Evaluation of Longevity Diet and Fasting Mimicking Diet programs on body composition, disease risk factors, and aging markers: a randomized clinical trial

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Protocol Summary

Protocol number	
Brief title	Longevity Diet and Fasting Mimicking Diet
Sponsor	Regione Calabria
Investigation type	Diet
Study type	Interventional
Primary Objective(s)	Change from baseline to end of study in body composition measured as percentage of fat mass.
Secondary Objectives	Change from baseline to end of study in blood pressure, serum lipids, glucose, HbA1C, CV biomarkers, body weight, BMI, insulin resistance, aging biomarkers, proportion of participants who receive antihypertensive/ hypoglycemic medications, sleep quality and duration.
Study design	This is a randomized, open-label trial in 501 adult subjects between the age of 30 and 65: 167 subjects randomized to the Fasting Mimicking Diet (FMD) arm with a 5-day meal program once every three months for a 6-month period (arm 1); 167 subjects randomized to follow the FMD plus a Longevity Diet program (FMD+LD) for a 6-month period (arm 2); 167 randomized to the control group (arm 3) that will continue their usual diet. Participants belonging to the control arm will be given an opportunity to follow a 6-month LD program starting at the end of 6 months.
Rationale	Fifteen years of extensive preclinical and clinical studies sponsored by the National Institutes of Health (NIH) and conducted at the Longevity Institute and Diabetes and Obesity Research Institute of the University of Southern California (USC) resulted in the development of two very promising dietary interventions effective in extending not only longevity but also the healthy life span: the Fasting Mimicking Diet (FMD) and the Longevity Diet (LD). The objective of the study is to determine the effects of two different dietary interventions, FMD and LD, on body composition and cardiovascular (CV) biomarkers (body weight,

	BMI, blood pressure, serum lipid levels and dysglycemia blood measurements).
Study population	Community Dwelling People 30-65 years old
Key Inclusion/exclusion criteria	Inclusion Criteria • Subjects of 30-65 years of age; •Body mass index equal to or greater than 25 kg/m^2. And at least one of the following: • Obesity (Body mass index equal to or greater than 30 kg/m2). • HbA1C greater than 5.6% (38 mmol/mol); • IGF-1 level greater than 200 $ngml^{-1}$; • SPB >130 mmHg and DBP>90 mmHg; • triglycerides >150 $mgdl^{-1}$; • C-reactive protein >1 mgL^{-1}; • total cholesterol>190 $mgdL^{-1}$ and LDL-cholesterol>129 $mgdL^{-1}$. Exclusion Criteria • individuals with a family member already included in the study; • individuals who are allergic to tree nuts, soy, oats, sesame, or celery/celeriac; • pregnant females; • any documented cancer diagnosis within the past 5 years; • documented myocardial infarction within past 5 years; • documented cerebrovascular accident within past 5 years; • chronic steroid use (longer than 45 consecutive days); • insulin-dependent diabetes mellitus; • individuals taking insulin or insulin-like drugs and individuals taking hypoglycemic agents other than metformin. In this last case, close attention will therefore be paid to the self-monitoring of blood glucose during the FMD cycles.
Study treatment	Fasting Mimicking Diet. A plant-based diet consists of ingredients which are Generally Regarded As Safe (GRAS). It was initially designed to attain fasting-like effects on the serum levels of IGF-1, IGFBP-1, glucose, and ketone bodies while providing both macro- and micronutrients to minimize the burden of fasting and adverse effects. Day 1 of the FMD supplies ~1000 kcal (~9% protein, 61% fat, and 30% carbohydrate), whereas days 2 to 5 provide ~700 kcal (~8% protein, 52% fat, and 40% carbohydrate) per day. The FMD comprises proprietary formulations of vegetable-based soups, energy bars, energy drinks, chip snacks, tea, and a supplement providing high levels of minerals, vitamins, and essential fatty acids. All items to be consumed per day were individually boxed to allow the subjects to choose when to eat while avoiding accidentally consuming components of the following day. Longevity Diet (LD). LD, described by Dr. Longo, is based on the common denominator of traditional foods that people

	consumed in longevity areas of the world including Okinawa, Southern Italy, Loma Linda (California) in the 1950's and 1960's and modifications based on epidemiological, clinical and laboratory longevity studies. LD is based on these following prescriptions: vegan based diet with the addition of fish 2-3 times a week, choosing fish with lowest mercury content; protein consumption should be 0.7-0.8 grams per kilogram of ideal weight (most of the protein coming from vegetables and legumes and only part from fish); vegetable monounsaturated and polyunsaturated fats are predominant and very low amounts of animal saturated and trans-fats are allowed; complex Carbohydrates from vegetables, whole grains and legumes; addition of vitamins, minerals and EPA, DHA (Omega3) fats; food selected between the traditional food consumed by our grandparents and ancestry; time-restricted feeding. All food must be consumed within 12 hours each day. for those that are overweight or are nearly overweight (BMI >24), substitute either lunch or dinner with a low calorie, low sugar snack (approximately 100 kcal: salad, nuts, fruit, etc.).
Efficacy assessments	Primary objectives: linear mixed models will be used to compare arm 1 (FMD) and arm 2 (FMD+LD) to the arm 3 (control group) sitting fat mass expressed in terms of change from baseline to the visit 3. Secondary objectives: linear mixed models will be used to compare FMD+LD and FMD to the control group sitting the following parameters expressed in terms of change from baseline to the visit 3: inflammatory biomarkers, IGF-1, blood pressure, serum lipids, glucose, CV biomarkers, body weight, BMI, body composition, insulin resistance, and aging biomarkers, proportion of participants who receive antihypertensive/ hypoglycemic medications, sleep quality and duration.
Key safety assessments	AEs and AEs leading to discontinuation
Key words	Longevity Diet, Fasting Mimicking Diet, Body Composition

1. Background Information and Scientific Rationale

1.1. Background Information

With a growing aging population all over the world, healthy ageing is an important goal for public health. Dietary restriction (DR), implemented as chronic and coordinate reduced intake of all dietary constituents except vitamins and minerals, was first shown more than 80 years ago to extend lifespan in model organisms and in humans. Dietary interventions that avoid unrealistic levels of self-deprivation, and pharmacological interventions that recapture beneficial effects of DR, are therefore important goals to improve human health during aging. Despite its potential for disease prevention and treatment, prolonged fasting is difficult to implement in human subjects and may exacerbate pre-existing nutritional deficiencies, making it not feasible and/or safe for children, the elderly, frail individuals, and even most of the healthy adults. Fifteen years of extensive preclinical and clinical studies sponsored by the National Institutes of Health (NIH) and conducted at the Longevity Institute and Diabetes and Obesity Research Institute of the University of Southern California (USC) resulted in the development of two very promising dietary interventions effective in extending not only longevity but also the healthy life span: the Fasting Mimicking Diet® (FMD) and the Longevity Diet (LD).

1.2. Fasting Mimicking Diet

The Fasting Mimicking Diet (FMD) is a 5-day meal program to be consumed every 1 to 6 months based on an authorized healthcare professional's recommendation. It is designed to promote the body's natural ability to protect, regenerate and rejuvenate itself. In clinical studies, FMD has been shown to reduce abdominal fat and maintain healthy levels of blood glucose, C-reactive protein (CRP), and insulin-like growth factor 1 (IGF-1) (Wei et al., 2017). Well-designed clinical trials, such as the study conducted by Houston et al. utilizing an Independent Review Board (IRB) approved protocol, have demonstrated the safety and short-term benefits of plant-based nutraceuticals and food restriction in individuals with chronic hypertension (Houston et al., 2014). Unpublished clinical trials indicate that FMD may have other positive health benefits. A detailed description of ingredients is provided in Appendix A.

