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Faculty of Sport

Research Center in Physical Activity, Health and Leisure

Beneficial role of regular physical exercise on tumor development and different susceptibility of muscle fibers to cancer-induced wasting in mice

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KEYWORDS: VOLUNTARY RUNNING, TUMOR MICROENVIRONMENT, VASCULARIZATION, IMMUNITY, MUSCLE WASTING.

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To my parents,
for giving me everything a son could need,
unconditional love and the best education.

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Resumo

A presente tese é suportada por uma revisão da literatura que reuniu o conhecimento teórico acerca da influência da vascularização no microambiente tumoral e do exercício físico como potencial agente modulador da vasculatura tumoral e por uma revisão sistemática com meta-análise, que permitiu sistematizar e quantificar a evidência da investigação produzida em modelos animais relativa aos efeitos da vascularização no crescimento tumoral. O trabalho experimental consistiu numa revisão sistemática com meta-análise de estudos realizados com animais, acerca dos efeitos do exercício físico na vasculatura tumoral e em dois protocolos, um com ratinhos machos C57BL/6 do qual resultaram dois estudos com o objetivo de investigar o efeito da corrida voluntária regular no crescimento tumoral, na sua vascularização e infiltração linfocitária e outro com ratinhos machos CR (CD1), que pretendeu descrever o impacto do desenvolvimento de cancro em diferentes tipos de músculo esquelético. A primeira revisão sistemática com meta-análise mostrou que a diminuição da vascularização tumoral induzida por terapia inibidora da angiogénese permitiu atenuar o crescimento tumoral, enquanto os estudos empíricos revelaram que a prática de exercício físico aumentou a vascularização dos tumores e que a corrida voluntária regular atenuou o crescimento tumoral através da diminuição da proliferação celular, do aumento da apoptose e melhor vascularização, da maior quantidade de linfócitos T CD8⁺ e de mais recetores NKG2D no tumor. Verificou-se também que a atrofia muscular induzida pelo desenvolvimento de cancro ocorreu preferencialmente nos músculos glicolíticos, constituídos maioritariamente por fibras tipo II, poupando os músculos oxidativos, compostos predominantemente por fibras tipo I e com um nível superior de atividade contráctil. Os resultados encontrados permitem concluir que no modelo animal a corrida voluntária regular melhora a vascularização tumoral e o acesso das células imunitárias, atenuando o crescimento tumoral e que os músculos glicolíticos são mais afetados pelo desenvolvimento de cancro.

PALAVRAS-CHAVE: CORRIDA VOLUNTÁRIA, MICROAMBIENTE TUMORAL, VASCULARIZAÇÃO, IMUNIDADE, ATROFIA MUSCULAR.

Abstract

The present thesis is supported by a literature review that overviewed the current theoretical knowledge about the implications of the vascularization on the tumor microenvironment, and by a systematic review with meta-analysis that allowed us to systematize and quantify the existing evidence about the effects of vascularization on tumor growth, in animal models. The experimental work was based on a systematic review with meta-analysis of animal studies, regarding the effects of physical exercise on tumor vascularization, and on two different protocols, one performed with C57BL/6 male mice, from which resulted two studies to investigate the effects of regular voluntary running on tumor growth, on its vascularization and lymphocytic infiltration, while the other involved CR (CD1) male mice, to describe the impact of cancer development on different types of skeletal muscle. The first systematic review with meta-analysis showed that the reduction of tumor vascularization induced by anti-angiogenic therapy contributed to the inhibition of its growth. The results from the empirical studies demonstrated that physical exercise improved the tumor vascularization and regular voluntary running attenuated tumor growth, through a decreased proliferation, an increased apoptosis and improved vascularization, a higher quantity of CD8⁺ T cells and more NKG2D receptors in tumor. We also found that the muscle wasting induced by cancer development occurred predominantly in glycolytic muscle, which are mostly composed by type II fibers, sparing the oxidative muscles, constituted mainly of type I fibers and with an increased contractile activity. These results allow us to conclude that in mice, regular wheel running attenuates tumor growth, improves tumor vascularization and the immune cells' access to the tumor, and the glycolytic muscles are more affected by cancer development.

KEYWORDS: VOLUNTARY RUNNING, TUMOR MICROENVIRONMENT, VASCULARIZATION, IMMUNITY, MUSCLE WASTING.

List of abbreviations

AiAs	Angiogenesis-inhibiting agents
ANS	Autonomic nervous system
APCs	Antigen presenting cells
ATP	Adenosine-5'-triphosphate
BBN	N-butyl-N-(4-hydroxybutyl) nitrosamine
BC	Breast cancer
BMI	Body mass index
BSA	Bovine serum albumin
BW	Body weight
Ca ²⁺	Calcium
CAMARADES	Collaborative Approach to Meta-Analysis and Review of Animal Data from Experimental Studies
CCL	CC-chemokine ligand
CO ₂	Carbon dioxide
CG	Control group
CRH	Corticotropin-releasing hormone
CSA	Cross-sectional area
CTLs	Cytotoxic T-lymphocytes
CXCL1	C-X-C Motif Chemokine Ligand 1
DAB	Diaminobenzidine tetrahydrochloride
DCs	Dendritic cells
DOX	Doxorubicin
ECM	Extracellular matrix
ECs	Endothelial cells
EDHF	Endothelium derived hyperpolarizing factor
EDL	Extensor digitorum longus
EG	Experimental group
EMT	Endothelial-to-mesenchymal transition
EX+T	Voluntarily exercised tumor induced
FOXO	Forkhead box O

GAST	Gastrocnemius
H ⁺	Hydrogen ions
H ₂ O ₂	Hydrogen peroxide
H&E	Haematoxylin and eosin
HIF	Hypoxia inducible factor
HPA	Hypothalamic-pituitary-adrenal
HR	Heart rate
ICAM	Intercellular adhesion molecule
IDO	Indoleamine 2,3-Dioxygenase
IFN	Interferon
IFP	Interstitial fluid pressure
IGF	Insulin-like growth factor
IGFBPs	Insulin-like growth factor-binding proteins
IL	Interleukin
K ⁺	Potassium
LIF	Leukemia Inhibitor Factor
LLC	Lewis lung carcinoma
M1	Type 1 macrophages
M2	Type 2 macrophages
MAFbx	Muscle atrophy F-box protein
MDSCs	Myeloid derived suppressor cells
MHC	Myosin heavy-chain
MMP	Matrix metalloproteases
MND	Myonuclear domain
MNU	N-methyl-N-nitrosourea
MuRF1	Muscle ring-finger protein 1
MVD	Microvessel density
MyoD	Myoblast determination protein 1
Na ⁺	Sodium
NFAT	Nuclear factor of activated T cells
NK	Natural Killer
·NO	Nitric oxide

NSAIDs	Non-steroidal anti-inflammatory drugs
O ₂	Oxygen
OS	Overall survival
PDAC	Pancreatic ductal adenomarcinoma
PCa	Prostate cancer
PD	Programmed Cell Death Protein
PD-L	Programmed Death-Ligand
PECAM-1	Platelet-endothelial cell adhesion molecule
PGC-1 α	Peroxisome proliferator-activated receptor-gamma co-activator
PGE ₂	Prostaglandin E ₂
PGI ₂	Prostacyclin
PHD2	Prolyl hydroxylase domain protein 2
pHe	Extracellular pH
pHi	Intracellular pH
PMNs	Polymorphonuclear leukocytes
pO ₂	Oxygen partial pressure
PRISMA	Preferred reporting items to systematic reviews and meta-analyses
RCTs	Randomized controlled trials
ROS	Reactive oxygen species
S1PR	Sphingosine-1-phosphate receptors
SCs	Satellite cells
SED+T	Sedentary tumor induced
SMA	α -smooth muscle actin
SMD	Standardized mean difference
SOL	Soleus
SR	Picrosirius Red
SYRCLE's RoB	Systematic Review Center for Laboratory animal Experiments risk of bias
TA	Tibialis anterior
TAMs	Tumor-associated macrophages

T-bet	T-box transcription factor
TdT	Terminal deoxynucleotidyl transferase
TER	Transcapillary escape rate
TGF	Transforming Growth Factor
Th	T helper
TILs	Tumor-infiltrative lymphocytes
TME	Tumor microenvironment
TNF	Tumor Necrosis Factor
Treg	T regulatory
TSP-1	Thrombospondin 1
TUNEL	Tdt-mediated dUTP-biotin Nick-End Labeling
UCC	Urothelial carcinoma
UPS	Ubiquitin–proteasome system
VCAM1	Vascular adhesion molecule 1
VDAs	Vascular disrupting agents
VEGF	Vascular Endothelial Growth Factor
VsMCs	Vascular smooth muscle cells
VTAs	Vascular targeting agents
vWF	von Willebrand Factor
WT	Wild type
α - SMA ⁺ /CD31	Vessel maturation index

CHAPTER I
INTRODUCTION

1.1. General introduction

Cancer incidence and mortality are growing worldwide and the reasons, although complex, partially reflect the growth and aging of the population, as well as exposure to major risk factors [1]. Only in 2018, about 18.1 million new cases were diagnosed and 9.6 million people died from cancer, making it one of the most debilitating diseases and the second leading cause of mortality in the world [2]. As such, during the last decades, efforts have been made to elucidate the molecular mechanisms involved in tumorigenesis, which today we know to be a multi-step process involving initiation, promotion, progression, and metastasis [3]. Briefly, initiation is characterized by a genomic damage, promotion involves an active proliferation of pre-neoplastic cells and progression occurs when cells increase the proliferative capacity and undergo further mutations with invasive and metastatic potential. Finally, metastasis encompasses the spread of cancer cells from the primary site to other parts of the body through bloodstream, lymph system, the peritoneal route or canaliculi system [4]. As such, during tumorigenesis cells acquire biological capabilities that include sustained cellular proliferation, evading growth suppressors, resisting cell death, replicative immortality, angiogenesis, reprogramming cell metabolism, activating invasion, metastasis and evading immune destruction [5]. The resultant neoplasms can be classified as benign or malignant. While benign neoplasms tend to grow slowly and have distinct borders, remaining in their primary location, malignant neoplasms, collectively known as cancers, grow uncontrollably and spread locally or to distant sites, displaying aggressive features that can affect the function of several organs and lead to the death of the host [6]. Consequently, contrary to benign lesions that can be surgical removed and are unlikely to recur, the treatment of malignant neoplasms may also require a systemic intervention [7].

However, neoplasms should not be considered just an accumulation of isolated cells, but a complex of cellular and extra-cellular components that constitute a unique microenvironment [8], mediating the interactions with the systemic milieu and regulating cells' growth [9, 10]. Indeed, the high proliferation rate of cancer cells and the unbalanced expression of pro- and anti-angiogenic factors

(angiogenic switch) present within the tumor microenvironment (TME) lead to the development of a structurally abnormal and dysfunctional vascular network, which induces a heterogeneous and variable intratumoral blood flow [11]. Consequently, there is an increased interstitial fluid pressure (IFP) that induces the collapse of vessels [12], thus impairing the perfusion of the tumors [13] and, consequently, limiting the delivery of nutrients, oxygen (O₂), anti-cancer drugs and immune cells [14, 15]. Mechanistically, existing evidence indicates that the defective tumor endothelium establishes a barrier that limits T cell adhesion and migration [16, 17] and, although cytotoxic T cells can be found at tumor's periphery, these cells often fail to infiltrate into the tumor's core in sufficient numbers, hindering an effective tumor elimination [18-21]. Interestingly, cancer patients with higher concentrations of intratumoral T cells experience an increased overall survival (OS) across multiple tumor types [22-24]. Comprehensively, tumor angiogenesis became the main target of several therapies in order to contain tumor growth [25]. Along this line, despite initial trials with anti-angiogenic drugs seemed to be promising [26-28], the clinical application of such agents revealed a limited effect in many tumors due to treatment resistance, vascular rarefaction and reduced intratumoral perfusion [29, 30]. Particularly, hypoperfusion-induced tumor hypoxia triggers several mechanisms involved in cell survival and proliferation, drug and radiation resistance, and immune evasion [31]. Therefore, the association between tumor vascularization and its progression continues to be a controversial point requiring further research **(Study I)**. In addition, considering that some systematic reviews and meta-analyses have reported that patients receiving anti-angiogenic treatment have a significant risk of developing hypertension and nephrotic syndrome [32-35], alternative approaches to remodel the tumor vasculature in order to change the immunosuppressive nature of the TME are still necessary [36]. Thus, physical exercise, as an important component of cancer rehabilitation, has been suggested as a potential strategy to achieve it **(Study II)** [37]. Indeed, although the main goal of cancer rehabilitation is to improve physical function and quality of life [38, 39], according to international organizations, the regular practice of physical activity or exercise is associated

with a delayed cancer progression, improved treatment response and better cancer-specific survival in several types of cancer [40, 41]. Specifically, data from breast, colon and prostate cancer (PCa) proposed the physical exercise-induced regulation of systemic factors such as sex and metabolic hormones, cytokines and immune function, as candidate mechanisms for its protective effect [42, 43]. Moreover, recent findings showed that regular physical exercise may also directly modulate the tumor biology to inhibit its growth [44], but the molecular mechanisms underpinning its impact have not been fully established **(Study III)** [45, 46]. Additionally, considering that some controversial results were found about the effects of physical exercise on tumor vascularization, hypoxia and immune function [45], the most important question continues to be how regular physical activity or exercise influences the modulation of the TME in order to impact cancer prognosis **(Research question I)**. Therefore, an improved understanding of the role of physical exercise on tumor vasculature is required to fully elucidate about its potential beneficial effects and limitations **(Study IV)**. In this regard it is tempting to hypothesize whether regular practice of physical activity or exercise induces an appropriate remodelling of tumor vasculature, to increase the number of intratumoral immune cells, in order to improve immune response and, ultimately, to inhibit tumor growth **(Study V)**.

