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Ľudmila Oreská * , Barbora Kundeková , Lukáš Varga , Katarína Stebelová , Monika Okuliarová , Juraj Payer , Milan Sedliak

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Ageing, Sex Differences, and REDs Risk in Endurance Runners: An Integrated Cross-Sectional Study Protocol

Ľudmila Oreská [1,2,*], Barbora Kundeková [1], Lukáš Varga [3], Katarína Stebelová [4], Monika Okuliarová [4], Juraj Payer [5] and Milan Sedliak [1]

[1] Department of Biological and Medical Sciences, Faculty of Physical Education and Sports, Comenius University in Bratislava, 814 69 Bratislava, Slovakia
[2] Department of Health Technologies, Faculty of Pharmacy, Comenius University in Bratislava, 832 32 Bratislava, Slovakia
[3] Department of Otorhinolaryngology Head and Neck Surgery, Faculty of Medicine and University Hospital Bratislava, Comenius University, Bratislava, Slovakia
[4] Department of Animal Physiology and Ethology, Faculty of Natural Sciences, Comenius University in Bratislava, Slovakia
[5] 5th Department of Internal Medicine, Faculty of Medicine, Comenius University in Bratislava, University Hospital, Bratislava, Slovak Republic
* Correspondence: ludmila.oreska@uniba.sk

Abstract

Endurance performance is influenced by age- and sex-specific physiological determinants, while emerging evidence indicates an increasing prevalence of Relative Energy Deficiency in Sport (REDs) among both young and master endurance runners. Despite its clinical relevance, limited data exist on how long-term endurance training modulates REDs risk, skeletal muscle characteristics, and physiological ageing in comparison with inactive individuals. **Methods:** This cross-sectional study protocol will examine 112 participants stratified by sex, age (20–35 vs. 65–80 years), and training status (endurance runners vs. inactive controls). Cardiorespiratory fitness (VO_2max) is defined as the primary outcome. Secondary outcomes include body composition, musculoskeletal function, biochemical and hormonal markers, and REDs–related screening variables. Assessments will comprise cardiorespiratory testing, DXA-based bone and body composition analysis, isometric knee dynamometry, mobility testing, validated REDs screening tools (LEAF-Q, LEAM-Q, and IOC REDs CAT2), seven-day dietary and training monitoring, venous blood sampling, and skeletal muscle biopsies from the vastus lateralis. **Results:** The study is designed to generate an integrated overview of physiological, nutritional, metabolic, and muscle-cell characteristics across sex-, age-, and training-specific subgroups. **Conclusions:** This protocol provides comprehensive insight into how ageing and sex influence endurance physiology and REDs susceptibility, and whether long-term endurance training preserves functional capacity across the lifespan. The findings aim to support evidence-based screening, prevention, and targeted interventions for REDs in endurance athletes.

Keywords: ageing; master athletes; endurance running; sex differences; relative energy deficiency

1. Introduction

Endurance running performance is shaped by a complex interplay of physiological, biomechanical, and sociocultural factors, with biological sex and ageing representing two of the most influential determinants across the lifespan. Consistently across all levels of competition, male runners consistently outperform female runners in events ranging from middle-distance to ultramarathons. This performance disparity is primarily attributed to sex-specific physiological characteristics, including higher maximal oxygen uptake (VO_2max), greater cardiac stroke volume, elevated haemoglobin concentrations, and larger skeletal muscle mass in men, which collectively enhance oxygen transport and utilisation during prolonged exercise. These differences largely reflect divergent hormonal environments, with testosterone promoting muscle hypertrophy, erythropoiesis, and aerobic capacity. Thereby supporting higher aerobic power and endurance performance in male athletes [1–3].

However, sex-based differences in endurance performance extend beyond aerobic capacity alone. Female runners exhibit sex-specific biomechanical and metabolic characteristics, including a greater reliance on lipid oxidation during submaximal exercise, a higher proportional area of type I muscle fibres, and pacing strategies that may be more even in prolonged events, which may contribute to performance in ultra-endurance contexts **[4,5]**. Despite these adaptations, males typically outperform females in endurance events, with the magnitude of the sex-based performance gap varying by discipline, event demands, and competitive level (approximately **10–30%** in many athletic contexts) **[2,4,5]**. Endogenous hormonal fluctuations across the menstrual cycle may influence selected physiological responses to exercise in female athletes; however, the current evidence linking menstrual-cycle phase to performance outcomes remains mixed and inconclusive. Findings are highly variable between individuals, and results differ across study designs and methodological approaches **[1]**. Ageing introduces an additional layer of complexity to endurance performance. Master athletes experience a progressive decline in physiological capacity, primarily driven by reductions in VO_2max, maximal heart rate, stroke volume, and skeletal muscle mass. After approximately 70 years of age, the rate of performance decline accelerates, exceeding 1.3% per year in both sexes. While older male runners generally retain higher absolute performance levels, they often exhibit steeper relative declines compared with female runners, suggesting that ageing may partially attenuate sex-based performance differences, particularly in long-duration endurance events [5–7].

Beyond performance-related determinants, endurance running is increasingly associated with the risk of Relative Energy Deficiency in Sport (REDs), a syndrome resulting from prolonged low energy availability and affecting multiple physiological systems, particularly endocrine, metabolic, musculoskeletal, and cardiovascular function. REDs has been documented in both male and female endurance runners across age groups, with sex-specific manifestations driven by hormonal and physiological variability. Female athletes tend to exhibit a greater number of affected physiological systems, with previous studies reporting an average of 2.9 affected systems in females (most frequently reproductive, endocrine, skeletal, and metabolic) compared with 1.6 in males (most frequently endocrine, skeletal and haematological) [8]. Furthermore, female runners more frequently report menstrual dysfunction, disordered eating behaviours, and compulsive training patterns, all of which are strongly linked to REDs development.