1.3. Longevity Diet (LD).

Longevity Diet (LD) program. LD program, described by Dr. Longo, is based on the common denominator of traditional foods that people consumed in longevity areas of the world including Okinawa, Southern Italy, Loma Linda (California) in the 1950's and 1960's and modifications based on epidemiological, clinical and laboratory longevity studies.

General guidelines of this nutritional regimen can be resumed as follow:

- vegan based diet with the addition of fish 2-3 times a week, choosing fish with lowest mercury content.
- Protein content should be 0.7-0.8 grams per kilogram of ideal weight (most of the protein coming from vegetables and legumes and only some from fishes).
- Vegetable monounsaturated and polyunsaturated fats are predominant and only very few amounts of saturated and trans-fats are allowed.
- Complex carbohydrates from vegetables, whole grains and legumes.
- Addition of vitamins, minerals and Eicosapentaenoic Acid (EPA), Docosahexaenoic Acid (DHA) (Omega3) fats.
- Food selected between the traditional food consumed by our grandparents and ancestry.
- Time-restricted eating. All food must be consumed within 12 hours each day.

- For those that are overweight or are nearly overweight (BMI >24 kg/m^2), substitute either lunch or dinner with a low calorie, low sugar snack (approximately 100 kcal: salad, nuts, fruit, etc.).

2. **Study Objectives/Endpoints**

2.1. **Objectives**

The objective of the study is to determine the effects of two different dietary interventions FMD alone or in combination with Longevity Diet (FMD + LD), on body composition.

Secondary objectives include the assessment of the effects of two different dietary interventions on: cardiovascular (CV) biomarkers, aging biomarkers, antihyperglycemic and antihypertensive medication, and sleep quality.

2.2. Endpoints

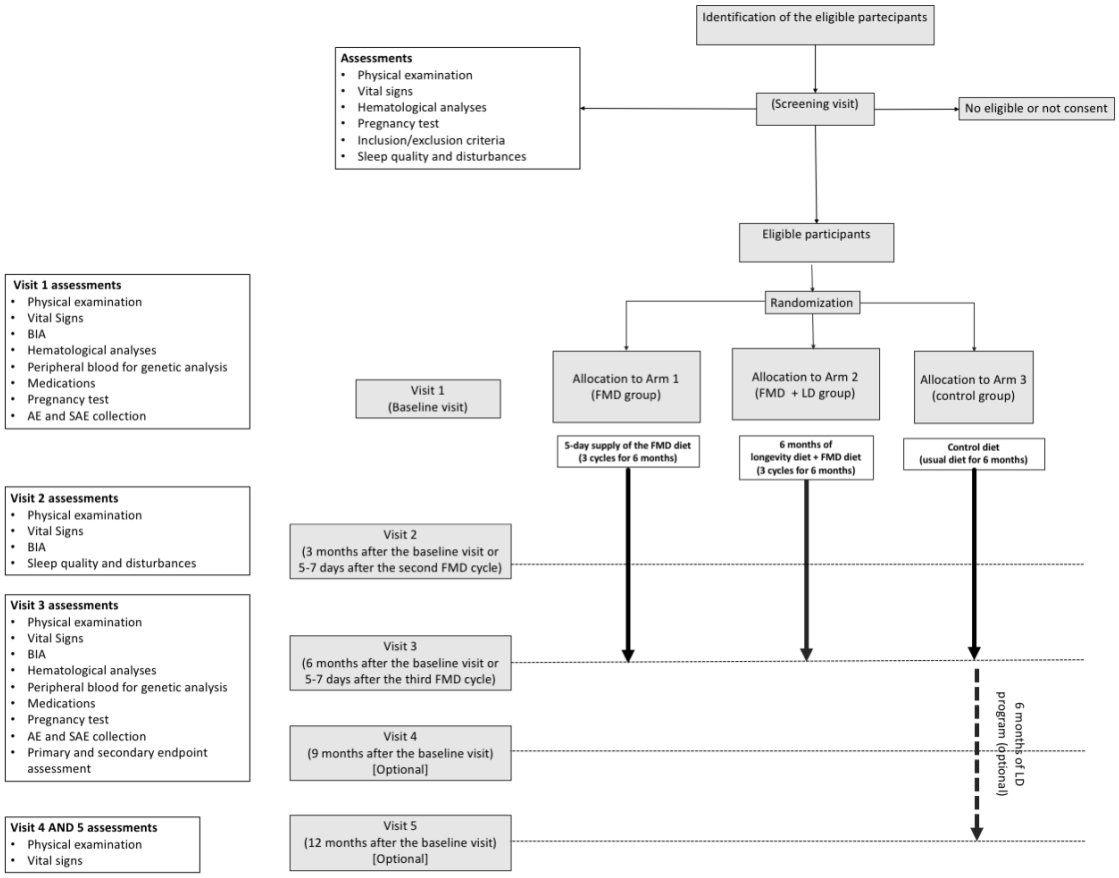
2.2.1. *Primary endpoint*: change from baseline to end of study in body composition measured as percentage of fat mass.

2.2.2. *Secondary endpoints*: change from baseline to end of study in blood pressure, serum lipids, glucose, HbA1C, CV biomarkers (body weight, BMI, blood pressure, serum lipid levels and dysglycemia blood measurements), body weight, BMI, insulin resistance, telomere length, proportion of participants who receive antihypertensive/hypoglycemic medications, and sleep quality and duration using the Pittsburgh Sleep Quality Index, and the Berlin questionnaire.

3. Study Design

This is a randomized, open-label 18-month trial in 501 adult subjects: 167 subjects randomized to follow a Fasting Mimicking Diet (FMD) plan with a 5-day meal program once every three months for a 6-month period (arm 1); 167 subjects randomized to follow the FMD plus a LD program for a 6-month period (FMD+LD, arm 2); 167 randomized to the control group (arm 3) that will be recommended to use a healthy dietary regime following the Italian guidelines to selecting healthy food or equivalent International guidelines (Figure 1). The enrolment period will last 12 months and the patients will be followed for 6 months from study entry. Participants belonging to the control arm will be given an opportunity to follow a 6-month LD program starting at the end of 6 months. This trial makes use of a control group to provide robust evidence on the effects of nutritional interventions on the primary and secondary endpoints.

Figure 1. Schematic representation of study design.



3.1. *Selection and withdrawal of subjects*

3.1.1.*Inclusion Criteria*

- Subjects of 30-65 years of age;
- Body mass index equal to or greater than 25 kg/m^2.
  And at least one of the following:

- obesity (Body mass index equal to or greater than 30 kg/m^2).
- HbA1C greater than 5.6% (38 mmol/mol);

- IGF-1 level greater than 200 ngml^{-1};
- systolic blood pressure >130 mmHg and diastolic blood pressure >90 mmHg; the inclusion of individuals currently taking antihypertensive medications is allowed. In this last case a careful attention will have to be paid to the self-monitoring of blood pressure during the FMD cycles;
  - triglycerides >150 mgdl^{-1};
  - C-reactive protein >1 mgL^{-1};

- total cholesterol >190 $mgdL^{-1}$ and LDL cholesterol >129 $mgdL^{-1}$.

