STUDY PROTOCOL “METABOLIC FLEXIBILITY IN PATIENTS WITH EARLY TRIPLE-NEGATIVE BREAST CANCER AND THE POSSIBLE EFFECT OF A PHYSICAL EXERCISE INTERVENTION”

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1. INTRODUCTION

1.1 CANCER INCIDENCE

Cancer is considered like a main worlwide issue. It was estimated about approximately 19,9 million of new cancer diagnoses in 2022 in the world and those will increase up to 28,00 million in the next two decades (1). Specifically, breast cancer (BC) represents the highest incidence worlwide, with approximately 2,3 million of new cases diagnosed in 2022 (1). According to *Sociedad Española de Oncología Médica* (SEOM) it was estimated about 37.682 new BC cases diagnoses in 2025 in Spain (1).

There is a higher BC incidence in developed countries that could be explained due to the higher obesity rates, alcohol and tobacco consumption, early onset of puberty, contraceptive use and hormone therapies, low levels of physical activity, and giving birth at an older age (2,3). Furthemore to everything mentioned above, hereditary factors and age are additional risk factors to cancer development (2,3). Last but not least important, the existence of relatives with BC and/or ovarian cancer who are carriers of mutations in the BRCA1 and BRCA2 genes, among others, which increase the likelihood of tumour proliferation, and being over 40 years of age, increment the likelihood of BC diagnosis too (2,3).

1.2 BREAST CANCER SUBTYPES

Currently, diferent types of BC are categorized based on molecular subtype (64,65). On the one hand, the luminal A molecular subtype is characterised by expressing oestrogen receptors (ER) and/or progesterone receptors (PR), absence of overexpression of human epidermal growth factor (HER2), and a Ki-67 cell proliferation index <20% (64,65). On the other hand, the luminal B molecular subtype has a inmunostoquimic profile compound by positive RE and/or RP, negative or positive HER2 and a Ki-67 ≥20% (64,65). Finally, the triple negative molecular subtype is defined as one that does not express ER, PR, and HER2 (64,65).

Specifically this last molecular subtype accounts for 10-20% of invasive BC, and they are biologically and clinically more agressive than other BC subtypes (64,65). Moreover, this subtype has a poorer prognosis and more limited treatment options than other BC subtypes (64,65).

In this line, in a study conducted in North Carolina on patients with triple-negative breast cancer (TNBC) a strong association was found between triple-negative tumor diagnoses in women and multiple births, early menarche, low breastfeeding rates, a high waist-to-hip ratio, and high adiposity (66). In contrast to other tumors, it appears to be the only molecular subtype in which increasing age is not a risk factor; rather, a high incidence of TNBC has been observed between the ages of 20 and 39 (66).

1.3 GENERAL BENEFITS OF PHYSICAL EXERCISE

It has been proven that exercise has beneficial effects on the immune system that could protect against the development of cancer (4,5). When physical exercise is performed, exerkines are detach, molecules that are released into the bloodstream by different organs and tissues (4).

There are two main types of exerkines: myokines, generated by muscle fibre contraction, and adipokines, secreted by adipose tissue (4).

With regard to these exerkines, specifically IL-6, a myokine released by muscle contraction, has an anti-tumor effect due to the way it is secreted in acute doses (6). However, the continuous secretion of IL-6 by other tissues has inflammatory effects and is considered pro-tumor as it increases the likelihood of tumor cell appearance and/or proliferation (6). Although these immunological benefits are not the target of study, the presence and release of these molecules through physical exercise directly influences the anti-inflammatory phenotype of the body's cells (6). It has been shown that cells with a high inflammatory profile may have mitochondrial dysfunction, greater oxidative stress, and a higher presence of pro-inflammatory cytokines. This could, in turn, alter the pathways of energy generation from the mitochondria (6).

Along the same lines, different studies show how physical exercise could change the cellular phenotype in different tissues of the body (13,14). Thus, a decrease in certain markers of inflammation such as IL-6, IL-1, TNF-α, M1 macrophages, etc. has been observed with different exercise programs, which would shift cells toward a more anti-inflammatory phenotype (13,14). Low-grade chronic inflammation is a risk factor for tumor proliferation (13,14). Therefore, exercise could interfere with tumor proliferation processes.

On the other hand, numerous authors reflect on the importance and potential effect that physical exercise can have on the metabolism of the body's cells. Various studies have been carried out that include concurrent training interventions in humans and evaluate certain metabolic parameters (15,16). The authors reported that physical exercise significantly influences glucose levels, insulin resistance, growth factors, fat oxidation ratio, and lactate, among others (15,16). Alteration of any of these markers could activate tumor signaling pathways, thus, posing a risk factor for tumor proliferation (15,16). Therefore, it could be concluded that modulation of metabolism through physical exercise plays a fundamental role in the development of cancer (5,15,16).

1.4 DEFINITION AND RISK FACTORS OF METABOLIC FLEXIBILITY

Metabolic flexibility is described as the body's ability to adapt to energy demands in different contexts (18,20). The availability of energy substrates in response to different activity requirements enables cell survival and homeostasis (18,20). Mitochondria, the main organelle in the cell, enable the oxidation of energy substrates for energy production in order to respond to the required intensity (20).

The main energy substrates used by muscle to generate energy are intramuscular glycogen, blood glucose, triglycerides (TG), and fatty acids (FA), which come mainly from abdominal white adipose tissue (WAT) (18). All of these provide the necessary energy supply for muscle fibre to generate muscle contraction (18).

In terms of rest, under normal health conditions, FAs and carbohydrates (CHO) are transported into the mitochondria to be oxidated into acetyl-CoA and enter the Krebs cycle (TCA) with its

corresponding process of obtaining adenosine triphosphate (ATP) through oxidative phosphorylation (OXPHOS) (20). Any alteration in the energy production process in the mitochondria could alters cellular homeostasis and the health of the individual (20).

The concept of metabolic inflexibility in muscle fibre refers to the inability of mitochondria to clear lactate, the low capacity to oxidate lipids, and the rapid transition from fat oxidation to CHO (21). Along the same lines, high metabolic function will mean lower lactate levels and a higher FA oxidation ratio at the same intensity. Thus, people with low metabolic function have a higher respiratory exchange ratio (RER) both at rest and during exercise, mainly due to the rapid transition from fat oxidation to CHO and/or mitochondrial dysfunction in fat oxidation (21).

Currently, many people suffer from metabolic inflexibility mainly due to unhealthy habits such as a diet high in processed foods, the consumption of toxins such as alcohol and drugs, regular use of medication for illnesses, inadequate rest and high levels of stress, and physical inactivity (19,20). This has a negative impact on a person's mitochondrial function and is associated with a high probability of developing type 2 diabetes (T2D), cardiovascular disease (CVD), metabolic syndrome (MS), cancer, and Alzheimer's disease (19,20). Other factors that could lead to mitochondrial dysfunction include age, infections, and DNA mutations (20).

Similarly, systemic mitochondrial dysfunction has a direct impact on skeletal muscle, which oxidates high levels of glucose and a minimal amount of FA (18,20). Some authors also indicate that defects in the oxidation of these alter energy production in the mitochondria, leading to an accumulation of intramyocellular lipids (18,20).

In this context, there is a new tool called near-infrared spectroscopy (NIRS), which allows the analysis of oxygenated haemoglobin (HbO2), deoxygenated haemoglobin (HHb) and the Tissue Saturation Index (TSI) (45,46). This measuring instrument has already been used to evaluate muscle mitochondrial capacity in numerous research (44,45,46,47,58). It therefore provides direct information on mitochondrial function, which indirectly determines an individual's metabolic flexibility.

1.5 METABOLIC INFLEXIBILITY IN THE ONCOLOGY POPULATION

On the one hand, some authors argue that in the presence of cancer there is generalized mitochondrial dysfunction in different tissues of the body, marked by the pathophysiology of the disease and by the toxicity induced by cancer treatments in these patients (11). Similarly, a reduction in PGC-1α levels has been observed in patients who received neoadjuvant (NA) chemotherapy (CTx), considered one of the coactivator proteins that control mitochondrial biogenesis. This would lead to an inability to produce and meet energy demands and, once again, to muscle dysfunction associated with both loss of function and muscle mass in cancer patients (7,11).

On the other hand, it should be noted that women with BC who have been under the effects of CTx are more likely to gain fat mass than women of the same age without BC (7,9,10,11,22). Body fat gain is a side effect that occurs with cancer treatments, but it is more pronounced in the context of adjuvant treatment and endocrine therapies in BC (7,9,10,11,22). Excess fatty tissue is associated with the presence of metabolic diseases and pro-inflammatory cytokines, which

again imply metabolic and mitochondrial dysfunction. Likewise, increased fat mass has been linked to an increased likelihood of recurrence, disease progression, and even mortality in studies of patients with BC (7,9,10,11,22).

