TEMPUS

Study Protocol

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1. Background and rationale

Obesity prevalence has steadily increased up to reach epidemic proportions and affecting around 603.7 million adults worldwide (1). The excess of triglycerides in the body is usually stored, apart from the subcutaneous adipose tissue (SAT), in other organs and tissues that are not otherwise designed for adipose storage (2). This process is known as ectopic fat deposition and may include organs and tissues such as the liver, pancreas, or skeletal muscle. Excessive accumulation of triglycerides in hepatocytes results in hepatic steatosis, a condition considered one of the diagnostic criteria (along with other metabolic dysregulatory factors) for metabolic dysfunction-associated steatotic liver disease (MASLD) (3,4). MASLD (which replaces non-alcoholic fatty liver disease - NAFLD) is a major public health problem considering its elevated prevalence (nearly 90% of adults with overweight/obesity) and its strong association with extrahepatic diseases (3,5). Therefore, implementing strategies to reduce hepatic steatosis in individuals with obesity may be a potential approach to mitigate/reduce the risk of liver dysfunction and cardiometabolic diseases (6).

Traditionally, low-calorie diets have been shown to be an effective strategy to reduce body weight and hepatic steatosis and, in turn, improve cardiometabolic health (7). However, energy-restricted approaches are still not a standard public health strategy due to their lack of long-term sustainability and some undesirable metabolic adaptations that certainly lead to weight regain even in highly motivated patients (8). Time-restricted eating (TRE) is a recently emerged intermittent fasting approach which has the potential to maximize the extensively reported beneficial metabolic effects of the energy intake restriction (9). TRE aims to maintain a consistent daily cycle of feeding (within a limited time window during ≤10 hours) and fasting (≥14 hours) to support healthy/consistent circadian rhythms (10). Irregular eating patterns and eating over an extended period of time may disrupt circadian rhythms and, thus, increase the risk of obesity and hepatic fat accumulation (11). Remarkably, recent studies in mice have concluded that TRE effectively reduces hepatic steatosis and improves cardiometabolic health (12), mainly through improved insulin sensitivity; yet, whether this strategy is similarly effective in humans remains still unclear.

Along with nutritional strategies, exercise has demonstrated its efficacy at reducing hepatic steatosis and at improving cardiometabolic health in humans (13–15). Furthermore, preliminary evidence has highlighted that the combination of TRE and exercise may normalize glucose homeostasis and improve lipid profile in women with overweight or obesity (16). Nevertheless, the differential effects of TRE combined with exercise and TRE or exercise alone on hepatic steatosis and cardiometabolic markers remain unknown.

Although promising, most preliminary pilot trials examining the effects of TRE combined with exercise in humans have important limitations: (i) the duration is shorter than 3 months (16–19) which may be insufficient to induce substantial changes in cardiometabolic health (20); (ii) the outcomes assessed were either body mass index (BMI) as a surrogate marker of obesity or bioelectrical impedance analysis which are not able to accurately assess hepatic or other ectopic fat depots (18); (iii) the majority of clinical trials have been focused solely on men (17–19), or have included an unevenness sample of men and women which limits the possibility to understand the important sex dimorphism in MASLD development; (iv) the published studies are limited to trained individuals (17–19) and have small sample size and, thus, have limited statistical power; (v) the previous work do not include follow-up of the participants to understand the maintenance of intervention effects; and, more importantly, (vi) have not studied or reported potential mechanisms through which TRE combined with exercise may result in health benefits. Therefore, as all these shortcomings limit generality and preclude to establish firm conclusions, a new approach is required to successfully translate findings into the community and clinical setting.

2. Aims

The overall aim of the TEMPUS randomized controlled trial is to investigate the effects of a 12-week TRE combined with a supervised exercise intervention, as compared with TRE or supervised exercise alone, and a usual care control group (UC), on hepatic fat (primary outcome) and cardiometabolic health (secondary outcomes) in men and women with obesity.

3. Methods/Design

3.1. Study design

The TEMPUS study is a randomized controlled trial (ClinicalTrials.gov under the identifier NCT05897073) with a four-arm parallel design. The protocol of the randomized trial has been written and reported based on the Recommendations for Interventional Trials (SPIRIT) guidelines (21). Later, the results will be reported following the CONSORT guidelines (REF). Consented participants will be randomly assigned to one of the four groups: UC, TRE alone (TRE), supervised exercise alone (Exercise), or TRE combined with supervised exercise (TRE + Exercise) group. The present study will focus on the recruitment of adults with obesity in Granada, a region located in southern Spain. The patient flow diagram from recruitment to randomization phases is shown in **Figure 1**.

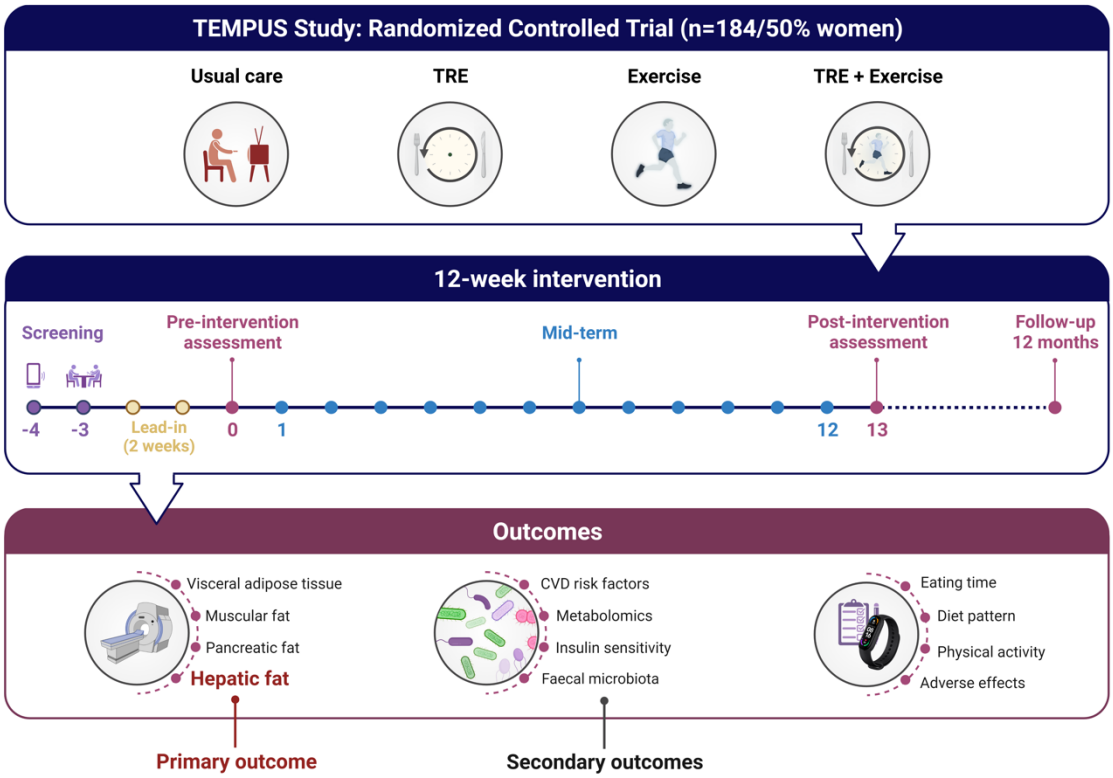

Figure 1. TEMPUS project design. TRE, time-restricted eating; CVD, cardiovascular disease.

3.2. Participants and eligibility criteria

The study will include both men and women (50%), with a BMI ranging from 30 to <40 kg/m^2, aged between 25 and 65 years, and with a habitual eating window of ≥11 hours. Detailed criteria for inclusion and exclusion can be found in **Table 1**. The screening phase, as shown in **Figure 1**, will involve assessing participants' medical history and vital signs to determine their eligibility for the study. During the physical examination, any existing conditions at the time and any pre-existing medical conditions will be thoroughly documented.

Table 1. Inclusion and exclusion criteria

Inclusion criteria
• Aged 25-65 years. • Body mass index between 30 and <40 kg/m^2. • Weight stability (within 3% of screening weight) for >2 months prior to study entry. • Habitual eating window ≥11 hours.
Exclusion criteria
• History of a major adverse cardiovascular event (e.g., acute myocardial infarction, ischemic or hemorrhagic stroke, or peripheral arterial ischemia, among others), kidney failure, chronic liver disease, or human immunodeficiency virus (HIV) / acquired immunodeficiency syndrome (AIDS). • Active endocrinological disease, innate errors of metabolism, myopathies or epilepsy. • Patients who have undergone bariatric surgery or other surgical techniques used for the treatment of other pathologies (e.g., "Roux-en-Y"). • Rheumatoid arthritis, Parkinson's disease, active cancer treatment in the past year or another medical condition in which fasting is contraindicated. • Use of medications that may affect the results of the study such as drugs for glycaemic control (e.g., antidiabetic, steroids, beta-blockers, antibiotics, prebiotics, probiotics and symbiotics). • Diagnosis of major sleep or eating disorders. • Caregiver for a dependent requiring frequent nocturnal care/sleep interruption or shift workers with nocturnal hours. • Metal or electrical prosthesis. • Foreign bodies in the eyes. • Fear of needles and claustrophobia to magnetic resonance imaging. • Active tobacco or illicit drug use or a history of alcohol abuse treatment (i.e., moderate or severe alcoholism). • Participating in a weight loss or a supervised exercise program (i.e., > 30 min in 3 times per week, or > 45 min in 2 or more times per week at moderate-to-vigorous intensity). • Pregnancy and lactation or planned pregnancy (within the study period). • Frequent travel over time zones during the study period. • Being unable to understand and to accept the instructions or the study objectives and protocol. • Not having or being able to use a smartphone with Apple iOS or Android OS. • Are deemed unsuitable by the investigator for any other reason.

3.3. Recruitment and screening

Recruitment of potential participants will be conducted through (i) newspaper advertisements, (ii) the Endocrinology and Nutrition Department of the San Cecilio' and Virgen de las Nieves' University Hospitals of Granada, and (iii) the community of the University of Granada. A pre-screening process will be implemented with an online form as well as via telephone interviews to assess the eligibility of potential participants and engage them in the study. Subsequently, the medical team will conduct telephone interviews, to review the patients' medical records and evaluate their medical history, verifying compliance with the inclusion and exclusion criteria. Potential participants who meet the criteria during the pre-screening phase will be provided with oral and written information about the study and will be scheduled for the first evaluation visit. During this session, oral and written informed consent will be obtained from potential participants, and measurements of body weight and height will be recorded. Additionally, participants will perform an incremental exercise test on a treadmill, supervised by the sports medicine staff from the *Centro Andaluz de Medicina del Deporte* and Sport and Health University Research Institute, to determine their aptitude for exercising. During the test and at rest, electrocardiogram, blood pressure, and capillary blood lactate measurements will be obtained.