3.1.2.*Exclusion Criteria*

- individuals with a family member already included in the study;
- individuals who are allergic to tree nuts (macadamia, cashew, almond, pecan), soy, oats, sesame, or celery/celeriac;
- pregnant females;
- any documented cancer diagnosis within the past 5 years;
- documented myocardial infarction within past 5 years;
- documented cerebrovascular accident within past 5 years;
- chronic steroid use (longer than 45 consecutive days);
- insulin-dependent diabetes mellitus;
- individuals taking insulin or insulin-like drugs and individuals taking hypoglycemic agents other than metformin. In this last case, close attention will therefore be paid to the self-monitoring of blood glucose during the FMD cycles;
- Individuals with severe hypertension (systolic greater than 200 mmHg and or diastolic greater than 105 mmHg.

3.1.3.Prohibited Medications, Medical Foods or Nutritional Supplements

- Change in prescription medications, over-the-counter (OTC) medications, medical foods, and nutritional supplements within 30 days prior to the start and for the duration of the study.
- Use of medications classified as narcotics 15 days prior start and for the duration of the study.
- Use of prescription medications and/or over-the-counter medications for acute and semi-acute medical conditions 15 days prior to start and for the duration of the study. Use of acetaminophen is permitted on an as-needed basis.
- Use of an investigational drug or participation in an investigational study within 30 days prior to the start and for the duration of the study.
- Use of oral or injectable corticosteroids within 30 days prior to the start and for the duration of the study.
- Use of anticoagulant medications (heparin compounds or warfarin) within 30 days prior to the start and for the duration of the study. Use of aspirin 81 mg or 325 mg once daily is permitted.
- Use of neuroactive prescription medications including major and atypical antipsychotic medications, anti-depressants, anti-anxiolytics, and epilepsy medications within 30 days prior to the start and for the duration of the study.
- Subjects will not be allowed to discontinue prohibited prescription medications to meet enrolment criteria.

3.1.4.*Medical History and Concurrent Diseases*

- A history of allergy or intolerance to study products. Detailed descriptions of study product are included in Section 4.1 and 4.2, appended to the Study Informed Consent.

- Clinically significant vital sign abnormalities (systolic blood pressure <90 mmHg or >200 mmHg, diastolic blood pressure <50 mmHg or >105 mmHg or resting heart rate of <50 or >100 bpm) at screening visit.
- A serious, unstable illness including cardiac, hepatic, renal, gastrointestinal, respiratory, endocrinologic, neurologic, immunologic, or hematologic disease.
- Known infection with HIV, TB or Hepatitis B or C.
- A current diagnosis or personal history of:
    - any cardiovascular disease including myocardial infarction, angina, cardiovascular surgery (within 5 years), congestive heart failure, cardiac arrhythmias or conduction abnormalities, cerebrovascular accident, transient ischemic attack (TIA), or peripheral vascular disease, deep vein thrombosis or pulmonary embolus. Diabetes mellitus requiring inhaled or injected insulin.
    - Any autoimmune disease such as inflammatory bowel disease (including Crohn's disease and/or ulcerative colitis), multiple sclerosis, rheumatoid arthritis, systemic lupus erythematosus, polymyositis, scleroderma and/or thyroiditis.
    - Any significant liver or kidney disease such as cirrhosis or non-alcoholic fatty liver disease, glomerulonephritis, and/or ongoing dialysis treatment.
    - Any malignancy (with the exception of adequately treated malignancies with no known recurrence for >2 years).
- Any serious mental illness including a history of attempted suicide.
- Any medical condition that in the opinion of the primary care doctor or a specialist would preclude safe participation in this study or interfere with compliance.

3.1.5.*Substance Use*

- Use of drugs of abuse (such as marijuana, cocaine, phencyclidine [PCP] and methamphetamine) 15 days prior to Day 1 and for the duration of the study.
- History of regular intake of >14 alcoholic drinks per week for females, and >21 drinks per week for males (1 drink = 35 cl. beer, 12 cl. wine, or 30 ml. hard liquor).

3.1.6.Technical reasons

Any condition in which bioelectrical impedance testing would be impossible or uninterpretable (e.g. prostheses in extremities on both sides, limb amputation, implanted pacemaker, inability to lay still or supine, or skin defects on preferred electrode placement sites.

3.1.7.*Other Exclusion Criteria*

Inability to comply with study and/or follow-up visits.

- Any concurrent condition (including clinically significant abnormalities in medical history, physical examination or laboratory evaluations) which, in the opinion of the PI, would preclude safe participation in this study or interfere with compliance.
- Any sound medical, psychiatric and/or social reason which, in the opinion of the PI, would preclude safe participation in this study or interfere with compliance.
- Abnormal laboratory findings including: abnormal blood counts (hematocrit < 33% or > 47%; WBC < 3.0 or > 12.0 x10^3/mm^3; platelets < 140 or > 500 x 10^9/L); abnormal kidney function test

(creatinine > 2.5 mg/dL) or liver function test(s) (AST, ALT, alkaline phosphatase) > 1.5X the upper limit of normal; serum calcium > 11 mg/dL); serum K < 3.5 mEq/L; Na < 134 or > 148 $mmolL^{-1}$

3.1.8.*Women of Childbearing Potential*

Contraception: the effects of the study products on the developing human fetus have not been studied extensively. For this reason, women of childbearing potential must agree to use adequate contraception (hormonal or barrier method of birth control; abstinence) prior to study entry and for the duration of study participation. Females of childbearing potential will have a pregnancy test prior to receiving study products. Should a woman become pregnant or suspect she is pregnant while participating in this study, she should inform study staff and her primary care physician immediately.

Pregnancy: because there is an unknown but potential risk for adverse events in pregnant women during treatment with the study products, pregnant women are not eligible for study participation.

Breast-feeding: Because there is an unknown but potential risk for adverse events in nursing infants secondary to treatment of the mother with the study products, breastfeeding mothers are not eligible for study participation.

3.1.9.*Co-enrollment Guidelines*

Co-enrollment in other trials is restricted other than for observational studies. Study staff should be notified of co-enrollment as it may require the approval of the investigator.

3.2. Recruitment

Participants will be identified through population registers of the municipalities under investigation (Varapodio, Molochio, Oppido Mamertina and additional towns within of a range of 20 km from Varapodio). In particular, according to the Italian National Institute of Statistics (ISTAT) data, in 2020 about 4612 people 30-65 years old lived in these three villages. Therefore, assuming that the proportion of subjects who will refuse to participate is 50%, and that the prevalence of people with BMI>25 kg/m^2 in this age-range is around 50% we will expect to screen around 1150 residents of the three villages. Assuming that in this group the prevalence of the other exclusion criteria is 10%, we expect that at least 1000 residents will be potentially eligible for the study. Once the approval will be obtained, we will work with the primary care doctors and health professionals and several community organizations of the involved villages to establish a network of collaborators for the identification of eligible individuals. Participants with an interest in clinical trial participation will be invited to contact their primary care doctor to inform him/her about the trial they will be participating in to be sure that there are no reasons why they cannot participate to the study, and to obtain a prescription of blood exams required for the assessment of the eligibility for the study. If interested, participants will be than contacted by the study staff by telephone call to determine their initial eligibility (pre-screening phone call visit). Final confirmation of eligibility and subsequent consenting will be carried out at the screening visit. The recruitment activities will be performed in the Varapodio clinic and in the additional surrounding villages. The recruitment period will last approximately 12 months (approximately 42 participants per month).

