Response to physical activity of females with multiple sclerosis throughout the menstrual cycle: a protocol for a randomised crossover trial (EMMA Project)


ABSTRACT
The relationship between multiple sclerosis (MS) and females is a crucial aspect in the development of the disease, with the oestrogen hormonal cycle being a sensitive stage, especially in females with relapsing-remitting multiple sclerosis. The objectives of the study are to identify moderating variables that modify satisfaction with physical activity practice throughout the menstrual cycle (MC) in females in or out of their MC, during high-intensity interval training (HIIT) and strength training sessions and to compare the acute effects of different types of physical activity sessions in females with and without MS. This protocol is the methodology used in the EMMA Study, a randomised, single-blind crossover trial study conducted in females with MS who were matched 1:1, based on age, lifestyle factors and country of residence, with females without MS, to analyse the effect of physical activity practice on satisfaction, functionality, fatigue and inflammatory profile through their MC. Participants will visit the facilities approximately 10 times (4 preliminary familiarisation visits and 6 visits to carry out a physical activity session in each phase of the MC) for 3–4 months. A total sample of 30 females (15 females without MS and 15 with MS) is necessary for the study. The evaluation will comprise clinical, nutritional and psychological interviews, including different variables. It is hypothesised during the luteal phase, females with MS are expected to exhibit different acute responses to HIIT and strength training sessions as compared with females without the disease. Before starting the study, all participants will read and sign an informed consent form. Trial registration number: This research protocol is registered with ClinicalTrials.gov to ensure transparency and accessibility of study information (NCT06105463). The university’s ethics committee number for this study is UALBIO2022/048.

WHAT IS ALREADY KNOWN ON THIS TOPIC
⇒ Multiple sclerosis (MS) predominantly affects females and exhibits a complex relationship with the menstrual cycle. Hormonal fluctuations during the menstrual cycle have been linked to changes in MS symptoms, but the acute responses to physical activity in different menstrual cycle phases are not well understood. Existing knowledge emphasises the importance of gender and hormonal factors in the clinical management of MS.

WHAT THIS STUDY ADDS
⇒ This study, part of the EMMA Project, aims to investigate how satisfaction with physical activity varies throughout the menstrual cycle in females with and without MS. It also explores the acute effects of different physical activity sessions (high-intensity interval training and strength training). The study examines hormonal, functional and psychological aspects, shedding light on the interplay between MS, gender and the menstrual cycle, with implications for tailoring exercise regimens for individuals with MS.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY
⇒ The EMMA Study can contribute to more personalised exercise programmes for females with MS by considering their menstrual cycle phase. The findings may provide insights into the role of hormonal fluctuations in symptom exacerbation and the effects of high-intensity interval training and strength training. This knowledge could enhance MS management strategies, emphasising gender-specific approaches and optimising outcomes for individuals with MS. The study’s results are expected to have significant implications for research, practice and policy, contributing to a better understanding of how the menstrual cycle affects the acute response to exercise in females with MS.

INTRODUCTION
Multiple sclerosis (MS) is one of the most prevalent neurological diseases that affect young adults, typically occurring in individuals aged...
between 20 and 40 years old, with a higher incidence in females, in a ratio of approximately 2.3:3.5. The total prevalence is 33 cases per 100 000 people, affecting more than 2 million individuals worldwide. Interestingly, the global incidence and prevalence of MS are increasing, even in regions previously considered to be low risk. This chronic disease significantly affects individuals, leading to disability and impairment, making their personal, social and occupational lives very challenging.

Although the aetiology of MS is unknown, it is believed to be a multifactorial disease resulting from the interaction of genetic, infectious and environmental factors (such as smoking, the ubiquitous γ-herpes virus, vitamin D deficit and exposure to sunlight). These factors, along with hormonal influences, contribute to the sexual differences observed in MS. Females experience more frequent relapses in relapsing-remitting MS (RRMS), particularly during their perimenopausal stage (i.e., with active ovarian function). They also develop more inflammatory lesions and have an earlier onset. Furthermore, the age of menarche is inversely associated with the risk of MS, and a lower relapse rate has been observed during pregnancy and menopause. In comparison, a higher relapse rate occurs after delivery. These findings highlight the importance of sex-specific approaches in the clinical management of MS, suggesting meaningful connections between gender, hormonal status and disease progression.

More specifically, menstruating females with MS during their premenstrual phase, and especially those with RRMS, could experience a worsening of symptoms due to exacerbation (new demyelinating plaques) and pseudoevacabration (worsening of pre-existing deficits due to previous plaques). Additionally, body temperature fluctuates during the menstrual cycle (MC), increasing shortly after ovulation and remaining elevated throughout the luteal phase. This temperature increase can lead to the Uhthoff phenomenon, a temporary worsening or exacerbation of neurological symptoms due to heat. Furthermore, hormonal changes during the MC also moderate symptom fluctuations in females with MS.

The long-term immunomodulatory effect of exercise is well-known. It has been shown that it can lead to a reduction in inflammation and the partial regulation of neuroimmune parameters in T cell behaviour. On the other hand, in healthy females, it has been observed that during physical activity (PA), hormonal fluctuations also lead to differences in performance and muscle damage. The perception of delayed-onset muscle soreness (DOMS) and loss of strength in response to PA is lower during the luteal phase when sex hormone concentrations are high. At the same time, during the early follicular phase (EFP), a greater perception of DOMS and a more significant loss of strength are observed. In this sense, the acute effects of exercise lead to an increase in T cell subsets 30 min after completing PA, followed by a decrease between 1 and 2 hours in healthy females. Training intensity modifies the immunomodulatory effect of T cell subpopulations and cytokines in healthy populations, generating a chronic response to immune system impairment. Therefore, PA could induce acute adverse events in females with MS during their reproductive years, especially during some phases of the MC, due to temperature changes and the acute inflammatory response to PA. However, to date, the acute response to exercise in females with MS and its relationship with hormonal fluctuations is still unclear.

Exercise has been proposed as a potential approach to improve MS symptoms, with aerobic exercise and strength training being the most commonly used and effective exercise modalities within the female population. Furthermore, it is worth noting that high-intensity interval training (HIIT) shows promise in enhancing fitness outcomes for individuals with MS.

In this context, HIIT and strength training emerge as promising exercise strategies. However, achieving substantial benefits requires a minimum number of exercise sessions, underscoring the importance of adherence to the exercise programme when demonstrating positive effects. While the exact dropout and adherence rates in exercise programmes for MS remain unclear, several factors, such as age, gender distribution, disability level, exercise modality (HIIT vs strength training), intervention duration and specific exercises included, could potentially influence the dropout rate in physical exercise programmes. Hence, studying satisfaction with practising these exercises following a session is crucial.