Nevertheless, although the continuous production of extracellular matrix (ECM) components and inflammatory chemo-attractants by the tumor takes place locally [47], it often leads to systemic manifestations such as chronic inflammation, insulin resistance, hypogonadism and anaemia [48, 49]. Of these, chronic inflammation has become a hallmark of cancer-induced cachexia, a multifactorial syndrome that is defined by an ongoing loss of skeletal muscle mass, with or without loss of fat mass, which cannot be fully reversed by conventional nutritional support, leading to progressive functional impairment [50]. Cancer-associated cachexia is considered a continuum spanning the categories of pre-cachexia, cachexia and refractory cachexia, affecting up to 80% of cancer patients [50]. Specifically, patients that demonstrate a substantial involuntary weight loss of 5% or less and any systemic manifestation of cancer are classified as having pre-cachexia; a weight loss superior to 5% over the

past 6 months, or higher than 2% in patients that have a body mass index (BMI) less than 20 kg/m² or skeletal muscle mass depletion (male < 7.26kg/m² and female < 5.45kg/m²) corresponds to cachexia, while refractory cachexia is a resistant catabolic state, associated with a poor performance status and a life expectancy inferior to 3 months [50]. Although no single agent was identified as a suitable standard treatment for cancer-related cachexia and its molecular mechanisms are not completely understood, research in the last years found that the up-regulation of pro-inflammatory cytokines and acute phase proteins triggers a cascade of downstream inflammatory signalling that directly activate the ubiquitin–proteasome system (UPS) and autophagy in skeletal muscles, which promote protein hypercatabolism and inhibit the synthesis of myofibrillar proteins, inducing muscle wasting [51]. In addition, the oxidative capacity of skeletal muscle is also disrupted during cancer progression [52], worsening the loss of muscle mass, which is associated with shorter time to tumor progression, increased postoperative complications, patients morbidity and higher mortality [53]. Indeed, cachectic patients have impaired ventilatory capacity, partially derived from respiratory muscles weakness, which increases the susceptibility to pulmonary infections [54] and reduced responsiveness to anticancer treatments, since, frequently, they cannot cope with standard treatment schedules, requiring a dosage reduction or treatment interruption [55-57]. In addition, patients with cachexia are prone to develop severe toxicities from systemic anticancer agents [58], because as many chemotherapeutic drugs are distributed to the body fat-free compartment [59], low muscle mass results in a reduced area available for drug distribution and, consequently, in higher systemic drug concentrations [60]. As such, the discovery that a contracting skeletal muscle produces and secretes cytokines (myokines) changed its concept from a peripheral tissue, exclusively responsible for voluntary contractile activity, into a highly dynamic endocrine organ, which can influence energy and protein metabolism in other tissues and organs [61, 62]. Particularly, myokines mediate the crosstalk between skeletal muscles and adipose tissue, liver and bone [63], and the effects include the regulation of systemic inflammation, immune function, insulin sensitivity, cell growth,

myogenesis and osteogenesis [64]. Therefore, given the clinical relevance of the skeletal muscle's integrity, there is increasing interest in developing ways to identify and limit the loss muscle mass in cancer patients [56]. However, when addressing cancer-induced muscle wasting, the general approach has been to assess the impact of the disease during the occurrence of its clinical manifestations, which hinders an appropriate evaluation of the initially induced muscle changes [65-69]. Considering that cancer-associated cachexia is often irreversible once weight loss begins [70] and that pathological changes precede the installation of clinical manifestations [49], a better understanding of skeletal muscle behaviour before reaching this threshold is mandatory [71]. Additionally, as skeletal muscles comprise myofibers that differ in respect to size, contractile function and metabolism [72], the crosstalk between the systemic and the local environment of each skeletal muscle may influence its susceptibility to wasting [73]. However, taking into account that previous studies have yielded controversial results [65, 66, 74, 75], whether skeletal muscles are equally affected by cancer-induced muscle wasting is unknown **(Research question II)**. With this in mind, understanding how cancer development impacts on different types of skeletal muscle will be highly valuable as a fundamental basis for future studies to explore, in order to address potential interventions that may prevent or overcome muscle wasting **(Study VI)**.

1.2. Aims

Given the above described state-of-the-art, the main aims of the present research project were:

- 1- To systematize and quantify the existing pre-clinical evidence on the effect of vascularization on tumor growth.
- 2- To overview the current theoretical knowledge regarding the implications of an abnormal tumor vasculature on TME.
- 3- To report the candidate mechanisms underlying the importance and effectiveness of physical exercise as a therapeutic intervention for remodelling tumor vasculature.
- 4- To determine if the regular practice of voluntary running can attenuate tumor growth in mice inoculated with a suspension of RM1 androgen-independent murine prostate carcinoma cells.
- 5- To systematize and quantify the existing pre-clinical evidence about the effects of physical exercise and physical activity on tumor vasculature.
- 6- To analyse if regular voluntary running is able to improve tumor vascularization, allowing a better intratumoral infiltration of immune cells in mice inoculated with a suspension of RM1 androgen-independent murine prostate carcinoma cells.
- 7- To describe the impact of cancer development on microscopic morphometric properties of three skeletal muscles with distinct phenotypic characteristics (Tibialis Anterior, Soleus and Diaphragm) of mice exposed to N-butyl-N-(4-hydroxybutyl) nitrosamine (BBN).

Based on the latest evidence approaching the effect of physical exercise on tumor biology and the impact of cancer in skeletal muscles, the working hypothesis to be tested were:

- 1- By improving tumor vascularization, regular voluntary running may favourably modulate the TME, increasing the intratumoral density and action of immune cells, thus contributing to a reduced tumor growth.

2- Fast-glycolytic muscles are more severely affected by cancer-associated wasting than chronically active oxidative muscles, which is demonstrated by a reduced cross-sectional area (CSA), higher collagen deposition and increased myonuclear domain (MND).

The present research document is structured according to the Scandinavian model and organized in six chapters. A general introduction (Chapter I) is first provided with an overview of the two research questions, specifically, how regular physical activity or exercise modulates the TME to impact cancer prognosis and how cancer-induced muscle wasting affects different skeletal muscles, as well as the research aims and the study's hypothesis. A background (Chapter II) approaches the effect of vascularization on tumor growth through a systematic review with meta-analysis (Study I), and introduces the research question I through a literature review about the implications of an abnormal tumor vasculature on the TME and the potential physical exercise-associated mechanisms involved in its remodelling (Study II). The chapter III (Empirical studies) is composed of four studies structured to explore the two research questions. Study III was designed to assess whether regular practice of voluntary running was able to attenuate tumor growth, by inhibiting proliferation and inducing apoptosis of tumor cells. Through meta-analytic procedures, the Study IV quantified the impact of physical exercise on tumor vasculature. Study V explored the modulation of the TME by regular wheel running, specifically, its effects on tumor vasculature and on intratumoral T cells infiltration. Finally, Study VI aimed to evaluate the impact that urothelial carcinoma (UCC) could have in three different skeletal muscles, regarding the CSA, fibrotic tissue infiltration and MND. Then, a general discussion about the methods and the main findings of the present research project and its clinical applicability are outlined in Chapter IV, followed by a conclusion with future research directions (Chapter V). The last chapter (Chapter VI) encompasses the references used in Chapters I and IV.

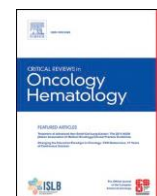
CHAPTER II

BACKGROUND

2.1. Study I

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The effects of vascularization on tumor development: A systematic review and meta-analysis of pre-clinical studies

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ABSTRACT

Purpose: This review aimed to systematize and quantify the existing evidence about the effect of tumor vascularization on its growth, in preclinical studies.

Methodology: A computerized research on databases PubMed, Scopus and EBSCO was performed to identify studies that evaluate both the vascularization parameters and the development of the tumors in animal models and the mean differences were calculated through a random effects model.

Results: Thirteen studies met the inclusion criteria and were included in the systematic review, of which, 6 studies were included in the meta-analysis. Besides tumor vascular density that all studies evaluated, 3 studies analysed the tumor perfusion, 2 studies the tumor hypoxia and 3 studies assessed the grade of vessel maturation. Most of the studies (11) related decreased tumor vascularization and a concomitant inhibition of tumor growth or metastasis development. Quantitatively, the decrease in tumor vascularization contributed to a significant decrease in the tumor growing rate of 5.23 (-9.20, -1.26).

Conclusion: A reduced level of tumor vascularization seems to be able to inhibit tumor growth and progression.

1. Introduction

Small tumors (diameter < 2 mm) are perfused by vasculature originating from surrounding host tissues and further growth is usually accompanied by newly formed microvessels (angiogenesis), for an adequate nutrients supply, oxygenation and removal of waste products (Folkman, 1995). Angiogenesis is stimulated even before the occurrence of a malignant phenotype and increases during tumor progression, in order to sustain growth and dissemination (Menakuru et al., 2008; Bossi et al., 1995). The dependence of tumor growth on angiogenesis has led to the concept of antiangiogenic therapy (Jain, 2001). Indeed, it has been assumed that an agent capable of inhibiting the formation of new blood vessels in tumors and/or that causes the regression of recently formed neovasculature and consequently, the decrease of vessel count in tumors, would be expected to reduce the levels of oxygen (O₂) and nutrients within tumors and thus inhibit its growth (Franco et al., 2006). Nonetheless, although it was already shown that inhibition of the

angiogenic process can inhibit tumor growth in a number of tumor models (Horsman and Siemann, 2006), the impact of this therapy on overall survival (OS) has been merely superficial (Mittal et al., 2014). As such, more recent investigations have raised doubts regarding the theoretical assumptions of antiangiogenic therapy, because the inhibition of tumor vasculature could hinder the perfusion of the tumor, compromising the cytotoxic functions of immune cells and increasing the potential for tumor growth (Multhoff et al., 2014; Anderson et al., 2017; Renner et al., 2017). As a result, an increasing number of pre-clinical studies have reported that after the initially reduction of tumor mass in response to the decrease of vascularization, the levels of tumor hypoxia increased, leading to more aggressive phenotypes (Ulivi et al., 2016; Van der Veldt et al., 2012; Mehta et al., 2011). Indeed, there is evidence supporting the association between the enhancement of tumor hypoxia with an increased expression of mesenchymal phenotypes and tumor cell dissemination and invasiveness (Xu et al., 2015; Jahangiri et al., 2013; Piao et al., 2013; Carbone et al., 2011).