In patients with any type of cancer, sarcopenia is associated with lower success rates of antineoplastic treatments, greater CTx-induced toxicity, and rapid tumor progression (7,9,11). Furthermore, taking into account the previous paragraph, the aforementioned predisposing factors could alter the body composition of cancer patients. Thus, sarcopenic obesity, characterized by an increase in fat mass and a decrease in muscle mass, is quite common in BC (7,9,11).

The changes in weight and body composition in patients with BC are not only induced by CTx and the disease itself, but could also be associated with physical inactivity, a decrease in resting metabolic rate (TMB), excessive food intake, and/or hormonal changes (9). These changes can persist for years after treatments (9).

Another factor to consider in cancer patients is the phase angle (PhA). This is defined as the correlation between the resistance (R) of the electrolytes contained in total body water and the reactance (Xc) present in cell membranes (42). Cancer treatments are known to decrease PhA as they may affect cell membrane integrity, causing inflammation and, in some cases, cancer-associated cachexia (42). PhA is considered a prognostic indicator of survival and quality of life in cancer survivors in the clinical setting (42)Thus, low PhA values indicate low Xc and high R, or, in other words, poor cell membrane integrity (42). High PhA values result in high Xc and low R, indicating that cell membranes remain intact and that there are high levels of electrolytes and total body water (42).

Therefore, PhA is considered an indirect marker of a person's metabolic flexibility due to its direct relationship with membrane integrity, which involves the cell itself and could lead to mitochondrial dysfunction (42).

In summary, it could be concluded that the metabolic flexibility of muscle fibre in patients with TNBC could be reduced, mainly in patients undergoing systemic treatment, with possible complications in adapting to different intensities and energy demands in different everyday contexts. The decrease in muscle metabolic flexibility would also imply a decrease in the patient's strength levels and physical function, significantly impairing their quality of life.

1.6 PHYSICAL EXERCISE AS A TOOL FOR IMPROVING METABOLIC FLEXIBILITY

When faced with an increase in glycolytic flux, high-intensity exercise, or high CHO intake, the pyruvate molecule is not reduced to acetyl-CoA, but rather prefers to be reduced to lactate outside the mitochondria in order to obtain energy more quickly (18,20). High-intensity exercise is known to increase glycolysis, thereby increasing glucose oxidation (20). It has also been observed that the only mechanism that allows glucose to enter muscle fibre and other tissues, regardless of the presence of insulin, is muscle contraction, via the GLUT 4 transporter (18,20). Whether or not glucose enters the mitochondria will depend on the energy requirements of the activity for the individual and their metabolic flexibility (18,20).

As the intensity decreases and the duration of exercise increases, FA make a significant contribution to energy production, as they provide large amounts of energy more slowly (18).

Physical exercise is key to activating the AMPK signalling pathway, an enzyme that is released in response to high energy requirements. When this mechanism of action is initiated, GLUT 4 translocation occurs, and glycolysis, FA oxidation and mitochondrial biogenesis increase (with an increase in the coactivator protein that controls mitochondrial biogenesis, PGC-1α) (23,25). It has been observed that extensive continuous exercise activates AMPK hours after activity and that there are greater benefits as the duration of activity increases (23,24). Similarly, high-intensity cardiovascular exercise programmes also appear to increase AMPK hours after activity (23,24). In addition, high-intensity sprint training has been found to increase PGC-1α expression, and this increase appears to be greater than with other types of training. The benefits of AMPK activation from both types of cardiovascular training appear to increase when the person performs the activity while fasting (23,24).

In relation to the above, physical exercise is considered a primary tool for improving metabolic flexibility in muscle fibre and WAT (18,19,21). When AMPK and PGC-1α increases with training, increases in mitochondrial content and function in muscle fibre have been observed (18,19,21,25). This has a positive effect on the ability to oxidise FA in the mitochondria, improving insulin sensitivity (18,19,21). All of this would improve the ability of muscle fibre, now with a greater number of mitochondria, to oxidise different substrates and thus improve metabolic flexibility (18,19,21,26).

Numerous studies reflect the high muscle oxidative capacity of trained endurance athletes, which translates into a preference for FA oxidation, preserving existing glycogen (21,26). These adaptations to prolonged training make them functionally more efficient in energy expenditure and production, with improved mitochondrial health (21,26). Athletes are able to maintain activity for longer and with less fatigue than the normal untrained population due mainly to the high mitochondrial function and density of muscle fibres (21,26).

Another study conducted on professional cyclists who underwent an incremental exercise test on a cycle ergometer showed that they had a greater capacity for fat oxidation and delayed lactate accumulation due to the high capacity of the mitochondria to continue clearing the substrate, compared to the healthy active population and those with MS (21). Different authors have observed a practically perfect inverse correlation between fat oxidation and lactate concentration, and therefore this relationship has been considered an indirect marker of mitochondrial function and metabolic flexibility (20,21).

There are different physiological markers that determine a person's cardiorespiratory fitness in an incremental exercise test on a cycle ergometer: maximum oxygen consumption ($VO2_{máx.}$), aerobic threshold or 1 (VT1), anaerobic threshold or 2 (VT2), maximum power (P_{max}), CHO oxidation (CHO_{ox}), and fat oxidation (FAT_{ox}), among others (21).

An improvement in these markers indicates a greater capacity to oxidise FA before switching to the main glycolytic pathway, and greater lactate clearance (15,21,27). Thus, professional cyclists have higher $VO2_{máx.}$values than the normal active population, as well as a higher number of watts (W) generated during the incremental test (15,21,27). The results obtained in the CHO_{ox} test are similar, with higher values in the healthy active population and populations with metabolic

dysfunctions compared to professional cyclists, and with higher values in the FAT_{ox} in professional cyclists throughout the test. These results once again reflect the high oxidative capacity of professional athletes (15,21,27).

Scientific evidence shows the importance of physical exercise programmes for improving body composition at all stages of the disease in cancer patients (22,28). Specifically, reducing or maintaining the percentage of fat mass and maintaining or increasing muscle mass gains importance in the different interventions proposed in the research (22,28,29). Improved body composition is directly associated with lower CTx-induced toxicity and a higher probability of completing treatment, which could lead to higher survival rates and lower disease progression rates (22,28).

Currently, there are few studies analysing the relationship between metabolic dysfunction and the different molecular subtypes in patients with BC (67). However, in a study conducted in Nigeria with 296 women diagnosed with BC and 259 healthy control women, a strong association was found between MS and triple-negative molecular subtype diagnoses (67). Therefore, it could be assumed that any alteration in metabolic markers such as hyperinsulinemia or hyperglycaemia could increase the risk of developing TNBC.

Along the same lines, new research shows that increasing muscle strength and preventing fat mass gain is essential to good metabolic health and adequate response to treatment, as it has been associated with improved metabolic markers (insulin sensitivity, glycaemic control, acute anti-inflammatory response, etc.) (29,31). The benefits of physical exercise on mitochondrial and metabolic health have been shown to be independent of weight loss associated with fat loss (29,31).

In summary, cancer patients, specifically those with TNBC, may suffer from systemic metabolic dysfunction marked by the pathophysiology of the disease, the toxicity of cancer treatments, and unhealthy habits (4,5,7,8,10,11,12,17,18,19,20,27). However, to date, no studies have described the metabolic response to exercise in this type of patient.

Therefore, the aim is to describe the metabolic flexibility presented in participating patients with TNBC in the different phases of early disease. In this way, the energy substrate could be verified indirectly with a greater preference for muscle fibre in the different phases of early disease, and determine the intensity at which FA oxidation could be most effective in improving the metabolic profile in these patients.

Similarly, the second aim is to verify the effects of two cardiovascular training interventions, on the one hand, and a strength intervention, on the other, on the metabolic profile in patients with TNBC in the early stages of the disease.

2. OBJECTIVES

2.1 MAIN OBJECTIVES

- Describe the metabolic function of women diagnosed with TNBC at different stages of early-onset disease.

- Assess the effects of different training interventions on the metabolic function of women diagnosed with early-onset TNBC.

2.2 SECONDARY OBJECTIVES

- Analyse whether the heterogeneity of treatments in women newly diagnosed with TNBC modifies metabolic function in the different phases of early-stage disease.
- Analyse whether the accumulation of cancer treatments and the toxicity induced by them modifies metabolic function in patients diagnosed with TNBC in early-stage disease.
- Analyse the possible relationship between patient-reported outcomes (PROs) in patients diagnosed with early-stage TNBC and metabolic flexibility.
- Compare whether possible changes in metabolic flexibility with different exercise interventions are associated with improvements in PROs in patients diagnosed with early-stage TNBC.
- Check the most effective exercise intervention on the different parameters of metabolic flexibility in women with TNBC.