Understanding patients’ experiences and satisfaction levels can contribute to designing more effective and sustainable exercise programmes for those living with MS.

Even though age and gender are key factors that influence adherence and optimising outcomes, studies on MS and exercise often involve mixed groups comprised of both men and women without considering age or the biological phase. Additionally, there is a lack of exploration of acute responses during the different MC phases.

Although some studies have examined the influence of MC on symptomatology, as well as on disease progression and rhythm, to our knowledge, no studies have specifically determined the acute response to PA in females based on the hormonal profile derived from the MC phases. Therefore, studies exclusively focused on females with MS are essential to understand better the potential relationship between MC, PA practice and MS. Furthermore, the relationship between MS and females is a crucial aspect in the development of the disease, with the ovarian hormonal cycle being a sensitive stage, especially in females with RRMS.

Thus, the objectives of the study are: (a) to identify moderating variables that modify satisfaction with HIIT and strength training sessions throughout the MC in females with and without MS, and (b) to compare the acute effects of different types of PA sessions (HIIT and strength training) throughout the MC, in females with and without MS.
The central hypotheses of this study are the following: first, during the luteal phase of the MC, females with MS are expected to show a stronger association between inflammation and perception (fatigue and energy) and hormonal levels, as compared with females without the disease. Furthermore, HIIT and strength training sessions are expected to impact satisfaction with PA practice in both populations throughout the MC. During the luteal phase of the MC, females with MS are expected to exhibit different acute responses to HIIT and strength training sessions as compared with females without the disease.

**MATERIAL AND METHODS**

**Design**

The present protocol describes the methodology of the EMMA Study, a randomised, single-blind crossover trial conducted on females with MS, matched 1:1 based on age, lifestyle factors (smoking, PA) and country of residence, with females without MS, to analyse the effect of PA practice (HIIT vs strength training) throughout the MC. To achieve this, the Consolidated Standards of Reporting Trials recommendations and protocols and previous studies that analysed the impact of the MC in females without MS will be followed. Participants will visit the facilities approximately 10 times (4 preliminary familiarisation visits and 6 visits to carry out HIIT and strength training session in each phase of the MC) over 3–4 months (figure 1).

**Participants**

Females diagnosed with RRMS according to McDonald’s criteria and certified by their neurologist will be invited to participate in the study voluntarily. Recruitment will be carried out in collaboration with Regional Multiple Sclerosis Associations and hospitals in the provinces of Almería, Alicante, Jaén, Toledo and Murcia. The established inclusion and exclusion criteria will be analysed for all females who voluntarily express their interest in participating.

An a priori sample size estimate was performed using G*Power V.3.1 software (Heinrich-Heine-University, Düsseldorf, Germany) to estimate the number of females required for the study. An a priori sample size estimation was performed, setting the significance level at α=0.05, the power at β=0.95, a large effect size of f(v)=0.80 and a sphericity correction of 0.2 (1/repetitions−1). Additionally, a minimum dropout rate of 25% was considered following Hopkins et al’s recommendations. Since the study aims to analyse the effect of hormonal fluctuations, a previous study that analysed hormonal differences in females with regular cycles was used. A sample of 60 females (30 females without MS and 30 with MS) is necessary for the study. In the current study, the sample size is larger than in previous clinical trials that analysed the acute effects of PA on MS. Nevertheless, several studies have employed sample sizes ranging from 15 to 20 individuals with sclerosis and 15–20 without sclerosis. Therefore, the study will consider a minimum of 15 per control group.

The inclusion criteria will be as follows: (a) women aged between 18 and 40 years old; (b) females with an MC length of ≥21 days and ≤35 days of natural menstruation; (c) absence of iron deficiency anaemia (serum ferritin >20 µg/L, haemoglobin >115 µg/L and transferrin saturation >16%); (d) being in a stable phase of the disease; and (e) to ambulate autonomously for more than 100 m. Participants will be excluded if they have: (a) a score <2 or >6 on the Expanded Disability Status Scale; (b) experienced a relapse in the 12 months before enrolment; (c) received corticosteroid treatment in the previous 2 months; (d) participated in a structured exercise programme in the past 6 months; (e) secondary amenorrhoea (absence of ≥3 consecutive periods despite not being pregnant and having previous menstruation); (f) used or currently using hormonal contraceptives for 3 months before recruitment; (g) reported musculoskeletal or neurological injuries not associated with MS, recent surgical interventions or pregnancies in the previous year; (h) diseases unrelated to MS. Furthermore, exercise and dietary restrictions and recommendations will be established 24 hours before the sessions, on the intervention day, and 24, 48 and 72 hours after, following the recommendations outlined in Peinado et al. Participants will be instructed not to modify their lifestyle during their participation in the project.
In addition to these inclusion and exclusion criteria, participants will be required to provide information, following the recommendations of Elliott-Sale et al., pertaining to the number of pregnancies (single or multiple pregnancies); age at menarche and any complications; previous MC irregularities; contraceptive use in the 12 months before the study, specifying the type (implants, injections, intrauterine devices/hormonal intrauterine systems, vaginal rings, transdermal patches) and formulation; the number of pregnancies resulting in gestational age of 24 weeks or more, regardless of whether the child was born alive or stillborn. Additionally, data on habitual PA (International Physical Activity Questionnaire), daily caffeine intake, dietary intake and nutritional supplements, alcohol consumption and smoking will be collected. Participants will also be asked to indicate the date of their MS diagnosis and the number of relapses.

Once all females with MS have been recruited, the enrolment of females without MS will begin, using a 1:1 matching methodology based on age, geographical area, lifestyle factors, and inclusion and exclusion criteria related to the MC. Before starting the study, all participants will read and sign an informed consent form. Furthermore, the project will adhere to the ethical principles of medical research involving human subjects described in the Declaration of Helsinki of the World Medical Association. This project and its procedures have also been registered in the US National Library of Medicine’s ClinicalTrials.gov database (NCT06105463).

Procedure

The methodological guidelines established by Elliott-Sale et al. for research in sports and exercise sciences with female participants will be followed to conduct the study. In addition, the recommendations proposed by Schmalenberger et al. and Janse de Jonge et al. for studying the MC will also be considered.

Familiarisation, MC monitoring and determination of MC phases

For this study, three out of the widely considered four phases of the MC will be selected: phase 1 (EFP), when oestrogen and progesterone are at their lowest blood concentrations; phase 2 (late follicular phase: LFP), when the highest oestrogen concentration occurs while progesterone remains low; and phase 4 (mid-luteal phase: MLP), the highest concentration of progesterone and a high concentration of oestrogen occur (figure 2). These phases will be identified following the protocols proposed by Peinado et al. and Janse de Jonge et al.