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However, although these findings are contrary to the premise that the reduction of tumor vascularization will inhibit tumor growth, recent studies suggested that a decreased vascularization cannot ensure a proper oxygenation, promoting the development of tumor hypoxia (Ribatti et al., 2019; Petrova et al., 2018; Jing et al., 2019). In turn, intratumoral hypoxia can induce proteome and genome changes, increasing the invasive potential of cancer cells by boosting the production of pro-migratory proteins and pro-invasive extracellular matrix (ECM) molecules (Finger and Giaccia, 2010). As a result, cancer cells can overcome nutritive deprivation and escape from the hostile environment, favouring tumor progression and unrestricted growth (Vaupel et al., 2004). Indeed, Hendriksen and collaborators, analysed the progression of glioblastoma, and found that, at the initial stage, small tumors were well vascularized and perfused, however, as they grew, tumors became less vascularized and poorly perfused, which promoted the development of extensive hypoxic areas (Hendriksen et al., 2009). Thus, the possibility of the abnormal tumor vasculature to not provide a necessary perfusion and, consequently, to induce tumor hypoxia, decreasing the accessibility for immune response and anti-cancer drugs, may support concerns about the best therapeutic modality to adopt (Ebos et al., 2009; Paez-Ribes et al., 2009). Therefore, accurate and controlled investigations are particularly necessary in this field, highlighting the importance of rigorous research based on animal models (Vandamme, 2014). Indeed, pre-clinical studies involving controlled and homogeneous conditions, with higher rates of reproducibility compared to clinical research, allow the identification of potential molecular mechanisms underlying the effect of vascularization on tumor behaviour (Cekanova and Rathore, 2014). Additionally, cancer clinical research expresses some limitations regarding the size and number of samples, availability, time and resources constraints (Bell et al., 2009). As such, animal experiments remain essential to understand the fundamental mechanisms underlying the onset of malignancies and to discover improved methods to diagnose, treat and prevent the disease (Vandamme, 2014).

Therefore, having in mind that tumor blood supply appears to be a double-edged sword and that animal research seems to be the better way to study it, the aim of this review is to analyse the link between the level of vascularization and the grade of tumor growth, in animal models.

2. Methods

2.1. Search strategy

An extensive literature search was performed in May of 2019 and again in January of 2021, in the following electronic databases: Medline, Scopus and EBSCO. The search terms were based on a combination of the following keywords: (cancer OR tumor) AND (vascularization OR perfusion OR hypoxia OR angiogenesis) AND (animals). Reference lists of the identified studies were then searched to find further articles relating to the topic of the review and to ensure that appropriate studies were obtained. The present review was reported according to preferred reporting items to systematic reviews and meta-analyses (PRISMA) guidelines (Moher et al. 2009).

2.2. Selection criteria

All studies retrieved from our search needed to meet the following inclusion criteria: fullpapers published as original articles; written in English; animal models with cancer; evaluated microvessel density (MVD); analysed tumor size, growth or metastasis development; were randomized controlled trials (RCTs). Studies were excluded if were: letters or editorial articles; review papers, studies involving intervention other than of vascular nature; studies that analysed only vascular outcomes or studies that evaluated just the tumor growth.

2.3. Study selection

Duplicates were first removed. Then, the title, abstract and keywords were analyzed to assess the type and relevance of the publication for the scope of the review. If the publication was potentially relevant, the full-text was screened for content to decide its inclusion. Fig. 1 shows the process of the study identification and selection.

2.4. Data extraction

Data from the included articles were extracted in a structured table-format, meaning: author's last name and year of publication, sample, tumor model, intervention, vascular outcomes, results and findings.

2.5. Methodological quality assessment

The methodological quality of each study was assessed using the The Collaborative Approach to Meta-Analysis and Review of Animal Data from Experimental Studies (CAMARADES) (Macleod et al., 2009). CAMARADES is a 7-point study quality checklist created to support the use of similar approaches to those used by the Cochrane Collaboration, to data from animal studies (Vesterinen et al., 2014). The following criteria was evaluated: (1) sample size calculation; (2) random allocation of treatment/control group; (3) blinded assessment of outcome; (4) appropriate animal species and models; (5) publication in a peer-reviewed journal; (6) compliance with animal welfare regulations and (7) statement of potential conflicts of interest. Each study was given a quality score out of a maximum total of seven points, according to Zhang and co-workers (Zhang et al., 2020).

2.6. Statistical analysis

We performed the statistical analysis using Review Manager 5.3 software. Mean differences are presented in both groups due to the existence of continuous data, which were grouped through a random effects model because the risk to overestimate the effect size is smaller. A *p* value lower than 0.05 was considered statistically significant.

3. Results

3.1. Study selection

A total of 272 studies were identified in the electronic databases search. After reviewing the title and abstract, 12 papers were excluded for being duplicates (coming from the two different databases). During the title, keywords and abstract screening 227 articles were further discarded. The full-text of the 33 potentially relevant articles was assessed and 20 papers were excluded due to the following reasons: clinical condition other than cancer (1), no RCTs (4), intervention directed to parameters other than vascular (6), outcomes did not include markers of vascularization or tumor development measures (9). Therefore, 13 original articles were included in this review (Fig. 1).

3.2. Methodological quality assessment

The methodological quality of the included studies ranged from 3 to 5 out of a total 7 points. All studies were conducted with proper animal models, involving a random allocation to treatment group and were published in peer-reviewed journals. Inversely, sample size calculation was not reported by any study and only two referred a blinded assessment of outcomes. In addition, nine studies mentioned compliance with animal welfare regulations but only three studies included a potential conflict of interests' statement (Table 1).

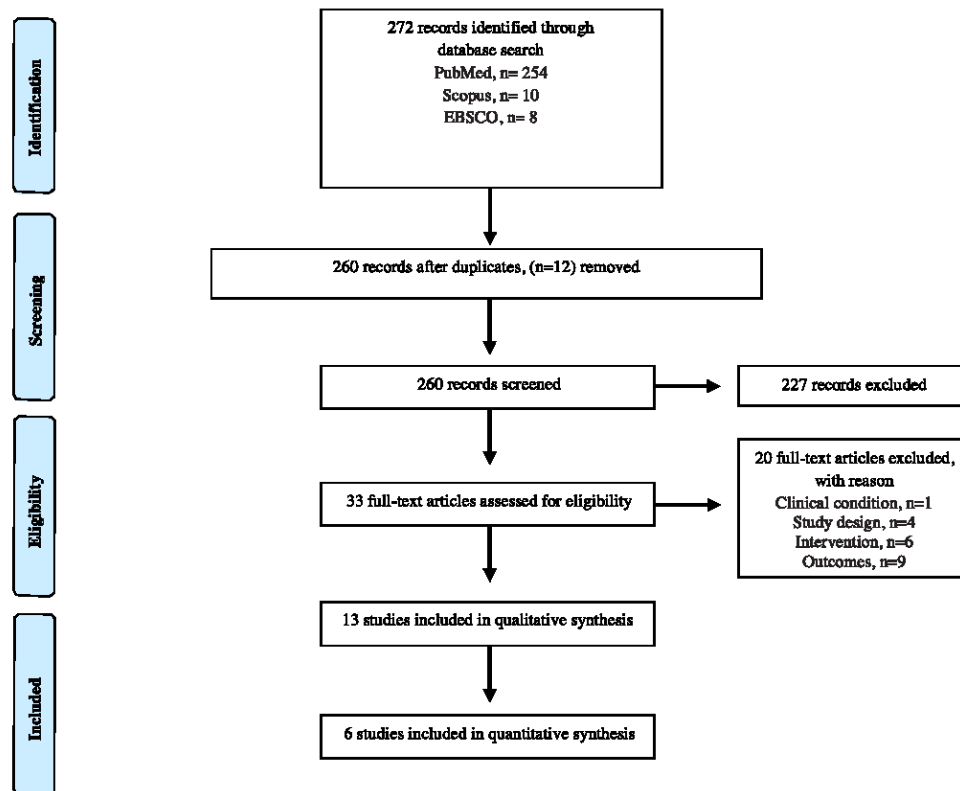


Fig. 1. Study flow diagram (PRISMA).

Table 1

Methodological quality of included studies (The Collaborative Approach to Meta-Analysis and Review of Animal Data from Experimental Studies (CAMARADES)).

CAMARADES	Total	1	2	3	4	5	6	7
(Yang et al., 2020)	5	X	✓	X	✓	✓	✓	✓
(La Porta et al., 2018)	5	X	✓	X	✓	✓	✓	✓
(Li et al., 2017)	5	X	✓	X	✓	✓	✓	✓
(Li et al., 2014)	4	X	✓	X	✓	✓	✓	X
(Mazzone et al., 2009)	4	X	✓	X	✓	✓	✓	X
(Franco et al., 2006)	3	X	✓	X	✓	✓	X	X
(Huang et al., 2003)	3	X	✓	X	✓	✓	X	X
(Dreves et al., 2002)	5	X	✓	✓	✓	✓	✓	X
(Wedge et al., 2002)	3	X	✓	X	✓	✓	X	X
(Beecken et al., 2001)	5	X	✓	✓	✓	✓	✓	X
(Kunkel et al., 2001)	3	X	✓	X	✓	✓	X	X
(Shaheen et al., 2001a)	4	X	✓	X	✓	✓	✓	X
(Shaheen et al., 2001b)	4	X	✓	X	✓	✓	✓	X
		0	13	2	13	13	9	3

Assessment criteria: (1) sample size calculation; (2) random allocation to treatment group; (3) blinded assessment of outcome; (4) appropriate animal species and models; (5) publication in a peer-reviewed journal; (6) compliance with animal welfare regulations; (7) statement of potential conflict of interests.

3.3. Study characteristics

The 13 original selected articles enrolled a total of 419 animals, ranged from 4 weeks (Li et al., 2017) to 12 weeks old (Mazzone et al., 2009; Kunkel et al., 2001), with a sample size ranging from 16 (Yang et al., 2020) to 78 animals (Beecken et al., 2001). Of these, 5 studies used immune-competent mice (La Porta et al., 2018; Li et al., 2017; Mazzone et al., 2009; Dreves et al., 2002; Shaheen et al., 2001a) and 8 studies an immune-compromised murine model (Li et al., 2014; Yang et al., 2020; Franco et al., 2006; Huang et al., 2003; Wedge et al., 2002; Kunkel et al., 2001; Shaheen et al., 2001b). Of the total studies included in the present

review, 6 studies used an allograft model (Li et al., 2017; Mazzone et al., 2009; Dreves et al., 2002; Kunkel et al., 2001; Shaheen et al., 2001a; La Porta et al., 2018) and 7 studies a xenograft one (Li et al., 2014; Franco et al., 2006; Huang et al., 2003; Wedge et al., 2002; Beecken et al., 2001; Shaheen et al., 2001b; Yang et al., 2020). Additionally, 6 studies applied a subcutaneous tumor cells inoculation (Li et al., 2017; Wedge et al., 2002; Beecken et al., 2001; Li et al., 2014; Yang et al., 2020; La Porta et al., 2018), 5 studies an orthotopic inoculation (Mazzone et al., 2009; Franco et al., 2006; Huang et al., 2003; Dreves et al., 2002; Kunkel et al., 2001) and 2 studies an intra-peritoneal inoculation (Shaheen et al., 2001a, b). Studies presented some heterogeneity regarding the type of tumor since 3 studies involved colon carcinoma (Shaheen et al., 2001a, b; Li et al., 2014), 2 studies renal carcinoma (Huang et al., 2003; Dreves et al., 2002), 1 study a mammary tumor (Franco et al., 2006) and another study glioblastoma (Kunkel et al., 2001). The remaining studies comprised lung carcinoma (Mazzone et al., 2009; Wedge et al., 2002; La Porta et al., 2018), melanoma (Li et al., 2017), bladder (Beecken et al., 2001) and gastric (Yang et al., 2020) carcinoma. Taking into account the tumor vascular assessment, besides MVD that all studies evaluated, 3 studies also analysed tumor perfusion (Franco et al., 2006; Mazzone et al., 2009; Li et al., 2017), 2 studies evaluated intratumoral hypoxia (Li et al., 2017; Mazzone et al., 2009) and 3 studies assessed the grade of vessel maturation (Franco et al., 2006; Mazzone et al., 2009; La Porta et al., 2018). Regarding intervention, the 13 studies applied specific procedures capable of influence the vascular feature of the tumor in order to verify the connexion between the increase or decrease of tumor vascularization and its growth. To do that, all studies had two groups, where a single experimental group (EG) was compared to a control group (CG).