3. MATERIALS AND METHODS

3.1 STUDY DESIGN

This is a pilot study, initially descriptive and longitudinal, followed by a randomized, open-label experimental phase conducted in patients with early-onset TNBC. It will involve a descriptive observational analysis in the first instance, followed by an experimental analysis with four groups using pre-post testing, including a control group (CG).

The recruited patients will begin the descriptive part of the study upon diagnosis and, once they have recovered from surgery for the condition, they will begin the experimental part.

Therefore, there will be a group consisting solely of newly diagnosed TNBC patients (D1) with the inclusion criteria specified in the following section. This group will only receive the general physical exercise recommendations of the World Health Organization (WHO) throughout neoadjuvant treatment and prior to breast surgery.

In the experimental part, the initial D1 group of the sample will be divided into subgroups to carry out the physical exercise intervention protocol. Thus, the CG will receive the WHO's general physical exercise recommendations; experimental group 1 (E1) will complete 12 weeks of cardiovascular training around maximum fat oxidation ($FAT_{máx}$) twice a week; experimental group 2 (E2) will perform 12 weeks of cardiovascular training at maximum aerobic power (MAP) twice a week; and finally, experimental group 3 (E3) will complete 12 weeks of strength training twice a week. The four groups in the experimental design will be composed of patients with TNBC who have received neoadjuvant treatment and undergone surgery for their BC (**Table 1**).

Table 1. Summary of groups and interventions.

STUDY PHASE	DESCRIPTIVE	EXPERIMENTAL			
GROUP	D1	D1→ CG	D1→ E1	D1→E2	D1→E3
INTERVENTION	Metabolic flexibility description during neoadyuvant treatment	WHO recommendations	12 weeks of cardiovascular training around $FAT_{máx}$	12 weeks of cardiovascular training at MAP	12 weeks of strength training

3.2 SAMPLE SELECTION AND PARTICIPANTS

The sample will consist of 40 patients with TNBC selected for convenience from the Severo Ochoa University Hospital (Av. de Orellana, s/n, 28914 Leganés, Madrid), the Infanta Leonor University Hospital (Av. Gran Vía del Este, 80, Vallecas, 28031 Madrid), and the la Princesa University Hospital (Calle de Diego de León, 62, Salamanca, 28006 Madrid), belonging to the Community of Madrid. The sample size is small due to the low number of diagnoses in this molecular subtype of TNBC and the estimated number of patients diagnosed each year at the aforementioned hospitals. Therefore, the study has limited power, as the results obtained cannot be extrapolated to the general oncology population or to breast cancer patients. For sample selection, a direct referral methodology will be used from the oncologists at the aforementioned hospitals to all patients with TNBC who wish to participate in the study and are seen at a medical consultation at the time of recruitment. Recruitment will last for one year, taking into account the diagnosis rates reported by the described hospitals.

The inclusion and exclusion criteria that will determine the sample selection for the study are:

Inclusion criteria:

- Female between 20-55 years of age.
- Confirmed histological new diagnosis of stage I to III triple-negative breast cancer and a candidate to receive systemic neoadjuvant treatment with chemotherapy +/- immunotherapy.
- Must not have started systemic treatment for the neoplastic disease.
- Participating in any external physical exercise program or sports activities during the experimental study phase.
- Inability to understand and provide written Informed Consent (IC) (Appendix 1).

Exclusion Criteria:

- Having neurological or orthopedic disease at the time of recruitment or during the study evaluation and intervention.
- Inability to understand Spanish language.
- Presenting absolute contraindications for performing a cardiopulmonary exercise test (CPET) such as heart failure, myocarditis, acute pericarditis, severe aortic stenosis, aortic dissection, vascular toxicity, uncontrolled severe arterial hypertension, uncontrolled severe cardiac arrhythmias, pulmonary thromboembolism, or severe anemia.

- Have relative contraindications for performing a CPET such as bradyarrhythmias or tachyarrhythmias, moderate valvular stenosis, inability to perform physical or mental exertion, chronic infectious diseases, musculoskeletal disabilities, ventricular aneurysm, second- or third-degree atrioventricular block, or severe arterial hypertension.

3.3 MEASURING INSTRUMENTS

➔ INDIRECT RESTING CALORIMETRY: Q-RNG

Resting calorimetry is an assessment tool that allows us to determine resting energy expenditure (REE), RER and some oxidation variables of different energy substrates (57). A study has validated this assessment method in adults with different diseases and has proven its effectiveness in estimating REE in relation to standard predictive formulas (57). Specifically, it will be performed with the Q-RNG (COSMED, Rome, Italy), which has already been validated in various publications (61,62,63).

➔ BIOCHEMISTRY: AFINION 2

The Afinion 2 is a multi-assay analyser that provides valuable immediate diagnostic tests (55,56). This tool has been validated for measuring glycosylated haemoglobin (HbA1c) and lipid profile (TG, total cholesterol, HDL, LDL) in the healthcare systems of Canada and Italy with populations with metabolic dysfunction (55). Similarly, a study evaluating the analytical performance of C-reactive protein (CRP) using the Afinion 2 showed that its results were closer and more similar to the central laboratory method, considered the gold standard, than the other evaluation methods used in the study (56).

➔ INDIRECT CALORIMETRY DURING EXERCISE: GERATHERM ERGOSTIK

This tool allows the measurement of energy expenditure, CHO_{ox} and FAT_{ox} during the test, among other variables, through a gas exchange mask that estimates and predicts the above based on oxygen used (VO_2) and carbon dioxide expelled (VCO_2) (32,33).

Specifically, the Geratherm Ergostik analyser is based on the breath-by-breath methodology. In a study conducted on healthy men, the Ergostik was considered an analyser with good reproducibility and reliability when comparing the results obtained on different days (33). Another study compared fifteen analysers that allow cardiorespiratory fitness to be assessed, including the Geratherm Ergostik. An error of less than 5% was observed between the analysers, which were found to be highly accurate (60).

➔ CYCLE ERGOMETER: ERGOLINE ERGOSELECT 100

A cycle ergometer is a lower limb cycling tool that estimates different variables related to power. It also allows for the improvement and evaluation of both cardiac and respiratory parameters (34).

Various studies have used a conventional cycle ergometer to evaluate and intervene with exercise protocols in populations with different cardiorespiratory and metabolic diseases (21,35,36,37).

➔ DEXA: HOLOGIC DISCOVERY QDR Wi

Dual-energy X-ray absorptiometry (DEXA) is an imaging test that mainly allows the evaluation of bone mineral density (BMD) for the diagnosis of osteoporosis, and body composition (39).

Specifically, recent research has demonstrated the accuracy of this DEXA model for measuring BMD. However, no significant differences in this variable have been found compared to conventional models (39,40).

➔ BIOIMPEDANCE: INBODY 770

Electrical bioimpedance (BIO) is a technique used to estimate body composition through different frequencies that travel through the body and indirectly estimate the compartments of fat mass, muscle mass and body water, among others (41).

Various studies have confirmed the reliability of this model for estimating body composition in healthy populations to estimate the percentage of fat mass and fat-free mass (41,42). In addition, this tool has been used to assess body composition and TMB as well as PhA in a study conducted on BC survivors (42) and, in another study, to assess Xc and R in trained athletes (43).

➔ NEAR-INFRARED SPECTROSCOPY: NIRS (PortaMon)

Over the last decade, this tool has been used to determine muscle oxygenation in different sporting contexts, thus providing interesting information on the oxidative capacity of the muscle (44,45,46,47). Its reliability has been proven in different studies in both trained and untrained populations (44,45,46,47,58).

➔ LACTATE ANALYZER: LACTATE PLUS

Blood lactate has been considered an indirect indicator of muscle fibre metabolic function (20,21). Thus, some authors have conducted various studies to evaluate blood lactate in cardiorespiratory assessment tests in healthy populations (48). This assessment method has spread throughout the high-performance sports community, mainly in endurance sports, to evaluate certain parameters related to the metabolic function and performance of athletes (49).

High inter- and intra-device reliability and adequate accuracy have been observed in the Lactate Plus device in both humans and murine models (50,59).

➔ KFORCE: KINVENT

Isometric strength will be measured using a pressure dynamometer (KFORCE, KInvent, Montpellier, France) that records the kilograms of pressure completed (68,69). This assessment tool has been validated in healthy subjects with no history of previous injury to determine the isometric strength of the pectoral muscles and the isometric strength of the anterior thigh muscles (68,69).

➔ HEART RATE MONITOR: POLAR H10

According to a study conducted with recreational athletes at rest and during an incremental test, the H10 heart rate (HR) sensor was shown to be highly reliable and valid for measuring both HR and HR variability (HRV) (51).

➔ FATIGUE QUESTIONNAIRE: PERFORM

This questionnaire is the only one validated for determining fatigue in cancer patients (52). Its reliability, validity and sensitivity for quantifying improvements or detriments in variables associated with fatigue have been proven (52).