During the 2–3 months before the intervention, familiarisation with the tests and PA sessions will be conducted. This familiarisation period will start with MC monitoring, allowing the appointment setting for/in each MC phase with little margin of error. For the monitoring of the MC and the setting of the appointments, the experimental considerations described below will be followed:

- Monitoring will be carried out starting 3 months before starting the intervention. Participants will be requested to provide information regarding the starting date of their six previous menstruations to estimate their MC duration. Then, during the two to three subsequent MCs, period starting dates will be collected concurrently with the familiarisation stage.
- Luteinising hormone (LH) peak detection urine kits will detect LH surge, ovulation, and, retrospectively, the LFP. This test will allow us to confirm that the MCs included in the study are ovulatory, as anovulatory cycles can occur even in normal cycles with regular menstruation.
- In addition, serum hormone concentrations (see Reproductive hormonal profile subsection) will be measured for each training session retrospectively since this is a direct method and is considered the gold standard for research purposes.

Therefore, a three-step method will be used for MC phase confirmation, combining the calendar method, qualitative determination of LH peak in urine and analysis of serum hormones, following the protocols described by Peinado et al. and Janse de Jonge et al. After the familiarisation period, a posteriori exclusion of participants and/or cycles who/which do not fulfil the levels of oestrogen and/or progesterone concentrations expected for each phase will be applied.

Additionally, familiarisation sessions will be conducted, employing the exercises used in the HIIT and strength training sessions, and these sessions will also be used to estimate the 1-RM (repetition maximum) and maximal oxygen consumption (VO$_2$max) of each participant.

Randomisation

In this crossover study, the evaluation order will be counterbalanced according to the MC phases (EFP, LFP and MLP) using codes 1–3. Participants and their counterparts will undergo evaluations in different sequences: 1–2–3; 2–3–1; 3–1–2; 2–1–3; and 1–3–2. Additionally, the type of exercise (HIIT: 1 or strength training: 2) they will perform in each phase will be randomised. This randomisation approach will ensure an equitable distribution of the effects of the MC and the types of exercise among participants, contributing to obtaining more robust and reliable results for the analysis. The ‘OxMaR’ software will be used for the participant randomisation process.

Experimental procedures

All sessions will be conducted at the same time of day to avoid different responses to changes in circadian rhythm, and the temperature (21–22°C) and humidity (45–60%) of the evaluation areas will be controlled. In the first visit, fasting (4 hours) and resting without exercising in the previous 24 hours will be required. First, participants will be informed about the study’s objectives, sign the informed consent forms, complete the health and lifestyle questionnaires, and the inclusion and exclusion criteria will be assessed. Subsequently, descriptive data
will be collected, and body composition and anthropometric tests will be performed. Finally, blood samples will be collected. Second and third visits will be performed to familiarise the participants with the maximal ramp test on a cycle ergometer and 1-RM exercise to individualise the physical exercise sessions and to familiarise them with the different tests. Subsequent visits will be scheduled according to the timing of the MC phases estimated in the monitoring months. A pre-intervention, immediate post-intervention, 24, 48 and 72-hour post-intervention assessment will be performed at each MC phase (hormone profile). A pre-intervention and post-intervention assessment will be performed at each MC phase (hormone profile). A HITT and strength training session will be performed between the pre-assignment and post-assignment. Table 1 shows the activities to be performed in each MC. This procedure will be performed twice (HITT and strength training sessions). All outcomes will be assessed by study staff blinded to group assignments as far as possible.

**Exercise training sessions**

The characteristics of each HITT and strength training session that participants will perform have been designed according to the recommendations provided by guidelines and results reported in recent meta-analyses published by Andreu-Caravaca et al. and Campbell et al., to optimise the long-term results of physical exercise programmes.

**HITT session protocol**

The proposed protocol for the HITT session is based on previous studies conducted with this population on a cycle ergometer, with the following distribution: 5 min of warm-up at 50% of peak power+20 min of HITT consisting of 30 s at 90% of peak power, followed by 30 s of passive rest+5 min of cool-down at 35–40% of peak power. Participants will be asked to maintain a cadence between 50 and 80 revolutions per minute (rpm); if participants do not achieve 50 rpm, they will be encouraged to cycle as fast as they can (but not less than 40 rpm). Peak power will be previously determined through an incremental cycle ergometer test using the cycle ergometer (Ergoselect 200, Ergoline, Germany), which will include 5 min of warm-up with unloaded cycling followed by a ramp incremental test with 25 W increments every 2 min until...
exhaustion. The test will be terminated when the participants reach volitional exhaustion or the cadence drops by 10 rpm.25 32 During the test and the sessions, the heart rate will be measured by an H10 heart rate monitor (Polar Electro, Kempele, Finland), and the cardiorespiratory response will be analysed using a gas analyser VO2 Master Pro (VO2 Master Health Sensors, Vernon, British Columbia, Canada). Peak power will also be established during each of the MC phases. Peak power will serve as a baseline at each of the hormone phases.

**Strength training session protocol**

Participants will perform a standardised warm-up protocol (5 min on a stationary bicycle, mobility of the lower extremities and 5 repetitions at 40% 1-RM on each machine) before each strength training session. Subsequently, participants will perform four lower limb exercises, including bilateral leg press, unilateral leg extension, unilateral hip extension and bilateral seated calf raise on conventional weight machines (Fittech, Viseu, Portugal). The session’s intensity will be 70–75% of 1-RM, performing a volume of 4 sets of 10 repetitions, leaving 1–2 repetitions in reserve (RIRs) and 120-second rest between sets. The 1-RM will be calculated for each exercise in the familiarisation phase.

The 1-RM load will be estimated using the following protocol: 1 set of 10 repetitions at 50% of the estimated 1-RM, 1 set of 5 repetitions at 75% of the estimated 1-RM, finishing with 1 set of 1 repetition at 100% of 1-RM. A 5-minute rest period will be allowed between sets. If a participant can complete more than one repetition in
the last set, the 1-RM will be estimated based on recommendations published in the literature. Supervisors will instruct participants to lower the weight in a controlled manner, pause briefly at the end of the movement and then contract the muscle as fast as possible (concentric phase) to maximise the neural component. In the training session, the load will be increased by 2–5% when the participants can achieve two more repetitions than the predetermined ones, always with two RIRs.

