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Long-Term Exercise Training Modulates Breast Cancer Outcomes and Attenuates Cancer-Induced Muscle Wasting in Animals

Academic dissertation submitted with the purpose of obtaining a doctoral degree in Physical Activity and Health from the Faculty of Sport of University of Porto, according to the Law 74/2006 from March 24th.

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To my family

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RESUMO

Esta tese é suportada por duas revisões sistemáticas da literatura com meta-análise, que perspetivaram a sistematização e a quantificação da investigação produzida em animais, e por dois estudos experimentais com ratos Sprague-Dawley que objetivaram a avaliação dos efeitos de 35 semanas de exercício físico regular de intensidade moderada, no desenvolvimento de neoplasias mamárias induzidas por carcinogénese química, no microambiente tumoral das lesões infiltrativas e no grau de atrofia muscular esquelética. As revisões sistemáticas com os respetivos procedimentos de meta-análise revelaram que a prática regular de exercício físico com caraterísticas específicas reduz a carga tumoral, a proliferação celular e a inflamação sistémica, aumentando as caraterísticas pro-apoptóticas das células tumorais e regulando o nível circulante das hormonas sexuais e dos fatores associados ao metabolismo da glicose. Não se encontraram evidências convincentes da associação entre exercício físico e angiogénese tumoral. Os resultados dos estudos experimentais revelaram, nos animais exercitados, uma redução no desenvolvimento dos tumores e no número de lesões infiltrativas. Nas lesões infiltrativas observou-se, também com o exercício físico regular, uma melhoria no equilíbrio entre a proliferação celular e a morte celular, bem como uma redução de tecido conjuntivo, o que sugere uma menor agressividade tumoral. Constatou-se ainda que o exercício físico regular inibiu a ativação de vias de sinalização associadas ao catabolismo proteico muscular esquelético, situação que conduziu a um aumento da área de secção transversal das fibras musculares e a uma melhoria do seu metabolismo oxidativo. Os resultados encontrados permitem concluir que, no modelo animal com cancro da mama induzido quimicamente, o exercício físico regular atenua a carga tumoral e favorece o estadio microscópico, limitando a agressividade infiltrativa e a progressão tumoral, reduzindo também as suas repercussões orgânicas sistémicas, particularmente a nível muscular esquelético.

ABSTRACT

The research for this thesis involved two systematic reviews of the literature guided by meta-analyses, which intend to summarize and quantify the effects of exercise on breast tumor outcomes in animal research. It also entailed two experimental studies with Sprague–Dawley rats to evaluate the effects of 35 weeks of regular physical exercise of moderate intensity not only on the development of chemically induced breast neoplasms, and in the tumor microenvironment of infiltrative lesions, but also on the degree of skeletal muscular atrophy. Guided by meta-analytic procedures, the systematic reviews revealed that regular physical exercise under specific conditions can reduce tumor burden, cell proliferation, and systemic inflammation, as well as increase the proapoptotic features of tumors, regulating the circulating levels of sex hormones and of glucose-related factors. By contrast, no convincing evidence was found to indicate a positive association between exercise and tumor angiogenesis. Moreover, the results of the experimental studies revealed a reduction in tumors development and in the number of infiltrative lesions in animals that exercised. In the infiltrative lesions of those animals, the improved balance of cell proliferation and cell death was observed, as was a reduction of connective tissue, which suggests lower tumor aggressiveness. Among other observations, regular physical exercise inhibited the activation of signaling pathways associated with catabolic effects in skeletal muscle proteins, which prompted increases in the cross-sectional area of muscle fibers, accompanied by improvements in their oxidative metabolism. Altogether, findings indicate that in animal models with chemically induced breast cancer, regular exercise training attenuates tumor burden and favorably affects tumor staging by limiting infiltrative aggressiveness and tumor progression, as well as by reducing systemic organic consequences in skeletal muscle.

LIST OF ABBREVIATIONS

ACSM [American college of sports medicine]

ACS [American cancer society]

AET [Aerobic exercise training]

AMP [Activated protein kinase

AMPK [Adenosine monophosphate-activated protein kinase]

Akt [Protein kinase B]

Apaf-1 [Apoptotic peptidase activating factor-1]

ATP [Adenosine triphosphate

Bax [BCL-2-associated x protein]

Bak [BCL-2-associated k protein]

BCL-2 [B-cell lymphoma 2]

BMI [Body mass index]

BRCA1 [Breast cancer 1]

BRCA2 [Breast cancer 2]

BT [Breast tumor]

CAF [Cancer-associated fibroblasts]

CC [Collagen content]

CD31 [Cluster of differentiation 31]

CI [Confidence interval]

CMA [Comprehensive meta-analysis]

CONT [Control]

CRP [C reactive protein]

CSA [Cross Sectional Area]

d [Day]

DAB [Diamonibenzidine]

DCIS [Ductal carcinoma in situ]

DMBA [7,12-Dimethylbenz[a]anthracene]

DNA [Deoxyribonucleic acid]

DPX [Distryene Palsticizer xylene]

ECM [Extra-cellular matrix]

E2F-1 [Transcription factor]

ER [Estrogen receptor]

ESM [Electronic supplementary material]

EX [Exercise Training]

FAS [First apoptosis signal]

FOXO [Forkhead box class O]

FW [Free wheel]

GF [Growth factor]

GHM [Glucose homeostasis and metabolism]

g. i. [Gastric intubation]

H&E [Hematoxylin and eosin]

IARC [International agency for research on cancer]

IC [Inflammation and cytokines]

IDC [Invasive ductal cancer]

IFN-γ [Interferon-gamma]

IGF [Insulin-like growth factor]

IGFBP [Insulin-like growth factor binding protein]

IL-6 [Interleukin-6]

IL-10 [Interleukin-10]

i.p. [Intraperitonial administration]

Ki67 [Marker of proliferation Ki-67]

KO [Knockout]

Ld [Long distance]

L [Long]

LCIS [Lobular carcinoma in situ]

LDL [Low density lipoprotein]

M [Mean]

M1 [Macrophages type 1]

M2 [Macrophages type 2]

MAFBx [Muscle atrophy F-box protein]

MCP-1 [Monocyte chemoattractant protein]

MeSH [Medical subject headings]

Med [Medium]

min [Minutes]

MNU [N-methyl-N-nitrosoureia]

Mod [Moderate]

mTOR [Mammalian target of rapamycin]

MURF1 [Muscle RING-finger protein 1]

MW [Motorized wheel]

NK [Natural killer cells]

NF-κB [Nuclear factor kappa B]

OR [Odds ratio]

p27 [Cyclin kinases inhibitors]

p27kip1 [Cyclin kinases inhibitors]

PBS [Phosphate-buffered saline]

PBS-T [Phosphate-buffered saline with tween]

PER VESS [Perfused vessels]

PRISMA [Preferred Reporting Items for Systematic Reviews and Meta-Analyses]

PSR [Picrosirius red]

QoL [Quality of life]

RCT [Randomized control trial]

RoB [Risk of bias]

RPA [Regular Physical Activity]

RET [Resistance exercise training]

REX [Regular exercise training]

SAP [Serum amyloid P]

SD [Standard deviation]

Sd [Short distance]

SED [Sedentary]

SH [Sex steroid hormones]

Sh [Short]

SIGLE [System for information on Grey literature in Europe]

SYRCLE [Systematic review center for laboratory animal experimentation]

TGF- β [Transforming growth factor- β]

Th [T helper cells]

TME [Tumor microenvironment]

TNF [Tumor necrosis factor]

TRDM [Treadmill]

TUNEL [Terminal deoxynucleotidyl transferase-mediated d-UTP Nick End Labeling]

TWEAK [TNF-like weak inducer of apoptosis]

V [Volume]

VEGF [Vascular endothelial growth factor]

Vig [Vigorous]

Vl [Very long]

VWR [Voluntary wheel running]

W [Weight]

WHO [World health organization]

wk [Week]

wks [Weeks]

XIAP [X-linked inhibitor of apoptosis]

CHAPTER I

GENERAL INTRODUCTION AND AIMS

1. GENERAL INTRODUCTION

Cancer remains not only one of the most daunting diseases worldwide but also a major public health concern. Although extensive research has been conducted on cancer prevention, diagnosis, and treatment, statistics of cancer's effects are disheartening. In 2012 alone, cancer killed roughly 8.2 million people worldwide [343]. Trends no doubt similar in more recent years clearly suggest that cancer continues to rank foremost among public health problems, one without any projected solutions in the near future [344].

Among cancer's various types, breast cancer is the second-most common and the most common among women. In 2012, an estimated 1.7 million women had breast cancer, more than 520,000 women died from the disease, and breast cancer accounted for 25% of all cases of cancer and 15% of all cancer-related deaths among women. Cases of breast cancer in developed countries account for about half of all cases of breast cancer worldwide and 38% of all deaths due to breast cancer [371]. The greater incidence of breast cancer in such countries reflects not only the use of breast cancer screening but also the higher prevalence of risk factors of the disease [372].

The International Agency for Research on Cancer (IARC) has estimated that 1.97 million new cases of breast cancer will be diagnosed in 2020, which represents 25% of all cases of cancer in women. In Portugal, breast cancer is the leading cause of cancer-related death among women, and the IARC estimates that the country will experience 6,749 new cases of breast cancer in

2020, 1,714 of which will be fatal [125]. Worldwide, it is assumed that one of every eight women will develop breast cancer at some point in their lives [214]. For that reason, cancer researchers have focused on seeking new preventive as well as therapeutic approaches that can reduce the number of new cases of breast cancer, more effectively treat cases that do occur, decrease the number of deaths related to breast cancer, improve the quality of life of individuals with breast cancer, and reduce the disease's recurrence in breast cancer survivors [344].

1.1 Breast cancer: Risk and prevention

The multifactorial origin of breast cancer encompasses dietary factors, reproductive factors, hormonal factors, and factors of physical activity, among others. Breast cancer's etiology begins *in utero* and, throughout life, is informed by various kinds of exposure that modulate risk at different times [124]. Several factors, including age, race, sex, estrogen exposure, reproductive factors, genetic alterations, and lifestyle, are associated with the risk of developing breast cancer [345, 381]. Dependent upon age, the development of breast cancer is nearly nonexistent before the age of 30 years and has far higher incidence in women older than 50. Once women enter their 70s, one in every 15 will probably develop breast cancer [342, 343]. Also sex dependent, breast cancer is far more frequent in women than in men, who account for less than 1% of all diagnosed cases [144]. By race, remarkable differences in the incidence and mortality of breast cancer have also been reported, with the

highest rates among Caucasian women and the lowest among Asian women [251, 341].

Evidence from epidemiologic and laboratory data indicate that the sex steroid hormones play a determining role in breast cancer etiology and that the greater the exposure to estrogens, the greater the risk of developing breast cancer [165]. Thus, the early onset of regular menstruation and late entry into menopause, both of which increase lifetime exposure to estrogens, expand the risk of developing breast cancer [353]. Indeed, a 2-years delay of menarche can reduce the risk by as much as 10%, and each year of menopause delays can increase the risk in 3% [30]. Consequently, another possible factor of increased estrogen exposure is the use of oral contraceptives and hormone replacement therapy, and indeed, the risk of breast cancer is greater following such therapy, especially when coupled with factors of density of breast tissue, body composition, and genetic mutations [63, 68, 244, 277]. Reproductive factors can also increase the risk of developing breast cancer. Women who have never given birth have an overall higher risk of breast cancer compared to parous women, and multiparous women reduce their risk by 7% with each additional birth [66, 67, 208, 238, 317]. Last, more than in other types of cancer, mammary neoplasms show familial clustering, and inherited mutations in tumor-suppressing genes *BRCA1* and *BRCA2* are also well-documented risk factors of the disease, since both are involved in DNA repair to ensure the stability of genetic components. Even if only 5–10% of all cases of breast cancer are

dependent upon genetics, individuals carrying mutations in those genes have 40–80% greater risk of developing the disease [74, 111, 145, 276, 278, 279].

Among lifestyle factors that increase the risk of breast cancer, alcohol consumption [73, 187, 234, 250], tobacco use [59, 87, 138, 275], unhealthy diet [260], and reduced levels of daily physical activity or exercise are rank among the most major [129]. Alcohol consumption and tobacco use, especially from initial menstruation to first full-term pregnancy, are consistently associated with the risk of breast cancer, for they are both considered to promote damage to deoxyribonucleic acid (DNA) [186, 193, 235]. Evidence of an association between alcohol consumption and smoking with reduced survival time following a diagnosis with breast cancer has also been reported [36, 153]. Due to increases in insulin and insulin-like growth factors (IGFs), increased body mass index (BMI), overweight or obesity, and especially visceral fat are also considered to be important risk factors for breast cancer [95, 96, 179, 205, 268, 309, 356].

By contrast, among lifestyle factors, higher daily levels of physical activity and exercise training are associated with a decreased risk of breast cancer. Unlike determined genetic factors, regular physical activity (RPA) and regular structured exercise training (REX) are modifiable risk factors that can contribute to reducing the risk of breast cancer, as convincing evidence suggests [128]. In general, RPA and/or REX (RPA/REX) can contribute to overall health, with undeniable benefits for cardiorespiratory fitness (CRF), muscle strength, insulin resistance, immune function, and BMI maintenance, which can particularly

extended to the prevention of several diseases, including breast cancer [131, 334]. As highlighted by prevention studies, their benefits occur under both premenopausal and postmenopausal conditions [105, 133, 135, 249, 290, 373], in a dose-responsive manner [129, 367]. Other evidence has recently indicated that the benefits of the RPA/REX occur independently of race or ethnicity. Moreover, stronger protection against breast cancer has been reported in women who maintain consistently high levels of physical activity from menarche to adulthood [35, 247].

Nevertheless, the biological mechanisms underlying the protective effect of RPA/REX on breast cancer remain poorly understood. Biomarkers proposed to support that association include the modulation of circulating levels of both metabolic hormones (e.g. insulin and IGFs) and sex steroid hormones (e.g. estradiol and progesterone), the reduction of proinflammatory factors (e.g. interleukins, IL-6 and IL-10; tumor necrosis factor-α, TNF-α; C-reactive protein, CRP), the enhancement of anti-inflammatory factors (e.g. adiponectin and IL-1) and immune function, by increasing the number and improving the function of innate and acquired immune cells (e.g., natural killer cells and leukocytes), and the reduction of oxidative stress (i.e. reactive oxygen species) [54, 134, 253]. Moreover, the benefits indicated by those biomarkers related to RPA/REX are closely associated with improved BMI [301]. However, it is unclear whether RPA/REX directly acts to modulate them or whether their modulation is indirectly achieved via improvements in BMI [270], even despite evidence suggesting the association of RPA/REX and the decreased risk of breast cancer

in women with normal BMI who perform exercise of moderate or vigorous intensity [129, 308].

1.2 Tumorigenesis and the hallmarks of cancer

With the potential to progress over several years, tumorigenesis is a multistage process involving initiation, promotion, progression and metastization, during which a succession of genetic changes occurs that promote the alteration of normal cells into cancer cells [161, 285]. Initiation is characterized by DNA damage as normal cells are converted into initiated ones; whereas promotion is considered to be a relatively long, reversible phase, in which altered cells proliferate and consequently generate a cumulative population of preneoplastic cells. During progression, genetic and phenotypic alterations occur along with cell proliferation, all of which increase tumor size. Last, metastization involves the spread of malignant cells from the site of origin to other distant sites [381], meaning that to form tumors, incipient cancer cells have to break the barrier that normally limits their proliferative potential. By some means, they need to become able to multiply during an exceptionally large number of growth and division cycles, so that they can complete the multiple steps of tumor development [381].

In 2001 [161], Weinberg *et al.* proposed several hallmarks of cancer to identify essential alterations in cell physiology that collectively dictate malignant growth. In 2011 [162], they updated their list to include eight hallmarks: sustained proliferative signaling, the evasion of growth suppressors, resistance

to cell death, replicative immortality, angiogenesis, the activation of invasion and metastasis, the reprogramming of energy metabolism, and the evasion of immune destruction. A notable characteristic of tumor cells is their ability to sustained proliferation by producing growth factors (GFs) and by signaling normal cells within tumor-associated stroma, which supplies them with several GFs (e.g. transforming GF-β, TGF- β) [39, 40, 61]. Another feature of cancer cells is their capacity to inhibit cell death. Their apoptotic machinery is regulated by extrinsic (i.e. the extrinsic apoptotic program) and intrinsic signals (i.e. the intrinsic apoptotic program) both types of which are guided by the activation of normally latent proteases—namely caspase 8 and 9, respectively [162]. Mediated by mitochondria, the intrinsic pathway is initiated by countless signals that triggered the proapoptotic members of the B cell lymphoma 2 (BCL-2) family (e.g. Bax, BCL-2-associated x protein and Bak, BCL-2-associated k protein) and activate a cascade of events including the release of cytochrome C to cytoplasm and the activation of caspase 9, that culminate in cell death. By contrast, the extrinsic pathway, which involves death receptors (e.g. FAS-first apoptosis signal ligand, and the FAS receptor), culminates in cell death via the activation of caspase 8 [106].

Another hallmark of cancer is angiogenesis, which tumors have to induce in order to growth. A compelling body of evidence indicates that the capacity of tumor-induced angiogenesis relies mostly on the upregulation of the vascular endothelial growth factor (VEGF) [211]. The best-characterized angiogenic factor, VEGF modulates vessel permeability and thereby promotes endothelial

cell survival, proliferation, and migration [85, 160]. Because those processes are typically overexpressed in cancer cells, VEGF is a common target of antitumor therapies [162, 337]. High levels of VEGF result in the formation of new vessels with abnormal and disorganized architectures that consequently hamper its function [239]. However, recent findings have suggested that the process of angiogenesis is far from fully understood. For patients with some types of cancer, including pancreatic cancer and breast cancer, the addition of an antiangiogenic component to chemotherapy has not produced meaningful improvement in overall survival [56]. Nevertheless, other evidence indicates that the antiangiogenic therapy has helped to normalize the tumor vasculature and thus affords an opportunity for the delivery of chemotherapy [240].

Many of the mentioned hallmarks of cancer, including the abilities to induce proliferation, inhibit apoptosis, stimulate angiogenesis avoiding hypoxia and immune detection, and confiscate immune cells to their benefit via invasion and metastasis [58], relate to tumor microenvironment (TME). During the progression of cancer, intricate crosstalk develops between malignant cells and the TME, which consists of both stromal cells and the extracellular matrix (ECM). The TME cultivates different types of altered cells (e.g. fibroblasts, endothelial cells, and leucocytes) that demonstrate permanent interaction [210], and the interaction of tumor cells and their TMEs can determine the phenotype of tumors. Cancer cells can alter their surrounding stroma to promote support for tumor development [178], and in particular, the subpopulation of cancer-associated fibroblasts (CAFs) determines cancer growth and progression [267].

The fibroblasts at the tumor site remain permanently activated, similar to their state when repairing of normal tissue [206]. In breast cancer, 80% of stromal fibroblasts, also known as myofibroblasts, are believed to have achieved the activated phenotype [206]. Such so-called “reactive stroma” is composed of a great many fibroblasts, type 1 collagen, and fibrin deposition, along with highly dense capillaries, epithelial cells, fat cells, and immune cells [225]; the ECM is also rich in type 1 collagen and fibronectin. Invasive carcinoma is often associated with the expansion of tumor stroma that corrupts the tumor basement membrane, together with increased deposition of the ECM (e.g. type 1 collagen) [338]. Given the importance of the TME in the progression of mammary neoplasms, it stands to reason that the TME could represent a therapeutic target that can be manipulated to improve the antitumor immune response by stifling the potential of malignancy.

1.3 Breast cancer-associated biomarkers

Breast cancer is a highly heterogeneous disease that encompasses a great number of biologically distinct entities with specific pathologic features and biological behaviors that inevitably prompt the host systemic response [88]. Biological evidence suggests that glucose and other factors related to glucose metabolism, including insulin, the IGF family, and IGF-binding proteins (IGFBPs), may contribute to the development of breast cancer by promoting a more favorable environment for cancer cells to grow [84, 90, 149, 274, 302, 307, 392]. Ample data highlight that cancer cells express insulin and IGF

receptors, whose active role in signaling specific networks in neoplastic tissue suggests their key role in the regulation of cellular proliferation and apoptosis [303]. Insulin may signal a cascade of proliferative and antiapoptotic event that, improve the cell cycle capacity in malignant tissue and decrease their proapoptotic properties, which could diminish the survival rate of patients with breast cancer [376]. Furthermore, mounting evidence suggests that insulin-related factors increase the risk of breast cancer recurrence and death, and adverse prognoses have been reported in relation to the levels of fasting insulin [100, 150-152, 180, 288].

Compelling proof associating inflammation and the progression of breast cancer suggests another certainty [4]. Inflammation is a function of the immune system that aims to protect the organism from pathological aggressions by inducing chemical mediators to destroy infective agents and repair the damaged tissue [24]. In 1863, Virchow first detected the presence of leukocytes in tumors, which suggested a possible connection between inflammation and cancer (for refs see [25]). At present, a wide range of evidence from epidemiological and preclinical studies supports the consensus that inflammation and cancer are related [4, 86, 243]. More than likely, tumor-related inflammation plays a significant role in the development and progression of cancer by influencing the host immune response. Indeed, the interplay of inflammatory cells and immune cells is a determinant of tumor development [155]. In response to persistent inflammation amid breast cancer, innate immune cells (e.g. macrophages and natural killer cells) and adaptive immune cells (T and B lymphocytes) are

components of TME, along with cancer cells and their surrounding stroma (e.g. fibroblasts, endothelial cells, and mesenchymal cells) [26]. Consequently, crosstalk among those various cell types determines the direction of the path that will be done [156]. The host systemic milieu is clearly affected by such interactions in the TME, which reacts to continuous inflammation that resembles a wound that never heals with unceasing surveillance [83, 101]. The permanent state of inflammation and the host–tumor interplay promote deregulation in a considerable number of systemic biomarkers. Among them, the serum diminishing of albumin synthesis has been underscored as an independent predictor of worst prognoses, even in early stages of the disease, and mortality in several types of cancer including breast cancer [159, 166, 231, 233]. Furthermore, increased levels of lactate dehydrogenase (LDH) have been implicated as an independent predictor of breast tumor progression and mortality. Some studies have even shown that LDH can be used to estimate tumor size and thereby predict treatment responses and prognoses [48, 233].

Another marker of the development and progression of mammary neoplasms is the circulating levels of sex hormones [254]. Estrogens, of which 17β-estradiol is the most common form in circulation, are steroid hormones once thought to be produced only by the ovaries but can be also produced by fat tissue, the liver and the adrenal glands [157]. Deriving from cholesterol, estrogens act at the cellular level by binding to specific nuclear estrogen receptors (ERs). In the breast tissue, estrogens stimulate the growth and differentiation of the ductal epithelium, which not only induce mitotic activity and

the growth of connective tissue but during breast cancer also stimulates the growth of breast cancer cells [282]. Of the two known subtypes of ERs—namely, ERα and ERβ—the cells of some types of breast cancer express mostly ERα, which is responsible for many of estrogen effects in normal and in cancerous breast tissue [94, 157, 282]. As mentioned, the risk of the development and progression of breast cancer relates to the circulating levels of sex hormones and is greater in women with sustained exposure to estrogen [94, 163, 204].

In contrast to estrogen, progesterone is believed to be antiproliferative, and to offer protection against the development and progression of breast cancer by reducing the estrogen-induced proliferation of breast cancer cells by way of progesterone receptors (PRs) [23]. However, whereas some studies have associated progesterone with the onset of breast tumors in response to carcinogens, others have suggested that progesterone reduces estrogen-induced proliferation in breast epithelial cells [7, 8, 46, 52, 164, 165, 185, 220, 228, 259, 326]. Such conflicting results have been observed in both human and preclinical studies. However, whereas the preclinical data can be explained, at least in part, by the use of different models for breast cancer, the divergence of results in human studies remains without explanation [46, 273].

1.4 Classification of mammary neoplasms

In women, mammary glands contain two distinct types of cells: epithelial (i.e. luminal) and myoepithelial (i.e. basal) cells. Although most breast tumors

originate in epithelial cells, the 3–15% of all breast cancers arising from basal cells feature a more aggressive type of tumor [388]. The earliest changes in breast tissue are proliferative, due to either an imbalance of factors that promote proliferation with ones that inhibit it or decreased apoptosis [381]. About 95% of breast cancers are adenocarcinomas, which can be further divided into ones that have not penetrated the limiting basement membrane (i.e. noninvasive) and ones that have (i.e. invasive or infiltrative) [216, 219].

The extraordinary diversity of histological types and subtypes of mammary tumors complicates the improvement of treatment strategies that target each one of them individually [10]. In the last decade, many efforts have focused on complementing the histological classification of breast tumors with molecular variables that could clarify their heterogeneity and thus their behavior, which would facilitate better decisions in strategizing therapies [339].

Based on similarities in gene expression profile, Perou *et al.'s* [295] classification of breast cancer into distinct subgroups has provided new insights into the biology of breast tumors. In their molecular approach, breast tumors are divided into four major classes—luminal A, luminal B, *HER2*-overexpressing, and basal-like—according to the tumors hormonal status, namely the expression of ERs and PRs, and the expression of a proto-oncogene, namely human epidermal GF receptor-type 2 (*HER2/neu*), also known as *Erbb2* in rodents and *ERBB2* in humans [295]. Luminal B tumors differ from luminal A tumors given the lower quantitative content of hormone receptors in the former; by contrast basal-like tumors are triple negative ($ER^-/PR^-/HER2^-$), and *HER2*-

overexpressing tumors cluster near them [295, 348, 349]. Hormonal status (ER+/ER-; PR+/PR-) and the expression of *HER2* (+/-) determine the aggressiveness of a given subtype [347]. Studies have shown that basal-like and *HER2*-overexpressing tumors have a more aggressive character, which results in less favorable outcome for patients than either luminal tumor type does [109]. Luminal A tumors account for 50% of invasive breast cancer tumors, luminal B tumors for 20 %, *HER2* overexpressed tumors for 15% of all invasive breast neoplasms, and the basal-like tumors for 15% of all invasive breast cancer tumors [347]. Currently, the choice of adjuvant systemic therapy is based on the patient's age, the tumor size, the histological grade, lymph node involvement, the hormone receptor status, and H*ER2* status [9, 47, 219]. However, the only predictive markers with an associated targeted therapy are the ER and *HER2* [57].

The plethora of histological types and subtypes of mammary neoplasms makes breast cancer a complex, heterogeneous disease, for which successful treatment options depend upon knowledge of the biological and molecular features of each phenotype. Accordingly, animal models are considered to be useful for investigating the mentioned phases and for understanding the biology of such an intricate disease in order to promote the development of new therapeutic targets [375]. The chief forms of carcinoma of the breast are briefly classified in Table 1 [216, 219, 318].

In both humans and animals, carcinoma *in situ* refers to a neoplastic proliferation limited to the ducts or lobes by the basement membrane that does

not invade the stroma or lymphovascular channels. Different histologic types and subtypes of mammary tumors presenting different histological patterns can be found in humans and rats, and a single tumor may comprise either a single histologic pattern or a set of different ones [216, 219, 318].

Table 1: Histological classification of mammary tumors in humans and in rats

Humans mammary gland tumors	Rats mammary gland tumors
I. Epithelial tumors	I. Epithelial tumors
A. Benign epithelial proliferations 1. Sclerosing adenosis 2. Apocrine adenosis 3. Microglandular adenosis 4. Radical scar/complex sclerosing lesion 5. Adenomas (a) Tubular (b) Lactating (c) Apocrine 6. Papillary lesions (a) Intraductal papilloma - Intraductal papilloma with atypical hyperplasia B. Intraductal proliferative lesions 1. Usual ductal hyperplasia 2. Columnar cell lesions 3. Atypical ductal hyperplasia	A. Benign lesions 1. Adenoma (a) Tubular (b) Lactating 2. Papillary lesions (a) Intraductal papilloma (b) Papillary cystadenoma B. Premalignant lesions 1. Intraductal proliferation
C. Precursor lesions 1. Ductal carcinoma in situ (DCIS)	C. Malignant lesions 1. Ductal carcinoma in situ

2. Lobular neoplasia (LCIS) 3. Papillary lesions (b) Intraductal papillary carcinoma (c) Encapsulated papillary carcinoma (e) Solid papillary carcinoma In situ	2. Papillary lesions (a) Ductal papillary carcinoma (b) Ductal solid and cribriform carcinoma (c) Ductal comedo carcinoma
D. Invasive breast carcinoma 1. Invasive ductal carcinoma, no special type (NST) 2. Invasive lobular carcinoma 3. Tubular carcinoma 4. Cribriform carcinoma 5. Mucinous carcinoma 6. Carcinoma with medullary features 7. Carcinoma with apocrine differentiation 8. Carcinoma with signet-ring-cell differentiation 9. Invasive micropapillary carcinoma 10. Metaplastic carcinoma NST 11. Papillary lesions	D. Invasive breast carcinoma 1. Tubular carcinoma 2. Cribriform carcinoma 3. Papillary carcinoma 4. Comedo carcinoma
II. Mesenchymal tumors	II. Stromal neoplasms
A. Benign 1. Nodular fasciitis 2. Myofibroblastoma 3. Benign vascular lesions 4. Pseudoangiomatous stromal hyperplasia 5. Granular cell tumor 6. Benign peripheral nerve-sheath tumors 7. Lipoma 8. Leyomioma	A. Benign 1. Fibroma
B. Premalignant lesions	

1. Desmoid-type fibromatosis 2. Inflammatory myofibroblastic tumor	
C. Malignant 1. Liposarcoma 2. Angiosarcoma 3. Rhabdomyosarcoma 4. Osteosarcoma 5. Leiomyosarcoma	B. Malignant 1. Fibrosarcoma
III. Fibroepithelial tumors	III. Epithelial stromal neoplasms
A. Benign 1. Fibroadenoma 2. Phyllodes tumor 3. Hamartoma	A. Benign 1. Fibroadenoma
B. Preneoplastic lesions 1. Phyllodes tumor	
C. Malignant 1. Phyllodes tumor 2. Periductal stromal tumor, low grade	B. Malignant 1. Carcinosarcoma

Accounting for 55% of breast cancer, invasive ductal carcinoma (IDC) is the most common form of invasive breast cancer, while ductal carcinoma *in situ* (DCIS) is the most frequent noninvasive breast neoplasms, which accounts for 20–25% of all newly diagnosed cases [103, 212]. Two thirds of all cases of breast cancer show hormone dependency upon and express hormone receptors for both estrogen ER^+ and for progesterone PR^+ [147], have better prognoses, and are linked with longer survival times as well as lower rates of recurrence [115, 177, 350].

Rodent models of breast cancer have shown intratumoral heterogeneity that can align with that of human subtypes at the molecular level [171]. Although no individual model can be expected to fully illustrate a disease as complex as breast cancer [375], mammary cancer in female rats resembles that in women in its hormone responsiveness, histological patterns, biochemical properties, and molecular and genetic characteristics [316, 318]. Consequently, rat models represent a fundamental tool for testing new therapeutic, pharmacological, and nonpharmacological approaches as well as their systemic and organic repercussions [375]. Although several models are suitable to mimic human cancer, cell-line derived models (i.e. subcutaneous or orthotropic allografts, and xenografts), genetically engineered models, and chemical models, have singular peculiarities, and the choice of any of them depends on both the goals of research and the availability of resources [139].

Among them, the chemically induced tumors pose certain advantages, particularly their short latency period and high reproducibility. Given their similarities with carcinogenesis in humans, including tumor development via a series of progressive steps (i.e. initiation, promotion, progression, and metastasis), such *in vivo* animal models provide highly relevant information about carcinogenesis in a target organ and the possibility of evaluating new preventive and therapeutic agents [209]. Therefore, chemically induced animal models of breast tumors have been extensively used to evaluate preventive and therapeutic agents for human breast cancer. Two such models, both involving Sprague–Dawley rats, are the most frequently used to study the rat mammary

tumorigenesis: tumors induced with either 7,2-dimethylbenz[a]-anthracene (DMBA) or with *N*-methyl-*N*-nitrosourea (MNU) [319]. MNU is a specific carcinogenic that, unlike DMBA does not require metabolic activation but acts directly to induce irreversible changes in DNA and thereby promotes tumors that are locally aggressive and can metastasized [271]. In general chemically induced mammary tumors are hormone-dependent adenocarcinomas [232].

Breast cancer is typically described in stages, and the most widely used system for staging breast carcinomas is the TMN system, which accounts for the size of the tumor and its metastasis in the axillary lymph nodes [219]. In the system's name, *T* refers to the tumor size (i.e. >2 cm alters the T stage), *N* refers to *nodus status*, which changes as the tumor spreads into lymph nodes, and *M* refers to metastasis, which indicates whether the cancer has spread to other sites. [219]. Histological grading is also considered to be an effective prognostic factor, and invasive breast carcinomas are routinely graded based on an assessment of tubular and glandular formation, nuclear pleomorphism and mitotic count [107, 108]. Many studies have demonstrated a significant association between those histological grades and the survival of patients with invasive breast carcinoma [41].

1.5 Cancer-induced muscle wasting

The loss of skeletal muscle in cancer is a well-documented process that affects most cancer patients, albeit to different degrees [6]. The most abundant organic system in human body, the musculoskeletal system affords the basic

functions of locomotion, strength generation, and respiration. For that reason, maintenance of muscle mass whether in health or with disease is crucial [17]. The preservation of muscle mass and muscle fiber size depends on protein turnover, in which the balance between protein synthesis and protein breakdown should be preserved. A network of signaling pathways regulates that balance, and under pathological conditions, such regulation can be compromised and result in muscle atrophy [321]. In oncology, interest in muscle function relies on the study of cancer-associated cachexia. Systemic inflammation and metabolic disorder caused by tumors seem to affect protein turnover by promoting wasting in muscle mass involving reduced protein content, muscle fiber diameter, force production, and fatigue resistance [98].

Although the understanding of the mechanisms that can underlie cancer-related cachexia remains incomplete, cachexia is considered to be a multifactorial syndrome characterized by the continuous waste of skeletal muscle, with or without loss of fat mass, primarily via the ubiquitin–proteasome system, often associated with inflammation and insulin resistance [13, 110, 120]. Cachexia can occur even in the absence of anorexia and cannot be completely reversed by nutritional support, both of which seem to suggest the presence of catabolic promoters perhaps driven through the tumor or the host, if not both [43, 346]. One possible explanation focuses on metabolic abnormalities caused by the disease that elevate the resting energy expenditure in cancer patients as a result of the high, constant demands of glucose from tumors, which prompts a negative energy balance that promotes muscle

wasting. In short, the greater the tumor mass the greater the energy demand [136, 169].

Mounting evidence suggests that the decline in muscle mass in cancer patients is an inherent condition regardless of the disease's stage. The loss of more than 5–10% of body weight is generally held as the basis of a cachectic state, although the physiological changes might emerge before that threshold is reached [102, 118, 121]. Some researchers have viewed diagnostic criteria for cancer cachexia based on weight loss as arbitrary measures that should take into account other criteria. In response to those concerns, in 2011 a panel of experts reached a consensus about the parameters for staging cachexia in cancer patients according to three levels: precachexia, cachexia and refractory cachexia. Other than weight loss, the presence of systemic inflammation, reduced muscle strength, and altered body composition, especially regarding muscle mass, should also be measured [120]. Moreover, to facilitate the classification of patients according to the severity of cachexia, Argilés *et al* have constructed the Cachexia Score (CASCO) to categorize patients according to degree of cachexia, ranging from mild to terminal, by taking the mentioned parameters (i.e., weight loss, systemic inflammation, body composition and muscle strength) into account during diagnoses [19]. However, despite all efforts it seems that weight loss continues to be the major reference for the diagnosis of a cachectic state [245].

As the physiological condition of patients with cancer deteriorates, their functional status to cope with possible pharmacological treatments deteriorates

as well. Patients with such altered body composition are highly prone to treatment-related toxic effects, even when drugs are administrated in relation to body mass or surface area [18]. Ultimately, cachectic conditions are estimated to account for 20% of all cancer deaths [368], and although certain cancers are more associated with cachexia than others, variation in the prevalence of cachexia detected among patients seems to indicate that some individuals are more susceptible to the development of cachexia than others, which suggests a genotypic dependency regardless of cancer site [192].

Skeletal muscle atrophy in cancer-related cachexia is regulated by signaling pathways that are activated via cytokines produced by tumors and stromal cells within the TME, and by cells of the host immune system [191]. As mentioned, cancer cells depend on inflammatory mediators to grow, receive protection from apoptosis, and promote angiogenesis, all of which allow them to initiate a cascade of events that has several consequences, including the triggering of the degradation of muscular proteins [190]. Studies in animals and humans have consistently shown that the ubiquitin–proteasome system is the chief regulator of protein breakdown under cancer conditions [369]. The activation of the system prompts the destruction of myofibrillar proteins that consequently impairs the contractile function of skeletal muscles and promotes atrophy [136]. Thus, the production of proinflammatory cytokines by the host and tumors, including IL-1, IL-6, TNF-α, interferon-gamma (INF-γ), TNF-like weak inducer of apoptosis (TWEAK), and myostatin, can serve to activate intracellular signals that precipitate protein degradation via nuclear factor–κB

(NF–κB) pathways [297, 298]. The presence of those proinflammatory cytokines increases the expression of muscle-specific ubiquitin ligases E3, muscle atrophy F-box protein (*MAFBx*), also known as atrogin 1, and muscle RING finger-containing protein 1 (*MuRF1*), all of which promote the ubiquitylation of myofibrillar proteins [16, 28, 29, 141]. By contrast, the activation of the phosphatidylinositol-3 kinase/Akt signaling pathway prevents muscle atrophy by inhibiting Forkhead box class O (FOXO) activity transcription factors, which augments protein synthesis [328, 395].

TWEAK and its receptor fibroblast GF inducible 14 (Fn14), in the so-called TWEAK–Fn14 system, are major regulators of skeletal muscle mass in many catabolic conditions [322, 359]. Additionally, peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α) is key in regulating skeletal muscle fiber composition, mitochondrial content, and oxidative metabolism [11]. Mitochondrial biogenesis is essential to prevent muscle loss under pathological conditions such as cancer [222, 314].

Skeletal muscles are composed of muscle fibers classified according to their speed of contraction and predominant type of energy metabolism. The phenotype and functional capacity of any given muscle depends of the quantity of the different types of fibers that compose it [329]. As mentioned, during atrophic processes, a reduction in fiber size should be expected that inevitably reduces functionality [327]. A particularity of cancer-associated muscle waste is that muscles seem to be selectively targeted [2]. Indeed, cancer stimuli seem to preferentially affect the myosin heavy chain (MyHC) of type 2 fibers with the

relative preservation of the MyHC of type 1 fibers [2, 65, 393]. Available data regarding humans and rodents seem to confirm the loss of MyHC and the consequent reduction in cross sectional area (CSA) driven by cancer stimuli [1, 21, 104, 119, 330, 379, 380, 394].

1.6 Exercise training along the breast cancer continuum

As mentioned, the risk of developing breast cancer relies upon several factors. However, unlike other risk factors such as age, family history, early menarche, and late menopause, RPA/REX is a modifiable one [308]. A considerable number of reviews have reported epidemiological evidence that establishes a connection between RPA/REX and cancer prevention by associating the amount of exercise performed with a decreased risk of developing cancer [134, 263, 312, 387]. Although the role of RPA/REX following the diagnosis of cancer has received less attention from researchers, its importance in controlling and reducing the side effects of cancer therapy is evident [79, 80, 194, 195, 199, 229]. Such evidence encourages health professionals to recommend RPA/REX as an adjuvant in treatment conditions to improve cardiorespiratory fitness that, in turn, can increase the rate of completion of pharmacologic therapies, reduce cancer-related fatigue, and improve the quality of life (QoL) of patients [78, 126, 197, 229, 272, 289, 293, 331, 334, 340, 357].

Early detection and improved treatments are vital to promoting the survival of patients with breast cancer [305]. However, better prognoses relate

not only to earlier detection but also to tumor phenotype and lifestyle behaviors [182, 347]. In recent years, researchers in exercise oncology have begun to investigate the association between active behaviors and the cellular and molecular mechanisms underlying that association [237]. Research in that field has several concerns regarding therapies that best suit specific cases, minimize the number of deaths, and reduce recurrence [60, 76, 218, 312].

Literature provides sufficient evidence to suggest that RPA/REX, when performed at moderate to vigorous intensity for at least 30 min/day, is safe and well tolerated by patients both during and after therapy [50, 182, 198, 311]. After a diagnosis of breast cancer, the American College of Sport Medicine (ACSM) recommends that patients avoid inactivity. Patients should be as active as allowed by their conditions and, if possible, follow the guidelines for healthy individuals that recommend 150 min/week of exercise training at moderate intensity or 70 min/week of exercise training at vigorous intensity, either of which should combined endurance and resistance exercises [137, 333]. Moreover, the guidelines of the American Cancer Society (ACS) support that recommendation [311]. However, the patient's overall status should always be taken into account to ensure an individually adjusted amount of activity by defining individual thresholds of activity established on a symptom-based approach [200, 284, 389].