3.3. Scheduled clinical visits

The assessment schedule (Table 1) lists all the assessments and when they are to be performed. Subjects will meet with study staff. When the visit doesn't include blood collection or BIA the visit can be made remotely. During the clinical visits, staff will review calendars, assess for signs and symptoms of adverse events, review compliance to the study product and answer any questions from the subject. Eligible participants will receive 5 different visits (pre-screening phone call visit, screening visit, baseline visit (t_0), and two follow-up visits (after 3 and 6 months from the baseline). Participants belonging to the control arm that will decide to follow a 6-month LD program at the last follow-up visit, will receive two additional (optional) visits at month 9 (visit 4) and at month 12 (visit 5). In particular, eligible participants belonging to the arm 1 and arm 2 (FMD or FMD+LD) will be telephonically contacted at the end of each FMD cycle (day 6) and every 2 months in order to assess signs and symptoms of adverse events, compliance to the nutritional interventions (see Appendix B,D and N), to answer to the questionnaire for dietary recall (see Appendix F) and to assess sleep quality and disturbances with the Pittsburgh Sleep Quality Index (Appendix G) and the risk associated with sleep apnea with the Berlin Questionnaire (Appendix H). Subjects belonging to the arm 3 (control group) will be telephonically contacted every 2 months in order to administer the questionnaire for dietary recall (see Appendix F), to evaluate the adherence to the Mediterranean diet (Appendix C).

Table 1 reports the timeline of the clinical visits and the activities scheduled. All data obtained from these assessments must be supported in the participant's source documentation.

Table 1. Scheduling of the activities.

	Screening Visit	Baseline visit (T_0)	Visit 2 (T_1)	Visit 3(T_2)
Informed Consent	√			
Review Eligibility Criteria	√			
Start of intervention				
Physical examination[1]		√	√	√
Vital Signs[2]	√	√	√	√
BIA		√	√	√
Peripheral Blood for genetic analysis	√	√		√
Hematological analyses[3]	√	√		√
Pregnancy test	√	√		√
AE and SAE collection		√	√	√
Primary and secondary endpoints assessment		√	√[4]	√
Medications	√	√		√

[1] height, weight (dress weight), waist and hip circumferences. [2] Body temperature, blood pressure, pulse, respiratory rate. [3] Lipid profile (total cholesterol, LDL-cholesterol, HDL-cholesterol and triglycerides, serum/plasma, albumin, alkaline phosphatase, insulin-like growth factor 1 (IGF-1), Insulin-like growth factor-binding protein 1 (IGFBP1), creatinine, insulin, C-reactive protein (CRP), aspartate aminotransferase (AST), alanine aminotransferase (ALT), homocysteine, glucose, glycosylated hemoglobin (HbA1c). [4] Secondary endpoints only include the assessment of sleep quality and disturbances.

Subjects in the control group (arm 3) and those between two FMD cycles of arm 1 will be asked to make no changes in dietary programs, exercise programs, caffeine and tobacco consumption and in prescription and Over-the-Counter (OTC) medications and nutritional supplements during the course of the study. The expectation is that subjects will maintain their current weight and do not engage in behaviours intended to produce weight loss.

3.3.1.Pre-screening phone call visit

The purpose of the pre-screening phone call visit is to quickly determine if potential participants fit within the study's core inclusion criteria (age and BMI) and doesn't meet some of its permanent exclusion criteria (e.g. morbidities, pregnancy, known allergies, etc.). Only participants with existing blood exams carried out in the previous 3 months or able to obtain a prescription of blood tests required for the assessment of the eligibility for the study will be evaluated. Once a potential participant is cleared through the pre-screening visit and confirms interest in participation, they may proceed through the screening visit.

3.3.2.Screening visit

The purpose of the screening visit is to definitely determine volunteer eligibility for study participation. Subjects will be screened for eligibility for the study, ascertaining that all inclusion and exclusion criteria are met. Once participant met all inclusion/exclusion criteria, will be offered by study staff the opportunity to learn more about the research opportunity before any procedure is performed. Screening will include measurement of height, weight, and vital signs (body temperature, blood pressure, pulse, respiratory rate), completion of medical history questionnaire, review of medical history and current medications, assessment of sleep quality and disturbances with the Pittsburgh Sleep Quality Index (Appendix G) and the risk associated with sleep apnea with the Berlin Questionnaire (Appendix H), and collection of fasting blood (12 mL: 6 mL for serum, 6 mL for plasma). Blood testing will include: complete blood count (CBC), glycated hemoglobin (HbA1c), glucose, insulin, lipid profile (total cholesterol, LDL-cholesterol, HDL-cholesterol and triglycerides), AST (GOT), ALT (GTP), alkaline phosphatase, creatinine, reactive C protein (PCR), albumin, insulin, IGF-1, IGFBP1, homocysteine as well as a urine pregnancy test in females of child-bearing potential. Blood exams carried out in the last 3 months will be considered valid for the ascertaining of the inclusion/exclusion criteria. Fasting is defined as 12 hours of refraining from consumption of food and beverages though unlimited consumption of water is allowed and encouraged. Upon review of screening data acceptable for inclusion, an in-person or telephone interview with a study investigator (or designated staff) will be completed to confirm eligibility, and document absence of contraindications to participation. During this visit, ring sizes of participants will be obtained for all fingers, from which a ratio of appropriate ring sizes for this population group will be established. Participants will be then randomised into either one of the intervention arms (LD+FMD or LD) or in the control arm using a web-based randomisation system that will be controlled by the researchers of the Valter Longo Foundation in Milan (see section 8.1)

3.3.3.Baseline visit (T_0)

Within a week from the screening visit, subjects in the arm 1 and 2 (FMD or FMD+LD) will be given one box of FMD together with a ketone test strips to self-monitor urinary ketone bodies during the FMD cycles. Subjects in the arm 2 will be given informative material and instructions during the Longevity Diet. Detailed information on the respective diet plans will be given to subjects during the visit. During the visit, staff will administer the questionnaire for assessing life style and dietary habits (appendix B). Visit 1 will also include

body composition assessment using Bioelectrical Impedance Analysis (BIA), measurement of height, weight, and vital signs, medical history, current medications and a collection of fasting blood for the genetic analysis (25 mL: 7.5 mL for serum, 7.5 mL for plasma, 10 mL for white blood cells). Note, the typical draw volume for blood donation in Italy is ~450 mL. Blood testing information will be also retried from the screening visit.

30 subjects for each study group reporting a sleep problem on the basis of the score obtained at the Pittsburgh Sleep Quality Index questionnaire (Appendix G) will also receive an Oura Ring device for the continuous monitoring of sleep state and wakefulness for a 2-week period (90 participants in total). These three study groups will be balanced for age, sex, BMI and for the score obtained at the Pittsburgh Sleep Quality Index. Study staff will ensure the participants wear the rings properly and that the ring and Oura mobile app will be connected the following 2 weeks to upload the data. Thirty second epoch by epoch data will be then obtained from Oura's cloud. Participants in the arm 3 will undergo the same testing measures as the intervention groups and will be recommended to continue their usual diet.

3.3.4. Visit 2 (after three months or 5-7 days after the second FMD cycle, T_1)

After 3 months from the randomization, subjects in the arm 1 and 2 will be contacted to receive the FMD boxes for the second and the third FMD cycles, respectively, together with the ketone test strips to self-monitor urinary ketone bodies during the FMD cycles. Visit 2 will also include body composition assessment using Bioelectrical Impedance Analysis (BIA), measurement of height, weight, and vital signs and to assess sleep quality and disturbances with the Pittsburgh Sleep Quality Index (Appendix G) and the risk associated with sleep apnea with the Berlin Questionnaire (Appendix H).