Importantly, emerging evidence suggests that the prevalence of REDs may be increasing among ageing master endurance runners, potentially due to cumulative training loads, age-related changes in energy requirements, and inadequate nutritional compensation [9]. Although long-term endurance training may attenuate certain aspects of physiological ageing, insufficient energy intake relative to expenditure may exacerbate hormonal disturbances, impaired bone health, and maladaptive musculoskeletal changes. Targeted training strategies combined with evidence-based nutritional interventions, therefore, represent a critical component in mitigating REDs risk across the lifespan, not only in master athletes but also as a preventive strategy in younger endurance runners.

Consequently, a multidisciplinary approach integrating sports medicine, nutrition, and training science is essential for the early identification and management of REDs Such an approach enables timely screening, individualised intervention, and improved long-term health and performance outcomes in endurance athletes of both sexes.

Therefore, the main aim of this study protocol is to comprehensively examine how biological sex and ageing influence physiological performance, skeletal muscle characteristics, bone health, and REDs risk in young and master endurance runners. The secondary aim is to compare endurance

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runners with their age- and sex-matched inactive counterparts to determine the extent to which long-term endurance training preserves physiological function and mitigates age-related decline across the lifespan. To address these aims, the primary outcome of this study is cardiorespiratory fitness, assessed as VO_2max, while musculoskeletal, metabolic, endocrine, and REDs related parameters are treated as secondary outcomes, providing complementary physiological context

2. Materials and Methods

Study Design

The overall study design, illustrated in Figure 1, follows a cross-sectional approach. Publishing this protocol enhances methodological transparency, preregistration of outcomes, and reproducibility of complex multimodal assessments involving invasive procedures. The study protocol was developed in accordance with the SPIRIT guidelines and is comprehensively documented using the SPIRIT checklist (Appendix A1), ensuring that all essential items required for reporting study protocols are systematically addressed. All measurements will be conducted under standardised laboratory conditions using calibrated equipment and trained personnel to ensure data quality and reproducibility across all study groups.

FIGURE 1: The cross-sectional study design. (Created with BioRender.com)

Sample Size

Based on the planned between-group comparisons across eight study groups (sex × age category × training status), an a priori sample size calculation for an omnibus factorial ANOVA (fixed effects) was performed ($\alpha = 0.05$, power = 0.90). Assuming a mean effect size of Cohen's $f \approx 0.42$, the required total sample size was $n = 112$, corresponding to 14 participants per group. The sample size estimation was performed using G*Power software (version 3.1.9.2).

Study Subjects

A total of 112 subjects will be enrolled in this cross-sectional study and stratified into eight groups according to sex, age, and athletic status: (1) Young Male Endurance Runners, (2) Master Male Endurance Runners, (3) Young Male Age-Matched Controls, (4) Elderly Male Age-Matched Controls, (5) Young Female Endurance Runners, (6) Master Female Endurance Runners, (7) Young Female Age-Matched Controls, and (8) Elderly Female Age-Matched Controls. The young adult groups will include subjects aged 20–35 years, while the master and elderly groups will include individuals aged 65–80 years. Endurance runner groups will include elite-level Slovak athletes actively competing in national and international long-distance events. Young endurance runners will be defined as those with ≥3 years of national-level competitive experience, whereas master runners will be characterised by >5 years of continuous competitive participation. Group inclusion will be further validated using performance benchmarks of ≤35 min (young) and ≤55 min (masters) in the 10-km run. Control groups will comprise non-athletic individuals matched by sex and age, reporting <150 min of moderate and < 75 of vigorous weekly activity during the past five years. This distinction reflects sustained physiological performance and adaptation in the context of ageing between both sexes [10].

The study will be conducted in accordance with the Declaration of Helsinki. All participants will receive detailed information about the study aims, procedures, and potential risks, and written informed consent will be obtained prior to enrolment. Ethical approval has been granted by the Ethical Committee of the University Hospital Bratislava – Hospital of Ladislav Dérer (No. 31/2020).

All data will be anonymised and stored securely following applicable data-protection regulations, with access restricted to authorised research staff. The protocol adheres to SPIRIT guidelines, and anonymised datasets will be made available upon reasonable request in line with institutional and ethical requirements.

Inclusion Criteria

Subjects will be considered eligible if they meet all the following criteria. Young adult groups will comprise individuals aged 20–35 years, whereas older adult groups will comprise individuals aged 65–80 years. Endurance-trained participants must perform ≥300 min·week^{-1} of structured endurance running, consistently maintained for at least three years, and compete at the national or international level. Physically inactive participants must report ≤150 min·week^{-1} of moderate-intensity activity and ≤75 min·week^{-1} of vigorous-intensity activity over the same period. Eligible participants must have a BMI between 18.5 and 35.0 kg·m^{-2} and sufficient Slovak language proficiency to understand study procedures and instructions (Appendix A2.1). All participants will provide written informed consent and will be required to comply with all study requirements.

Female-Specific Considerations (Menstrual Status and Hormonal Contraception)

Menstrual status and hormonal contraceptive (HC) use will be recorded at screening. HC users (if any) will be eligible only with a stable regimen for ≥3 months, and the type and formulation of HC (including dose/route where available) will be reported. If HC users are enrolled, analyses will consider naturally menstruating women and HC users separately (e.g., combined vs progestin-only) in sensitivity analyses where appropriate. In the present cohort, no endurance-trained female runners using HC enrolled; therefore, analyses in female runners reflect naturally menstruating participants only.