Several studies provide evidence supporting those recommendations. Among them, a meta-analysis with 14 randomized controlled trials (RCTs) involving 715 patients with breast cancer showed that aerobic exercise training

(AET) and resistance exercise training (RET) improved self-esteem, physical fitness, body composition, and the rate of chemotherapy completion [252]. A few years later, a prospective study with more than 4,000 patients revealed that being active during and after treatment for breast cancer can reduce mortality among women regardless of age, state of the disease, and the BMI [170], while another one involving more than 14,000 women demonstrated that high levels of cardiorespiratory fitness were strongly associated with a decreased number of deaths [294]. Similar results were found in another meta-analysis of six prospective cohort studies involving more than 12,000 breast cancer survivors; those findings showed that physical activity after the diagnosis of breast cancer reduced death and recurrence by 34% and 24%, respectively, regardless of BMI, while pre-diagnosis physical activity reduced the risk of mortality only among women with a BMI <25 kg/m^2 [176].

More recently, Lahart *et al*. [218] and Lipsett *et al*. [229] conducted two meta-analyses to quantify the effects of exercise training on breast cancer outcomes during adjuvant therapy. Both meta-analyses revealed that patients with breast cancer benefited by engaging in exercise training activities. Lahart *et al*. [218] concluded that a combination of AET and RET affords significant benefits by reducing fatigue among women with breast cancer, while Lipsett *et al*. [229] reported an inverse relationship between RPA levels and breast cancer-related deaths and recurrence. Nevertheless, results observed in human populations, most of which have stemmed from epidemiological evidence, have highlighted a positive relationship between RPA/REX and breast tumor-

associated deaths and recurrence, that seems to be dose-dependent—that is, the more activity, the more protection. Yet, confusion remains about the ideal amount of exercise in response to breast cancer-associated mechanisms that can prompt the best outcomes.

However, if studies in clinical contexts have provided extensive evidence showing that RPA/REX promotes breast tumor survival and reduced recurrence, then such linearity in animal studies has not been found. In fact, divergent results have been reported regarding the development and progression of breast tumors amid RPA/REX. Furthermore, although evidence showing a positive relationship between RPA/REX and the development of mammary tumors [69, 70, 116, 188, 189, 221, 241, 242, 264, 364-366, 385, 396-398] exists, the opposite have also been reported by several researchers [71, 241, 363, 390].

Given all of the above, it is clear that interest in understanding whether RPA/REX plays a major role in tumorigenesis-related outcomes by modulating tumor behavior has increased [82, 176]. Researchers have sought to confirm a link between RPA/REX and concurrent biological changes in order to establish better outcomes. That association addresses the type, intensity, and duration of exercise training bouts, potential pathophysiological pathways, and breast cancer-associated mechanisms within the context of the beneficial effects of exercise [77].

As mentioned, the modulation of metabolic hormones, including markers of glucose–insulin homeostasis (e.g. glucocorticoids, IGFs, and insulin), sex

steroid hormones (e.g. progesterone and estrogens), systemic inflammation (e.g. IL-6, TNF-α, CRP and INF-γ), and improvements in immune response are reported to be connected to exercise [27, 97, 162, 312, 315]. Tumors are recognized to have an altered cellular metabolism that favors aerobic glycolysis in order to support high-energy turnover and rapid cell proliferation [246]. The markedly increased consumption of glucose by some tumors, such as those in the breast, which result in enhanced lactate production (i.e. the Warburg effect), is a well-described mechanism [374, 377]. Thus, limiting glucose availability should restrict the capacity of GFs to maintain cellular viability, thereby leading to cell death, in a process in which RPA/REX might be pivotal. In other research, RPA/REX has been hypothesized as an inducer of perturbations in the insulin–glucose axis, improved insulin sensitivity, and, consequently, a reduction in the circulating levels of insulin and glucose [42, 183].

Several studies, mostly RCTs, conducted in the past few years have sought to elucidate whether RPA/REX has an active role in the modulation of glucose-related factors of improved outcome projections in women diagnosed with breast cancer. Fairey *et al*. [112] conducted a RCT to determine the effects of REX in glucose-related markers of 53 postmenopausal breast cancer survivors. The exercise group trained on cycle ergometers for 15 weeks (3 days/week for 35 min) at a moderate intensity. Although no significant changes occurred in fasting insulin, glucose, insulin resistance, or IGFPB-1, REX markedly improved the levels of IGF-1 and IGFBP-3. Theoretically, increases in in IGF-1 imply improvements in cell division and the inhibition of cell death [54,

302, 325]; however, also theoretically, because IGFBP-3 is responsible for binding the majority of IGF-1, increased levels of IGFBP-3 should be a good sign [392]. Nevertheless, in the authors' opinion, the clinical implications of the results remain to be clarified [112]. In a different exercise paradigm, Schmitz *et al.* [332] also conducted a RCT involving 85 postmenopausal breast cancer survivors who underwent a twice-weekly (60 min/session) weight training program for 12 months. Training sessions were supervised for 6 months during which participants learned how to work and how to increase their workload. Thereafter, they continued to work unsupervised for another 6 months. Although positive results were found regarding IGF-2 levels, no evidence was detected concerning improvements in insulin sensitivity and glucose levels [332]. Those results, which are both discouraging and challenging, also do not indicate whatsoever that REX can effectively improve insulin levels, glucose levels, or insulin resistance. Some evidence of a positive relationship was revealed regarding IGF-2 levels that could related to the different lengths of exercise exposure in the studies, differences in the types of exercise performed, or reductions in women's BMI, as reported in the latter but not in the former study.

A few years later, Ligibel *et al.* [226] randomly assigned 101 sedentary and overweight breast cancer survivors to either a 16-week program of unsupervised AET combined with RET (2 days/week for 50 min with supervision) or to a control group in an attempt to analyze the influence of REX on insulin concentrations. The intervention group was asked to complete 90 min/week of AET. Positive changes were reported for fasting insulin with some

evidence for improvement in insulin resistance but not for fasting glucose [226]. Similarly, Irwin *et al.* [183] studied 75 postmenopausal breast cancer survivors who were subjected to an exercise program involving three weekly supervised sessions and twice-weekly unsupervised sessions, both lasting 30 min, of moderate AET for 6 months. Findings underscored decreased insulin, IGF-1 and IGFBP-3, in women with higher exercise levels. Intriguingly, albeit despite other evidence [320], the authors concluded that the decrease in IGFBP-3 probably related to similarly reduced levels of IGF-1.

Along similar lines, another RCT conducted by Guinan *et al.* [158] involve a group of 26 breast cancer survivors to verify the effects of a light-to-moderate AET program on the levels of glucose and insulin. However, the combined supervised and home-based program twice weekly for 60 min in either case produced no changes after 8 weeks [158]. Likewise, Thomas *et al.* [360] submitted 65 postmenopausal breast cancer survivors to an intervention program that combined supervised (3 days/week) and unsupervised (2 days/week) 30-min training sessions of moderate exercise with the goal of reaching 150 min/week during a 6-month period. They observed a significant, seemingly dose-dependent reduction in fasting glucose among women in the intervention, and more active women (>120 min/week) achieved better outcomes [360]. Again however, differences in their results and the results of other studies could have derived from the different exercise designs used, which casts doubts on which type and amount of exercise are best to prescribe to patients with breast cancer in order to achieve the best outcomes.

Two recently published meta-analyses did not shed any clarifying light on the discrepancies, either. Although their results demonstrated that exercise reduced fasting insulin levels [207] and IGFs [257] in the breast cancer survivors, differences in the exercise programs prevent attempting strength subgroup analysis in one of the meta-analysis [257], whereas in the other, the heterogeneity in exercise designs was mentioned as a limitation, and no subgroup analysis was performed [207]. Clearly, upholding the ethical principles that should guide any research in the field and harmonizing those concerns with results sought rank among the greatest challenges in research in exercise oncology.

Similarly to trends in human studies, contrasting results also characterize preclinical data. Several reports have associated RPA/REX with improvements in the levels of glucose-related factors [116, 398], whereas others have reported the opposite [140]. Moreover, the use of different exercise programs precludes any clear understanding of the amount of exercise desirable to enhance the glucose-related markers [366, 397].

As mentioned, chronic inflammation is a key factor of the development and progression of breast cancer [299]. RPA/REX could counteract that permanent state of inflammation by promoting a systemic anti-inflammatory environment. Among the mechanisms by which exercise can reduces cancer-induced inflammation, the working skeletal muscles by increasing the production of anti-inflammatory myokines is one of them [248, 296]. Such reductions in cancer-induced systemic inflammation could relate to the type,

intensity, duration and frequency of the exercise performed [143, 367]. Indeed, it seems that higher levels of RPA/REX intensity (i. e. moderate or vigorous) can induce the reduction of the circulating levels of proinflammatory cytokines, and improve immune function [143, 270, 281, 292, 300].

Data correlating cancer-induced inflammation with exercise in human patients is relevant to illustrate the need for more studies. In a RCT with 52 breast cancer survivors exposed to 15 weeks of moderate cycle ergometer exercise, Fairey *et al.* [114] observed significant improvements in immune function expressed by exercise-induced natural killer cell activity but did not detected any association between REX and the expression of either proinflammatory (i.e. IL-1, IL-6 and TNF-α) or anti-inflammatory (i.e. IL-4 and IL-10) cytokines [113]. In another article published a few months later with the same intervention group, the authors reported positive associations between REX and the CRP levels [114]. Similarly, in a study involving 28 breast cancer survivors exposed to moderate treadmill exercise training combined with resistance training for 6 months, Hutnick *et al.* [175] reported no association between REX and levels of plasma IL-6 and IFN-γ, although the improved activation of lymphocyte in women who exercised showcased a positive association between exercise and immune function. Gómez *et al.* [148] conducted another RCT that involved subjecting 16 breast cancer survivors to an 8-week, three-times-weekly combined aerobic and resistance exercise program but found no significant changes in their inflammation-related systemic markers (e.g. IL-6, IL-10 and TNF-α). In still another RCT Jones *et al.* [202] had

75 breast cancer survivors complete a 6-month exercise program of three weekly supervised and twice-weekly unsupervised sessions of moderate AET (i.e. running or use of different ergometers, if not both); although they observed beneficial outcomes in plasma IL-6 concentrations they detected no effects in CRP and TNF-α levels. Following a highly similar approach, Scott *et al.* [335] randomly assigned 90 breast cancer survivors to a control group or to three weekly 30-min supervised sessions of moderate exercise with different ergometers, accompanied by 15 minutes of RET (intervention group) for 24 weeks, accompanied also by individualized nutritional information about healthy diets. Although in the intervention group they detected improvements in levels of leptin, another protein with proinflammatory functions [283], they observed no changes in CRP. Likewise, Rogers *et al.* [313] found a positive relationship between REX and leptin levels, along with evidence of the benefits induced by REX in some proinflammatory cytokines (i.e. IL-6 and TNF-α) in a 3-month training program combining AET and RET in breast cancer survivors.

Despite several additional studies that have collectively addressed the spectrum of RPA and breast cancer, no associations in the levels of inflammatory parameters measured (e.g. IL-6, IL-1 and CRP) have been observed. Payne *et al.* [291] assessed the levels of RPA measured by pedometers in 20 postmenopausal women with breast cancer for 14 weeks, while Demark–Wahnefried *et al.* [95] evaluated RPA plus resistance training in a home-based program among 90 premenopausal patients for 6 months. The lack of association between RPA and inflammation parameters in both studies

likely related to the interventions implemented, which were based on patients' adherence without supervision, as well as to nonadherence of participants, as the researchers acknowledge. Similarly, Campbell *et al.* [55] did not find any association between a 24-week home-based program of moderate exercise and the outcomes of CPR as an inflammatory marker in 37 postmenopausal breast cancer survivors. However, their exceptionally small sample size, which limited the power to detect causal relationships, likely influenced their results.

Even given the considerable amount of research performed in the last decade, the relative importance of RPA/REX to changes in the systemic repercussions of breast cancer currently remains unknown. Again, differences among studies complicate discerning whether RPA/REX programs truly benefit inflammation-related markers or enhances immune response. In response to that uncertainty, researchers highlighted the positive effects of chronic exercise training in low-grade inflammation among women with breast cancer in a recent meta-analysis of different types of exercise and inflammatory mediators in breast cancer survivors. They also reported that the benefits related to intervention length (>11 weeks) and duration (>45 minutes/session) and that significant decrease in TNF- α levels were associated with decreased levels of adiposity [256]. Although such results are encouraging, they should be interpreted with caution given the number of correlations performed, which in some cases were quiet few.

In like manner and regarding preclinical data the benefits of RPA/REX to modulate inflammation are also diverse, including the improved expression of

several inflammation markers (e.g. TNF-α, CRP, INF-γ, IL-6, monocyte chemoattractant protein 1 [MCP1], serum amyloid P [SAP], leptin and spleen weight) [116, 146, 184, 269, 366, 397, 398], although other data has reported the opposite, primarily regarding in IL-6 regulation [366, 398]. In that case, the considerable variety of exercise designs and breast tumor models might have significantly influenced the outcomes.

In response to breast cancer, RPA/REX has been linked to lower levels of circulating estrogen, which could explain its favorable association with breast cancer [255]. Circulating levels of estrogen have been linked to increased cell proliferation and to the inhibition of apoptosis via ER-mediated mechanisms [224]. Lower estrogen levels among physically active women with breast cancer improve survival, particularly for ones with tumors overexpressing ER^+ and PR^+, although few data from human studies exist to support that hypothesis [176]. Irwin *et al.* [182] conducted a prospective observational study with 933 breast cancer survivors stratified by levels of self-reported physical gathered with questionnaires. They concluded that higher levels of RPAREX correlated significantly with a lower risk of death, although only among women who presented ER^+ tumors. Also using participants' physical activity self-reported via questionnaire, Dal Maso *et al.* [89] verified that the levels of RPA/REX did not relate to mortality levels regardless of the hormonal type of tumor. Similarly, Sternfeld *et al.* [352] used the data of 1970 women from a previous cohort study to assess levels of RPA/REX self-reported on a questionnaire; however, they found no differences in levels of RPA/REX, the number of breast cancer deaths,

or tumors' hormonal status. Interestingly, the number of all-cause deaths was significantly lower among women who presented ER^+ and PR^+ tumors and had engaged in RPA/REX of moderate intensity. In 2011, Irwin *et al.* [181] assessed the self-reported data of 2,910 women with breast cancer regarding their RPA/REX behaviors to determine whether such behaviors influenced mortality but found inconsistent evidence of any positive relationship between the RPA/REX levels and mortality in women with ER^+ and HER^- tumors. Conversely Chen *et al.* [60] observed that levels of RPA/REX were associated with reduced total mortality only among women with ER^- or PR^- tumors, but not with ER^+ or PR^+ ones. Although the methodological approach was identical, the results differed starkly. Referring also to self-reported levels of RPA/REX Bradshaw *et al.* [45] used the data of 1,423 women with breast cancer to gauge the relationship between those levels and the mortality index. The positive association of RPA/REX with mortality was more pronounced among women with ER^+ or PR^+ tumors than ones with ER^- or PR^-.

Altogether human studies have also presented divergent evidence even though most have used the same methodological approach. Notably lacking are studies on the relationship between circulating levels of estrogen and RPA/REX in the development and progression of breast cancer, particularly because those levels are associated with the growth of tumor cells [62, 254].

In preclinical studies, the lack of data also hampers any sure understanding of the role of RPA/REX in regulating the circulating levels of estrogen and progesterone. Indeed, RPA/REX has been shown to have a

positive association with sex hormone circulating levels in some studies [184, 366, 397, 398], although the opposite can be also found [116].

Overall, such findings suggest that methodological limitations, including small sample sizes, heterogeneous cohorts, and randomized characteristics, could explain conflicting results regarding the impact of RPA/REX on breast cancer-associated biomarkers. Moreover, the lack of data concerning some biomarkers consistently associated with the progression of mammary tumors in which improvements have been linked to RPA/REX is remarkable. And if this is true for breast cancer-associated systemic markers, then it is even more marked regarding intratumoral markers.

As mentioned, the highly adaptable architecture and integrity of TME are susceptible to permanent reshuffle in response to host signaling [213]. Host exposure to different stimuli, including exercise, can alter the systemic milieu and thereby impose a reaction of the TME [213]. However, despite of the mentioned findings linking RPA/REX with breast cancer and reflecting the epidemiological associations, to the best of our knowledge no studies with humans have involved analyzing that association with TME. Although some evidence from animal studies indicates that RPA/REX can alter the TME, such evidence is sparse and refers only to some of TME milestones, namely angiogenesis [37, 117, 184, 201, 396], apoptosis [37, 188, 189, 396] and proliferation [184, 188, 189, 396]. Notably, results of the mentioned research have also revealed divergences that mark a clear limitation to understanding the benefits of RPA/REX in the TME. Moreover, they seem to conflict regarding

angiogenesis. In fact, exercise may increase VEGF levels and at once promote a shift towards a more normalized TME by improving intratumoral perfusion and vascularization [201].

Knowing the role of physical exercise in the muscle atrophy associated with cancer is paramount. To reiterate, the loss of muscle mass throughout the disease process of cancer undoubtedly reduces the functional capacity of patients. In response, considerable efforts has been made in the past few years to find an effective anticachectic agent, and exercise has been proposed as a possible measure to mitigate or reverse muscle wasting and dysfunction, if not both [14, 15, 44, 120, 223, 230]. Several studies with humans with different types of cancer have reached conclusions that, though varied, generally favor exercise. Unfortunately, regarding breast cancer, results are scarce due to the lower incidence of breast cancer patients who suffer from cachexia [53]. As mentioned, Schmitz *et al.* [332] conducted a RCT with 85 postmenopausal breast cancer survivors divided in either an immediate treatment or delayed treatment group, which underwent a twice-weekly (60 min/session) weight training program for 12 and 6 months, respectively. Although both groups ultimately exhibited increased lean mass, the finding was more expressed in the immediate treatment group, whereas the delayed treatment group did not show variations in lean mass across the study period [332]. Courneya *et al.* [81] obtained similar results after randomizing 242 patients with breast cancer receiving ongoing treatments into three groups: a control group, a group that received AET with different ergometers of moderate-to-vigorous intensity 45

min/day three times weekly, and another that received RET involving two sets of 8/12 repetitions in different muscle groups three times weekly. The study lasted approximately 17 weeks in order to follow the entire treatment period. Both exercise groups ultimately demonstrated increased lean mass, although the result was more expressed in women who received RET, whereas the control group did not show any variation in lean mass during the study period [81]. Such lack of variation in lean mass observed between the groups prompted the underestimation of the results in a published review [6]; however, given the limited number of studies on the topic, definite conclusions about the effectiveness of REX in people with breast cancer cannot be made.

Similar to human studies research conducted with animals has also highlighted the benefits of exercise against the depletion of muscle mass in cancer context. Al-Majid *et al.* [5] reported the benefits of REX in the muscles of the lower limb of tumor-bearing animals subjected to eight sessions of electrical stimulation modeling RET. Puppa *et al.* [306] additionally reported the beneficial effects of REX on muscle mass in cancer-induced cachectic animals overexpressing systemic IL-6. REX involving moderate treadmill exercise of 55 min/day 6 days/week for 11 weeks, blocked the reduction in the quadriceps mass. Using the same animals, White *et al.* [386] observed that similar to the quadriceps, the gastrocnemius also suffered a reduction under the action of elevated systemic IL-6 that was completely reversed by exercise training. By contrast, after measuring 11 days of daily physical activity along with skeletal muscle strength, Toledo *et al.* observed the reduced physical activity and

contractile functionality of upper limb skeletal muscles, both of which effects were associated with increased tumor burden [370]. However, none of those studies were conducted with breast tumor models.

In one of two recent studies with mammary neoplasm models [127, 286], Franjacomo *et al.* [127] sought to determine whether their model of mammary neoplasms could be adequate to pursue the aims of studying cachexia in mammary models of cancer. They concluded that the model, involving Ehrlich carcinoma cells inoculation could feature systemic inflammation and the muscle wasting of cachexia in a less aggressive manner suitable for studying new pharmacological approaches. Padilha *et al.* [286], using an inoculation model of Walker-256 cancer cells subjected rats to a 6-week RET program commenced before inoculation and continued for 12 days afterward. RET performed prior to tumor implantation prevented the development of cachexia by attenuating tumor-induced systemic proinflammatory conditions, oxidative stress in the muscles, and muscle damage, which seems to suggest the benefits of exercise training prior to tumor onset.

Considering the reported findings, the heterogeneity of research designs in clinical and animal models seems to be a pivotal shortcoming in research on cancer-induced muscle wasting. The incidence and severity of cachexia depends on a reasonable range of factors and can vary according to the type, site and mass of the tumor, interindividual variations in susceptibility to cachexia, and reductions in food intake or abnormal metabolism [14, 31, 32, 34, 191, 192]. Clearly, additional studies are needed in the context of cancer-

induced muscle wasting. In a recently published systematic review and meta-analysis of RCTs involving exercise for patients with cancer cachexia, insufficient data in published literature precluded the possibility of determining whether the patients met precachexia or cachexia criteria [154]. To date, no medical intervention has completely reversed cachexia, and no approved drug therapies are available [31, 33].

However, promising results, though currently scarce, considering more local approaches have been reported. Indeed, evidence from animal studies demonstrates that RPA/REX might play an important role in attenuating cancer-induced muscle wasting by regulating or inhibition, if not both, of several factors at the molecular level [321]. As mentioned earlier, the TWEAK– Fn14 system, is the major regulator of skeletal muscle mass in many catabolic conditions [322, 359]; whereas the system's activation causes skeletal muscle wasting, PGC-1α preserves muscle mass [167]. As Hindi *et al.* [167] have shown TWEAK can significantly reduce the expression of PGC-1α along with mitochondrial content (≈ 50%), while levels of PGC-1α can be significantly increased in TWEAK-knockout and Fn14-knockout mice. Furthermore, the transgenic overexpression of PGC-1α can inhibit muscle wasting in TWEAK-transgenic mice, and TWEAK-induced activation of NF–κB (≈ 50%) can considerably reduce (≈ 90%) the expression of the atrogenes *MAFBx* and *MuRF1*. Similarly, Sato *et al.* [323] reported that TWEAK- knockout mice presented a higher expression of PGC-1α and other molecules involved in oxidative metabolism in the muscles, which consequently enhanced the muscles' mitochondrial biogenesis, oxidative

capacity, and tolerance to exercise (i.e. treadmill running at a speed of 15 m/min for 90 min). Additionally, levels of VEGF and angiogenesis also improved. In another study conducted by the same research team in TWEAK-transgenic mice, a reduction in voluntary physical activity was observed together with several other metabolic abnormalities, including glucose intolerance and insulin insensitivity [324]. Apparently, exercise activates a network of transcription factors, kinases, and coregulator proteins that cap change in gene expression and prompt increases in mitochondrial biogenesis, which in turn cause metabolic reprogramming in skeletal muscle. The neutralization of TWEAK therefore seems to be a potential approach to prevent skeletal muscle wasting [322]. In the past few years, several studies in rodents have been conducted to understand the active role of exercise in modulating transcription factors that could influence muscle metabolism and, in turn, alter the fiber phenotype in cancer [11]. Consistent evidences shows that endurance training induces mitochondrial biogenesis and a fast-to-slow fiber-type switch in skeletal muscles, in which PGC-1α is highly expressed in either type 1 and type 2A fibers [12, 227].

Taken together, the results demonstrate that a range of discrepancies in the field need clarification and that several lines of evidence need to be supported by more powerful studies in human populations. Regarding animal studies, such divergence could be overcome with protocols that realistically mimic the human contexts. In general, considering the diversity of exercise protocols of published research and the lack of consensus, we believe that if

such diversity affords the possibility of establishing whether the variables under study changed amid various degrees of exposure to exercise, then it also limits perceiving characteristics arguably essential to prescribe exercise in patients with breast cancer. Clearly, the dynamic of that diversity poses major challenges to research in exercise oncology, both with humans and animals.

2. AIMS

Considering all of the above, particularly the divergence found in published research, the specific objectives of the research conducted for this thesis were:

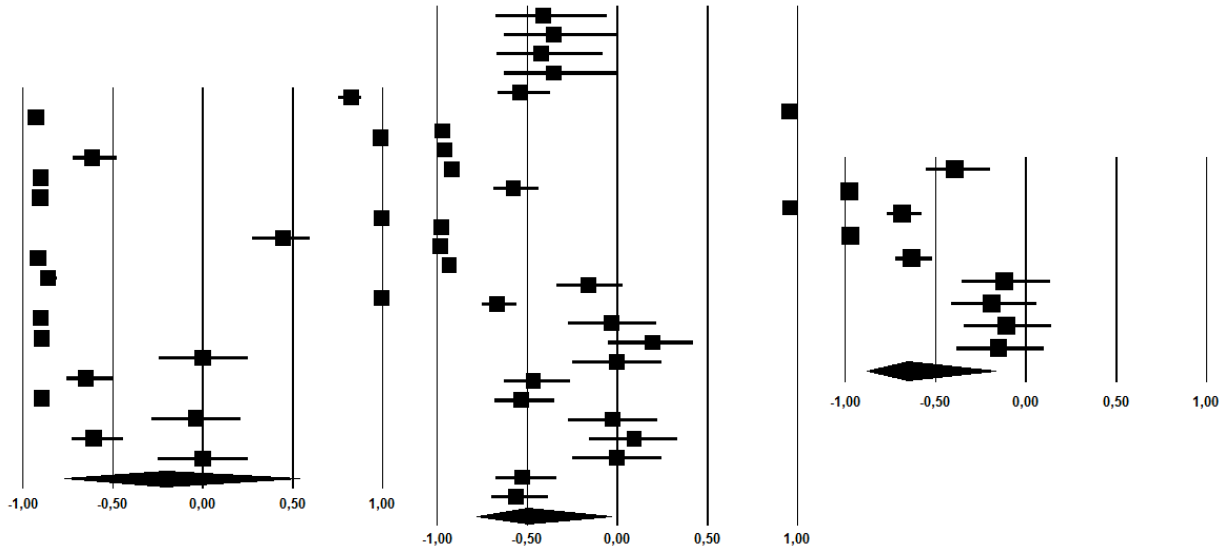
1. To summarize and quantify knowledge in preclinical data about the effects of regular exercise training and regular physical activity in breast cancer outcomes, specifically concerning tumor burden and tumor microenvironment, as well as on circulating levels of cancer-associated biomarkers;
2. To verify whether long-term moderate exercise training can attenuate breast cancer progression and malignancy in rats exposed to MNU;
3. To assess the influence of moderate exercise training on the rate of cell proliferation and death within tumors, as well as on the amount of connective tissue; and
4. To analyze the contribution of TWEAK signaling to cancer-induced muscle wasting and evaluate whether long-term exercise training alters such signaling and prevents such muscle waste.

This thesis is structured according to the Scandinavian model and is divided into five chapters. Chapter 1 includes a general, enlarged introduction to breast cancer, including its classification, etiology and prevention, that analyzes

the role of regular exercise in instances of breast cancer along the disease's continuum and in cancer-associated muscle wasting. Chapter 1 ends by stating the objectives of the thesis and its research. Chapter 2 constitutes the experimental part of the thesis and includes a systematic review and meta-analysis of the efficacy of exercise training in breast cancer outcomes in animal studies; a systematic review and meta-analysis of the effects of exercise training in breast cancer-related systemic biomarkers; an experimental study on the efficiency of exercise training in modulating the infiltrative neoplasm microenvironment; and an experimental work regarding the attenuation of muscle wasting under the influence of exercise training. Next, Chapter 3 is dedicated to a general discussion of methods and results obtained in the original studies reported in the previous chapter. Chapter 4 details the major conclusions along with the implications of the studies. Last, Chapter 5 presents a bibliography of work that supports Chapter 1 and 3.

CHAPTER II

ORIGINAL STUDIES



1. Study 1

Figueira, ACC; Cortinhas, A; Soares, JP; Leitão, JC; Ferreira, R and Duarte, JA. (2018). Efficacy of exercise on breast cancer outcomes: a systematic review and meta-analysis of preclinical data. International Journal of Sports Medicine. 39: 1–16. doi.org/10.1055/s-0044-101149. Reprinted here with the kind permission of George Thieme Verlag KG.

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Efficacy of Exercise on Breast Cancer Outcomes: A Systematic Review and Meta-analysis of Preclinical Data

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tumor burden, tumor microenvironment, proliferation, apoptosis, angiogenesis, effect size

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ABSTRACT

The use of preclinical models to investigate antitumor effects of exercise on breast tumor (BT) development and progression are critical. However, published results have not been quantitatively summarized or examined for potential exercise-moderating variables. We conducted this review to summarize and quantify the effect-size of exercise on BT outcomes in preclinical studies. A literature search was performed in MEDLINE, PubMed, Web of Science and System for Information on Grey Literature in Europe (SIGLE) databases. Risk of bias was assessed using SYRCLE's RoB tool. A total of 116 correlations were performed to analyze 28 preclinical studies published through December 2016, which included 2,085 animals and 51 exercise programs. Positive effects of small, medium and large magnitude were observed in tumor incidence, growth and multiplicity, respectively. In the tumor microenvironment, positive effects of large magnitude were also observed in proliferation and apoptosis but not in angiogenesis. Moderator variables correlated with higher intervention effects were identified along with a considerable heterogeneity in exercise protocols that precluded us from clearly perceiving the benefits of exercise exposure. In conclusion, exercise performed under specific conditions benefits BT outcomes. Preclinical studies with exercise designs mimicking exercise exposure that can be used in clinical contexts are needed.

Introduction

Breast cancer is one of the most commonly diagnosed malignancies among women, and it is the leading cause of death by cancer in women in several countries. As a result, breast cancer is also an increasingly common field of study [16]. In recent years, researchers in the field of exercise oncology have begun to investigate the association between active behaviors and cancer, as well as the cellular and molecular mechanisms underlying that association [43, 52].

The beneficial effects of regular physical activity and structured exercise training (considered here to be exercise) on breast cancer prevention and survivorship are now widely recognized [11, 17, 18, 20, 41]. Furthermore, although a few aspects of the beneficial association between exercise and tumor outcomes remain uncertain, being active is highly recommended during and after cancer treatment [3, 4, 57]. Exercise is important to control and reduce the side effects of cancer therapy [10, 36, 38, 56, 58, 59],

and being active during and after treatment appears to reduce mortality among women [26, 29, 30, 48].

Published studies have sought to confirm a link between exercise and biological changes that promote better tumor outcomes possibly achieved via the modulation of tumor behavior [49]. Indeed, knowledge of cell proliferation, the cellular apoptosis rate and proangiogenic and antiangiogenic events are important for predicting the behavior of tumors and their metastatic potential, and there is evidence that exercise may exert a beneficial effect by altering these processes [13, 45, 54].

The current literature suggests that exercise, when performed at a moderate to vigorous intensity for 75–150 min per week, is safe and well tolerated by patients during and after therapy [35, 57]. However, the majority of investigations focusing on humans have ascertained an appropriate activity level using subjective (i. e., self-reported) measures, which obscure knowledge about the type, intensity, duration and frequency of exercise that confers optimal benefits [19].

Research on the topic using animal models to mimic the disease in humans is essential to reveal the biological mechanisms by which exercise exerts its beneficial effects, identify the amounts of exercise that yield better outcomes and provide information about tumor physiology [64]. However, the results of preclinical research are still heterogeneous, likely due to the diversity of exercise experimental protocols that preclude a clear understanding of the strength of exercise effects, as well as the type, duration, intensity and frequency of exercise that most effectively prevent and control breast cancer in animals [63].

Although several reviews addressing the effects of exercise on breast cancer in animal models have been conducted, the results of such reviews have not been assessed quantitatively; no meta-analytic procedures were used to report effect sizes or to scrutinize potential moderators of intervention effects.

Our study addressed this substantial gap in the literature by quantifying the effects of exercise on breast cancer outcomes in animals. This meta-analysis focuses primarily on the effect size of exercise on tumor outcomes as assessed by changes in incidence (i. e., the total of tumors per group of animals, active vs. sedentary), multiplicity (i. e., the number of tumors per animal, active vs. sedentary), tumor weight, tumor volume and changes in the tumoral microenvironment (i. e., markers of cellular proliferation and apoptosis and markers of angiogenesis). This quantitative assessment also examines features of exercise designs that influence the exercise-cancer relation in different ways, by analyzing which ones are associated with better intervention effects.

Methods

This meta-analysis was conducted according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses statement [50]. This article does not contain any studies with human participants or animals [24].

Search strategy

A literature search, conducted once in April 2016 and again in December 2016, was performed on the following databases: MEDLINE, PubMed, Web of Science (Web of Science; Current Contents Connect), and System for Information on Grey Literature in Europe (SIGLE), and in the search we used MeSH terms and keywords related to breast cancer, exercise and animals [Electronic supplementary material (ESM) **Table S1**]. Booleans were also used, when possible, to form search expressions combining the MeSH terms (ESM **Fig. S1**). We also conducted an additional manual search of the references in the manuscripts retrieved.

Inclusion criteria

We included studies, if they satisfied the following criteria: (1) they were performed on animals and written in English; (2) they compared the effects of an active and a sedentary lifestyle on tumor outcomes; (3) they investigated the effects of exercise following tumor induction. We excluded studies, if: (1) they addressed the cancer-preventive effects of exercise; (2) they endeavored to determine the acute effects of exercise; (3) they combined exercise exposure with chemical therapy exposure; (4) they described data that made it impossible to calculate effect sizes.

Two sources were excluded because full-text versions were unavailable for purchase and could not be obtained; the authors of those sources were contacted and the manuscripts requested, but no response was received.

Data collection process

Three authors (ACCF, RF and JAD) independently assessed the eligibility of the studies and graded their methodological quality using the SYstematic Review Centre for Laboratory animal Experimentation (SYRCLE)'s Risk of Bias (RoB) tool (Cochrane RoB tool adapted for animals). This quality assessment tool rates the studies' procedures as "high." "unclear" or "low" risk of bias in six dimensions (selection, performance, detection, attrition, reporting and other bias), adapted for aspects of bias that play a specific role in animal studies (ESM **Fig. S2**) [28, 74]. Any disagreements were resolved by consensus or by involving a fourth reviewer (JPS). Three reviewers (ACCF, AC, JCL) extracted and interpreted the data and, when necessary, contacted the authors to request additional information.

The following information was extracted from each manuscript: name of the first author, year of publication, sample size and type of animal(s) used, as well as tumor induction protocol, tumor outcome data and exercise design features.

Statistical analysis

We performed the statistical analysis using Comprehensive Meta-Analysis (CMA) software version 2.2.057 (Biostat Inc, Englewood, New Jersey, USA).

To estimate the effect size of the exercise-cancer relation, we used the correlation coefficient r when available. When r was unavailable, we used averages, standard deviations and percentages and converted these statistics into r values. In comparative studies, we converted to r values using exact p values [55]. Considering the different methodologies adopted in each study a random-effects model was used and Cohen's (1988) criteria for small (r = 0.10), medium (r = 0.30) and large (r = 0.50) effects were considered to quantify the magnitude of the results [6].

▶ **Table 1** Codification of moderators.

Moderator	Values	Coding Description
Exercise type	0 = Treadmill; 1 = Free-wheel; 2 = Motorized-wheel	Type of exercise performed. Protocols in treadmill were considered as forced exercise. For voluntary exercise, protocols in free-wheel or in motorized-wheel were considered.
Exercise intensity	0 = ND; 1 = Low; 2 = Moderate; 3 = Vigorous	0: Not defined; 1: Until 50 % of maximum speed; 2: 50 %-70 % of maximum speed; 3: Above 70 % of maximum speed.
Distance covered	0 = NR; 1 = Short distance; 2 = Long distance	Distance covered during the entire program 0: Not registered; 1: Group of animals who covered shorter distances; 2: Group of animals who covered longer distances.
Exercise duration	0 = NR; 1 = Short duration; 2 = Medium duration; 3 = Long duration; 4 = Very long Duration	Daily exercise duration. 0: Not registered; 1: Under 30 min; 2: Between 30 and 45 min; 3: Between 46 and 60 min; 4: Above 60 min.
Exercise frequency	0 = 5 d/week; 1 = 6 d/week; 2 = 7 d/week	Weekly frequency. In voluntary exercise protocols, if frequency was not defined by the authors, a frequency of 7 days is always assumed.

Heterogeneity and risk of bias

We used Cochran's Q and I^2 tests to gauge heterogeneity and performed a sensitivity analysis omitting one study at a time from the initial meta-analysis. We also scrutinized potential moderators of intervention effects (e. g. voluntary or forced exercise, intensity, distance covered, duration and frequency) selected based on theory and prior findings [64]. We then accordingly coded intervention features (▶ **Table 1**).

To test for bias, we examined funnel plots and used Egger's regression and Begg's rank correlation.

Results

Study characteristics

As shown in ▶ **Fig. 1**, we identified 271 articles in the above-mentioned databases plus 90 in searches of grey literature. After an initial analysis, fifty-one articles remained and were examined in detail; however, only 28 of the articles [2, 7–9, 14, 15, 21, 23, 31, 33, 34, 39, 42, 46, 47, 51, 53, 60, 66–70, 72, 73, 75–77] fulfilled all of the inclusion criteria.

Descriptive data regarding the selected studies are presented in ▶ **Table 2**.

In total, we evaluated 51 programs, 18 of which used voluntary exercise (51.0 %), 11 used a free wheel (27.4 %) [7–9, 23, 39, 47, 60, 69, 70, 75, 76] and 7 a motorized wheel (21.5 %) [33, 34, 42, 47, 51, 69, 77]. Forced exercise on a treadmill was used in 13 studies (49.0 %) [2, 9, 14, 15, 21, 31, 46, 53, 66–68, 72, 73].

Exercise intensity was measured in 33 protocols (64.7 %). Moderate-intensity exercise was the most frequently used (45.5 %), followed by low-intensity exercise (30.3 %) and vigorous-intensity exercise (24.2 %). Only 54.4 % of the experimental protocols have defined exercise bout durations (very long: 3.5 %; long: 25.0 %; medium: 53.6 %; short: 17.9 %).

The duration of the experiments varied from 2 to 35 weeks, and 27 of these lasted more than one month [2, 7–9, 14, 15, 21, 23, 31, 33, 34, 39, 42, 46, 47, 51, 53, 60, 66–70, 72, 75–77], with 17 lasting more than two months [7–9, 14, 15, 21, 23, 33, 42, 46, 51, 53, 60, 66–68, 77].

Chemical models for breast cancer were the most frequently used (67.86 %) [7, 8, 14, 15, 21, 33, 34, 42, 46, 47, 51, 66–69, 72, 75–77], followed by transgenic models (14.29 %) [9, 23, 53, 60].

Twenty studies analyzed tumor incidence [7–9, 14, 21, 33, 34, 42, 46, 47, 51, 66–69, 72, 73, 75–77], and five of those studies (25 %) reported adverse effects of exercise [9, 21, 46, 66, 73]. Of the 14 studies that analyzed tumor multiplicity [8, 9, 14, 23, 33, 34, 46, 47, 53, 60, 69, 75–77], two (14.3 %) reported negative data associated with exercise [9, 60]. Tumor weight was observed in nine studies [2, 14, 33, 34, 60, 72, 73, 76, 77], and tumor volume was observed in 11 studies [2, 7, 9, 14, 23, 31, 46, 53, 60, 70, 72]. Negative effects of exercise were reported by one study in tumor weight (11.1 %) [14] and by three studies in tumor volume (27.3 %) [7, 14, 46].

The tumor microenvironment was analyzed in only six studies [15, 31, 33, 34, 39, 75], and negative data were reported in two studies, but only in tumor angiogenesis [15, 39].