3.3.5. Visit 3 (after six months or 5-7 days after the third FMD cycle, T_2)

Subjects will meet with the study staff. During the clinical visit, staff will assess for signs and symptoms of adverse events, review compliance to the diet programs. Visit 3 will also include body composition assessment using Bioelectrical Impedance Analysis (BIA), measurement of height, weight, and vital signs, medical history and current medications and a collection of fasting blood for both hematological and genetic analyses as previously described for the baseline visit (see Table 1). Only subjects that received the Oura Ring device at the baseline visit will also receive an Oura Ring device for the continuous monitoring of sleep state and wakefulness for a 2-week period as previously mentioned. After this period, study staff will assess sleep quality and disturbances with the Pittsburgh Sleep Quality Index (Appendix G), the risk associated with sleep apnea with the Berlin Questionnaire (Appendix H) and will answer any questions from the subject. At this point, subjects belonging to the control arm will be given an opportunity to follow a LD program for a 6-month period (optional).

3.3.6. Visit 4 and 5 (month 9 and 12, optional)

Subjects belonging to the arm 3 that will decide to follow a LD program for a 6-month period will meet with the study nutritionist. During the clinical visit at month 9 (optional visit 4) and month 12 (optional visit 5), staff will review compliance to the LD diet and answer any questions from the subject.

3.4. Additional assessments

3.4.1. Exploratory Genetics: Telomere length

The study includes genetic sample at the screening visit (see Table 1) that requires a separate informed consent form if the participant agrees to participate.

Exploratory genetic research studies are planned following study completion with the objectives of evaluating the impact of the two nutritional programs on telomere length used as a biomarker of individual ageing.

3.4.2. Oura Rings

The study will also include a continuous monitoring of sleep state and wakefulness by providing Oura Ring devices: the rings are already documented in the literature for their potential in the measurement of sleep parameters with results comparable to polysomnography, the gold standard for the assessment of sleep disturbances, but making it also available on a larger scale and for a longer period of time (The Sleep of the Ring: Comparison of the ŌURA Sleep Tracker Against Polysomnography; Behav Sleep Med. Mar-Apr 2019;17(2):124-136.). The ring is a "sleep tracker" that comes in different sizes (US standard ring size 6–13), it is waterproof, weights approximately 15 g and its battery life lasts approximately 3 days. It automatically connects via Bluetooth and transfers data to a mobile platform in the dedicated App. The ring continuously collects data and processes them through an algorithm, to determine the stages of sleep (wakefulness, "light", "deep" and REM sleep): each night it monitors the latency sleep onset, time spent in "deep sleep", time spent in "light sleep", total time spent asleep and periods of wakefulness after starting sleep. It also adds information regarding sleep patterns, an analysis of heart rate and its variations, physical activity and body temperature. Oura ring fulfils the essential requirements of the applicable CE marking directives and regulations.

The design plans to randomly allocate 20 rings among 90 subjects in the 3 study groups that will result with sleep problems at the screening visit on the basis of the score obtained at the Pittsburgh Sleep Quality Index questionnaire (Appendix G). These three study groups will be balanced for age, sex, BMI and for the score obtained at the Pittsburgh Sleep Quality Index. The continuous monitoring of each parameter and sleep marker will last over a 2-week period. At each check point, questionnaires will also be distributed to assess sleep quality and disturbances with the Pittsburgh Sleep Quality Index (Appendix G) and the risk associated with sleep apnea with the Berlin Questionnaire (Appendix H).

3.5. *Prohibited Medications and Procedures*

No concomitant prescription medications, over-the-counter medications, medical foods, and nutritional supplements are to be started or doses changed during the study unless they are prescribed by the PI (or the subject's primary care giver) for treatment of a specific clinical event.

3.6. *Rescue Medications*

Acetaminophen, may however, be used for mild headache or myalgia at a dose of 650 mg three times daily as needed.

3.7. *Subject Compensation*

Subjects will not be compensated for participation in this trial.

4. **Study Product:** *Formulation, Packaging, Labeling, Preparation, Administration, and Dosage of Study Products*

4.1. Arm 1 (FMD plan)

The subjects will be instructed to follow their usual diet plan, except for 5 days when they eat the foods in the FMD box. The FMD diet is made up of *nut* bars, dehydrated soups, tea, olives, kale crackers, electrolyte beverages, and a chocolate crisp bar.

• Nut bars: Almond, macadamia nuts, pecans, vegetable inulin fibre (chicory root fiber), honey, coconut flour, flaxseed, natural flavour, sea salt, rosemary extract.

• Almond and Kale crackers: almonds, sesame seeds, tapioca flour, chia seeds, golden flax seeds, sunflower seeds oil, kale, sea salt, coconut sugar, coconut vinegar, onion powder, chili pepper, cumin seeds, black pepper, antioxidant: extract rich in tocopherol, garlic, origan, acidifier: citric acid.

• Electrolyte beverages containing water, natural vegetable glycerin, natural flavor,

• Olives.

• Spearmint tea, spearmint lemon tea, hibiscus tea.

• There are 5 different soups, which contain various combinations of rice flour, rice starch, potato flakes, tomato concentrate, sweet red pepper, basil, onion, leek, extra virgin olive oil, parsley, peas, yeast extract, savoy cabbage, carrot, garlic, spinach, celery, turmeric, black and white beans, champignon mushroom powder: minestrone, mushroom, tomato, vegetable, black beans soup;

• Chocolate crisp bar: inulin, almond butter, brown rice, cocoa powder, almonds, chocolate chips (brown sugar, cocoa mass, cocoa butter), rolled oats, brown rice syrup, flaxseed oil, rice dextrin, grape juice, salt.

In order to evaluate adherence to the FMD and its metabolic effects, patients in arm 1 will receive ketone test strips together with each FMD kit to self-monitor urinary ketone bodies.

4.1.1.Study Product Storage and Stability

Subjects in the arm 1 will be given one box of FMD at the end of the baseline visit and other 2 boxes before the starting of the other FMD cycles during the study. Subjects in the arm 2 (FMD+LD) will be also given a copy of the longevity diet at screening visit. Information on the respective diet plans will be given to subjects after the screening visit. The study products will be handled and dispensed to subjects at ambient temperature at the study site according to good clinical practices.

4.1.2.*Study Product Accountability Procedures*

It is the responsibility of the PI to ensure that a current record of study product disposition is maintained at the Study Site, and that records and logs include:

- amount of study product received and placed in storage area;
- dates and initials of individual responsible for investigational product inventory entry/movement;
- amount dispensed to and returned by each subject, including unique subject identifiers;
- amount transferred to another area for dispensing or storage;
- non-study disposition (e.g. lost, broken, wasted);
- amount destroyed at the site (if applicable).

The unused study products for this study will be disposed of according to good clinical practices.

4.2. *Arm 2 (FMD + LD plans)*

The subjects will be instructed to follow their longevity diet plan, except for 5 days when they eat the foods in the FMD box. Subjects in the arm 2 will be instructed to follow a longevity diet based on these following prescriptions:

- vegan based diet with the addition of fish 2-3 times a week, choosing fishes with lowest mercury content;
- protein content should be 0.8 grams per day per kilogram of weight (most of the protein coming from vegetables and legumes and only some from fishes);
- monounsaturated and polyunsaturated fats are predominant and only very few amounts of saturated and trans-fats are allowed;
- complex Carbohydrates from vegetables, whole grains and legumes;
- addition of vitamins, minerals and EPA, DHA (Omega3) fats;
- food selected between the traditional food consumed by our grandparents and ancestry;
- time-restricted feeding. All food must be consumed within 12 hours each day.
- for those that are overweight or are nearly overweight (BMI >24), substitute either lunch or dinner with a low calorie, low sugar snack (approximately 100 kcal: salad, nuts, fruit, etc.).

The subjects will be instructed to follow the FMD program with the same procedures previously described for participants in the FMD arm. Also in this case, in order to evaluate adherence to the FMD and its metabolic effects, patients will receive ketone test strips together with each FMD kit to self-monitor urinary ketone bodies.