Exclusion Criteria

Subjects will be excluded if they have musculoskeletal conditions that limit mobility or prevent participation in study assessments; acute or chronic infection; or diagnosed cardiovascular, neurological, metabolic, oncological, autoimmune, or other systemic diseases that contraindicate study involvement. Individuals with nutritional disorders (including malnutrition) or BMI < 18.5 kg·m^{-2} will also be excluded. The use of medications or substances within the past six months that may interfere with biological analyses (e.g., systemic corticosteroids, immunosuppressants, chemotherapeutic agents, hormonal treatments, or other immunomodulatory drugs) will constitute an exclusion criterion. In addition, the use of non-steroidal anti-inflammatory drugs (NSAIDs) within 24 h prior to any study visit will not be permitted and will result in rescheduling or exclusion, depending on frequency and clinical necessity. Any current or prior use of doping or performance-enhancing substances, major surgery or hospitalisation within the previous six months, and pregnancy or lactation will also result in exclusion (Appendix A2.2).*Female-specific exclusions (menstrual status and hormonal contraception)*

Young adult female subjects will be excluded if they have initiated, discontinued, or changed the type, dose, or delivery method of hormonal contraception within the past 3 months, or if the contraceptive formulation and delivery method cannot be reliably documented (where applicable).

Familiarisation and Pre-Participation Medical Screening

Prior to enrolment, all prospective participants will complete a familiarisation session at the testing facility to ensure full understanding of study procedures, equipment, and the laboratory environment. This step is implemented to reduce procedural anxiety, improve compliance, and minimise learning effects during subsequent assessments.

Following familiarisation, a licensed sports medicine physician will conduct a comprehensive pre-participation medical evaluation, including medical history, resting heart rate and blood pressure measurements, physical examination, and resting and graded-exercise electrocardiography and assessment of maximal oxygen uptake (VO_2max) to screen for latent cardiovascular abnormalities. Additional assessments, such as spirometry, will be performed when clinically indicated.

Only participants who are medically cleared and present no contraindications to maximal or submaximal exertion will proceed to the study. This two-stage pre-inclusion process—familiarisation followed by medical clearance—ensures participant safety, strengthens the reliability of

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physiological measurements, and adheres to established ethical standards for human research [11,12].

Primary Outcomes

Cardiorespiratory fitness expressed as estimated (VO_2max) is defined as the primary outcome of interest, with secondary outcomes encompassing body composition, musculoskeletal function, biochemical and hormonal markers, and REDs–related screening variables. While VO_2max ($mL \cdot kg^{-1} \cdot min^{-1}$) will serve as the primary clinical outcome, estimated VO_2max normalised to fat-free mass (FFM) will be analysed as a secondary, mechanistically informative indicator of aerobic capacity relative to metabolically active tissue.

Cardiorespiratory Fitness Assessment

Maximal oxygen uptake (VO_2max) will be estimated indirectly from the maximal power output (Wmax) achieved during a continuous incremental cycling test using the American College of Sports Medicine (ACSM) metabolic equation for leg cycling:

$$\boldsymbol{VO_2max\ (mL \cdot kg^{-1} \cdot min^{-1}) = \frac{11.016 \cdot W_{max}}{BM} + 7}$$

Where Wmax represents the maximal achieved power output in watts and BM denotes body mass in kilograms. This equation is derived from the standard ACSM relationship between work rate and oxygen uptake during cycle ergometry and will be applied to estimate VO_2max at peak exercise intensity. VO_2max will serve as the primary physiological outcome, representing a validated indicator of aerobic capacity and cardiorespiratory health [13,14]. All testing will be conducted at the SPORTMED Sports Medicine Centre under medical supervision and in accordance with ACSM Guidelines for Exercise Testing and Prescription [12]. The test will begin with a 1-minute rest and a warm-up at 20 W, followed by a continuous ramp protocol with workload increments of 20 W/min until volitional exhaustion or clinical termination. Increment size may be adjusted slightly (e.g., 25–30 W/min) based on sex, age, and predicted capacity to achieve an optimal test duration of 8–12 minutes, consistent with validated ramp protocols [15].

Heart rate and blood pressure will be monitored continuously via 12-lead ECG (Quark T12, Cosmed, Rome, Italy), and brachial blood pressure will be assessed before, during, and after exercise (Metronik BL-6, STOLL Medizintechnik, Germany). Maximum workload (in W and $W \cdot kg^{-1}$) will also be recorded. Tests will be terminated upon volitional fatigue, abnormal ECG responses, or safety-related criteria, following ACSM termination guidelines [12].

Secondly, to account for differences in body composition between sexes and age groups, VO_2max will be normalised to fat-free mass obtained by dual-energy X-ray absorptiometry (DXA) by using the following equation:

$$\boldsymbol{VO_2max\_FFM\ (mL \cdot kg^{-1}\ FFM \cdot min^{-1}) = \frac{VO_2max\_abs}{2aFFM}}$$

All assessments will be administered by a licensed sports physician and a trained exercise physiologist to ensure participant safety, standardisation, and data quality.

Secondary Outcomes

Anthropometric and Body Composition Assessment

Basic anthropometric variables, including standing height and body weight, will be collected as input parameters for subsequent body composition analyses. Standing height will be measured to the nearest 0.1 cm using a digital stadiometer (BSM 170, InBody Co., Ltd., Cerritos, CA, USA), with participants barefoot and positioned upright. Body weight will be assessed to the nearest 0.1 kg using a calibrated bioelectrical impedance analyser (InBody 230, InBody Co., Ltd., Cerritos, CA, USA) with subjects wearing minimal clothing.