3.4. Outcome measures

A 2-week lead-in period will be implemented prior to pre-intervention assessments and group allocation (see **Figure 1**). During this period, potential participants will be instructed to maintain their usual dietary and physical activity habits. They will be provided with a mobile phone application (Tempus: com.nnbi.app_extreme_granada; NNBi2020 S.L., Navarra, Spain) to record daily information regarding their eating time, sleeping patterns, naps, and any adverse events experienced. Additionally, physical activity and sleep quality will be evaluated using accelerometry, and glucose excursions over day and night will be assessed using continuous glucose monitoring (CGM) devices over the course of 2 weeks. This data collection will be used to verify that the participants' habitual eating windows are ≥11 hours and perform< 30 min in 3 times per week, or < 45 min in 2 or more times per week at moderate-vigorous intensity activity (see inclusion and exclusion criteria, **Table 1**). Pre-intervention measurements, after 12-week intervention measurements (±3 days), and after 12 months post-intervention measurements will be conducted by a dedicated team of trained staff to ensure consistency and reliability. In addition, we will assess anthropometry and body composition at week 6 (mid-term in **Figure 1**). By including these intermediate measurements, we will potentially capture changes over time and evaluate the trajectory of outcomes during the 12-week intervention period. A detailed overview of the study's outcomes is shown in **Table 2**, which provides a comprehensive summary of the variables assessed.

The study primary outcome will be changes from baseline to 12 weeks in hepatic fat. Secondary outcome variables include changes from baseline to 12 weeks in other ectopic fat depots (i.e., abdominal visceral and subcutaneous adipose tissue, pancreatic fat and mid-thigh intermuscular and intramuscular adipose tissue), anthropometry and body composition (i.e., fat mass and fat-free mass, bone mineral density and content), cardiometabolic markers (i.e., glucose, insulin, hemoglobin A1c, lipid profile, vitamin D, alkaline phosphatase and calcium, liver and kidney function markers, estimated glomerular filtration rate and steroid and thyroid hormones), 24h diurnal and nocturnal mean glucose (via CGM), fecal microbiota, sleep and physical activity patterns, psychological outcomes (i.e., depression, stress and anxiety), quality of life, dietary habits and eating behaviour assessment, and fitness (i.e. cardiorespiratory fitness and muscular strength). After the screening examination and the 2-week lead-in period, pre-intervention assessments will be held within 3 face-to-face sessions:

Day 1: participants will perform physical fitness tests and will be required to complete several web-based questionnaires. These questionnaires will gather information on several outcomes such as quality of life, sleeping patterns, psychological status and eating behaviour. Dietary intake will be also assessed by qualified nutritionists where participants will be asked to provide the first 24-hour recall detailing their food consumption.

Day 2: fasting venous blood samples will be collected at the San Cecilio University Hospital (Granada, Spain). Thereafter, anthropometric and body composition measurements will be conducted at the Sport and Health University Research Institute (iMUDS - 300 meters apart). Finally, oral glucose tolerance tests will be also conducted as part of the glycaemic assessment.

Day 3: magnetic resonance imaging (MRI) and elastography will be performed in the San Cecilio University Hospital by the TEMPUS' medical staff (Granada, Spain). To ensure consistency and standardization during the assessment, participants will be instructed to maintain a fasting period of 8-10 hours prior to the appointment, during which they will be advised to consume only water and abstain from solid food or other beverages. In addition, participants will be advised not to consume alcohol or diuretics 24 hours before the test and to avoid stimulants like caffeine for 12 hours before the test.

Table 2. Overview of the study outcomes.

Outcomes	Pre-Intervention	Mid-term	Post-Intervention	12-month follow-up
Primary outcome				
Hepatic fat	✔		✔	✔
Secondary outcomes				
Ectopic fat depots	✔		✔	✔
Anthropometry and body composition	✔	✔	✔	✔
Cardiometabolic markers	✔		✔	✔
Glycaemic control	✔		✔ *	
Fecal microbiota	✔		✔ *	
Plasma and fecal metabolomics	✔		✔	
Sleep and physical activity	✔		✔ *	✔
Psychosocial	✔		✔	
Quality of life	✔		✔	✔
Eating behavior and dietary habits	✔		✔	✔
Fitness	✔		✔	

*Assessment will be conducted during the last 2 weeks of the intervention.

3.5. Primary outcome: hepatic fat

The quantification of hepatic fat and iron content will be performed using MRI with a Siemens 3T Magnetom Vida scanner located at San Cecilio University Hospital in Granada. Additionally, liver steatosis, viscosity and fibrosis severity will be assessed using attenuation imaging coefficient, shear wave elastography and shear wave dispersion with a Canon Aplio i800 (located at San Cecilio University Hospital in Granada). The Fibrosis-4 (FIB-4) index will be calculated as an indicator of liver fibrosis severity (22).

3.6. Secondary outcomes

Ectopic fat depots: abdominal subcutaneous, visceral and intermuscular adipose tissue, as well as pancreatic fat content, will be obtained using MRI. To assess subcutaneous, visceral and intermuscular adipose tissue in all 3D abdominal volume (i.e., volume, cross-sectional area at selected levels, and mean/median fat fraction), a semiautomatic software program will be employed for tissue segmentation. These valuable image markers will be derived from a standard 6 echo Dixon series, ensuring accurate characterization of abdominal adipose tissue distribution and composition. We will also measure image markers from mid-thigh using a 6 echoes Dixon series, and will obtain cross-sectional area, muscular tissue, subcutaneous adipose tissue, intramuscular adipose tissue, intermuscular adipose tissue, fat fraction, and bone marrow fat fraction. The segmentation for all these structures will be performed with a semiautomatic proprietary algorithm.

Anthropometry and body composition: body weight and height measurements will be obtained using a stadiometer and scale (Seca model 799, Electronic Column Scale, Hamburg, Germany). Participants will be instructed to be barefoot and wear lightweight clothing during these measurements. Neck, waist, and hip circumferences, as well as calf girth, will be determined following the procedures outlined by the International Society for the Advancement of Kinanthropometry (ISAK) by certified personnel (ID's ISAK: #637686045477240182 and #638227824311214767) (23). Furthermore, fat mass, fat free mass, and overall adipose mass will be assessed by bioelectrical bioimpedance (Tanita MC 980-MA Plus, Tanita Corporation, Tokyo, Japan), with measurements taken after voiding the bladder to ensure accurate readings. Bone mineral density and content will be assessed by dual-energy X-ray absorptiometry scans (Discovery Wi, Hologic, Inc., Bedford, MA, USA). Participants will be instructed to maintain a fasting period of 8-10 hours prior to the appointment, during which they will be advised to consume only water and abstain from solid food or other beverages. In addition, participants will be advised to abstain from alcohol or diuretics 24 hours before the test and stimulants like caffeine for 12 hours before the test, and avoid engaging in moderate exercise or physical activity for 24 hours, or vigorous exercise for 48 hours, prior to the test.

Cardiometabolic risk markers*:* venous blood samples will be carefully preserved at a temperature of -80°C to ensure their integrity for subsequent analysis. We will collect a full set of cardiometabolic risk markers, as these parameters may inform about the metabolic dysregulation in liver disease (24). These set will include: fasting glucose (Alinity C system analyzer, Abbott Laboratories, IL, USA), insulin (UniCel DxI 800 access immunoassay system, Beckman Coulter Inc., CA, USA), hemoglobin A1c (automated glycohemoglobin G11 analyser, Horiba) and lipid profile (i.e., total cholesterol, high-density lipoprotein cholesterol, triglycerides, apolipoprotein A1 and B) using Alinity C system analyzer (Abbott Laboratories, IL, USA). Low-density lipoprotein cholesterol will be calculated using a previously validated equation (LDL-c = CT – HDL-c – (TG/5)) (25). Furthermore, we will measure vitamin D, alkaline phosphatase and calcium (Alinity C system analyzer, Abbott Laboratories, IL, USA). Liver and kidney function markers (i.e., alanine transaminase, gamma-glutamyl transferase, bilirubin, creatinine) will be also measured using an Alinity C system analyzer, while estimated glomerular filtration rate, steroid hormones (i.e., estradiol, progesterone, testosterone, follicle stimulating hormone and luteinizing) and thyroid hormones (i.e., thyrotropin, thyroxine, triiodothyronine) will be also assessed using a UniCel DxI 800 access immunoassay system (Beckman Coulter Inc., CA, USA). Blood count and biochemistry and inflammatory markers (i.e., iron, ferritin, folic acid, C-reactive protein and interleukin-6) will be measured using a AU5800 automated analyzer (Beckman Coulter Inc., CA, USA). Additionally, we will calculate insulin resistance surrogates, such as the homeostatic model of assessment for insulin resistance (HOMA-IR) and the quantitative insulin-sensitivity check index (QUICKI). Finally, we will also

conduct metabolomics analysis on plasma samples. The omics analyses will allow the identification of: (i) metabolites strongly associated to hepatic fat content (baseline – cross-sectional analysis); (ii) metabolites predictors of hepatic fat content changes after the intervention. In addition, systolic and diastolic blood pressure will be measured using an automated monitor (M3-Comfort, Omron Healthcare Europe B.V., Hoofddorp, The Netherlands), following the established guidelines outlined by the 2021 European Society of Hypertension (26).

Glycaemic control: due to the crucial role of insulin resistance in MASLD development, through fatty acid accumulation in hepatocytes, CGM and analysis of acute response to glucose will be conducted. Participants will be instructed to wear a CGM device (FreeStyle LibrePro, Abbott Laboratories, Abbott Park, IL) during 2 weeks before the intervention (lead-in period) and during the last 2 weeks of the intervention (weeks 11 and 12). The CGM data obtained will be used to calculate different variables related to glycaemic control (i.e., 24-hour mean glucose), following the guidelines outlined in the most recent international consensus statement (27). Additionally, oral glucose tolerance tests will also be conducted as part of the glycaemic assessment using a 75-g oral glucose dose (NUTER TEC: orange flavour, Toulouse, France).

Fecal microbiota*:* for the comprehensive identification and quantification of fecal microbiome diversity and composition, pre-intervention and during the last 2 weeks of the intervention, fecal samples will be collected. Stool microbial DNA will be isolated from participants and subsequently, 16S rRNA gene amplicon sequencing, with the possibility of employing shotgun methodology pending final budget considerations, will be performed. Furthermore, a fecal metabolomic fingerprint analysis will be conducted to determine the metabolic profile among different groups of patients.