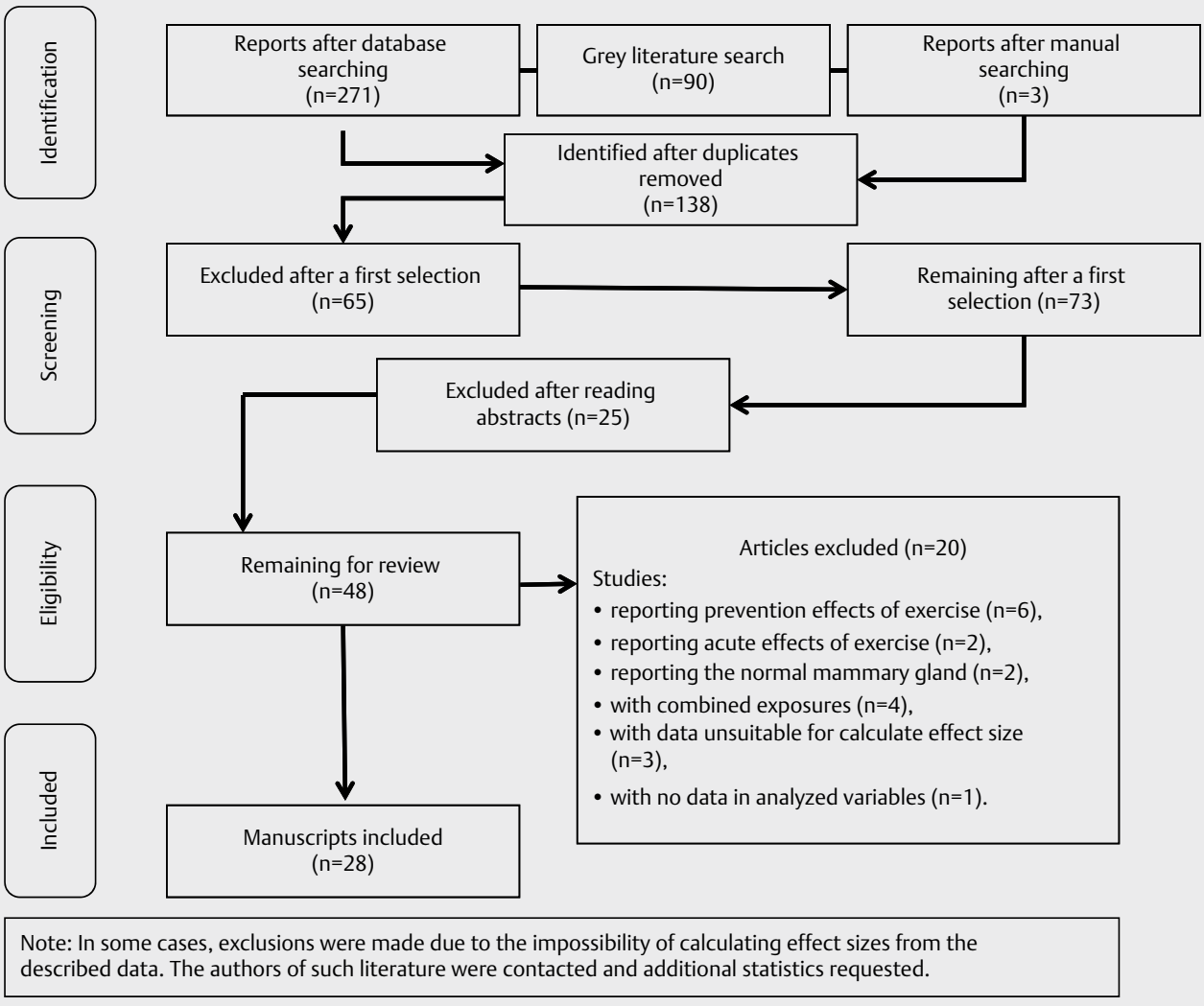
Risk of bias and quality evidence

We analyzed the proportion of "low", "unclear" or "high" risk of bias, among studies, for each item in the tool [25]. In 89.29 % (n = 25) of the studies the randomization, the baseline characteristics and the allocation concealment were adequate. The housing conditions were similar between groups in 85.71 % of the cases (n = 24), and in 96.43 % (n = 27) of the studies all the animals were used in reported outcomes. The outcomes were completely reported in 71.43 % (n = 20) of the cases. High risk of bias (64.29 %, n = 18) was observed in the "detection bias" dimension, mainly due to the unreported conditions in which the animals of the different experimental groups were sacrificed. Individually, only one study [51] was rated with high (66.67 %) and unclear (22.22 %) risk of bias. The remaining studies were rated with low risk of bias (ESM **Table S3**).

Effect size of exercise on tumor outcomes

After meta-analytic procedures the results showed that exercise significantly reduces tumor burden in animals. We performed a total of 116 correlations, 37 for incidence (▶ **Fig. 2a**), 20 for multiplicity (▶ **Fig. 2b**), 14 for tumor weight (▶ **Fig. 2c**) and 15 for volume (▶ **Fig. 2d**), and relevant correlations were noted. Regarding tumor microenvironment, ten correlations were made for proliferation (▶ **Fig. 3a**), ten for angiogenesis (▶ **Fig. 3b**) and 12 for apoptosis (▶ **Fig. 3c**). We also found significant and positive results in proliferation and apoptosis but not in angiogenesis.

The positive impact of exercise was noted in incidence with a small magnitude (r = −0.202, p = 0.000; 95 % CI = −0.295; −0.106;



▶ **Fig. 1** Flowchart depicting the selection of studies for meta-analysis.

n = 1,871; k = 37), and with a large magnitude in multiplicity (r = −0.632, p = 0.000; 95 % CI = −0.766; −0.446; n = 1,034; k = 20). Medium-level benefits were observed in tumor weight (r = −0.366, p = 0.002; 95 % CI = −0.557; −0.139; n = 639; k = 14) and tumor volume (r = −0.443, p = 0.005; 95 % CI = −0.668; −0.143; n = 508; k = 15).

Significant large-magnitude benefits were observed in tumor proliferation (r = −0.794, p = 0.000; 95 % CI = −0.881; −0.655; n = 280; k = 10) and apoptosis (r = −0.798, p = 0.000; 95 % CI = −0.872; −0.690; n = 264; k = 12). In tumor angiogenesis (r = −0.256, p = 0.448; 95 % CI = −0.734;0.392; n = 110; k = 10), small positive benefits were observed, but they were not statistically significant.

We noticed significant heterogeneity in incidence [Q(36) = 82.696, p = 0.000 (I^2 = 57 %)], multiplicity [Q(20) = 530.844, p = 0.000 (I^2 = 96 %)], tumor weight [Q(13) = 191.797, p = 0.000 (I^2 = 93 %)], tumor volume [Q(14) = 284.087, p = 0.000 (I^2 = 95 %)], proliferation [Q(9) = 51.840, p = 0.000 (I^2 = 83 %)], apoptosis [Q(11) = 53.059, p = 0.000 (I^2 = 79 %)] and angiogenesis [Q(7) = 154.379, p = 0.000 (I^2 = 96 %)].

Sensitivity analysis

For incidence, removing the study judge at high risk of bias did not reveal different results (r = −0.193, p = 0.000; 95 % CI = −0.285; −0.097) in exercise effects (ESM **Fig. S2a**). Moreover, in the remaining variables, removing one study at a time for the analysis did not substantially alter our results (ESM **Fig. S2b, c, d and S3a, b, c**).

Moderator variables analysis

Because substantial variability in effect sizes can relate to exercise features, we also analyzed moderator variables. Moderation induced by exercise features was observed in the majority of the variables that we analyzed (▶ **Table 3**).

Voluntary exercise and forced exercise

The type of exercise performed is a moderating variable of exercise effects that acts on tumor burden by decreasing it. In fact, tumor burden exhibited benefits irrespective of whether the type of ex-

▶ **Table 2** List of all the publication used with the moderator variables considered for analysis and the main tumor outcomes.

Study [Reference]	Animal/BC model	Exercise design							Tumor outcomes
		n	Study duration	Type	Intensity	Distance covered	Duration	Frequency	
Moore et al, 1973 [51]	Sprague-Dawley rats (50 days old) DMBA (1.2 mg per animal) Intravenous administration	10 Ex + 11 Sed	19 wks	MW	–	466.288 Km	30 min/d	7 days/wk	- ↓ Incidence in the active animals.
Cohen et al, 1988 [7]	F344 rats (50 days old) MNU (37.5 mg/Kg) i.p.	30 Ex + 36 Sed	20 wks	FW	–	411 260 km	–	7 days/wk	- ↓ Incidence in the active animals; - ↑ Tumor volume in the active animals.
Thompson et al, 1988 [66]	Sprague-Dawley rats (50 days old) DMBA (5 mg per animal) g.i.	35 Ex + 28 Sed	18wks	TRDM	Moderate	–	15 min/d	5 days/wk	- ↑ Incidence in the active animals.
Lane et al, 1991 [42]	BALB/c mice (42 days old) DMBA (1 mg per animal during 6 wks) g.i.	33 Ex + 32 Sed 38 Ex + 48 Sed 31 Ex + 29 Sed	34 wks	TRDM	Low	–	60 min/d	5 days/wk	- ↓ Incidence in all the active animals under standard, restricted, and high fat diets.
Cohen et al, 1993 [8]	Sprague-Dawley rats (50 days old) DMBA (10 mg per animal)	30 Ex + 30 Sed	19 wks	FW	–	–	–	7 days/wk	- ↓ Incidence in the active animals under low and high fat diets;
	DMBA (5 mg per animal) g.i.	30 Ex + 30 Sed	11 wks		–	–	–		- ↓ Multiplicity in the active animals under low and high fat diets.
Woods et al, 1994 [73]	C3H/HEN mice (52 days old) subcutaneously inoculated (SCA-1)	31 Ex + 27 Ex + 30 Sed	2 wks	TRDM	Moderate Vigorous	– –	30 min/d 125 min/d	7 days/wk	- ↑ Incidence in the active animals in both intensities; - ↓ Tumor weight in the active animals in both intensities.
Welsch et al, 1995 [70]	Sprague-Dawley rats (60 days old) Transplantation of MDA-MB231 human BC	27 Ex + 27 Ex + 27 Sed	5 wks	FW	–	4.344 to 7.562 km/day >7.562 km/day	–	7 days/wk	- ↓ Tumor volume in the active animals more pronounced in long distance runners.
Thompson et al, 1995 [67]	F-344 rats (50/57 days old) MNU (50 mg/Kg) i.p.	30 Ex + 30 Ex 29 Ex + 29 Ex + 28 Sed	12 wks	TRDM	Low Moderate	– –	20 min/d 40 min/d 20 min/d 40 min/d	5 days/wk	- ↓ Incidence in all the active animals; - ↓ Incidence even lower in the active animals under low intensities and exercise bouts of medium duration; and under moderate intensities and exercise bouts of short duration.

▶ **Table 2** Continued.

Study [Reference]	Animal/BC model	Exercise design							Tumor outcomes
		n	Study duration	Type	Intensity	Distance covered	Duration	Frequency	
Thompson et al, 1995 (1) [68]	F344 rats (50/57 days old) MNU (37.5 mg/Kg) i.p.	30 Ex + 29 Ex + 28 Ex + 30 Sed	26 wks	TRDM	Low Moderate Vigorous	– – –	40 min/d	5 days/wk	- ↓ Incidence in the active animals in all intensities.
Gillette et al, 1997 [21]	F-344 rats (50/57 days old) MNU (50 mg/Kg) i.p.	32 Ex + 30 Sed 32 EX + 30 Sed	20.5 wks	TRDM	Moderate	–	30 min/d	5 days/wk	- ↑ Incidence in the active animals under standard and restricted diets.
Westerlind et al, 2003 [72]	MNU (50 mg/Kg) i.p. Sprague-Dawley rats (21 days old) MNU (50 mg/Kg)	44 Ex + 40 Sed	2/4/6/8wks	TRDM	Moderate	–	30 min/d	5 days/wk	- = Tumor incidence in the active animals; - ↓ Tumor weight in the active animals; - ↓ Tumor volume in the active animals.
	MNU (25 mg/Kg) i.p.	79 Ex + 80 Sed	2/4/6/8wks	TRDM	Moderate	–	30 min/d	5 days/wk	- ↓ Incidence in the active animals; - ↓ Tumor weight in the active animals; - ↓ Tumor volume in the active animals.
Zhu et al, 2008 [76]	Sprague-Dawley rats (21 days old) MNU (50 mg/Kg) i.p.	52 Ex + 52 Sed	8 wks	FW	–	428.400 Km	–	7 days/wk	- ↓ Incidence in the active animals; - ↓ Multiplicity in the active animals; - ↓ Tumor weight in the active animals.
Zhu et al, 2009 [75]	Sprague-Dawley rats (21 days old) MNU (50 mg/Kg) i.p.	27 Ex + 27 Sed	8 wks	FW	–	–	–	7 days/wk	- ↓ Incidence in the active animals; - ↓ Number of tumors in the active animals; - ↓ VEGF in the active animals; - ↑ BAX, Caspase 3 and Apaf-1 in the active animals; - ↓ XIAP and BCL-2 in the active animals; - ↓ p21cip1, Cyclin D1 and E2F-1 in the active animals.
Jiang et al, 2009 [34]	Sprague-Dawley rats (21 days old) MNU (50 mg/Kg) i.p.	45 Ex + 45 Sed	6 wks	MW	Low	299.250 Km	–	7 days/wk	- ↓ Incidence in the active animals; - ↓ Multiplicity in the active animals; - ↓ Tumor weight in the active animals; - ↑ BAX, Caspase 3 and Apaf-1 in the active animals; - ↓ BCL-2 and XIAP in the active animals; - ↑ p27kip and p21cip1; ↓ Cyclin D1 and E2F-1, in the active animals.
Colbert et al, 2009 [9]	MMTV-Wnt-1 Transgenic mouse ($p53^{+/-}$)	20 Ex + 21 Ex + 22 Sed	24 wks 21 wks	TRDM	Moderate Vigorous	– –	45 min/d	5 days/wk	- ↑ Incidence in active animals in both intensities.
		20 Ex + 21 Sed	23 wks	FW	–	430.500 Km	–	7 days/wk	- ↑ Multiplicity in the active animals; - ↓ Tumor volume in TRDM animals.
Jones et al, 2010 [39]	Athymic mice Orthotopically injected cells (MDA-MB-231)	25 Ex + 25 Sed	6 wk	FW	–	168/252 Km	–	7 days/wk	- ↑ Perfused vessels in the active animals; - ↓ VEGF in the active animals; - ↑ CD31 in the active animals.

▶ **Table 2** Continued.

Study [Reference]	Animal/BC model	Exercise design							Tumor outcomes
		n	Study duration	Type	Intensity	Distance covered	Duration	Frequency	
Mann et al, 2010 [47]	Sprague-Dawley rats MNU (50 mg/Kg) i.p.	27 Ex 23 Ex + 50 Sed	8 wks	MW FW	Vigorous –	459.900 Km –	– –	7 days/wk	- ↓ Incidence in the active animals more pronounced under motorized wheel exercise; - ↓ Multiplicity in the active animals more pronounced under motorized wheel exercise.
Thompson et al, 2010 [69]	Sprague-Dawley rats MNU (50 mg/Kg) i.p.	22 Ex + 33 Ex + 55 Sed	8 wks	MW FW	Vigorous –	– 391.000 Km	– –	7 days/wk	- ↓ Incidence in the active animals; - ↓ Multiplicity in the active animals.
Murphy et al, 2011 [53]	C3(1) Transgenic mouse model of breast tumor	12 Ex + 14 Sed	20 wks	TRDM	Moderate	–	60 min/d	6 days/wk	- ↓ Multiplicity in the active animals; - ↓ Tumor volume in the active animals.
Zhu et al, 2012 [77]	Sprague Dawley rats (21 days old) MNU (50 mg/Kg) i.p.	30 Ex + 30 Ex + 30 Sed	10 wks	MW MW	Low Vigorous	125.000 Km 245.000 Km	– –	7 days/wk	- ↓ Incidence in active the animals in both intensities; - ↓ Multiplicity in the active animals in both intensities; - ↓ Tumor weight in the active animals.
Goh et al, 2013 [23]	PyMT Transgenic mouse of invasive breast cancer	13 Ex + 12 Sed	10 wks	FW	–	< 150 Km/ > 150 km	–	7 days/wk	- ↓ Multiplicity in the active animals; - ↓ Tumor volume in the active animals more pronounced in long distance runners.
Steiner et al, 2013 [60]	C3(1)/SV40 Tag Transgenic mouse model of breast tumor	12 Ex + 15 Sed	20 wks	FW	–	–	–	7 days/wk	- ↑ Multiplicity in the active animals - ↓ Tumor weight in the active animals - ↓ Tumor volume in active animals
Jiang et al, 2013 [33]	Sprague-Dawley rats (21 days old) MNU (50 mg/Kg) i.p.	30 Ex + 30 Ex + 30 Sed	10 wks	MW	Moderate Vigorous	245.000 Km 125.000 Km	– –	7 days/wk	- ↓ Incidence in the active animals in both intensities; - ↓ Multiplicity in the active animals in both intensities; - ↓ Tumor weight in the active animals; - ↑ BAX, ↓ BCL-2 in the active animals; - ↑ p27 ↓ Cyclin D1 in the active animals.
Malicka et al, 2015 [46]	Sprague-Dawley rats (42 days old) MNU (180 mg/Kg) i.p.	12 Ex + 10 Ex + 7 Ex + 14 Sed	12 wks	TRDM	Low Moderate Vigorous	– – –	28 min/d 35 min/d 42 min/d	5 days/wk	- ↑ Incidence in the active animals under low intensity; - ↓ Incidence in the active animals under moderate and vigorous intensities; - ↓ Multiplicity in the active animals in all intensities; - ↓ Tumor volume in the active animals under moderate and vigorous intensities; - ↑ Tumor volume in the active animals under low intensity.
Aveseh et al, 2015 [2]	BALB/c mice (35 days old) (MC4-L2) Subcutaneously inoculated	10 Ex + 10 Sed	7 wks	TRDM	Moderate	–	55 min/d	5 days/wk	- ↓ Tumor weight in the active animals; - ↓ Tumor volume in the active animals.

▲ **Table 2** Continued.

Study [Reference]	Animal/BC model	Exercise design						Tumor outcomes
		n	Study duration	Type	Intensity	Distance covered	Duration	Frequency	
Faustino-Rocha et al, 2016 [15]	Sprague-Dawley rats (50 days old) MNU (50 mg/Kg) i.p.	10 Ex+11 Sed	35 wks	TRDM	Moderate	–	60 min/d	5 days/wk	- ↑ Vessels density in the active animals; - ↑ VEGF in the active animals.
Faustino-Rocha et al (1), 2016 [14]	Sprague-Dawley rats (50 days old) MNU (50 mg/Kg) i.p.	10 Ex+11 Sed	35 wks	TRDM	Moderate	–	60 min/d	5 days/wk	- ↓ Incidence in the active animals; - ↓ Multiplicity in the active animals; - ↑ Tumor weight in the active animals; - ↑ Tumor volume in the active animals.
Isanejad et al 2016 [31]	BALB/c mice (42/48 days old) Cell inoculation (MC4-L2 human BC)	8 Ex+8 Sed	5 wks	TRDM	Low	–	16/18 min/d	5 days/wk	- ↓ Tumor volume in the active animals; - ↓ VEGF and CD31 in the active animals; - ↓ Ki67 in the active animals.

Abbreviations: DMBA, 7,12-Dimethylbenz(a)anthracene; MNU, 1-methyl-1-nitrosoureia. BC, breast cancer; i.p., intraperitoneal; g.i., gastric intubation. Ex, active animals; Sed, sedentary animals. TRDM, treadmill; FW, Free-wheel; MW, motorized-wheel. Wks, weeks; wk, week; min, minutes; d, day. Proliferation family: E2F-1, transcription factor; p27 and p27kip1, cyclin kinases inhibitors family members and Ki67. Apoptosis family: Apaf-1, apoptotic peptidase activating factor-1; BAX, BCL-2-associated X protein; CASP 3, caspase 3 and XIAP, X-linked inhibitor of apoptosis. Angiogenesis family: CD31, cluster; VEGF, vascular endothelial growth factor. ↑ Increase, ↓ decrease.

ercise performed was forced or voluntary. In incidence (34.8 % vs. 26.9 %) and multiplicity (87.0 % vs. 73.1 %), motorized-wheel exercises appeared to be more advantageous than free-wheel or treadmill exercise (34.8 % vs. 9.5 % and 87.0 vs. 27.5 %, respectively). In terms of tumor weight, running in a free wheel (79.4 %) was more beneficial than motorized-wheel (48.5 %) or treadmill (44.6 %) exercise. Tumor volume was significantly decreased with treadmill exercise (70.7 %) compared with free-wheel exercise (33.8 %). No research has been conducted about tumor volume under motorized-wheel conditions.

Exercise performed on a motorized wheel favored an antiangiogenic environment (85 %), and the positive effects highlighted in proliferation and apoptosis were not influenced by the modality of the exercise performed. Moreover, no studies have been conducted to assess markers of apoptosis with forced exercise.

Intensity

Tumor burden appears to accumulate benefits from all levels of intensity of training but to differing degrees. Increased benefits to incidence (39.8 %) and multiplicity (88.1 %) were associated with vigorous exercise training, and moderate-intensity exercise exerted a stronger influence on tumor weight (50.7 %). Reductions in tumor volume were higher for low-intensity exercise (84.7 %).

Positive and large effects were found between moderate-intensity exercise and tumor proliferation (88.6 %); apoptosis (85.6 %) and angiogenesis (80.7 %) exhibited better results with low-intensity exercise. No studies have thoroughly examined proliferation and angiogenesis markers under vigorous exercise conditions.

Distance covered

The proliferation and apoptosis outcomes were not moderated by the distance the animals covered. Covering long distances appears to confer moderate benefits in cancer incidence (35.8 %), tumor volume (46.1 %) and angiogenesis (45.3 %); the effects were more pronounced for tumor multiplicity (85.0 %) and tumor weight (68.5 %). Additionally, covering short distances also significantly affected tumor multiplicity (89.0 %), tumor weight (53.1 %) and tumor volume (79.2 %).

Duration

The positive benefits found in tumor microenvironment markers of proliferation and apoptosis were not moderated by the duration of exercise bouts.

Performing exercise for 30–45 min has a small positive influence on tumor incidence (15.9 %) and multiplicity (27.6 %) and a large positive influence on tumor weight (55.3 %) and tumor volume (69.0 %). In addition, short bouts of exercise confer large benefits on tumor volume (98.7 %) and angiogenesis (80.7 %). Surprisingly, being active for more than 45 min appears to be unfavorable in terms of tumor incidence (17.8 %), tumor weight (37.1 %) and angiogenesis (75.5 %).

Frequency

Exercise performed weekly is meaningfully correlated with incidence, multiplicity, tumor volume and angiogenesis. However, weekly exercise does not moderate the exercise-tumor weight relationship or any of the other biomarkers families that were stud-

▶ **Table 3** Moderators of the relationship between exercise and tumor outcomes.

		Incidence			Multiplicity			Weight			Volume			Proliferation			Angiogenesis			Apoptosis		
Moderator		**k**	**r**	**p**	**k**	**r**	**p**	**k**	**r**	**p**	**k**	**r**	**p**	**k**	**r**	**p**	**k**	**r**	**p**	**k**	**r**	**p**
Exercise type	Trdm	19	-0.095	0.032	5	−0.275	0.002	9	−0.446	0.000	9	−0.695	0.000	1	a)		4	0.045	0.644	0	a)	
	Fw	7	−0.269	0.000	8	−0.731	0.000	2	−0.794	0.000	6	−0.338	0.000	0			3	−0.204	0.110	10		
	Mw	11	−0.348	0.000	7	−0.870	0.000	3	−0.485	0.000	0	-	-	9			1	−0.850	0.000	2		
Exercise intensity	Low	9	−0.191	0.001	3	−0.715	0.000	1	−0.345	0.004	2	−0.847	0.000	5	−0.746	0.000	2	−0.807	0.000	5	−0.856	0.000
	Mod	13	−0.107	0.052	4	−0.764	0.000	9	−0.507	0.000	6	−0.685	0.000	5	−0.886	0.000	3	−0.354	0.001	5	−0.776	0.000
	Vig	6	−0.398	0.000	5	−0.881	0.000	1	−0.378	0.001	1	−0.202	0.342	0	-	-	0	-	-	2	−0.747	0.000
Distance covered	Sd	8	−0.215	0.001	2	−0.890	0.000	3	−0.531	0.000	2	−0.792	0.000	0	a)		0	-	-	0	a)	
	Ld	15	−0.358	0.000	9	−0.850	0.000	5	−0.685	0.000	4	−0.461	0.000	9			4	−0.453	0.000	2		
Exercise duration	Sh	3	−0.076	0.438	0	-	-	0	-	-	1	−0.987	0.000	5	a)		2	−0.807	0.000	0	b)	
	Med	13	−0.159	0.003	3	−0.276	0.015	6	−0.553	0.000	6	−0.690	0.000	5			0	-	-	0		
	L	3	0.178	0.280	2	−0.273	0.049	2	0.371	0.008	3	−0.078	0.450	0			2	0.755	0.000	0		
	Vl	1	0.166	0.423	0	-	-	1	−0.053	0.691	0	-	-	0			0			0		
Exercise frequency	5d	16	−0.133	0.004	4	−0.235	0.019	4	a)		7	−0.720	0.000	0	a)		4	0.054	0.644	0	a)	
	6d	0	-	-	6	−0.847	0.000	0			3	−0.434	0.000	0			0	-	-	0		
	7d	21	−0.268	0.000	10	−0.789	0.000	7			5	−0.269	0.000	9						12		

NOTE: Exercise intensity, distance covered, duration and exercise frequency as moderators of tumor incidence, multiplicity, weight, volume, proliferation, angiogenesis and apoptosis.

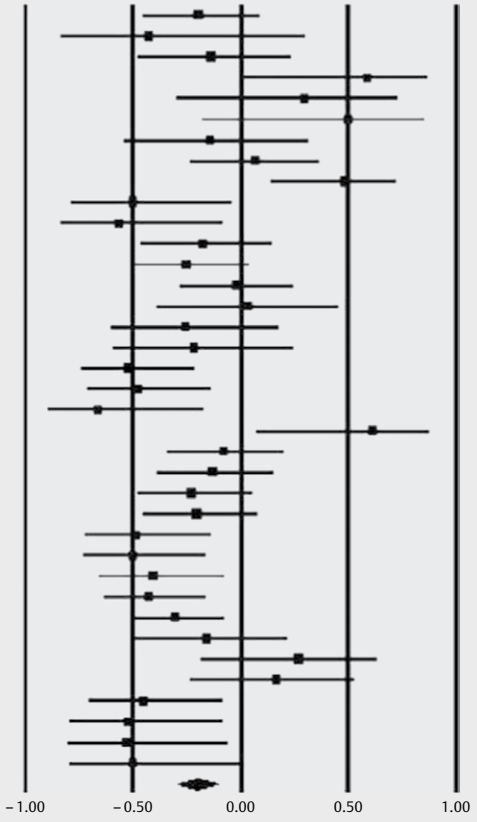
Number of cases (k) for correlation (r), and respective p-value.

a) Is not moderated by this exercise condition. b) Was not observed.

Trdm, treadmill; Fw, free-wheel; Mw, motorized-wheel; Mod, moderate; Vig, vigorous; Sd, short distance; Ld, long distance; Sh, Short; Med, medium; L, long; Vl, very long; d, days.

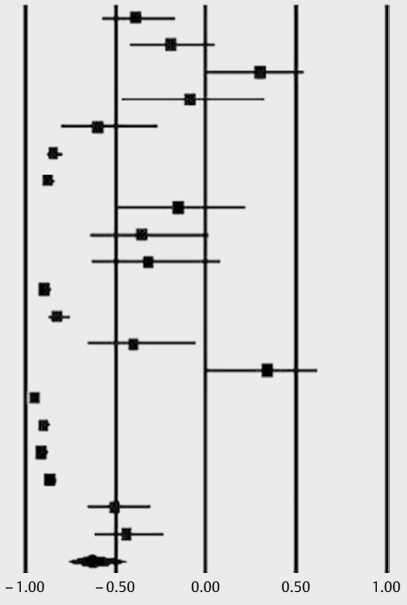
a

Study name	Correlation	Lower limit	Upper limit	Z-Value	p-Value
Cohen, Chol and Wang, 1988	−0.197	−0.455	0.091	−1.346	0.178
Cohen, Kendall Meschter et al, 1993 a	−0.428	−0.840	0.297	−1.174	0.240
Cohen, Kendall Meschter et al, 1993 b	−0.140	−0.478	0.234	−0.730	0.465
Colbert, Westerlind, Hursting et al, 2009 a	0.586	0.009	0.870	1.988	0.047
Colbert, Westerlind, Hursting et al, 2009 b	0.297	−0.301	0.727	0.973	0.330
Colbert, Westerlind, Hursting et al, 2009 c	0.496	−0.178	0.853	1.472	0.141
Faustino-Rocha et al, 2016 (1)	−0.143	−0.547	0.316	−0.598	0.550
Gillette, Zhu, Thompson et al, 1997 a	0.069	−0.241	0.366	0.432	0.666
Gillette, Zhu, Thompson et al, 1997 b	0.480	0.135	0.722	2.644	0.008
Jiang, Zhu and Thompson, 2009	−0.502	−0.787	−0.040	−2.113	0.035
Jiang, Zhu and Thompson, 2013	−0.566	−0.835	−0.081	−2.242	0.025
Lane, Teer, Strahan et al, 1991 a	−0.176	−0.464	0.145	−1.079	0.281
Lane, Teer, Strahan et al, 1991 b	−0.253	−0.508	0.042	−1.687	0.092
Lane, Teer, Strahan et al, 1991 c	−0.022	−0.283	0.242	−0.162	0.871
Malicka et al, 2015 a	0.036	−0.388	0.448	0.160	0.873
Malicka et al, 2015 b	−0.258	−0.606	0.173	−1.177	0.239
Malicka et al, 2015 c	−0.217	−0.598	0.244	−0.922	0.357
Mann, Jiang, Thompson et al, 2010 a	−0.523	−0.738	−0.210	−3.098	0.002
Mann, Jiang, Thompson et al, 2010 b	−0.479	−0.718	−0.140	−2.687	0.007
Moore and Tittle, 1973	−0.664	−0.892	−0.170	−2.495	0.013
Thompson, Ronan, Meeker et al, 1988	0.611	0.071	0.874	2.179	0.029
Thompson, Westerlind Singh et al, 1995 a	−0.077	−0.346	0.204	−0.534	0.594
Thompson, Westerlind Singh et al, 1995 b	−0.132	−0.393	0.150	−0.917	0.359
Thompson, Westerlind Singh et al, 1995 c	−0.229	−0.478	0.053	−1.595	0.111
Thompson, Westerlind Singh et al, 1995 d	−0.204	−0.457	0.079	−1.420	0.155
Thompson, Westerlind Snedden et al, 1995 (1) a	−0.487	−0.727	−0.140	−2.669	0.008
Thompson, Westerlind Snedden et al, 1995 (1) b	−0.501	−0.734	−0.162	−2.791	0.005
Thompson, Westerlind Snedden et al, 1995 (1) c	−0.407	−0.656	−0.077	−2.384	0.017
Thompson, Wolfe, Mctiernan et al, 2010 a	−0.427	−0.632	−0.165	−3.088	0.002
Thompson, Wolfe, Mctiernan et al, 2010 b	−0.306	−0.505	−0.077	−2.595	0.009
Westerlind, McCarty, Strange et al, 2003	−0.160	−0.497	0.220	−0.820	0.412
Woods, Davis, Pate et al, 1994 a	0.266	−0.192	0.629	1.143	0.253
Woods, Davis, Pate et al, 1994 b	0.166	−0.238	0.522	0.801	0.423
Zhu, Jiang, Thompson et al, 2009	−0.451	−0.712	−0.080	−2.347	0.019
Zhu, Jiang, Thompson et al, 2008	−0.525	−0.794	−0.084	−2.292	0.022
Zhu, Jiang, Thompson et al, 2012 a	−0.530	−0.807	−0.061	−2.187	0.029
Zhu, Jiang, Thompson et al, 2012 b	−0.499	−0.798	−0.003	−1.973	0.049
	−0.202	−0.295	−0.106	−4.084	0.000



b

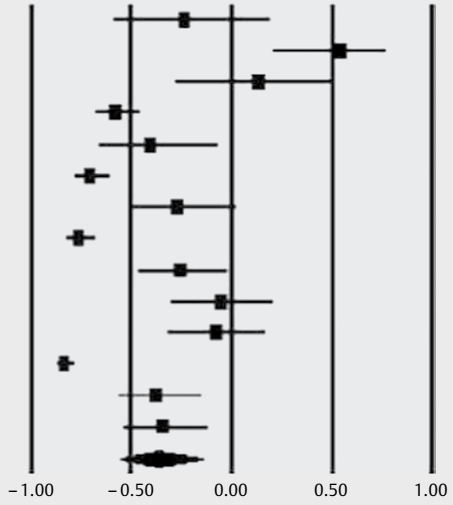
Study name	Correlation	Lower limit	Upper limit	Z-Value	p-Value
Cohen, Kendall, Meschter et al, 1993 a	−0.389	−0.574	−0.166	−3.307	0.001
Cohen, Kendall, Meschter et al, 1993 b	−0.195	−0.420	0.053	−1.545	0.122
Colbert, Westerlind, Hursting et al, 2009	0.301	0.012	0.544	2.037	0.042
Faustino-Rocha et al, 2016	−0.087	−0.473	0.327	−0.400	0.689
Goh, Tsai, Bammier et al, 2013	−0.600	−0.804	−0.269	−3.251	0.001
Jiang, Zhu and Thompson, 2009	−0.845	−0.886	−0.791	−14.662	0.000
Jiang, Zhu and Thompson, 2013	−0.881	−0.909	−0.845	−19.310	0.000
Malicka et al, 2015 a	−0.154	−0.491	0.223	−0.794	0.427
Malicka et al, 2015 b	−0.355	−0.640	0.016	−1.880	0.060
Malicka et al, 2015 c	−0.322	−0.635	0.083	−1.570	0.116
Mann, Jiang, Thompson et al, 2010 a	−0.899	−0.928	−0.861	−16.698	0.000
Mann, Jiang, Thompson et al, 2010 b	−0.825	−0.876	−0.756	−12.347	0.000
Murphy, Davis, Barrileaux et al, 2011	−0.400	−0.660	−0.055	−2.254	0.024
Steiner, Davis, Murphy et al, 2013	0.342	−0.009	0.619	1.910	0.056
Thompson, Wolfe, Mctiernan et al, 2010 a	−0.953	−0.966	−0.936	−22.182	0.000
Thompson, Wolfe, Mctiernan et al, 2010 b	−0.902	−0.928	−0.867	−18.056	0.000
Zhu, Jiang, Thompson et al, 2009	−0.916	−0.943	−0.877	−15.092	0.000
Zhu, Jiang, Thompson et al, 2008	−0.868	−0.901	−0.825	−17.099	0.000
Zhu, Jiang, Thompson et al, 2012 a	−0.505	−0.660	−0.309	−4.611	0.000
Zhu, Jiang, Thompson et al, 2012 b	−0.442	−0.614	−0.231	−3.875	0.000
	−0.632	−0.766	−0.446	−5.514	0.000



▶ **Fig. 2** **a** Forest plot of the meta-analysis depicting the influence of exercise on tumor incidence. Correlation: effect size (r) for each study. CI = confidence interval. **a, b, c, d**: different measures in different exercise protocols within the same study. **b:** Forest plot of the meta-analysis depicting the influence of exercise on tumor multiplicity. Correlation: effect size (r) for each study. CI = confidence interval. **a, b**: different measures in different exercise protocols within the same study.

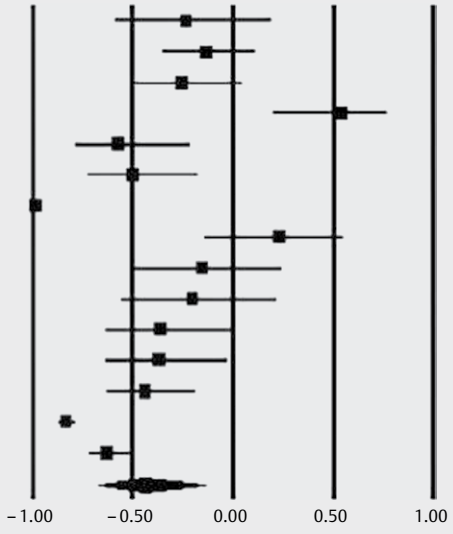
c

Study name	Correlation	Lower limit	Upper limit	Z-Value	p-Value
Aveseh et al, 2015	−0.233	−0.585	0.192	−1.078	0.281
Faustino-Rocha et al, 2016 a	0.543	0.210	0.763	3.019	0.003
Faustino-Rocha et al, 2016 b	0.135	−0.282	0.509	0.625	0.532
Jiang, Zhu and Thompson, 2013	−0.581	−0.679	−0.462	−7.967	0.000
Steiner, Davis Murphy et al, 2013	−0.404	−0.658	−0.067	−2.322	0.020
Westerlind, McCarty, Strange et al, 2003 b	−0.705	−0.782	−0.607	−9.962	0.000
Westerlind, McCarty, Strange et al, 2003 c	−0.271	−0.519	0.019	−1.835	0.066
Westerlind, McCarty, Strange et al, 2003 d	−0.763	−0.827	−0.679	−11.227	0.000
Westerlind, McCarty, Strange et al, 2003 a	−0.254	−0.463	−0.019	−2.113	0.035
Woods, Davis, Pate et al, 1994 b	−0.053	−0.302	0.204	−0.397	0.691
Woods, Davis, Pate et al, 1994 a	−0.078	−0.318	0.170	−0.615	0.539
Zhu, Jiang, Thompson et al, 2008	−0.834	−0.876	−0.781	−15.179	0.000
Zhu, Jiang, Thompson et al, 2012 a	−0.378	−0.566	−0.152	−3.193	0.001
Zhu, Jiang, Thompson et al, 2012 b	−0.345	−0.540	−0.113	−2.870	0.004
	−0.366	−0.557	−0.139	−3.083	0.002



d

Study name	Correlation	Lower limit	Upper limit	Z-Value	p-Value
Aveseh et al, 2015	−0.233	−0.585	0.192	−1.078	0.281
Cohen, Choi and Wang, 1988	−0.130	−0.355	0.109	−1.070	0.284
Colbert, Westerlind, Hursting et al, 2009	−0.254	−0.509	0.041	−1.694	0.090
Faustino-Rocha et al, 2016	0.540	0.197	0.765	2.924	0.003
Goh, Tsai, Bammier et al, 2013 a	−0.570	−0.791	−0.216	−2.967	0.003
Goh Tsai, Bammier et al, 2013 b	−0.499	−0.727	−0.172	−2.867	0.004
Isanejad et al, 2016	−0.987	−0.994	−0.974	−14.049	0.000
Malicka et al, 2015 a	0.233	−0.141	0.549	1.226	0.220
Malicka et al, 2015 b	−0.155	−0.504	0.237	−0.771	0.441
Malicka et al, 2015 c	−0.202	−0.557	0.215	−0.949	0.342
Murphy, Davis Barrilleaux et al, 2011	−0.359	−0.634	−0.004	−1.980	0.048
Steiner, Davis, Murphy et al, 2013	−0.371	−0.637	−0.025	−2.095	0.036
Welsch M., Cohen and Welsch C., 1995	−0.437	−0.631	−0.192	−3.346	0.001
Westerlind, McCarty, Strange et al, 2003 a	−0.834	−0.868	−0.792	−18.879	0.000
Westerlind, McCarty, Strange et al, 2003 b	−0.629	−0.719	−0.518	−8.701	0.000
	−0.443	−0.668	−0.143	−2.812	0.005



▶ **Fig. 2** Continued. **c:** Forest plot of the meta-analysis depicting the influence of exercise on tumor weight. Correlation: effect size (r) for each study. CI = confidence interval. **a, b, c, d**: different measures in different exercise protocols within the same study. **d:** Forest plot of the meta-analysis depicting the influence of exercise on tumor volume. Correlation: effect size (r) for each study. CI = confidence interval. **a, b**: different measures in different exercise protocols within the same study.

ied. Tumor incidence (26.8 %), multiplicity (84.7 %) and angiogenesis (45.3 %) benefit from higher frequencies of exercise. However, exercising five days per week appears to be sufficient for conferring benefits to tumor volume (72 %).

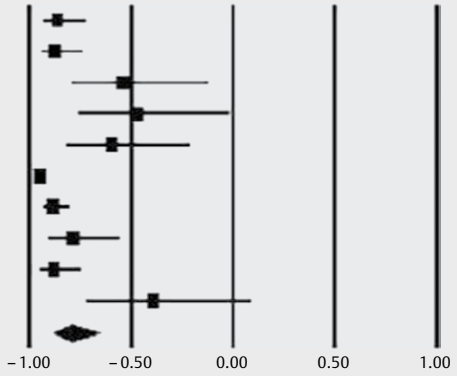
After observing funnel plots and considering Egger's regression and Begg's rank correlation, we verified the absence of study bias in tumor incidence (p = 0.41712), multiplicity (p = 0.00569), tumor weight (p = 0.01240), tumor volume (p = 0.14913), angiogenesis (p = 0.50000) and apoptosis (p = 0.27426). However, bias was found in proliferation (p = 0.00037).