The bioimpedance system (InBody 230, InBody Co., Ltd., Cerritos, CA, USA) will be used to obtain standardised body weight and descriptive estimates of body fat percentage and skeletal muscle mass for the calculation of strength normalised to body mass and/or skeletal muscle mass, whereas DXA will serve as the reference method for detailed regional body composition, fat-free mass (FFM), and all bone-related outcomes [16,17].Therefore to obtain a comprehensive and accurate assessment of body composition, all participants will undergo a whole-body Dual-Energy X-ray Absorptiometry (DXA). Scans will be performed using standardised positioning procedures in the supine position, with careful alignment of the head, trunk, and limbs to minimise movement artefacts. All assessments will be conducted in the radiology department under the supervision of a certified radiologist and according to manufacturer guidelines to ensure measurement reliability and between-subject consistency.

DXA will provide a full set of bone-related parameters, including bone mineral content (BMC; g), bone mineral density (BMD; $g\cdot cm^{-2}$), and T-scores for whole body, proximal femur, and lumbar spine, enabling evaluation of bone health status. Additionally, body composition outcomes will be obtained, including total and regional lean soft tissue mass, fat mass, body fat percentage, appendicular lean mass (ALM), and indices relevant for functional and clinical assessment such as the appendicular lean mass index (ALMI; $kg\cdot m^{-2}$) and fat-free mass index (FFMI). Regional compartmental analysis (arms, legs, trunk) will also be performed. Furthermore, in accordance with the recent viewpoint advocating for sport-specific bone mineral density reference values for athletes rather than general population thresholds, we will compare our runners' bone health classifications using both conventional clinical criteria and the updated athlete-oriented cut-offs [18].

Assessment of Lower Body Strength

Lower-body strength will be assessed using an isometric knee dynamometer (ARS dynamometry, S2P Ltd., Ljubljana, Slovenia) to determine maximal voluntary contraction (MVC) of the knee extensors and flexors, following validated procedures [19,20]. Testing will be performed in a custom-designed isometric chair adjusted individually to ensure optimal joint alignment and reproducibility, in accordance with established dynamometry guidelines [21,22].

Prior to MVC assessment, subjects will complete two submaximal warm-up contractions at approximately 50% and 80% of perceived maximal effort, separated by 30 seconds of rest, to minimise variability and enhance measurement accuracy [22]. Subjects will then perform three maximal isometric trials for both knee extension and flexion. Each contraction will involve a rapid force production sustained for five seconds, with strong verbal encouragement provided. A 90-second rest will be given between trials and a 3-minute rest between muscle groups to limit fatigue and potentiation effects [23,24].

Peak torque and the rate of torque development (RTD) will be recorded at 0–50 ms, 0–100 ms, 0–150 ms, and 0–200 ms to capture early-phase neuromuscular performance relevant for athletic capacity and age-related functional decline [25,26]. The highest-performing trial will be retained for analysis. To normalise relative MVC, the following formulas will be used to enable better interindividual comparisons among groups:

(1) *peak torque (PT; Nm) to body mass (m; kg):*

$$\boldsymbol{RelativePT(Nm\cdot kg^{-1}) = \frac{PT(Nm)}{m\ (kg)}}$$

(2) *peak torque (PT; Nm) to total lean body mass (LBM; kg):*

$$\boldsymbol{RelativePT(Nm\cdot kg\ LBM^{-1}) = \frac{PT(Nm)}{LBM\ (kg)}}$$

(3) *peak torque (PT; Nm) to thigh lean mass from DXA segment analysis (thigh LBM; kg):*

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$$\boldsymbol{RelativePT}(\boldsymbol{Nm}\cdot\boldsymbol{kg\ thigh\ LBM^{-1}}) = \frac{\boldsymbol{PT}(\boldsymbol{Nm})}{\boldsymbol{thigh\ LBM}\ (\boldsymbol{kg})}$$

Mobility

Y-Balance Test

Dynamic balance will be evaluated using the Y-Balance Test (YBT) [27,28]. Subjects will perform the test barefoot, completing one familiarisation trial per limb to minimise learning effects, followed by one recorded trial for each leg.

During testing, subjects will maintain a single-leg stance on the central footplate while reaching maximally with the contralateral limb in the anterior, posteromedial, and posterolateral directions. Balance must be maintained without shifting the stance foot or using the reaching foot for support. Reach distances will be measured in centimetres and normalised to limb length, assessed from the anterior superior iliac spine to the distal medial malleolus [28].

Screening of Low Energy Availability: LEAM and LEAF questionnaires

Risk of low energy availability (LEA) will be screened during the familiarisation session using sex-specific, validated questionnaires designed to detect early indicators of Relative Energy Deficiency in Sport (REDs). Male participants will complete the Low Energy Availability in Males Questionnaire (LEAM-Q), whereas female participants will complete the Low Energy Availability in Females Questionnaire (LEAF-Q).

The LEAM-Q focuses on physiological and behavioural domains relevant to men, including training load, dietary habits, gastrointestinal symptoms, libido, injury history, and psychological attitudes related to eating and body image [29]. It demonstrates acceptable reliability (Cronbach's α = 0.83) and preliminary construct validity, particularly in relation to testosterone concentrations and markers of bone turnover [30]. Screening for LEA in males is essential, as REDs in men is often subclinical and may present with less overt symptoms compared with females [31].