Sleep and physical activity*:* sleep quality and chronotype will be evaluated through the administration of validated questionnaires, including the Pittsburgh Sleep Quality Index (PSQI) (28), Munich Chronotype Questionnaire (MCTQ) (29), and Horne and Östberg Questionnaire for Morning-Evening type assessment (MEQ-SA) (30). Objective measures of sleep and physical activity levels will be obtained using triaxial accelerometer (ActiGraph GT3X+, Pensacola, FL, USA) that participants will wear on their non-dominant wrist during two-week periods: before the beginning of the intervention (lead-in period) and during the last two weeks of the intervention (weeks 11 and 12).

Psychosocial assessment*:* as weight loss intervention may improve emotional well-being and psychosocial functioning, several key psychological dimensions will be evaluated through the administration of validated questionnaires: the Beck Depression Inventory Fast Screen for depression (BDI-FS) (31), the Perceived Stress Scale for stress (PSS) (32), and the State-Trait Anxiety Inventory for anxiety (STAI) (33).

Quality of life: quality of life may also improve from weight loss intervention (34) and will be evaluated using the EuroQol 5 dimensions 5 levels (EQ-5D-5L) questionnaire (35) and Rand Short Form 36 (SF-36) (36). In addition, participants will be asked to complete an adverse events questionnaire to identify any potential adverse effects or complications experienced during the intervention.

Eating behavior and dietary habits*:* to assess participants' adherence to the Mediterranean dietary pattern, validated questionnaires such as the PREDIMED questionnaire will be administered. This questionnaire provides a reliable measure of adherence to the specific dietary components and guidelines of the Mediterranean diet (37). Food Frequency Questionnaire (FFQ) (38) will be administered to assess the frequency that participants have consumed each specific

food during the previous 4 weeks. Food Craving Inventory (FCI) (39) and the Adult Eating Behavior Questionnaire (AEBQ) for appetite traits (40) will be also administered.

Physical fitness: an incremental treadmill exercise test until exhaustion will be performed to determine cardiorespiratory fitness. The modified Balke protocol (41) will be applied, which has been extensively used and validated (42–44). The exercise electrocardiogram and heart rate (HR) will be monitored continuously and reviewed by a cardiologist. Furthermore, capillary blood lactate will be measured at rest and along different stages of the test (Lactate Pro™ 2 LT-1730, Arkray, Kyoto, Japan). Supervised exercise proposed in the present study could restore or mitigate adverse effects of diet-induced weight loss on muscle strength (45). Thus, upper muscular strength will be assessed through hand grip strength test (46) using a digital hand dynamometer (TKK 5401 Grip-D; Takei, Tokyo, Japan), whereas lower body muscular strength will be assessed through the 30-s sit-to-stand muscle power test (47), and walking speed to assess functional capacity with gait speed test (48).

Confounding: as unintentional reductions in energy intake (10-30% or ~300-500 kcal/day) have been reported when participants confine their eating windows to 4-10 h/day (49,50), we will control and analyze changes in energy intake over the intervention period. Participants will undergo dietary assessments through the completion of 3 non-consecutive 24-hour dietary recalls (2 working days and 1 non-working day) (51). These recalls will be conducted via face-to-face or telephone interviews by qualified and trained research nutritionists. Total energy intake, carbohydrates, fat and protein intake will be calculated. In addition, to understand the important sex dimorphism in MASLD development, at baseline and after the intervention, specific reproductive-profile questions will be asked regarding detailed information on menstrual cycle history and hormonal contraceptive use and type, as well as regarding any gynecological condition. This will help in categorizing participants into different hormonal profiles, following the recent consensus (52–54).

3.7. Intervention description

3.7.1. Usual care group

Participants randomly assigned to the UC group will be indicated to continue with their dietary eating time schedule. All participants will receive monthly in-person nutritional education sessions, lasting approximately 90 minutes, throughout the intervention for weight management and cardiovascular health promotion based on Mediterranean dietary patterns (55) and physical activity recommendations from the World Health Organization (56). Key points in nutritional counseling will be: a) to correctly interpret food labels and to plan grocery, b) to recognize and include high satiety foods, and c) to fight against some extended myths in Spanish culture such as “alcohol in small quantities is healthy”, “it is unhealthy to skip breakfast”, or “it is important to eat 5 meals per day”.

3.7.2. Time-restricted eating intervention

Before the beginning of the intervention, participants allocated to TRE groups will select their preferred 8-hour eating window before the intervention and will be required to maintain the same 8-hour eating window during the 12-week intervention. They will be advised that the last meal should be completed before or at 21:00. Participants assigned to the TRE groups will be strictly prohibited from consuming any calorie-containing food or beverage outside their designated 8-hour eating window. However, they will be allowed to consume water, coffee, and tea without sugar or artificial sweeteners during the fasting period. Instructions for the TRE intervention will emphasize its daily implementation, meaning participants are expected to adhere to the eating window restriction (±30 minutes) throughout all seven days of the week.

3.7.3. Supervised exercise intervention

The objective of the supervised exercise program will be to ensure its transferability and feasibility for the target population. We will follow the Consensus on Exercise Reporting Template to facilitate replicability and transparency (57) and record the timing when participants are training (58). Thus, the physical activity recommendations for adults proposed by the World Health Organization serve as the foundation for determining the specific exercise dosage in the TEMPUS study (59). Given that both resistance and aerobic training modalities have shown improvements in hepatic steatosis among patients with MASLD (60,61), the TEMPUS study will combine both supervised resistance and aerobic high-intensity interval training (HIIT) sessions. Qualified sports scientists from outside the research group will carefully supervise the exercise sessions and work with groups of no more than six people to ensure that participants perform the exercise technique correctly, and at proper intensity. Moreover, participants will receive an individualized moderate-intensity goal-setting aerobic (i.e., walking) program consisting of increasing 10% daily steps per week based on their daily steps logs. Goal-setting aerobic will be updated weekly using a smart band (Xiaomi Mi Band 7, Xiaomi, Pekin, China) that participants enrolled in the exercise groups will wear on their wrists during the intervention period.

Volume: participants will engage in a 12-week intervention program including 2 supervised exercise sessions per week ranging from 60 to 90 minutes per session. Furthermore, participants will receive instructions to complete their personalized daily step goals established for each day.

Intensity: supervised sessions will start with a supervised circuit-based resistance training that focuses on upper and lower body exercises that target major muscle groups. To prescribe intensity, the rate of perceived exertion (RPE) scale ranging from 0 to 10 will be utilized, using the evaluations and first sessions to properly train the participants on this scale (62). The target intensity for resistance training will be set at an RPE level greater than 7, which will vary depending on the week number of the intervention (from 7 to 10). The participants will be encouraged to achieve this intensity through all the exercises performed in each lap of the circuit. Moreover, training sessions will include HIIT as the vigorous-intensity aerobic exercise component. HIIT will consist of 3-4 sets of 4-minute intervals at >85% of the individual heart rate reserve (HRR) with 4 minutes of active recovery at 65-75% HRR. HRR will be calculated considering the peak of HR achieved in the incremental treadmill test and the rest HR lying in bed recorded during pre-intervention assessments. HR will be continuously monitored during all exercise sessions using the Polar H10 band (Polar Products Inc., Stow, OH, USA).

Frequency: participants will perform 2 supervised exercise training sessions per week, with a resting period of at least 48 hours between them. Additionally, participants will be instructed to accomplish the prescribed daily step goal. If a participant misses a training session, it will be rescheduled and recovered considering a minimum resting period of 48 hours between sessions.

Type of exercise: the resistance training sessions will be composed of major upper and lower body muscle groups including (63): horizontal/vertical pull exercises (i.e., seated low row and lat pulldown with resistance elastic bands), hip-dominant exercises (i.e., deadlift with resistance elastic bands and weight-bearing glute bridge), knee-dominant exercises (i.e., weight-bearing squats and lunges), and horizontal/vertical push exercises (i.e., weight-bearing push-ups and shoulder press with resistance elastic bands). We will propose 4 levels of exercises' difficulty based on the resistance elastic band and the progressive complexity of the basic movement patterns involved. After the resistance circuit training, HIIT will be preferably performed on a treadmill to ensure a suitable progressive overload. As alternative modality, elliptical ergometer will be used.

Training load variation: we are aware that participants might not be immediately capable of exercising at high intensities and volumes. Therefore, there will be a gradual progression to the assigned exercise dose in 3 phases (see **Figure 2**) and proper technique will be a priority for the difficulty level progression to avoid potential injuries.

Training periodization: the supervised exercise intervention will be divided into 3 phases (**Figure 2**). The initial phase will have a length of 6 weeks and will start with a first week of familiarization. During this week, participants will learn the structure and organization of the sessions and the movement patterns that constitute the base of the different exercises. Considering that an inactive person may be unable to immediately train at the selected doses, the familiarization phase will prepare participants to gradually increase the workload until the required dose is achieved. On the other hand, the resistance training will set light loads using resistance elastic bands or weight-bearing exercises. They will increase the load and coordinative difficulty (through 4 levels for each exercise) as soon as participants perform the exercises with a proper technique.

We expect a parallel increase in the intensity of both resistance training and HIIT as participants' fitness levels improve. Specifically, adjustments (i.e., greater speed or power) to HIIT will be controlled based on the HRR. This approach is essential to maintain a targeted percentage of HRR, particularly as participants exhibit enhanced fitness capacities.

Training session: a training session will be organized as follow: (i) a warm-up of one circuit training lap with light loads using resistance elastic bands or weight-bearing exercises (20 seconds work: 20 seconds rest), (ii) 2 to 4 circuit training laps of eight exercises (30 seconds work: 30 seconds rest) at 7-10 RPE, with a between-set rest of 3-5 min, and (iii) 3 or 4 sets of 4-min intervals at 85-95% HRR interposed by 4 min of active recovery at 65-75% of HRR, with 2 min prior of warm-up. A cool-down protocol of 5 min will be performed at the end including anterior and/or posterior chain exercises for muscle elongation and/or relaxation. The total duration of exercise sessions will be 55 minutes in the familiarization week, 65 minutes during the first phase, 75 minutes in the second phase, and 85 minutes in the third phase.