3.4. Synthesis of the results

The results were summarized in two categories: tumor vascularization and tumor development, which includes size and metastasis

formation. Detailed information about each study is provided in Table 2.

3.4.1. Tumor vascularization and tumor development

For MVD analysis, most of the studies used the CD31 marker (Li et al., 2017, 2014; Mazzone et al., 2009; Franco et al., 2006; Huang et al., 2003; Drevs et al., 2002; Wedge et al., 2002; Beecken et al., 2001; Shaheen et al., 2001a, b) and 1 study the von Willebrand Factor (vWF) immunoexpression (Kunkel et al., 2001). MVD was defined by counting the number of vessels in 8 studies (La Porta et al., 2018; Yang et al., 2020; Li et al., 2014, 2017; Mazzone et al., 2009; Drevs et al., 2002; Franco et al., 2006; Beecken et al., 2001) and by measuring the total area covered by vessels in 2 studies (Wedge et al., 2002; Kunkel et al., 2001). Huang and colleagues assessed MVD through the positive pixel counting (Huang et al., 2003) and Shaheen and co-workers through endothelial cells (ECs) count (Shaheen et al., 2001b, a). Eleven studies reported a decreased tumor vascularization and a concomitant inhibition of tumor growth (Yang et al., 2020; Franco et al., 2006; Huang et al., 2003; Drevs et al., 2002; Wedge et al., 2002; Beecken et al., 2001; Kunkel et al., 2001; Shaheen et al., 2001b, a) or metastasis development (Li et al., 2017; La Porta et al., 2018), 1 study observed an increase in tumor vascularization and a consequent stimulation of tumor growth (Li et al., 2014) and 1 study, involving an immuno-competent mouse model, did not show differences related to vascular density or to tumor volume (Mazzone et al., 2009). All studies involving immuno-compromised mice showed a significant impact of vascular change in tumor growth.

Besides MVD, 3 studies evaluated the level of tumor perfusion. Mazzone et al. (2009) and Li et al. (2017) used the lectin marker and Franco et al. (2006) intravenously injected the perfusion marker Hoechst 33342. Contrary to Franco et al. (2006), that observed a decrease on the perfused area, Mazzone et al. (2009) and Li et al. (2017) found a significantly increase in tumor perfusion. In addition, vessel maturity was also assessed by 3 studies through the vessel maturation index (Franco et al. (2006); La Porta et al., 2018) or the percentage of laminin⁺ vessels Mazzone et al. (2009). All the three studies found a higher amount of mature tumour vessels. Finally, two studies evaluated the grade of tumor hypoxia by using the pimonidazole marker and it was observed a significant decrease of the hypoxic levels Li et al. (2017); Mazzone et al. (2009).

3.4.2. Results of the meta-analysis

Six studies were included in the meta-analysis involving a total of 183 animals. The EG received anti-angiogenic therapy with a mean duration of 25 days (19–35), showing a significant decrease of tumor vascularization, while the CG did not receive any manipulation of the tumor vasculature. The reduction in tumor vascularization in EG contributed to a decrease in the tumor growing rate of 5.23 (–9.20, –1.26), with a significant test for overall effect ($p = 0.01$) (Fig. 2).

4. Discussion

Typically, tumors can only grow to a few millimetres in size before tumor cells begin to exist beyond the diffusion distance of O₂ and other essential nutrients (500 μ m) (Multhoff et al., 2014). So, further growth requires the formation of a vascular system to meet the demands of the growing tumor mass (Horsman and Siemann, 2006). In this way, inhibition of tumor vessel formation and consequently the interruption of O₂ and nutrients supply could, theoretically, suppress the tumor growth process (Maj et al., 2016). Indeed, the results of the present meta-analysis support this notion. Using an antiangiogenic therapy, the studies of Franco et al. (2006); Huang et al. (2003); Wedge et al. (2002); Beecken et al. (2001) and Shaheen et al. (2001a, b), successfully decreased the tumor MVD. The reduced tumor vascularization limited the supply of O₂ and nutrients to tumor, resulting in a less evident growth rate (–5.23). This is in line with the evidence showing that tumor growth beyond 2 mm requires the formation of new blood vessels for an adequate provision of O₂ and nutrients (Bergers and Benjamin, 2003).

According to Hockel & Vaupel (Hockel and Vaupel, 2001), without a proper supply, the O₂ partial pressure (PO₂) falls below 45–50 mmHg, causing a decrease of O₂ consumption and ATP production, that hinders the active transport process in tumor cells, reducing proliferation and tumor growth. The study of La Porta et al. (2018) corroborated these findings by showing that the deletion of Tiel, an essential promoter of vascular development, reduced MVD of LLC and, consequently, decreased the number of lung metastasis. Similarly, Yang et al. (2020) also observed that the administration of Oridonin, a kaurene-type diterpenoid, reduced the MVD and induced apoptosis of gastric carcinoma, inhibiting its growth. In turn, Li et al. (2017) used a gold nanoparticles therapy to inhibit tumor angiogenesis on a melanoma allograft model. Although gold nanoparticles are customarily used as a vehicle for delivering biomolecules to target organs, it was shown that monotherapy by gold nanoparticles can inhibit blood vessels formation in hepatic tumors (Pan et al., 2013). Based on this assumption, Li and collaborators Li et al. (2017) observed a significant reduction in tumor MVD which resulted in a lower number of lung metastasis. Indeed, for a tumor cell to metastasize it must infiltrate into the blood vessels which is easier within the tumor milieu because tumor capillaries present fragmented and leaky basement membranes, making a considerable percentage of blood flow to stay in direct contact with tumor cells (Hashizume et al., 2000). However, the therapy with gold nanoparticles also resulted in less vascular permeability and consequently in increased tumor blood perfusion. This means that although there were less capillaries after the gold nanoparticles therapy, they were more capable of deliver blood and O₂, resulting in the decrease of tumor hypoxia. As such, it is possible that the action of gold nanoparticles that limited the possibility of cancer cells to infiltrate into the blood flow and then metastasize was driven by the modulation of tumor vasculature, allowing a higher level of tumor perfusion and an effective inhibition of tumor development.

To study the influence of MVD on tumor development, Li et al. (2014) injected PKM2 intraperitoneally for 8 days in mice with colon cancer and verified that the increased MVD favoured tumor growth. PKM2 is a glycolytic enzyme that co-activates the transcription factor HIF-1 α for angiogenesis stimulation, to support tumor growth (Luo et al., 2011). Nevertheless, in this study, the higher MVD promoted by PKM2 was accompanied by an increase in the number of vessel branches points that often coincides with blind vascular endings, with fewer pericytes and perivascular smooth muscle, leading to the extravasation of blood plasma and red blood cells. The result is an increase in the interstitial fluid pressure (IFP) and a consequent decrease of tumor perfusion (Multhoff et al., 2014; Yonenaga et al., 2005). In this line, Mazzone et al. (2009) studied the effect of PHD2 haploinsufficiency on tumor oxygenation and metastasis. PHD2 is considered to be the key O₂ sensor, as knockdown of PHD2 results in stabilization of HIF protein and a consequent angiogenic response (Chan et al., 2009). However, the authors verified that, although the haploinsufficiency of PHD2 did not affect the number of tumor vessels or the lumen size, they became more mature, with enhanced perivascular smooth muscle coverage, increasing the levels of tumor perfusion. As a result there was an improvement in tumor oxygenation that led to a reduction in the areas of hypoxia and to a decreased number of liver metastasis. Indeed, although MVD is an important therapy target in several types of cancer (Hasan et al., 2002; Sharma et al., 2005), as tumor vessels often possesses poorly developed intimal and medial layers (Baluk et al., 2003; Morikawa et al., 2002), the structural and functional features of tumor vasculature must be considered. Tumor angiogenesis is associated to excessive growth-promoting signals, which means that, contrary to other tissues, after ECs proliferation, there is not a resolution phase characterized by the loss of ECs proliferation and the recruitment of pericytes and vascular smooth muscle cells (VsMCs), that provides stabilization to endothelial tubes and induce the maturation of blood vessels (Hellstrom et al., 2001).

The maintenance of an optimal number of pericytes is indispensable

Table 2
Summary of the characteristics of identified studies.

Author, year	Sample	Tumor model	Intervention	Vascular outcomes	Results	Findings
(Yang et al., 2020)	16 female BALB/c nude mice (6 weeks)	Human gastric carcinoma BGC823 cell line Xenograft model Subcutaneous inoculation	Oridonin (80 mg kg ⁻¹ ·d ⁻¹ every day for 14 days)	MVD (CD31)	MVD (vessels/mm ²): EG = 9.14 ± 4.30 vs CG = 26 ± 3.79, p < 0.05 MVD (vessels/mm ²): EG = 150 ± 10 vs CG = 210 ± 90, p < 0.05	Tumor volume (mm ³): EG = 160 ± 50 vs CG = 280 ± 100, p < 0.05
(La Porta et al., 2018)	17 male and female Tie1fl/fl VE-cadherin-CreERT2 mice (7–9 weeks)	LLC cells Allograft model Subcutaneous inoculation	Tie1 deletion (5 days of tamoxifen (2 mg))	MVD (CD31) Vessel maturation index (α-SMA/ CD31)	α-SMA/ CD31: EG = 55 ± 10 vs CG = 45 ± 10, p < 0.05 MVD (vessels/field): EG = 1 ± 0.5 vs CG = 3 ± 0.5, p < 0.01	Lung metastasis: CG = 10 mice vs EG = 1 mouse
(Li et al., 2017)	30 female C57BL6 mice (4–6 weeks)	Melanoma B16F10 cell line Allograft model Subcutaneous inoculation	Gold nanoparticles (1 mg/kg) daily for 4 days and then intermittently for an additional 10 days	Vascular permeability (Dextran) Vascular perfusion (Lectin) Hypoxia (Pimonidazole)	Vascular permeability: EG = 0.3 ± 0.05 vs CG = 0.8 ± 0.1, p < 0.05 Vascular perfusion: EG = 0.45 ± 0.1 vs CG = 0.15 ± 0.05, p < 0.05 Hypoxia (% tumor area): EG = 20 ± 0.2 vs CG = 29 ± 0.1, p < 0.01	Lung metastasis: CG = 10 mice vs EG = 2 mice
(Li et al., 2014)	Athymic nude mice (5–6 weeks)	Human colon cancer SW620 cell line Xenograft model Subcutaneous inoculation	PKM2 (5 mg/kg) for 8 days, started 5 days post tumor inoculation	MVD (CD31)	MVD (vessels/mm ²): EG = 90.6 ± 19.5 vs CG = 41.4 ± 7.1, p < 0.01	Tumor volume (mm ³): EG = 330 ± 10 vs CG = 230 ± 20, p < 0.05
(Mazzone et al., 2009)	50 C57BL/6 mice (8–12 weeks old)	LLC cells Allograft model Orthotopic inoculation	Generation of PHD2 ^{+/-} mice	MVD (CD31) Perfusion (Lectin) Vessel maturity (Laminin) Hypoxia (Pimonidazole)	MVD (vessels/mm ²): EG = 42 ± 5 vs CG = 45 ± 8, p > 0.05 Perfusion (lectin ⁺ vessels/field): WT = 5.2 ± 0.5 vs PHD2 ^{+/-} = 7.9 ± 0.1, p = 0.003 Vessel maturity (% laminin ⁺ vessels): WT = 68 ± 4% vs PHD2 ^{+/-} = 89 ± 2%, p < 0.0001 Hypoxia (% tumor area): WT = 21 ± 2% vs PHD2 ^{+/-} = 7 ± 3%, p = 0.01	Tumor volume (mm ³): EG = 800 ± 200 vs CG = 750 ± 150, p = 0.33
(Franco et al., 2006)	30 CB-17 SCID female mice (6 weeks)	Human breast carcinoma MDA-MB-231 Xenograft model Orthotopic inoculation	DC101 (800 µg/mouse) twice a week over 3 weeks, started on day 27 of tumor growth	Perfusion (Hoechst 33342) Vessel maturation index (α-SMA ⁺ / CD31)	MVD (vessels/field): EG = 12.56 ± 6.16 vs CG = 41.94 ± 15.91, p < 0.0001 Perfusion (% tumor): EG = 3.97 ± 1.42 % vs CG = 9.69 ± 3.84 %, p < 0.05 Vessel maturation index : EG = 7.32 ± 0.44% vs CG = 1.33 ± 0.26%, p < 0.0005. 1 day after: MVD (pixel count): EG = 37.60 ± 23.43 vs CG = 81.17 ± 39.36, p = 0.037 Day 15 almost complete absence of vessels	Tumor volume (mm ³): EG = 600 ± 150 vs CG = 1700 ± 400, p < 0.001
(Huang et al., 2003)	70 female NCr nude mice (4–6 weeks)	Human SK-NEP-1 Wilms tumor cells Xenograft model Orthotopic inoculation	VEGF-Trap (500 µg), twice weekly for 5 weeks, initiated 5 weeks after tumor inoculation	MVD (CD31)	MVD (vessels/field): EG = 37.60 ± 23.43 vs CG = 81.17 ± 39.36, p = 0.037	Tumor weight (g): EG = 2.7 ± 0.8 vs CG = 13 ± 1.8, p < 0.001
(Dreves et al., 2002)	30 female BALB/c mice (6–8 weeks)	RENCA cells (obtained from a spontaneously BALB/c mice renal tumor) Allograft model		MVD (CD-31)	MVD (vessels/field): decrease by 48 % (p = 0.00031)	24 % primary tumor growth inhibition