➔ QUALITY OF LIFE QUESTIONNAIRE: EORTC QLQ-C30

The European Organisation for Research and Treatment of Cancer (EORT) designed the Quality of Life Questionnaire C30 (QLQ-C30), which addresses aspects related to functional health, cancer symptoms, and other aspects of quality of life (53). Its validity has been demonstrated in patients at all stages of the disease (53).

➔ QUALITY OF LIFE QUESTIONNAIRE: EORTC QLQ-BR45

There is a complementary module to the general questionnaire above depending on the type of tumour diagnosed, so patients with BC have an additional section for providing different tumour-specific responses (54). Some studies have already confirmed its reliability and validity in other countries for assessing quality of life in patients with BC (54).

3.4 ANALYSED VARIABLES

The following variables will be evaluated after diagnosis, at the end of neoadjuvant treatment, after recovery from breast surgery, and at the end of the physical exercise programme.

- ➔ Indirect calorimetry at rest: $FAT_{ox,}$ CHO_{ox}, REE, RER.
- ➔ Cycle ergometer CPET: W, CHO_{ox}, FAT_{ox}, $VO2_{máx}$, RER, ventilatory volume (VE), VO_2, VCO_2, partial pressure of carbon dioxide (PETCO2), ventilatory oxygen equivalent (VE/VO2), ventilatory carbon dioxide equivalent (VE/CO2), HR, HRV, watts at maximum fat oxidation point ($WFAT_{máx}$), and lactate in peripheral blood sample.
- ➔ Assessment of muscle oxygenation during cycle ergometer CPET: HbO2, HHb, %TSI, HbO2 resaturation and %TSI resaturation.
- ➔ Body composition: body mass index (BMI), % total fat mass, muscle mass, bone mass, BMD, total visceral fat volume (cm3 VAT), TMB, PhA, Xc and R.
- ➔ Biochemical parameters: glucose, lactate, HDL, LDL, TG, total cholesterol, HbA1c, and CRP.
- ➔ Metabolic function: takes into account RER, FAT_{ox} y CHO_{ox}, kilocalories (KCAL) from fat and KCAL from CHO, and lactate concentration.
- ➔ Isometric strength test of the pectoral muscles and anterior thigh muscles: maximum upper limb strength (SmaxUL), maximum upper limb strength time (TSmaxUL), upper limb rate of force development (RFD_UL), maximum right lower limb strength (SmaxRL), maximum left lower limb strength (SmaxLL), lower limb RFD (RFD_LL), and lower limb asymmetry (AsymLL).

3.5 STUDY PROCEDURE

Prior to the inclusion of patients and their initial assessment, the IC (Annex 1) will be completed and formalised in the aforementioned hospitals. Data will be collected from the analyses associated with each assessment stage of the descriptive protocol (diagnosis, post-NA, post-surgery recovery and post-intervention) and from routine analyses containing some of the biochemical parameters mentioned above, as requested by the oncology team at their respective hospitals. If this is not possible, these parameters will be taken at the European University of Madrid (C/ Tajo, s/n, Urbanización El Bosque, Edificio D, 28670 Villaviciosa de Odón, Madrid) prior to each assessment.

Next, DEXA and BIO will be performed to determine the body composition of all patients in the study, carried out at the European University of Madrid.

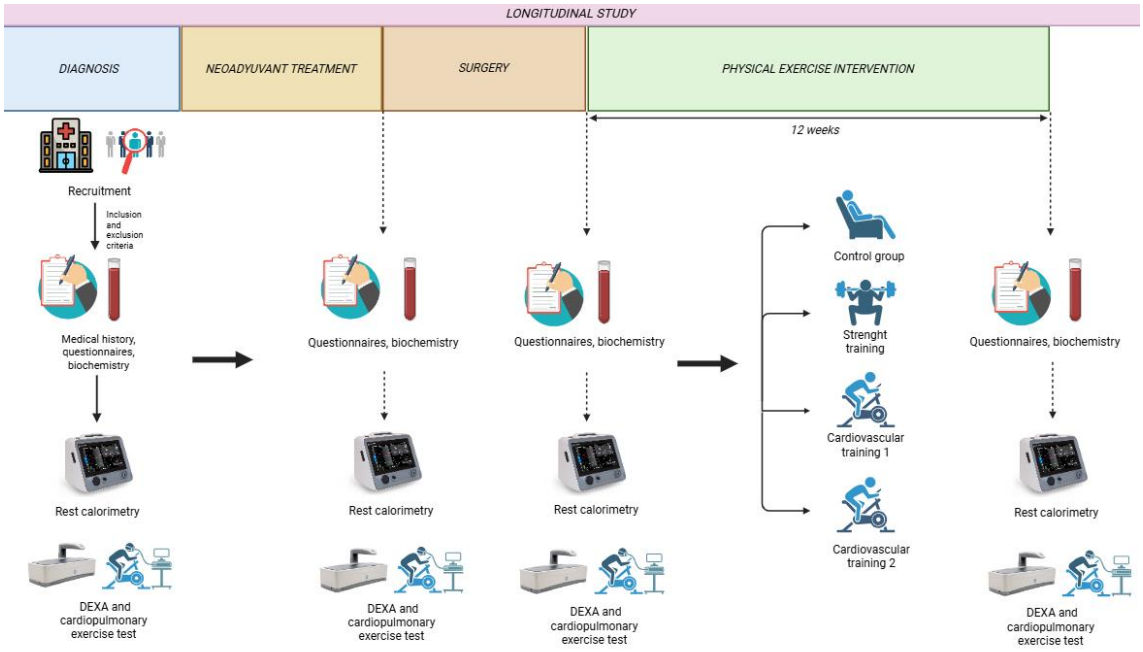
Similarly, before starting the resting calorimetry and CPET, the EORTC QLQ-C30, EORTC QLQ-BR45 and FATIGA PERFORM questionnaires related to the quality of life and health of patients with BC will be administered at the European University of Madrid.

Once all of the above has been carried out, resting calorimetry will be performed. This test will begin with the patient in a supine position on a examination table and will last approximately 15 minutes. The chamber surrounding the patient will rest on the mid-abdominal area and the front of the shoulders, so that the gases are directed exclusively towards the umbilical region. The patient will be instructed to ensure correct gas exchange by not altering their normal breathing, not falling asleep and not speaking until the test is complete. Similarly, there will be no external disturbances that could interfere with the proper completion of the test. To this end, the room will be silent and the ambient temperature will be between 18 and 22 degrees celsius. The test will be carried out on the premises of the European University of Madrid by the study researchers.

Once the resting calorimetry has been completed, the isometric strength tests will be carried out. Firstly, the pectoral muscle strength test will consist of a 5-second isometric action to analyse the neuromuscular capacities of the aforementioned muscles at 90° of shoulder flexion. Secondly, the anterior thigh muscle strength test will consist of performing an isometric knee extension in a seated position for 5 seconds, starting with a 90° knee flexion. The procedure will be repeated 3 times for each test.

All participating patients will perform an CPET on a cycle ergometer using a incremental stepwise protocol to indirectly assess metabolic flexibility through gas exchange. Subsequently, a maximum ramp protocol will also be performed on a cycle ergometer to estimate $VO2_{máx}$. These two protocols will be repeated at three key moments in the newly diagnosed BC patients selected for the study: before NA treatment; after NA treatment; and after surgery. Patients will then enter the physical exercise intervention, either through the CG or the experimental groups (E1, E2, or E3), and the final assessment of CPET will be carried out using the aforementioned protocols (**Figure 1**).

Figure 1. Longitudinal study procedure.



The CPET will follow a incremental step protocol in which participants will begin with a load of 0 W for three minutes to stabilise gas exchange data. The load will then be increased based on each participant's training level and the previously estimated relative power that takes into account the subject's weight (**Table 2**). To this end, a P_{max} value of 175W is estimated for untrained subjects, a P_{max} value of 245W for physically fit subjects, and a P_{max} value of 315W for trained subjects (**Table 2**). At least four three-minute periods representing between 20 and 60% of PAM must be completed, where the intensity at which fat oxidation is highest (FAT_{max}) is expected to be found. The three-minute per step protocol will be continued until the RER is greater than 1. In addition, pedalling must be maintained between 60 and 90 revolutions per minute (RPM) during the test. The CPET with the incremental step protocol will have a total duration of between 15 and 21 minutes depending on the cardiovascular and metabolic capacity of each patient.

After a 5-minute rest, participants from the stepwise protocol will perform the maximum ramp protocol. The test begins with the last load imposed in the step test and will increase by 3 W every 12 seconds until the patient is exhausted. It is not possible to know the approximate total duration of the CPET with this protocol due to differences in the physical condition of each patient.

Table 2. Estimation of MAP based on training level and weight. Example: subject weighing 60 kilograms.