Discussion

The impact of exercise was observed in the reduction of the total number of tumors in the active animals (20.2 %). We also noted a reduction in the number of tumors per animal (63.2 %), a decrease in tumor weight (36.6 %) and a decrease in tumor volume (44.3 %).

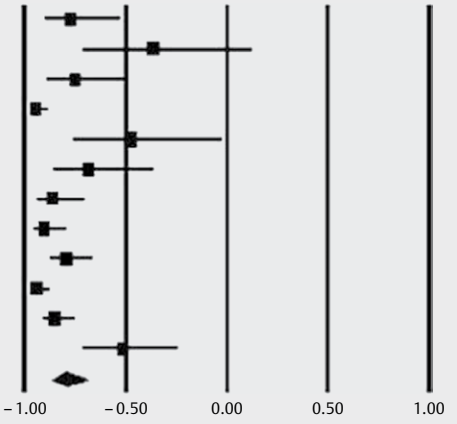
a

Study name	Biomarkers	Correlation	Lower limit	Upper limit	Z-Value	p-Value
Isanejad et al, 2016	ki67	−0.864	−0.935	−0.726	−6.608	0.000
Jiang, Zhu and Thompson, 2009 a	CYCLIN D1	−0.876	−0.944	−0.738	−6.472	0.000
Jiang, Zhu and Thompson, 2009 a	E2F-1	−0.543	−0.798	−0.124	−2.466	0.014
Jiang, Zhu and Thompson, 2009 b	p27kip1	−0.468	−0.762	−0.013	−2.013	0.044
Jiang, Zhu and Thompson, 2009 c	p27kip1	−0.596	−0.822	−0.209	−2.838	0.005
Jiang, Zhu and Thompson, 2013	CYCLIN D1	−0.948	−0.968	−0.914	−13.780	0.000
Jiang, Zhu and Thompson, 2013	p27	−0.882	−0.929	−0.805	−10.010	0.000
Zhu, Jiang, Thompson et al, 2009	CYCLIN D1	−0.787	−0.905	−0.556	−4.783	0.000
Zhu, Jiang, Thompson et al, 2009	E2F-1	−0.881	−0.946	−0.749	−6.603	0.000
Zhu, Jiang, Thompson et al, 2009	p27kip1	−0.393	−0.725	−0.087	−1.620	0.105
		−0.794	−0.881	−0.655	−7.132	0.000



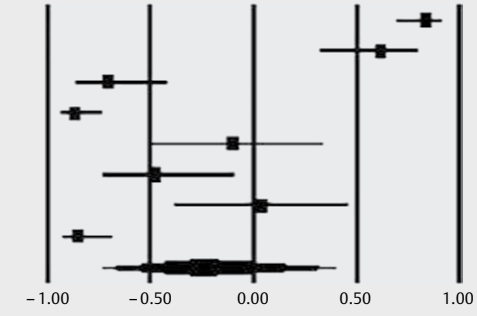
b

Study name	Biomarkers	Correlation	Lower limit	Upper limit	Z-Value	p-Value
Zhu, Jiang, Thompson et al, 2009	Apaf-1	−0.773	−0.899	−0.529	−4.589	0.000
Zhu, Jiang, Thompson et al, 2009	BAX	−0.384	−0.710	0.124	−1.477	0.140
Zhu, Jiang, Thompson et al, 2009	BCL-2	−0.752	−0.890	−0.438	−4.316	0.000
Zhu, Jiang, Thompson et al, 2009	CASP 3	−0.946	−0.975	−0.836	−9.030	0.000
Zhu, Jiang, Thompson et al, 2009	XIAP	−0.474	−0.765	−0.022	−2.046	0.041
Jiang, Zhu and Thompson, 2009	Apaf-1	−0.685	−0.861	−0.362	−3.582	0.000
Jiang, Zhu and Thompson, 2009	BAX	−0.862	−0.937	−0.710	−6.144	0.000
Jiang, Zhu and Thompson, 2009	BCL-2	−0.904	−0.958	−0.797	−7.270	0.000
Jiang, Zhu and Thompson, 2009	CASP 3	−0.795	−0.879	−0.663	−7.423	0.000
Jiang, Zhu and Thompson, 2009	XIAP	−0.939	−0.972	−0.872	−8.677	0.000
Jiang, Zhu and Thompson, 2013	BAX	−0.850	−0.911	−0.753	−8.895	0.000
Jiang, Zhu and Thompson, 2013	BCL-2	−0.516	−0.713	−0.244	−3.471	0.001
		−0.798	−0.872	−0.690	−3.726	0.000



c

Study name	Biomarkers	Correlation	Lower limit	Upper limit	Z-Value	p-Value
Faustino-Rocha et al, 2016 a	PER VESS	0.833	0.692	0.913	6.793	0.000
Faustino-Rocha et al, 2016 b	VEGF	0.616	0.322	0.802	3.657	0.000
Isanejad et al, 2016 a	VEGF	−0.703	−0.861	−0.420	−4.025	0.000
Isanejad et al, 2016 b	CD31	−0.867	−0.936	−0.732	−6.686	0.000
Jones, Viglianti, Tashjian et al, 2010	CD31	−0.102	−0.501	0.333	−0.448	−0.654
Jones, Viglianti, Tashjian et al, 2010	PER VESS	−0.477	−0.737	−0.096	−2.406	0.016
Jones, Viglianti, Tashjian et al, 2010	VEGF	0.041	−0.387	0.455	0.179	0.858
Zhu, Jiang, Thompson et al, 2009	VEGF	−0.850	−0.932	−0.684	−5.877	0.000
		−0.258	−0.734	0.392	−0.759	0.448



▶ **Fig. 3** **a:** Forest plot of the meta-analysis depicting the influence of exercise on biomarkers of proliferation. Correlation: effect size (r) for each study in proliferation. CI = confidence interval. **a, b, c**: different measures within the same study. **Abbreviations:** E2F-1, transcription factor; p27 and p27kip1, cyclin kinases inhibitors family members and Ki67. **b:** Forest plot of the meta-analysis depicting the influence of exercise on biomarkers of apoptosis. Correlation: effect size (r) for each study in. CI = confidence interval. **Abbreviations:** Apaf-1, apoptotic peptidase activating factor-1; BAX, BCL-2-associated X protein; CASP 3, caspase 3; XIAP, X-linked inhibitor of apoptosis. **c:** Forest plot of the meta-analysis depicting the influence of exercise on biomarkers of angiogenesis. Correlation: effect size (r) for each study in. CI = confidence interval. **Abbreviations:** CD31, cluster; PER VESS, number of perfused vessels; VEGF, vascular endothelial growth factor.

In contrast to some preclinical research linking an increased tumor burden to exercise [7, 9, 14, 21, 46, 60, 66, 73], these data provide a step forward by quantifying the benefits highlighted in the majority of previous preclinical data [2, 7–9, 14, 23, 31, 33, 34, 42, 46, 47, 51, 53, 60, 67–70, 72, 73, 75–77]. Tumor incidence was the only variable associated with small benefits, which can be partly related to the breast cancer models used. The negative results found in this variable were reported in five studies [9, 21, 46, 66, 73]. Each of them used a different tumor model that may have resulted in differences in tumor phenotypes and consequently in different behaviors when exposed to exercise [22, 27, 37]. However, no data were available regarding the different histological types of the developed tumors, and so we cannot know for sure whether this could be the case. Yet, three of the five studies that have reported negative results in tumor incidence, also reported a beneficial effect of exercise in tumor burden, namely, in tumor weight [73] and in tumor volume [9, 46]. Additionally, the characteristics of exercise protocols and the total duration of the experiments can also be a factor that might explain this negative data. Indeed, the negative data reported for tumor incidence in one of the experiments, only occurred in animals that were exposed to exercise of low intensity [46]. Nevertheless, several reports have noted a reduction of malignant and invasive types of tumor in active rodents compared with sedentary ones [14, 15]. Our results confirm the evidence of a strong association between exercise and cancer outcomes in human patients [3, 29, 59, 61].

Exercise improved the deregulation of tumor proliferation (79.4 %) and apoptosis (79.8 %). Furthermore, there is some evidence, albeit inconsistent, for positive effects in tumor angiogenesis (25.6 %), which is consistent with some previous research [33, 34, 49, 53, 54, 64, 65, 71, 75, 77]. Nevertheless, only a few preclinical studies have examined changes in tumor biology regarding proliferation [33, 34, 75], angiogenesis [15, 31, 39, 75] and apoptosis [33, 34, 75]. Moreover, experimental protocols with voluntary exercise were the most often used; considering the intermittent features of voluntary exercise in animals, this situation is a clear limitation to speculating about similar results in a clinical context.

It is clear that increased proliferation is a hallmark of malignant tumors and a key feature of tumor progression that is associated with a higher risk of recurrence and a shorter survival duration [62]. Additionally, suppression of apoptosis is believed to play a central role in the development and progression of breast cancer [5, 12, 13]. The present data strongly support the recommendation for exercise in order to modulate the proliferative and the apoptotic rate in a tumor environment.

The present results do not allow us to confirm a consistent relation between exercise and angiogenesis. However, in spite of the undoubtedly important role that angiogenesis plays in the growth, progression and metastasis of a tumor [45, 54], recent research has shown that the process of tumor angiogenesis is far from clearly understood [32]. In fact, it seems that the antiangiogenic therapy more than reduced tumor vessels, normalized tumor vasculature reducing tumor vessel permeability and as a result may improve chemotherapeutic delivery (window of opportunity) [44]. Could exercise create a window of opportunity for establishing optimal conditions for chemical therapy? This might explain some of the results in the studies used in this review in which the active animals showed high levels of VEGF (vascular endothelial growth factor) expression but, at the same time, developed tumors histologically less aggressive [15].

Another finding of this review is that the diversity of exercise protocols used in the selected studies might be an obstacle to creating an accurate definition of the type and amount of exercise necessary to induce better tumor outcomes.

Voluntary exercise appears to exert more influence on the incidence, multiplicity and weight of tumors than forced exercise. However, forced exercise exhibited better tumor volume results. Favorable influences were also confirmed in multiplicity and tumor weight under forced-exercise conditions. Nevertheless, it is worth noting that even though voluntary exercise more strongly influenced tumor outcomes, this situation persisted in motorized-wheel exercise, which appears to be an argument in favor of protocols that can manipulate exercise intensity. Forced exercise also proved to be effective at inhibiting tumors [53, 67, 68, 72], and it may be worthwhile to gathering information about exercise prescriptions in a clinical context.

Moderate- or vigorous-intensity exercise favorably affected all the variables that we considered. For tumor volume, angiogenesis and proliferation, the benefits were even more pronounced for low-intensity exercise. Similar results can be found in the previously published literature: a reduction in cancer recurrence and survival are associated with moderate and vigorous exercise. However, in general, the results of the majority of the studies, mostly meta-analyses, failed to present significant results about the ideal amount of exercise (i. e., intensity, duration and frequency) to achieve benefits. The vast majority of cases used studies that poorly reported the amount of exercise performed or only reported levels of physical activity estimated by self-response [17, 19, 26, 29, 48, 59].

Interestingly, in this study we found benefits associated with low-intensity exercise, a level of intensity that is not usually associated with large effects and is therefore not usually recommended in international guidelines [41]. Again, this may be due to the small number of studies that evaluated the microenvironment of the tumor. Nevertheless, intensity appears to be a determining factor in modulating tumor outcomes [8, 9, 46, 67, 68, 73, 77]. There should accordingly be a preference for study designs in which the intensity can be controlled. Such studies are most likely to yield important information that advances this field of research [1].

In contrast to some preclinical results [23, 70], the distances covered during exercise do not appear to affect tumor outcomes. In fact, based on the present results, it appears that intensity instead of distance may have more of an influence on tumor outcomes.

Tumor burden accrues more benefits when exercise is performed for 30–45 min. Only tumor volume and angiogenesis [31] benefitted from short periods of exercise. Unexpectedly, bouts of exercise longer than 45 min adversely impacted tumor incidence, tumor weight and angiogenesis [14, 15, 73].

Higher exercise frequencies (6 or 7 days per week) are correlated with better tumor outcomes in incidence, multiplicity and angiogenesis; for tumor volume, running 5 days per week is preferred [7–9, 23, 33, 34, 39, 47, 51, 53, 60, 69, 70, 73, 75–77].

Curiously, the benefits highlighted by these quantitative proliferation and apoptosis data do not appear to be influenced by any of the moderating variables, with the exception of intensity. This finding may be related to the limited number of studies that used forced exercise protocols to examine the tumor microenvironment [14, 15, 31]. However, a key question still remains: does only intensity matter?

The present results highlight the importance of new research that makes use of mechanisms to assess the intensity, frequency and duration of performed exercise. It will then be possible to test the amount of exercise necessary to improve either, tumor outcomes or an aggressive intratumoral environment.

Given the diversity of exercise protocols used in the studies included in this review, accompanied by the fact that only a minority of studies clearly defined the exercise model in humans that they wished to reproduce, it is impossible to extrapolate specific and accurate recommendations to a clinical context.

In general, additional research is necessary that takes into account a methodological approach mimicking the exercises that can be performed by humans with breast cancer. Understanding the effects of specific (intensity, duration and frequency) exercise prescriptions on physiological outcomes across different stages of cancer will allow researchers to personalize exercise prescriptions.

Study limitations

There are several limitations to this study. First, there was significant heterogeneity among the studies that we attempted to measure performing a sensitivity analysis and analyzing exercise-related moderators. Furthermore, publication bias was found in one of the variables studied (i. e., proliferation). However, after performing the sensitivity analysis we verified that the overall effect had a small variation (3.3 % vs. 2.5 %) in this variable.

Finally, studies published in languages other than English were not considered. Although the exclusion of non-English-language studies might have resulted in smaller intervention effects, the language bias is generally small [40].

Conclusions

To the best of our knowledge, this meta-analysis is the first attempt to quantify the results of preclinical research addressing the exercise-breast cancer relationship. Our primary findings were: 1) there is convincing evidence from the preclinical data that exercise is associated with tumor burden reduction; 2) there is evidence suggesting that exercise may result in beneficial changes in the levels of proliferation and apoptotic biomarkers in the tumoral microenvironment; and 3) there is insufficient evidence regarding the association between exercise and a positive modulation of the angiogenic events in the tumoral environment.

Finally, there is evidence that the intensity, duration and frequency of exercise are important determinants of tumor outcomes. Nevertheless, uniform preclinical exercise designs are necessary to facilitate comparisons of results from different studies, and define the amounts of exercise required to alter carcinogenesis.

Future research directions include the need for more preclinical studies, particularly in tumor biology, and for exercise conditions that make it possible to manipulate the amount of exercise performed.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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Supplementary Material

▶ **Table S1** Search strategy.

Search strategy	"Exercise"	"Breast cancer"	"Animals"
MeSH terms	Exercise Running	Breast neoplasms	Animals
Text words	Physical activity Voluntary running Treadmill running	Breast tumor Mammary cancer	

▶ **Table S2** SYRCLE's RoB tool for assessing risk of bias.

Item	Bias domain	Source of bias	Support for judgments	Review author's judgment
1	**Selection bias**	Sequence generation	Describe the methods used, if any, to generate the allocation sequence in sufficient detail to allow an assessment whether it should produce comparable groups.	Y = The investigators described a random component. U = The investigators mention the randomization but its not clear how. N = No randomization were used or is not reported.
2	**Selection bias**	Baseline characteristics	Describe all the possible prognostic factors or animal characteristics, if any, that are compared in order to judge whether or not intervention and control groups were similar at the start of the experiment.	Y = Similarity in baseline characteristics (ex. animal type, animal age). The disease was induced before randomization or a transgenic model was used. U = The similarity its not clear or/and the disease was induced before randomization. N = The baseline characteristics are not similar or not clear, and the disease was induced after randomization.
3	**Selection bias**	Allocation concealment	Describe the method used to conceal the allocation sequence in sufficient detail to determine whether intervention allocations could have been foreseen before or during enrolment.	Y = The allocation was adequately concealed if the baseline characteristics were similar. U = The methods used to allocation are not clear neither are the similarity of baseline characteristics. N = Not reported.
4	**Performance bias**	Random housing	Describe all measures used, if any, to house the animals randomly within the animal room.	Y = The housing conditions were similar between groups (ex. temperature, humidity, lighting); In protocols of forced exercise the sedentary animals were placed in a stationary treadmill during the same time that exercised animals. U = The housing conditions were similar between groups (ex. temperature, humidity, lighting); In protocols of forced exercise the sedentary animals remain in the cages during the exercise period. N = The housing conditions were not similar between groups (ex. temperature, humidity, lighting); In protocols of forced exercise the sedentary animals remain in the cages during the exercise period or is not reported.
5	**Performance bias**	Blinding	Describe all measures used, if any, to blind trial caregivers and researchers from knowing which intervention each animal received. Provide any information relating to whether the intended blinding was effective.	To investigate the effects of exercise exposure blindness is not possible. The investigators and/or the caregivers must be aware of the compliance of the animals with the exercise conditions in order to reduce the outcomes bias. Thus, this condition is not applicable.
6	**Detection bias**	Random outcome assessment	Describe whether or not animals were selected at random for outcome assessment, and which methods to select the animals, if any, were used.	Y = The animals were selected at random for outcome assessment, or the animals were not selected at random for outcome assessment, but all the animals were used and the outcomes is not likely to be influenced. U = The animals were not selected at random for outcome assessment but the investigators pinpoint the reasons why. N = The animals were not selected at random for outcome assessment; or its not clear why the investigators choose a specific number of animals; or its not reported.

▶ **Table S2** Continued.

Item	Bias domain	Source of bias	Support for judgments	Review author's judgment
7	**Detection bias**	Blinding	Describe all measures used, if any, to blind outcome assessors from knowing which intervention each animal received. Provide any information relating to whether the intended blinding was effective.	Y = The outcome assessment methods were the same in both groups, or if they were not, a valid reason was defined (ex. to measure the effects of different amounts of exercise exposure). U = The outcome assessment methods were the same in both groups but its not clear if a random component were used. N = Differences in the outcome assessment (e. g., different times in sacrificed, differences in blood sample collection); or if not reported.
8	**Attrition bias**	Incomplete outcome data	Describe the completeness of outcome data for each main outcome, including attrition and exclusions from the analysis. State whether attrition and exclusions were reported, the numbers in each intervention group (compared with total randomized animals), reasons for attrition or exclusions, and any re-inclusions in analyses for the review.	Y = All the animals were included in the analysis; or if not, the reasons for missing data are well explain (ex. animals that did not develop tumors). U = The analysis do not include all the animals or all the tumors, but the reasons to do that are not clear. N = The analysis do not include all the animals or all the tumors and no reasons were reported.
9	**Reporting bias**	Selective outcome reporting	State how selective outcome reporting was examined and what was found.	Y = The study protocol is not registered but the published report included all expected outcomes. U = The study protocol is not registered and some results are not presented. N = The study protocol is not registered and the presented results are not in accordance with all the study questions.
10	**Other**	Other sources of bias	State any important concerns about bias not covered by other domains in the tool.	Y = The financial support are clearly stated; no additional animals were added to replace the missing ones; U = The financial support is not clearly stated. The animals used were males. N = No references is made regarding the funders; additional animals were added to replace the missing ones.

▶ **Table S3** Quality analysis.

STUDY	SYRCLE's RoB tool										RESULTS BY STUDY
	Q1	Q2	Q3	Q4	Q5	Q6	Q7	Q8	Q9	Q10	
	Selection bias			Performance bias		Detection bias		Attrition bias	Reporting bias	Other bias	
Moore, 1973	N	N	N	N	NA	U	N	Y	U	N	**LRB = 11.11 % URB = 22.22 % HRB = 66.67 %**
Thompson, 1988	Y	Y	Y	Y	NA	Y	N	Y	Y	Y	**LRB = 88.89 % URB = 0.00 % HRB = 11.11 %**
Cohen, 1988	Y	Y	Y	Y	NA	Y	N	Y	Y	Y	**LRB = 88.89 % URB = 0.00 % HRB = 11.11 %**
Lane, 1991	Y	N	U	N	NA	Y	N	Y	Y	Y	**LRB = 55.56 % URB = 11.11 % HRB = 33.33 %**
Cohen, 1993	Y	Y	Y	Y	NA	Y	N	Y	Y	U	**LRB = 77.78 % URB = 11.11 % HRB = 11.11 %**
Woods, 1994	Y	Y	Y	Y	NA	Y	N	Y	Y	U	**LRB = 77.78 % URB = 11.11 % HRB = 11.11 %**
Thompson, 1995	Y	Y	Y	Y	NA	Y	N	Y	Y	Y	**LRB = 88.89 % URB = 0.00 % HRB = 11.11 %**
Thompson, 1995(1)	Y	Y	Y	Y	NA	Y	N	Y	Y	Y	**LRB = 88.89 % URB = 0.00 % HRB = 11.11 %**
Welsch, 1995	N	Y	Y	N	NA	Y	N	N	Y	Y	**LRB = 55.56 % URB = 0.00 % HRB = 44.44 %**
Gillette, 1997	Y	Y	Y	Y	NA	Y	N	U	Y	Y	**LRB = 77.78 % URB = 11.11 % HRB = 11.11 %**
Westerlind, 2003	Y	Y	Y	Y	NA	Y	U	Y	Y	Y	**LRB = 88.89 % URB = 11.11 % HRB = 0.00 %**
Zhu, 2008	Y	Y	Y	Y	NA	Y	Y	U	Y	Y	**LRB = 88.89 % URB = 11.11 % HRB = 0.00 %**
Colbert, 2009	Y	Y	Y	Y	NA	Y	Y	U	Y	Y	**LRB = 88.89 % URB = 11.11 % HRB = 0.00 %**
Zhu, 2009	Y	Y	Y	Y	NA	Y	Y	U	Y	Y	**LRB = 88.89 % URB = 11.11 % HRB = 0.00 %**
Jiang, 2009	Y	Y	Y	Y	NA	Y	Y	N	Y	Y	**LRB = 88.89 % URB = 0.00 % HRB = 11.11 %**
Thompson, 2010	Y	Y	Y	Y	NA	Y	Y	Y	Y	Y	**LRB = 100.0 % URB = 0.00 % HRB = 0.00 %**
Mann, 2010	Y	Y	Y	Y	NA	Y	N	Y	Y	Y	**LRB = 88.89 % URB = 0.00 % HRB = 11.11 %**
Jones, 2010	Y	Y	Y	Y	NA	Y	Y	Y	Y	Y	**LRB = 100.0 % URB = 0.00 % HRB = 0.00 %**
Murphy, 2011	Y	Y	Y	Y	NA	Y	U	Y	Y	Y	**LRB = 88.89 % URB = 11.11 % HRB = 0.00 %**
Zhu, 2012	Y	Y	Y	Y	NA	Y	N	Y	Y	Y	**LRB = 88.89 % URB = 0.00 % HRB = 11.11 %**
Jiang, 2013	Y	Y	Y	Y	NA	Y	U	Y	Y	Y	**LRB = 88.89 % URB = 11.11 % HRB = 0.00 %**
Steiner, 2013	Y	Y	Y	Y	NA	Y	N	Y	Y	U	**LRB = 77.78 % URB = 11.11 % HRB = 11.11 %**
Goh, 2013	Y	Y	Y	Y	NA	Y	N	Y	Y	Y	**LRB = 88.89 % URB = 0.00 % HRB = 11.11 %**
Malicka, 2015	N	Y	Y	U	NA	Y	Y	U	Y	Y	**LRB = 66.67 % URB = 22.22 % HRB = 11.11 %**
Aveseh, 2015	Y	Y	Y	U	NA	Y	N	Y	Y	U	**LRB = 66.67 % URB = 22.22 % HRB = 11.11 %**
Faustino-Rocha, 2016	Y	Y	Y	Y	NA	Y	Y	U	Y	Y	**LRB = 88.89 % URB = 11.11 % HRB = 0.00 %**
Faustino-Rocha, 2016(1)	Y	Y	Y	Y	NA	Y	Y	Y	Y	Y	**LRB = 100.0 % URB = 0.00 % HRB = 0.00 %**
Isanejad, 2016	Y	U	U	Y	NA	Y	Y	Y	Y	Y	**LRB = 77.78 % URB = 22.22 % HRB = 0.00 %**

Legend: Y = Low risk of bias (LRB); N = High risk of bias (HRB); U = Unclear risk of bias (URB). NA = Not applicable.

Results by dimension: **(Q1, Q2 & Q3)** – **HRB** = 7.14 %; **URB** = 3.58 %; **LRB** = 89.28 %; **(Q4)** – **HRB** = 10.71 %; **URB** = 7.13 %; **LRB** = 82.13 %; **(Q6 & Q7)** – **HRB** = 26.79 %; **URB** = 8.93 %; **LRB** = 64.29 %; **(Q8)** – **HRB** = 7.14 %; **URB** = 21.43 %; **LRB** = 71.43 %; **(Q9)** – **HRB** = 0.00 %; **URB** = 3.57 %; **LRB** = 96.43 %; **(Q10)** – **HRB** = 3.57 %; **URB** = 14.29 %; **LRB** = 82.14 %;

Translations:

Exercise	"exercise"[MeSH Terms] OR "exercise"[All Fields]
Physical activity	"exercise"[MeSH Terms] OR "exercise"[All Fields] OR ('physical"[All Fields] AND "activity"[All Fields]) OR "physical activity"[All Fields]
running	"running"[MeSH Terms] OR "running"[All Fields]
breast tumor	"breast neoplasms"[MeSH Terms] OR ("breast"[All Fields] AND "neoplasms"[All Fields]) OR "breast neoplasms"[All Fields] OR ("breast"[All Fields] AND "tumor"[All Fields]) OR "breast tumor"[All Fields]
breast neoplasms	"breast neoplasms"[MeSH Terms] OR ("breast"[All Fields] AND "neoplasms"[All Fields]) OR "breast neoplasms"[All Fields]
mammary cancer	"breast neoplasms"[MeSH Terms] OR ("breast"[All Fields] AND "neoplasms"[All Fields]) OR "breast neoplasms"[All Fields] OR ("mammary"[All Fields] AND "cancer"[All Fields]) OR "mammary cancer"[All Fields]
animals	"animals"[MeSH Terms:noexp] OR animals[All Fields]

Database:

PubMed

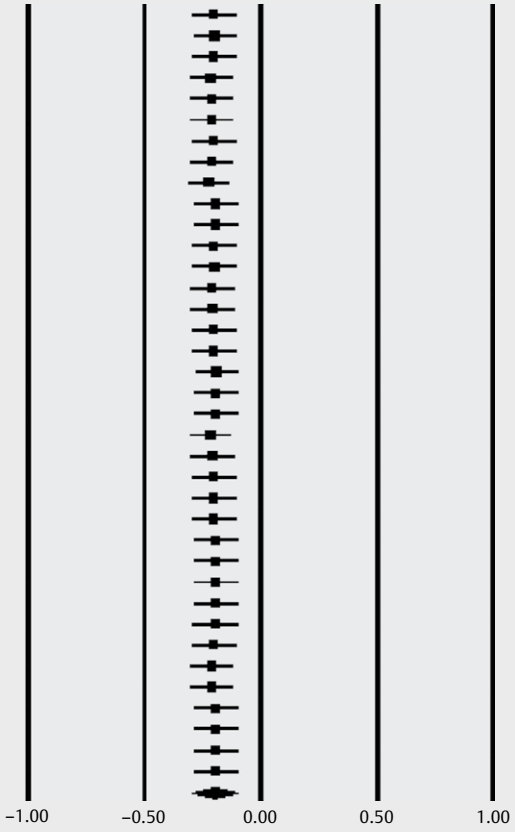
User query:

[(Exercise OR Physical activity) OR (Voluntary running OR treadmill running)] AND [(breast tumor OR breast neoplasms OR mammary cancer) AND (animals)

▶ **Fig. S1** Example of electronic search material.

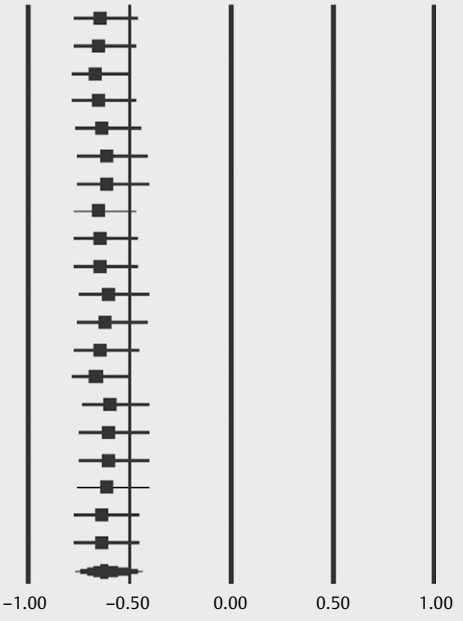
a

Study name	Point	Lower limit	Upper limit	Z-Value	p-Value
Cohen, Choi and Wang, 1988	−0.202	−0.298	−0.102	−3.931	0.000
Cohen, Kendall, Meschter er al, 1993 a	−0.199	−0.293	−0.102	−3.971	0.000
Cohen, Kendall, Meschter er al, 1993 b	−0.204	−0.299	−0.105	−4.004	0.000
Colbert, Westerlind, Hursting et al, 2009 a	−0.216	−0.305	−0.123	−4.478	0.000
Colbert, Westerlind, Hursting et al, 2009 b	−0.211	−0.303	−0.115	−4.258	0.000
Colbert, Westerlind, Hursting et al, 2009 c	−0.212	−0.303	−0.118	−4.338	0.000
Faustino-Rocha er al, 2016 (1)	−0.204	−0.298	−0.105	−4.018	0.000
Gillette, Znu, Thompson er al, 1997 a	−0.212	−0.305	−0.114	−4.210	0.000
Gillette, Znu, Thompson er al, 1997 b	−0.224	−0.309	−0.135	−4.841	0.000
Jiang, Zhu and Thompson, 2009	−0.195	−0.289	−0.098	−3.890	0.000
Jiang, Zhu and Thompson, 2013	−0.194	−0.287	−0.097	−3.894	0.000
Lane, Teer, Strahan et al, 1991 a	−0.203	−0.298	−0.104	−3.962	0.000
Lane, Teer, Strahan et al, 1991 b	−0.200	−0.295	−0.101	−3.898	0.000
Lane, Teer, Strahan et al, 1991 c	−0.209	−0.303	−0.110	−4.102	0.000
Malicka et al, 2015 a	−0.203	−0.302	−0.111	−4.134	0.000
Malicka et al, 2015 b	−0.201	−0.295	−0.102	−3.952	0.000
Malicka et al, 2015 c	−0.202	−0.296	−0.103	−3.979	0.000
Mann, Jiang, Thompson et al, 2010 a	−0.191	−0.285	−0.094	−3.836	0.000
Mann, Jiang, Thompson et al, 2010 b	−0.193	−0.287	−0.095	−3.847	0.000
Moore and Tittle, 1973	−0.193	−0.285	−0.097	−3.904	0.000
Thompson, Ronan, Meeker et al, 1988	−0.217	−0.305	−0.125	−4.539	0.000
Thompson, Westerlind, Singh et al, 1995 a	−0.207	−0.302	−0.107	−4.038	0.000
Thompson, Westerlind, Singh et al, 1995 b	−0.205	−0.300	−0.105	−3.983	0.000
Thompson, Westerlind, Singh et al, 1995 c	−0.201	−0.297	−0.101	−3.906	0.000
Thompson, Westerlind, Singh et al, 1995 d	−0.202	−0.298	−0.102	−3.923	0.000
Thompson, Westerlind, Snedden et al, 1995(1) a	−0.193	−0.287	−0.096	−3.849	0.000
Thompson, Westerlind, Snedden et al, 1995(1) b	−0.193	−0.286	−0.095	−3.845	0.000
Thompson, Westerlind, Snedden et al, 1995(1) c	−0.195	−0.290	−0.097	−3.855	0.000
Thompson, Wolfe, Mctiernan et al, 2010 a	−0.193	−0.288	−0.095	−3.821	0.000
Thompson, Wolfe, Mctiernan et al, 2010 b	−0.198	−0.294	−0.097	−3.827	0.000
Westerlind, McCarty, Strange et al, 2003	−0.203	−0.298	−0.105	−3.992	0.000
Woods, Davis, Pate et al, 1994 a	−0.214	−0.305	−0.118	−4.330	0.000
Woods, Davis, Pate et al, 1994 b	−0.212	−0.305	−0.116	−4.267	0.000
Zhu, Jiang, Thompson et al, 2009	−0.195	−0.289	−0.097	−3.863	0.000
Zhu, Jiang, Thompson et al, 2008	−0.194	−0.288	−0.097	−3.881	0.000
Zhu, Jiang, Thompson et al, 2012 a	−0.195	−0.288	−0.097	−3.890	0.000
Zhu, Jiang, Thompson et al, 2012 b	−0.196	−0.289	−0.098	−3.901	0.000
	−0.202	−0.295	−0.106	−4.084	0.000



b

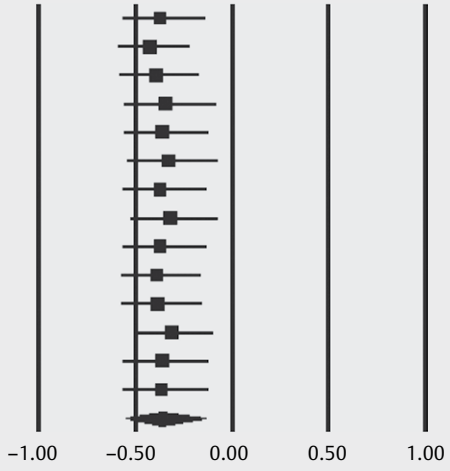
Study name	Point	Lower limit	Upper limit	Z-Value	p-Value
Cohen, Kendall, Meschter et al, 1993 a	−0.643	−0.775	−0.457	−5.541	0.000
Cohen, Kendall, Meschter et al, 1993 b	−0.650	−0.778	−0.471	−5.741	0.000
Colbert, Westerlind, Hursting et al, 2009	−0.666	−0.784	−0.501	−6.224	0.000
Faustino-Rocha et al, 2016	−0.651	−0.780	−0.470	−5.688	0.000
Goh, Tsai, Bammler et al, 2013	−0.634	−0.770	−0.442	−5.372	0.000
Jiang, Zhu and Thompson, 2009	−0.615	−0.762	−0.407	−4.941	0.000
Jiang, Zhu and Thompson, 2013	−0.610	−0.759	−0.400	−4.874	0.000
Malicka et al, 2015 a	−0.650	−0.779	−0.468	−5.666	0.000
Malicka et al, 2015 b	−0.643	−0.776	−0.457	−5.533	0.000
Malicka et al, 2015 c	−0.644	−0.776	−0.458	−5.550	0.000
Mann, Jiang, Thompson et al, 2010 a	−0.607	−0.755	−0.402	−4.954	0.000
Mann, Jiang, Thompson et al, 2010 b	−0.617	−0.763	−0.412	−5.003	0.000
Murphy, Davis, Barrilleaux et al, 2011	−0.642	−0.775	−0.454	−5.505	0.000
Steiner, Davis, Murphy et al, 2013	−0.666	−0.786	−0.496	−6.087	0.000
Thompson, Wolfe, Mctiernan et al, 2010 a	−0.595	−0.737	−0.402	−5.177	0.000
Thompson, Wolfe, Mctiernan et al, 2010 b	−0.607	−0.754	−0.401	−4.942	0.000
Zhu, Jiang, Thompson et al, 2009	−0.605	−0.751	−0.401	−4.991	0.000
Zhu, Jiang, Thompson et al, 2008	−0.612	−0.760	−0.403	−4.898	0.000
Zhu, Jiang, Thompson et al, 2012 a	−0.638	−0.773	−0.448	−5.417	0.000
Zhu, Jiang, Thompson et al, 2012 b	−0.641	−0.774	−0.453	−5.485	0.000
	−0.632	−0.766	−0.446	−5.514	0.000



▶ **Fig. S2 a:** Incidence (sensitivity analysis). **b:** Multiplicity (sensitivity analysis)

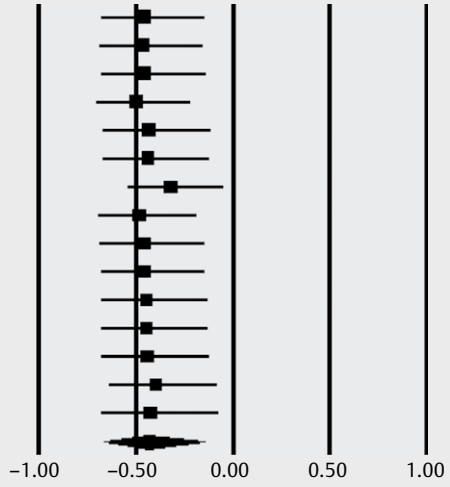
c

Study name	Point	Lower limit	Upper limit	Z-Value	p-Value
Aveseh er al, 2015	−0.375	−0.570	−0.139	−3.039	0.002
Faustino-Rocha et al, 2016 a	−0.427	−0.596	−0.222	−3.872	0.000
Faustino-Rocha et al, 2016 b	−0.397	−0.584	−0.170	−3.319	0.001
Jiang, Zhu and Thompson, 2013	−0.344	−0.560	−0.084	−2.563	0.010
Steiner, Davis, Murphy et al, 2013	−0.363	−0.563	−0.123	−2.900	0.004
Westerlind, McCarty, Strange et al, 2003 b	−0.329	−0.542	−0.077	−2.533	0.011
Westerlind, McCarty, Strange et al, 2003 c	−0.373	−0.571	−0.134	−2.989	0.003
Westerlind, McCarty, Strange et al, 2003 d	−0.321	−0.529	−0.077	−2.552	0.011
Westerlind, McCarty, Strange et al, 2003 a	−0.374	−0.572	−0.135	−2.989	0.003
Woods, Davis, Pate et al, 1994 b	−0.389	−0.579	−0.160	−3.226	0.001
Woods, Davis, Pate et al, 1994 a	−0.387	−0.578	−0.157	−3.198	0.001
Zhu, Jiang, Thompson et al, 2008	−0.311	−0.497	−0.097	−2.814	0.005
Zhu, Jiang, Thompson et al, 2012 a	−0.364	−0.567	−0.120	−2.864	0.004
Zhu, Jiang, Thompson et al, 2012 b	−0.367	−0.569	−0.124	−2.897	0.004
	−0.366	−0.557	−0.139	−3.083	0.002



d

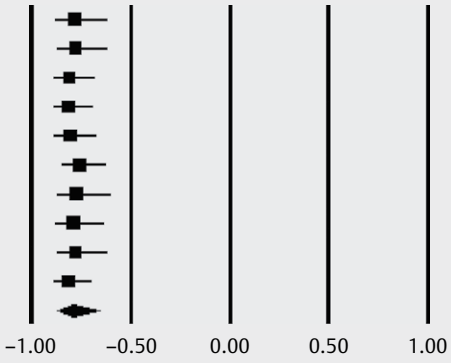
Study name	Point	Lower limit	Upper limit	Z-Value	p-Value
Aveseh et al, 2015	−0.456	−0.685	−0.145	−2.786	0.005
Cohen, Choi and Wang, 1988	−0.463	−0.690	−0.153	−2.830	0.005
Colbert, Westerlind, Hursting et al, 2009	−0.455	−0.686	−0.140	−2.750	0.006
Faustino-Rocha et al, 2016	−0.501	−0.704	−0.224	−2.336	0.001
Goh, Tsai, Bammler et al, 2013 a	−0.433	−0.671	−0.114	−2.604	0.009
Goh, Tsai, Bammler et al, 2013 b	−0.438	−0.676	−0.119	−2.629	0.009
Isanejad et al, 2016	−0.322	−0.548	−0.054	−2.336	0.019
Malicka et al, 2015 a	−0.482	−0.697	−0.188	−3.072	0.002
Malicka et al, 2015 b	−0.460	−0.688	−0.151	−2.824	0.005
Malicka et al, 2015 c	−0.458	−0.686	−0.147	−2.801	0.005
Murphy, Davis, Barrilleaux et al, 2011	−0.448	−0.681	−0.132	−2.708	0.007
Steiner, Davis, Murphy et al, 2013	−0.448	−0.681	−0.131	−2.699	0.007
Welsch M., Cohen and Welsch C., 1995	−0.443	−0.681	−0.119	−2.618	0.009
Westerlind, McCarty, Strange et al, 2003 a	−0.397	−0.638	−0.085	−2.459	0.014
Westerlind, McCarty, Strange et al, 2003 b	−0.426	−0.684	−0.073	−2.334	0.020
	−0.443	−0.668	−0.143	−2.812	0.005



▶ **Fig. S2** Continued. **c:** Tumoral mass (sensitivity analysis). **d:** Tumoral volume (sensitivity analysis)

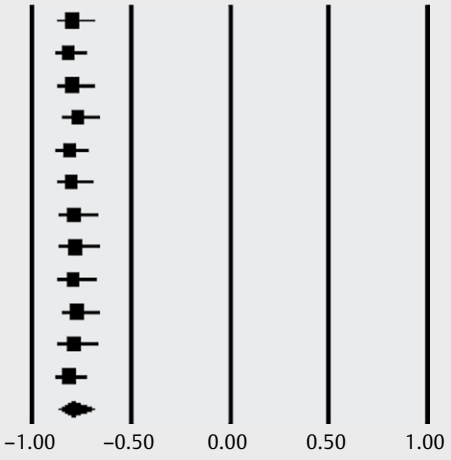
a

Study name	Biomarkers	Point	Lower limit	Upper limit	Z-Value	p-Value
Isanejad et al, 2016	ki67	−0.783	−0.882	−0.617	−6.202	0.000
Jiang, Zhu and Thompson, 2009 a	CYCLIND1	−0.781	−0.880	−0.616	−6.227	0.000
Jiang, Zhu and Thompson, 2009 a	E2F-1	−0.812	−0.893	−0.680	−7.321	0.000
Jiang, Zhu and Thompson, 2009 b	p27kip1	−0.816	−0.894	−0.690	−7.573	0.000
Jiang, Zhu and Thompson, 2009 c	p27kip1	−0.809	−0.892	−0.673	−7.142	0.000
Jiang, Zhu and Thompson, 2013	CYCLIND1	−0.761	−0.851	−0.627	−7.493	0.000
Jiang, Zhu and Thompson, 2013	p27	−0.777	−0.882	−0.600	−5.883	0.000
Zhu, Jiang, thompson et al, 2009	CYCLIND1	−0.794	−0.887	−0.638	−6.487	0.000
Zhu, Jiang, thompson et al, 2009	E2F-1	−0.780	−0.880	−0.614	−6.218	0.000
Zhu, Jiang, thompson et al, 2009	p27kip1	−0.819	−0.894	−0.699	−7.830	0.000
		−0.794	−0.881	−0.655	−7.132	0.000



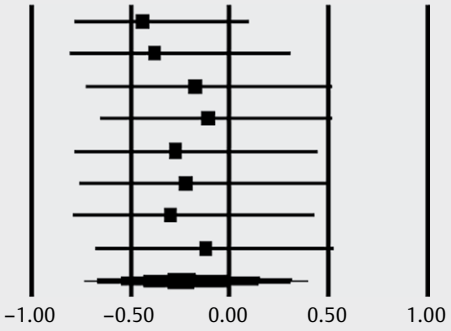
b

Study name	Biomarkers	Point	Lower limit	Upper limit	Z-Value	p-Value
Zhu, Jiang, Thompson et al, 2009	Apaf-1	−0.800	−0.878	−0.682	−8.100	0.000
Zhu, Jiang, Thompson et al, 2009	BAX	−0.818	−0.884	−0.721	−9.338	0.000
Zhu, Jiang, Thompson et al, 2009	BCL-2	−0.802	−0.878	−0.685	−8.158	0.000
Zhu, Jiang, Thompson et al, 2009	CASP 3	−0.775	−0.853	−0.662	−8.576	0.000
Zhu, Jiang, Thompson et al, 2009	XIAP	−0.815	−0.883	−0.713	−8.994	0.000
Jiang, Zhu and Thompson, 2009	Apaf-1	−0.806	−0.880	−0.693	−8.355	0.000
Jiang, Zhu and Thompson, 2009	BAX	−0.791	−0.872	−0.669	−7.930	0.000
Jiang, Zhu and Thompson, 2009	BCL-2	−0.785	−0.866	−0.663	−7.997	0.000
Jiang, Zhu and Thompson, 2009	CASP 3	−0.798	−0.879	−0.672	−7.707	0.000
Jiang, Zhu and Thompson, 2009	XIAP	−0.777	−0.856	−0.661	−8.395	0.000
Jiang, Zhu and Thompson, 2009	BAX	−0.792	−0.875	−0.664	−7.625	0.000
Jiang, Zhu and Thompson, 2009	BCL-2	−0.817	−0.883	−0.721	−9.378	0.000
		−0.798	−0.872	−0.690	−8.726	0.000



c

Study name	Biomarkers	Point	Lower limit	Upper limit	Z-Value	p-Value
Faustino-Rocha et al, 2016 a	PER VESS	−0.441	−0.782	0.102	−1.612	0.107
Faustino-Rocha et al, 2016 b	VEGF	−0.382	−0.809	0.309	−1.094	0.274
Isanejad et al, 2016 a	VEGF	−0.173	−0.727	0.517	−0.459	0.647
Isanejad et al, 2016 b	CD31	−0.109	−0.659	0.518	−0.313	0.754
Jones, Viglianti, Tashjian et al, 2010	CD31	−0.277	−0.784	0.451	−0.724	0.469
Jones, Viglianti, Tashjian et al, 2010	PER VESS	−0.222	−0.760	0.496	−0.574	0.566
Jones, Viglianti, Tashjian et al, 2010	VEGF	−0.296	−0.790	0.432	−0.778	0.436
Zhu, Jiang, Thompson et al, 2009	VEGF	−0.119	−0.677	0.525	−0.334	0.738
		−0.256	−0.734	0.392	−0.759	0.448



▶ **Fig. S3 a:** Proliferation (sensitivity analysis). **b:** Apoptosis (sensitivity analysis). **c:** Angiogenesis (sensitivity analysis)

PRISMA 2009 Checklist.