(continued on next page)

Table 2 (continued)

Author, year	Sample	Tumor model	Intervention	Vascular outcomes	Results	Findings
(Wedge et al., 2002)	20 female athymic (nu/nu genotype) mice (8 weeks)	Orthotopic inoculation	ZD6474 (100 mg/kg/day) once daily for 24 days, from the day of tumor implantation	MVD (CD31)	MVD (CD31 ⁺ area/500 μm^2): EG = 5 ± 2 vs CG = 45 ± 6 , $p < 0.001$	30 % lung metastases decreased
		Xenograft model				Tumor volume (mm^3): EG = 500 ± 100 vs CG = 1400 ± 200 , $p < 0.01$
	78 female	Subcutaneous inoculation	TNP-470: 30 mg/kg every day for 3 weeks, started when tumor reached 1% of the mouse body weight	MVD (CD31)	MVD (vessels/field)	MGH-U1 tumor volume (mm^3)
(Beecken et al., 2001)	SCID mice (7–8 weeks)	RT-4 - slowly growing, well-differentiated human bladder carcinoma cell line	Angiostatin: 20 mg/kg every day for 3 weeks, started when tumor reached 1% of the mouse body weight		MGH-U1 tumors TNP-470: Decreased MVD ($p < 0.001$)	TNP-470: EG = 250 ± 100 vs CG = 1000 ± 400 , $p < 0.001$
		MGH-U1, a quickly growing, poorly differentiated, highly aggressive human bladder carcinoma cell line			mFc-m-angiostatin: Decreased MVD ($p < 0.001$)	mFc-m-angiostatin: EG = 200 ± 50 vs CG = 1000 ± 400 , $p < 0.001$
		Xenograft model			RT-4 tumors TNP-470	RT-4 tumors TNP-470: EG = 75 ± 25 vs CG = 130 ± 50 , $p < 0.001$
(Kunkel et al., 2001)	Female SCID mice (11–12 weeks)	Glioblastoma G55	DC101 (800 μg) every day for 16 days, starting on the day of tumor cell inoculation	MVD (vWF)	Decreased MVD ($p = 0.004$)	mFc-m-angiostatin: EG = 20 ± 25 vs CG = 130 ± 50 , $p < 0.001$
		cell line				Tumor volume (mm^3): EG = 3 ± 0.5 vs CG = 10.5 ± 2 , $p < 0.005$
		Allograft model				
(Shaheen et al., 2001a)	37 male	Orthotopic inoculation	DC101 (800 μg) every third day for 19 days, beginning 3 days after the injection of tumor cells	MVD (CD31)	MVD: (CD31 ⁺ /field): EG = 10 ± 2 vs CG = 22 ± 2 , $p < 0.001$	Tumor size (mm): EG = 1.9 ± 0.05 vs CG = 2.3 ± 0.1 , $p < 0.05$
	BALB/c mice (8 weeks)	CT-26 murine colon carcinoma cells				
(Shaheen et al., 2001b)	40 male athymic nude mice (8 weeks)	Allograft model	DC101 (0.8 mg) every third day, for 30 days, started 3 days after tumor cells inoculation	MVD (CD31)	MVD: (CD31 ⁺ /field): EG = 9.5 ± 1.5 vs CG = 16.5 ± 2 , $p < 0.001$	Tumor size (mm): EG = 1.8 ± 0.2 vs CG = 3.4 ± 0.5 , $p < 0.05$
		Intra-peritoneal inoculation				

CG: control group; EG: experimental group; LLC: Lewis lung carcinoma; MVD: microvessel density; ns: not significant; PHD2: Prolyl hydroxylase domain protein2; SCID, severe combined immunodeficient; VEGF, vascular endothelial growth factor; vWF: von Willebrand Factor; WT: wild type.

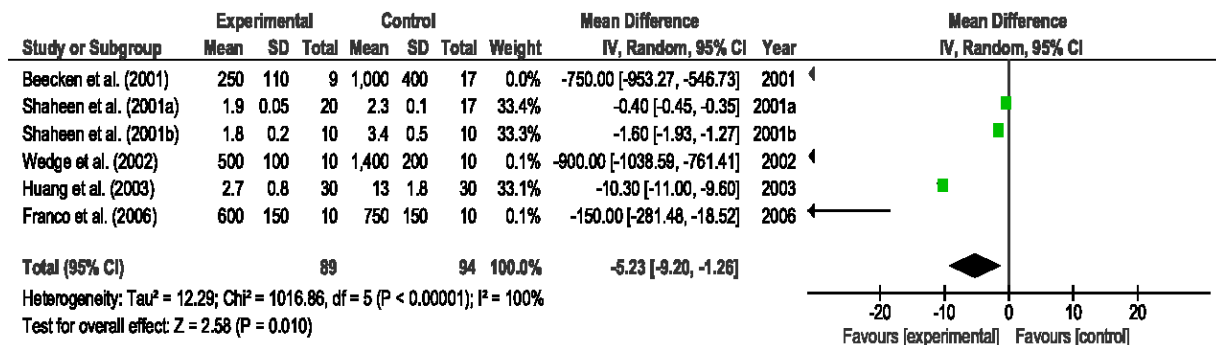


Fig. 2. Forest plot.

to stabilize newly formed endothelial tubes, because it supports an adequate blood flow and prevents further sprouting (Chung et al., 2010). Indeed, during angiogenesis, pericytes generate long cytoplasmic processes on the surface of ECs, creating tight connexions that are important for the stability and function of capillaries (Armulik et al., 2005).

However, whereas normal vessels present an appropriate number of pericytes, in tumor these cells are less abundant and show abnormal phenotypes (Abramsson et al., 2002; Hashizume et al., 2000). Indeed, during cancer progression, pericytes frequently change its shape and the cytoplasmic processes do not always colocalize with ECs, making them,

quite often, entirely detached from the endothelium (Abramsson et al., 2002; Morikawa et al., 2002). Considering that cell–cell communication is of major importance for vessels to act as a synchronized unit along their longitudinal axis and to inform upstream feeding vessels about the perfusion status of downstream tissues (Pries et al., 2010), these findings suggest that deficient pericyte–ECs interactions in tumor capillaries may induce hematogenous and lymphatic tumor cell spreading (Xian et al., 2006), contributing to high IFP and reduced delivery of drug molecules to solid tumors (Libutti et al., 2018).

Additionally, Ashraf and co-workers found, in human liver metastases, that the tunica media of blood vessels is composed by cells with a fibroblast-like phenotype that cannot generate contractile forces similar to those that are produced by the smooth muscle cells and, therefore, are unable to induce changes in tumor blood circulation in response to vasoactive stimuli (Ashraf et al., 1996). Consequently, the average blood flow in tumors is lower than in normal tissues, with insufficient O₂ and nutrients supply and reduced clearance of by-products of metabolism, resulting in intratumoral hypoxia and acidification (Petrova et al., 2018; Jing et al., 2019). This hypoxic and acidic microenvironment enhances the expression of pro-angiogenic factors, such as collagenase and plasminogen activators, that further disrupts the basement membrane of vessels and triggers angiogenesis (Ribatti et al., 2007). Together these events prolong the angiogenic cascade and ECs become progressively unstable, heterogeneous and resistant (Akino et al., 2009), attenuating the effect of the antiangiogenic agents over time (Kerbel, 2001; Masoud and Pages, 2017; van Beijnum et al., 2015).

Therefore, vascular remodelling appears to be a key promoter of intratumoral perfusion and oxygenation, preventing tumor invasiveness and metastasis (van Beijnum et al., 2015). Interestingly, besides growth factor signalling, blood vessels are also regulated by mechanical stimuli created by changes in blood flow, velocity and pressure (Qiu et al., 2019). Indeed, blood flow creates shear stress, the mechanical force on endothelium that is critical for the establishment of mature and functional vasculature during development (Schadler et al., 2016). In response to shear stress, ECs hypertrophy, the density of actin microfilaments and focal adhesions increases and the amount of adhesion-associated proteins is greater than in cells that do not experience shear stress (Ballermann and Ott, 1995). Curiously, one of the many effects of endurance exercise is a systemic increase in blood flow that stimulates the mechanical force on ECs (Green et al., 2017). During physical exercise, there is a re-distribution of cardiac output, with shunting of blood flow from non-active tissues to the contracting muscles (Rowell, 1974). Partially, this shunt occurs through the enhancement of sympathetic system activity and the release of norepinephrine, that bind to α -adrenergic receptors on the vascular smooth muscle inducing vasoconstriction (Mishra et al., 2018). However, the reduced α -adrenergic vasoconstriction and the loss of myogenic tone at higher intraluminal pressures of tumor arterioles inhibit the action of the sympathetic nervous system, resulting in the increase of tumor blood flow during physical exercise (McCullough et al., 2014). There are several benefits to improve tumor blood perfusion; first, there is an increased oxygenation, preventing the development of intratumoral hypoxia; second, the accessibility of circulating immune cells is greater; third, the delivery of anti-neoplastic medication to tumors is facilitated (McGee et al., 2010; Missiaen et al., 2018; Martin et al., 2016). Indeed, tumor blood supply plays an important role in the delivery of therapeutic agents to solid tumors (Seynhaeve et al., 2020) and recent studies found that exercise training improves the penetration of Gemcitabine and Doxorubicin, in pancreatic adenocarcinoma (Schadler et al., 2016) and Ewing's Sarcoma (Morrell et al., 2019) of mice, respectively. Clinically, this suggests that physical exercise may have the potential to enhance tumor perfusion by promoting the remodelling of tumor vessels and, therefore, it may be considered, as co-adjuvant non-pharmacologic therapy, in the oncologic field (Morrell et al., 2019; Betof et al., 2015; McCullough et al., 2014).

4.1. Limitations

This systematic review has some limitations that need to be considered. First, two studies did not report the sample size (Li et al., 2014; Kunkel et al., 2001). Second, the studies included in the present review involved a comparison between different cancer models and from a more rigorous point of view, the assessment of the vascularization should be done within the same tumor type. Third, from the 13 studies, only 6 were included in the meta-analysis due to missing information related to the sample size (Kunkel et al., 2001; Li et al., 2014), study results (Dreves et al., 2002) and intervention (Mazzone et al., 2009; Li et al., 2017; Yang et al., 2020; La Porta et al., 2018). Finally, another point that may represent an important limitation is the fact that the studies included in the meta-analysis involved anti-angiogenic therapy with a mean duration of only 25 days. This means that we can observe the acute and sub-acute effects of the therapy but, first, we cannot analyse the long term results on tumor vascularization and, second, makes impossible to know if an acute and a potential chronic decrease in tumor vascularization have the same effect on tumor growth. Therefore, to attain a better methodological quality, good RCTs should be designed in the future. The design should be double blinded, with a long term follow up and the clinical condition should be the most homogeneous possible. This will result in higher quality studies that can help us to better understand the influence of tumor vascularization on its growth.