Phase		FIRST						SECOND				THIRD	
Week		1*	2	3	4	5	6	7	8	9	10	11	12
Supervised Exercise Volume (min/week)		110	130	130	130	130	130	150	150	150	150	170	170
Warm-up (RT)	Volume (min/session)	5	5	5	5	5	5	5	5	5	5	5	5
Resistance training	Total volume (min/week)	32	32	32	32	32	32	48	48	48	48	64	64
	Intensity (RPE 1-10 scale)	7	7 - 8	7 - 8	7 - 8	8 - 9	8 - 9	8 - 9	8 - 9	9 - 10	9 - 10	9 - 10	9 - 10
	Secs work/Secs rest (per exercise)	30/30	30/30	30/30	30/30	30/30	30/30	30/30	30/30	30/30	30/30	30/30	30/30
	Number of laps (per session)	2	2	2	2	2	2	3	3	3	3	4	4
	Rest between laps (min)	2	2	2	2	2	2	2	2	2	2	2	2
	Number of exercises (per session)	8	8	8	8	8	8	8	8	8	8	8	8
Rest between RT & AET	Volume (min/session)	3	5	5	5	5	5	5	5	5	5	5	5
Warm-up (HIIT)	Volume (min/session)	2	2	2	2	2	2	2	2	2	2	2	2
HIIT	Vigorous-intensity intervals / Moderate-intensity intervals	3/3	4/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4
	Total volume vigorous-intensity (min/week)	24	32	32	32	32	32	32	32	32	32	32	32
	Intensity (%HRR)	80 - 85	85 - 95	85 - 95	85 - 95	85 - 95	85 - 95	85 - 95	85 - 95	85 - 95	85 - 95	85 - 95	85 - 95
	Total volume moderate-intensity (min/week)	24	32	32	32	32	32	32	32	32	32	32	32
	Intensity (%HRR)	60 - 65	65 - 75	65 - 75	65 - 75	65 - 75	65 - 75	65 - 75	65 - 75	65 - 75	65 - 75	65 - 75	65 - 75
Cool down	Volume (min/session)	5	5	5	5	5	5	5	5	5	5	5	5

Figure 2. Supervised exercise periodization of the TEMPUS project. *Week of familiarization. HIIT, high-intensity interval training; HRR, heart rate reserve; RPE, rate of perceived exertion; RT, resistance training. AET, aerobic endurance training.

3.8. Participant retention, adherence and sustainability

The Principal Investigator and study team will exert every effort to facilitate participants' completion of all study visits and ensure overall study retention. The following strategies will be implemented to maximize participants' retention and minimize loss to follow-up: a) implementing a proactive retention plan that focuses on building close participant relations and ensuring participant satisfaction, b) giving the opportunities for participants and their families to ask questions and voice any concerns related to their condition throughout the study, c) reinforcing comprehension of the objectives and protocol of the study during study visits or conducting question and answer sessions after each visit, and d) evaluating each likelihood of dropout and implementing appropriate interventions to maintain their interest and motivation to continue participating in the study.

All supervised exercise sessions will be performed in a well-lit and airy room, providing to the participants the opportunity to choose their own music. The training specialists and other study staff will consistently offer support to participants throughout the duration of the study.

During the 12-week intervention period, participants will be required to record their daily sleep and eating times (i.e., exact times of the beginning of the first meal and of the end of the last meal), as well as any potential adverse events in a mobile phone app specifically designed for the study. These data will be revised 2 to 3 times every week, asking the participant for missing records, and will provide insights into their adherence to the prescribed eating window. Participants in the exercise groups and TRE groups will be labeled as “adherent” if they perform >80% of training sessions and eat within their allocated window of 8h (±30 minutes) >80% of days. Finally, we will assess the long-term adherence to the intervention (at the 12-month follow-up). This will allow us to evaluate the sustainability of participants' adherence over an extended period.

3.9. Adverse events

The study coordinators and project managers will oversee the collection of data and monitor the frequency of reported adverse events on a weekly basis using the mobile phone app (Tempus: com.nnbi.app_extreme_granada; NNBi2020 S.L., Navarra, Spain). Additionally, participants will complete validated questionnaires that assess gastrointestinal and autonomic symptoms, well-being, eating behavior, sleep quality, stress levels, mood, anxiety, and depression. These questionnaires will offer valuable information on any potential adverse effects and overall health-related outcomes. If a serious adverse event or an unanticipated problem occurs, the study coordinators will immediately notify both the principal investigators and the medical staff. Subsequently, a collective decision will be made and, if needed, the ethics committee will be properly informed. Moreover, appropriate measures will be taken to address and manage the reported event effectively.

3.10. *Data management*

Data collected will be directly entered into REDCap (Research Electronic Data Capture), a secure web-based platform specifically designed to create and manage research-related databases and surveys. This platform will ensure data security and confidentiality. For any data not recorded in REDCap, strict access control measures will be implemented to securely store the data on university computers, maintaining confidentiality and data integrity.

To ensure data quality and integrity, regular quality control checks will be conducted to identify any potential data anomalies, such as missing data or forms, data that falls outside the expected range, erroneous data entries, illogical dates over time, data inconsistencies across different forms and study visits, and incomplete fields on completed forms without a valid explanation for the

missing data. Any identified issues will be promptly reviewed and addressed by the data monitoring committee to ensure the accuracy and reliability of the data. The data monitoring committee will be independent from the sponsor. There are no auditing procedures planned.

3.11. Patient and public involvement

TEMPUS will stimulate patient and public involvement throughout the entire process. Potential participants have actually been involved in the preparation of the intervention and in the development of the mobile app, organization of the outcome measures, as well as are helping in the recruitment of new participants. Once we have the results ready, we will actively involve the participants in the reporting and advocacy of the study results.

4. Statistical Analysis Plan

4.1. Randomization and blinding

We expect to recruit and randomize ~184 participants using both stratification and permuted blocks with random block sizes, after completing the pre-intervention measurements. Randomization will be stratified based on sex (men-women), resulting in 2 strata. Prior to the beginning of the trial, randomization lists will be generated for each strata. The block sizes will be randomly determined, with allowable sizes of 4 and 8. Within each block, random selection will be used to assign participants to one of the four possible groups (i.e., UC, TRE, Exercise or TRE + Exercise) using a parallel design with a 1:1:1:1 allocation ratio. The sequential assignment of participants will follow the predetermined randomization list specific to their strata. The utilization of random block sizes ensures that the next assignment cannot be predicted. Stratification by sex ensures that the intervention groups will be balanced for this important characteristic. Blinding procedures will be rigorously applied to all personnel responsible for assessing primary outcomes, specifically hepatic fat depots and other ectopic fat depots derived from MRI. This blinding will also extend to those analyzing cardiometabolic risk factors, conducting glucose monitoring, examining fecal microbiota, evaluating physical activity and sedentary time, as well as those engaged in the statistical analysis of data. Conversely, personnel responsible for other measurements and intervention administration will not be blinded to the group assignment (open-label). Participants will receive clear information about the group they will be assigned to, along with details about the study hypotheses. In order to ensure practicality and feasibility, and based on previous research experience (64–66), the study will be conducted in successive waves of participants, each of them including a maximum of 16 participants.

4.2. Sample size

Based on previous findings from a recent trial on the combination of alternate day fasting and exercise on hepatic fat content (67), we anticipate approximately 5.0% reduction in hepatic fat content in the TRE + Exercise group, 2.5% in the TRE group, 2.5% in the exercise group, and no significant change in this outcome in the usual-care group. Assuming a pre-post correlation of 0.8 and a standard deviation of 6 points in the main outcome, we estimate a medium effect size of 0.45. To detect this effect size as statistically significant in a one-way ANOVA with $\alpha = 0.05$ and a power of 0.8, a minimum of 19 patients per group is required. Accounting for subgroup analyses based on sex and a maximum dropout rate of 20%, we will aim to recruit ~46 participants for each trial group, resulting in a total sample size of ~184 participants, with ~92 women included. To ensure a balanced representation of both sexes and maintain an adequate sample size, we will implement several strategies:

- Recruitment Process: A specific recruitment process will be utilized to aim for an equal enrollment of men and women. For each woman recruited, we will encourage her to invite one man to participate in the study. This approach has been successfully employed in our previous intervention studies and has proven effective in achieving a balanced sex

distribution (68–70).

- Sample size calculations: Our sample size calculations have taken into account subgroup analyses by sex. We have conservatively estimated a maximum dropout rate of approximately 20%. By considering this dropout rate, we have ensured that our study is adequately powered to detect the specified effect size even if there are differential dropout rates between men and women. For example, if men have a dropout rate of 5% and women have a dropout rate of 15%, our study will still have sufficient power to analyze the data separately in men and women.
- Expected dropout rate: While we have conservatively estimated the maximum dropout rate, we anticipate that the actual dropout rate will be relatively lower and similar between both sexes. This expectation is based on our previous studies and the measures we have to promote participant engagement and adherence (68–70).

4.3. Statistical analysis

The effects on primary and secondary outcomes in response to the present 12-week intervention will be assessed based on repeated-measures linear mixed-effects multilevel models. Individual measures of change will be therefore modeled as a function of the randomly assigned group, assessment time, and their interaction terms. All the analyses will also be conducted separately for men and women. Model-based estimations will be performed with an intention-to-treat approach (primary analyses) using the restricted maximum-likelihood method, the model assuming that missing values are missing-at-random. Analyses and estimations will also be performed with a per-protocol approach, and an attrition propensity will be calculated using a logistic model predicting attrition with baseline values of allocation group, age, sex, and BMI. Additional models will be conducted including energy intake, physical activity, or reproductive status in women. In addition to the conventional approach of assessing intervention effects based on statistical and practical significance, it is important to highlight that this study will employ a practical benefit approach. This approach emphasizes the reporting of unadjusted values that are intuitive to human judgment and easily replicable, considering the design and methodology of the study.

The main statistical analyses will be performed by an independent researcher (Dr. Almudena Carneiro-Barrera), who is not involved in the recruitment, evaluations, and interventions, and will be performed blinded to the treatment allocation by coding the intervention arms (e.g., A, B, C, D).

5. Ethics and dissemination

The study has received ethical approval from the Granada Provincial Research Ethics Committee (CEI Granada - 0365-N-23) and will be performed following the ethical guidelines of the Declaration of Helsinki. Before their inclusion in the study, participants will be required to provide oral and written informed consent, with further details available in the Online Supporting Information.

Results will be presented in peer-reviewed, scientific journals and at international conferences. We aim to publish a main paper with the primary outcome data. Since it is a large study with numerous secondary outcomes, other specific manuscripts on each topic will be published.