Section/topic	#	Checklist item	Reported on page #
TITLE			
Title	1	Identify the report as a systematic review, meta-analysis, or both.	1
ABSTRACT			
Structured summary	2	Provide a structured summary including, as applicable: background; objectives; data sources; study eligibility criteria, participants, and interventions; study appraisal and synthesis methods; results; limitations; conclusions and implications of key findings; systematic review registration number.	1
INTRODUCTION			
Rationale	3	Describe the rationale for the review in the context of what is already known.	2
Objectives	4	Provide an explicit statement of questions being addressed with reference to participants, interventions, comparisons, outcomes, and study design (PICOS).	3
METHODS			
Protocol and registration	5	Indicate if a review protocol exists, if and where it can be accessed (e. g., Web address), and, if available, provide registration information including registration number.	4
Eligibility criteria	6	Specify study characteristics (e. g., PICOS, length of follow-up) and report characteristics (e. g., years considered, language, publication status) used as criteria for eligibility, giving rationale.	5, 6
Information sources	7	Describe all information sources (e. g., databases with dates of coverage, contact with study authors to identify additional studies) in the search and date last searched.	5, 6
Search	8	Present full electronic search strategy for at least one database, including any limits used, such that it could be repeated.	▶ **Table 4**; ▶ **Fig. 4**
Study selection	9	State the process for selecting studies (i. e., screening, eligibility, included in systematic review, and, if applicable, included in the meta-analysis).	5, 6
Data collection process	10	Describe method of data extraction from reports (e. g., piloted forms, independently, in duplicate) and any processes for obtaining and confirming data from investigators.	5
Data items	11	List and define all variables for which data were sought (e. g., PICOS, funding sources) and any assumptions and simplifications made.	6
Risk of bias in individual studies	12	Describe methods used for assessing risk of bias of individual studies (including specification of whether this was done at the study or outcome level), and how this information is to be used in any data synthesis.	6, ▶ **Table 5**
Summary measures	13	State the principal summary measures (e. g., risk ratio, difference in means).	6
Synthesis of results	14	Describe the methods of handling data and combining results of studies, if done, including measures of consistency (e. g., I^2) for each meta-analysis.	6
Risk of bias across studies	15	Specify any assessment of risk of bias that may affect the cumulative evidence (e. g., publication bias, selective reporting within studies).	6
Additional analyses	16	Describe methods of additional analyses (e. g., sensitivity or subgroup analyses, meta-regression), if done, indicating which were pre-specified.	6
RESULTS			
Study selection	17	Give numbers of studies screened, assessed for eligibility, and included in the review, with reasons for exclusions at each stage, ideally with a flow diagram.	6, ▶ **Fig. 1**
Study characteristics	18	For each study, present characteristics for which data were extracted (e. g., study size, PICOS, follow-up period) and provide the citations.	6, 7, ▶ **Table 2**
Risk of bias within studies	19	Present data on risk of bias of each study and, if available, any outcome level assessment (see item 12).	▶ **Table 6**
Results of individual studies	20	For all outcomes considered (benefits or harms), present, for each study: (a) simple summary data for each intervention group (b) effect estimates and confidence intervals, ideally with a forest plot.	▶ **Table 6**
Synthesis of results	21	Present results of each meta-analysis done, including confidence intervals and measures of consistency.	8, 9, 10, 11
Risk of bias across studies	22	Present results of any assessment of risk of bias across studies (see Item 15).	8
Additional analysis	23	Give results of additional analyses, if done (e. g., sensitivity or subgroup analyses, meta-regression [see Item 16]).	9 ▶ **Fig. 5**a,b,c,d and ▶ **6**a, b, c

DISCUSSION			
Summary of evidence	24	Summarize the main findings including the strength of evidence for each main outcome; consider their relevance to key groups (e. g., healthcare providers, users, and policy makers).	12, 13, 14, 15, 16
Limitations	25	Discuss limitations at study and outcome level (e. g., risk of bias), and at review-level (e. g., incomplete retrieval of identified research, reporting bias).	16
Conclusions	26	Provide a general interpretation of the results in the context of other evidence, and implications for future research.	17
FUNDING			
Funding	27	Describe sources of funding for the systematic review and other support (e. g., supply of data); role of funders for the systematic review.	-

From: Moher D, Liberati A, Tetzlaff J, Altman DG, The PRISMA Group (2009). Preferred Reporting Items for Systematic Reviews and Meta-Analyses: The PRISMA Statement. PLoS Med 6(7): e1000097. doi:10.1371/journal.pmed1000097

For more information, visit: www.prisma-statement.or

2. Study 2

Figueira, ACC; Cortinhas, A; Soares, JP; Leitão, JC; Ferreira, R and Duarte, JA. (2018). Exercise-induced changes in systemic biomarkers of breast cancer: systematic review with meta-analysis. (Submitted in Scandinavian Journal of Medicine and Science in Sports; SJMSS-R-933-17 has been submitted)

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Exercise-induced changes in systemic biomarkers of breast cancer: systematic review with meta-analysis

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Keywords:	Inflammation and cytokines, Glucose metabolism, Sex hormone, Exercise intensity, Preclinical data

Exercise-induced changes in systemic biomarkers of breast cancer: systematic review with meta-analysis

Running title: Exercise-induced benefits in breast cancer

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Abstract

Background: Investigating the mechanisms by which exercise training can positively influence breast carcinogenesis is one of the main goals in exercise-oncology research. There is strong evidence that exercise exposure after a diagnosis of breast cancer may induce major advantages in tumor development by decreasing inflammation and sex hormone concentrations in addition to favorable changes in insulin resistance. Nevertheless, the published preclinical data are varied and have never been quantitatively summarized, and the exercise-moderating variables with the greatest potential influence on carcinogenesis have not yet been examined. **Objective:** With this review we intend to investigate the effectiveness of exercise in modulation of host systemic biomarkers. **Method searches:** PubMed, MEDELINE, Web of science and System for Information on Gray Literature in Europe databases were searched up to December 2016. We performed 61 correlations in order to analyze 10 exercise programs that involved 1224 animals in nine studies. Effect sizes were calculated for three dependent variables (i.e., inflammation and cytokines, glucose homeostasis and metabolism, and sex hormones). **Results:** The positive impact of exercise upon host systemic responses observed with respect to the three dependent variables was of large magnitude. We also identified the exercise-moderating variables that correlated with better outcomes, in which we emphasized the vigorous intensity as a determinant. However, the ideal amount of exercise needed to improve the outcomes was hampered by the diversity of experimental protocols used in the analyzed studies. **Conclusions:** Exercising can significantly improve tumor-related systemic inflammation, glucose metabolism, and sex hormones levels.

Keywords: Inflammation and cytokines, glucose metabolism, sex hormone, exercise intensity, preclinical data.

Introduction

Breast cancer is the most frequently diagnosed and extensively studied cancer among women [1,2]. The increasing number of survivors is due to several factors, including the adoption of an active lifestyle [3].

Regular physical activity and exercise training (considered exercise in this study) has been pointed out as one of the non-pharmacological approaches to improve breast cancer outcomes [4-11]. However, the way in which exercise can improve breast cancer survival remains poorly understood.

A number of mechanisms have been proposed to explain how exercise can potentially lower disease-specific mortality and recurrence. A considerable amount of data has established that cancer-induced inflammation, in conjunction with insulin resistance and high circulating estradiol levels are key determinants in breast cancer progression, recurrence and death [12-17]. Thus, over the past several years, the efficacy of exercise in altering the circulating levels of specific key biomarkers that are supposed to be involved in the exercise-cancer relationship has been studied using animal models [18].

Given the possibility of preclinical trials to clarify the biological mechanisms that may underlie associations between exercise and cancer, and evaluating the behavior of key biomarkers after exposure to specific amounts of exercise, is important to quantify the extent of evidences from previously published data. This was the first aim of the present review. Accordingly, we analyzed circulating biomarkers classified into three families: (1) inflammation and cytokines, (2) glucose homeostasis and metabolism, and (3) sex steroid hormones [19].

Additionally, we also examined the exercise designs used in order to determine the exercise parameters that would contribute to improved outcomes.

Methods

We followed the recommendations of the PRISMA statement for systematic reviews and meta-analyses [20].

Information sources and search strategy

In both April and December 2016, we search for published reports in PubMed, MEDELINE, Web of Science (Web of Knowledge), and SIGLE (System for Information on Gray Literature in Europe) databases [Eletronic upplementary material (ESM) figure F1). Relevant MeSH terms and text expressions were used to construct the search strategy (ESM Table S1). Additionally, the reference lists of included manuscripts were screened for potential eligible studies.

Study selection criteria

Three authors independently assessed full-text versions (ACCF, RF and JAD) and disagreements were resolved by consensus.

We included the manuscripts if they fulfilled the next set of inclusion criteria: (1) they were written in English and performed on animals; (2) they compared the effects of an active versus a sedentary lifestyle on at least one host systemic biomarkers; and (3) they examined the effects of exercise following tumor induction.

We excluded manuscripts for several reasons: (1) they examined the cancer-preventive effects of exercise: (2) they evaluated acute effects of exercise; (3) they only included combined exposures (e.g., exercise plus chemical therapy); and (4) the reported results do not allow calculation of effect sizes.

Data extraction and quality assessment

Four reviewers (ACCF, AC, JS and JCL) extracted the baseline characteristics and outcomes data and contacted the authors for missing data. In order to make it possible to correlate the eighteen biomarkers found, they were grouped into three families: (1) inflammation and cytokines, (2) glucose homeostasis and metabolism, and (3) sex

steroid hormones, accordingly with the host-related determinant processes in which they participate in the context of cancer [19].

Three reviewers (ACCF, RF and JAD) evaluate the Risk of Bias of included studies using the SYRCLE's Risk of Bias (RoB) tool (SYstematic Review Centre for Laboratory animal Experimentation Risk of Bias tool), a Cochrane RoB tool adapted for animals [21,22]. Each article was graded (low, unclear or high risk of bias) in the six domains (selection, performance, detection, attrition, reporting and other bias) of SYRCLE's RoB tool. However, we decided to abolish the question 5 (blinding) of performing bias dimension. In fact, to investigate the effects of exercise is absolutely essential that both, investigators and caregivers, be aware of the animals compliance with the exercise conditions in order to reduce the outcome bias (ESM Table S2).

Disagreements were resolved by consensus and a fourth author (JS) was consulted if consensus could not be reached.

Data synthesis and analysis

Data were pooled using random-effects model. As the included studies used different scales to assess the same outcome, we used the correlation coefficient, *r*, to estimate the effect size of its value in exercise-cancer associations. When *r* was unavailable, we converted the available data (averages, standard deviations, percentages and exact p values) into *r* values [23]. Presence of heterogeneity was tested using Cochran's Q and I^2 tests, and Cohen's (1988) criteria for small, medium and large *r* (r = .10, .30, and .50 respectively) effects were used [24] to estimate the magnitude of the results. In order to explore potential sources of heterogeneity, and the influence of different factors on the estimate of effect size we select, relied on theory and previous findings, potential moderating variables (e.g., voluntary or forced exercise, intensity, distance covered, duration and frequency) [25], and we coded them to categorize the analyses (Table 1).

To test the robustness of the overall weighted effect sizes, a sensitivity analysis was conducted by extracting the results of one study at a time from the initial meta-analysis.

Determination of bias was made observing the funnel plot and applying Egger's regression and Begg's rank correlation. We used the Comprehensive Meta-Analysis software version 2.2.057 (CMA, Englewood, New Jersey, USA) to perform all of the statistical analyses.

Table 1

Results

Study selection and characteristics

At the end of the search, we identified 282 reports (Fig. 1), from which only nine [19,26-33] fulfilled the inclusion criteria and blocked any exclusion criteria.

Fig. 1

A total of ten experimental exercise (Table 2) protocols that were equally divided into forced exercise (50.0%) [26,27,29-31] and voluntary (50.0%) exercise [19,28,32,33] modalities were used. In voluntary exercise designs, the free-wheel was used the most when compared with the motorized-wheel (30.0% versus 20.0%).

Intensity was measured in eight protocols (80.0%). Moderate intensity was the most frequently used, while vigorous and low intensities were equally distributed over the remaining two protocols (40.0% versus 20.0% versus 20.0%).

Only 50.0% of the experimental protocols had defined exercise bout durations (long: 60.0%; medium: 20.0%; short: 20.0%). In the remaining 50.0% of the designs, no data was provided regarding the length of the daily exercise period.

Chemical-induced breast cancer (equal doses and administration routes) was the most frequently used model (66.7%) [19,26,27,31-33], followed by a transgenic model [28,30] (66.7%

versus 22,2%). Only one study used the human breast cancer cell inoculation (11.1%) [29].

Table 2

In general, a positive balance in the exercise-tumor relationship can be observed in the analyzed publications regarding the three families of systemic biomarkers.

Exercise prevented tumor-induced systemic inflammation in the majority of the published studies [19,26,28-33], but negative data concerning circulating levels of IL-6 [19,33] and albumin content [31] were reported in three studies.

Positive modulation of glucose metabolism biomarkers by exercise was also observed in the majority of the studies [19,26,27,32,33]. Nevertheless, a negative influence was referred in one study in which the association of high levels of circulating glucose with the free-wheel exercise modality was reported [19].

Exercise also prevents the exposure to higher levels of estradiol [19,32,33] although the opposite is described in one study [26].

Risk of bias and quality of evidence

In 92.59% of the cases the sequence generation, the baseline characteristics and the allocation concealment were adequate (low risk of bias). Housing conditions were similar in 88.89% of the cases, and the outcome assessment methods were also adequate in 72.22%. Incomplete outcome data (the analysis do not include all the animals) was unclear in only 22.22% of the analyzed studies. 100% (low risk of bias) of the published reports included all the expected outcomes and clearly stated the financial support. Overall, the nine studies were judge at low risk of bias (ESM Table S3).

Effect size of exercise on systemic biomarkers levels

To quantify the relationship between exercise and the evaluated 18 biomarkers, a total of 65 correlations were made, using 33 of them to examine the effects of exercise on the inflammatory process and cytokines levels (Fig. 2a), 21 of them to analyze exercise effects with respect to glucose homeostasis and metabolism (Fig. 2b), and 11 to determine exercise effects on the sex hormone family (Fig. 2c).

Fig. 2 a, b, c

The positive impact of exercise on the modulation of all of the selected biomarkers could be observed. Exercise promotes large benefits on tumor-related systemic inflammation (r = -.519, p = .011; 95% CI = -.768; -. 133; n = 413; k = 33), on exposure to sex hormones (r = -.697, p = .002; 95% CI = -.887; -.308; n = 470; k = 11), and on the regulation of glucose-related factors (r = -.745, p = .000; 95% CI = -.898; -.433; n = 341; k = 21).

We identified high heterogeneity among collected study data [34] with respect to dependent variables, namely with respect to inflammation and cytokines family [$Q(32)$ = 4104.799, p = .000 (I^2 = 99.220)], glucose homeostasis metabolism [$Q(20)$ = 2934.269, p = .000 (I^2 = 99.318)], and sex hormone families [$Q(10)$ = 699.156, p = .000 (I^2 = 98.570)]. Removing on study at a time did not substantially alter our results in any of the variables. In systemic-induced inflammation the variations in global effect size (r = -.479, p = .019, 95% CI = -.738, -.064; r = -.575, p = .001, 95% CI = -.779 -.260) were low than 10% (ESM figure F2a). Similar results (r = -.698, p = .000, 95% CI = -.873, -.361; r = -.799, p = .000, 95% CI = -.909 -.584) can be observed for glucose-related levels (ESM figure F2b), and for sex hormone levels (ESM figure F2c: r = -.615, p = .005, 95% CI = -.839, -.213; r = -.734, p = .001, 95% CI = -.906 -.353).

High variability in effect sizes could also be related to different parameters in the experimental exercise designs. Accordingly, we also tried to identify the strength of the influence of the different exercise parameters on the exercise-tumor relationship.

Potential moderating factors

To examine how the outcomes with respect to the three dependent variables could be affected by the exercise designs, we performed separate analyses. With some exceptions, exercise-induced moderation could be observed (Table 3).

Table 3

Voluntary exercise versus forced exercise: The benefits of exercise in tumor-related inflammation, circulating levels of glucose-related factors, and sex hormone concentrations seem to occur whether or not the exercise type was forced or voluntary. Inflammation biomarkers (72.5% versus 58.9%) and sex hormones (86.9% versus 86.6%) benefit from both exercise modalities. Additionally, glucose metabolism shows small beneficial effects when exercise is performed on a treadmill (25.1%), and larger beneficial effects when performed on motorized (82.7%) or free-wheel equipment (94.4%).

Intensity: Vigorous exercise intensity appears to be essential in order to positively alter systemic biomarker concentrations. In spite of all levels of intensity have exerted positive influence in the three studied biomarker families, the highest scores were obtained with vigorous followed by moderate intensity (inflammation and cytokines: 58.3% - 43,1%; glucose homeostasis and metabolism: 88.8% - 25,1%; and sex hormones: 80.5% - 47.9%).

Distance covered: When considering the distance covered, the beneficial effects observed on biomarkers outcomes did not seem to depend on this variable. Moderation was found only in glucose homeostasis and metabolism , but the

beneficial effects were significant regardless of the distance that was covered (85.7% versus 92.4%).

Duration: Short and medium exercise bouts exerted significant effects on circulating marker concentrations. Short exercise bouts showed a large effect on inflammation when compared with long exercise bouts (97.9% versus 43.1%), and the same effect could be observed on sex hormone levels (96.0% versus 47.9%). The biomarkers associated with glucose metabolism seem to have gained more benefits from medium length exercise bouts (32.2%). However, it is worth noting that none of the other members of the biomarker families were examined under medium exercise bouts conditions.

Frequency: Weekly exercise did not moderate the observed benefits in circulating sex hormone levels. In tumor-related inflammation, an exercise frequency of 5 or 7 days has similar and significant effects (67.1% versus 62.0%) with respect to the observed improvements, while for glucose metabolism a 7-day frequency (90.6%) appeared to be more beneficial.

We found no evidences of publication bias after observing funnel plots and considering Egger's regression and Begg's rank correlation, with respect to inflammation and cytokines (p = .16742), glucose homeostasis and metabolism (p = .24057), and sex hormone families (p = .04655).

Discussion

In this review we first aimed to summarize and quantify the current knowledge about the effects of exercise on the levels of tumor-related biomarkers of inflammation, glucose metabolism, and sex hormones. To the best of our knowledge, no meta-analytical study has been done to quantify this association in breast cancer preclinical models.

In contrast to some preclinical studies that link the deregulation of some systemic markers to exercise [19,26,31,33], our meta-analysis showed that engaging in approximately 85 min per week (useful days) of vigorous forced exercise after cancer induction, was associated with 61.5% tumor-reduced systemic inflammation biomarkers, and with 69.7% improvement in steroid hormones circulating levels. Similar outcomes can be obtained with vigorous motorized-wheel and in free-wheel modalities but in the latter, it is not possible to point the exercise parameters.

We also found that engaging in voluntary exercise modalities (free-wheel or vigorous motorized-wheel) is associated with 86.6% improvement in the circulating levels of glucose metabolism biomarkers. Small beneficial effects can also be achieved by participating in about 200 minutes of vigorous treadmill exercise per week (useful days).

Our results also indicated a positive influence of moderate intensity in all biomarkers outcomes but in a small degree. In fact, participating in approximately 85 min per week (useful days) of moderate treadmill exercise can also have positive effects on tumor-related inflammation (43.1%) and in the circulating sex hormone levels (47.9%). In glucose metabolism, the positive effects of moderate intensity (25.1%) can also be observed but only to a small degree.

Exercise's significant impact on inflammation and cytokines family and on the sex hormone family was achieved with the similar amounts of exercise than those prescribed in the guidelines for cancer patients regarding vigorous activity (i.e., 75 min vigorous activity per week) [35].

The best results in glucose homeostasis and metabolism family appear to be associated with voluntary exercise, which indicates that we can not determine the exact amount of exercise performed that made possible to achieve those results. On the other hand,

the results obtained when exercise is performed under forced modalities, hardly reproducible during breast cancer continuum (i.e. 200 minutes of vigorous exercise every week), even positive, were smaller.

In contrast to what has been reported in several studies [19,32,33], we found that the distance covered by the animals did not seems to be important for modulating selected biomarker levels. It seems that exercise intensity is the determining variable that improved the outcomes. This finding seems to be a strong argument favoring exercise designs in which the intensity levels can be manipulated.

It is clear that inflammation plays a significant role in the development and progression of cancer by influencing the host immune response [36-40]. Despite inconsistent results about an inverse relationship between exercise and inflammation described in the literature [41], the present data from animal studies strongly supports this inverse relationship. It remains to be clarified as to whether exercise can exert a direct influence on inflammatory pathways or if the beneficial effects are achieved thought indirect ways such as the modulation of body composition [42]. Evidence that improvements in circulating sex hormone levels are associated with exercise, especially in postmenopausal breast cancer, is more consistent in the literature [43-45]. Accordingly, our data demonstrates a strong and inverse association between exercise and the modulation of steroid hormone levels. Additionally, there is sufficient current evidence to justify interventions, such as exercise, that lower insulin levels, as targeted treatments in breast cancer [46,47]. Several studies have demonstrated that elevated insulin and insulin-like growth factors levels are associated with poor prognosis in breast cancer patients [4,14,15,39,48-50], and that exercise might have an important action on regulating these levels. The present data confirmed this finding, however, the fact that

the best results were obtained with voluntary exercise modalities limited any kind of information about the exact amount of exercise needed to reach this endpoint.

Another finding in this review lies in the diversity of exercise designs used, which imposes clear limitations in extending the results obtained by these studies to other populations and other contexts.

Animal studies provide an important contribution to manipulating exercise variables in order to establish the ideal amount to modulate the biological pathways involved with the underlying exercise-cancer relationship mechanisms (e.g., insulin sensitivity, chronic inflammation, steroid hormone levels, and innate immunity). Nevertheless, the lack of studies in the present analysis shows that these studies have been underused. Furthermore, the differences in exercise designs create serious difficulties for quantitative reviews. Additionally, half of the studies do not clearly define the exercise model that they want to reproduce in humans. This is a strong limitation with respect to relating preclinical results to clinical contexts.

Strengths and limitations of this review

As far as we know our meta-analysis is the first one to provide a comprehensive and quantitative overview in preclinical data related to the effects of exercise in breast cancer biomarkers. We used current recommendation to judge the risk of bias and rated the reports with an overall low risk of bias.

We analyzed potential moderators that could have influenced our effect estimates and we found the amounts of exercise training that better improved cancer-induced inflammation and sex hormone levels. We also performed a sensitivity analysis to examine the impact of each study in the overall effect size and we observed small variations. We do not found publication bias in none of the variable studied.

There are limitations in this study. First, studies published in languages other than English were not considered. Additionally, some manuscripts were excluded (n = 3) because they did not present all the necessary data.

Perspectives

We found considerable improvements in cancer-related inflammation, in the regulation of glucose-related factors, and in the sex hormone concentrations, among the animals exposed to exercise. It became clear that performing 85 min per week of vigorous forced exercise reduced systemic inflammation, and improved sex hormone levels. It was impossible to pinpoint the exercise amount needed to regulate host glucose-related factors. Based on the proposed framework, we recommend future preclinical studies including similar exercise parameters with specific endpoints evaluation that can be reproduce in humans.

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Figure legends

Fig. 1 Flow chart depicting the selection of studies for the meta-analysis.

Fig. 2a Forest plot of the meta-analysis about exercise effects on systemic levels of inflammation and cytokines. Correlation: effect size (r) for each study. CI= confidence interval. a, b: different measures within the same study.

Abbreviations: SPWEIGHT, spleen weight; IL-6, interleukin-6; TNF-α, tumor necrosis factor-α; CRP, C reactive protein; IFN-Y, interferon-Y; MCP-1, monocyte chemoattractant protein; SAP, serum amyloid P; TWEAK, TNF-like weak inducer of apoptosis.

Fig. 2b Forest plot of the meta-analysis about the influence of exercise on systemic levels of glucose homeostasis and metabolism biomarkers. Correlation: effect size (r) for each study in. CI= confidence interval. a, b, c: different measures within the same study.

Abbreviations: CORT, corticosterone; GLUC, glucose; IGF-1, insulin like growth factor; INSUL, insulin; LDH, lactate dehydrogenase.

Fig. 2c Forest plot of the meta-analysis addressing the influence of exercise on systemic levels of sex steroid hormone biomarkers. Correlation: effect size (r) for each study CI= Confidence Interval. a, b: different measures within the same study.

Abbreviations: ESTR, estradiol; PROG, progesterone.

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Table 1: Moderators codification

Moderator	Values	Coding Description
Exercise type	0=Treadmill; 1=Free-wheel; 2=Motorized-wheel	Type of exercise performed. Protocols in treadmill were considered as forced exercise. For voluntary exercise, protocols in free-wheel or in motorized-wheel were considered.
Exercise intensity	0=ND; 1=Low; 2=Moderate; 3=Vigorous	0: Not defined; 1: Until 50% of maximum speed; 2: 50%-70% of maximum speed; 3: Above 70% of maximum speed.
Distance covered	0= NR; 1=Short distance; 2=Long distance	Distance covered during the entire program 0: Not registered; 1: Group of animals who covered shorter distances; 2: Group of animals who covered longer distances.
Exercise duration	0=NR; 1=Short duration; 2=Medium duration; 3=Long duration; 4=Very long Duration	Daily exercise duration. 0: Not registered; 1: Under 30 minutes; 2: Between 30 and 45 minutes; 3: Between 46 and 60 minutes; 4= Above 60 minutes.
Exercise frequency	0=5 d/week; 1=6 d/week; 2=7 d/week	Weekly frequency. In voluntary exercise protocols, when the frequency has not been defined by the authors a frequency of 7 days was always assumed.

Table 2: List of all the publication used with the exercise-moderating variables considered for analysis, and the main outcomes in the 3 families of biomarkers.

Study (Reference)	Animal /BC model	Exercise design							Inflammation and cytokines main outcomes
		n	Study duration	Type	Intensity	Distance covered	Duration	Frequency	
Zhu et al, 2008 (49)	Sprague-Dawley rats (21 days old) MNU (50mg/Kg) i.p.	52 Ex+52 Sed	8 wks	FW	-	428.400 Km	-	7 days/wk	- ↓ CRP and Leptin in the active animals.
Thompson et al, 2010 (51)	Sprague-Dawley rats MNU (50mg/Kg) i.p.	22 Ex+33 Ex + 55 Sed	8 wks	MW FW	Vigorous -	- 391.000 Km	- -	7 days/wk	- ↓ CRP, Leptin, TNF-α; and ↑ INF-Y in the active animals. - ↑ IL-6 in the active animals.
Murphy et al, 2011 (40)	C3(1) Transgenic mouse model of breast tumor	12 Ex+14 Sed	20 wks	TRDM	Moderate	-	60 min/d	6 days/wk	- ↓ MCP-1, IL-6 and Spleen weight in the active animals.
Zhu et al, 2012 (57)	Sprague Dawley rats (21 days old) MNU (50mg/Kg) i.p.	30 Ex+30 Ex + 30 Sed	10 wks	MW MW	Low Vigorous	125.000 Km 245.000 Km	- -	7 days/wk	- ↓ CRP, Leptin, TNF-α and SAP in the active animals. - ↑ IL-6 in the active animals.
Goh et al, 2013 (52)	PyMT Transgenic mouse of invasive breast cancer	13 Ex+12 Sed	10 wks	FW	-	<150 Km/>150 km	-	7 days/wk	- ↓ Spleen weight in the active animals.
Faustino-Rocha et al (1), 2016 (43)	Sprague-Dawley rats (50 days old) MNU (50mg/Kg) i.p.	10 Ex+11 Sed	35 wks	TRDM	Moderate	-	60 min/d	5 days/wk	- ↓ CPR, IL-6 and Spleen weight in the active animals.
Isanejad et al 2016 (45)	BALB/c mice (42/48 days old) Cell inoculation (MC4-L2 human BC)	8 Ex+8 Sed	5 wks	TRDM	Low	-	16/18 min/d	5 days/wk	- ↓ TNF-α in the active animals.
Pdrão et al 2016	Sprague-Dawley rats (50 days old) MNU (50mg/Kg) i.p.	10 Ex+11 Sed	35 wks	TRDM	Moderate	-	60 min/d	5 days/wk	- ↓ TWEAK and Myostatin in the active animals. - ↑ Albumin in the active animals.

Table 2 (cont.)

Study (Reference)	Animal /BC model	Exercise design							Glucose homeostasis and metabolism main outcomes
		n	Study duration	Type	Intensity	Distance covered	Duration	Frequency	
Gillette et al, 1997 (37)	F-344 rats (50/57 days old) MNU (50mg/Kg) i.p.	32 Ex+30 Sed 32 EX+30 Sed	20.5 wks	TRDM	Moderate	-	40 min/d	5 days/wk	- ↑ Corticosterone in the active animals.
Zhu et al, 2008 (49)	Sprague-Dawley rats (21 days old) MNU (50mg/Kg) i.p.	52 Ex+52 Sed	8 wks	FW	-	428.400 Km	-	7 days/wk	- ↓ Insulin and IGF-1 in the active animals. - ↑ Corticosterone in the active animals.
Thompson et al, 2010 (51)	Sprague-Dawley rats MNU (50mg/Kg) i.p.	22 Ex+33 Ex + 55 Sed	8 wks	MW FW	Vigorous -	- 391.000 Km	- -	7 days/wk	- ↓ Insulin and IGF-1 in the active animals. - ↓ Glucose in the active animals under motorized-wheel exercise. - ↑ Glucose in the active animals under free-wheel exercise. - ↑ Corticosterone in all the active animals.
Zhu et al, 2012 (57)	Sprague Dawley rats (21 days old) MNU (50mg/Kg) i.p.	30 Ex+30 Ex + 30 Sed	10 wks	MW MW	Low Vigorous	125.000 Km 245.000 Km	- -	7 days/wk	- ↓ Glucose, IGF-1 and Insulin in the active animals.
Faustino-Rocha et al (1), 2016 (43)	Sprague-Dawley rats (50 days old) MNU (50mg/Kg) i.p.	10 Ex+11 Sed	35 wks	TRDM	Moderate	-	60 min/d	5 days/wk	- ↓ Glucose in the active animals.
Pdrão et al 2016	Sprague-Dawley rats (50 days old) MNU (50mg/Kg) i.p.	10 Ex+11 Sed	35 wks	TRDM	Moderate	-	60 min/d	5 days/wk	- ↓ LDH in the active animals.

Table 2 (cont.)

Study (Reference)	Animal /BC model	n	Study duration	Exercise design: Type	Intensity	Distance covered	Duration	Frequency	Sex Hormones main outcomes
Zhu et al, 2008 (49)	Sprague-Dawley rats (21 days old) MNU (50mg/Kg) i.p.	52 Ex+52 Sed	8 wks	FW	-	428.400 Km	-	7 days/wk	- ↓ Estradiol in the active animals.
Thompson et al, 2010 (51)	Sprague-Dawley rats MNU (50mg/Kg) i.p.	22 Ex+33 Ex + 55 Sed	8 wks	MW FW	Vigorous -	- 391.000 Km	- -	7 days/wk	- ↓ Estradiol in the active animals. - ↑ Progesterone in the active animals.
Zhu et al, 2012 (57)	Sprague Dawley rats (21 days old) MNU (50mg/Kg) i.p.	30 Ex+30 Ex + 30 Sed	10 wks	MW MW	Low Vigorous	125.000 Km 245.000 Km	- -	7 days/wk	- ↓ Estradiol in the active animals. - ↑ Progesterone in the active animals.
Faustino-Rocha et al (1), 2016 (43)	Sprague-Dawley rats (50 days old) MNU (50mg/Kg) i.p.	10 Ex+11 Sed	35 wks	TRDM	Moderate	-	60 min/d	5 days/wk	- ↑ Estradiol in the active animals.
Isanejad et al 2016 (45)	BALB/c mice (42/48 days old) Cell inoculation (MC4-L2 human BC)	8 Ex+8 Sed	5 wks	TRDM	Low	-	16/18 min/d	5 days/wk	- ↓ Estradiol in the active animals.

NOTE: (1) Inflammation and cytokines family - Spleen weight; IL-6, interleukin-6; TNF-α, tumor necrosis factor-α; CRP, C reactive protein; IFN-Y, interferon-Y; MCP-1, monocyte chemoattractant protein; SAP, serum amyloid P; Albumin; TWEAK, TNF-like weak inducer of apoptosis; Myostatin; Leptin. (2) Glucose homeostasis and metabolism family – Corticosterone, glucose; IGF-1, insulin like growth factor; insulin; LDH, lactate dehydrogenase. (3) Sex hormones family – Estradiol, progesterone. ↑ Increase, ↓ decrease.

Abbreviations: DMBA, 7,12-Dimethylbenz(a)anthracene; MNU, 1-methyl-1-nitrosoureia. BC, breast cancer; i.p., intraperitoneal; g.i., gastric intubation. Ex, active animals; Sed, sedentary animals. TRDM, treadmill; FW, Free-wheel; MW, motorized-wheel. Wks, weeks; wk, week; min, minutes; d, day.

Table 3: Moderators of the relationship between exercise, and host systemic biomarkers.

		IC			GHM			SH		
Moderator		k	r	p	k	r	p	k	r	p
Exercise type	Trdm	10	-.589	.000	4	-.251	.000	2	-.866	.000
	Fw	8	-.725	.000	7	-.944	.000	3	-.869	.000
	Mw	15	-.512	.000	10	-.827	.000	6	-.697	.000
Exercise intensity	Low	6	-.408	.000	3	-.272	.000	3	-.455	.000
	Mod	9	-.431	.000	4	-.251	.000	1	-.479	.011
	Vig	10	-.583	.000	7	-.888	.000	4	-.805	.000
Distance covered	Sd	11	a)		7	-.857	.000	4	a)	
	Ld	12			10	-.924	.000	5		
Exercise duration	Sh	1	-.979	.000	0	-	-	1	-.960	.000
	Med	0	-	-	2	-.322	.000	0	-	-
	L	9	-.431	.000	2	-.110	.475	1	-.479	.011
	Vl	0	-	-	0	-	-	0	-	-
Exercise frequency	5d	7	-.671	.000	4	-.251	.000	2		
	6d	4	-.385	.000	0	-	-	0	a)	
	7d	22	-.620	.000	17	-.906	.000	9		

NOTE: Exercise intensity, distance covered, duration and exercise frequency as moderators of selected biomarkers. Number of cases (k) for correlation (r), and respective p-value.
a) Is not moderated by this exercise condition.
Abbreviations: IC, inflammation and cytokines; GHM, glucose homeostasis and metabolism; SH, sex hormones; Trdm, treadmill; Fw, free-wheel; Mw, motorized-wheel; Mod, moderate; Vig, vigorous; Sh, short; Med, medium; L, long; Vl, very long; Sd, short distance; Ld, long distance; d, days.

Conflict of interest: The authors declare that they have no conflict of interest.

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Ethical approval: This article does not contain any studies with human participants or animals.