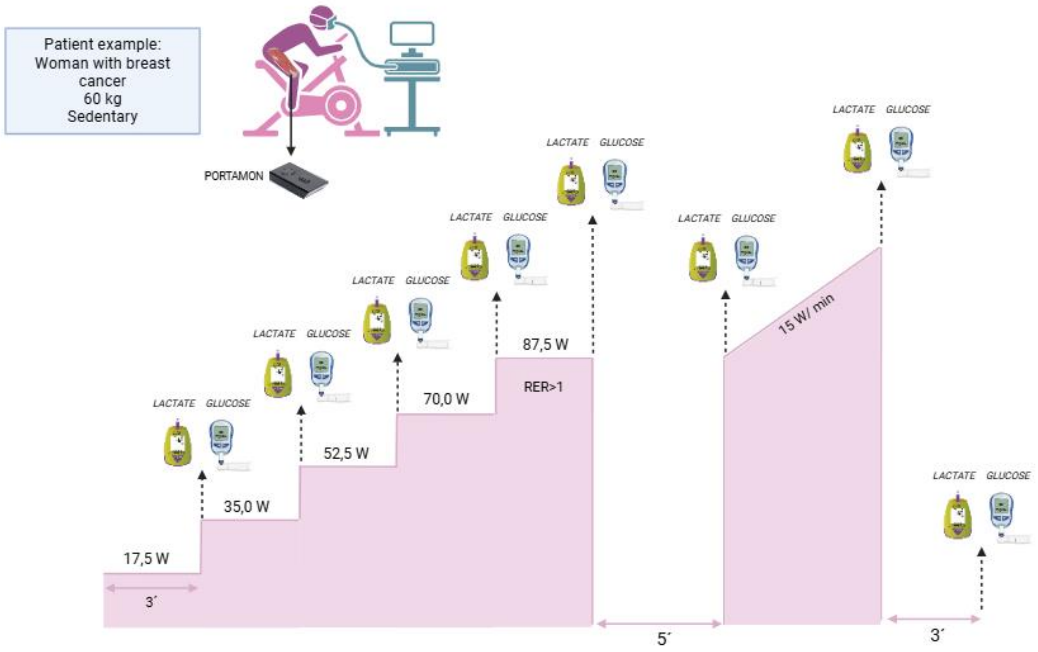
Poorly trained			
MAP estimated (w/kg)	Increase (W/kg/stage)	Subject weight	Pmax
2,92	0,29	60,00	175,00
Stage	**Load (W/kg)**	**Absolut load**	**%MAP**
1,00	0,29	17,50	0.1
2,00	0,58	35,00	0.2
3,00	0,88	52,50	0.3
4,00	1,17	70,00	0.4
5,00	1,46	87,50	0.5
6,00	1,75	105,00	0.6

Physically fit			
MAP estimated (w/kg)	Increase (W/kg/stage)	Subject weight	Pmax
4,08	0,41	60,00	245,00
Stage	**Load (W/kg)**	**Absolut load**	**%MAP**
1,00	0,41	24,50	0.1
2,00	0,82	49,00	0.2
3,00	1,23	73,50	0.3
4,00	1,63	98,00	0.4
5,00	2,04	122,50	0.5
6,00	2,45	147,00	0.6

Trained			
MAP estimated (w/kg)	Increase (W/kg/stage)	Subject weight	Pmax
5,25	0,53	60,00	315,00
Stage	**Load (W/kg)**	**Absolut load**	**%MAP**
1,00	0,53	31,50	0.1
2,00	1,05	63,00	0.2
3,00	1,58	94,50	0.3
4,00	2,10	126,00	0.4
5,00	2,63	157,50	0.5
6,00	3,15	189,00	0.6

HbA1c, CRP, and lipid profile samples (in cases where hospital test results are not available) will be collected once for each complete patient evaluation at the European University of Madrid, under resting conditions. Similarly, lactate and glucose samples will be taken at rest before starting the CPET; however, this procedure will be repeated at the end of each 3-minute period during the stepwise protocol, at the beginning and end of the ramp protocol, and after 3 minutes of active recovery without resistance from the ramp protocol (**Figure 2**).

Figure 2. Measurement of lactate and glucose samples during step and ramp protocols.



➔ EXERCISE PROGRAM

With regard to the intervention, there will be three experimental groups (E1, E2, and E3) and a CG, resulting from the D1 initial sample. For the assignment of the experimental groups and the CG, the D1 group will be randomized.

On the one hand, the participants in E1 will join a 12-week cardiovascular training program on a stationary bike at the W associated with $FAT_{máx}$, which was determined in the CPET after recovery from surgery, two days a week. Thus, there will be two weeks of familiarization in which the session will last 60 minutes at the intensity described above. Weeks three and four of the intervention will be marked by an increase in volume in minutes while maintaining the intensity, with a total session time of 65 minutes. This will be followed by 75 minutes of work at $FAT_{máx}$ W over the next eight weeks. To monitor the internal load, we will work at around 3 RPE (Rate of Perceived Exertion) throughout the intervention. Finally, the session will end with 10 minutes of cool-down with joint mobility exercises and active stretching. Numerous stationary bikes and a functional room will be available for the cardiovascular training of group E1 at the European University of Madrid.

On the other hand, participants in E2 will carry out a 12-week cardiovascular training program on a stationary bike at MAP, which was determined in the CPET after recovery from surgery, two days per week. Thus, during the first two weeks of familiarization, ten 1-minute intervals at MAP will be carried out with a 1-minute rest between intervals at 10% of MAP. Subsequently, the interval time will be increased in the following two weeks (weeks 3 and 4), with six 2-minute intervals at MAP with a 2-minute rest between intervals at 10% of MAP. Finally, four 3-minute intervals at MAP will be reached in the remaining eight weeks, with a 3-minute rest between intervals at 10% of MAP. To monitor internal load, we will work at around 7-8 RPE throughout

the intervention. Prior to the main interval training, there will be a 5-minute warm-up at 60% of MAP. The session will end with a 10-minute cool-down with joint mobility exercises and active stretching. Numerous stationary bikes and a functional room will be available for the cardiovascular training of group E2 at the European University of Madrid.

Both cardiovascular training programs (E1 and E2) will have the same perceived exertion index per session ($_S$RPE), considering this to be the RPE at the end of the session, multiplied by the total minutes of work. In this way, the total training load per patient will be obtained for each session (**Table 3**).

Table 3. Total load of the training groups throughout the intervention.

INTERVENTION	E1- CONTINUOUS TRAINING				E2- INTERVAL TRAINING						
	WORKING TIME	INTERVALS	sRPE	LOAD	WARP-UP	INTERVAL TIME	NUMBER OF INTERVALS	TIME BETWEEN INTERVALS	NUMBER OF BREAKS	sRPE	LOAD
1ª/2ª WEEK	60	1	3	180	5	1	10	1	10	7	175
3ª/4ª WEEK	65	1	3	195	5	2	6	2	6	7	203
5ª - 12ª WEEK	75	1	3	225	5	3	4	3	4	8	232

Participants in E3 will follow a 12-week strength training program with progressive loads, twice a week. The intervention in this group will be based on the key training principles in the field of health: the principle of individualization, the principle of supercompensation, and the principle of specificity.

Each strength training session will begin with joint mobility and muscle activation exercises. The latter will consist of core exercises (shoulder girdle, lumbopelvic girdle, and gluteal activation) and plyometrics. There will be a two-week familiarization period that will take into account the patient's initial level, starting with moderate loads around 6 RPE and a high number of repetitions (12) with a high repetitions in reserve (RIR) (4). Between weeks three and four, patients should perform 10 repetitions per strength exercise with a moderate RIR (3) and an RPE of 7. The following eight weeks will progress to high loads with an RPE between 8-9 and a lower number of repetitions (8-6) with a low RIR (2-1), thus attempting to maximize adaptations to training. They will start with 3 sets during the first six weeks, reaching 4 sets in the remaining six weeks of the intervention (**Table 3**).

The exercises to be implemented during the intervention are as follows: two knee-dominant lower limb exercises, hex bar squats and front lunges; two hip-dominant lower limb exercises, bilateral glute bridge and unilateral hamstring bridge; two upper limb pushing exercises, bench press and unilateral landmine in knight position; and two upper limb pulling exercises, neutral pull-ups with elastic band and unilateral dumbbell row.

Table 3. Progression of experimental group 3 (E3) throughout the strength training programme.