During the session, the supervisors will fill out a control sheet where the weight lifted in each exercise, the completed repetitions and sets, and RIR will be recorded. All sessions will be supervised by the same certified trainer, specialised in strength training. The 1-RM will be established during the control phases of the MC, and a baseline will be set at different points in the hormonal phase for the study.

Outcome measures
All outcomes will be conducted and interpreted by study personnel who are blinded to group allocation (table 1).

Primary outcomes
Satisfaction
Satisfaction with PA will be assessed using an eight-item scale adapted from previous studies. The questionnaire begins with the statement ‘When I am doing PA’, followed by the items ‘I am satisfied with the results of my PA’, ‘I enjoy/feel good when I have done PA’, ‘PA has many advantages’ and ‘I find PA nice/difficult’. Responses to this single-item question will be measured on a 5-point Likert scale, ranging from 1 (very dissatisfied) to 5 (very satisfied). Satisfaction will be assessed before, immediately following the training session, and at 24, 48 and 72 hours.

Visual Analogue Scale for Fatigue
Visual Analogue Scale for Fatigue (VAS-F): to measure fatigue, the VAS-F will be used. The VAS-F will be assessed before, immediately following the training session, and at 24, 48 and 72 hours. This scale, developed by Lee et al., is subdivided into two subscales: fatigue and energy. The VAS-F presents a horizontal line measuring 100 mm in length, with the term ‘none’ at one end and ‘very severe’ at the opposite end. Participants must mark the point on the line corresponding to their perception of fatigue severity between these two endpoints. The fatigue subscale is organised, from the most positive to negative items. Conversely, the energy subscale ranges from the most negative to the most positive items. A high score on the VAS-F indicates a low score on the energy subscale and a high severity level on the fatigue subscale. This scale is widely used in the general population and clinical patients due to its brevity, ease of use and comprehensibility. The fatigue subscale of the VAS-F shows a Cronbach’s α of internal consistency of 0.90. The Cronbach’s α for the energy subscale is 0.74. Participants will be instructed to consider their overall level of fatigue when responding to the scale rather than a specific moment.

Secondary outcomes
Secondary outcomes will be assessed at various time points, with specific protocols outlined in online supplemental material.

Baseline outcomes measured in each MC (control outcomes)
Baseline assessments will be conducted in each MC, including evaluations of abdominal obesity and anthropometric variables, dietary and nutritional monitoring, monitoring of PA levels and walking endurance.

Measurements in each phase of the MC
Throughout different phases of the MC, various measurements will be carried out. These assessments include the evaluation of physical self-perception, assessment using the Catastrophizing Pain Scale, analysis of the Modified Fatigue Impact Scale, examination of the Multiple Sclerosis Quality of Life-54, evaluation of state anxiety and trait anxiety, monitoring of body temperature and bioimpedance analysis.

Assessments before and after each physical exercise training session
Before and after each physical exercise training session, the following evaluations will take place: measurement of reproductive hormones, including 17β-oestradiol, progesterone, prolactin, I.H, follicle-stimulating hormone, thyroid-stimulating hormone and testosterone; analysis of inflammatory markers, such as interleukin (IL)-6 and IL-10; assessment of cognitive function, including brain-derived neurotrophic factor; examination of the blood profile; evaluation of neuromuscular strength, voluntary activation and contractile properties, including rate force development, maximal voluntary isometric strength of lower limbs, muscle contractile function and central activation ratio; and analysis of maximal upper limb strength. Functional assessment includes evaluating spasticity, gait speed, balance, the sit-to-stand test, the timed up and go test, pain levels and the rating of perceived exertion.

Evaluations during intervention sessions (per session)
During each intervention session, the following evaluations will be conducted: measurement of muscle oxygen saturation, analysis of lactate levels, assessment of VO2max and monitoring of heart rate variability (HRV).

Assessing residual effects of exercise training sessions
Sleep quality will be evaluated over the three nights following each intervention to assess the lingering effects of exercise training sessions. This assessment will use the Subjective Sleep Quality Questionnaire, sleep quality measured by actigraphy and HRV.

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Contributors  The protocol was conceived and designed by members of the EMMA Project. The statistical analysis plan was designed by JAR-A, AM-R and McD-C. The protocol was initially drafted by JAR-A. Subsequent versions were reviewed by DJR-C, LA-C, RL-L, GM-T, AM-B, AR-M, MC, McD-C, MV-M, RC, NR-P and PE-G. Revisions were made by JAR-A, RC, DJR-C and PE-G. All members of the EMMA Project read and accepted the submitted version of the manuscript.

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Competing interests  None declared.

Patient and public involvement  Patients and/or the public were not involved in the design, conduct, or reporting, or dissemination plans of this research.

Patient consent for publication  Not required.

Ethics approval  An institutional review board (University of Almería) has approved the research protocol (notification no: UALBIO2022/048). Informed consent is required before inclusion. Persons with direct access to the data must respect their confidentiality. All data collected during the study will be anonymised, and only the inclusion number will appear in the database. The results of this research are liable to be disseminated in conferences and published in conference proceedings and academic journal articles.

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Data availability statement  No data are available. All data relevant to the study are included in the article or uploaded as online supplemental information.

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REFERENCES  


Supplementary Material 1. Description of the procedures for the secondary variables

1. Secondary outcomes

1.1. Baseline outcomes measured on each menstrual cycle (control outcomes)

**Abdominal Obesity and Anthropometric Variables**

Body mass, stature, relaxed arm, mid-thigh, calf, waist and hip girths, and three skinfolds (triceps, front thigh and medial calf) will be measured, following the protocol established by The International Society for the Advancement of Kinanthropometry (ISAK), by an ISAK level 1 certified anthropometrist. The following will be calculated with the data obtained: BMI (Weight (kg)/ (Height (m))², waist-to-hip ratio (WHR) and waist-to-height ratio (WHtR). Abdominal obesity will be identified based on WHtR when it is over 0.5.

In addition, relative fat mass (RFM), will also be estimated using the following equation: RFM (%) = 64 - (20 · height/waist girth) + 12. Additionally, Skeletal muscle mass (SMM) will be determined by applying [PEG2] the equation “SMM (kg) = 0.566 * FFM, followed by the calculation of the skeletal muscle mass index (SMMI) using the formula “SMMI = SMM (kg) / height squared (m²). Finally, the prevalence of sarcopenia among the participants will also be calculated following the criteria of the European Working Group on Sarcopenia in Older People (EWGSOP).