Fig. 1

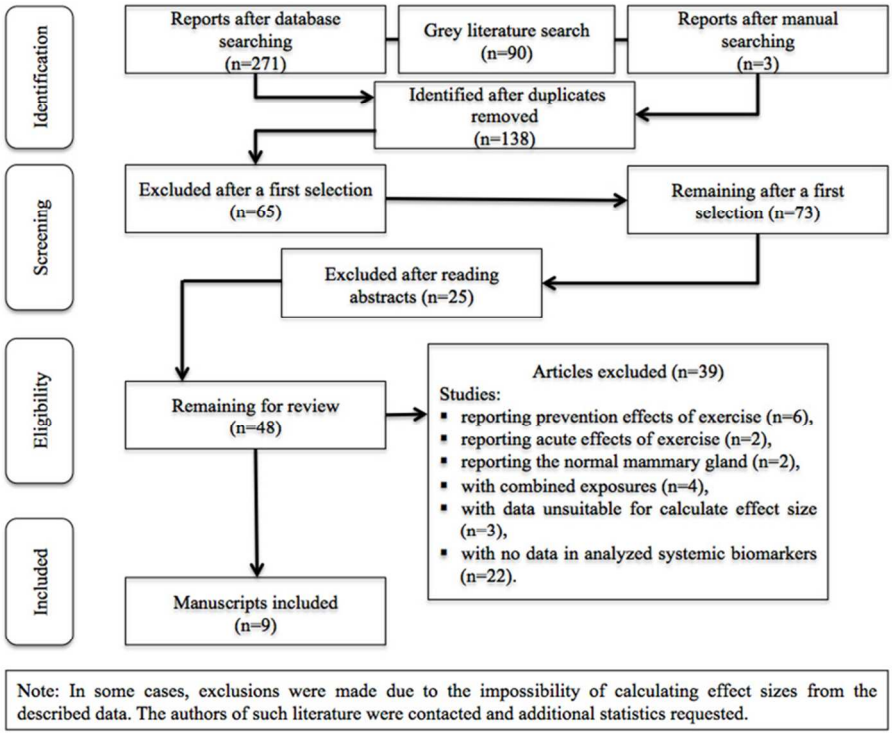

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Fig. 2 a

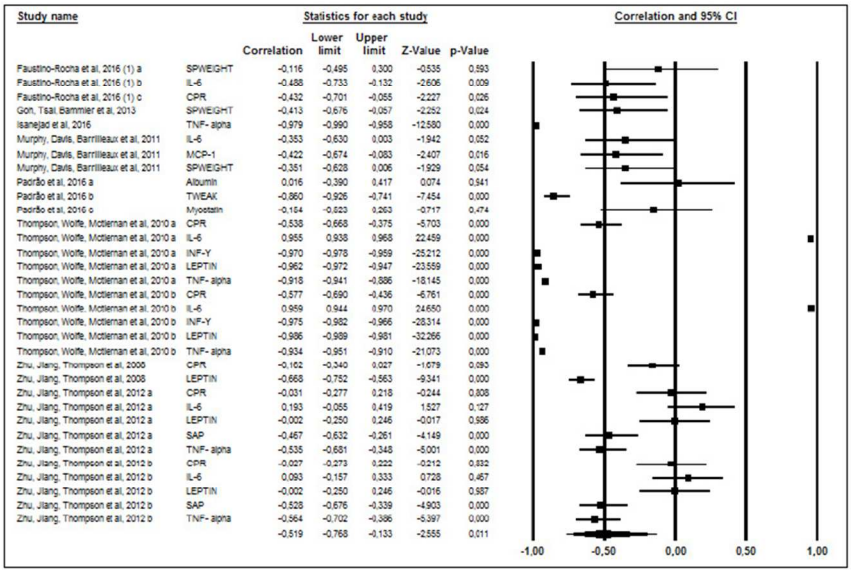

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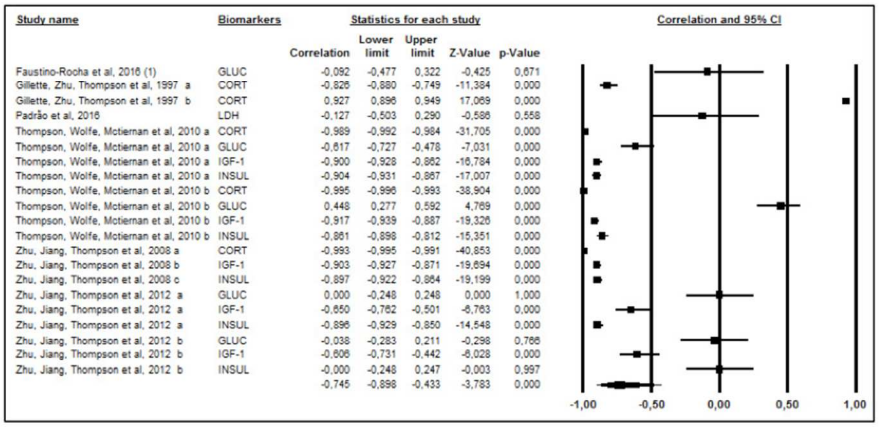
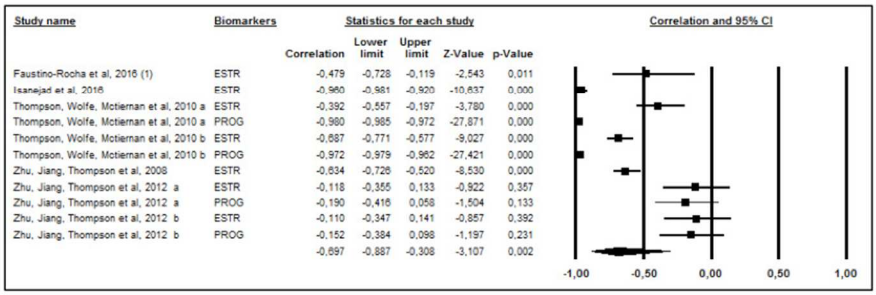
Fig. 2 b



Study name	Biomarkers	Correlation	Lower limit	Upper limit	Z-Value	p-Value
Faustino-Rocha et al, 2016 (1)	GLUC	-0,092	-0,477	0,322	-0,425	0,671
Gillette, Zhu, Thompson et al, 1997 a	CORT	-0,826	-0,880	-0,749	-11,384	0,000
Gillette, Zhu, Thompson et al, 1997 b	CORT	0,927	0,896	0,949	17,069	0,000
Padrão et al, 2016	LDH	-0,127	-0,503	0,290	-0,586	0,558
Thompson, Wolfe, Mctiernan et al, 2010 a	CORT	-0,989	-0,992	-0,984	-31,705	0,000
Thompson, Wolfe, Mctiernan et al, 2010 a	GLUC	-0,617	-0,727	-0,478	-7,031	0,000
Thompson, Wolfe, Mctiernan et al, 2010 a	IGF-1	-0,900	-0,928	-0,862	-16,784	0,000
Thompson, Wolfe, Mctiernan et al, 2010 a	INSUL	-0,904	-0,931	-0,867	-17,007	0,000
Thompson, Wolfe, Mctiernan et al, 2010 b	CORT	-0,995	-0,996	-0,993	-38,904	0,000
Thompson, Wolfe, Mctiernan et al, 2010 b	GLUC	0,448	0,277	0,592	4,769	0,000
Thompson, Wolfe, Mctiernan et al, 2010 b	IGF-1	-0,917	-0,939	-0,887	-19,326	0,000
Thompson, Wolfe, Mctiernan et al, 2010 b	INSUL	-0,861	-0,898	-0,812	-15,351	0,000
Zhu, Jiang, Thompson et al, 2008 a	CORT	-0,993	-0,995	-0,991	-40,853	0,000
Zhu, Jiang, Thompson et al, 2008 b	IGF-1	-0,903	-0,927	-0,871	-19,694	0,000
Zhu, Jiang, Thompson et al, 2008 c	INSUL	-0,897	-0,922	-0,864	-19,199	0,000
Zhu, Jiang, Thompson et al, 2012 a	GLUC	0,000	-0,248	0,248	0,000	1,000
Zhu, Jiang, Thompson et al, 2012 a	IGF-1	-0,650	-0,762	-0,501	-6,763	0,000
Zhu, Jiang, Thompson et al, 2012 a	INSUL	-0,896	-0,929	-0,850	-14,548	0,000
Zhu, Jiang, Thompson et al, 2012 b	GLUC	-0,038	-0,283	0,211	-0,298	0,766
Zhu, Jiang, Thompson et al, 2012 b	IGF-1	-0,606	-0,731	-0,442	-6,028	0,000
Zhu, Jiang, Thompson et al, 2012 b	INSUL	-0,000	-0,248	0,247	-0,003	0,997
		-0,745	-0,898	-0,433	-3,783	0,000

338x151mm (72 x 72 DPI)

Fig. 2 c



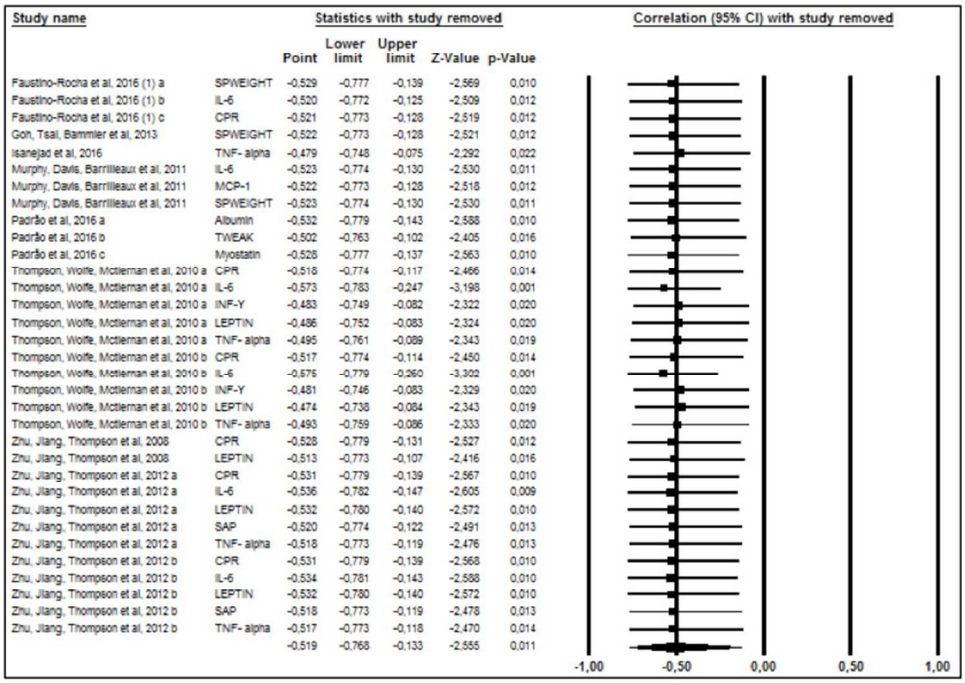
339x105mm (72 x 72 DPI)

Supplemental Fig. F1: Example of electronic search material

Translations:	
Exercise	"exercise"[MeSH Terms] OR "exercise"[All Fields]
Physical activity	"exercise"[MeSH Terms] OR "exercise"[All Fields] OR ("physical"[All Fields] AND "activity"[All Fields]) OR "physical activity"[All Fields]
running	"running"[MeSH Terms] OR "running"[All Fields]
breast tumor	"breast neoplasms"[MeSH Terms] OR ("breast"[All Fields] AND "neoplasms"[All Fields]) OR "breast neoplasms"[All Fields] OR ("breast"[All Fields] AND "tumor"[All Fields]) OR "breast tumor"[All Fields]
breast neoplasms	"breast neoplasms"[MeSH Terms] OR ("breast"[All Fields] AND "neoplasms"[All Fields]) OR "breast neoplasms"[All Fields]
mammary cancer	"breast neoplasms"[MeSH Terms] OR ("breast"[All Fields] AND "neoplasms"[All Fields]) OR "breast neoplasms"[All Fields] OR ("mammary"[All Fields] AND "cancer"[All Fields]) OR "mammary cancer"[All Fields]
animals	"animals"[MeSH Terms:noexp] OR animals[All Fields]
Database:	
PubMed	
User query:	
[(Exercise OR Physical activity) OR (Voluntary running OR treadmill running)] AND [(breast tumor OR breast neoplasms OR mammary cancer) AND (animals)	

339x170mm (72 x 72 DPI)

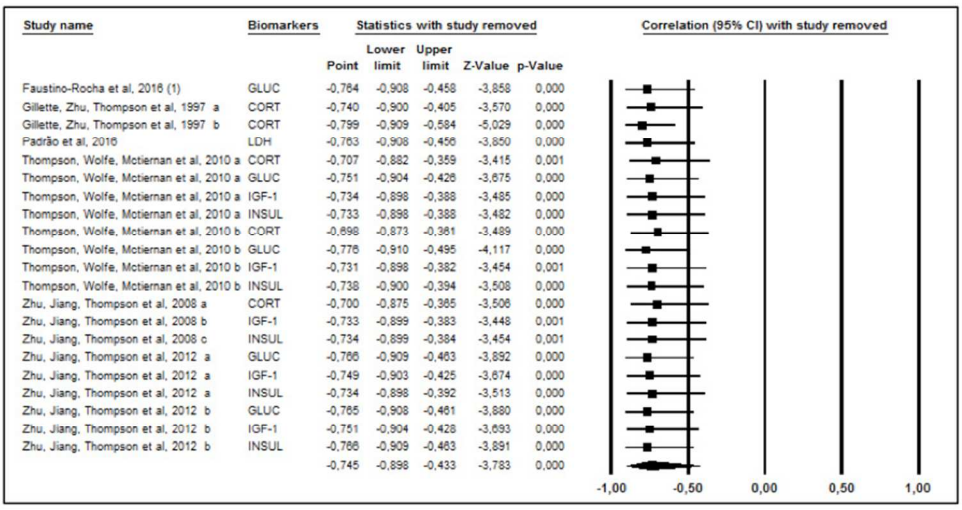
Supplemental Fig. F2 a: Inflammation and Cytokines (sensitivity analysis)



Study name		Statistics with study removed					Correlation (95% CI) with study removed
		Point	Lower limit	Upper limit	Z-Value	p-Value	
Faustino-Rocha et al, 2016 (1) a	SPWEIGHT	-0,529	-0,777	-0,139	-2,569	0,010	
Faustino-Rocha et al, 2016 (1) b	IL-6	-0,520	-0,772	-0,125	-2,509	0,012	
Faustino-Rocha et al, 2016 (1) c	CPR	-0,521	-0,773	-0,128	-2,519	0,012	
Goh, Tsai, Bammler et al, 2013	SPWEIGHT	-0,522	-0,773	-0,128	-2,521	0,012	
Isanejad et al, 2016	TNF- alpha	-0,479	-0,748	-0,075	-2,292	0,022	
Murphy, Davis, Barrilleaux et al, 2011	IL-6	-0,523	-0,774	-0,130	-2,530	0,011	
Murphy, Davis, Barrilleaux et al, 2011	MCP-1	-0,522	-0,773	-0,128	-2,518	0,012	
Murphy, Davis, Barrilleaux et al, 2011	SPWEIGHT	-0,523	-0,774	-0,130	-2,530	0,011	
Padrão et al, 2016 a	Albumin	-0,532	-0,779	-0,143	-2,588	0,010	
Padrão et al, 2016 b	TWEAK	-0,502	-0,763	-0,102	-2,405	0,016	
Padrão et al, 2016 c	Myostatin	-0,528	-0,777	-0,137	-2,563	0,010	
Thompson, Wolfe, Mctiernan et al, 2010 a	CPR	-0,518	-0,774	-0,117	-2,466	0,014	
Thompson, Wolfe, Mctiernan et al, 2010 a	IL-6	-0,573	-0,783	-0,247	-3,198	0,001	
Thompson, Wolfe, Mctiernan et al, 2010 a	INF-Y	-0,483	-0,749	-0,082	-2,322	0,020	
Thompson, Wolfe, Mctiernan et al, 2010 a	LEPTIN	-0,486	-0,752	-0,083	-2,324	0,020	
Thompson, Wolfe, Mctiernan et al, 2010 a	TNF- alpha	-0,495	-0,761	-0,089	-2,343	0,019	
Thompson, Wolfe, Mctiernan et al, 2010 b	CPR	-0,517	-0,774	-0,114	-2,450	0,014	
Thompson, Wolfe, Mctiernan et al, 2010 b	IL-6	-0,575	-0,779	-0,260	-3,302	0,001	
Thompson, Wolfe, Mctiernan et al, 2010 b	INF-Y	-0,481	-0,746	-0,083	-2,329	0,020	
Thompson, Wolfe, Mctiernan et al, 2010 b	LEPTIN	-0,474	-0,738	-0,084	-2,343	0,019	
Thompson, Wolfe, Mctiernan et al, 2010 b	TNF- alpha	-0,493	-0,759	-0,086	-2,333	0,020	
Zhu, Jiang, Thompson et al, 2008	CPR	-0,528	-0,779	-0,131	-2,527	0,012	
Zhu, Jiang, Thompson et al, 2008	LEPTIN	-0,513	-0,773	-0,107	-2,416	0,016	
Zhu, Jiang, Thompson et al, 2012 a	CPR	-0,531	-0,779	-0,139	-2,567	0,010	
Zhu, Jiang, Thompson et al, 2012 a	IL-6	-0,536	-0,782	-0,147	-2,605	0,009	
Zhu, Jiang, Thompson et al, 2012 a	LEPTIN	-0,532	-0,780	-0,140	-2,572	0,010	
Zhu, Jiang, Thompson et al, 2012 a	SAP	-0,520	-0,774	-0,122	-2,491	0,013	
Zhu, Jiang, Thompson et al, 2012 a	TNF- alpha	-0,518	-0,773	-0,119	-2,476	0,013	
Zhu, Jiang, Thompson et al, 2012 b	CPR	-0,531	-0,779	-0,139	-2,568	0,010	
Zhu, Jiang, Thompson et al, 2012 b	IL-6	-0,534	-0,781	-0,143	-2,588	0,010	
Zhu, Jiang, Thompson et al, 2012 b	LEPTIN	-0,532	-0,780	-0,140	-2,572	0,010	
Zhu, Jiang, Thompson et al, 2012 b	SAP	-0,518	-0,773	-0,119	-2,478	0,013	
Zhu, Jiang, Thompson et al, 2012 b	TNF- alpha	-0,517	-0,773	-0,118	-2,470	0,014	
		-0,519	-0,768	-0,133	-2,555	0,011	

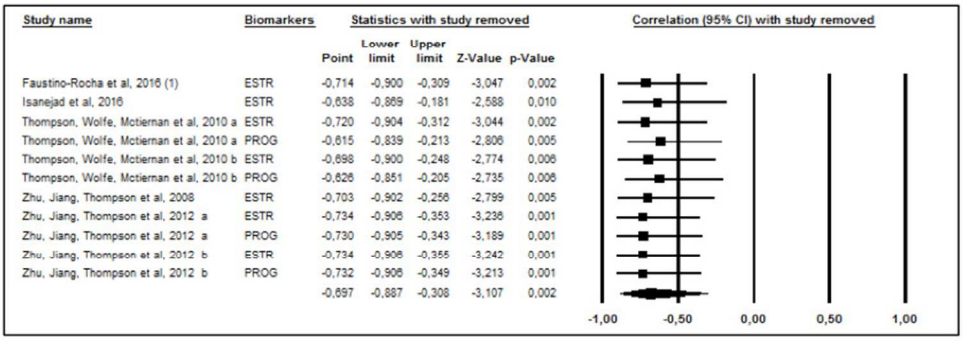
307x231mm (72 x 72 DPI)

Supplemental Fig. F2 b: Glucose homeostasis and metabolism (sensitivity analysis)



308x177mm (72 x 72 DPI)

Supplemental Fig. F2 c: Sex hormones (sensitivity analysis)



307x129mm (72 x 72 DPI)

Supplemental Table S1: Search strategy

Search strategy	"Exercise"	"Breast cancer"	"Animals"
MeSH terms	Exercise Running	Breast neoplasms	Animals
Text words	Physical activity Voluntary running Treadmill running	Breast tumor Mammary cancer	

Supplemental Table S2: SYRCLE's RoB tool for assessing risk of bias

ITEM	BIAS DOMAIN	SOURCE OF BIAS	SUPPORT FOR JUDGMENTS	REVIEW AUTHOR'S JUDGMENT
1	**Selection bias**	Sequence generation	Describe the methods used, if any, to generate the allocation sequence in sufficient detail to allow an assessment whether it should produce comparable groups.	Y= The investigators described a random component. U= The investigators mention the randomization but its not clear how. N= No randomization were used or is not reported.
2	**Selection bias**	Baseline characteristics	Describe all the possible prognostic factors or animal characteristics, if any, that are compared in order to judge whether or not intervention and control groups were similar at the start of the experiment.	Y= Similarity in baseline characteristics (ex. animal type, animal age). The disease was induced before randomization or a transgenic model was used. U= The similarity its not clear or/and the disease was induced before randomization. N= The baseline characteristics are not similar or not clear, and the disease was induced after randomization.
3	**Selection bias**	Allocation concealment	Describe the method used to conceal the allocation sequence in sufficient detail to determine whether intervention allocations could have been foreseen before or during enrolment.	Y= The allocation was adequately concealed if the baseline characteristics were similar. U= The methods used to allocation are not clear neither are the similarity of baseline characteristics. N= Not reported.
4	**Performance bias**	Random housing	Describe all measures used, if any, to house the animals randomly within the animal room.	Y= The housing conditions were similar between groups (ex. temperature, humidity, lighting); In protocols of forced exercise the sedentary animals were placed in a stationary treadmill during the same time that exercised animals. U= The housing conditions were similar between groups (ex. temperature, humidity, lighting); In protocols of forced exercise the sedentary animals remain in the cages during the exercise period. N= The housing conditions were not similar between groups (ex. temperature, humidity, lighting); In protocols of forced exercise the sedentary animals remain in the cages during the exercise period or is not reported.
5	**Performance bias**	Blinding	Describe all measures used, if any, to blind trial caregivers and researchers from knowing which intervention each animal received. Provide any information relating to whether the intended blinding was effective.	To investigate the effects of exercise exposure blindness is not possible. The investigators and/or the caregivers must be aware of the compliance of the animals with the exercise conditions in order to reduce the outcomes bias. Thus, this condition is not applicable.
6	**Detection bias**	Random outcome assessment	Describe whether or not animals were selected at random for outcome assessment, and which methods to select the animals, if any, were used.	Y= The animals were selected at random for outcome assessment, or the animals were not selected at random for outcome assessment, but all the animals were used and the outcomes is not likely to be influenced. U= The animals were not selected at random for outcome assessment but the investigators pinpoint the reasons why. N= The animals were not selected at random for outcome assessment; or its not clear why the investigators choose a specific number of animals; or its not reported.

Supplemental Table S2: SYRCLE's RoB tool for assessing risk of bias (cont.)

7	**Detection bias**	Blinding	Describe all measures used, if any, to blind outcome assessors from knowing which intervention each animal received. Provide any information relating to whether the intended blinding was effective.	Y= The outcome assessment methods were the same in both groups, or if they were not, a valid reason was defined (ex. to measure the effects of different amounts of exercise exposure). U= The outcome assessment methods were the same in both groups but its not clear if a random component were used. N= Differences in the outcome assessment (e. g., different times in sacrificed, differences in blood sample collection); or if not reported.
8	**Attrition bias**	Incomplete outcome data	Describe the completeness of outcome data for each main outcome, including attrition and exclusions from the analysis. State whether attrition and exclusions were reported, the numbers in each intervention group (compared with total randomized animals), reasons for attrition or exclusions, and any re-inclusions in analyses for the review.	Y= All the animals were included in the analysis; or if not, the reasons for missing data are well explain (ex. animals that did not develop tumors). U= The analysis do not include all the animals, but the reasons to do that are not clear. N= The analysis do not include all the animals and no reasons were reported.
9	**Reporting bias**	Selective outcome reporting	State how selective outcome reporting was examined and what was found.	Y= The study protocol is not registered but the published report included all expected outcomes. U= The study protocol is not registered and some results are not presented. N= The study protocol is not registered and the presented results are not in accordance with all the study questions.
10	**Other**	Other sources of bias	State any important concerns about bias not covered by other domains in the tool.	Y= The financial support are clearly stated; no additional animals were added to replace the missing ones; U= The financial support is not clearly stated. The animals used were males. N= No references is made regarding the funders; additional animals were added to replace the missing ones.

Supplemental Table S3: Quality analysis

	SYRCLE's RoB tool												
	Q1	**Q2**	**Q3**	**Q4**	**Q5**	**Q6**	**Q7**	**Q8**	**Q9**	**Q10**			
STUDY	Selection bias			Performance bias		Detection bias		Attrition bias	Reporting bias	Other bias	RESULTS BY STUDY		
Gillette, 1997	Y	Y	Y	Y	NA	Y	N	U	Y	Y	**LRB = 77.78%**	**URB = 11.11%**	**HRB = 11.11%**
Zhu, 2008	Y	Y	Y	Y	NA	Y	Y	U	Y	Y	**LRB = 88.89%**	**URB = 11.11%**	**HRB = 0.00%**
Thompson, 2010	Y	Y	Y	Y	NA	Y	Y	Y	Y	Y	**LRB = 100.0%**	**URB = 0.00%**	**HRB = 0.00%**
Murphy, 2011	Y	Y	Y	Y	NA	Y	U	Y	Y	Y	**LRB = 88.89%**	**URB = 11.11%**	**HRB = 0.00%**
Zhu, 2012	Y	Y	Y	Y	NA	Y	N	Y	Y	Y	**LRB = 88.89%**	**URB = 0.00%**	**HRB = 11.11%**
Goh, 2013	Y	Y	Y	Y	NA	Y	N	Y	Y	Y	**LRB = 88.89%**	**URB = 0.00%**	**HRB = 11.11%**
Faustino-Rocha, 2016(1)	Y	Y	Y	Y	NA	Y	Y	Y	Y	Y	**LRB = 100.0%**	**URB = 0.00%**	**HRB = 0.00%**
Isanejad, 2016	Y	U	U	Y	NA	Y	Y	Y	Y	Y	**LRB = 77.78%**	**URB = 22.22%**	**HRB = 0.00%**
Padrão, 2016	Y	Y	Y	Y	NA	Y	U	Y	Y	Y	**LRB = 100.0%**	**URB = 0.00%**	**HRB = 0.00%**

Legend: Y = Low risk of bias (LRB); N = High risk of bias (HRB); U = Unclear risk of bias (URB). NA = Not applicable.

Results by dimension: **(Q1, Q2 & Q3)** – **HRB** = 0.00%; **URB** = 7.41%; **LRB** = 92.59%; **(Q4) - HRB** = 0.00%; **URB** = 11.11%; **LRB** = 88.89%; **(Q6 & Q7) - HRB** = 16.67%; **URB** = 11.11%; **LRB** = 72.22%; **(Q8) - HRB** = 0.00%; **URB** = 22.22%; **LRB** = 77.78%; **(Q9) - HRB** = 0.00%; **URB** = 0.00%; **LRB** = 100%; **(Q10) - HRB** = 0.00%; **URB** = 0.00%; **LRB** = 100%;

3. Study 3

Figueira, ACC; Figueira, MC; Silva, C. Padrão, AI; Oliveira, PA; Ferreira, R; and Duarte, JA. (2018). Exercise training-induced modulation of microenvironment in rat mammary neoplasms. (Submitted in International Journal of Sports Medicine; IJSM-03-2018-6869-pb)

International Journal of Sports Medicine
Thieme

Exercise training-induced modulation in microenvironment of rat mammary neoplasms

Journal:	*International Journal of Sports Medicine*
Manuscript ID	IJSM-03-2018-6869-pb
Manuscript Type:	Physiology & Biochemistry
Key word:	Breast tumor, Ki67, Cell proliferation, TUNEL assay, Cell death, Colagem content
Abstract:	Despite the importance attributed to exercise training in the breast cancer (BC) continuum, the underlying mechanisms modulating tumor behavior are unknown. We evaluated the effects of long-term moderate-exercise in the development of mammary tumors, and studied the microenvironment of infiltrative lesions, the amount of connective tissue, and balance between cellular proliferation/death. Fifty Sprague-Dawley rats, randomly assigned into four groups: two control groups (sedentary and exercised) and two models of BC groups (sedentary and exercised) induced by N-methyl-N-nitrosoureia (MNU), were sacrificed after 35 weeks of moderate-exercise, and all perceptible tumors were removed for histological and immunohistochemistry analysis. The median number of infiltrative-lesions per animal was lower in the MNU exercised animals (p=0.02). More than one histological pattern was identified, and papillary carcinoma was the most frequent in both groups. Within infiltrative-lesions, the number of immunopositive cells per μm2 of Ki67 was lower in exercised animals (p=0.002). This presents increased cell death per μm2 (p=0.019). Tumors from sedentary animals had a higher expression of collagen deposition (p=0.027). Long-term moderate-exercise has beneficial effects in tumor development with a diminished prevalence of malignancy. Within infiltrative-lesions, moderate-exercise improves the balance between cell-proliferation and cell-death with decreased connective tissue that suggests lower tumor aggressiveness.

Exercise training-induced modulation in microenvironment of rat mammary neoplasms

Abstract

Despite the importance attributed to exercise training in the breast cancer (BC) continuum, the underlying mechanisms modulating tumor behavior are unknown. We evaluated the effects of long-term moderate-exercise in the development of mammary tumors, and studied the microenvironment of infiltrative lesions, the amount of connective tissue, and balance between cellular proliferation/death.

Fifty Sprague-Dawley rats, randomly assigned into four groups: two control groups (sedentary and exercised) and two models of BC groups (sedentary and exercised) induced by N-methyl-N-nitrosoureia (MNU), were sacrificed after 35 weeks of moderate-exercise, and all perceptible tumors were removed for histological and immunohistochemistry analysis.

The median number of infiltrative-lesions per animal was lower in the MNU exercised animals ($p=0.02$). More than one histological pattern was identified, and papillary carcinoma was the most frequent in both groups. Within infiltrative-lesions, the number of immunopositive cells per μm^2 of Ki67 was lower in exercised animals ($p=0.002$). This presents increased cell death per μm^2 ($p=0.019$). Tumors from sedentary animals had a higher expression of collagen deposition ($p=0.027$).

Long-term moderate-exercise has beneficial effects in tumor development with a diminished prevalence of malignancy. Within infiltrative-lesions, moderate-exercise improves the balance between cell-proliferation and cell-death with decreased connective tissue that suggests lower tumor aggressiveness.

Keywords: Breast tumor, exercise training, ki67, cell death, TUNEL assay, cell proliferation, collagen content.

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Introduction

Breast cancer is the second most common cancer in world and the most common in women [39]. The International Agency for Research on Cancer (IARC) has estimated that 1.97 million new cases will be diagnosed worldwide in 2020 (25% of all cancers) [16]. Early detection, improved treatments, and active lifestyles increase breast cancer survival [38]. Several studies have shown that exposing breast cancer patients to regular physical exercise benefits prevention, recurrence, and survival but the biological mechanisms underlying these benefits remain unclear [17, 34]. The role of exercise in the breast cancer continuum is unclear despite convincing clinical epidemiological data confirming its efficacy with contrasting results in animal models [9]. In addition, knowledge about the tumor microenvironment (TME) during exercise is lacking [2].

The proliferative capacity of a tumor is one of the most important variables that determines tumors' progression along with its ability to undergo apoptosis [8, 31]. In fact, what defines the growth rate of a given cell population is the balance between cellular proliferation and death. Thus, improve the competency of the apoptotic machinery in malignancy is an important target in breast cancer. [10]. Furthermore, a relationship is established between malignant cells and the TME during cancer progression. This is composed of the extracellular matrix (ECM) and cellular components. Moreover, it is now well-documented that neoplastic cells are influenced by the surrounding microenvironment and vice-versa [36]. The crosstalk between cancer cells and the other TME-associated cells (e.g. fibroblasts and macrophages) may underlie the tumor's capability to grow and metastize [22].

It is unclear how mammary tumors in animals respond to exercise training. The few research reports in this area show promising results by associating exercise training exposure with reductions in proliferation-associated proteins together with increased

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expression of apoptosis-associated proteins, but this needs confirmation [25-27, 47]. It remains unknown if exercise training modulates neoplastic stroma.

Here, we used a well-characterized model of breast cancer induced in rats by N-methyl-N-nitrosourea (MNU) to determine the long-term exercise effects in the microenvironment of neoplastic tissue. Previously, our research team has shown that exercise training can reduce malignancy [15]. Here, we hypothesized that exercise training could favorably modulate the TME via the following: (1) reducing their proliferative capacity; (2) improving their ability to undergo cell death; and (3) remodeling the TME stroma.

Materials and methods

Animals

Fifty female Sprague-Dawley rats (38 days old; 289±17g body weight) from Harlan Interfauna Inc. (Barcelona, Spain) were randomly housed in collective cages (4 animals *per* cage) and maintained under controlled atmospheric conditions (21-22°C; 60 ± 5% humidity) in a 12/12 hours light/dark cycle with free access to food (standard laboratory diet 4RF21® Mucedola, Italy) and water. All the ethical principles of research involving animals were met [23]. The Portuguese Ethics Committee for Animal Experimentation (Direção Geral de Alimentação e Veterinária) approved the animal protocol with license number 008961, and the experimental protocol was performed in accordance to European Commission Recommendation 2007/5266/CE.

Experimental design

After two weeks of acclimatization, the animals were randomly assigned to one of four groups: sedentary injected with MNU (MNU+SED, n=15); exercised injected with MNU (MNU+EX, n=15); sedentary control, injected with sterile saline–solution

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(CONT+SED, n=10); and exercised control, injected with sterile saline solution (CONT+EX, n=10). Animals from the MNU groups were injected intraperitoneally (i.p.) with 1-methyl-1-nitrosureia (ISOPAC®, Sigma Chemical Co., Madrid, Spain) given in a dose of 50 mgkg; the control groups received i.p. injection of vehicle (sterile saline solution 0.9%). Two days after carcinogen injection, animals from the exercised groups started a treadmill running program (Treadmill Control LE 8710, Harvard Apparatus, USA) for 35 weeks. The duration and intensity of running were gradually increased over the first two weeks until it reached 60 m/day, 5 days/week and 20 m/min of speed (estimated work rate of 70% maximum oxygen consumption) [32]. This was maintained until the end of the experimental protocol. Sedentary animals were manipulated daily to establish identical conditions to active animals.

At the end of experimental protocol, animals were euthanized by i.p. injection of ketamine (75mg/Kg, Imalgen® 1000, Merial SAS, Lyon, France) and xylazine (10 mg/Kg, Rompun® 2%, Bayer Healthcare S. A., Kiel, Germany). In each animal, all perceptible tumors were removed, counted, weighed, and prepared for histological and immunohistochemical analysis.

Histological and immunohistochemical analysis

Briefly, samples were fixed with paraformaldehyde, dehydrated in graded ethanol solutions, cleared in xylene, and embedded in paraffin. Paraffin blocks were then cut into 5 µm sections with a Leica 2125 rotary microtome (Leica Microsystems Inc.). The paraffin embedded slides were deparaffinized in xylene and hydrated using a series of graded ethanol. They were stained with hematoxylin and eosin (H&E) for histological classification and with Picrosirius Red (PSR) to assess collagen content (CC). Tumors were classified according to Russo and Russo [37] and presented different histological patterns (here designated as lesions). After being identified and quantified, all

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infiltrative lesions were further analyzed via the TUNEL assay and Ki67 immunostaining.

TUNEL assay: A TUNEL test (Terminal deoxynucleotidyl transferase-mediated d-UTP Nick End Labeling) was performed (In Situ Cell Death Detection Kit, Ap, 11684809910 from Roche®, Germany) according to manufacturer's instruction on paraffin embedded tissues using the citrate buffer (pH=6.0). Samples were then heat-treated (in a pressure cooker) for 5 minutes for antigen retrieval. Appropriate positive and negative controls were used throughout. After Fast Red application, the slides were rinsed in water, counterstained with hematoxylin for 5 minutes, and mounted with Crystal Mount (aqueous-based). Imaging detected and quantified the cell death per μm^2.

Ki67 immunostaining: Rabbit monoclonal [SP6] antibody to Ki67 (rabbit ab16667 from Abcam®, England) was used to quantify cells into cell cycle per μm^2. Slides were immersed in citrate buffer (pH=6.0) and heat-treated (in a pressure cooker) for antigen retrieval for 15 minutes. This was then cooled down in citrate buffer for 30 minutes. Slides were rinsed in PBS (pH=7.4) twice between applications of each of the following reagents. Endogenous peroxidase activity was blocked applying a solution with methanol, hydrogen peroxide, and PBS-Tween (phosphate-buffered saline) for 30 minutes. Bovine serum albumin (3%) was applied 30 minutes for non-specific blocking. Primary and secondary (goat anti-rabbit HRP, ab 97069 from Abcam ®, England; HRP-conjugated secondary antibody) antibodies were applied for 120 minutes and 60 minutes, respectively, in a humidified 37°C chamber. After DAB (diaminobenzidine) application for 7 minutes, the slides were rinsed in water, counterstained with methyl-green for 3 minutes, dehydrated using a series of graded ethanol, cleared in xylene, and mounted with DPX (Distryene Plasticizer Xylene). Negative controls for primary and secondary antibodies were used throughout.

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After all of these procedures, slides from each malignant lesion were scanned with a Virtual Slide System VS110 (Olympus, Japan), and the digital slides were analyzed using Fiji ImageJ Pro and Image Pro (Media Cybernetics, version 6.0). Several parameters were assessed from each analyzed section including: (1) the number of Ki67-positive nuclei per μm^2 [5]; (2) the number of TUNEL-positive nuclei per μm^2 [11] and; (3) the overall percentage of collagen within the tissue section [24].

Statistical analysis

All quantitative variables were expressed as median and percentiles (25^{th} and 75^{th}) due to their abnormal distribution as previously assessed with the Shapiro-Wilk test. All categorical variables were expressed as an absolute and/or relative frequency. For quantitative variables, data comparison between groups was done with a non-parametric Mann-Whitney test one-tailed; inter-group comparison of categorical variables was performed with the Chi squared test. The odds ratio (OR) with 95% CIs were also calculated (Fisher's exact test) to establish the association between sedentary and exercise behaviors with the odds of developing an infiltrative lesions. A p value < 0.05 was considered statistically significant. Graph Pad Prism software (version 7.0) was used for all analysis.

Results

All MNU animals developed tumors at the end of the experimental protocol; no tumors were detected in the control groups. The number of tumors developed was similar in both groups [14]. Table 1 shows no significant differences between groups in the infiltrative tumor's total weight. Non-infiltrative lesions were slightly higher in the MNU sedentary animals, but this was not significant. However, the positive impact of exercise training can be observed in the prevalence of infiltrative lesions per animal—these were significantly lower in the exercised MNU animals (p=0.020).

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The frequency of distribution for the different types of lesions between groups (Fig. 1) was significantly different (p=0.0005). Different histological patterns of non-infiltrative (Fig. 1A) and infiltrative (Fig. 1B) lesions were identified. The most frequent infiltrative type for both groups, although lower in exercised animals, was the papillary type followed by the cribriform pattern. No invasive comedocarcinoma pattern was detected in MNU-exercised animals. This was the most aggressive lesion identified in MNU sedentary animals. Similar rates of pre-neoplastic lesions (intraductal proliferation) were found in both MNU groups.

Insert Table 1

Insert Figure 1

Additionally, the computed OR revealed that the odds of appearance of an infiltrative lesion in sedentary animals are higher (OR=1.27; CI: 0.67 - 2.37) than the odds of this appearance in exercised animals. The opposite is seen relative to non-infiltrative lesions (OR=0.79; CI: 0.41, 1.51). These are less likely to appear in MNU sedentary animals. This means that the probability of a MNU sedentary animal developing an infiltrative lesion is 27% higher.

Immunohistochemical analysis

The positive influence exerted by long-term (35 wks.) moderate exercise training in malignant lesions was noted in their density of cell proliferation and cell death. Cell proliferation was evaluated by Ki67 staining by counting the positive nuclei in all sections of the scanned slides (Fig. 2A and 2B). An increase in cell proliferation (322 [265.5-442.5]) was observed in sedentary animals comparatively to exercised animals (194 [98.5-284.5]; Figure 2C).

Insert Fig. 2

Cell death was assessed by TUNEL staining, and the positive nuclei for DNA fragmentation were counted using all sections of the scanned slides. Figure 3 shows the collected data for cellular death fraction—a decrease in positive nuclei (19 [15.75-39.5]) can be observed in MNU sedentary (Fig. 3A) animals compared to the MNU exercised animals (Fig. 3B, 38 [27.5-70.5]). Significant differences were observed between MNU groups (Fig 3C).

Insert Fig. 3

Collagen content analysis

The collagen content was measured as the percentage of pixels containing the red signaling in tissue images. Exercised animals (Fig. 4B; 6.16 [3.76-9.48]) had a lower amount of connective tissue in the TME of active animals' tumors versus sedentary animals (Fig. 4A, 7.41 [4.97-10.22]) (Fig. 4C).