	WEEK1	WEEK2	WEEK3	WEEK4	WEEK5	WEEK6	WEEK7	WEEK8	WEEK9	WEEK10	WEEK11	WEEK12
EXERCISE	Hex bar squat, front lunge, bilateral glute bridge, unilateral hamstring bridge, bench press, unilateral landmine, pull-ups and unilateral dumbbell row.											
SETS	3	3	3	3	3	3	4	4	4	4	4	4
REPETITIONS	12	12	10	10	8	8	8	8	7	7	6	6
RIR	4	4	3	3	2	2	2	2	1	1	1	1
RPE	6	6	7	7	8	8	8	8	9	9	9	9

Finally, there will be a CG that will not be included in the intervention programs. However, they will be informed about general exercise recommendations for cancer patients. Like groups E1, E2, and E3, they will be evaluated at the end of the exercise intervention (**Figure 1**). In addition, they will subsequently be offered the program that has obtained the best results in the metabolic flexibility parameters and the rest of the variables analyzed.

To determine the most effective physical exercise intervention for improving metabolic flexibility and metabolic function, the following will be taken into account: the intervention group with the highest number of patients who decrease RER and lactate during CPET, defined at the end of each step in the stepwise protocol during the evaluation to be carried out after surgery, compared to the evaluation to be carried out after the exercise intervention; the intervention group with the highest number of patients who increase FAT_{ox} and CHO_{ox}, as well as the respective kilocalories (KCAL), at the points defined at the end of each step mentioned, obtained from the CPET after surgery, in relation to the results of the CPET performed at the end of the exercise intervention; the experimental group with a statistically significant inference in the changes produced in the variables mentioned above.

➔ ETHICAL AND CONFIDENTIALITY ISSUES

Informed consent

All participants will be informed about their inclusion in the study by means of an informed consent form, which will explain what their participation in the study entails.

Voluntary participation

Participation by patients is, of course, entirely voluntary, and they may decide whether or not to participate without this affecting their medical care in any way.

Data protection

The Data Controller (the Principal Investigator, Dr Lidia B. Alejo), in compliance with Organic Law 3/2018 of 5 December on the Protection of Personal Data and the guarantee of digital rights and Regulation (EU) 2016/679 of the European Parliament and of the Council of 27 April 2016 on the protection of natural persons with regard to the processing of personal data and on the free movement of such data, undertakes to accept its confidentiality responsibilities.

The data will be included in a file owned by UNIVERSIDAD EUROPEA DE MADRID, S.L.U, with registered office at C/ Tajo s/n, Villaviciosa de Odón, Madrid, which is the recipient of the information provided, for the purpose of being managed by the OTRI – Office for the Transfer of Research Results of the European University of Madrid, for the call for external research projects.

The information obtained will be stored in a computerised database and the data will be processed statistically in coded form. Donors will have the right to access, rectify or cancel the data stored in the database at any time, provided they expressly request to do so. To do so, they must contact the principal investigator. The data will be kept under the responsibility of the Principal Investigator of the Study, Dr Lidia B. Alejo.

Obtaining and using biological samples

The extraction of glucose, lactate, glycosylated haemoglobin, C-reactive protein, and the lipid profile will be carried out by professionals in the field and in an environment that complies with the specific regulations for doing so, at the European University of Madrid. The second drop of blood will be extracted, discarding the first, for each biochemical parameter by capillary puncture of the middle or ring finger of the non-dominant hand, if possible.

The samples of these biochemical parameters will be used exclusively for the indirect determination of metabolic flexibility. The biological samples will be used only for this project and will be destroyed at the end of the analysis at the time of collection.

Risks and discomfort associated with participation in the study

The discomfort associated with finger pricking refers to mild pain at the time of the prick, redness at the puncture site, residual blood droplets after extraction, small bruising, and/or minimal local inflammation.

Strength training may carry a certain risk of joint, tendon, and muscle stress, which increases the risk of injury and muscle overload (70,71). Similarly, training with heavy weights may raise blood pressure and increase the risk of acute cardiovascular events (70,71).

Strenuous, intense, prolonged physical exercise or exercise with a high demand for eccentric contractions could lead to delayed onset muscle soreness (DOMS) and muscle fatigue, causing a temporary loss of maximum voluntary contraction strength (74).

There may be a high risk of acute cardiovascular events when exercising if the person is sedentary and has known or hidden heart disease during high-intensity exercise (72).

Performing an CPET can cause certain changes such as abnormal blood pressure, fainting, or irregular, slowed, or accelerated heart rhythm (73). There is a minimal risk of sudden cardiac death, specifically 0 to 5 per 100.000 tests, or 0.005% (73). Similarly, the risk of complications requiring hospitalisation (including severe arrhythmias), acute myocardial infarction or sudden cardiac death during or immediately after the test is less than 0.2% in the first case and 0.04% and 0.01% respectively (73).

➔ STUDY RESEARCH TEAM

Table 4. Research team work schedule.

Task	Involved and responsible members	MONTH 1	MONTH 2	MONTH 3	MONTH 4	MONTH 5	MONTH 6	MONTH 7	MONTH 8	MONTH 9	MONTH 10	MONTH 11	MONTH 12	MONTH 13	MONTH 14	MONTH 15	MONTH 16	MONTH 17	MONTH 18	MONTH 19	MONTH 20	MONTH 21	MONTH 22	MONTH 23	MONTH 24
Recruitment	María José Echarri, Ana Isabel Ballesteros, Dulce Bañón, Berta Obispo and nursing team	X	X	X	X	X	X	X	X	X	X	X	X												
Informed consent	María José Echarri, Ana Isabel Ballesteros, Dulce Bañón, Berta Obispo and nursing team	X	X	X	X	X	X	X	X	X	X	X	X												
Biochemistry	María José Echarri, Ana Isabel Ballesteros, Dulce Bañón, Berta Obispo Andrea Calderón	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X				
Completion of questionnaires, resting calorimetry, bone densitometry, and exercise test	Lidia Brea, Itziar Pagola Aldazabal, David Barranco, Adriana Gallo	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X				
Exercise programmes	Adriana Gallo									X	X	X	X	X	X	X	X	X	X	X					
Data analysis	Blanca Romero, Lidia Brea , Itizar Pagola Aldazabal, David Barranco, Adriana Gallo																			X	X	X	X		
Publication of the results	The whole team																							X	X

➔ PATIENT TIMELINE

Table 5. Example of a schedule for a patient recruited in the study.

		MONTH 1				MONTH 2				MONTH 3				MONTH 4				MONTH 5				MONTH 6				MONTH 7				MONTH 8				MONTH 9				MONTH 10				MONTH 11				MONTH 12			
		W1	W2	W3	W4	W1	W2	W3	W4	W1	W2	W3	W4	W1	W2	W3	W4	W1	W2	W3	W4	W1	W2	W3	W4	W1	W2	W3	W4	W1	W2	W3	W4	W1	W2	W3	W4	W1	W2	W3	W4	W1	W2	W3	W4	W1	W2	W3	W4
E1, E2, E3, CG	RECRUITMENT AND INFORMED CONSENT																																															
	BIOCHEMISTRY RECORD																																															
	IMPLEMENTATION OF QUESTIONNAIRES, RESTING CALORIMETRY, DEXA, EXERCISE TEST																																															
	NEOADYUVANT TREATMENT																																															
	SURGERY																																															
	CARDIOVASCULAR INTERVENTION GROUP (E1)																																															
	CARDIOVASCULAR INTERVENTION GROUP (E2)																																															
	STRENGHT INTERVENTION GROUP (E3)																																															

DEXA: bone densitometry; E1: cardiovascular training group around $FAT_{máx}$; E2: cardiovascular training group around MAP; E3: strength training group; CG: control group.

➔ STUDY BUDGET

It is estimated that between 9 and 10 lactate strips will be used per patient per assessment, depending on the number of steps required to complete the stepwise protocol. Therefore, considering that four assessments with lactate collection will be carried out throughout the study, 36 lactate strips will be used per patient. The initial sample will be 40 patients, therefore the total number of lactate strips is 1,440, which multiplied by the individual cost of €3, gives a total value of €4,320.

It is estimated that between 9 and 10 glucose strips will be used per patient per assessment, depending on the number of steps required to complete the stepwise protocol. Therefore, considering that four assessments with glucose collection will be carried out throughout the study, 36 lactate strips will be used per patient. The initial sample will be 40 patients, so the total number of glucose strips is 1,440, which multiplied by the individual cost of €0.28, gives an estimated total value of €403.20.

One lipid profile per patient per assessment will be used, and considering that four assessments will be carried out throughout the study, four lipid profiles per patient will be used. This multiplied by the 40 patients in the sample equals a total of 160 lipid profiles. Thus, 160 multiplied by the cost of each lipid profile of €9 gives a total value of €1,440.

One HbA1c test per patient per assessment will be used, and, considering that they will undergo four assessments throughout the study, four HbA1c tests per patient will be used. Multiplying this by the 40 patients in the sample gives a total of 160 HbA1c tests. Thus, 160 multiplied by the cost of each HbA1c analysis of €6 gives a total value of €960.

One CRP analysis per patient per assessment will be used, and considering that they will undergo four assessments throughout the study, four CRP analyses per patient will be used. This multiplied by 40 patients in the sample gives a total of 160 CRP analyses. Thus, 160 multiplied by the cost of each CRP analysis of €6 gives a total value of €960.