4.3. Arm 3 (control group)

Participants in the arm 3 will undergo the same testing measures as the intervention groups and will be recommended to continue their usual diet. Participants belonging to this group will be given an opportunity to follow a 6-month LD program at month 6 with two additional (optional) visits at month 9 (visit 4) and at month 12 (visit 5).

4.4. *Potential Risks*

There is a possibility, however, that a subject could be sensitive to food ingredients in one or more of the food formulas and have an allergic or intolerance reaction. Potentially serious reactions may include face and

throat swelling, trouble breathing, anaphylaxis (shock), convulsions, and death. Such reactions are possible if a subject has an intolerance or allergy (known or unknown) to the ingredients in the FMD. Longevity diet will be based on a selection of the traditional food of Southern Italy and thus the possibility of allergic reactions is very low.

4.5. Potential Benefits

The FMD is designed to achieve the beneficial effects of fasting while providing micronutrient nourishment (vitamins, minerals and others) of which the body is deprived during fasting. It minimizes the psychological burden of pure fasting. The Longevity diet has the advantage of being easy to follow as a lifestyle element, with appropriate foods being available in multiple venues. The subjects will receive extensive medical testing and these results will be available to the subjects after completion of their participation for their ongoing care.

4.6. *Assessment of Subject Compliance with Study Product*

Compliance will be measured using both a subject calendar provided by the investigator and direct verification of consumption of the FMD and adherence to the Longevity diet. Subjects will not be asked to return FMD boxes.

5. Description of Specific Testing Modalities

5.1. Hematological analyses

Complete metabolic and lipid panels (overnight fasting) will be completed at the Istituto Clinico "Prof. Dr. R. De Blasi" srl, Via Torrione Prol.to n. 55, in Reggio Calabria and analyzed immediately after the blood draw of each visit. Clinical and biochemical evaluations include lipid profile (total cholesterol, LDL-cholesterol, HDL-cholesterol and triglycerides), serum/plasma, albumin, alkaline phosphatase, insulin-like growth factor 1 (IGF-1), Insulin-like growth factor-binding protein 1 (IGFBP1), insulin, C-reactive protein (CRP), aspartate aminotransferase (AST), alanine aminotransferase (ALT), homocysteine, glucose, glycosylated hemoglobin (HbA1c).

5.2. Blood collection and storage

Blood samples will be collected and stored for future biomarker analyses. In particular, two 6.0 ml ethylenediaminetetraacetic acid tube of whole blood samples will be collected for each participant. One tube will be spun at 1300 g for 20 minutes at room temperature to generate supernatant (plasma), buffy coat and red blood cells which will be individually aliquoted into 1 ml, polypropylene, cryovials and stored at -80 °C for later analysis. The other 6.0 ml tube of whole blood will individually aliquoted into 1 ml and stored at -80 °C for later analysis as previously mentioned (see Table 1 and Figure 1).

5.3. Blood Pressure Measurements

Subjects will not consume any caffeine or alcohol or use tobacco products within 6 hours of the blood pressure measurement. Subjects will have their blood pressure measured using a mercury

sphygmomanometer on each visit. Subject's blood pressure will be measured in the left arm sitting position three times at 2-minute intervals following the American Heart Association (AHA) guidelines for blood pressure measurement. Heart rate will be measured with each blood pressure measurement.

5.4. *Body Impedance Analysis*

The BIA technique is based on the fact that lean tissues have a high water and electrolyte content, and thus provide a good electrical pathway. Fat contains a lower percentage of body water, and thus is a poor conductor of the electrical signal. Utilizing a low energy, high frequency, electrical signal (50 kHz, 500 micro amp), a measurement of the baseline resistance and reactance to the flow of electrical current can be made. The measurement relates directly to the volume of the conductor, which is used to determine total body water, lean body mass, and finally, fat mass and percent body fat measurement on the Tanita® scale.

5.5. Telomere length

Telomere length will be measured in genomic DNA extracted from stored buffy coat samples by quantitative polymerase chain reaction (qPCR) using a method adapted from the one originally described by Cawthon (2009). Telomere length will be performed on the blood samples collected before the dietary intervention and at the end of the two treatments (visit 2, month 6)(see Table 1). Telomere length measurements will be carried out at the laboratory of Genetics of Department of Biology, Ecology and Earth Sciences, University of Calabria.

5.6. Clinical and Research Laboratory Evaluations and Specimen Collection

5.6.1. Biohazard Containment

As the transmission of HIV and other blood-borne pathogens can occur through contact with contaminated needles, blood, and blood products; appropriate blood and secretion precautions will be employed by all personnel in the drawing of blood and shipping and handling of all specimens for this study, as currently recommended by the Centers for Disease Control and Prevention and the National Institutes of Health.

6. **Safety**

6.1. *Definition of an Adverse Event (AE)*

An AE is any unanticipated or unintended medical occurrence or worsening of a sign or symptom (including an abnormal laboratory finding) or disease in a study subject, including those events which do not necessarily have a causal relationship with the study condition, procedures or Study Product(s), that occurs after the informed consent is obtained.

Pre-existing conditions or illnesses which are expected to exacerbate or worsen are not considered adverse events and will be accounted for in the subject's medical history.

6.2. *Definition of a Serious Adverse Event (SAE)*

A SAE is defined as an AE meeting one of the following outcomes:

- Death during the period of protocol defined surveillance.

- Life Threatening Event (defined as a subject at immediate risk of death at the time of the event).
- Inpatient hospitalization or prolongation of existing hospitalization during the period of protocol defined surveillance.
- A congenital anomaly or birth defect
- A persistent or significant disability/incapacity.
- Any other important medical event that may not result in one of the above outcomes, may be considered a serious adverse event when, based upon appropriate medical judgment, the event may jeopardize the subject and may require medical or surgical intervention to prevent one of the outcomes listed above. Examples of such medical events include allergic bronchospasm requiring intensive treatment in an emergency room or at home, blood dyscrasias or convulsions that do not result in inpatient hospitalization, or the development of drug dependency or drug abuse.

6.3. *Assessing, Recording, Analyzing, and Managing Safety Parameters*

6.3.1.*Methods and Timing for Assessment*

Adverse or clinical events will be assessed at each clinical visit. Each visit is conducted by a clinician. All reported events will be recorded in the subject's medical record.

6.3.2.*AE Severity — Grading Scale*

Each adverse event will be graded for severity. All laboratory and clinical AEs that occur in a subject, will be assessed for severity and classified into one the categories below following a standard criterion of Mild, Moderate or Severe.

- Mild Adverse Event — event requires minimal or no treatment and does not interfere with the subject's daily activities.
- Moderate Adverse Event — event results in a low level of inconvenience or concern with the therapeutic measures. Moderate events may cause some interference with functioning.
- Severe Adverse Event — event interrupts a subject's usual daily activity and may require systemic drug therapy or other treatment. Severe events are usually incapacitating.

6.3.3.*AE/SAE Causality — Relatedness Scale*

For all collected AEs, the clinician who examines and evaluates the subject will determine the adverse event's causality based on temporal relationship and his/her clinical judgment. The degree of certainty about causality will be graded using the categories below:

- Definitely Related: There is clear evidence to suggest a causal relationship, and other possible contributing factors can be ruled out. The clinical event, including an abnormal laboratory test result, occurs in a plausible time relationship to study product administration and cannot be explained by concurrent disease or other drugs or chemicals. The response to withdrawal of the product (de-challenge) should be clinically plausible. The event must be pharmacologically or phenomenologically definitive, with use of a satisfactory re-challenge procedure if necessary.
- Possibly Related: there is some evidence to suggest a causal relationship (e.g., the event occurred within a reasonable time after administration of the study product). However, the influence of other factors may have contributed to the event (e.g., the subject's clinical condition, other concomitant events). Although an adverse event may rate only as "possibly related" soon after discovery, it can be flagged as requiring more information and later be upgraded to as appropriate.