Insert Fig. 4

Discussion

This study provides strong evidence for the protective effects of exercise training on tumor development and malignancy accompanied by substantial improvements in the features of the lesions. In contrast to previous reports that associated a reduction in tumor burden with exercise training, we found that breast tumors grew at comparable rates in sedentary and exercising animals [3, 19, 25-27, 33, 35, 40, 43-46, 48, 49]. Other researchers have reported that the increased rates of mammary tumor growth are associated with exercise exposure [6, 7, 14, 33]. These contrasting results can be explained by differences in the protocols.

For example, Zhu et al., Jiang et al., Goh et al., and Welsch et al., found positive results in tumor size associated with voluntary exercise, but the duration of the experiments

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ranged from 5 to 10 weeks [48, 49] [26, 27] [19] [43]. Only one report has associated voluntary exercise with a 40.0% decrease in tumor size, but this used a longer protocol (20 weeks) [40]. In contrast, Cohen et al., and Colbert et al., found the opposite results in animals subjected to voluntary exercise for a longer period (20 and 21 weeks respectively) [6] [7].

If we consider forced exercise designs, then we can verify the same tendency of exercise-induced reduction in tumor size associated with small duration experiments (between 2 and 12 weeks) [3, 33, 44, 46]. Exercise-induced tumor growth appears to be associated with longer experiments (20 and 35 weeks) under forced exercise designs [7, 14]. These differences might be partially explained in voluntary exercise experiments. There is an inevitable reduction in the amount of exercise performed when tumor burden increases. Whereas in forced exercise protocols like ours, the heavier and bigger tumors observed over longer duration experiments might result from the increased vascularization induced by exercise [15, 28, 29].

The benefits of regular exercise were also shown in the histology data. The tumor-bearing animals exposed to exercise training presented fewer non-infiltrative lesions and fewer infiltrative lesions. Exercise training might hamper the progression from noninvasive to invasive lesions, and this might explain at least partially explain the significant differences in the prevalence of malignant lesions. These differences toward a lower aggressiveness in the histological lesions of exercised animals are related to cancer-reduced systemic inflammation as we described previously [14]. Indeed, the levels of CRP and IL-6 were higher in sedentary animals. They also had a heavier spleen weight. This establishes an association between exercise training and decreased expression in systemic markers of inflammation that might be mediated by improvements in the host immune response, which is consistent with other results [18].

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It remains unclear if the same thing happened in the TME of exercised animals once we no longer measured intratumoral markers of inflammation or immune infiltration. Obviously, the expression of immune mediators (e.g. natural killer cells and macrophages) could be activated or suppressed as could the expression of pro-inflammatory cytokines [20]. IL-6 and other factors secreted by the tumor influence the behavior of TME-associated cells such as macrophages and fibroblasts, and these factors can lead to alternative activation pathways for these cells [41]. The conversion of Type 1 macrophages (M1) into Type 2 macrophages (M2), for example, produces IL-10 and TGF-β and alters the differentiation of T cells away from the cytotoxic Th1 response. This can cause the cancer cells to attain stem cell-like features and can increase the fibrosis content [4].

The high degree of proliferation in the infiltrative lesions of sedentary animals suggests an overexpression of pro-inflammatory markers. This could suppress the anti-tumor immune response and can lead to increased malignancy [20]. Exercise could also impact absorption of MNU. Indeed, if the dose, route, and age of administration were the same in both groups, then the differences in the outcomes might depend on variations in the carcinogenic's toxicokinetics and toxicodynamics as mediated by exercise training [1]. Interestingly, other papers do not describe differences in histology unlike our work [2]. Thus, is spite of our results being encouraging they can not be compared with others

The reduced malignancy seen here could also be via modulation of malignant tissue properties. Obviously, uncontrolled cell proliferation and decreased apoptosis are key features of malignancy [21]. Increased proliferation and suppression of apoptosis are hallmarks of malignant tumors, and they play a central role in the development and progression of cancer [13]. Ki67 is a prognostic and predictive marker in breast cancer, and higher values of this protein are normally associated with poor prognosis [12]. Our

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results show that the infiltrative tumors of the animals exposed to moderate exercise training have lower Ki67 expression versus sedentary animals.

Our findings indicate that exercise training positively affected the growth fraction of malignant tissue differently than what has been previously reported by Zhu and colleagues in an identical work [47]. They found that physical activity had no significant influence on the immunoexpression of Ki67 in mammary carcinomas Malicka and co-workers also saw no changes in the expressiveness of Ki67 [33]. On the contrary and similar to us, Isanejad and co-workers described significantly decreased expression of Ki67 protein in tumor-bearing animals subjected to interval training [25]. Other studies have analyzed different markers to evaluate the proliferation of neoplastic cells in breast tumors in animals. In general, the benefits of exercise training are associated with decreased levels of cell cycle regulatory proteins [26, 48]. We think that these conflicting results can be related to the differences in exercise design (i.e., amount of performed exercise and total experiment duration).

The increased cell survival via blocked programmed cell death is the other face of tumor development. It is well established that tumor cells can acquire resistance to apoptosis through different mechanisms including increased anti-apoptotic proteins or decreased pro-apoptotic proteins [42]. Here, lifelong moderate exercise training also impacted cell death and induced a pro-apoptotic environment for infiltrative lesions of the MNU active animals. Similarly to prior work our results show that exposure to moderate exercise training increases the number of apoptotic cells in exercised animals [27, 47]. Previous reports have studied markers of apoptosis in animal models of breast cancer exposed to exercise and found that some anti-apoptotic markers (e.g. Bcl-2, Xiap – X-linked inhibitor of apoptosis pathway) are decreased, and some pro-apoptotic (e.g. Bax, caspase 3, caspase 8, Apaf-1 – apoptosis peptidase-activating factor-1) markers are

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overexpressed in active animals. These findings suggest that exercise training can induce apoptosis via a mitochondrial pathway (intrinsic pathway) or via a death-receptor pathway (extrinsic pathway) [26]. It remains unclear what might trigger this chain of events through different pathways. This is explained, at least in part, by the response of the host's immune system to tumors with different histological types [22].

The tumor stroma is another cancer hallmark and is modulated by exercise as assessed by collagen content. The lower levels of collagen in the exercised animals speaks suggests less fibrotic stimuli (such as TFG-β) and less activity of cancer-associated fibroblasts (CAFs). CAFs are known to secrete IL-6 and matrix metalloproteinase (MMP) that break down the ECM to facilitate cancer cell migration and metastasis [4]. Therefore, exercise could be an advantage because it could modulate the TME away from tumorigenesis. Furthermore, the increased deposition of collagen type I and III by CAFs can alter the ECM microenvironment. This could provide additional oncogenic signs for cancer cell proliferation [30]. The lower expression of collagen seen in our exercised animals agrees with the equally low expression of cancer cell proliferation.

Conclusion

The main findings of our work are that long-lasting moderate exercise training can reduce tumor progression by significantly decreased malignancy, cell proliferation, and cell death. This leads to an improved tissue microenvironment. There is strong evidence for decreased collagen deposition. Although the biological mechanism by which these benefits were achieved are not known, these results are a valuable contribution and confirm that exercise is an important non-pharmacological approach to reduce the growth and metastatic potential of mammary neoplasms.

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Figure legends

Fig. 1 XY line graph depicting the frequency distribution of non-infiltrative lesions (A) and infiltrative lesions (B).

Abbreviations: (A) IDPA – Intraductal papilloma; PACY – Pappilary cystadenoma; TAD – Tubular adenoma; LAD – Lactating adenoma; FI – Fibroma; FIAD – Fibroadenoma; PRENP – Preneoplastic. (B) PAPC – Papillary carcinoma; CRIC – Cribriform carcinoma; COC – Comedo carcinoma.

Fig. 2 Representative light micrographs from malignant lesions of sedentary (A) and exercised animals (B) stained for ki67. Comparatively to A, it is notorious a lower density of brown marked nuclei in A. In C it is depicted a boxplot diagram of values observed in both groups.

Fig. 3 Representative light micrographs from malignant lesions of sedentary (A) and exercised animals (B) stained for Tunel. Comparatively to A, it is notorious a higher density of red marked nuclei in A. In C it is depicted a boxplot diagram of values observed in both groups.

Fig. 4 Representative light micrographs from malignant lesions of sedentary (A) and exercised animals (B) stained with Pricosirius red. Comparatively to A, it is notorious a lowers density of red stained area in A, which is indicative of a reduced collagen content. In C it is depicted a boxplot diagram of values observed in both groups.

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Table 1: Tumor burden and different types of lesions in both MNU groups (expressed as median quartiles [25th and 75th percentiles]).

	MNU groups	
	Sedentary	Exercised
Tumor weight (g)	4.20 [0.75 – 6.27]	6.45 [0.91 – 12.65]
Non-infiltrative lesions per animal	3 [1-5]	2 [2-4]
Infiltrative lesions per animal	4 [2-5]*	2 [1-3]
Total number of lesions per animal	5.5 [3.25-8.5]	4 [3-5]

* p = 0.02 vs. Exercised

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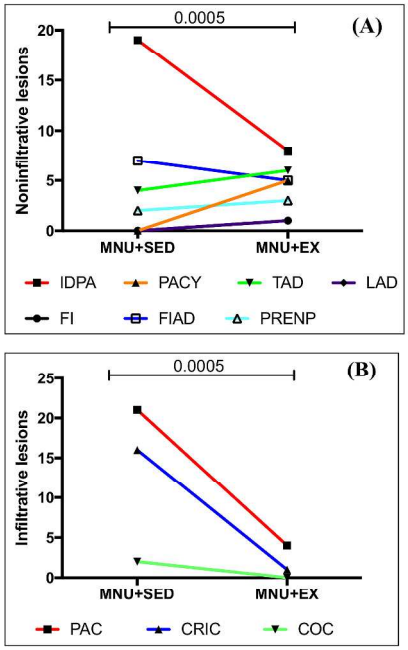
Fig. 1



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922x691mm (200 x 200 DPI)

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Fig. 2

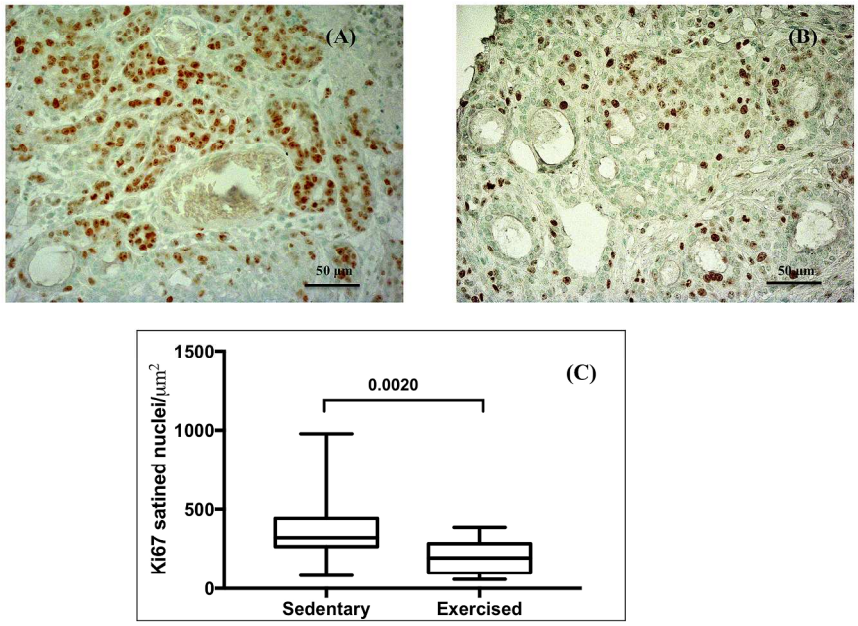
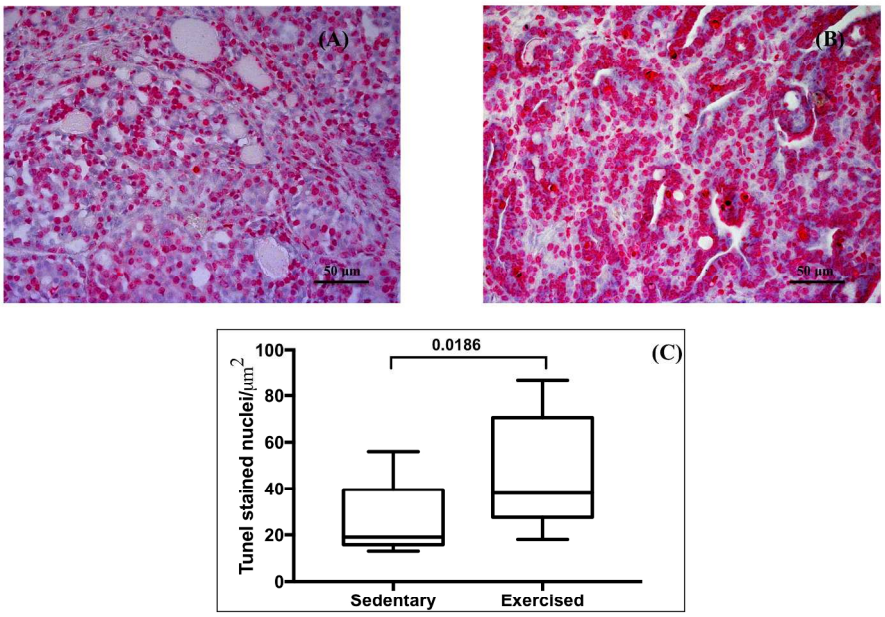


Fig. 2 Representative light micrographs from malignant lesions of sedentary (A) and exercised animals (B) stained for ki67. Comparatively to A, it is notorious a lower density of brown marked nuclei in A. In C it is depicted a boxplot diagram of values observed in both groups.

922x691mm (72 x 72 DPI)

Fig. 3



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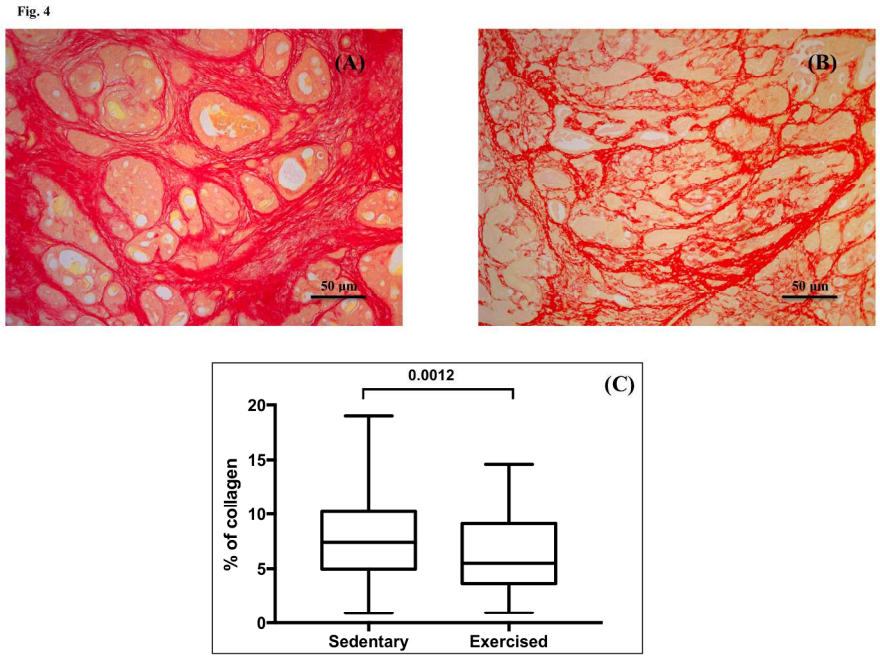
922x691mm (72 x 72 DPI)



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4. Study 4

Padrão, AI*; Figueira, ACC*; Faustino-Rocha, AI; Gama, A; Loureiro, MM; Neuparth, MJ; Moreira-Gonçalves, D; Vitorino, V; Amado, F; Santos, LL; Oliveira, PA; Duarte, JA and Ferreira, R. (2017). Long-term exercise training prevents mammary tumorigenesis-induced muscle wasting in rats through the regulation of TWEAK signaling. Acta Physiol (Oxf), 219, 803-813. (First published May 26, 2016). doi: 10.1111/apha.12721. Reprinted here with the kind permission of John Wiley & Sons Ltd. * Equally contributors.

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Long-term exercise training prevents mammary tumorigenesis-induced muscle wasting in rats through the regulation of TWEAK signalling

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Abstract

Aim: Exercise training has been suggested as a non-pharmacological approach to prevent skeletal muscle wasting and improve muscle function in cancer cachexia. However, little is known about the molecular mechanisms underlying such beneficial effect. In this study, we aimed to, firstly, examine the contribution of TWEAK signalling to cancer-induced skeletal muscle wasting and, secondly, evaluate whether long-term exercise alters TWEAK signalling and prevents muscle wasting.

Methods: Female Sprague-Dawley rats were randomly assigned to control and exercise groups. Fifteen animals from each group were exposed to *N*-Methyl-*N*-nitrosourea carcinogen. Animals in exercise groups were submitted to moderate treadmill exercise for 35 weeks. After the experimental period, animals were killed and *gastrocnemius* muscles were harvested for morphological and biochemical analysis.

Results: We verified that exercise training prevented tumour-induced TWEAK/NF-κB signalling in skeletal muscle with a beneficial impact in fibre cross-sectional area and metabolism. Indeed, 35 weeks of exercise training promoted the upregulation of PGC-1α and oxidative phosphorylation complexes. This exercise-induced muscle remodelling in tumour-bearing animals was associated with less malignant mammary lesions.

Conclusion: Data support the benefits of an active lifestyle for the prevention of muscle wasting secondary to breast cancer, highlighting TWEAK/NF- κB signalling as a potential therapeutic target for the preservation of muscle mass.

Keywords gastrocnemius, inflammation, mammary carcinogenesis, NF-κB signalling, oxidative metabolism.

Cachexia is a multi-factorial syndrome associated with several chronic diseases including cancer and involves changes in several metabolic pathways, in many tissues and organs. In the context of cancer, it was estimated that cachexia indirectly accounts for the death of at least 20% of all patients with cancer

(Fearon *et al.* 2011, Argiles *et al.* 2014). Skeletal muscle wasting is a major factor involved in cancer cachexia that contributes to physical disability, weakness, reduced tolerance to anticancer therapy and reduced survival. Despite its impact on the patient outcome, body weight loss is rarely assessed, recognized or even targeted in the cancer setting (Fearon *et al.* 2011, Julienne *et al.* 2012, Argiles *et al.* 2014). This could be attributed, in part, to the poor understanding of the underlying mechanisms in addition to the lack of approved therapeutic approaches.

Currently, there is strong evidence pointing to the unbalance between protein synthesis and degradation as the main mechanisms responsible for skeletal muscle atrophy in cancer cachexia (Padrao *et al.* 2013). Reduced ribosome formation and protein synthesis, and overexpression of atrogin-1 and muscle RING finger 1 (MuRF1), muscle-specific E3 ligases involved in protein ubiquitination, have all been shown to mediate muscle wasting in cancer cachexia (Julienne *et al.* 2012, Padrao *et al.* 2013). Pro-inflammatory cytokines secreted by either the host or tumour were shown to modulate the activation of the signalling pathways that upregulate muscle protein degradation (White *et al.* 2012, Johns *et al.* 2014, Onesti & Guttridge 2014). TWEAK is a member of the TNF superfamily of cytokines that was shown to mediate the overexpression of E3 ligases through the activation of NF-κB pathway in muscle fibres (Dogra *et al.* 2007, Mittal *et al.* 2010a, Bonaldo & Sandri 2013, Onesti & Guttridge 2014). TWEAK also seems to inhibit the activity of PI3K/Akt signalling pathway, boosting the catabolic action of this cytokine (Kumar *et al.* 2012), which makes this pro-inflammatory cytokine a putative therapeutic target for the management of cancer cachexia.

Different therapeutic approaches have been tested for the management of cancer cachexia-induced muscle mass loss; however, none was yet approved (Fearon *et al.* 2013). Beyond pharmacological strategies, exercise training has been suggested as the gold standard for increasing muscle function in cancer cachexia (Argiles *et al.* 2012, Morley *et al.* 2014). Endurance training was reported to reduce cancer-related inflammation, reactive oxygen species and protein catabolism and to increase protein synthesis (Gould *et al.* 2013). So, regular exercise training appears as an attractive therapeutic strategy to be included in the management of cancer to attenuate the adverse effects of cachexia. However, the molecular mechanisms modulated by exercise in cancer cachexia are poorly characterized, and even less are the ones underlying the preventive effect of lifelong exercise training.

To give new molecular insights on the impact of endurance training in the prevention of cancer-induced muscle wasting, we used an animal model of mammary tumorigenesis submitted to 35 weeks of moderate treadmill exercise since infancy. In the *gastrocnemius* muscle, we searched for the pathways modulated by the pro-inflammatory cytokine TWEAK and related it with tumour–host interplay.

Material and methods

Chemicals

N-Methyl-*N*-nitrosourea (MNU) was purchased from Sigma chemical Co (Madrid, Spain). All other reagents and chemicals used were of highest grade of purity commercially available. Rabbit polyclonal anti-GDF8 (myostatin; ab996), rabbit polyclonal anti-MURF1 (ab77577), rabbit polyclonal anti-TWEAK (ab37170), rabbit monoclonal anti-TWEAKr (ab109365), rabbit polyclonal anti-GAPDH (ab9485), rabbit polyclonal anti-PGC-1α (ab54481), rabbit monoclonal anti-NF-κB p105/p50 [E381] (ab32360), rabbit polyclonal anti-NF-κB p65 (ab16502), mouse monoclonal anti-NF-κB p100/52 (ab71108), rabbit polyclonal anti-RelB (ab150305) and mouse monoclonal anti-ATPB (ab14730) were purchased from Abcam (Cambridge, UK). MitoProfile® Total OXPHOS Western blotting kit (ab110413) was also purchased from Abcam. Rabbit polyclonal atrogin-1 antibody (AP2041) was obtained from ECM Biosciences (Versailles, KY, USA). Rabbit polyclonal antibodies for *p*-mTOR (#2971), *p*-Akt (#4058), mTOR (#2972) and Akt (#9272) and rabbit monoclonal antibody against *p*-4E-BP1 (#2855), *p*-p70S6K (#9234) and *p*-IκBα (Ser32) (#2859) were acquired from Cell Signalling Technology (Leiden, the Netherlands). Secondary peroxidase-conjugated antibodies (anti-mouse IgG and anti-rabbit IgG) were obtained from GE Healthcare (Buckinghamshire, UK), and anti-goat IgG (ab7125) was obtained from Abcam.

Animals

Fifty female Sprague-Dawley rats (aged 38 days) were obtained from Harlan (Barcelona, Spain) and randomly housed in groups of 4 rats/cage, in a controlled environment at 22 ± 2°C of temperature and 60 ± 5% of relative humidity with 12/12 h dark–light inverted cycle, with free access to food (standard diet 4RF21®; Mucedola, Settimo Milanese, Italy) and water. After 1 week of quarantine, the animals were randomly divided into four experimental groups: sedentary control (CONT + SED, $n = 10$), sedentary MNU (MNU + SED, $n = 15$), exercised control (CONT + EX; $n = 10$), and exercised MNU (MNU + EX, $n = 15$). Animals were observed daily

for health check. During the experimental protocol, four animals from MNU + SED group and five from MNU + EX group died and were not included for data analysis. The following protocol was approved by the Portuguese Ethics Committee for Animal Experimentation, *Direção Geral de Alimentação e Veterinária* (licence number 008961) and was performed in accordance with European Directive 2010/63/EU.

Induction of mammary tumorigenesis and implementation of exercise training

Mammary tumorigenesis was chemically induced in rats from MNU groups (MNU + SED and MNU + EX) by the intraperitoneal administration of one single dose of the carcinogen MNU (50 mg kg^{-1}) at 50 days of age. Rats from CONT groups (CONT + SED and CONT + EX) were i.p. injected with a single dose of vehicle.

Animals from EX groups started a treadmill training protocol (Treadmill Control LE 8710; Harvard Apparatus, Holliston, MA, USA) at 52 days of age. In the first two weeks, exercise duration and treadmill speed was gradually increased until reaching 60 min per day at 20 m per min, 5 days per week, which was maintained during thirty-five weeks. At the end of the experimental protocol, animals were killed with ketamine/xylazine (Imalgen® and Rompun® respectively). Blood samples were collected from the inferior vena cava and centrifuged for 5 min at 5000 g, and serum was obtained and stored at −80 °C for biochemical determinations. All noticeable tumours were counted and removed for histological analysis. *Gastrocnemius* was removed, weighted and immediately prepared for histological and biochemical analysis.

Histological analysis of gastrocnemius muscle and histological and immunohistochemical analysis of mammary tumours

Cubic pieces from *gastrocnemius* muscle and mammary tumours were fixed with buffered paraformaldehyde 4% (v/v) by diffusion during 24 h and subsequently dehydrated with graded ethanol and included in paraffin blocks. Serial cross sections (5 μm of thickness) of paraffin blocks were cut by a microtome and mounted on silane-coated slides. The slides were dewaxed in xylene and hydrated through graded alcohols finishing in phosphate-buffered saline solution (pH to 7.2). *Gastrocnemius* samples were stained with haematoxylin–eosin, dehydrated with graded alcohols through xylene and mounted with DPX for analysis in a photomicroscope (Zeis Phomi 3). Photographs of *gastrocnemius* cross sections of all experimental groups were digitalized and analysed with the NIH ImageJ (Image Processing and Analysis in JAVA, USA) software. An average of 1429 ± 217 fibres were analysed *per* group for cross-sectional area (CSA) quantification.

Deparaffinized sections of mammary tumours were stained for haematoxylin–eosin and classified according to Russo and Russo (Russo & Russo 2000). The immunohistochemical detection of TWEAK was performed using the standard protocol of Novolink Polymer Detection System (Leica Biosystems, Newcastle, UK). Sections were incubated with primary antibody for TWEAK (ab37170; Abcam) at a dilution of 1:50, overnight at 4 °C.

Blood tests

Serum albumin and lactate dehydrogenase (LDH) were measured in duplicate on an AutoAnalyzer (PRESTIGE 24i, Cormay PZ). Serum TWEAK levels were assayed by immunoblotting as described below.

Gastrocnemius homogenate preparations

Whole *gastrocnemius* muscle was homogenized in ice-cold solubilization buffer [50 mM Tris-HCl, 1 mM EDTA, 1 mM EGTA, 0.1% Triton X-100, 10 mM PMSF, phosphatase inhibitor cocktail (P0044 and P5726; Sigma), pH 7.5]. *Gastrocnemius* homogenate was aliquoted for subsequent biochemical analysis. Total protein content was determinate with RC-DC Protein Assay Kit (Bio-Rad, Hercules, CA, USA).

Citrate synthase activity

Citrate synthase (CS) activity was measured in muscle homogenate using the method proposed by Coore *et al.* (1971). In brief, the CoASH released from the reaction of acetyl-CoA with oxaloacetate was measured by its reaction with 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) at 412 nm (ε = 13.6 mM^{-1} cm^{-1}). A Multiskan GO Microplate Spectrophotometer (Thermo Scientific, Northumberland, UK) was used for CS activity measurement.

Immunoblotting analysis

Serum samples were diluted (1:20) in Tris-buffered saline (TBS; 100 mM Tris, 1.5 mM NaCl, pH 8.0), and 100 μL was slot-blotted into a nitrocellulose membrane (Whatman; Protan, Sigma-Aldrich, St. Louis, MO, USA). In parallel, equivalent amounts of whole *gastrocnemius* muscle proteins of each group were electrophoresed on a 12.5% SDS-PAGE as described by Laemmli (Laemmli 1970). Gels were

blotted onto a nitrocellulose membrane (Whatman®; Protan, Sigma-Aldrich) in transfer buffer (25 mM Tris, 192 mM glycine, pH 8.3 and 20% methanol) during 2 h (200 mA). Then, non-specific binding was blocked with 5% (w/v) dry non-fat milk in TBS-T (100 mM Tris, 1.5 mM NaCl, pH 8.0 and 0.5% Tween 20). Membranes were incubated with primary antibody diluted 1:1000 in 5% defatted bovine serum albumin in TBS-T (anti-*p*-p70S6K, anti-*p*-IκBα, anti-*p*-Akt, anti-*p*-mTOR) or in 5% (w/v) non-fat free milk in TBS-T (anti-TWEAK, anti-TWEAKr, anti-NF-κB p65, anti-NF-κB p105/p50, anti-NF-κB p100/p52, anti-RelB, anti-mTOR, anti-Akt, anti-atrogin-1, anti-MuRF-1, anti-4E-BP1, anti-ATPB, anti-PGC-1α and anti-GAPDH) for 2 h at room temperature, washed and incubated with secondary horseradish peroxidase-conjugated anti-rabbit or anti-mouse (1:10000; GE Healthcare). Mitochondrial subunits of oxidative phosphorylation proteins were semi-quantified with the MitoProfile® Total OXPHOS Western blotting kit (1:5000, containing NDUFB8-20 kDa of complex I, SDHB-30 kDa of complex II, core protein 2–48 kDa of complex III, subunit I- 40 kDa of complex IV and alpha subunit-55 kDa of ATP synthase). Immunoreactive bands were detected by enhanced chemiluminescence ECL (GE Healthcare) according to the manufacturer's procedure, and images were recorded using X-ray films (Kodak Biomax Light Film; Sigma, St. Louis, MO, USA). The films were scanned in Molecular Imager Gel Doc XR+ System (Bio-Rad) and analysed with QuantityOne software (v 4.6.3 Bio-Rad). Protein loading control was performed with Ponceau S staining (Romero-Calvo *et al.* 2010). Representative images of the results obtained are presented at Fig. S1.

Statistical analysis

The results are presented as mean ± SD for each experimental group. Kolmogorov–Smirnov test was performed to check the normality of the data. Kruskal–Wallis test followed by Dunns test was used for non-normal data (muscle fibres CSA). The statistical significance of the differences between the experimental groups for the remaining variables was determined using a two-way ANOVA followed by the Tukey multiple comparisons post hoc test. The significance level was set at $P \leq 0.05$. Statistical analysis was performed with GraphPad Prism Software (version 6.0; GraphPad Software, La Jolla, CA, USA).

Results

Characterization of rat's response to MNU administration and/or endurance training

The administration of MNU induced mammary lesions in 100% of the animals, which was accompanied by 8.4% of lower body weight (Table 1) without alterations in food consumption (data not shown). No histopathological changes in mammary tissue were observed in control animals (CONT + SED and CONT + EX groups). In MNU groups, animals' response to tumour burden was not homogeneous. Approximately 30% of animals with mammary tumours presented slight variations that range from

Table 1 Characterization of the animals' response to MNU-induced muscle wasting and/or endurance training regarding body weight, *gastrocnemius* mass, *gastrocnemius* to body weight, citrate synthase activity in *gastrocnemius* muscle, and serum albumin, lactate dehydrogenase (LDH), TWEAK and myostatin

	Experimental group			
	CONT + SED	MNU + SED	CONT + EX	MNU + EX
Body weight (g)	298.29 ± 13.59	273.20 ± 16.62*	313.74 ± 24.51	267.47 ± 27.77†
Gastrocnemius muscle weight (g)	3.94 ± 0.28	3.50 ± 0.31*	4.15 ± 0.245	3.36 ± 0.62†
Gastrocnemius to body weight (mg g^{-1})	13.38 ± 0.73	13.08 ± 0.65	13.39 ± 0.49	12.43 ± 1.89
Citrate synthase activity (nmol min^{-1} mg^{-1} muscle)	18.07 ± 1.53	17.2 ± 0.95	21.42 ± 4.06	29.25 ± 7.67†‡
Serum Albumin (g L^{-1})	40.73 ± 1.32	37.59 ± 2.05*	39.56 ± 2.77	37.46 ± 5.46
Serum LDH (U L^{-1})	328.11 ± 91.84	544.81 ± 307.48	483.05 ± 143.97	472.58 ± 251.34
Serum TWEAK (OD, arbitrary units)	130.87 ± 7.24	290.61 ± 34.39*	188.02 ± 49.80*	167.31 ± 38.93‡
Serum myostatin (OD, arbitrary units)	263.80 ± 9.18	340.00 ± 30.97*	266.27 ± 14.24	329.20 ± 38.16†

OD, Optical density.
Values are expressed as mean ± SD.
*$P < 0.05$ vs. CONT + SED.
†$P < 0.05$ vs. CONT + EX.
‡$P < 0.05$ vs. MNU + SED.

no differences of weight to <5%, and 25% of the animals evidenced a lower body weight in more than 10%. Such variation among individuals with the same type of tumour and burden was previously reported (Fearon *et al.* 2012). The MNU-related lower body weight was accompanied by a 11% diminishing of muscle mass (Table 1). Regarding *gastrocnemius* muscle, eight animals from MNU + SED group showed lower muscle mass, >7% (three of them more than 17%). No significant differences of muscle-to-body weight ratio were observed between groups (MNU + SED vs. CONT + SED, Table 1). Endurance training modulated tumour development with lower incidence of mammary lesions observed in the MNU + EX than in MNU + SED group (50 vs. 71 lesions respectively), and less malignant lesions. Long-term exercise training also prevented the lower weight body gain in 10% of animals, although without apparent effect on the average of body weight or *gastrocnemius* muscle mass (MNU + SED vs. MNU + EX groups, Table 1).

MNU administration prompted an inflammatory response in animals, which was characterized by a significant decrease of serum albumin and a significant increase of the pro-inflammatory cytokine TWEAK and myostatin. Increased serum levels of lactate dehydrogenase (LDH) were also noticed in these animals, similarly to the reported for patients with breast cancer (Liu *et al.* 2015). The impact of endurance training was mainly noticed in the serum levels of TWEAK, which were significantly lower in MNU + EX compared to MNU + SED (Table 1). To better clarify the impact of exercise training on tumour–host interplay that underlies the regulation of TWEAK levels, immunohistochemistry analysis of TWEAK was performed in tumour sections. Weak-to-moderate expression of TWEAK was noticed in the cytoplasm of epithelial cells in adjacent mammary glands, especially in those presenting secretory changes. Sporadically, TWEAK was also expressed by stromal cells, namely fibroblasts, especially in peri-neoplastic reactive stroma and by plasma cells. Exercise training had no significant impact on the levels and pattern of TWEAK immunostaining in mammary tumours (Fig. S2).

Analysis of gastrocnemius muscle adaptation to mammary tumours and/or endurance training

Morphometric analysis of *gastrocnemius* highlighted significantly decreased values of fibre cross-sectional area (CSA) in sedentary MNU-treated rats (895.24 ± 341.92 μm^2 vs. 1461.45 ± 904.19 μm^2 in CONT + SED group), which was accompanied by increased interstitial space (Fig. 1). Exercise training prevented the fibre CSA decrease induced by mammary tumorigenesis (1273.07 ± 648.31 μm^2 vs. 895.24 ± 341.92 μm^2 in MNU + SED).

The metabolic adaptation of the *gastrocnemius* muscle to mammary tumorigenesis was assessed by the analysis of metabolic enzymes. MNU + SED animals showed a significant decrease of citrate synthase (CS) activity in the *gastrocnemius* muscle (Table 1). No significant differences of the ratio ATP synthase/GAPDH expression were noticed in MNU-treated rats (Fig. 2c). Treadmill exercise training prevented the MNU-induced decrease of CS activity (Table 1) and prompted a shift towards oxidative metabolism given

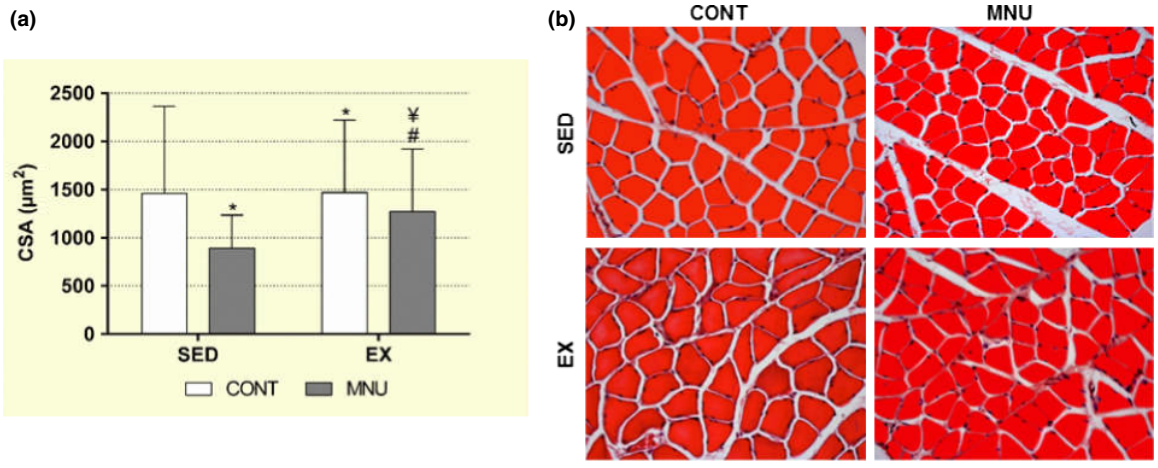

Figure 1 Effect of MNU-induced mammary tumorigenesis and/or endurance training on cross-sectional area (CSA) in *gastrocnemius* muscle. Values are presented as mean ± SD of results obtained from the analysis of 1429 ± 217 fibres *per* group (a). Histological appearance of *gastrocnemius* stained with haematoxylin and eosin evidences fibres' CSA in each experimental group (b). (*$P < 0.05$ vs. CONT + SED; $^{¥}P < 0.05$ vs. CONT + EX; $^{\#}P < 0.05$ vs. MNU + SED).

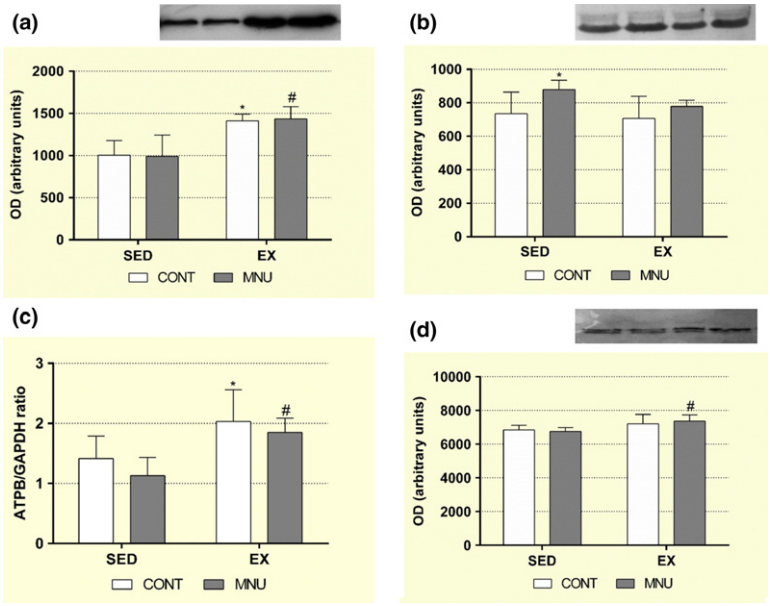

Figure 2 Effect of MNU-induced mammary tumorigenesis and/or endurance training on the muscle expression of ATPB (a), GAPDH (b), ATPB/ GAPDH ratio (c) and PGC-1α (d). Representative immunoblots are presented above the corresponding graphs. Values are expressed as mean ± SD. (*$P < 0.05$ vs. CONT + SED; #$P < 0.05$ vs. MNU + SED).

by the increase of ATP synthase/GAPDH ratio, mostly due to the overexpression of ATP synthase subunit β (Fig. 2a and c). This metabolic effect of long-term exercise training was further supported by the augmented expression of other subunits from the oxidative phosphorylation complexes I and III in the *gastrocnemius* muscle of exercised animals (CONT + EX and MNU + EX groups, Fig. 3). For instance, the content of NDUF88 subunit from OXPHOS complex I increased approximately 40% in MNU + EX compared to MNU + SED and 26% in CONT + EX compared to CONT + SED (Fig. 3b). The content of the subunit UQCRC2 from OXPHOS complex III increased approximately 45% in MNU + EX compared to MNU + SED (Fig. 3d). No expression changes were noticed among experimental groups for the subunits SDH8 and MTCD1 from OXPHOS complexes II and IV respectively. Altogether, data highlight an increase of skeletal muscle oxidative capacity stimulated by long-term endurance training, with concomitant increase of PGC-1α (Fig. 2d), a key player in the regulation of mitochondria biogenesis (Vitorino *et al.* 2015).