**Dietary and nutritional monitoring**

To establish both an initial baseline and a monthly evaluative measurement of dietary practices and adherence, the aforementioned 'Dietary and Nutritional Monitoring Protocol' will be administered at two critical junctures of our study. Initially, at the onset of the research protocol, this dietary assessment will be undertaken to capture the existing eating patterns, providing a pre-intervention description of participants' diets. This data will be instrumental in identifying any subsequent changes or trends potentially attributable to the intervention. At the conclusion of each month throughout the study, the same dietary assessment will be administered again, ensuring monthly post-intervention records of dietary habits. The comparison between these monthly datasets will provide a comprehensive perspective on shifts in dietary patterns and adherence throughout the study.

Following the outlined schedule for dietary assessment, participants will be guided through the core elements of the “Dietary and Nutritional Monitoring Protocol.” A significant component of this monitoring process will be the detailed questionnaires targeting the Mediterranean diet's principles (PREDIMED Questionnaire).
Mediterranean Diet Adherence Screener (MEDAS) \(^8,9\). This structured inquiry is tailored to distill the intricacies of each participant's adherence to the diet, shedding light on their individual dietary habits.

Subsequently, over a span of one week, participants will be instructed to maintain an exhaustive food diary. They will be asked to provide entries detailing not only the foods and beverages consumed, but also their precise quantities. The added requirement of a photographic record of each meal and snack is designed to further confirm portion sizes and the nature of foods consumed. This visual reinforcement seeks to add another dimension of accuracy to the data.

Weighing of food items will be highlighted as an essential practice. Through clear guidelines, participants will be made aware of the nuances of weighing foods, especially the disparities between the weights of raw versus cooked items. Anticipating potential challenges, we've also prepared an auxiliary guide on household measures, serving as an alternative when traditional weighing methods prove to be impractical.

Ensuring clarity and addressing uncertainties, participants will have the advantage of being in direct touch with a registered dietitian-nutritionist. This continuous professional support is aimed at guiding participants through the intricacies of accurate food recording. Ultimately, the dietitian-nutritionist will bear the responsibility of analysing the amassed data, validating its credibility.

**Monitoring of Physical Activity levels**

The International PA Questionnaire (IPAQ) \(^10,11\), will be administered once in each MC as a control variable. The telephone-based short version, consisting of 7 items, will be utilized. This version provides information about the time spent performing moderate and vigorous-intensity activities, time dedicated to walking, and time spent sitting during a workday. It is designed to be used with young people and adults aged 15-69 years old.

PA will be recorded in METs (Metabolic Equivalent of Task or METs Units) using the following equation, only at the beginning and end of the experimental design (control measure):

\[
\text{MET} = \text{walking} (3.3 \text{ MET} \times \text{minutes} \times \text{days per week}) + \text{moderate PA} (4 \text{ MET} \times \text{minutes} \times \text{days per week}) + \text{high PA} (8 \text{ MET} \times \text{minutes} \times \text{days per week}).
\]
In addition, the participants’ PA will be assessed with WGT3X-BT triaxial accelerometers (Cambridge Neurotechnology, Cambridge, UK) for 7 days of the experimental period to verify PA levels.

**Walking endurance**

Participants will undergo the 2-minute walk test (2-MWT), during which they will walk at their self-selected preferred speed to assess walking endurance. The testing track will be rectangular, with corners marked by cones. Participants will be allowed to rest during the test if needed, but the time will not pause during these resting periods. The total distance covered (in meters) will be recorded. An investigator will be present during the test to accompany the participants, although no conversations will take place.

**Measurements in each phase of menstrual cycle**

**Physical self-perception.**

The evaluation of physical self-perception will comprise six subscales aimed at gauging self-perception in various dimensions, including sports competence, physical condition, attractive body, physical strength, general physical self-perception, and general self-perception. The participants’ responses will be organized using a 5-point Likert scale, with each subscale score having the potential to obtain 6 to 36 points. A higher score will indicate a positive physical self-perception.

**Catastrophizing Pain Scale**

The Catastrophizing Pain Scale will be used to assess feelings of catastrophizing related to pain (such as painful experiences). Subscale scores encompassing rumination and helplessness will be examined. Each of the 13 questions will be rated on a 5-point Likert scale, with the endpoints ranging from “not at all” to “all the time.” A lower score will indicate minimal or no pain catastrophizing.

**Modified Fatigue Impact Scale (MFIS)**

The perception of fatigue will be measured using the MFIS. This scale is a 21-item multidimensional questionnaire that assesses the physical, cognitive, and psychosocial effects of fatigue on a five-point ordinal scale (with a maximum total score of 84).

**Multiple Sclerosis Quality of Life-54 (MSQoL-54)**

Participants will fill out the Multiple Sclerosis Quality of Life-54 (MSQoL-54) questionnaire. The MSQoL-54 is a structured, self-report questionnaire comprising 14 sub-scales: physical function, physical role limitations, emotional role limitations, pain, emotional well-being, energy, health perceptions, social function, cognitive function, health distress, sexual function, satisfaction with sexual function, change in health, and...
overall quality of life. Two summary scores, the physical health composite summary and the mental health composite summary, can be derived from the MSQoL-54 questionnaire. Elevated scores in each subscale or summary score will indicate an improved quality of life.

**State anxiety and trait anxiety (STAI)**

The STAI will be used to measure state and trait anxiety. This questionnaire assesses and discriminates the temporary psychological state in a given situation (state anxiety; 20 items), as well as the more stable character trait of attitudes and temperaments (trait anxiety; 20 items) \(^1^6\). This questionnaire is especially useful for the diagnosis of anxiety problems in non-psychiatric patients. It is based on a 4-point Likert scale, with scores in each subscale ranging from 10 to 40 points. A higher score in each of the subscales reflects a higher state or trait anxiety, respectively.

**Body temperature**

Body temperature will be measured through thermal images. To capture thermal images, a FLIR C5 thermographic camera (FLIR, Estonia) will be used, equipped with an infrared resolution of 19200 pixels and a thermal temperature range of -10 to 50°C (Thermal sensitivity/NETD <70 mK). The calibration of the camera will be performed according to the manufacturer's recommendations, as well as recommendations from previous studies \(^1^7,1^8\). The camera will be connected at least 30 min prior to all evaluations to allow its thermal sensor to stabilise.