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TEMPUS

Study Protocol

University of Granada, Spain
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1. Background and rationale

Obesity prevalence has steadily increased up to reach epidemic proportions and affecting around 603.7 million adults worldwide (1). The excess of triglycerides in the body is usually stored, apart from the subcutaneous adipose tissue (SAT), in other organs and tissues that are not otherwise designed for adipose storage (2). This process is known as ectopic fat deposition and may include organs and tissues such as the liver, pancreas, or skeletal muscle. Excessive accumulation of triglycerides in hepatocytes results in hepatic steatosis, a condition considered one of the diagnostic criteria (along with other metabolic dysregulatory factors) for metabolic dysfunction-associated steatotic liver disease (MASLD) (3,4). MASLD (which replaces non-alcoholic fatty liver disease - NAFLD) is a major public health problem considering its elevated prevalence (nearly 90% of adults with overweight/obesity) and its strong association with extrahepatic diseases (3,5). Therefore, implementing strategies to reduce hepatic steatosis in individuals with obesity may be a potential approach to mitigate/reduce the risk of liver dysfunction and cardiometabolic diseases (6).

Traditionally, low-calorie diets have been shown to be an effective strategy to reduce body weight and hepatic steatosis and, in turn, improve cardiometabolic health (7). However, energy-restricted approaches are still not a standard public health strategy due to their lack of long-term sustainability and some undesirable metabolic adaptations that certainly lead to weight regain even in highly motivated patients (8). Time-restricted eating (TRE) is a recently emerged intermittent fasting approach which has the potential to maximize the extensively reported beneficial metabolic effects of the energy intake restriction (9). TRE aims to maintain a consistent daily cycle of feeding (within a limited time window during ≤10 hours) and fasting (≥14 hours) to support healthy/consistent circadian rhythms (10). Irregular eating patterns and eating over an extended period of time may disrupt circadian rhythms and, thus, increase the risk of obesity and hepatic fat accumulation (11). Remarkably, recent studies in mice have concluded that TRE effectively reduces hepatic steatosis and improves cardiometabolic health (12), mainly through improved insulin sensitivity; yet, whether this strategy is similarly effective in humans remains still unclear.

Along with nutritional strategies, exercise has demonstrated its efficacy at reducing hepatic steatosis and at improving cardiometabolic health in humans (13–15). Furthermore, preliminary evidence has highlighted that the combination of TRE and exercise may normalize glucose homeostasis and improve lipid profile in women with overweight or obesity (16). Nevertheless, the differential effects of TRE combined with exercise and TRE or exercise alone on hepatic steatosis and cardiometabolic markers remain unknown.

Although promising, most preliminary pilot trials examining the effects of TRE combined with exercise in humans have important limitations: (i) the duration is shorter than 3 months (16–19) which may be insufficient to induce substantial changes in cardiometabolic health (20); (ii) the outcomes assessed were either body mass index (BMI) as a surrogate marker of obesity or bioelectrical impedance analysis which are not able to accurately assess hepatic or other ectopic fat depots (18); (iii) the majority of clinical trials have been focused solely on men (17–19), or have included an unevenness sample of men and women which limits the possibility to understand the important sex dimorphism in MASLD development; (iv) the published studies are limited to trained individuals (17–19) and have small sample size and, thus, have limited statistical power; (v) the previous work do not include follow-up of the participants to understand the maintenance of intervention effects; and, more importantly, (vi) have not studied or reported potential mechanisms through which TRE combined with exercise may result in health benefits. Therefore, as all these shortcomings limit generality and preclude to establish firm conclusions, a new approach is required to successfully translate findings into the community and clinical setting.

2. Aims

The overall aim of the TEMPUS randomized controlled trial is to investigate the effects of a 12-week TRE combined with a supervised exercise intervention, as compared with TRE or supervised exercise alone, and a usual care control group (UC), on hepatic fat (primary outcome) and cardiometabolic health (secondary outcomes) in men and women with obesity.

3. Methods/Design

3.1. Study design

The TEMPUS study is a randomized controlled trial (ClinicalTrials.gov under the identifier NCT05897073) with a four-arm parallel design. The protocol of the randomized trial has been written and reported based on the Recommendations for Interventional Trials (SPIRIT) guidelines (21). Later, the results will be reported following the CONSORT guidelines. Consented participants will be randomly assigned to one of the four groups: UC, TRE alone (TRE), supervised exercise alone (Exercise), or TRE combined with supervised exercise (TRE + Exercise) group. The present study will focus on the recruitment of adults with obesity in Granada, a region located in the southern Spain. The patient flow diagram from recruitment to randomization phases is shown in **Figure 1**.

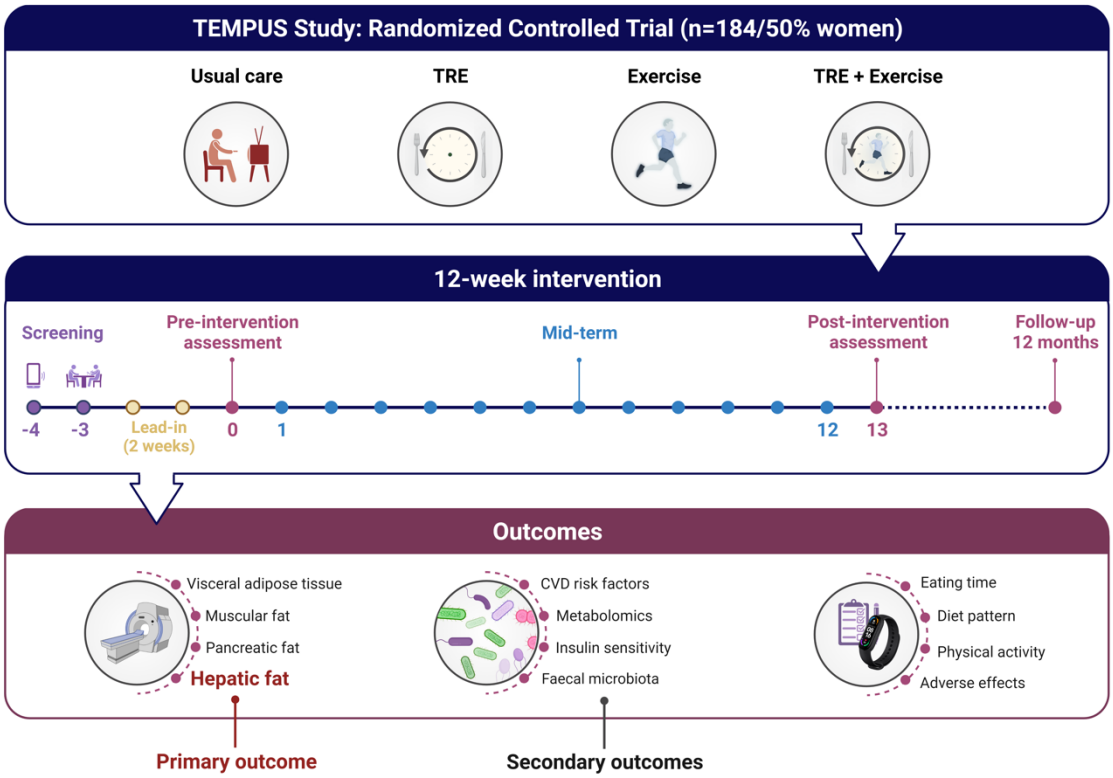

Figure 1. TEMPUS project design. TRE, time-restricted eating; CVD, cardiovascular disease.

3.2. Participants and eligibility criteria

The study will include both men and women (50%), with a BMI ranging from 30 to <40 kg/m^2, aged between 25 and 65 years, and with a habitual eating window of ≥11 hours. Detailed criteria for inclusion and exclusion can be found in **Table 1**. The screening phase, as shown in **Figure 1**, will involve assessing participants' medical history and vital signs to determine their eligibility for the study. During the physical examination, any existing conditions at the time and any pre-existing medical conditions will be thoroughly documented.

Table 1. Inclusion and exclusion criteria

Inclusion criteria
• Aged 25-65 years. • Body mass index between 30 and <40 kg/m^2. • Weight stability (within 3% of screening weight) for >2 months prior to study entry. • Habitual eating window ≥11 hours.
Exclusion criteria
• History of a major adverse cardiovascular event (e.g., acute myocardial infarction, ischemic or hemorrhagic stroke, or peripheral arterial ischemia, among others), kidney failure, chronic liver disease, or human immunodeficiency virus (HIV) / acquired immunodeficiency syndrome (AIDS). • Active endocrinological disease, innate errors of metabolism, myopathies or epilepsy. • Patients who have undergone bariatric surgery or other surgical techniques or used for the treatment of other pathologies (e.g., "Roux-en-Y"). • Rheumatoid arthritis, Parkinson's disease, active cancer treatment in the past year or another medical condition in which fasting is contraindicated. • Use of medications that may affect the results of the study such as drugs for glycaemic control (e.g., antidiabetic, steroids, beta-blockers, antibiotics, prebiotics, probiotics and symbiotics). • Diagnosis of major sleep or eating disorders. • Caregiver for a dependent requiring frequent nocturnal care/sleep interruption or shift workers with nocturnal hours. • Metal or electrical prosthesis. • Foreign bodies in the eyes. • Fear of needles and claustrophobia to magnetic resonance imaging. • Active tobacco or illicit drug use or a history of alcohol abuse treatment (i.e., moderate or severe alcoholism). • Participating in a weight loss or a supervised exercise program (i.e., > 30 min in 3 times per week, or > 45 min in 2 or more times per week at moderate-to-vigorous intensity). • Pregnancy and lactation or planned pregnancy (within the study period). • Frequent travel over time zones during the study period. • Being unable to understand and to accept the instructions or the study objectives and protocol. • Not having or being able to use a smartphone with Apple iOS or Android OS. • Are deemed unsuitable by the investigator for any other reason.

3.3. Recruitment and screening

Recruitment of potential participants will be conducted through (i) newspaper advertisements, (ii) the Endocrinology and Nutrition Department of the San Cecilio' and Virgen de las Nieves' University Hospitals of Granada, and (iii) the community of the University of Granada. A pre-screening process will be implemented with an online form as well as via telephone interviews to assess eligibility of potential participants and engage them in the study. Subsequently, the medical team will conduct telephone interviews, to review the patients' medical records and evaluate their medical history, verifying compliance with the inclusion and exclusion criteria. Potential participants who meet the criteria during the pre-screening phase will be provided with oral and written information about the study and will be scheduled for the first evaluation visit. During this session, oral and written informed consent will be obtained from potential participants, and measurements of body weight and height will be recorded. Additionally, participants will perform an incremental exercise test on a treadmill, supervised by the sports medicine staff from the *Centro Andaluz de Medicina del Deporte* and Sport and Health University Research Institute, to determine their aptitude for exercising. During the test and at rest, electrocardiogram and blood pressure measurements will be obtained.