One resting calorimetry test will be performed per patient per assessment, with four assessments throughout the study. Therefore, 4 resting calorimetries will be performed per patient during the research. Considering 40 patients as the initial sample, 160 resting calorimetries will be performed, which multiplied by the cost of €80, gives a total value of €12,800.

Two CPET will be performed per patient for each evaluation, with four evaluations throughout the study. Thus, eight CPET will be performed per patient during the investigation. Considering 40 patients as the initial sample, 320 CPET will be performed, which multiplied by the cost of the test of €145, reports a total value of €46,400.

Finally, one DEXA scan will be performed per patient for each assessment, with four measurements throughout the study. Thus, four DEXA scans will be performed per patient during the research. Considering 40 patients as the initial sample, 160 DEXA scans will be performed, which multiplied by the cost of the test of €120, reports a total value of €19,200.

<u>Accident insurance for participants:</u>

The cost of accident insurance per patient is €12. Taking into account the initial sample of 40 participants, €480 would be required.

Civil liability insurance:

The cost of civil liability insurance is €2,558.57.

Travel and subsistence expenses:

Travel between the different hospitals and the University will be required, for which the estimated cost is €800.

Publication and dissemination of results:

The aim is to publish at least two articles in Q2-Q1 journals, based on the team's previous experience in similar research. One of these articles is intended to be published in an Open Access (Q1) journal in the field of Sport Science, Physiology or Oncology, for which an associated budget is required. The estimated cost for this item is €2,100 (**Table 6**).

Table 6. Study Budget.

Test	Quantity	€/ unit	€Total
Lactate strips	1440	3	4320
Glucose strips	1440	0,28	403,2
Lipid profile	160	9	1440
HbA1c	160	6	960
CRP	160	6	960
Resting calorimetry	160	80	12800
Cardiopulmonary exercise test	320	145	46400
DEXA	160	120	19200
Accident insurance	40	12	480
Civil liability insurance	1	2558,57	2558,57
Travel and subsistence expenses	1	800	800
Publication of results	1	2100	2100
TOTAL			92421,77

3.6 STATISTICAL ANALYSIS

The data will be processed and analysed using *IMB SPSS Statistics version 29.0* (International Business Machines Corporation, Armonk, USA). Descriptive statistics will be expressed as percentages, means and standard deviations (SD) for all variables to be analysed. To analyse the distribution of all variables, the Kolmorgorov-Smirnov normality test will be performed before conducting inferential statistics. Thus, if the variable is parametric and follows a normal distribution, Student's t-tests or ANOVA will be used, and Pearson's correlation will be used to determine the relationships between two variables. On the other hand, if the variable is non-parametric and does not follow a normal distribution, Wilcoxon's or Mann-Whitney's U tests will be used, and Spearman's correlation will be used to determine the relationships between two variables.

During the statistical analysis, an effect size (ES) according to Cohen's d will be taken into account. To this end, a weak ES will be established with values between 0.2 and 0.5, a medium ES with values between 0.5 and 0.8, and a strong ES with values above 0.8. For all statistical analysis, the significance level will be $p \leq 0.05$.

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APPENDICES

APPENDIX 1

INFORMATION SHEET

Study entitled: "Metabolic flexibility in patients with early triple-negative breast cancer and the possible effect of a physical exercise intervention"

You are being asked to participate in a study entitled: "Metabolic flexibility in patients with early triple-negative breast cancer and the possible effect of a physical exercise intervention". This research has been approved by the Research Ethics Committee of Getafe University Hospital.

Voluntary participation

The healthcare personnel treating you have proposed that you participate in a study to determine the metabolic flexibility of muscle fibres, i.e., to verify the substrate with the highest preference in muscle fibre and the existence or absence of mitochondrial dysfunction due to cancer treatments and the pathophysiology of the disease. In addition, the aim is to find out whether the proposed physical exercise programmes can modify the aforementioned metabolic flexibility. Your participation is, of course, entirely voluntary and you can decide whether or not to participate without this affecting your medical care in any way.

General information

Some authors suggest that physical exercise has a significant influence on glucose levels, insulin resistance, growth factors, fat oxidation ratio, and lactate, among other factors. Alteration of any of these markers could activate tumour signalling pathways, thus representing a risk factor for tumour proliferation. Therefore, it could be concluded that the modulation of metabolism through physical exercise plays a fundamental role in the development of cancer.

Physical exercise is considered a key tool for improving metabolic flexibility in muscle fibre. In response to increased AMPK and PGC-1α activity with training, increases in mitochondrial content and function have been observed in muscle fibre. This has a positive effect on the ability to oxidise fatty acids in the mitochondria, improving insulin sensitivity. The above would improve the ability of muscle fibre, now with a greater number of mitochondria, to oxidise different substrates, thus improving metabolic flexibility and adapting to different energy requirements with activity.

Purpose of this study

This study has two main objectives:

a) To describe the metabolic function of women diagnosed with triple-negative breast cancer at different stages of early disease.

b) To verify the effects of different training interventions on the metabolic function of women diagnosed with triple-negative breast cancer.

Description of the tests

Your participation in the study will consist of undergoing the following assessment tests, which will be repeated four times throughout the study (after diagnosis, at the end of neoadjuvant cancer treatment, after surgery, and at the end of the physical exercise intervention): a biochemical test if you have not had one done at the hospital; a DEXA scan; an electrical bioimpedance test; three questionnaires, EORTC QLQ-C30, EORTC QLQ-BR45 and FATIGA PERFORM, related to quality of life and fatigue; a resting calorimetry test; two isometric strength tests of the pectoral muscles and the anterior thigh muscles; a cycle ergometer CPET using an incremental methodology; and a CPET using a ramp protocol. The complete assessment includes all of the above tests and will be carried out on the same day over approximately two hours for each patient at the European University of Madrid.

Following the patient's surgery, there will be three experimental groups (E1, E2, E3) and a control group (CG). If randomly selected for the experimental group, they will participate in moderate cardiovascular training two days per week, interval cardiovascular training two days per week, or strength training two days per week at the European University of Madrid.

On the one hand, participants in E1 will join a 12-week cardiovascular training programme on a stationary bike at the W associated with $FAT_{máx}$, which was determined in the incremental exercise test after recovery from surgery, two days a week. There will be two weeks of familiarisation, during which the session will last 60 minutes at the intensity described above. Weeks three and four of the intervention will be marked by an increase in volume in minutes while maintaining the intensity, with a total session time of 65 minutes. This will be followed by 75 minutes of work at the W associated with $FAT_{máx}$ over the next eight weeks. Finally, the session will end with 10 minutes of cool-down with joint mobility exercises and active stretching. There will be numerous exercise bikes and a functional room for cardiovascular training for group E1 at the European University of Madrid.

On the other hand, participants in E2 will carry out a 12-week cardiovascular training programme on a stationary bicycle at MAP, which was determined in the ramp exercise test after recovery from surgery, two days a week. Thus, during the first two weeks of familiarisation, ten 1-minute intervals at MAP will be carried out with a 1-minute rest between intervals at 10% of MAP. Subsequently, the interval time will be increased in the following two weeks (weeks 3 and 4), with six 2-minute intervals at MAP with a 2-minute rest between intervals at 10% of MAP. Finally, four 3-minute intervals at MAP will be reached in the remaining eight weeks, with a 3-minute rest between intervals at 10% of MAP. The session will end with a 10-minute cool-down with joint mobility exercises and active stretching. There will be numerous exercise bikes and a functional room for the cardiovascular training of group E2 at the European University of Madrid.

Participants in E3 will follow a 12-week strength programme with progressive loads, training twice a week. Each strength session will begin with joint mobility and muscle activation exercises. This activation exercises will be consisting of core exercises (shoulder girdle, lumbopelvic girdle, and gluteal activation) and plyometrics. There will be a two-week familiarisation period in which the patient's initial level will be taken into account, starting with moderate loads around 6 on the RPE and a high number of repetitions (12) with a high repetitions in reserve (RIR) (4). Between weeks three and four, patients should perform 10 repetitions per strength exercise with a moderate RIR (3) and an RPE of 7. The following eight weeks will progress to high loads with an RPE between 8-9 and a lower number of repetitions (8-6) with a low RIR (2-1), thus attempting

to maximise adaptations to training. They will start with 3 sets during the first six weeks, reaching 4 sets in the remaining six weeks of the intervention.

The exercises to be implemented during the strength intervention are as follows: two knee-dominant lower limb exercises, hex bar squats and front lunges; two hip-dominant lower limb exercises, bilateral glute bridge and unilateral hamstring bridge; two upper limb pushing exercises, bench press and unilateral landmine in knight position; and two upper limb pulling exercises, neutral pull-ups with elastic band and unilateral dumbbell row.

