to the research group by the CIBEROBN (Centro de Investigación Biomédica en Red – Fisiopatología de la Obesidad y Nutrición, Instituto de Salud Carlos III), as well as through competitive calls from public funding agencies (e.g., Generalitat de Catalunya**,** European Horizon 2020 programs**)** and private foundations (e.g., Recercaixa).

These combined resources will ensure adequate financial support for both the core activities and the additional sub-studies described in this protocol.

18. Publication Policy

The sponsor commits to ensuring that the results of this study are made publicly available, irrespective of whether the findings are positive, negative, or inconclusive. This commitment is fully aligned with the principles of transparency and scientific integrity set forth in the Declaration of Helsinki, the recommendations of the International Committee of Medical Journal Editors (ICMJE), and applicable European Union regulations governing clinical research.

A dedicated publication policy will be developed to define procedures for authorship attribution, data sharing, and dissemination of study results. Given the multidisciplinary

and collaborative nature of the project, this policy will also establish guidelines for joint publications involving investigators from other research groups and institutions, including international collaborators participate in the additional analyses described in this protocol.

All publications, presentations, or other communications arising from this study will appropriately acknowledge the sponsor**,** funding sources, and institutional affiliations, and will comply with all applicable ethical and legal requirements related to data protection and participant confidentiality.

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