The LEAF-Q, by contrast, is specifically validated for females and targets LEA-related dysfunction in three key physiological domains: menstrual function, gastrointestinal symptoms, and injury history—areas strongly linked to the Female Athlete Triad and REDs [32].The questionnaire shows high sensitivity (78%) and specificity (90%) for detecting LEA-related disturbances, with excellent test–retest reliability (r = 0.92) and good internal consistency (Cronbach's α = 0.71). Female athletes are particularly vulnerable to the consequences of chronic LEA, including menstrual irregularities, impaired bone health, and increased injury risk [33,34].

Together, the LEAM-Q and LEAF-Q allow for sex-specific assessment of LEA risk, reflecting the distinct physiological pathways through which REDs develops in males versus females. Questionnaire scores will be compared between male and female endurance athletes as well as their age-matched counterparts and correlated with hormonal, metabolic, physiological, and bone-health markers obtained from laboratory analyses and physical assessments, enabling an integrated evaluation of REDs-related outcomes.

Menstrual Function

As part of the LEAF-Q, menstrual function will be assessed using the questionnaire items addressing cycle regularity and self-reported menstrual disturbances. Menstrual status will be classified using LEAF-Q–consistent criteria, supported by standard clinical definitions of menstrual dysfunction [32]. Amenorrhoea will be defined as primary amenorrhoea (no menarche by age 15 years) or secondary amenorrhoea (absence of menses for >90 days). Eumenorrhoea will be defined as regular menstrual cycles occurring at intervals of approximately 21–35 days. For descriptive classification aligned with LEAF-Q responses, subjects will be categorised as eumenorrhoeic (normal menstruation with recent bleeding within ~0–4 weeks and regularity reported) or as having menstrual disturbances (oligomenorrhoea/irregular cycles and/or amenorrhoea). These categories may be collapsed into two analytical groups: normally menstruating (eumenorrhoea + regular cycles) and menstrual disturbance (oligomenorrhoea and/or amenorrhoea and/or irregular cycles. Subjects reporting current hormonal contraception will be excluded from menstrual-function analyses, as exogenous hormones can mask cycle-based outcomes. Postmenopausal master female runners (≥12 months without menses) will not complete cycle-based LEAF-Q menstrual items; menopausal status will be recorded, and menstrual-cycle classifications will be treated as not applicable in this subgroup.

The IOC REDs CAT2 Clinical Assessment Tool

To stratify the severity/risk of LEA and REDs development in endurance running groups, the IOC REDs CAT2 calculator tool will be used. The IOC REDs CAT2 is based on the 2023 IOC REDs [35] Consensus Statement, replacing the original REDs CAT [36]. The results of participants will be categorised by a standard “traffic light system”, where green colour represents no REDs or very low risk; yellow colour represents low to moderate risk; orange colour represents moderate to high risk; red colour represents high risk of REDs development. [37].

Dietary Intake Monitoring and Nutritional Analysis

Following the familiarisation session, subjects will be instructed to maintain their habitual dietary patterns during the seven-day monitoring period. They will receive standardised instructions for completing dietary records, including estimation of portion sizes and optional photographic documentation to enhance accuracy [38]. Dietary intake will be recorded for seven consecutive days (Monday–Sunday) using structured food logs.

A certified nutrition specialist will analyse all records using Planeat software (Planeat s.r.o., Bratislava, Slovakia). The analysis will include total daily energy intake, absolute and average daily macronutrient intake (carbohydrates, proteins, fats), and the temporal distribution of nutrient intake to identify within-day energy availability patterns [39,40].

Seven-day Training Monitoring Period

All endurance running groups of subjects will maintain a 7-day training diary throughout the monitoring period. For each training session, subjects will record exercise modality, session duration, distance covered, running pace, elevation gain, perceived intensity, and training frequency. Perceived intensity will be quantified using the session rating of perceived exertion (sRPE) method: subjects will report an overall session RPE on the modified Borg CR-10 scale (0–10) approximately 30 min after each session, following standardised written instructions and verbal anchoring. Participants will be familiarised with the CR-10 scale before the monitoring period using example sessions to ensure consistent interpretation. Internal training load will be calculated as sRPE training load (AU) = session RPE × session duration (min) and summarised as daily and 7-day cumulative training load.

To enhance data accuracy and completeness, training records will be complemented by session data exported from Strava (Strava Inc., USA), including time-stamped activity files and external workload parameters. Strava-derived metrics (duration, distance, pace, and elevation gain) will be used to characterise external training load and to cross-check diary entries, whereas sRPE-derived load will be used to capture internal load. These combined records will be used to characterise individual training load and to support the estimation of exercise energy expenditure.

To further characterise habitual physical activity patterns beyond structured training, daily movement behaviour will be objectively monitored using a wrist-worn triaxial accelerometer (MotionWatch 8, CamNtech). Participants will wear the device continuously for seven consecutive days, concurrent with the training diary period. Downloaded actigraphy data will be analysed using MotionWare software to derive daily wear time, to identify valid monitoring days, and to generate Day Activity Analysis summaries including counts and duration of activity across intensity thresholds. Time spent in sedentary, light, moderate, and vigorous intensity activity will be quantified (min·day^{-1}) based on the activity count thresholds configured in MotionWare, and moderate-to-vigorous physical activity (MVPA) will be calculated as the combined duration of moderate and vigorous activity. In addition, average daily activity counts will be extracted from MotionWare outputs as an index of overall movement volume independent of intensity categorisation. Given the wrist-based placement of the accelerometer, participants were explicitly instructed not to cover the device with clothing or accessories during the monitoring period, either indoors or outdoors, to minimise signal attenuation and ensure consistent detection of movement-related activity. These parameters will be used to complement training diary and Strava-derived data, allowing a comprehensive assessment of daily physical activity exposure.