Three measurements shall be performed following the Glamorgan protocol \(^1^8\), designed to provide accurate measurements of body thermography. This procedure will be repeated at each phase of the MC. Image collection will be performed in a laboratory with standardised conditions, and monitored according to the specific recommendations for this type of evaluation \(^1^7,1^9\). The procedure will start with a baseline thermography, followed by a thermography after mechanical stress (physical exercise), and finally a thermography after thermal stress. To ensure the thermal stability of the participants, they will be allowed a 10-minute acclimatisation period to the ambient temperature of the laboratory \(^2^0\). During the acclimatisation period, they will be asked to answer a questionnaire to ascertain the existence of any possible influencing factors affecting the results. The thermal imaging camera will be positioned perpendicular to the ground at a specific distance (2 meters), which will allow the subject to fit completely in the camera image, thus ensuring that all ROIs (Regions of Interest) can be satisfactorily assessed. The images will be analysed with the Irbis®3 software. The ROIs will be: trunk, hip and lower limbs following indications from previous studies.
**Bioimpedance analysis**

Body composition will be measured by bioelectric impedance analysis (BIA) using a portable device (InbodyS10® system, InBody Corp, Seoul, Korea), with an applied current of 100 μA (1 KHz) and 500 μA (5, 5, 50, 250, 500, and 1000 kHz), and a 100-240 VAC power supply, 50 / 60 Hz, 1.2 A, in five segments of the body (right and left arms, right and left legs, and trunk). The parameters obtained in the measurement will include the following: weight, lean muscle mass (muscle mass), skeletal muscle index (skeletal muscle mass/height [m²]), fat-free mass, skeletal muscle mass, body fat percentage, extracellular water/total body water ratio (ECW/TBW), basal metabolic rate, and phase angle arc tangent of \((Xc/R) \times 180°/\pi\), which will be automatically calculated by the device.

1.2. Before and after each physical exercise training session

**Reproductive hormones, inflammatory and cognitive function, blood profile**

Methodological considerations for performing blood collections: as a critical component of our upcoming research, we've established a 4-hour fasting protocol. This method ensures standardization across participants and aims to create a consistent metabolic baseline, allowing for more accurate and reliable study outcomes. In the upcoming study, participants will be informed about the significance and objectives of the fasting protocol. They will receive recommendations for a standard meal to consume before the fasting period begins, ensuring a controlled intake of calories and macronutrients. Furthermore, participants will be advised to avoid strenuous activities or exercises in the 24 hours leading up to the fasting period. For dinner, the total caloric intake will be set at 450 kcal. Carbohydrates make up 50% of this, which is equivalent to 225 kcal or 56 grams. Proteins contribute 25%, corresponding to 113 kcal or 28 grams. Similarly, fats also account for 25% of the meal's energy, amounting to 113 kcal or 12 grams.

Participants will also receive instructions regarding medications during this period. Some may need to take their medication with food, so consultation with a medical professional will be necessary to offer guidance. The use of non-essential supplements will be discouraged during the fasting period unless otherwise instructed. There will be a clear statement provided on the conditions under which participants should end their fast, for example, if they feel faint or unwell. After the study measurements are taken, a standardised post-fast meal or snack will be made available to participants. This will ensure that they can safely resume their regular dietary routines.

To verify adherence to the fasting protocol, participants will be provided with guidelines or a checklist. On the day of the study, researchers will verbally confirm with each
participant that they followed the fasting guidelines. To ensure the reliability and validity of our research findings, it is imperative to standardize the breakfast for all participants. By doing so, we eliminate potential variations in nutritional intake that could influence metabolic responses, thereby providing a consistent starting point for our study measurements. For breakfast, the total caloric intake will be set at 350 kcal. Carbohydrates constitute 60% of this, translating to 210 kcal or 53 grams. Proteins contribute 15%, which corresponds to 53 kcal or 13 grams. Fats, on the other hand, account for 25% of the meal’s energy, amounting to 88 kcal or 10 grams.

a) Reproductive hormonal profile: to monitor the MC and to assess sexual function, serum concentration of reproductive system-related hormones will be analysed: 17β-estradiol, progesterone, prolactin, LH, FSH, TSH and testosterone, before each training session. The female hormonal profile will be measured basally in each phase of the menstrual cycle.

b) Inflammatory profile: although MS has been historically thought to be primarily a T-cell-driven disease, the role of B cells in the pathophysiology of MS has been increasingly recognised and characterised in recent years. Therefore, IFN-γ, as a marker associated with the pathogenesis of the disease, IL-6 and TNF-α, as pro-inflammatory markers, and IL-10 and β1 (TGF-b1) as anti-inflammatory markers, will be assessed. All of these markers shall be assessed before and at 30 and 60 minutes immediately after the session. For clinical analyses, the protocols described by Alvarenga-Filho et al. (2016) will be followed. The inflammatory profile will be assessed pre-training, at 30 minutes, and 2 hours after the training session.

c) Cognitive function: brain-derived neurotrophic factor (BDNF) is a member of the neurotrophin family, and several studies have found associations between exercise, circulating levels of BDNF, hippocampal volume, and cognitive function in the general population and neurodegenerative disorders. For the analysis of BDNF, the protocol described by Briken et al. (2016) will be followed, and BDNF will be assessed before, 30 minutes and 2 hours after the end of the training session. The inflammatory profile will be assessed pre-training, at 30 minutes, and 2 hours after the training session.

Neuromuscular Strength, Voluntary Activation, and Contractile Properties

a) Rate Force Development (RFD) and Maximal Voluntary Isometric Strength of the lower limbs (MVIC): following the recommendations by Maffiuletti et al. (2021), the participants will be seated in a chair, with both legs flexed at a 90 degree angle and the ankle of those tested firmly strapped to a customised device with a load cell (MuscleLab Force Sensor, Ergotest AS, Langesund, Norway). Subsequently, participants will be
instructed to apply "as much force as possible, as fast as possible" on each trial. Participants will perform three maximal contractions lasting two seconds each, with a rest of three minutes between contractions. RFD and the time at which the value is observed will be analysed in the following ranges: 0-50 ms: RFD_{early}, 0-200 ms: RFD_{late} and peak slope: RFD_{peak}. The right leg will be tested first, and the repetition in which the highest peak value of each leg is observed will be analysed.

Ten minutes after the previous test and from the same position, in order to measure the maximum isometric force of each leg (MVIC), the participants will be verbally encouraged to apply “as much force as possible” during the course of 3 consecutive maximal contractions. Participants will perform three 5-second MVICs with 3 minutes of rest between contractions. The right leg will always be evaluated first, and the trial with the highest MVIC will be used for analysis.

To measure neural drive, the sEMG activity of the vastus lateralis of the right leg will be recorded during the MVIC (Delsys Trigno, Delsys Inc., Boston, MA). The preparation of the skin will involve shaving, abrasion, and cleansing with alcohol. Following the SENIAM Guidelines, the upper electrode of each pair will be placed over the largest part of the vastus lateralis. Transparent paper will be used to map exact electrode placements for subsequent measurements. EMG activity will be analysed using the following time intervals: 0–50 ms (EMG 0 to 50), 0 to 200 ms (EMG 0 to 200), peak of EMG and EMG time-to-peak. The EMG peak during MVIC will represent maximal neural drive.