Analysis of the anabolic/catabolic balance in gastrocnemius muscle in response to mammary tumours and/or endurance training

To assess the molecular mechanisms modulated by mammary tumorigenesis in skeletal muscle and the regulatory role of long-term exercise training, we evaluated the expression of the main mediators involved in the signalling pathway activated by the pro-inflammatory cytokine TWEAK. The expression of TWEAK in *gastrocnemius* muscle was related to the serum levels of this pro-inflammatory cytokine. Indeed, a significant increase of this pro-inflammatory cytokine and of its receptor Fn14 was noticed in the *gastrocnemius* of MNU + SED animals (Fig. 4a and b). These higher levels of TWEAK were related to the activation of NF-κB pathway, through the phosphorylation of IκBα (Fig. 4c) and overexpression of p50, RelB and p100/p52 subunits (Fig. 4d, f and g). No alterations in the expression of p65 subunit were noticed (Fig. 4e). Endurance training prevented the MNU-related increase of TWEAK but not of Fn14 receptor. The levels of phosphorylated IκBα and NF-κB subunit p50 also decreased in exercised tumour-bearing rats. Curiously, in healthy animals, exercise training promoted a significant increase of RelB and p52 subunits (Fig. 4f and g).

The expression of the E3 ligases MAFbx/atrogin-1 and MuRF1 was also evaluated by Western blotting. Data evidenced a statistically significant increase of atrogin-1 in the *gastrocnemius* muscle of MNU-treated animals (Fig. 5a). Regarding to MuRF-1, its expression was not altered in any of the groups excepting for MNU + EX where it was upregulated (Fig. 5b).

To assess the impact of tumour–host interplay in the anabolic activity of *gastrocnemius* muscle, we assessed the expression of the kinases Akt and mTOR, and the downstream *p*-4E-BP1 and *p*-p70S6K by Western blotting. Mammary tumours induced the decrease of total Akt but not of its phosphorylated form (Fig. 5c and d). An opposite trend was noticed for the phosphorylated form of mTOR, which increased in MNU-treated animals (Fig. 5f), although no

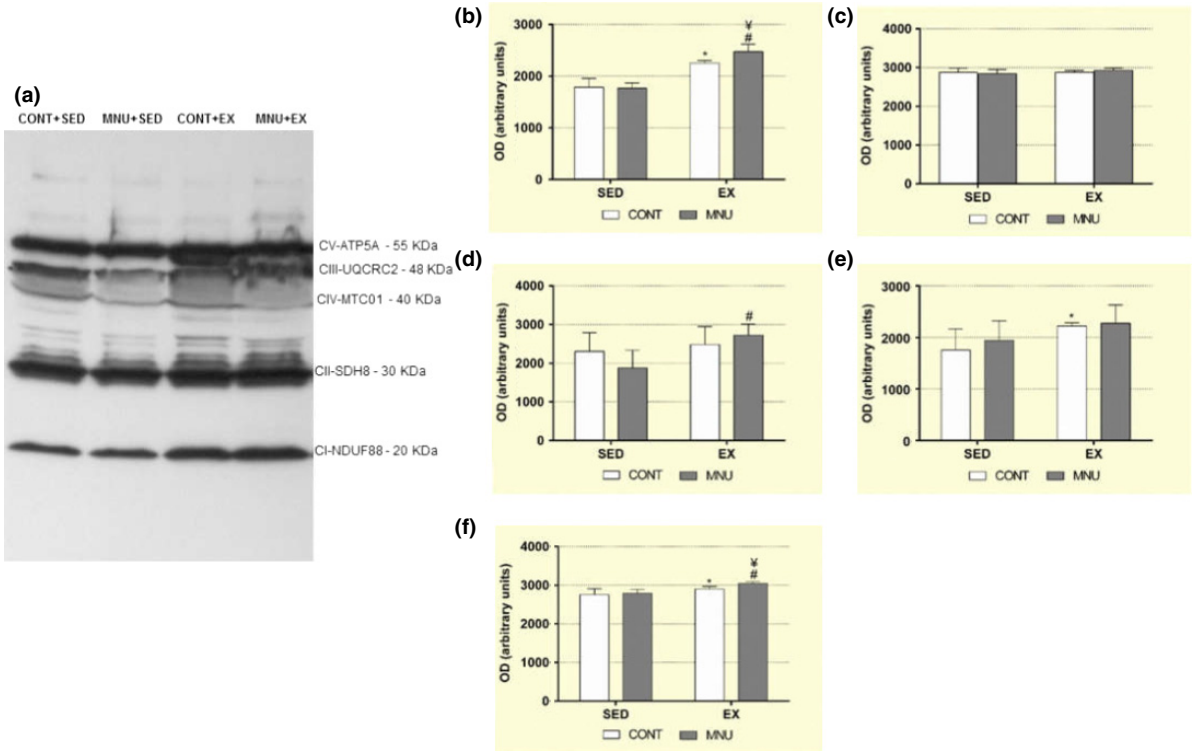

Figure 3 Effect of MNU-induced mammary tumorigenesis and/or endurance training on the levels of OXPHOS subunits in *gastrocnemius* muscle from animals of all experimental groups. A representative immunoblot is presented (a). Semi-quantitative analysis of Complex I, CI-NDUFB8 (b); complex II, CII-SDHB (c); complex III, CIII-UQCRC2 (d); complex IV, CIV-MTCO1 (e) and complex V, CV-ATP5A (f). Values are expressed as mean ± SD. (*$P < 0.05$ vs. CONT + SED; $^{¥}P < 0.05$ vs. CONT + EX; $^{\#}P < 0.05$ vs. MNU + SED).

differences were observed for total mTOR (Fig. 5g). Nevertheless, the levels of phosphorylated 4E-BP1 decreased and no differences were observed for phosphorylated p70S6K content. Exercise training induced the increase of phosphorylated Akt and so of phospho-Akt/total Akt ratio as well as of phosphorylated and total mTOR in MNU-treated animals (Fig. 5c–h). However, these differences were not translated in the levels of phosphorylated p70S6K and 4E-BP1 (Fig. 5i and j).

Discussion

The present study highlights the protective role of long-term moderate exercise training against cancer-related muscle wasting through the modulation of tumour–host interplay in the MNU animal model. This model of mammary tumorigenesis mimics human breast cancer in terms of the tumour's histopathology, origination from mammary ductal epithelial cells, and altered expression of TGFβ, oestrogen receptor and cyclin D1 (Perse *et al.* 2009, Soares-Maia *et al.* 2013). MNU-induced mammary cancer is also characterized by chronic inflammation (Soares-Maia *et al.* 2013). The long time-course of tumour response with the first tumours appearing after 10 weeks of carcinogen administration makes this animal model appropriate to study the impact of long-term exercise training on cancer-induced muscle wasting.

MNU administration to female rats at the age of 50 days resulted in histological signs of mammary carcinoma similarly to the observed in human breast cancer, the most incident and prevalent type of cancer in women worldwide (Jemal *et al.* 2011). MNU-induced mammary tumorigenesis was paralleled by 8% lower body weight (Table 1), a value that in humans is seen as a sign of mild-to-moderate cachexia according to the cachexia score (CASCO) (Argiles *et al.* 2011). This lower body weight was paralleled by a 11% diminishing of *gastrocnemius* mass (Table 1) and 39% less fibre CSA (Fig. 1), which are suggestive of altered muscle properties such as weakness and fatigue (Argiles *et al.* 2011, Shum *et al.* 2012). The low serum levels of albumin and high content of the pro-inflammatory and catabolic cytokines TWEAK and myostatin in MNU animals (Table 1) support the catabolic profile noticed in this group. Indeed, systemic inflammation is thought to be a major mediator of cancer cachexia (Fearon *et al.* 2012).

Exercise training has been prescribed as a tool to counteract systemic inflammation and apparently

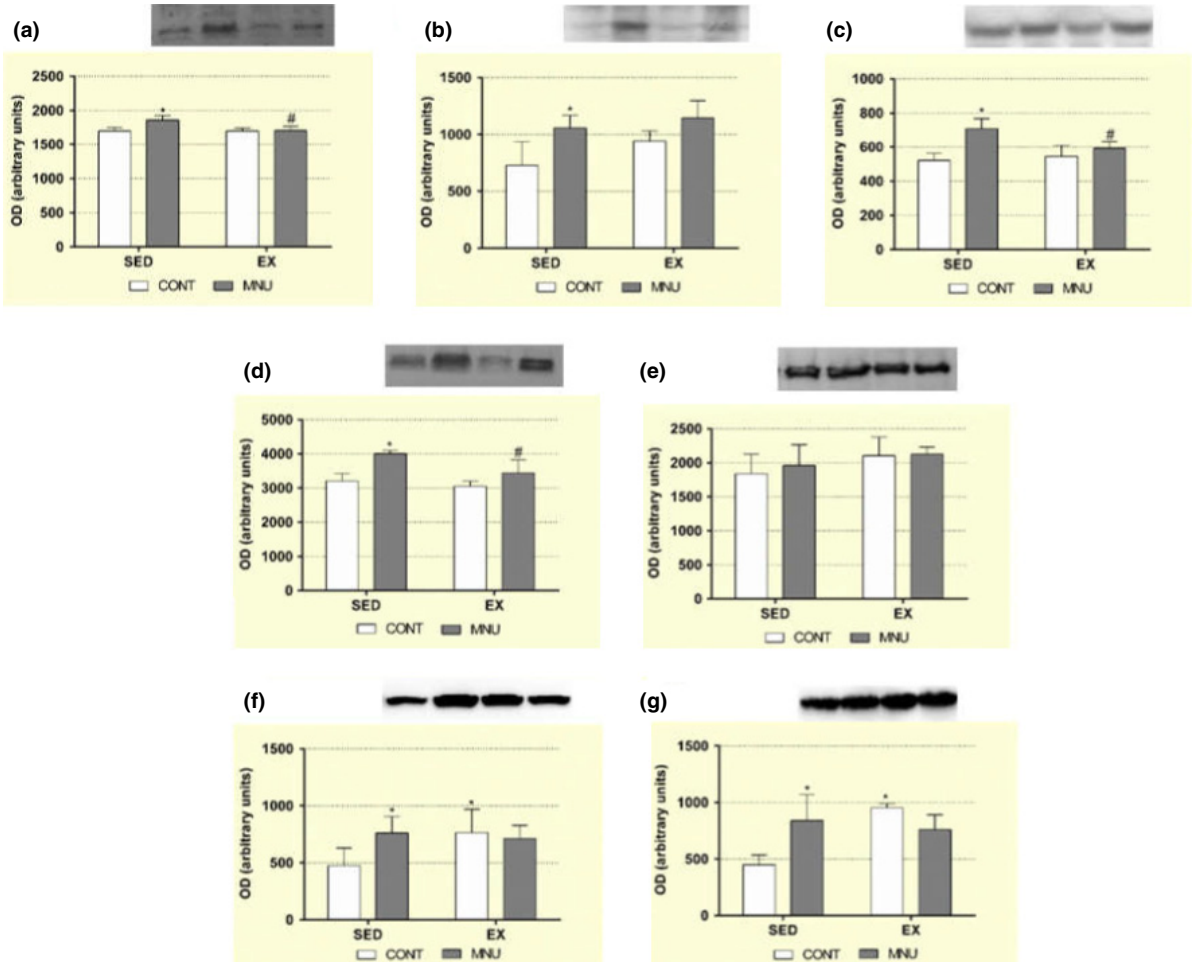

Figure 4 Effect of MNU-induced mammary tumorigenesis and/or endurance training on the muscle expression of TWEAK (a), Fn14 (b), *p*-IκBα (c), NF-kB p50 (d) and p65 (e), RelB (f) and NF-kB p100/p52 (g) subunits evaluated by Western blotting in whole *gastrocnemius* muscle from animals of all experimental groups. Representative immunoblots are presented above the corresponding graphs. Values are expressed as mean ± SD. (*$P < 0.05$ vs. CONT + SED; $^{\#}P < 0.05$ vs. MNU + SED).

represents a safe and effective strategy for the prevention/attenuation of cancer cachexia (Lira *et al.* 2014). In the present study, we verified that 35 weeks of treadmill exercise modulated the levels of the pro-inflammatory cytokine TWEAK, as recently reported (Padrao *et al.* 2015). TWEAK is a typical TNF superfamily member expressed by immune cells, with leucocytes as the major source. This cytokine was also observed in tumour specimens, some apparently produced by tumour cells themselves, with soluble TWEAK detected in the conditioned media of some tumour cell lines (Burkly 2014). TWEAK acts on cells via binding to the TNF receptor superfamily member fibroblast growth factor-inducible 14 (Fn14) (Willis *et al.* 2008), and TWEAK/Fn14 has protumorigenic activity associated with proliferation, invasion, angiogenesis and inflammation (Michaelson *et al.* 2005). TWEAK/Fn14 signalling regulates breast cancer cell invasive capacity in multiple biological contexts (Willis *et al.* 2008) and was previously correlated with higher tumour grade (Burkly 2014) and cachexia (Johnston *et al.* 2015). Our data highlight the preventive effect of chronic exercise training in mammary tumorigenesis-related increase of soluble TWEAK, with impact in tumour grade and body wasting. Indeed, less mammary lesions and less signs of malignancy (such as invasive carcinomas) were observed in exercised MNU-treated animals (Faustino-Rocha *et al.* 2016). Once no significant alterations in the tumour expression of TWEAK were noticed, we might suspect that the downregulation of soluble TWEAK reflects the host response to the interplay between tumour burden and exercise training.

Thirty-five weeks of exercise training prevented, to some extension, mammary tumorigenesis-induced muscle wasting by lowering the serum and muscle levels of TWEAK but not the muscle content of its receptor Fn14. TWEAK/Fn14 has been identified as a

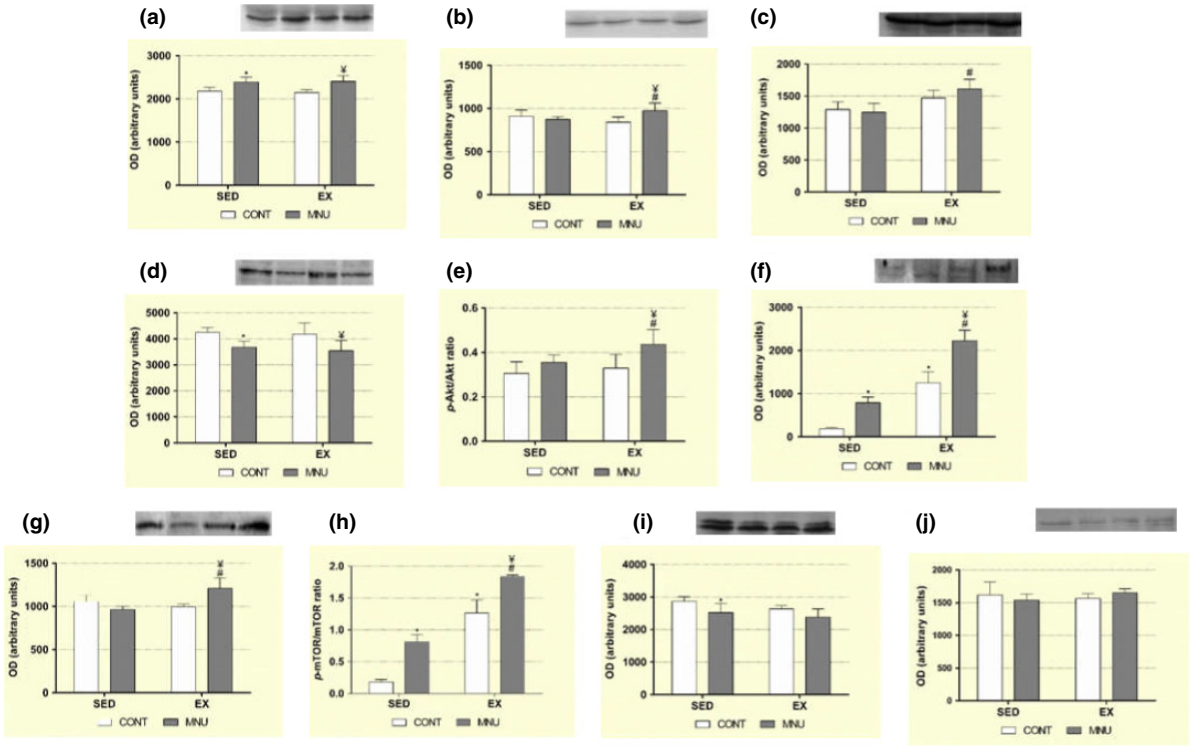

Figure 5 Effect of MNU-induced mammary tumorigenesis and/or endurance training on the muscle expression of MAFbx/atrogin-1 (a), MuRF-1 (b), *p*-Akt (c), total Akt (d), *p*-Akt-to-total Akt ratio (e), *p*-mTOR (f), total mTOR (g), *p*-mTOR-to-total mTOR (h), *p*-4E-BP1 (i) and *p*-p70S6K (j) evaluated by Western blotting in whole *gastrocnemius* muscle from animals of all experimental groups. Representative immunoblots are presented above the corresponding graphs. Values are expressed as mean ± SD (*$P < 0.05$ vs. CONT + SED; $^{¥}P < 0.05$ vs. CONT + EX; $^{\#}P < 0.05$ vs. MNU + SED).

critical regulator of skeletal muscle mass (Kumar *et al.* 2012), through the activation of NF-κB with the ensuing overexpression of ubiquitin–proteasome system (UPS) components (Guttridge 2004, Sato *et al.* 2014), and the inhibition of the PI3K/Akt signalling pathway (Dogra *et al.* 2007). Our data support the association between tumour-induced TWEAK/NF-κB signalling in the *gastrocnemius* muscle, with the involvement of the canonical and alternative NF-κB pathways. Despite the activation of Akt and mTOR, the reduced levels of *p*4E-BP1 in MNU-treated rats suggest decreased protein synthesis. So, mTOR does not seem to be involved in the mTORC1-4E-BP signalling in the skeletal muscle of rats with mammary tumours but can possibly be associated with mTORC2 signalling that regulates cellular processes as glucose uptake via not only Akt but also SGK and PKC kinases (Albert & Hall 2015). These findings might be explained by an overlap between the mechanisms of action of TWEAK and other cytokines. One of the features of TWEAK that is not shared with other cytokines is its effect on NF-κB signalling (Kumar *et al.* 2004, Bhatnagar & Kumar 2012). Exercise training prevented tumour-induced TWEAK/NF-κB signalling, particularly the canonical pathway, but without apparent effect on UPS system. The levels of phosphorylated Akt and mTOR also increased in the muscle of trained MNU rats although with no impact on the activity of its downstream targets p70S6K and 4E-BP1. Even so, exercise training prevented the decrease in MNU-related muscle fibre CSA (Fig. 1). Besides being involved in the regulation of protein synthesis, mTOR also modulates autophagy (Fritzen *et al.* 2016) and mitochondrial biogenesis in skeletal muscle (Albert & Hall 2015). Studies with TWEAK-KO mice also support the involvement of TWEAK in the regulation of metabolism through the activation of the canonical NF-κB signalling and the consequent repression of PGC-1α expression (Mittal *et al.* 2010b, Sato *et al.* 2013, Hindi *et al.* 2014). By preventing TWEAK/NF-κB signalling, long-term exercise training inhibits the suppression of PGC-1α, which regulates mitochondrial biogenesis, promoting an oxidative metabolism and fast-to-slow type fibre transition (Sato *et al.* 2013). Indeed, in the *gastrocnemius* muscle, exercise training increased the levels of PGC-1α, which might justify, at least in part, the muscle remodelling towards an oxidative phenotype (Figs 2 and 3). This metabolic adaptation has been related to enhanced muscle's ability to extract and utilize oxygen

from arterial blood (Rivera-Brown & Frontera 2012), and to improved aerobic performance (Hindi *et al.* 2014).

In conclusion, the present study provides molecular evidences that the interaction between endurance training and mammary tumorigenesis is mediated by TWEAK/NF-κB signalling. Long-term endurance training modulates the serum and muscle levels of TWEAK that despite not being related with its tumour levels seem to be associated with tumour grade. By preventing tumour-induced TWEAK/NF-κB signalling in skeletal muscle, endurance training impacts its metabolic status. Data support the benefits of an active lifestyle for the prevention of breast cancer-related side effects, highlighting the TWEAK signalling as a potential therapeutic target for the preservation of muscle performance in wasting conditions.

Conflict of interest

The authors declare no conflict of interests, financial or otherwise.

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Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Figure S1. Representative images of Ponceau S stained blotting membranes showing no differences among experimental groups.

Figure S2. Representative images of the immunohistochemical expression of TWEAK in mammary tumours from sedentary (a) and exercised (b) MNU-treated animals.

Supporting information

Figure S1. Representative images of Ponceau S stained blotting membranes showing no differences among experimental groups.

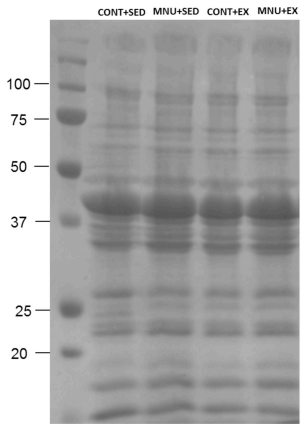
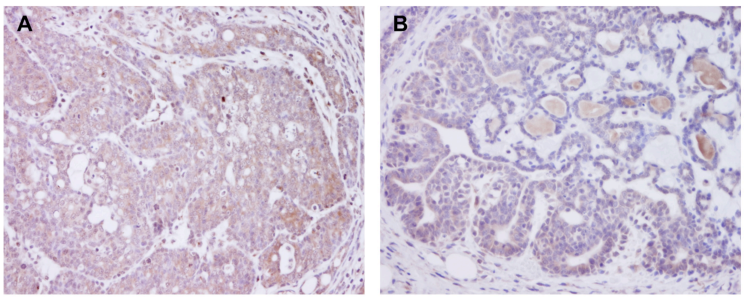


Figure S2. Representative images of the immunohistochemical expression of TWEAK in mammary tumours from sedentary (a) and exercised (b) MNU-treated animals.



CHAPTER III

GENERAL DISCUSSION

The present chapter is divided into two sections that respectively provide a discussion of the various methodological approaches and a general discussion of the results.

1. DISCUSSION OF METHODS

The mechanisms by which RPA/REX can improve the prognoses of patients with breast cancer have been subject to several studies that have identified numerous candidate mechanisms [3, 130]. However, the amount of RPA/REX that should be performed to favorably affect the modulation of those mechanisms remains unclear, mainly due to the approaches used to evaluate the exercise level performed. Nevertheless, it is generally accepted that 150–300 min/week of moderate exercise or 75min/week of vigorous exercise are required, with requisite attention to the patient's symptomatology [75, 137, 333, 389].

As the intermediate step between *in vitro* cell cultures and clinical assays in humans, animal models have often been used to study cancer. However, RPA/REX protocols have presented such diverse data that their results run the gamut from positive to negative [20]. To elucidate such divergence we sought to summarize and quantify the strength of published preclinical data regarding the different mechanisms within the relationship of RPA/REX with breast cancer. Second we aimed to scrutinize the features of exercise training designs that have better correlated with tumor outcomes. To that end, we conducted two systematic reviews involving meta-analyses, in which we analyzed the effects of

exercise from either a local perspective focused on the tumor burden (Study 1) or a systemic perspective focused on the host organic repercussions (Study 2). For target tumor burden, we examined several variables—tumor incidence, tumor multiplicity, tumor growth and TME, including cellular proliferation, cellular apoptosis, and angiogenesis—that permit measuring the effects of RPA/REX in tumorigenesis (i.e. incidence and multiplicity) and in tumor progression (i.e. tumor weight and tumor volume), as well as to verify the action of RPA/REX in TME hallmarks. Unfortunately, regarding TME collecting data was not an option but an unavoidable collection due to the lack of studies on the topic. For the systemic perspective, the targeted biomarkers were markers of inflammation, sex hormones, and glucose-related factors; analyzing those variables relied on the theoretical background about cancer-associated biomarkers mostly presented in Chapter 1. We also defined the RPA/REX variables (i.e. type of exercise performed, voluntary vs. forced; intensity, duration; frequency, and distance covered) that could moderate the interplay of exercise and breast cancer, as well as performed grouped analysis considering each of them.

In response to the heterogeneity of the collected data, we used a random effects model in both meta-analyses and performed a sensitivity analysis. Both meta-analyses were conducted according to the guidelines of the PRISMA statement (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) [262], and the quality of the analyzed studies was assessed using the Systematic Review Center for Laboratory animal Experimentation Risk of Bias tool (SYRCLE's RoB), adapted for animal studies [174].

Since most investigations in humans have ascertained the appropriate activity level by using subjective measures, which obscures knowledge of the amount (e.g. intensity, frequency and duration) of RPA/REX that confers optimal benefits, and because ethical principles do not permit the study of certain trends in humans, research on the topic using animal models to mimic disease in humans seems to be essential. Conceived to complement epidemiological studies and human clinical trials, animal studies can help to elucidate the cellular and molecular mechanisms by which RPA/REX exerts its beneficial effects, identify amounts of RPA/EXT that achieve better outcomes, and provide information about cancer initiation, progression, and metastization [361]. To furnish new insights into the field of research, the experimental work presented in this thesis was conducted in animals. We evaluated the effects of long-term (i.e. 35 weeks) moderate (i.e. 20m/min for 60 min every useful days) treadmill running on MNU-induced mammary cancer.

Published studies with animals (e.g. rodents) typically involve voluntary exercise via access to a free-spinning or motorized wheel, forced exercise on treadmills or swimming [20]. Although all methods have advantages and disadvantages, voluntary exercise has the advantage of relying on the animal's willingness, of apparently decreasing stress, and of allegedly imitating daily physical activity in humans. However, voluntary exercise in rodents promotes intermittent features (e.g., small bouts of intense activity, several times per day, every day) that cannot be unfeasible replicated in humans in some, if not all, stages of disease [168]. When tumor burden increases, a decline in animal

voluntary activity level is almost inevitable. Moreover, voluntary exercise does not allow inference about the adjusted training load for greater benefits, because it is not always possible to control the amount of exercise performed and sometimes impossible to control intensity (i. e. with a free-spinning wheel). With motorized wheels, it is possible to define intensity although the animals' willingness cannot be altered [361]. By contrast, forced exercise allows researchers to make inferences in a dose-responsive manner, yet it can induce stress on animals and influence the results of variables under consideration [195].

In the research reported here, we used forced exercise in order to allow the maintenance of the same levels of exercise in all animals and at once uphold guidelines for cancer patients [333]. To the best of our knowledge, the exercise design used, equivalent to 23 years [336], was the longest-lasting one ever made, which allowed the study of the effects of long-term exposure to exercise on cancer outcomes [383]. In addition, the option of a chemical model of breast cancer, even considering the major disadvantage of limited potential for metastization [383], yielded the well-documented advantages of chemically induced models, especially the ones that act directly (e.g. MNU) [168]. Such major advantages include the ability to investigate stage-specific effects, well-defined parallels between animal models and human disease, and insights into the cellular and molecular mechanisms that underlie tumor development [361]. Last, the model was considered to be a better alternative to cell-line derived models and a more economical option than genetically engineered ones [139].

Several methodological choices were made in order to conduct an objective evaluation of the impact of exercise on the progression of mammary neoplasms and muscle wasting. The histological type of the developed tumors was classified according to Russo and Russo's taxonomy (see Chapter 1) [318] by analyzing slides stained with hematoxylin and eosin (H&E). To characterize the infiltrative lesions, we measured cellular proliferation, cell death and collagen content. Cellular death was assessed with the terminal deoxynucleotidyl transferase-mediated dUTP *in situ* nick-end labeling (TUNEL) test, an increasingly common staining method used to detect apoptotic cells in tissue sections [91]. However, the fact that the TUNEL assay usually marks the apoptotic nucleus and necrotic cells [358, 378] could have problematized examining tumors whose histological character includes necrotic zones, particularly comedocarcinomas. Even with that limitation and considering that only one comedo-type tumor was detected, the protocol translated the amplitude of cellular death within the tumors [91].

Regarding the growth fraction of malignant tumor tissue, the number of cells stained by ki-67 antibody was considered. Uncontrolled proliferation is a hallmark of malignancy and may be assessed by a variety of methods, among which the immunohistochemical assessment of Ki67 antigen [99], recognized as a powerful prognosis marker in patients with breast cancer, is widely used [93, 236, 266, 391]. In addition, considering that collagen signature may be a promising cancer marker, since the content and distribution of collagen in cancer tissue, due to remodeling of the ECM during the malignant process,

differ from those aspects in corresponding normal tissue [173], tissue sections were stained with Picrosirius red to visualize collagen content [203, 354]. However, we did not analyze the collagen alignment, which is considered to be a tumor-associated collagen signature in breast cancer, and a particularly powerful one for tumor progression [72, 304].

Regarding muscle wasting, the choice to analyze gastrocnemius was based on its physiological characteristics as fast muscle, accompanied by what is known about the preferential target of cancer stimuli [65]. All methodological choices in that regard were made in an attempt to measure the impact of REX on circulating levels of relevant markers (i.e. mediators of cancer-induced muscle wasting) produced by both host and tumors and with both anabolic and catabolic actions.

Statistical procedures in both meta-analyses were performed with Comprehensive Meta-Analysis version 2.2.057 (BioStat Inc., Englewood, NJ, USA). All other statistical procedures were performed with GraphPad Prism version 6.0 and 7.0 (GraphPad Software, La Jolla, CA, USA). The choice to use parametric and nonparametric tests was based on the normal versus abnormal data distribution.

2. DISCUSSION OF RESULTS

In Study 1, a strong association was shown between RPA/REX and breast tumor outcomes. In contrast to some preclinical research associating the development and progression of mammary tumors with exercise [69, 70, 116, 140, 241, 351, 363, 390], our results after meta-analytic procedures showed the beneficial effects of RPA/REX on tumorigenesis. Cellular proliferation and apoptosis presented largely favorable changes amid exposure to RPA/REX, and tumor weight and tumor volume were shown to benefit moderately from RPA/REX. The sole variable that demonstrated small benefits was incidence. The meta-analytic procedures allowed us to identify the strength and the direction of the previously published findings and thereby confirm the positive ones [22, 69-71, 116, 146, 184, 188, 189, 221, 241, 242, 264, 269, 351, 364-366, 382, 385, 390, 396-398]. The findings of Study 3 also supported those results, for we observed evidence of a reduction in the number of tumors developed by MNU-exercised animals and in the number of tumors per animal. Our results also corroborate previous findings regarding multiplicity [70, 146, 188, 189, 241, 242, 269, 366, 396-398].

Moderate benefits of RPA/REX were found concerning tumor weight and tumor volume in meta-analysis 1, which support the results of most cited research on that variable [22, 146, 184, 188, 189, 241, 269, 351, 382, 385, 390, 397, 398]. However, we also found that the tumors grew at comparable rates in exercised and sedentary MNU animals (Study 3), arguably due to the length of the experimental design. Indeed, our study lasted longer than any of the other

previous study on the topic, which inevitably meant increased in tumor burden.

Meta-analytic procedures in Study 1 also revealed the strong influence of RPA/REX on the modulation of the TME in terms of cellular proliferation and death. RPA/REX largely improved the dysregulation between proliferation and apoptosis, which supports some previous findings [188, 189, 253, 269, 312, 361, 362, 384, 396, 398] as well as the results of Study 3 that clearly showed a reduction in intratumoral cellular proliferation accompanied by the increased expression of cellular death in MNU-exercised animals. Furthermore, the balance between cell proliferation and cell death in those animals was coupled with a significant reduction in their infiltrative lesions. Such results might have stemmed from improvements in the systemic markers of inflammation that we observed to be deregulated in Study 4. Indeed, we detected a significant reduction in the systemic expression of TWEAK in MNU-exercised animals, which corroborates knowledge that the TWEAK–Fn14 system demonstrates protumorigenic activity associated with proliferation, invasion, angiogenesis, and inflammation [258]. Such low malignancy—in fact, the lowest—revealed by the mammary tumors of MNU-exercised animals is probably due to improvements in host immune response reported by other studies [202, 248, 256, 313]. However, though promising, the results cannot be compared to findings from previous research, which lack data concerning the possible association between exercise and the lower aggressiveness in the histological patterns of mammary tumors [20]. Additionally, we also believe that the lower inflammatory condition revealed by the MNU-exercised animals induced

differences observed in Study 3 regarding the collagen content of the TME. The lower levels of collagen content of MNU-exercised animals (Study 3) speak in favor of a less fibrotic stimuli that can be associated with a lower activity of so-called “activated” fibroblasts inhibited by the reduction in systemic inflammation (Study 4) as has been previously reported [206].

In Study 1, we identified that the animal models for breast cancer used in the different studies were diverse, which could account for the comparatively slight differences obtained for incidence. Different tumor models might result in different tumor phenotypes and, consequently, in different behaviors when exposed to exercise [142, 172, 196]. Insufficient evidence of a positive association between RPA/REX and tumoral angiogenesis was also observed in Study 1. Notably, only six studies have involved analyzing TME (Study 1), which is a clear limitation to robust conclusions and to securely extrapolating the results to clinical contexts. Nevertheless, by showcase the importance of cellular proliferation [355] and apoptosis [49, 92, 106] as key features in tumor progression, the results of Study 1 and 3 strongly support the recommendation of RPA/REX in response to breast cancer. Regarding angiogenesis, we suspected that RPA/REX might act to normalize tumor vasculature by reducing tumor vessels permeability, which highlight an opportunity for chemotherapeutic delivery [239]. However, in Study 1, in attempting to identify the ideal amount of exercise to inform better results in tumor outcomes, we encountered considerable diversity in exercise protocols, which partly undermined our objectives. Yet, we also detected that exercise intensity seems to be a major

determinant of reducing tumor development and progression. Exercise training of moderate-to-vigorous intensity in bouts of 30–45 min, which upholds international guidelines [217, 311, 333], seemed to positively affect all the variables under study (Study 1). Also in Study 1 and unlike what is generally accepted regarding the duration of exercise bouts, longer exercise episodes (>45 min) had adverse effects on tumor incidence, tumor weight and tumoral angiogenesis. The exercise episodes used in our experimental research (60 min) fell into that range; however we did not find significant differences in tumor incidence or tumor weight (Study 3), although we did not study angiogenetic markers. Regarding moderate intensity, the effects observed in Meta-analysis 1 aligned with the outcomes of Study 3. Considering those results, future studies should preferentially choose exercise designs in which intensity can be measured along with exercise bouts in order to yield information that can enable advances in the field.

Findings in Study 2 consistently showed that REX is powerfully correlated with improvements in cancer-associated systemic biomarkers, which somewhat contradicts published research [116, 287, 366, 398]. Meta-analytic procedures enabled us to convincingly report that, for animals, engaging in approximately 85 min of vigorous treadmill exercise every week on useful days, reduced tumor-induced inflammation by 61.5%, and improved the circulating levels of sex hormones by 69.7%. Moreover, our results (Study 2) also showed that engaging in approximately 85 min of moderate treadmill exercise every week on useful days can also have positive, though in this case moderate,

effects upon cancer-related inflammation (43.1%) and the circulating levels of sex hormones (47.9%). The results in those two variables were achieved under conditions of exercise similar to ones internationally recommended for cancer patients [217, 311, 333].

Inflammation is another hallmark in the development and progression of cancer [83, 155, 156]. Regardless of inconsistent results concerning an inverse relationship between exercise and inflammation [55, 95, 114, 202, 291], our data from animals strongly supported that relationship, in accordance with the findings in other studies [116, 146, 148, 184, 256, 269, 287, 313, 335, 366, 397, 398]. As mentioned, they also sustained improvements in systemic inflammation observed in MNU-exercised animals in Study 4. Indeed, systemic inflammation is thought to be a major mediator of cancer-induced muscle wasting [122], and compelling evidence indicates that exercise activates a network of transcription factors that promote metabolic reprogramming in skeletal muscle. TWEAK inhibition appears to be a strategy to prevent the loss of skeletal muscle mass [322]. Our results also seem to suggest that REX is important for regulating the expression of mediators of skeletal muscle wasting. In fact, the impact of REX was primarily observed in the significant enlargement of the cross sectional area of muscle fibers and in the significant reduction in the serum and muscle levels of TWEAK in the gastrocnemius of MNU-exercised animals. The inhibition of TWEAK levels prompted a decreased in the expression of atrogenes and the inevitable suppression of NF–κb signaling. As previously reported in other studies, TWEAK induces *MuRF1* upregulation via

NF–κb and thereby causes MyHC loss [261]. The inhibition of NF–κb is sufficient to decrease significantly tumor-induced muscle loss by inhibiting the upregulation of *MuRF1* and *MAFBx* [38, 51, 215, 265].

The benefits of RPA/REX for the circulating levels of sex hormones are more consistently reported in previous research, particularly among postmenopausal women [132, 172, 280], and in preclinical data [366, 397, 398]. In contribution, our data from Meta-analysis 2 clearly indicated a strong association between exercise and the circulating levels of sex hormones. In glucose-related factors small benefits (25.1%) were also observed. In fact, the best results in glucose-related factors under treadmill exercise modality were achieved with aproximately 200 min/week of vigorous exercise, which hardly seems to be reproducible during breast cancer in humans. We also found that the best results in glucose-related markers (Study 2) were associated with voluntary exercise, which makes it impossible to determine the exact amount of exercise needed to achieve those benefits. Although much evidence supports a connection between RPA/REX and biomarkers related to glucose metabolism [149, 226], our results, though confirming that evidence, did not allows us to pinpoint the amount of REX required to alter glucose-related biomarker levels. Nevertheless, in Study 4, we did find local improvement in the metabolic profile of the gastrocnemius. Indeed, the increased expression of PGC-1α, citrate synthase, and other subunits of oxidative metabolism in that muscle of exercised animals appear to indicate a fiber shift toward the oxidative phenotype (fast-to-slow shift), which also reveals improvements in mitochondrial

activity, as reported in others studies [64, 167, 323]. That metabolic adaptation has been related to the enhanced ability of muscles to extract and use oxygen from arterial blood [310], as well as to improved aerobic performance [167].

Shown again in Study 2, albeit to a degree considerably slighter relatively than in Study 1, the diversity among experimental designs clearly limited the extension of results to other populations. Both studies revealed that the distance covered by the animals throughout the experiments did not seem to affect the outcomes, which contrasts what has been reported by other researchers [146, 366, 382, 397, 398]. Surprisingly, covering short or long distances resulted in similar outcomes. In that sense, what seems to be important is not how far we go, but instead in which intensity do we get there.

CHAPTER IV

GENERAL CONCLUSIONS AND IMPLICATIONS

1. CONCLUSIONS

Our results provided support for the following conclusions:

1. As preclinical data indicated, exercise is associated with reduced tumor burden in animals.
2. Regular exercise promotes beneficial changes in the levels of proliferation and apoptotic biomarkers in animals' TMEs.
3. In exercise programs, moderate-to vigorous intensity seems to be the most determinant variable in the modulation of tumor behavior.
4. Performing 85 min/week of vigorous forced exercise reduced systemic inflammation levels and improved circulating sex hormone levels in animals.
5. From preclinical data, it is impossible to identify the amount of exercise training needed to favorably regulate glucose-related factors.
6. Long-term moderate exercise training did not induce improvements in tumor growth.
7. Long-term moderate exercise training reduced tumor proliferation and the amount of infiltrative lesions in animals, in the latter of which long-term moderate exercise training promoted a more favorable TME by enhancing the rate of cell death and a decreasing collagen deposition.
8. Moderate exercise training prevented tumorigenesis by modulating of TWEAK–NF-κB signaling.
9. In mammary tumor-bearing animals, long-term moderate training

improved the metabolic status and prevented the skeletal muscle wasting of the gastrocnemius, likely by modulating the serum and skeletal muscle levels of TWEAK.

10. Evidence of the association between exercise and the positive modulation of angiogenic events in TMEs remains insufficient.

2. PRACTICAL IMPLICATIONS

Considering all of the findings collected in this thesis, first with preclinical contexts in mind, the research as presented sufficient data to inform exercise designs. By taking into account a methodological approach mimicking the exercise training conditions that can be performed by humans with breast cancer, the research allows the extrapolation of its results to clinical contexts. However, much work remains to be done to convincingly support evidence-based exercise training for breast cancer, especially as it pertains to TMEs.

Taking the clinical contexts into consideration, despite the diversity of results reported in the literature, evidence of the benefits of exercising after being diagnosed with breast cancer does exist. In light of our results, regular exercise training could serve to regulate the mechanisms by which tumors become more aggressive. Furthermore, exposure to programs of regular exercise training could diminish cancer-induced muscle wasting by modulating several associating factors. In particular, host response to the stimuli of cancer could be improved under the action of regular exercise training. Exercise training is important and should be performed throughout the course of breast cancer, although always by considering a symptom-based approach tailored to each patient. Moreover, considering our results, exercise training programs for patients with breast cancer should prescribe activities that allow them to achieve moderate-and-vigorous-intensity levels in their training plans. Altogether, we believe that breast cancer treatment teams need to be multidisciplinary and always include a specialist in exercise prescription.

CHAPTER V

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REFERENCES

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