5. Conclusion

The current meta-analysis provides evidence suggesting a relation between vascularization and tumor growth, meaning that the more vascularized the tumor is, the greater potential to grow appears to have. However, it seems that the lack of vascular efficiency may be also an important feature of tumor growth because is intimately related to the lack of tumor perfusion and consequently the inability of immune cells and anti-cancer drugs to infiltrate and to mitigate tumor proliferation. Therefore, the knowledge of the relationship between vascularization, perfusion and tumor growth is of particular interest because may give a new set of possibilities in order to optimize the evaluation and treatment of cancer patients, providing them with clinically relevant benefits.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

References

- Abramsson, A., Berlin, O., Papayan, H., Paulin, D., Shani, M., Betsholtz, C., 2002. Analysis of mural cell recruitment to tumor vessels. *Circulation* 105 (1), 112–117. <https://doi.org/10.1161/hc0102.101437>.
- Akino, T., Hida, K., Hida, Y., Tsuchiya, K., Freedman, D., Muraki, C., Ohga, N., Matsuda, K., Akiyama, K., Harabayashi, T., Shinohara, N., Nonomura, K., Klagsbrun, M., Shindoh, M., 2009. Cytogenetic abnormalities of tumor-associated endothelial cells in human malignant tumors. *Am. J. Pathol.* 175 (6), 2657–2667. <https://doi.org/10.2353/ajpath.2009.090202>.
- Anderson, K.G., Stromnes, I.M., Greenberg, P.D., 2017. Obstacles posed by the tumor microenvironment to t cell activity: a case for synergistic therapies. *Cancer Cell* 31 (3), 311–325. <https://doi.org/10.1016/j.ccell.2017.02.008>.
- Armulik, A., Abramsson, A., Betsholtz, C., 2005. Endothelial/pericyte interactions. *Circ. Res.* 97 (6), 512–523. <https://doi.org/10.1161/01.RES.0000182903.16652.d7>.
- Ashraf, S., Crowe, R., Loizidou, M.C., Turmaine, M., Taylor, I., Burnstock, G., 1996. The absence of autonomic perivascular nerves in human colorectal liver metastases. *Br. J. Cancer* 73 (3), 349–359. <https://doi.org/10.1038/bjc.1996.60>.
- Ballermann, B.J., Ott, M.J., 1995. Adhesion and differentiation of endothelial cells by exposure to chronic shear stress: a vascular graft model. *Blood Purif.* 13 (3–4), 125–134. <https://doi.org/10.1159/000170195>.
- Baluk, P., Morikawa, S., Haskell, A., Mancuso, M., McDonald, D.M., 2003. Abnormalities of basement membrane on blood vessels and endothelial sprouts in tumors. *Am. J. Pathol.* 163 (5), 1801–1815. [https://doi.org/10.1016/S0002-9440\(10\)63540-7](https://doi.org/10.1016/S0002-9440(10)63540-7).
- Beecken, W.D., Fernandez, A., Jousen, A.M., Achilles, E.G., Flynn, E., Lo, K.M., Gillies, S. D., Javaherian, K., Folkman, J., Shing, Y., 2001. Effect of antiangiogenic therapy on slowly growing, poorly vascularized tumors in mice. *J. Natl. Cancer Inst.* 93 (5), 382–387. <https://doi.org/10.1093/jnci/93.5.382>.

- Bell, W.C., Sexton, K.C., Grizzle, W.E., 2009. How to efficiently obtain human tissues to support specific biomedical research projects. *Cancer Epidemiol. Biomarkers Prev.* 18 (6), 1676–1679. <https://doi.org/10.1158/1055-9965.EPI-08-0820>.
- Bergers, G., Benjamin, L.E., 2003. Tumorigenesis and the angiogenic switch. *Nat. Rev. Cancer* 3 (6), 401–410. <https://doi.org/10.1038/nrc1093>.
- Betof, A.S., Lascola, C.D., Weitzel, D., Landon, C., Scarbrough, P.M., Devi, G.R., Palmer, G., Jones, L.W., Dewhirst, M.W., 2015. Modulation of murine breast tumor vascularity, hypoxia and chemotherapeutic response by exercise. *J. Natl. Cancer Inst.* 107 (5) <https://doi.org/10.1093/jnci/djv040>.
- Bossi, P., Viale, G., Lee, A.K., Alfano, R., Coggi, G., Bosari, S., 1995. Angiogenesis in colorectal tumors: microvessel quantitation in adenomas and carcinomas with clinicopathological correlations. *Cancer Res.* 55 (21), 5049–5053.
- Carbone, C., Moccia, T., Zhu, C., Paradiso, G., Budillon, A., Chiao, P.J., Abbruzzese, J.L., Melisi, D., 2011. Anti-VEGF treatment-resistant pancreatic cancers secrete proinflammatory factors that contribute to malignant progression by inducing an EMT cell phenotype. *Clin. Cancer Res.* 17 (17), 5822–5832. <https://doi.org/10.1158/1078-0432.CCR-11-1185>.
- Cekanova, M., Rathore, K., 2014. Animal models and therapeutic molecular targets of cancer: utility and limitations. *Drug Des. Dev. Ther.* 8, 1911–1921. <https://doi.org/10.2147/DDDT.S49584>.
- Chan, D.A., Kawahara, T.L., Sutphin, P.D., Chang, H.Y., Chi, J.T., Giaccia, A.J., 2009. Tumor vasculature is regulated by PHD2-mediated angiogenesis and bone marrow-derived cell recruitment. *Cancer Cell* 15 (6), 527–538. <https://doi.org/10.1016/j.ccr.2009.04.010>.
- Chung, A.S., Lee, J., Ferrara, N., 2010. Targeting the tumour vasculature: insights from physiological angiogenesis. *Nat. Rev. Cancer* 10 (7), 505–514. <https://doi.org/10.1038/nrc2868>.
- Dreys, J., Muller-Driver, R., Wittig, C., Fuxius, S., Esser, N., Hugenschmidt, H., Konerding, M.A., Allegrini, P.R., Wood, J., Hennig, J., Unger, C., Marme, D., 2002. PTK787/ZK 222584, a specific vascular endothelial growth factor-receptor tyrosine kinase inhibitor, affects the anatomy of the tumor vascular bed and the functional vascular properties as detected by dynamic enhanced magnetic resonance imaging. *Cancer Res.* 62 (14), 4015–4022.
- Ebos, J.M., Lee, C.R., Cruz-Munoz, W., Bjarnason, G.A., Christensen, J.G., Kerbel, R.S., 2009. Accelerated metastasis after short-term treatment with a potent inhibitor of tumor angiogenesis. *Cancer Cell* 15 (3), 232–239. <https://doi.org/10.1016/j.ccr.2009.01.021>.
- Finger, E.C., Giaccia, A.J., 2010. Hypoxia, inflammation, and the tumor microenvironment in metastatic disease. *Cancer Metastasis Rev.* 29 (2), 285–293. <https://doi.org/10.1007/s10555-010-9224-5>.
- Folkman, J., 1995. Angiogenesis in cancer, vascular, rheumatoid and other disease. *Nat. Med.* 1 (1), 27–31.
- Franco, M., Man, S., Chen, L., Emmenegger, U., Shaked, Y., Cheung, A.M., Brown, A.S., Hicklin, D.J., Foster, F.S., Kerbel, R.S., 2006. Targeted anti-vascular endothelial growth factor receptor-2 therapy leads to short-term and long-term impairment of vascular function and increase in tumor hypoxia. *Cancer Res.* 66 (7), 3639–3648. <https://doi.org/10.1158/0008-5472.CAN-05-3295>.
- Green, D.J., Hopman, M.T., Padilla, J., Laughlin, M.H., Thijssen, D.H., 2017. Vascular adaptation to exercise in humans: role of hemodynamic stimuli. *Physiol. Rev.* 97 (2), 495–528. <https://doi.org/10.1152/physrev.00014.2016>.
- Hasan, J., Byers, R., Jayson, G.C., 2002. Intra-tumoural microvessel density in human solid tumours. *Br. J. Cancer* 86 (10), 1566–1577. <https://doi.org/10.1038/sj.bjc.6600315>.
- Hashizume, H., Baluk, P., Morikawa, S., McLean, J.W., Thurston, G., Roberge, S., Jain, R.K., McDonald, D.M., 2000. Openings between defective endothelial cells explain tumor vessel leakiness. *Am. J. Pathol.* 156 (4), 1363–1380. [https://doi.org/10.1016/S0002-9440\(10\)65006-7](https://doi.org/10.1016/S0002-9440(10)65006-7).
- Hellstrom, M., Gerhardt, H., Kalen, M., Li, X., Eriksson, U., Wolburg, H., Betsholtz, C., 2001. Lack of pericytes leads to endothelial hyperplasia and abnormal vascular morphogenesis. *J. Cell Biol.* 153 (3), 543–553. <https://doi.org/10.1083/jcb.153.3.543>.
- Hendriksen, E.M., Span, P.N., Schuurin, J., Peters, J.P., Sweep, F.C., van der Kogel, A.J., Bussink, J., 2009. Angiogenesis, hypoxia and VEGF expression during tumour growth in a human xenograft tumour model. *Microvasc. Res.* 77 (2), 96–103. <https://doi.org/10.1016/j.mvr.2008.11.002>.
- Hockel, M., Vaupel, P., 2001. Tumor hypoxia: definitions and current clinical, biologic, and molecular aspects. *J. Natl. Cancer Inst.* 93 (4), 266–276. <https://doi.org/10.1093/jnci/93.4.266>.
- Horsman, M.R., Siemann, D.W., 2006. Pathophysiologic effects of vascular-targeting agents and the implications for combination with conventional therapies. *Cancer Res.* 66 (24), 11520–11539. <https://doi.org/10.1158/0008-5472.CAN-06-2848>.
- Huang, J., Frischer, J.S., Serur, A., Kadenhe, A., Yokoi, A., McCrudden, K.W., New, T., O'Toole, K., Zabski, S., Rudge, J.S., Holash, J., Yancopoulos, G.D., Yamashiro, D.J., Kandel, J.J., 2003. Regression of established tumors and metastases by potent vascular endothelial growth factor blockade. *Proc. Natl. Acad. Sci. U. S. A.* 100 (13), 7785–7790. <https://doi.org/10.1073/pnas.1432908100>.
- Jahangiri, A., De Lay, M., Miller, L.M., Carbonell, W.S., Hu, Y.L., Lu, K., Tom, M.W., Paquette, J., Tokuyasu, T.A., Tsao, S., Marshall, R., Perry, A., Bjorgan, K.M., Chaumeil, M.M., Ronen, S.M., Bergers, G., Aghi, M.K., 2013. Gene expression profile identifies tyrosine kinase c-Met as a targetable mediator of antiangiogenic therapy resistance. *Clin. Cancer Res.* 19 (7), 1773–1783. <https://doi.org/10.1158/1078-0432.CCR-12-1281>.
- Jain, R.K., 2001. Normalizing tumor vasculature with anti-angiogenic therapy: a new paradigm for combination therapy. *Nat. Med.* 7 (9), 987–989. <https://doi.org/10.1038/nm0901-987>.
- Jing, X., Yang, F., Shao, C., Wei, K., Xie, M., Shen, H., Shu, Y., 2019. Role of hypoxia in cancer therapy by regulating the tumor microenvironment. *Mol. Cancer* 18 (1), 157. <https://doi.org/10.1186/s12943-019-1089-9>.
- Kerbel, R.S., 2001. Clinical trials of antiangiogenic drugs: opportunities, problems, and assessment of initial results. *J. Clin. Oncol.* 19 (18 Suppl), 45S–51S.
- Kunkel, P., Ulbricht, U., Bohlen, P., Brockmann, M.A., Fillbrandt, R., Stavrou, D., Westphal, M., Lamszus, K., 2001. Inhibition of glioma angiogenesis and growth in vivo by systemic treatment with a monoclonal antibody against vascular endothelial growth factor receptor-2. *Cancer Res.* 61 (18), 6624–6628.
- La Porta, S., Roth, L., Singhal, M., Mogler, C., Speeg, C., Schieb, B., Qu, X., Adams, R.H., Baldwin, H.S., Savant, S., Augustin, H.G., 2018. Endothelial Tie1-mediated angiogenesis and vascular abnormalization promote tumor progression and metastasis. *J. Clin. Invest.* 128 (2), 834–845. <https://doi.org/10.1172/JCI94674>.
- Li, L., Zhang, Y., Qiao, J., Yang, J.J., Liu, Z.R., 2014. Pyruvate kinase M2 in blood circulation facilitates tumor growth by promoting angiogenesis. *J. Biol. Chem.* 289 (37), 25812–25821. <https://doi.org/10.1074/jbc.M114.576934>.
- Li, W., Li, X., Liu, S., Yang, W., Pan, F., Yang, X.Y., Du, B., Qin, L., Pan, Y., 2017. Gold nanoparticles attenuate metastasis by tumor vasculature normalization and epithelial-mesenchymal transition inhibition. *Int. J. Nanomed.* 12, 3509–3520. <https://doi.org/10.2147/IJN.S128802>.
- Libutti, S.K., Tamarkin, L., Nilubol, N., 2018. Targeting the invincible barrier for drug delivery in solid cancers: interstitial fluid pressure. *Oncotarget* 9 (87), 35723–35725. <https://doi.org/10.18632/oncotarget.26267>.
- Luo, W., Hu, H., Chang, R., Zhong, J., Knabel, M., O'Meally, R., Cole, R.N., Pandey, A., Semenza, G.L., 2011. Pyruvate kinase M2 is a PHD3-stimulated coactivator for hypoxia-inducible factor 1. *Cell* 145 (5), 732–744. <https://doi.org/10.1016/j.cell.2011.03.054>.
- Macleod, M.R., Fisher, M., O'Collins, V., Sena, E.S., Dirnagl, U., Bath, P.M., Buchan, A., van der Worp, H.B., Traystman, R., Minematsu, K., Donnan, G.A., Howells, D.W., 2009. Good laboratory practice: preventing introduction of bias at the bench. *Stroke* 40 (3), e50–52. <https://doi.org/10.1161/STROKEAHA.108.525386>.
- Maj, E., Papiernik, D., Wietrzyk, J., 2016. Antiangiogenic cancer treatment: the great discovery and greater complexity (Review). *Int. J. Oncol.* 49 (5), 1773–1784. <https://doi.org/10.3892/ijo.2016.3709>.
- Martin, J.D., Fukumura, D., Duda, D.G., Boucher, Y., Jain, R.K., 2016. Reengineering the tumor microenvironment to alleviate hypoxia and overcome cancer heterogeneity. *Cold Spring Spring. Perspect. Med.* 6 (12) <https://doi.org/10.1101/cshperspect.a027094>.
- Masoud, V., Pages, G., 2017. Targeted therapies in breast cancer: new challenges to fight against resistance. *World J. Clin. Oncol.* 8 (2), 120–134. <https://doi.org/10.5306/wjco.v8.i2.120>.
- Mazzone, M., Dettori, D., de Oliveira, R.L., Loges, S., Schmidt, T., Jonckx, B., Tian, Y.M., Lanahan, A.A., Pollard, P., de Almodovar, C.R., De Smet, F., Vinckier, S., Aragonés, J., Debackere, K., Luttun, A., Wyns, S., Jordan, B., Pisacane, A., Gallez, B., Lampugnani, M.G., Dejana, E., Simons, M., Ratcliffe, P., Maxwell, P., Carmeliet, P., 2009. Heterozygous deficiency of PHD2 restores tumor oxygenation and inhibits metastasis via endothelial normalization. *Cell* 136 (5), 839–851. <https://doi.org/10.1016/j.cell.2009.01.020>.
- McCullough, D.J., Stabley, J.N., Siemann, D.W., Behnke, B.J., 2014. Modulation of blood flow, hypoxia, and vascular function in orthotopic prostate tumors during exercise. *J. Natl. Cancer Inst.* 106 (4), djuo36 <https://doi.org/10.1093/jnci/dju036>.
- McGee, M.C., Hamner, J.B., Williams, R.F., Rosati, S.F., Sims, T.L., Ng, C.Y., Gaber, M.W., Calabrese, C., Wu, J., Nathwani, A.C., Duntsch, C., Merchant, T.E., Davidoff, A.M., 2010. Improved intratumoral oxygenation through vascular normalization increases glioma sensitivity to ionizing radiation. *Int. J. Radiat. Oncol. Biol. Phys.* 76 (5), 1537–1545. <https://doi.org/10.1016/j.ijrobp.2009.12.010>.
- Mehta, S., Hughes, N.P., Buffa, F.M., Li, S.P., Adams, R.F., Adwani, A., Taylor, N.J., Levitt, N.C., Padhani, A.R., Makris, A., Harris, A., 2011. Assessing early therapeutic response to bevacizumab in primary breast cancer using magnetic resonance imaging and gene expression profiles. *J. Natl. Cancer Inst. Monogr.* 2011 (43), 71–74. <https://doi.org/10.1093/jncimonographs/lgr027>.
- Menakuru, S.R., Brown, N.J., Staton, C.A., Reed, M.W., 2008. Angiogenesis in pre-malignant conditions. *Br. J. Cancer* 99 (12), 1961–1966. <https://doi.org/10.1038/sj.bjc.6604733>.
- Mishra, R.C., Rahman, M.M., Davis, M.J., Wulff, H., Hill, M.A., Braun, A.P., 2018. Alpha1-adrenergic stimulation selectively enhances endothelium-mediated vasodilation in rat cremaster arteries. *Physiol. Rep.* 6 (9), e13703 <https://doi.org/10.14814/phys2.13703>.
- Missian, R., Mazzone, M., Bergers, G., 2018. The reciprocal function and regulation of tumor vessels and immune cells offers new therapeutic opportunities in cancer. *Semin. Cancer Biol.* 52 (Pt 2), 107–116. <https://doi.org/10.1016/j.semcancer.2018.06.002>.
- Mittal, K., Ebos, J., Rini, B., 2014. Angiogenesis and the tumor microenvironment: vascular endothelial growth factor and beyond. *Semin. Oncol.* 41 (2), 235–251. <https://doi.org/10.1053/j.seminoncol.2014.02.007>.
- Moher, D., Liberati, A., Tetzlaff, J., Altman, D.G., Group, P., 2009. Preferred reporting items for systematic reviews and meta-analyses: the PRISMA statement. *PLoS Med.* 6 (7), e1000097 <https://doi.org/10.1371/journal.pmed.1000097>.
- Morikawa, S., Baluk, P., Kaidoh, T., Haskell, A., Jain, R.K., McDonald, D.M., 2002. Abnormalities in pericytes on blood vessels and endothelial sprouts in tumors. *Am. J. Pathol.* 160 (3), 985–1000. [https://doi.org/10.1016/S0002-9440\(10\)64920-6](https://doi.org/10.1016/S0002-9440(10)64920-6).
- Morrell, M.B.G., Alvarez-Florez, C., Zhang, A., Kleiner, E.S., Savage, H., Marmonti, E., Park, M., Shaw, A., Schadler, K.L., 2019. Vascular modulation through exercise improves chemotherapy efficacy in Ewing sarcoma. *Pediatr. Blood Cancer* 66 (9), e27835. <https://doi.org/10.1002/pbc.27835>.