In addition, two bipolar stimulating electrodes (10 × 15 cm) will be positioned and secured on the proximal and distal portions of the quadriceps of the right leg. Signal 6.0 software (CED, Cambridge, England) will control the electrical stimulation characteristics, which will be 100 Hz, 50 pulses, length 0.009 s, and interval 0.01 s. The stimulus intensity will be established at 40–50% of MVIC. Muscle contractile function and central activation ratio (CAR) will be assessed using the following protocol (Figure 4): a supramaximal twitch, a 100 Hz tetanic train (50 pulses), an MVIC with a superimposed 100 Hz train during steady maximal force, another potentiated 100-Hz train, and finally, a potentiated supramaximal twitch. This sequence will be repeated twice, providing 2 minutes of rest between the sequences. Twitch-to-tetanus ratio (Tw/Tet), peak MVIC, peak force obtained by the superimposed twitch and tetanus stimulation will be determined. The following formula will be used to calculate CAR:

\[ \text{CAR} = \frac{\text{MVIC}}{\text{MVIC} + \text{superimposed train}} \times 100 \]
b) **Maximal upper limb strength (MULS):** Participants will stand with their elbows fully extended and separated from the trunk. In this position, isometric handgrip strength will be measured for 5 seconds using the electronic hand dynamometer (K-Force Grip®, Kinvent, Monpellier, France). Participants will perform three trials in each hand (right and left), and a rest interval of 30 seconds will be given between attempts. The highest value achieved will be recorded.

### Functional assessment

a) **Spasticity:** The pendulum test will be used for spasticity assessment. Metrics including the relaxation index (RI), the count of oscillations, duration of oscillations, and the extent of the first swing excursion will be computed. Participants will sit with their torso reclined approximately 30° (to prevent biceps femoris stretching), and their legs will hang over the seat's edge. Participants will be barefoot and wearing shorts. Markers will be placed on the major trochanter of the femur, lateral epicondyle of the femur, and lateral malleolus of the fibula. A high-speed camera will record the pendulum test.

Participants will be instructed to close their eyes and ensure complete relaxation of their leg muscles throughout the testing. The examiner will hold the heel and move the leg from its resting position (approximately 90° knee flexion) to full extension (approximately 180° knee extension). Subsequently, the heel will be released to allow passive oscillation until the leg comes to rest. The examiner will continuously monitor the participant's relaxed leg during the test to verify trial validity. Rest periods of thirty seconds will be provided between trials. The analysis will be based on the mean of two valid trials.
Knee angles will be determined using video recordings (Iphone 14-pro) for each pendulum test (Image J software, version 1.42; National Institutes of Health, Bethesda, MD, USA). The initial angle and the first inversion angle of the swinging limb will define the first swing excursion. The initial angle will be taken when the examiner releases the participant's heel. The count of swings will be ascertained from the number of sine wave peaks produced by the swinging limb following heel release. Each oscillation will be considered valid if it exhibits a shift of at least 3° towards extension. The oscillations' duration (in seconds) will also be determined from heel release until the end of the final oscillation defined by the aforementioned criterion. The RI will be calculated using the formula: RI = (initial angle - first angle) / (initial angle - rest angle). The rest angle will denote the knee joint's position after oscillatory motion concludes.

In addition, we will be using portable muscle diagnostic equipment (MyotonPRO, Myoton AS, Estonia) to assess muscle characteristics, including measurements of muscle frequency, muscle stiffness and muscle decline. The Myoton®PRO measures the deformation properties of damped natural oscillations that occur after a brief mechanical impact (15 ms) with a pre-compression of 0.18 N and an impulse of 0.40 N (total = 0.58 N) on the skin's surface. Anatomical locations will be selected to measure tone, stiffness and elasticity of the quadriceps and triceps surae muscles. The points defined by SENIAM for EMG will be chosen for their reliability and reproducibility. First, a mark shall be made with a dermographic pen and then the marked point shall be measured. To ensure reproducibility, a permanent marker pen shall be used and the mark shall be checked by the patient himself every day to avoid erasure.

b) Gait speed: Gait speed will be measured using the 10-meter walk test (10-MWT), for which 2 photocells (Ergotest Technology AS, Langesund, Norway) will be placed at 5 and 10 meters to record the time. Participants will perform the test as fast as possible without running, completing it twice with a 2-minute rest in between. Participants will be consistently encouraged throughout the 10-MWT. The recorded time for the slowest walking trial (in seconds) will be utilized for analysis.

c) Balance: during the static balance measurements, participants will stand quietly on a portable force platform (Ergotest Technology AS, Langesund, Norway) while barefoot, maintaining a shoulder-width stance, and letting their arms hang at their sides. Each participant will perform two trials lasting 30 seconds each, with both eyes open, and another two trials of the same duration with their eyes closed. A rest period of two minutes will be allotted between trials. The trial yielding the most favourable balance outcomes for each test will be subjected to analysis. The outcome measures will be: the mean anterior/posterior displacement (MAPD; mm), mean medial/lateral displacement
(MMLD; mm), total sway displacement (TSD; mm), sway area (SA; mm2), mean total velocity (MTV; mm/s), phase plane portrait anterior/posterior (PPPAP; a.u.), medial/lateral (PPPML; a.u.) and anterior/posterior–medial/lateral (PPPAP-ML; a.u.), standard deviation of velocity anterior/posterior (SDVAP; mm/s) and medial/lateral (SDVML; mm/s), and standard deviation of amplitude anterior/posterior SDAAP (mm) and medial/lateral SDAML (mm). Balance will be tested before and after each session.

d) Sit-to-Stand Test (STS): Participants will be positioned in an upright manner on an adjustable chair (considering lower limb length; 90° knee flexion) with arms crossed over the chest. They will be instructed to rise to a standing position as swiftly as possible. Video recording with an Iphone ® 14 will be employed to determine the conclusion of the movement, when both the participant's trunk and knees are fully extended. This test will be conducted twice, and the most successful trial will be utilized for analysis.

e) Timed Up-and-Go Test (TUG): In a prompt manner, participants will transition from a seated to a standing position. They will then walk a distance of 3 meters forward, execute a turn, walk back, and resume the seated posture. This test will be repeated twice. A video recording will be used to ascertain the fastest time between the two trials, which will subsequently be used for analysis.