Energy Intake and Energy Balance

Energy intake (EI) will be assessed using a 7-day dietary intake monitoring based on participant-completed food diaries. Daily body mass (BM, kg) will be recorded, and fat-free mass (FFM, kg) will be obtained from dual-energy X-ray absorptiometry (DXA). From MotionWatch-derived actigraphy data, daily wear time and valid monitoring days will be identified, and time spent in sedentary, light, moderate, and vigorous intensity activity (t_sed, t_low, t_mod, t_vig; min·day^{-1}) will be extracted using MotionWare based on predefined activity count thresholds; total accumulated time across all epochs will be used for energetic calculations.

Resting metabolic rate (RMR) will be predicted using a fat-free mass–based equation (Cunningham):

$$\boldsymbol{RMR_{day} = 500 + 22 \times FFM}$$

Physical activity energy expenditure (PAEE) will be estimated from actigraphy-derived time spent in light, moderate, and vigorous intensity activity by assigning standard metabolic equivalent (MET) values to each intensity category (light: 2.0 METs; moderate: 4.0 METs; vigorous: 8.0 METs) and subtracting 1 MET to account for resting energy expenditure. PAEE will be calculated as:

$$\boldsymbol{PAEE_{day} = BM \times [(2.0 - 1)\frac{t_{low}}{60} + (4.0 - 1)\frac{t_{mod}}{60} + (8.0 - 1)\frac{t_{vig}}{60}]}$$

The thermic effect of food (TEF) will be estimated as 10% of daily energy intake:

$$\boldsymbol{TEF_{day} = 0.10 \times EI_{day}}$$

Estimated total daily energy expenditure (eTEE) will be calculated as:

$$\boldsymbol{eTEE_{day} = RMR_{day} + PAEE_{day} + TEF_{day}}$$

Daily estimated energy balance (EB) will be calculated as:

$$\boldsymbol{eEB_{day} = EI_{day} - TEE_{day}}$$

and further normalised to fat-free mass (FFM) obtained from DXA as:

$$\boldsymbol{EB_{F}FM, day = \frac{EI_{day} - TEE_{day}}{FFM}}$$

expressed in kcal·kg FFM^{-1}·day^{-1}.

Mean estimated energy balance across the 7-day monitoring period will serve as the primary indicator of chronic energetic stress and will be interpreted in combination with energy availability estimates and validated REDs screening tools (LEAF-Q, LEAM, and IOC REDs CAT2).

Low Energy Availability Assessment

In this study, estimated energy balance (eEB) serves as the primary energy-related outcome, while classical energy availability (EA) is considered a secondary and exploratory measure, reflecting current methodological challenges in accurately quantifying exercise energy expenditure in endurance runners. EA will be estimated only where sufficient exercise energy expenditure data are available and interpreted in conjunction with REDs screening tools.

Low energy availability (LEA) will be assessed using the classical EA framework. Energy intake (EI) will be assessed using the same 7-day dietary intake monitoring as described above. Exercise energy expenditure (EEE) will be quantified based on a 7-day structured training diary of each participant and training session records exported from Strava (Strava Inc., USA), including exercise modality, duration, and intensity. These data will be used to identify exercise bouts and estimate daily exercise energy expenditure. FFM will be obtained using DXA.

Daily energy availability will be calculated using the following equation:

$$\boldsymbol{EA_{day}} = \frac{\boldsymbol{EI_{day} - EEE_{day}}}{\boldsymbol{FFM}}$$

and will be expressed as kcal·kg $\mathrm{FFM}^{-1}\cdot\mathrm{day}^{-1}$.

Mean daily EA across the 7-day monitoring period will be used to characterise the habitual energy availability of participants. EA values below ~30 kcal·kg $\mathrm{FFM}^{-1}\cdot\mathrm{day}^{-1}$ will be considered indicative of low energy availability (LEA), based on established physiological thresholds [41,42]. Interpretation will follow the International Olympic Committee (IOC) consensus framework [31,35]. Calculated EA values will be interpreted in conjunction with validated screening tools (LEAF-Q, LEAM, and IOC REDs CAT2). These tools will not be used diagnostically but rather as screening instruments within the REDs risk framework.

Blood Sample Collection, Biochemical and Metabolic Analyses

Venous blood samples will be collected 13 days after the physical performance testing session. Performance testing will be followed immediately by a free-living monitoring period, during which dietary intake and daily physical activity are recorded. Upon completion of monitoring, participants will observe a short period without structured training to minimise acute exercise-induced alterations in biochemical, metabolic, and endocrine markers. Blood sampling will therefore reflect habitual physiological status rather than short-term post-exercise responses.

To minimise acute exercise–related fluctuations in circulating biomarkers, participants will refrain from strenuous activity for at least 48 hours prior to sampling [43,44,47,48]. During this period, young and master endurance runners will be allowed one low-intensity training session in heart rate zone 2 (60–70% HRmax), limited to 60 minutes for young adults and 45 minutes for masters, to maintain habitual physiological states without provoking acute metabolic or endocrine responses [31].

Blood sampling will be performed by a certified nurse at the hospital between 7:00 and 7:30 a.m. Subjects will arrive by private transport, rested, and in an overnight fasted state (≥10 hours) to standardise hormonal and metabolic conditions across groups.

Biochemical, metabolic, and endocrine analyses will be conducted in ISO-accredited commercial laboratories. The assay panel will include a comprehensive panel of haematological, biochemical, metabolic, inflammatory, and hormonal markers, as well as markers of energy availability, bone metabolism, and endocrine status, with particular attention to biomarkers relevant to the Female Athlete Triad and Relative Energy Deficiency in Sport (REDs) [33,34].