- Multhoff, G., Radons, J., Vaupel, P., 2014. Critical role of aberrant angiogenesis in the development of tumor hypoxia and associated radioresistance. *Cancers (Basel)* 6 (2), 813–828. <https://doi.org/10.3390/cancers6020813>.
- Paez-Ribes, M., Allen, E., Hudock, J., Takeda, T., Okuyama, H., Vinals, F., Inoue, M., Bergers, G., Hanahan, D., Casanovas, O., 2009. Antiangiogenic therapy elicits malignant progression of tumors to increased local invasion and distant metastasis. *Cancer Cell* 15 (3), 220–231. <https://doi.org/10.1016/j.ccr.2009.01.027>.
- Pan, Y., Ding, H., Qin, L., Zhao, X., Cai, J., Du, B., 2013. Gold nanoparticles induce nanostructural reorganization of VEGFR2 to repress angiogenesis. *J. Biomed. Nanotechnol.* 9 (10), 1746–1756.
- Petrova, V., Annicchiarico-Petruzzelli, M., Melino, G., Amelio, I., 2018. The hypoxic tumour microenvironment. *Oncogenesis* 7 (1), 10. <https://doi.org/10.1038/s41389-017-0011-9>.
- Piao, Y., Liang, J., Holmes, L., Henry, V., Sulman, E., de Groot, J.F., 2013. Acquired resistance to anti-VEGF therapy in glioblastoma is associated with a mesenchymal transition. *Clin. Cancer Res.* 19 (16), 4392–4403. <https://doi.org/10.1158/1078-0432.CCR-12-1557>.
- Pries, A.R., Hopfner, M., le Noble, F., Dewhirst, M.W., Secomb, T.W., 2010. The shunt problem: control of functional shunting in normal and tumour vasculature. *Nat. Rev. Cancer* 10 (8), 587–593. <https://doi.org/10.1038/nrc2895>.
- Qiu, Y., Myers, D.R., Lam, W.A., 2019. The biophysics and mechanics of blood from a materials perspective. *Nat. Rev. Mater.* 4 (5), 294–311. <https://doi.org/10.1038/s41578-019-0099-y>.
- Renner, K., Singer, K., Koehl, G.E., Geissler, E.K., Peter, K., Siska, P.J., Kreutz, M., 2017. Metabolic hallmarks of tumor and immune cells in the tumor microenvironment. *Front. Immunol.* 8, 248. <https://doi.org/10.3389/fimmu.2017.00248>.
- Ribatti, D., Nico, B., Crivellato, E., Roccaro, A.M., Vacca, A., 2007. The history of the angiogenic switch concept. *Leukemia* 21 (1), 44–52. <https://doi.org/10.1038/sj.leu.2404402>.
- Ribatti, D., Annese, T., Ruggieri, S., Tamma, R., Crivellato, E., 2019. Limitations of anti-angiogenic treatment of tumors. *Transl. Oncol.* 12 (7), 981–986. <https://doi.org/10.1016/j.tranon.2019.04.022>.
- Rowell, L.B., 1974. Human cardiovascular adjustments to exercise and thermal stress. *Physiol. Rev.* 54 (1), 75–159. <https://doi.org/10.1152/physrev.1974.54.1.75>.
- Schadler, K.L., Thomas, N.J., Galie, P.A., Bhang, D.H., Roby, K.C., Addai, P., Till, J.E., Sturgeon, K., Zaslavsky, A., Chen, C.S., Ryeom, S., 2016. Tumor vessel normalization after aerobic exercise enhances chemotherapeutic efficacy. *Oncotarget* 7 (40), 65429–65440. <https://doi.org/10.18632/oncotarget.11748>.
- Seynhaeve, A.L.B., Amin, M., Haemmerich, D., van Rhoon, G.C., Ten Hagen, T.L.M., 2020. Hyperthermia and smart drug delivery systems for solid tumor therapy. *Adv. Drug Deliv. Rev.* <https://doi.org/10.1016/j.addr.2020.02.004>.
- Shaheen, R.M., Tseng, W.W., Vellagas, R., Liu, W., Ahmad, S.A., Jung, Y.D., Reinmuth, N., Drazan, K.E., Bucana, C.D., Hicklin, D.J., Ellis, L.M., 2001a. Effects of an antibody to vascular endothelial growth factor receptor-2 on survival, tumor vascularity, and apoptosis in a murine model of colon carcinomatosis. *Int. J. Oncol.* 18 (2), 221–226.
- Shaheen, R.M., Ahmad, S.A., Liu, W., Reinmuth, N., Jung, Y.D., Tseng, W.W., Drazan, K.E., Bucana, C.D., Hicklin, D.J., Ellis, L.M., 2001b. Inhibited growth of colon cancer carcinomatosis by antibodies to vascular endothelial and epidermal growth factor receptors. *Br. J. Cancer* 85 (4), 584–589. <https://doi.org/10.1054/bjoc.2001.1936>.
- Sharma, S., Sharma, M.C., Sarkar, C., 2005. Morphology of angiogenesis in human cancer: a conceptual overview, histopathologic perspective and significance of neoangiogenesis. *Histopathology* 46 (5), 481–489. <https://doi.org/10.1111/j.1365-2559.2005.02142.x>.
- Uliivi, P., Marisi, G., Passardi, A., 2016. Relationship between hypoxia and response to antiangiogenic therapy in metastatic colorectal cancer. *Oncotarget* 7 (29), 46678–46691. <https://doi.org/10.18632/oncotarget.8712>.
- van Beijnum, J.R., Nowak-Sliwinski, P., Huijbers, E.J., Thijssen, V.L., Griffioen, A.W., 2015. The great escape; the hallmarks of resistance to antiangiogenic therapy. *Pharmacol. Rev.* 67 (2), 441–461. <https://doi.org/10.1124/pr.114.010215>.
- Van der Veldt, A.A., Lubberink, M., Bahce, I., Walraven, M., de Boer, M.P., Greuter, H.N., Hendrikse, N.H., Eriksson, J., Windhorst, A.D., Postmus, P.E., Verheul, H.M., Serne, E.H., Lammertsma, A.A., Smit, E.F., 2012. Rapid decrease in delivery of chemotherapy to tumors after anti-VEGF therapy: implications for scheduling of anti-angiogenic drugs. *Cancer Cell* 21 (1), 82–91. <https://doi.org/10.1016/j.ccr.2011.11.023>.
- Vandamme, T.F., 2014. Use of rodents as models of human diseases. *J. Pharm. Bioallied Sci.* 6 (1), 2–9. <https://doi.org/10.4103/0975-7406.124301>.
- Vaupel, P., Mayer, A., Hockel, M., 2004. Tumor hypoxia and malignant progression. *Methods Enzymol.* 381, 335–354. [https://doi.org/10.1016/S0076-6879\(04\)81023-1](https://doi.org/10.1016/S0076-6879(04)81023-1).
- Vesterinen, H.M., Sena, E.S., Egan, K.J., Hirst, T.C., Churolov, L., Currie, G.L., Antonic, A., Howells, D.W., Macleod, M.R., 2014. Meta-analysis of data from animal studies: a practical guide. *J. Neurosci. Methods* 221, 92–102. <https://doi.org/10.1016/j.jneumeth.2013.09.010>.
- Wedge, S.R., Ogilvie, D.J., Dukes, M., Kendrew, J., Chester, R., Jackson, J.A., Boffey, S.J., Valentine, P.J., Curwen, J.O., Musgrove, H.L., Graham, G.A., Hughes, G.D., Thomas, A.P., Stokes, E.S., Curry, B., Richmond, G.H., Wadsworth, P.F., Bigley, A.L., Hennequin, L.F., 2002. ZD6474 inhibits vascular endothelial growth factor signaling, angiogenesis, and tumor growth following oral administration. *Cancer Res.* 62 (16), 4645–4655.
- Xian, X., Hakansson, J., Stahlberg, A., Lindblom, P., Betsholtz, C., Gerhardt, H., Semb, H., 2006. Pericytes limit tumor cell metastasis. *J. Clin. Invest.* 116 (3), 642–651. <https://doi.org/10.1172/JCI25705>.
- Xu, H., Rahimpour, S., Nesvick, C.L., Zhang, X., Ma, J., Zhang, M., Zhang, G., Wang, L., Yang, C., Hong, C.S., Germanwala, A.V., Elder, J.B., Ray-Chaudhury, A., Yao, Y., Gilbert, M.R., Lonser, R.R., Heiss, J.D., Brady, R.O., Mao, Y., Qin, J., Zhuang, Z., 2015. Activation of hypoxia signaling induces phenotypic transformation of glioma cells: implications for bevacizumab antiangiogenic therapy. *Oncotarget* 6 (14), 11882–11893. <https://doi.org/10.18632/oncotarget.3592>.
- Yang, Q., Ma, W., Yu, K., Zhang, Q., Ye, Z., Xia, W., Li, S., 2020. Oridonin suppresses human gastric Cancer growth in vitro and in vivo via inhibition of VEGF, integrin beta3, and PCNA. *Biol. Pharm. Bull.* 43 (7), 1035–1045. <https://doi.org/10.1248/bpb.b19-00839>.
- Yonenaga, Y., Mori, A., Onodera, H., Yasuda, S., Oe, H., Fujimoto, A., Tachibana, T., Imamura, M., 2005. Absence of smooth muscle actin-positive pericyte coverage of tumor vessels correlates with hematogenous metastasis and prognosis of colorectal cancer patients. *Oncology* 69 (2), 159–166. <https://doi.org/10.1159/000087840>.
- Zhang, S., Zhu, Q., Chen, J.Y., OuYang, D., Lu, J.H., 2020. The pharmacological activity of epigallocatechin-3-gallate (EGCG) on Alzheimer's disease animal model: a systematic review. *Phytomedicine* 79, 153316. <https://doi.org/10.1016/j.phymed.2020.153316>.