3.4. Outcome measures

A 2-week lead-in period will be implemented prior to pre-intervention assessments and group allocation (see **Figure 1**). During this period, potential participants will be instructed to maintain their usual dietary and physical activity habits. They will be provided with a mobile phone application (Tempus: com.nnbi.app_extreme_granada; NNBi2020 S.L., Navarra, Spain) to record daily information regarding their eating time, sleeping patterns, naps, and any adverse events experienced. Additionally, physical activity and sleep quality will be evaluated using accelerometry, and glucose excursions over day and night will be assessed using continuous glucose monitoring (CGM) devices over the course of 2 weeks. This data collection will be used to verify that the participants' habitual eating windows are ≥11 hours and perform < 30 min in 3 times per week, or < 45 min in 2 or more times per week at moderate-vigorous intensity activity (see inclusion and exclusion criteria, **Table 1**). Pre-intervention measurements, after 12-week intervention measurements (±3 days), and after 12 months post-intervention measurements will be conducted by a dedicated team of trained staff to ensure consistency and reliability. In addition, we will assess anthropometry and body composition at week 6 (mid-term in **Figure 1**). By including these intermediate measurements, we will potentially capture changes over time and evaluate the trajectory of outcomes during the 12-week intervention period. A detailed overview of the study's outcomes in shown in **Table 2**, which provides a comprehensive summary of the variables assessed.

The study primary outcome will be changes from baseline to 12 weeks in hepatic fat. Secondary outcome variables include changes 12 weeks to 12 months in hepatic fat, changes from baseline to 12 weeks and change from 12 weeks to 12 months in other specific fat depots (i.e., visceral and abdominal subcutaneous adipose tissue, pancreatic fat and mid-thigh intermuscular and intramuscular adipose tissue), elastography (i.e., liver stiffness, liver steatosis and liver viscosity), anthropometry and body composition (i.e., fat mass and fat free mass, bone mineral density and content and skeletal muscle tissue – via MRI-), cardiometabolic markers (i.e., fating glucose, fating insulin, hemoglobin A1c and the homeostatic model of assessment for insulin resistance [HOMA-IR], lipid profile, liver markers, blood pressure and inflammatory biomarkers), 24h, diurnal and nocturnal mean glucose (via CGM), fecal microbiota, sleep and physical activity patterns,

psychological outcomes (i.e., depression, stress and anxiety), quality of life, dietary habits and eating behaviour assessment, and physical fitness (i.e. cardiorespiratory fitness, lower muscular strength, upper muscular strength and walking speed). After the screening examination and the 2-week lead-in period, pre-intervention assessments will be held within 3 face-to-face sessions:

Day 1: participants will perform physical fitness tests and will be required to complete several web-based questionnaires. These questionnaires will gather information on several outcomes such as quality of life, sleeping patterns, psychological status and eating behaviour. Dietary intake will be also assessed by qualified nutritionist where participants will be asked to provide the first 24-hour recall detailing their food consumption.

Day 2: fasting venous blood samples will be collected at the San Cecilio University Hospital (Granada, Spain). Thereafter, anthropometric and body composition measurements will be conducted at the Sport and Health University Research Institute (iMUDS - 300 meters apart). Finally, oral glucose tolerance tests will be also conducted as part of the glycaemic assessment.

Day 3: magnetic resonance imaging (MRI) and elastography will be performed in the San Cecilio University Hospital by the TEMPUS' medical staff (Granada, Spain). To ensure consistency and standardization during the assessment, participants will be instructed to maintain a fasting period of 8-10 hours prior to the appointment, during which they will be advised to consume only water and abstain from solid food or other beverages. In addition, participants will be advised not to consume alcohol or diuretics 24 hours before the test and to avoid stimulants like caffeine for 12 hours before the test.

Table 2. Overview of the study outcomes.

Outcomes	Pre-Intervention	Mid-term	Post-Intervention	12-month follow-up
Primary outcome				
Hepatic fat	✔		✔	✔
Secondary outcomes				
Ectopic fat depots	✔		✔	✔
Anthropometry and body composition	✔	✔	✔	✔
Cardiometabolic markers	✔		✔	✔
Glycaemic control	✔		✔ *	
Fecal microbiota	✔		✔ *	
Plasma and fecal metabolomics	✔		✔	
Sleep and physical activity	✔		✔ *	✔
Psychosocial	✔		✔	
Quality of life	✔		✔	✔
Eating behavior and dietary habits	✔		✔	✔
Physical Fitness	✔		✔	

*Assessment will be conducted during the last 2 weeks of the intervention.

3.5. Primary outcome: hepatic fat

The quantification of hepatic fat will be performed using MRI with a Siemens 3T Magnetom Vida scanner located at San Cecilio University Hospital in Granada. Additionally, liver steatosis, viscosity and fibrosis severity will be assessed using attenuation imaging coefficient, shear wave elastography and shear wave dispersion with a Canon Aplio i800 (located at San Cecilio University Hospital in Granada). The Fibrosis-4 (FIB-4) index will be calculated as an indicator of liver fibrosis severity (22).

3.6. Secondary outcomes

Specific fat depots: subcutaneous, visceral and intermuscular adipose tissue, as well as pancreatic fat content, will be obtained using MRI. To assess subcutaneous, visceral and intermuscular adipose tissue in all 3D abdominal volume (i.e., volume, cross-sectional area at selected levels, and mean/median fat fraction), a semiautomatic software program will be employed for tissue segmentation. These valuable image markers will be derived from a standard 6 echo Dixon series, ensuring accurate characterization of abdominal adipose tissue distribution and composition. We will also measure image markers from mid-thigh using a 6 echoes Dixon series, and will obtain cross-sectional area, muscular tissue, intramuscular adipose tissue, intermuscular adipose tissue, fat fraction, subcutaneous adipose tissue, and bone marrow fat fraction. The segmentation for all these structures will be performed with a semiautomatic proprietary algorithm.

Anthropometry and body composition: body weight and height measurements will be obtained using a stadiometer and scale (Seca model 799, Electronic Column Scale, Hamburg, Germany). Participants will be instructed to be barefoot and wear lightweight clothing during these measurements. Neck, waist, and hip circumferences, as well as calf girth, will be determined following the procedures outlined by the International Society for the Advancement of Kinanthropometry (ISAK) by certified personnel (ID's ISAK: #637686045477240182 and #638227824311214767) (23). Furthermore, fat mass, fat free mass, and overall adipose mass will be assessed by bioelectrical bioimpedance (Tanita MC 980-MA Plus, Tanita Corporation, Tokyo, Japan), with measurements taken after voiding the bladder to ensure accurate readings. Bone mineral density and content will be assessed by dual-energy X-ray absorptiometry scans (Discovery Wi, Hologic, Inc., Bedford, MA, USA). Participants will be instructed to maintain a fasting period of 8-10 hours prior to the appointment, during which they will be advised to consume only water and abstain from solid food or other beverages. In addition, participants will be advised to abstain from alcohol or diuretics 24 hours before the test and to avoid stimulants like caffeine for 12 hours before the test, and avoid engaging in moderate exercise or physical activity for 24 hours, or vigorous exercise for 48 hours, prior to the test.

Cardiometabolic risk markers*:* venous blood samples will be carefully preserved at a temperature of -80°C to ensure their integrity for subsequent analysis. We will collect a full set of cardiometabolic risk markers, as these parameters may inform about the metabolic dysregulation in liver ease (24). These set will include: fasting glucose (Alinity C system analyzer, Abbott Laboratories, IL, USA), fasting insulin (UniCel DxI 800 access immunoassay system, Beckman Coulter Inc., CA, USA), hemoglobin A1c (automated glycohemoglobin G11 analyser, Horiba) and lipid profile (i.e., total cholesterol, high-density lipoprotein cholesterol, triglycerides, apolipoprotein A1 and B) using Alinity C system analyzer (Abbott Laboratories, IL, USA). Low-density lipoprotein cholesterol will be calculated using a previously validated equation (LDL-c = CT – HDL-c – (TG/5) (25). Furthermore, we will measure alkaline phosphatase (Alinity C system analyzer, Abbott Laboratories, IL, USA). Liver markers (i.e., alanine transaminase,

aspartate aminotransferase and gamma-glutamyl transferase) will be also measured using an Alinity C system analyzer. Inflammatory markers (i.e., C-reactive protein and interleukin-6) will be measured using a AU5800 automated analyzer (Beckman Coulter Inc., CA, USA). Additionally, we will calculate insulin resistance surrogates, such as HOMA-IR. We will also conduct multi-omics (metabolomics, proteomics) analysis on plasma samples according to budget considerations. The omics analyses will allow the identification of: (i) metabolites/proteins strongly associated to hepatic fat content (baseline – cross-sectional analysis); (ii) metabolites/proteins predictors of hepatic fat content changes after the intervention. In addition, fasting urine samples will be collected for targeted metabolomic analysis to quantify the profile of essential and non-essential amino acids, providing complementary data on nitrogen balance and potential indicators of metabolic stress or muscle protein catabolism. Finally, systolic and diastolic blood pressure will be measured using an automated monitor (M3-Comfort, Omron Healthcare Europe B.V., Hoofddorp, The Netherlands), following the established guidelines outlined by the 2021 European Society of Hypertension (26).

Glycaemic control: due to the crucial role of insulin resistance in MASLD development, through fatty acid accumulation in hepatocytes, CGM and analysis of acute response to glucose will be measured. Participants will be instructed to wear a CGM device (FreeStyle LibrePro, Abbott Laboratories, Abbott Park, IL) during 2 weeks before the intervention (lead-in period) and during the last 2 weeks of the intervention (weeks 11 and 12). The CGM data obtained will be used to calculate different variables related to glycaemic control (i.e., 24-hour mean glucose), following the guidelines outlined in the most recent international consensus statement (27). Additionally, oral glucose tolerance tests will also be conducted as part of the glycaemic assessment using a 75-g oral glucose dose (NUTER TEC: orange flavour, Toulouse, France). Furthermore, capillary blood lactate will be measured using a stationary lactate analyzer (Biosen C-Line, EKF Diagnostic GmbH, Germany).

Fecal microbiota*:* for the comprehensive identification and quantification of fecal microbiome diversity and composition, pre-intervention and during the last 2 weeks of the intervention, fecal samples will be collected. Stool microbial DNA will be isolated from participants and subsequently, 16S rRNA gene amplicon sequencing, with the possibility of employing shotgun methodology pending final budget considerations, will be performed. Furthermore, a fecal metabolomic fingerprint analysis will be conducted to determine the metabolic profile among different groups of patients (pending final budget considerations).