- Unlikely: a clinical event, including an abnormal laboratory test result, whose temporal relationship to the study product makes a causal relationship improbable (e.g., the event did not occur within a reasonable time after administration of the study product) and in which other drugs or chemicals or underlying diseases provide plausible explanations (e.g., the subject's clinical condition, other concomitant treatments).
- Unrelated/None: the AE is completely independent of study product administration, and/or evidence exists that the event is definitely related to another etiology. There must be an alternative, definitive etiology documented by the clinician.
- Expected Events Related to Disease Process: expectedness refers to the awareness of adverse events previously observed, not on the basis of what might be anticipated from the pharmacological properties of the Study Product.

6.3.4.*Recording/Documentation*

At each contact with the subject, information regarding adverse events will be elicited by appropriate questioning and examinations and will be immediately recorded on a source document. Source documents will include: progress notes, laboratory reports, consult notes, phone call summaries, survey tools and data collection tools. Source documents will be reviewed in a timely manner by the research team. All reportable adverse events that are identified will be recorded on an appropriate case report form (CRF) if applicable. The start date, the stop date, the severity of each reportable event, and the Pl's judgment of the AEs relationship to the Study Product/intervention will also be recorded in the Subject's progress notes or on the CRF if applicable.

6.3.5.*Specific Serious Adverse Event Requirements*

SAEs will be handled according to good clinical practices associated with Serious Adverse Events. SAEs will be recorded in the study's SAE form and clinician progress notes, and require expeditious handling and reporting to the Sponsor.

Follow-up information which becomes available as the SAE evolves, as well as supporting documentation, will be collected subsequently and reported to the Sponsor. The PI will report SAE events to the IRB if applicable, in compliance with IRB requirements.

6.3.6.*Reporting of Pregnancy*

Should a woman become pregnant or suspects she is pregnant while participating in this study, she is instructed to inform study staff and her primary care provider immediately.

6.4. *Type and Duration of the Follow-up of Subjects after Adverse Events*

6.4.1.*Monitoring of Subjects*

At each contact with the subject, the investigator must seek information on adverse events by specific questioning and, as appropriate, by examination.

AEs may be observed by the Investigator and/or study staff, elicited from the subject and/or family member, or volunteered by the study subject. Adverse events that had previously been reported by study subject will

also be reassessed for duration, intensity and possible reoccurrence. Assessment of safety will include clinical observation and monitoring of hematological, chemical, and immunologic parameters.

Any AE that occurs between the times a study Subject signs the informed consent form and the time s/he departs the study at the end of the final visit (or at the time of early discontinuation of the subject from the study for any reason) will be captured and recorded.

Due to the nature and composition of the study product, no delayed toxicities or withdrawal effects are expected after a subject has discontinued participation in the study. Therefore, no collection of safety information will be done after subject's discontinuation from the study granted that the subject does not have any unresolved AEs.

6.4.2.*Follow-up of Subjects after Adverse Events*

All SAES and non-serious AEs reported in this study will be followed until resolution or until the investigator and the clinical/medical monitor are in agreement that the AE/SAE has stabilized and no more follow-up is required. This requirement indicates that follow-up may be required for some events after the patient discontinues participation from the study.

6.4.3.*Modification of Study Product(s)/Intervention(s) for a Subject*

No food substitutes are permitted. A reduction of food products, a hold of food or discontinuation of food product is allowed at the discretion of the study clinician.

6.4.4.*Halting Rules for the Protocol*

The PI will closely monitor and analyze study data as it becomes available and will make determinations regarding the presence and grading of adverse events. Evaluation of adverse events will be analyzed for the study products and with regard to the known complications associated with administration. The study will be halted (no new enrollments and no further administration of product) by the investigators and a report will be submitted to the IRB (if applicable) if a safety issue is identified.

6.4.5.*Stopping Rules for an Individual Subject*

A study subject will be discontinued from further Study Product(s) administration for:

a significant reduction of BMI greater than 15% from baseline measurement and in any case of a BMI measurement lower than 22 Kg/m^2 for women and 23 Kg/m^2 for men during the follow-up visits; any clinical adverse event, laboratory abnormality, concurrent illness, other medical condition.

6.4.6.*Premature Withdrawal of a Subject*

A subject may decide to withdraw informed consent for any reason. Premature discontinuation by a subject will be evaluated to assess the status of the subject at termination, and will be recorded in the subject's medical record and in the CRF.

6.4.7. Replacement of a Subject Who Discontinues Study Treatment

Subjects may be replaced at the PI's discretion.

6.5. *Research Use of Stored Human Samples, Specimens or Data*

6.5.1.*Use of Stored Data*

Any other research or experimental treatments will be done under additional protocols for which separate signed informed consent documents will be obtained.

6.5.2.*Disposition of Stored Samples and Data*

Data will be stored using codes assigned by the investigators. Data will be kept in password-protected computers. Only authorized members of the study team will have access to the samples and data.

6.6. *Assent or Informed Consent Process in Case of a Minor*

Minors are excluded from this study.

6.7. *Subject Confidentiality*

The investigator will ensure that the subject's anonymity is maintained. Subjects will not be identified in any publicly released reports of this study. All records will be kept confidential to the extent provided by federal, state and local law. The study monitors and other authorized representatives of the Sponsor may inspect all documents and records required to be maintained by the Investigator, including but not limited to, medical records.

The investigator will inform the subjects that the above-named representatives will review their study-related records without violating the confidentiality of the subjects. All laboratory specimens, evaluation forms, reports, and other records that leave the site will be identified only by a coded number in order to maintain subject confidentiality. All records will be kept locked and all computer entry and networking programs will be done with coded numbers only. Clinical information will not be released without written permission of the subject, except as necessary for monitoring by the FDA, the Sponsor, or the sponsor's designee.

6.8. *Study Discontinuation*

The study will be discontinued for the following reasons:

- any clinical adverse event or serious adverse event, laboratory abnormality or inter-current illness which, in the opinion of the PI, indicates the continued treatment with study therapy is not in the best interest of the subjects.
- Termination of the study by the Sponsor.

7. **Statistical Analysis plan**

7.1. *Study Randomization*

This is a randomized, open-label three-arm exploratory study. After the screening visit, participants will be randomized through a pre-stratified randomization method to achieve balance between the three groups

(interventions and control group) according to baseline characteristics (covariates) that include gender, age (30-50; 51-65), and BMI (25-29,9; 30 and over) obtaining 8 strata. The block-based randomization will be then performed separately within each layer using a web-based randomisation system (https://www.project-redcap.org/) that will be controlled by the researchers not involved in the study from the Valter Longo foundation in Milan.

7.2. *Study Records Retention*

The PI will retain investigational product disposition records, electronic database files, and source documents for the maximum period required by applicable regulations and guidelines, or as specified by the Sponsor, whichever is longer. Records with individual patient identifiers will be maintained for 10 years only. After this date, all records will be identified by subject number only with no connection to individual patients. The Sponsor will inform the PI when records are no longer needed.

7.3. *Clinical Monitoring Plan*

Representatives of Sponsor will be allowed to visit the study site periodically to assess the data, quality and study integrity. This will include: reviewing study records, compare records with source documents, discussing conduct of study with the PI and verifying the conditions of the facility. In addition, the study may be evaluated by Sponsor's auditors and government inspectors. They will be allowed access to source documents, electronic database, and other study files.