Hormonal status will be evaluated through measurement of sex hormones and related indices, including estradiol, total testosterone, free testosterone, biologically available testosterone, sex hormone–binding globulin (SHBG), and derived androgen indices. In female participants, menopausal status was recorded and reported to account for age-related endocrine changes. Hormonal data will be interpreted in relation to sex, age category, and training status in order to control for potential confounding effects of hormonal variability and to support physiologically meaningful interpretation of endocrine outcomes.

Insulin resistance as a metabolic parameter will be estimated using the Homeostatic Model Assessment of Insulin Resistance (HOMA-IR), calculated as:

$$\textit{HOMA-IR} = [\textit{Fasting Insulin } (\mu U/mL) \times \textit{Fasting Glucose } (mmol/L)] / 22.5$$

Menopausal Status

In female participants, menopausal status will be assessed using a structured questionnaire, including the age at natural menopause onset and current bleeding status. Self-reported menopausal status will be contextualised using circulating sex hormone concentrations obtained from venous blood analyses to provide objective endocrine support for age-related hormonal status. Menopausal status will be recorded and reported as a descriptive participant characteristic and considered in the interpretation of hormonal, metabolic, and bone-related outcomes, without being used as a diagnostic or primary grouping criterion.

Muscle Biopsy

Muscle biopsies will be obtained at 08:00 a.m. following fasting blood collection. Muscle biopsy procedures are clearly described in the informed consent provided prior to enrolment, and participants will be informed that participation in this procedure is voluntary. Biopsies will be performed by a licensed physician with nursing assistance at the hospital. Under local anaesthesia (2% lidocaine), a percutaneous biopsy will be taken from the mid-portion of the right vastus lateralis using a 5-mm Bergström needle [49] with manual suction to optimise tissue yield [45],

followed by standardised processing procedures for immunohistological analyses [48].

Post-collection, visible connective and adipose tissue will be removed. A sample will be embedded in OCT, snap-frozen in isopentane cooled in liquid nitrogen for cryosectioning, and all samples will be stored at −80 °C until analysis [48].

Variables derived from muscle biopsy analyses will be treated as secondary exploratory outcomes and interpreted within the broader physiological context of endurance training, ageing, and REDs risk.

Immunohistochemical Analysis of Skeletal Muscle Tissue

Frozen muscle samples will be cryosectioned into 8 μm transverse sections at −20 °C using a calibrated cryostat (CM3050 S; Leica Microsystems, Wetzlar, Germany). Sections will be mounted on adhesive slides (Superfrost Plus; Thermo Fisher Scientific, Waltham, MA, USA), air-dried, and stored at −80 °C until immunohistochemical analysis.

Immunohistochemistry will be used to evaluate myonuclear content, satellite cells identified by Pax7, capillary density via endothelial markers (e.g., CD31), and muscle fibre type distribution using myosin heavy chain isoforms [50–52]. Analyses of the samples will be carried out using ImageJ and TEMA software.

Statistical Analysis

Statistical analyses will be performed using standard statistical software (version 10; GraphPad Software, San Diego, CA, USA). Data will be inspected for completeness, normality, and homogeneity of variance prior to analysis. Normality of distribution will be assessed using the Shapiro–Wilk test, and homogeneity of variances will be evaluated using Levene's test. Descriptive statistics will be reported as mean ± standard deviation (SD) for normally distributed variables or median (interquartile range) for non-normally distributed variables.

To ensure a clear statistical hierarchy and reduce the risk of multiplicity, study outcomes were classified *a priori* as primary and secondary. Primary outcomes include measures of cardiorespiratory fitness, reflecting the main physiological adaptations to long-term endurance training and ageing. Secondary outcomes comprise additional anthropometrical, functional, cardiometabolic, biochemical and skeletal muscle variables intended to provide complementary physiological context.

Between-group differences in primary and secondary outcomes will be examined using factorial analysis of variance (ANOVA) with sex (female vs. male), age category, and training status (endurance-trained vs. sedentary) included as fixed factors. Where appropriate, interaction effects between sex, age category, and training status will be explored. In the presence of significant main or interaction effects, post hoc comparisons with appropriate correction for multiple comparisons will be applied to control the family-wise error rate. Given the exploratory nature of some secondary outcomes, no formal adjustment for multiplicity is applied beyond post hoc family-wise error control.

For variables that violate assumptions of normality or homogeneity, non-parametric alternatives will be used as appropriate. In addition to p-values, effect sizes will be systematically reported to facilitate the interpretation of the magnitude and practical relevance of observed differences. Partial eta squared (η^2_p) will be reported for main and interaction effects derived from ANOVA models, while Cohen's d will be calculated for pairwise comparisons. Effect sizes will be interpreted according to established conventions.

Associations between selected physiological, performance, and body composition variables will be explored using correlation analyses, with the choice of correlation coefficient determined by data distribution. Statistical significance will be set at $p < 0.05$.

3. Discussion

This study aims to examine how sex and ageing jointly influence physical fitness, physiological adaptations, endurance performance, and REDs risk in young and master endurance runners. A secondary aim is to determine whether long-term endurance running can preserve physiological function and mitigate age-related decline.

Extensive scientific evidence suggests that long-term endurance running in advanced age is effective in attenuating age-related physiological decline [53–55]. However, its effects differ between male and female runners due to various underlying, yet unclear biological and physiological mechanisms. Therefore, a deeper understanding of the physiological changes associated with ageing and sex differences in endurance runners is crucial not only for their training optimisation and performance [56–58].