Sleep and physical activity*:* sleep quality and chronotype will be evaluated through the administration of validated questionnaires, including the Pittsburgh Sleep Quality Index (PSQI) (28), Munich Chronotype Questionnaire (MCTQ) (29), and Horne and Östberg Questionnaire for Morning-Evening type assessment (MEQ-SA) (30). Objective measures of sleep, physical activity levels and rest-activity rhythms will be obtained using triaxial accelerometer (ActiGraph GT3X+, Pensacola, FL, USA) that participants will wear on their non-dominant wrist during two-week periods: before the beginning of the intervention (lead-in period) and during the last two weeks of the intervention (weeks 11 and 12). Also, steps counts will be assessed by activity band, at baseline and the last 2 weeks of intervention.

Psychosocial assessment*:* as weight loss intervention may improve emotional well-being and psychosocial functioning, several key psychological dimensions will be evaluated through the administration of validated questionnaires: the Beck Depression Inventory

Fast Screen for depression (BDI-FS) (31), the Perceived Stress Scale for stress (PSS) (32), and the State-Trait Anxiety Inventory for anxiety (STAI) (33).

Quality of life: quality of life may also improve from weight loss intervention (34) and will be evaluated using the EuroQol 5 dimensions 5 levels (EQ-5D-5L) questionnaire (35) and Rand Short Form 36 (SF-36) (36). In addition, participants will be asked to complete an adverse events questionnaire to identify any potential adverse effects or complications experienced during the intervention.

Eating behavior and dietary habits*:* to assess participants' adherence to the Mediterranean dietary pattern, validated questionnaires such as the PREDIMED questionnaire will be administered. This questionnaire provides a reliable measure of adherence to the specific dietary components and guidelines of the Mediterranean diet (37). Food Frequency Questionnaire (FFQ) (38) will be administered to assess the frequency that participants have consumed each specific food during the previous 4 weeks.

Physical fitness: an incremental treadmill exercise test until exhaustion will be performed to determine cardiorespiratory fitness. The modified Balke protocol (41) will be applied, which has been extensively used and validated (42–44). The exercise electrocardiogram and heart rate (HR) will be monitored continuously and reviewed by a cardiologist. Supervised exercise proposed in the present study could restore or mitigate adverse effect of diet-induced weight loss on muscle strength (45). Thus, upper muscular strength will be assessed through hand grip strength test (46) using a digital hand dynamometer (TKK 5401 Grip-D; Takei, Tokyo, Japan), whereas lower body muscular strength will be assessed through the 30-s sit-to-stand muscle power test (47), and walking speed to assess functional capacity with gait speed test (48).

Confounding: as unintentional reductions in energy intake (10-30% or ~300-500 kcal/day) have been reported when participants confine their eating windows to 4-10 h/day (49,50), we will control and analyze changes in energy intake over the intervention period. Participants will undergo dietary assessments through the completion of 3 non-consecutive 24-hour dietary recalls (2 working days and 1 non-working day) (51). These recalls will be conducted via face-to-face or telephone interviews by qualified and trained research nutritionists. Total energy intake, carbohydrates, fat and protein intake will be calculated. In addition, to understand the important sex dimorphism in MASLD development, at baseline and after the intervention, specific reproductive-profile questions will be asked regarding detailed information on menstrual cycle history and hormonal contraceptive use and type, as well as regarding any gynecological condition. This will help in categorizing participants into different hormonal profiles, following the recent consensus (52–54).

3.7. Intervention description

3.7.1. Usual care group

Participants randomly assigned to the UC group will be indicated to continue with their dietary eating time schedule. All participants will receive monthly in-person nutritional education sessions, lasting approximately 90 minutes, throughout the intervention for weight management and cardiovascular health promotion based on Mediterranean dietary patterns (55) and physical activity recommendations from the World Health Organization (56). Key points in nutritional counseling will be: a) to correctly interpret food labels and to plan grocery, b) to recognize and include high satiety foods, and c) to fight against some extended myths in Spanish culture such as “alcohol in small quantities is healthy”,

"it is unhealthy to skip breakfast", or "it is important to eat 5 meals per day".

3.7.2. Time-restricted eating intervention

Before the beginning of the intervention, participants allocated to TRE groups will select their preferred 8-hour eating window before the intervention and will be required to maintain the same 8-hour eating window during the 12-week intervention. They will be advised that the last meal should be completed before or at 21:00. Participants assigned to the TRE groups will be strictly prohibited from consuming any calorie-containing food or beverage outside their designated 8-hour eating window. However, they will be allowed to consume water, coffee, and tea without sugar or artificial sweeteners during the fasting period. Instructions for the TRE intervention will emphasize its daily implementation, meaning participants are expected to adhere to the eating window restriction (±30 minutes) throughout all seven days of the week.

3.7.3. Supervised exercise intervention

The objective of the supervised exercise program will be to ensure its transferability and feasibility for the target population. We will follow the Consensus on Exercise Reporting Template to facilitate replicability and transparency (57) and record the timing when participants are training (58). Thus, the physical activity recommendations for adults proposed by the World Health Organization serve as the foundation for determining the specific exercise dosage in TEMPUS (59). Given that both resistance and aerobic training modalities have shown improvements in hepatic steatosis among patients with MASLD (60,61), the TEMPUS study will combine both supervised resistance and aerobic high-intensity interval training (HIIT) sessions. Qualified sports scientist from outside the research group will carefully supervise the exercise sessions and work with groups of no more than six people to ensure that participants perform the exercise technique correctly, and at proper intensity. Moreover, participants will receive an individualized moderate-intensity goal-setting aerobic (i.e., walking) program consisting of increasing 10% daily steps per week based on their daily steps logs. Goal-setting aerobic will be updated weekly using a smart band (Xiaomi Mi Band 7, Xiaomi, Pekin, China) that participants enrolled in the exercise groups will wear on their wrists during the intervention period.

Volume: participants will engage in a 12-week intervention program including 2 supervised exercise sessions per week ranging from 60 to 90 minutes per session. Furthermore, participants will receive instructions to complete their personalized daily step goals established for each day.

Intensity: supervised sessions will start with a supervised circuit-based resistance training that focuses on upper and lower body exercises that target major muscle groups. To prescribe intensity, the rate of perceived exertion (RPE) scale ranging from 0 to 10 will be utilized, using the evaluations and first sessions to properly train the participants on this scale (62). The target intensity for resistance training will be set at an RPE level greater than 7, which will vary depending on the week number of the intervention (from 7 to 10). The participants will be encouraged to achieve this intensity through all the exercises performed in each lap of the circuit. Moreover, training sessions will include HIIT as the vigorous-intensity aerobic exercise component. HIIT will consist of 3-4 sets of 4-minute intervals at >85% of the individual heart rate reserve (HRR) with 4 minutes of active recovery at 65-75% HRR. HRR will be calculated considering the peak of HR achieved in the incremental treadmill test and the rest HR lying in bed recorded during pre-intervention assessments. HR will be continuously monitored during all exercise sessions using the Polar H10 band (Polar Products Inc., Stow, OH, USA).

Frequency: participants will perform 2 supervised exercise training sessions per week, with a resting period of at least 48 hours between them. Additionally, participants will be instructed to accomplish the prescribed daily step goal. If a participant misses a training session, it will be rescheduled and recovered considering a minimum resting period of 48 hours between sessions.

Type of exercise: the resistance training sessions will be composed of major upper and lower body muscle groups including (63): horizontal/vertical pull exercises (i.e., seated low row and lat pulldown with resistance elastic bands), hip-dominant exercises (i.e., deadlift with resistance elastic bands and weight-bearing glute bridge), knee-dominant exercises (i.e., weight-bearing squats and lunges), and horizontal/vertical push exercises (i.e., weight-bearing push-ups and shoulder press with resistance elastic bands). We will propose 4 levels of exercises' difficulty based on the resistance elastic band and the progressive complexity of the basic movement patterns involved. After the resistance circuit training, HIIT will be preferably performed on a treadmill to ensure a suitable progressive overload. As alternative modality, elliptical ergometer will be used.

Training load variation: we are aware that participants might not be immediately capable of exercising at high intensities and volumes. Therefore, there will be a gradual progression to the assigned exercise dose in 3 phases (see **Figure 2**) and proper technique will be a priority for the difficulty level progression to avoid potential injuries.

Training periodization: the supervised exercise intervention will be divided into 3 phases (**Figure 2**). The initial phase will have a length of 6 weeks and will start with a first week of familiarization. During this week, participants will learn the structure and organization of the sessions and the movement patterns that constitute the base of the different exercises. Considering that an inactive person may be unable to immediately train at the selected doses, the familiarization phase will prepare participants to gradually increase the workload until the required dose is achieved. On the other hand, the resistance training will set light loads using resistance elastic bands or weight-bearing exercises. They will increase the load and coordinative difficulty (through 4 levels for each exercise) as soon as participants perform the exercises with a proper technique.

We anticipate that both resistance training and HIIT load will be parallelly higher with the participants fitness increase. In this context, HIIT will be controlled based on HRR. Higher speed or power will need to be selected to achieve a determined percentage of HRR when fitness levels are increasing.

Training session: a training session will be organized as follow: (i) a warm-up of one circuit training lap with light loads using resistance elastic bands or weight-bearing exercises (20 seconds work: 20 seconds rest), (ii) 2 to 4 circuit training laps of eight exercises (30 seconds work: 30 seconds rest) at 7-10 RPE, with a between-set rest of 3-5 min, and (iii) 3 or 4 sets of 4-min intervals at 85-95% HRR interposed by 4 min of active recovery at 65-75% of HRR, with 2 min prior of warm-up. A cool-down protocol of 5 min will be performed at the end including anterior and/or posterior chain exercises for muscle elongation and/or relaxation. The total duration of exercise sessions will be 55 minutes in the familiarization week, 65 minutes during the first phase, 75 minutes in the second phase, and 85 minutes in the third phase.