Pain

To measure Delayed-Onset Muscle Soreness (DOMS), a 10-point Likert scale will be employed to evaluate pain, where 1 = no pain and 10 = unbearable pain. Participants will assess their level of DOMS in nine different muscle areas: 1) posterior leg; 2) posterior thigh; 3) anterior thigh; 4) lower back and glutes; 5) abdominals; 6) upper back; 7) shoulders; 8) anterior arm; 9) posterior arm and total pain. A daily average score will be calculated by summing all pain scores and dividing it by the number of areas, to reflect overall DOMS. Additionally, an FPIX algometer (Wagner's instruments, USA) will be used to quantify pain sensitivity in specific muscle regions (same anatomical areas where spasticity is measured). The algometer will be calibrated according to the manufacturer's guidelines. Participants will be instructed to indicate their initial pain threshold. The rounded tip of the algometer will ensure an even application of pressure.

Rating of Perceived Exertion (RPE)

Participants will be instructed and familiarized with the use of the RPE scale during the familiarisation phase. RPE will be assessed before, during (after each of the proposed exercises within the session), and after the training session using the Borg 6-20 RPE.

1.3. Evaluations during intervention sessions (per)
**Muscle oxygen saturation (SMO2)**

SMO2 of the right and left lateral quadriceps will be measured during sessions using a near-infrared spectroscopy system, the Moxy 3-Sensor Bundle (Fortiori Design LLC, Hutchinson, MN, USA). The average SMO2 will be calculated throughout the entire training session on both legs simultaneously.

**Lactate**

A portable lactate analyser (Lactate Scout system, RedMed, Warsaw, Poland), which measures lactate concentration using the principle of enzymatic determination by photometric reflection, will be used. During the exercise sessions, capillary blood samples from the fingertip will be collected before warm-up (i.e. basal level) within the training session and after the end of cool down.

**VO2max**

Oxygen consumption (VO2) will be measured breath by breath throughout the session, and EPOC will be analysed for 20 minutes after the end of it, using a breath-by-breath gas analyser (VO2 Master Pro (VO2 Master Health Sensors Inc., Vernon, British Columbia, CA)). The gas analyser system will be calibrated before each test using the manufacturer’s recommendations.

**Heart rate variability**

Autonomic nervous system adaptation. The fluctuation of the autonomic nervous system through the training session will be recorded. Blood pressure, oxygen saturation (SAT), heart rate (HR) and heart rate variability (HRV) will be recorded. The systolic (SBP) and diastolic blood (DBP) pressure will be obtained by a single indirect measurement performed by an automatic oscillometric blood pressure measuring device (OMRON, Kyoto, Japan) positioned on the left arm of the patient. Oxygen saturation will be measured using a portable finger oximeter (9500, Onix-Indumeda, Nonin Fernbrook Lane North Plymouth, USA) positioned on the third finger of the participant. Resting, reactivity and recovery HR and HRV will be recorded using a Polar H10 heart rate sensor (Kempele, Finland). HRV time-domain and frequency-domain will be recorded and logarithm-transformed as described in the quality of sleep section. For that purpose, the participant will be in a supine position for 10 minutes before and after the training session. Resting HR and HRV will correspond with the 10 minutes of rest before the training session, reactivity HR and HRV will comprise the period of exercise, removing the first and last 5 minutes, and recovery HR and HRV will correspond to the 10 minutes of rest after the training session, which will be subdivided into two phases: the first (HR_{rec1} and HRV_{rec1}) and the last (HR_{rec2} and HRV_{rec2}) 5 minutes of recovery.
1.4. Residual effect of exercise training sessions

Quality of sleep will be measured during the 3 nights following the intervention.

a) Subjective Sleep Quality Questionnaire: Subjective sleep quality will be measured using the Karolinska Sleep Diary questionnaire. The questionnaire includes the following items: a) sleep quality (very poor [1] – very good [5]), b) sleep tranquillity (very restless [1] – very calm [5]), c) ease of falling asleep (very difficult [1] – very easy [5]), d) awakenings (awakened much too early [1] – did not wake up early [3]), e) ease of awakening (very difficult [1] – very easy [5]), f) feeling of restfulness (did not rest at all [1] – completely rested [3]), and g) sleep sufficiency (no, definitely too little [1] – yes, definitely enough [5]).

b) Sleep Quality Measured by Actigraphy: Actigraphy-based sleep quality will be assessed using the Actiwatch wGT3X-BT activity monitoring system (Cambridge Neurotechnology, Cambridge, UK). This device employs a piezoelectric accelerometer to measure activity. Participants will wear the Actiwatch on their non-dominant wrist. The lower threshold of actigraphic sensitivity will be set at 80 counts/epoch. The Actiwatch sleep analysis software will be utilized. Data analysis will begin at the start of the nocturnal rest (bedtime) and conclude at the beginning of daytime activity (wake time). Sleep efficiency (%), percentage of time spent asleep, time in bed (minutes), actual sleep time (minutes), actual wake time (minutes), number of awakenings, and average duration of each awakening (minutes) will be measured.

A minimum of 2 nights is required to evaluate sleep percentage, at least 5 nights for sleep efficiency, and 7 nights for total sleep time using Actigraphy. Self-reported questionnaires require a minimum of 6 nights.

c) HRV: HRV measurements will be taken using a Polar H10 heart rate sensor (Kempele, Finland) to record heartbeats during sleep. Analysis of HRV variables will be conducted using Kubios HRV software (version 3.0). This software will also be used to apply threshold filters (very low, low, or medium) to eliminate artefacts where necessary. Poincaré plot, time-domain, and frequency-domain analyses will be performed. The analysed time-domain variables include: mean heart rate (HR), mean R-R interval (RR; ms), standard deviation of consecutive R-R intervals (SDNN), square root of the mean squared differences of successive R-R intervals (RMSSD; ms), and the proportion of consecutive intervals differing by more than 50 ms (pNN50). Fast Fourier Transformation (FFT) will be employed to calculate frequency-domain spectral components. High-frequency (HF; 0.15-1.0 Hz) and low-frequency (LF; 0.04-0.15 Hz) power components will be computed as integrals of their respective power spectral density curves.
variables will be expressed as natural logarithm-transformed values (HFln and LFln). Lastly, Poincaré plot variables, standard deviation of instantaneous beat-to-beat variability (SD1), and standard deviation of continuous long-term R-R interval variability (SD2) will be calculated. Stress score (SS) will be analysed using the equation 1000x1/SD2, and the sympathetic/parasympathetic ratio (S/PS) will be calculated using SS/SD1.

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