Finally, there will be a CG that will not be included in the intervention programmes. However, they will be informed about the general exercise recommendations for cancer patients. Like groups E1, E2 and E3, they will be evaluated at the end of the exercise intervention. In addition, they will subsequently be offered the programme that has obtained the best results in the metabolic flexibility parameters and the rest of the variables analysed.

The incremental exercise test will be carried out by exercise professionals (physical education and sports instructors) and in the presence of a doctor whenever there is a medium or high risk of cardiovascular events, although it is unusual for any type of setback to occur during these tests.

The exercise sessions will be supervised and designed by physical education instructors, meaning registered professionals who have completed a degree in Physical Activity and Sports Sciences.

Collection and use of biological samples

Glucose, lactate, glycosylated haemoglobin, C-reactive protein and lipid profile tests will be carried out by professionals in the field and in an environment that complies with the specific regulations for their performance, at the European University of Madrid. The second drop of blood will be extracted, discarding the first, for each biochemical parameter by capillary puncture of the middle or ring finger of the non-dominant hand, if possible.

The samples of these biochemical parameters will be used exclusively for the indirect determination of metabolic flexibility. The biological samples will be used solely for this project and will be destroyed at the end of the analysis at the time of collection.

Anticipated risks

The discomfort associated with capillary puncture refers to mild pain at the time of puncture, redness at the puncture site, residual blood drop after extraction, small bruising, and/or minimal local inflammation.

Strength training may pose a certain risk of joint, tendon and muscle stress, which increases the risk of injury and overload (Chen et al., 2021; Kambič et al., 2024). Similarly, training with heavy weights could raise blood pressure and increase the risk of acute cardiovascular events (Chen et al., 2021; Kambič et al., 2024).

There may be a high risk of acute cardiovascular events when exercising if the person is sedentary and has known or hidden heart disease during high-intensity exercise (Riebe et al., 2015).

Strenuous, intense, prolonged physical exercise or exercise with a high demand for eccentric contractions could lead to delayed onset muscle soreness (DOMS) and muscle fatigue, causing a temporary loss in maximum voluntary contraction strength (Dupuy et al., 2018).

Under conditions of prolonged fasting or a high contribution of glucose to energy generation during exercise, there is a certain risk of hypoglycaemia (Davis et al., 2014). However, this risk occurs mainly in diabetic patients (Davis et al., 2014).

Performing a CPET can cause certain changes such as abnormal blood pressure, fainting, or irregular, slowed, or accelerated heart rate (Thompson et al., 2013). There is a minimal risk of sudden cardiac death, specifically 0 to 5 per 100,000 tests, or 0.005% (Thompson et al., 2013; Myers et al., 2009). Similarly, the risk of complications requiring hospitalisation (including severe arrhythmias), acute myocardial infarction, or sudden cardiac death during or immediately after the test is less than 0.2% in the first case and 0.04% and 0.01%, respectively (Myers et al., 2009).

Patients with absolute contraindications for CPET, such as heart failure, myocarditis, acute pericarditis, severe aortic stenosis, aortic dissection, vascular toxicity, severe uncontrolled hypertension, severe uncontrolled cardiac arrhythmias, pulmonary thromboembolism, or severe anaemia, may not be included in the study (Fletcher et al., 2001).

Similarly, patients with relative contraindications for CPET, such as bradyarrhythmias or tachyarrhythmias, moderate valvular stenosis, inability to perform physical or mental exertion, chronic infectious diseases, musculoskeletal disabilities , ventricular aneurysm, second- or third-degree atrioventricular block, or severe hypertension (Fletcher et al., 2001).

Finally, there are some absolute contraindications for strength training. These include uncontrolled hypertension, unstable cardiovascular disease, and acute musculoskeletal injuries. Relative contraindications for this type of training include chronic kidney disease, moderate hypertension, diabetes mellitus, and proliferative diabetic retinopathy (Thompson et al., 2013; Williams et al., 2007; Paulo et al., 2020).

Duration of the study

The duration of the longitudinal study will be a maximum of 2 years, including the descriptive phase and the physical exercise intervention phase, depending on the protocol followed by each patient in their respective hospital in relation to the cancer treatment plan, planned surgery, etc.

The cardiovascular exercise interventions and strength training intervention will have a total duration of 12 weeks, with a training frequency of 2 days per week and a recovery period of at least 48 hours between sessions.

Confidentiality and Data Protection

The Data Controller (Principal Investigator, Dr Lidia B. Alejo), in compliance with Organic Law 3/2018 of 5 December on the Protection of Personal Data and the guarantee of digital rights and Regulation (EU) 2016/679 of the European Parliament and of the Council of 27 April 2016 on the protection of natural persons with regard to the processing of personal data and on the free movement of such data, undertakes to accept its confidentiality responsibilities.

The data will be included in a file owned by UNIVERSIDAD EUROPEA DE MADRID, S.L.U, with registered office at C/ Tajo s/n, Villaviciosa de Odón, Madrid, which is the recipient of the information provided, for the purpose of being managed by the OTRI – Office for the Transfer of Research Results of the European University of Madrid, for the call for external research projects.

The information obtained will be stored in a database, in computer format, and the data will be processed statistically in a coded form. At any time, the donor shall have the right to access, rectify or cancel the data stored in the database, provided that they expressly request it. To do so, they must contact the principal investigator. The data will be kept under the responsibility of the Principal Investigator of the Study, Dr Lidia B. Alejo.

Insurance

In accordance with current legislation, you will be insured under a policy taken out by the study sponsor to cover any potential risks that may arise from your participation in the study.

At the start of the study, accident insurance will be taken out for the participants. In addition, all members of the research team are registered with their respective professional associations, which means that they all have civil liability insurance covering their professional activities. This insurance is associated with their professional work and covers the institution responsible for the project.

On the one hand, in the event of an accident, immediate attention must be given, the accident must be recorded and reported in the policy's group register, Mapfre must be notified of the accident as soon as possible, and the patient's details and documentation relating to the accident must be provided. This policy covers all types of accidents that occur during the insured person's professional activity within the insured group. In turn, accident insurance covers accidental death (€12,000) and permanent disability according to a scale of up to €12,000. The maximum limit per claim for all insured persons is €1,500,000.

On the other hand, civil liability insurance covers everything that may be claimed from the insured as a result of damages caused unintentionally to clients and third parties due to events arising from their activity, such as: Metabolic flexibility in patients with early triple-negative breast cancer and the possible effect of a physical exercise intervention.

Costs

Participating in this project will not incur any costs.

You will be provided with a copy of this information sheet and your informed consent form to keep.

The researcher providing this information is:

_____

RESEARCHER TELEPHONE NUMBER

INFORMED CONSENT

I, Mr/Mrs/Ms……
ID/Passport No……Date of birth……..
Address (Street/Road)……..
Town/City……Postcode……..
Country……Email address……

I declare that:

1. I have understood the information contained in the information sheet for the research project "Metabolic flexibility in patients with early triple-negative breast cancer and the possible effect of a physical exercise intervention" that has been given to me, and I have had the opportunity to resolve any questions I may have had about participating in this study.
2. The personal data collected in the study will be used solely for the purposes of the aforementioned project.
3. I may withdraw from the study at any time:
   - Whenever I wish.
   - Without having to provide any explanation.
   - Without this having any negative repercussions.

We inform you that your personal data will be incorporated into a file for which Dr Lidia Brea Alejo is responsible, in order to provide you with the service that is the subject of this communication. If you wish to exercise your rights of access, rectification, cancellation or opposition, you may contact her by email at lidia.brea@universidadeuropea.es

We inform you that you may access, rectify, oppose and/or cancel the personal data in our databases at any time. To do so, you must contact the person responsible for the file and send us a signed request to the address given in the previous paragraph.

We also inform you that recordings or photographs are occasionally taken and used as evidence to justify the project, in which you may appear as a participant. By signing this document, you give your express consent to this.

<u>Data protection:</u>

☐ I consent to the processing of my personal data in accordance with the information provided.

<u>Image rights authorisation:</u>

☐ I consent to the taking, collection and processing of images and/or recordings in accordance with the information provided.

☐ I do not consent to the taking, collection and processing of images and/or recordings in accordance with the information provided.

I wish to give my consent to participate in the study entitled “Metabolic flexibility in patients with early triple-negative breast cancer and the possible effect of a physical exercise intervention” and, to this end, I sign this informed consent document in duplicate.

SIGNATURE OF THE PARTICIPANT/LEGAL REPRESENTATIVE At ……..on the……day of……..in 202…

Signature: …….

I have discussed this project with the patient in understandable language. I believe that I have informed the patient of the nature of the study and that the patient has understood this explanation. I have provided a copy of the information sheet.

SIGNATURE OF THE PERSON RESPONSIBLE FOR THE STUDY At ……..on the……day of……..in 202…

Signature: ……