Current research highlights several critical gaps in knowledge, particularly regarding female endurance runners and the ageing process. The scientific evidence remains limited or inconsistent concerning long-term cardiovascular and musculoskeletal remodulations and adaptations in master female runners, endocrine system fluctuations associated with prolonged excessive endurance training, and the complex interactions between excessive endurance exercise, chronic low energy availability, and physiological maladaptation across the lifespan. These mechanisms warrant further investigation from physiological, health, and performance perspectives. Importantly, such research should not be limited only to female athletes, as growing evidence indicates REDs also affects male endurance runners, underscoring the need for sex-inclusive research approaches.

Clinical and Practical Implications

The outcomes of this study protocol will have direct implications for clinical and applied practice in endurance sports. By integrating cardiorespiratory fitness, musculoskeletal health, endocrine status, and REDs screening, the study framework may support sports physicians and sports nutrition professionals in distinguishing healthy long-term endurance adaptations from early maladaptive responses, particularly in ageing athletes. This approach may facilitate earlier identification of REDs risk and more individualised preventive strategies across sex and age categories.

Expected Outcomes of the Study

It is expected that endurance-trained master athletes will demonstrate more favourable cardiorespiratory fitness and body composition profiles compared with age-matched sedentary controls. Differences between training status, age category, and sex are anticipated to emerge across selected physiological and cardiometabolic outcomes, reflecting long-term adaptations to endurance exercise and the ageing process. Given the growing recognition of low energy availability as a key factor underlying REDs, this study may also provide indirect insight into how chronic endurance training, in combination with age- and sex-specific energy demands, relates to physiological health markers in older athletes. Although causal relationships cannot be established due to the cross-sectional design, the findings are expected to contribute to a better understanding of the interaction between endurance training, energy availability, and healthy ageing, thereby informing future longitudinal studies and preventive strategies in master athletes.

Strengths of the Study

The main strength of this cross-sectional study is its comprehensive and multimodal assessment strategy. The comprehensive methodology enables a holistic linkage between physical performance, nutritional status, and overall health status across age and sex. The study further benefits from a robust design that includes young and master endurance runners alongside with their age- and sex-matched inactive counterparts, allowing precise evaluation of the effects of ageing, long-term endurance training, and sex differences. Moreover, the use of standardised screening tools for LEA strengthens the ability to detect REDs risk in a clinically relevant age and sex-specific manner in endurance runners.

Limitations of the Study

On the other hand, this study also has several important limitations. An important limitation of this study is that its cross-sectional design limits the ability to capture long-term physiological and health changes in the subgroups of the endurance runners. This short observational time frame is only focused on a single one-week period, where all assessments of subjects' habitual week are conducted. Therefore, this study design does not allow for longitudinal monitoring of training load, dietary patterns, hormonal fluctuations, or menstrual cycle–related variability in female participants, which may influence energy availability, hormonal responses, and REDs manifestation over longer time scales. Additionally, the REDs screening tools used (LEAF-Q, LEAM-Q, IOC REDs CAT2) rely only on self-reported symptoms. Thus, these tools may be vulnerable to recall bias; however, their use in combination with objective physiological and biochemical measures strengthens overall interpretability. Moreover, the REDs risk categories, which derive from questionnaires, must be interpreted with caution, complemented by other objective physiological and biochemical markers collected in this study and afterwards interpreted by a responsible medical specialist.

4. Conclusions

This study protocol presents a comprehensive cross-sectional framework to investigate the combined effects of ageing, biological sex, and long-term endurance running on physiological performance, musculoskeletal and bone health, nutritional status, and the risk of REDs development. The protocol is designed to generate a multidimensional dataset across young adult and master endurance runners and their age-matched inactive counterparts. Importantly, this protocol has clear clinical and practical relevance, providing a structured framework for translational application in endurance athletes across the lifespan.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki, and approved by the Ethics Committee of the University Hospital Bratislava – Hospital of Ladislav Dérer (No. 31/2020, date 2022-12-13).

Informed Consent Statement: Informed consent will be obtained from all subjects involved in the study.

Data Availability Statement: Data will be available upon request from the authors of the article.

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Abbreviations

ACSM	American College of Sports Medicine
ALM	Appendicular Lean Mass
ALMI	Appendicular Lean Mass Index
BMC	Bone Mineral Content
BMD	Bone Mineral Density
BMI	Body Mass Index
CAT2	IOC REDS Clinical Assessment Tool 2
CD31	Cluster of Differentiation 31 (endothelial marker)
DXA	Dual-Energy X-Ray Absorptiometry
EA	Energy Availability
EB	Energy Balance
ECG	Electrocardiography
EEE	Exercise Energy Expenditure
EI	Energy Intake
FFM	Fat-Free Mass
FFMI	Fat-Free Mass Index
HOMA-IR	Homeostatic Model Assessment of Insulin Resistance
HRmax	Maximum Heart Rate
IOC	International Olympic Committee
LEA	Low Energy Availability
LEAF-Q	Low Energy Availability in Females Questionnaire
LEAM-Q	Low Energy Availability in Males Questionnaire
LBM	Lean Body Mass
MVC	Maximal Voluntary Contraction
OCT	Optimal Cutting Temperature Compound
Pax7	Paired Box Protein 7 (satellite cell marker)
PT	Peak Torque
REDs	Relative Energy Deficiency in Sport
RER	Respiratory Exchange Ratio
RTD	Rate of Torque Development
SPIRIT	Standard Protocol Items: Recommendations for Interventional Trials
TEE	Total Energy Expenditure
VE	Ventilation
VO_2	Oxygen Uptake
VO_2max	Peak Oxygen Uptake
YBT	Y-Balance Test

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