7.4. *Sample Size Consideration*

Sample size determination was based on the power to detect a significant effect of FMD nutritional intervention on fat mass during the treatment period (6 months) with respect to the control group. The primary hypothesis of this trial can be formally stated as a test of whether there is a time × intervention interaction. In particular, assuming a low effect size of the FMD intervention (Cohen's F = 0.10) and a correlation coefficient among the repeated measures equals to 0.5, a sample size of 107 participants per group, would give a power of at least 0.9 for testing the hypothesis of whether there is a time × intervention interaction at a significance level of 0.05. With an estimated dropout rate of 30%, at least 153 participants would need to be recruited in each arm. For this reason, we will enrol 501 participants that will be randomly assigned to each of the three arms (167 for each arm).

7.5. *Source Documents and Access to Source Data/Documents*

Study data will be collected on CRFs designed for the study if required by the Pl. The PI is responsible for assuring that the data collected is complete, accurate, and recorded in a timely manner. Source documentation (the point of initial recording of a piece of data) should support the data collected on the case report form. The CRFs will be signed and dated by the person recording and/or reviewing the data. Source documents include all recordings of observations or notations of clinical activities and all reports and records necessary for the evaluation and reconstruction of the clinical trial. Data for CRFs will be collected during patient visits, phone calls with subjects and health care providers, and subject diaries (if applicable). The CRF form may act as the source document for the following study procedures: Subject Scheduling, Vital Signs, Clinical Assessments (adverse event and medication logs). It is not acceptable for the CRF to be the only record of a patient's participation in the study. This is to ensure that anyone who would access the patient medical record has adequate knowledge that the patient is participating in a event based upon the

2016 American Heart Association and American College of Cardiology recommendations, compliance and age are planned. Additional subgroup analyses may be performed.

7.6. *Safety Review*

A review of the collected safety data will be ongoing by the study coordinator. These reports will be reviewed by the PI.

7.7. *Study conclusions*

Collected data from subjects will be reviewed to demonstrate that favorable changes are noted in biomarkers of body composition and age-related biomarkers with administration of the proprietary food products. Data will be evaluated for change from baseline over time within arms and for differences between the intervention arm and the control arm.

7.7.1.*Analysis supporting primary objectives*

Generalized mixed linear models will be used to compare arm 1 (FMD) to the arm 3 (control group) sitting fat mass expressed in terms of change from baseline to the end of the 3 FMD cycles (month 6, visit 3). The analysis will include the fixed categorical effect of treatment, a fixed term modelling changes between baseline and other time periods, as well as a random term accounting for repeated measurements on the same patients. The same analyses will be used to compare arm 2 to the arm 3, sitting fat mass expressed in terms of change from baseline to the end of the study (month 6).

7.7.2.*Analysis supporting secondary objectives*

Generalized mixed linear models will be used as previously described to compare arm 1 and arm 2 vs arm 3, sitting the following parameters expressed in terms of change from baseline to the end of the study (month 6): inflammatory biomarkers (CRP, etc), IGF-1, blood pressure, serum lipids, glucose, CV biomarkers and, body weight, BMI, body composition, insulin resistance, aging biomarkers, sleep quality and duration, and number of participants who receive antihypertensive/hypoglycaemic medications.

We will also evaluate the effect of FMD and FMD+LD treatments on risk factors for CVD and metabolic syndrome, defined as three of five of the following conditions: abdominal obesity, elevated fasting glucose, elevated blood pressure, high serum triglycerides, and low HDL cholesterol.

We will select clinically relevant cutoffs and compared normal and at-risk subjects for each risk factor: total cholesterol >199 mg/dl and LDL cholesterol levels >130 mg/dl are associated with an increased risk for CVD, a fasting glucose >99 mg/dl indicates impaired fasting glucose/prediabetes, and triglyceride levels >100 mg/dl as well as CRP >1 mg/liter are associated with increased risk for CVD. For serum IGF-1, no clinically relevant risk level has been established, but a number of epidemiological studies have associated IGF-1 levels above 200 ng/ml with various cancers (Levine et al., 2014; Pollack et al., 2007). We will therefore compare the effect of FMD/FMD + LD cycles on subjects in the highest quartile of IGF-1 expression (>225 ng/ml) with that on subjects with IGF-1 levels ≤225 ng/ml. The effect of FMD and FMD+LD treatments on these risk factors will be assessed also stratifying our sample by baseline BMI, SBP and DBP, fasting glucose, cholesterol, CRP and IGF-

7.7.3.*Safety and Tolerability/Acceptability Evaluation*

Collected data from subjects will be reviewed for occurrence of AEs and for data concerning hedonics, tolerability and acceptability of products. CMP and CBC data will be tallied and evaluated. This information will be considered in drafting any product instructions and warnings.

7.7.4.*Subgroup analyses*

To explore the effect of treatment in subgroups, the estimated effect of the nutritional interventions will be estimated for each of the subgroups listed below will be derived. No adjustment for multiple comparisons will be made. Additionally, the frequency and percentage of subjects reaching the primary endpoint will be presented by treatment group for each of the subgroups based on the baseline measurements: baseline fat mass (≥ 20% and ≥25% for men; ≥ 30% and ≥ 35% for women), age groups (30-50, 51-65 years), gender, baseline BMI (25-29.9; 30 and over);

8. **Ethical Considerations**

8.1. *Declaration of Helsinki*

The investigator will ensure that this study is conducted in full conformity with the current revision of the Declaration of Helsinki, or with the International Conference for Harmonization Good Clinical Practice regulations and guidelines, whichever affords the greater protection to the subject.

8.2. *Informed Consent Process*

Informed consent is a process that is initiated prior to the individual agreeing to screen for and participate in the study and continuing throughout the individual's study participation. Extensive discussion of risks and possible benefits of this therapy will be provided to the subjects. Consent forms describing in detail the Study Product, the study procedures and associated risks will be given to the Subject and written documentation of informed consent is required prior to screening for the study and if qualified, prior to starting the study. The subject will be asked to read and review the document. Upon reviewing the document, the investigator or designee will explain the research study to the Subject and answer any questions that may arise. The subjects will sign the informed consent document prior to any procedures being done specifically for the screening process and if qualified, for the study. The subjects will have sufficient opportunity to discuss the study and process the information in the consent process prior to agreeing to participate. The subjects may withdraw consent at any time throughout the course of the trial. A copy of all informed consent documents will be given to the subjects for their records.

The acquisition of informed consent will be documented in the subject's medical records. The informed consent form will be signed and personally dated by the subject and the person who conducted the informed consent discussion. The original signed informed consent form will be retained in the medical chart and a copy will be provided to the subject.

The study poses minimal risk for the participants. The anonymized information collected in the study will be used for research and publication purposes, and always safeguarding the right of privacy and anonymity according to the rules of General Data Protection Regulation (GDPR) of the European Union (UE) 2016/679.

This study will be initiated only after all required legal documentation has been reviewed and approved by the University of Calabria Ethic Committee, according to the national and international regulations. The same applies for the implementations of changes introduced by amendments.

Individual patient medical information obtained as a result of this study is considered confidential and disclosure to third parties is prohibited with the exception noted below. Patient confidentiality will be ensured by using patient identification code number.

The biological samples will be stored for 15 years under liquid nitrogen at the Laboratory of Genetics of the University of Calabria and thereafter destroyed. They will undergo a coding process with measures taken to ensure that specimens are kept under correct conditions always when it is stored. Each sample will be assigned to an identification code, which will be used by researchers and will prevent third parties from identifying the study participants from their samples. Encryption and access passwords will be adopted to protect and safeguard the stored samples.

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