Phase		FIRST						SECOND				THIRD	
Week		1*	2	3	4	5	6	7	8	9	10	11	12
Supervised Exercise Volume (min/week)		110	130	130	130	130	130	150	150	150	150	170	170
Warm-up (RT)	Volume (min/session)	5	5	5	5	5	5	5	5	5	5	5	5
Resistance training	Total volume (min/week)	32	32	32	32	32	32	48	48	48	48	64	64
	Intensity (RPE 1-10 scale)	7	7 - 8	7 - 8	7 - 8	8 - 9	8 - 9	8 - 9	8 - 9	9 - 10	9 - 10	9 - 10	9 - 10
	Secs work/Secs rest (per exercise)	30/30	30/30	30/30	30/30	30/30	30/30	30/30	30/30	30/30	30/30	30/30	30/30
	Number of laps (per session)	2	2	2	2	2	2	3	3	3	3	4	4
	Rest between laps (min)	2	2	2	2	2	2	2	2	2	2	2	2
	Number of exercises (per session)	8	8	8	8	8	8	8	8	8	8	8	8
Rest between RT & AET	Volume (min/session)	3	5	5	5	5	5	5	5	5	5	5	5
Warm-up (HIIT)	Volume (min/session)	2	2	2	2	2	2	2	2	2	2	2	2
HIIT	Vigorous-intensity intervals / Moderate-intensity intervals	3/3	4/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4
	Total volume vigorous-intensity (min/week)	24	32	32	32	32	32	32	32	32	32	32	32
	Intensity (%HRR)	80 - 85	85 - 95	85 - 95	85 - 95	85 - 95	85 - 95	85 - 95	85 - 95	85 - 95	85 - 95	85 - 95	85 - 95
	Total volume moderate-intensity (min/week)	24	32	32	32	32	32	32	32	32	32	32	32
	Intensity (%HRR)	60 - 65	65 - 75	65 - 75	65 - 75	65 - 75	65 - 75	65 - 75	65 - 75	65 - 75	65 - 75	65 - 75	65 - 75
Cool down	Volume (min/session)	5	5	5	5	5	5	5	5	5	5	5	5

Figure 2. Supervised exercise periodization of the TEMPUS project. *Week of familiarization. HIIT, high-intensity interval training; HRR, heart rate reserve; RPE, rate of perceived exertion; RT, resistance training. AET, aerobic endurance training.

3.8. Participant retention, adherence and sustainability

The Principal Investigator and study team will exert every effort to facilitate participants' completion of all study visits and ensure overall study retention. The following strategies will be implemented to maximize participants' retention and minimize loss to follow-up: a) implementing a proactive retention plan that focuses on building close participant relations and ensuring participant satisfaction, b) giving the opportunities for participants and their families to ask questions and voice any concerns related to their condition throughout the study, c) reinforcing comprehension of the objectives and protocol of the study during study visits or conducting question and answer sessions after each visit, and d) evaluating each likelihood of dropout and implementing appropriate interventions to maintain their interest and motivation to continue participating in the study.

All supervised exercise sessions will be performed in a well-lit and airy room, providing to the participants the opportunity to choose their own music. The training specialists and other study staff will consistently offer support to participants throughout the duration of the study.

During the 12-week intervention period, participants will be required to record their daily sleep and eating times (i.e., exact times of the beginning of the first meal and of the end of the last meal), as well as any potential adverse events in a mobile phone app specifically designed for the study. These data will be revised 2 to 3 times every week, asking the participant for missing records, and will provide insights into their adherence to the prescribed eating window. For participants in the TRE + Exercise group and TRE alone group, each day will be labeled as adherent if the participant meets their self-selected 8-hour (±30 minutes) eating window. Adherence will then be defined as the percentage of days on which the eating window was met. Finally, we will assess the long-term adherence to the intervention (at the 12-month follow-up). This will allow us to evaluate the sustainability of participants' adherence over an extended period. Attendance will be defined as percentage of exercise sessions attended by the participant (TRE + Exercise

and Exercise alone groups), recorded by the trainers, divided by the actual number of exercise sessions offered (12 weeks x 2 sessions / week = 24). Finally, we will assess the long-term adherence to the intervention (at the 12-month follow-up). This will allow us to evaluate the sustainability of participants' adherence over an extended period.

3.9. Adverse events

The study coordinators and project managers will oversee the collection of data and monitor the frequency of reported adverse events on a weekly basis using the mobile phone app (Tempus: com.nnbi.app_extreme_granada; NNBi2020 S.L., Navarra, Spain). Additionally, participants will complete validated questionnaires that assess gastrointestinal and autonomic symptoms, well-being, eating behavior, sleep quality, stress levels, mood, anxiety, and depression. These questionnaires will offer valuable information on any potential adverse effects and overall health-related outcomes. If a serious adverse event or an unanticipated problem occurs, the study coordinators will immediately notify both the principal investigators and the medical staff. Subsequently, a collective decision will be made and, if needed, the ethics committee will be properly informed. Moreover, appropriate measures will be taken to address and manage the reported event effectively.

3.10. *Data management*

Data collected will be directly entered into REDCap (Research Electronic Data Capture), a secure web-based platform specifically designed to create and manage research-related databases and surveys. This platform will ensure data security and confidentiality. For any data not recorded in REDCap, strict access control measures will be implemented to securely store the data on university computers, maintaining confidentiality and data integrity.

To ensure data quality and integrity, regular quality control checks will be conducted to identify any potential data anomalies, such as missing data or forms, data that falls outside the expected range, erroneous data entries, illogical dates over time, data inconsistencies across different forms and study visits, and incomplete fields on completed forms without a valid explanation for the missing data. Any identified issues will be promptly reviewed and addressed by the data monitoring committee to ensure the accuracy and reliability of the data. The data monitoring committee will be independent from the sponsor. There are no auditing procedures planned.

3.11. Patient and public involvement

TEMPUS will stimulate patient and public involvement throughout the entire process. Potential participants have actually been involved in the preparation of the intervention and in the development of the mobile app, organization of the outcome measures, as well as are helping in the recruitment of new participants. Once we have the results ready, we will actively involve the participants in the reporting and advocacy of the study results.

4. Statistical Analysis Plan

4.1. Randomization and blinding

We expect to recruit and randomize ~184 participants using both stratification and permuted blocks with random block sizes, after completing the pre-intervention measurements. Randomization will be stratified based on sex (men-women), resulting in 2 strata. Prior to the beginning of the trial, randomization lists will be generated for each strata. The block sizes will be randomly determined, with allowable sizes of 4 and 8.

Within each block, random selection will be used to assign participants to one of the four possible groups (i.e., UC, TRE, Exercise or TRE + Exercise) using a parallel design with a 1:1:1:1 allocation ratio. The sequential assignment of participants will follow the predetermined randomization list specific to their strata. The utilization of random block sizes ensures that the next assignment cannot be predicted. Stratification by sex ensures that the intervention groups will be balanced for this important characteristic. Blinding procedures will be rigorously applied to all personnel responsible for assessing primary outcomes, specifically hepatic fat depots and other ectopic fat depots derived from MRI. This blinding will also extend to those analyzing cardiometabolic risk factors, conducting glucose monitoring, examining fecal microbiota, evaluating physical activity and sedentary time, as well as those engaged in the statistical analysis of data. Conversely, personnel responsible for other measurements and intervention administration will not be blinded to the group assignment (open-label). Participants will receive clear information about the group they will be assigned to, along with details about the study hypotheses. In order to ensure practicality and feasibility, and based on previous research experience (64–66), the study will be conducted in successive waves of participants, each of them including a maximum of 16 participants.

4.2. Sample size

Based on previous findings from a recent trial on the combination of alternate day fasting and exercise on hepatic fat content (67), we anticipate approximately 5.0% reduction in hepatic fat content in the TRE + Exercise group, 2.5% in the TRE group, 2.5% in the exercise group, and no significant change in this outcome in the usual-care group. Assuming a pre-post correlation of 0.8 and a standard deviation of 6 points in the main outcome, we estimate a medium effect size of 0.45. To detect this effect size as statistically significant in a one-way ANOVA with $\alpha = 0.05$ and a power of 0.8, a minimum of 19 patients per group is required. Accounting for subgroup analyses based on sex and a maximum dropout rate of 20%, we will aim to recruit ~46 participants for each trial group, resulting in a total sample size of ~184 participants, with ~92 women included. To ensure a balanced representation of both sexes and maintain an adequate sample size, we will implement several strategies:

- Recruitment Process: A specific recruitment process will be utilized to aim for an equal enrollment of men and women. For each woman recruited, we will encourage her to invite one man to participate in the study. This approach has been successfully employed in our previous intervention studies and has proven effective in achieving a balanced sex distribution (68–70).
- Sample size calculations: Our sample size calculations have taken into account subgroup analyses by sex. We have conservatively estimated a maximum dropout rate of approximately 20%. By considering this dropout rate, we have ensured that our study is adequately powered to detect the specified effect size even if there are differential dropout rates between men and women. For example, if men have a dropout rate of 5% and women have a dropout rate of 15%, our study will still have sufficient power to analyze the data separately in men and women.
- Expected dropout rate: While we have conservatively estimated the maximum dropout rate, we anticipate that the actual dropout rate will be relatively lower and similar between both sexes. This expectation is based on our previous studies and the measures we have to promote participant engagement and adherence (68–70).

4.3. Statistical analysis

The main analyses will consist of the intention-to-treat analyses for the primary and

secondary outcomes using a constrained baseline (meaning baseline adjusted) linear mixed model, which accounts for baseline differences among the study groups. The model will include fixed effects for time (three levels) and treatment (four levels) as well as the unique participant identifier as a random effect, and sex will be included as a covariate. Model assumptions will be checked. In case we detect model violations, we will take appropriated measures by e.g., conducting data transformations. If the global test of significance indicates between-group differences, pairwise comparisons will be explored. Although no adjustments for multiplicity will be performed, family-wise type 1 error rate on the primary outcome will be retained by using a hierarchical analytic approach. The prespecified hierarchical hypotheses will be tested using the prespecified sequence: TRE + Exercise versus UC, TRE versus UC, Exercise versus UC, TRE + Exercise versus TRE, TRE + Exercise versus Exercise and TRE versus Exercise. The main statistical analyses will be performed by an independent researcher (Dr. Almudena Carneiro-Barrera), who is not involved in the recruitment, evaluations, and interventions, and will be performed blinded to the treatment allocation by coding the intervention arms (e.g., A, B, C, D).

It should also be noted that the intervention effect assessments will not only be based on statistical and practical significance (as usually done), but also on a practical benefit approach emphasizing and reporting unadjusted values that are intuitive to human judgment and readily replicable considering the design and methodology of this project.

5. Ethics and dissemination

The study has received ethical approval from the Granada Provincial Research Ethics Committee (CEI Granada - 0365-N-23) and will be performed following the ethical guidelines of the Declaration of Helsinki. Before their inclusion in the study, participants will be required to provide oral and written informed consent, with further details available in the Online Supporting Information.

Results will be presented in peer-reviewed, scientific journals and at international conferences. We aim to publish a main paper with the primary outcome data. Since it is a large study with numerous secondary outcomes, other specific manuscripts on each topic will be published.

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7. Summary of changes to Study Protocol

Date	Amendment
01/07/2024	Review and improvement of the statistical analysis methods, including constrained baseline (meaning baseline-adjusted) linear mixed model.
01/07/2024	Update of secondary outcomes including new outcomes derived from magnetic resonance imaging, elastography, fasting urine, and fasting blood samples.