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2.2. Study II

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Role of Regular Physical Exercise in Tumor Vasculature: Favorable Modulator of Tumor Milieu

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Abstract

The tumor vessel network has been investigated as a precursor of an inhospitable tumor microenvironment, including its repercussions in tumor perfusion, oxygenation, interstitial fluid pressure, pH, and immune response. Dysfunctional tumor vasculature leads to the extravasation of blood to the interstitial space, hindering proper perfusion and causing interstitial hypertension. Consequently, the inadequate delivery of oxygen and clearance of by-products of metabolism promote the development of intratumoral hypoxia and acidification, hampering the action of immune cells and resulting in more aggressive tumors. Thus, pharmacological strategies targeting tumor vasculature were developed, but the overall outcome was not satisfactory due to its transient nature and the higher risk of hypoxia and metastasis. Therefore, physical exercise emerged as a potential favorable modulator of tumor vasculature, improving intratumoral vascularization and perfusion. Indeed, it seems that regular exercise practice is associated with lasting tumor vascular maturity, reduced vascular resistance, and increased vascular conductance. Higher vascular conductance reduces intratumoral hypoxia and increases the accessibility of circulating immune cells to the tumor milieu, inhibiting tumor development and improving cancer treatment. The present paper describes the implications of abnormal vasculature on the tumor microenvironment and the underlying mechanisms promoted by regular physical exercise for the re-establishment of more physiological tumor vasculature.

Introduction

The vascular feature of solid tumors was first described in the 19th century with the discovery of blood vessels present in different tumor types (for references, see [1]). Later, tumors were categorized into four regions based on tumor vasculature: (1) avascular necrotic region; (2) seminecrotic region with capillaries, pre-capillaries, and post-capillaries disposed along the avascular necrotic

region; (3) stabilized microcirculation region characterized by many venular and venous drainage vessels with few arteriolar vessels; and (4) tumor advance front region with blood flow similar to the host tissue [2]. Generally, peripheral regions show higher blood vessel density than central regions and the ratio of avascular and seminecrotic regions to well-perfused regions is a function of tumor size, with larger avascular regions present in bigger tumors [3, 4].

Today, the defective tumor blood vessel network is recognized as a critical factor impacting variations in tumor microenvironment (TME) and supporting tumor evasion [5]. Indeed, non-functional tumor vessels increase vascular permeability leading to the extravasation of blood to the interstitial space, which precludes effective tumor perfusion [6]. As a result, there is insufficient oxygen (O_2) supply and waste product removal, which promote the elevation of the interstitial fluid pressure (IFP) and the development of hypoxic and acidic regions [7]. Consequently, the perfusion and action of immune cells progressively become more inefficient and tumor continues to grow, assuming more aggressive phenotypes [7]. Therefore, various modalities of vascular targeting therapies have been explored in order to delay tumor growth [8, 9]. However, since all drugs present side effects and the outcomes regarding vascular therapies have been largely disappointing, non-pharmacological approaches for tumor vascular remodeling have emerged as an important therapeutic field [10]. In this way, the assumption that physical exercise can prevent cancer initiation was suggested in the 20th century and, since then, many investigations have reported that the rate of cancer mortality is inversely related to the levels of physical exercise accomplished [11, 12]. However, although exercise training has the capacity to modulate multiple biological effects, including metabolism, vascular function, and immunity, little is known about the effects on TME [10]. Therefore, understanding how physical exercise impacts vascular density and tumor perfusion, can create better conditions to improve therapeutic outcomes and potentiate patient care [13]. Thus, the focus of the present review is to describe the implications of abnormal tumor vasculature on TME and to report the candidate mechanisms that may account for the importance and effectiveness of exercise training as a valid therapeutic modality for the reestablishment of more physiological tumor vasculature.

Morphology of Tumor Vessels

Contrary to physiological angiogenesis, in which new vessels rapidly mature and become stable, tumor blood vessels do not become quiescent, allowing the continuous growth of new blood vessels [14]. As a result, tumor vasculature develops a large series of severe structural and functional abnormalities that are quite distinct from the normal blood vessel network [15]. Indeed, whereas normal vessels are organized in a hierarchical fashion with arterioles, capillaries, and venules that are easily differentiated, tumor vessels lack this orderly branching hierarchy that feed a regularly spaced capillary bed [16]. Instead, tumor vessels are often dilated and tortuous, ranging from capillaries to glomeruloid vessel outgrowths and vascular malformations [17]. As such, the concept of specifically targeting tumor vasculature was proposed and the use of vascular targeting agents (VTAs) was established as a potential therapeutic strategy [18, 19]. First, vascular disrupting agents (VDAs) were developed to compromise the function of the already established tumor blood vessels [20]. Indeed, VDAs induce direct damage to the endothelium by disrupting its cytoskeleton and adhesion to the matrix and activating local coagulation [21]. The destruction of the endothelium would result in the occlusion of blood vessels and capillary sprouts causing widespread necrosis and secondary ischemic cell death [22]. VDAs are mostly effective against

vessels in the central region of the tumor, possibly because the higher IFP in these regions contributes to vascular collapse [23]. However, although the characteristic pattern of central necrosis produced by VDAs can extend up to 95% of the tumor, a thin viable rim of tumor cells survives at the periphery, which later regrows [24]. Because at the tumor periphery nutritional support is derived from the vasculature of the adjacent normal tissues, these cells can survive VDA treatment, making it an ineffective strategy to eradicate the total tumor mass [25].

Consequently, instead of disrupting the already established tumor blood vessels, the belief that blocking tumor angiogenesis as much as possible would starve tumors to death led to the concept of antiangiogenic therapy [8]. As a sustained growing vasculature is necessary for tumor survival and development, angiogenesis-inhibiting agents (AIAs) seek to inhibit new tumor vessel formation by blocking the angiogenic stimuli through the inhibition of the angiogenic factors and receptor action, thus preventing endothelial cell proliferation, migration, and tube formation [26]. Due to the importance of vascular endothelial growth factor (VEGF) and its receptor VEGFR2 in angiogenesis, there was hope that blocking this pathway would eradicate the tumor vasculature [27]. As such, anti-VEGF antibodies were developed to re-establish the balance between pro- and anti-angiogenic factors and the vascular network of tumors by pruning immature vessels [28–32]. Indeed, some pre-clinical and clinical studies showed a reduction in vascular density and permeability after angiogenesis-inhibiting treatment [33–35]. However, the effects of such therapy in cancer patients during clinical trials have not fulfilled the expected hopes as the improvement in survival by AIAs was modest, with controversy arising from the finding that after antiangiogenic treatment the levels of tumor hypoxia increased [9, 36, 37]. Indeed, other experimental studies have also demonstrated that AIAs decreased tumor perfusion and the overall distribution of large macromolecules, such as antibodies [38–40]. Following these findings, another two pre-clinical studies presented intriguing evidence showing that VEGF-targeted agents inhibited primary tumor growth but shortened the overall survival by promoting tumor invasiveness and metastasis [41, 42]. In addition, the use of AIAs is likely to mean long-term therapy because after treatment conclusion, dormant tumor cells may regain pro-angiogenic activity and proliferate [43, 44]. Therefore, the continuous blockade of the VEGF pathway can lead to vascular collapse and poorly vascularized regions, inducing a more aggressive tumor phenotype [34].

So, modulating the TME by repairing the function of tumor vessels instead of inhibiting it is likely to be a more efficacious strategy to slow tumor progression and enhance cancer treatment [45]. Indeed, the pioneering work of Algire & Chalkley showed that capillaries in quickly growing tumors have about five times the diameter of those in normal tissue and rarely differentiate into arterioles or venules (for references, see [46]). This difference occurs because vessel walls are compressed by tumor or stroma cells, with some vessels being oversized and others being immature smaller vessels, while in other regions vessels are completely absent [47]. Additionally, tumor vessel walls may have fenestrations, discontinuous or absent basement membranes, fewer pericytes, and less perivascular smooth muscle than normal vessels (**Fig. 1**) [17]. Interestingly, it seems that the judicious application of AIAs might revert much

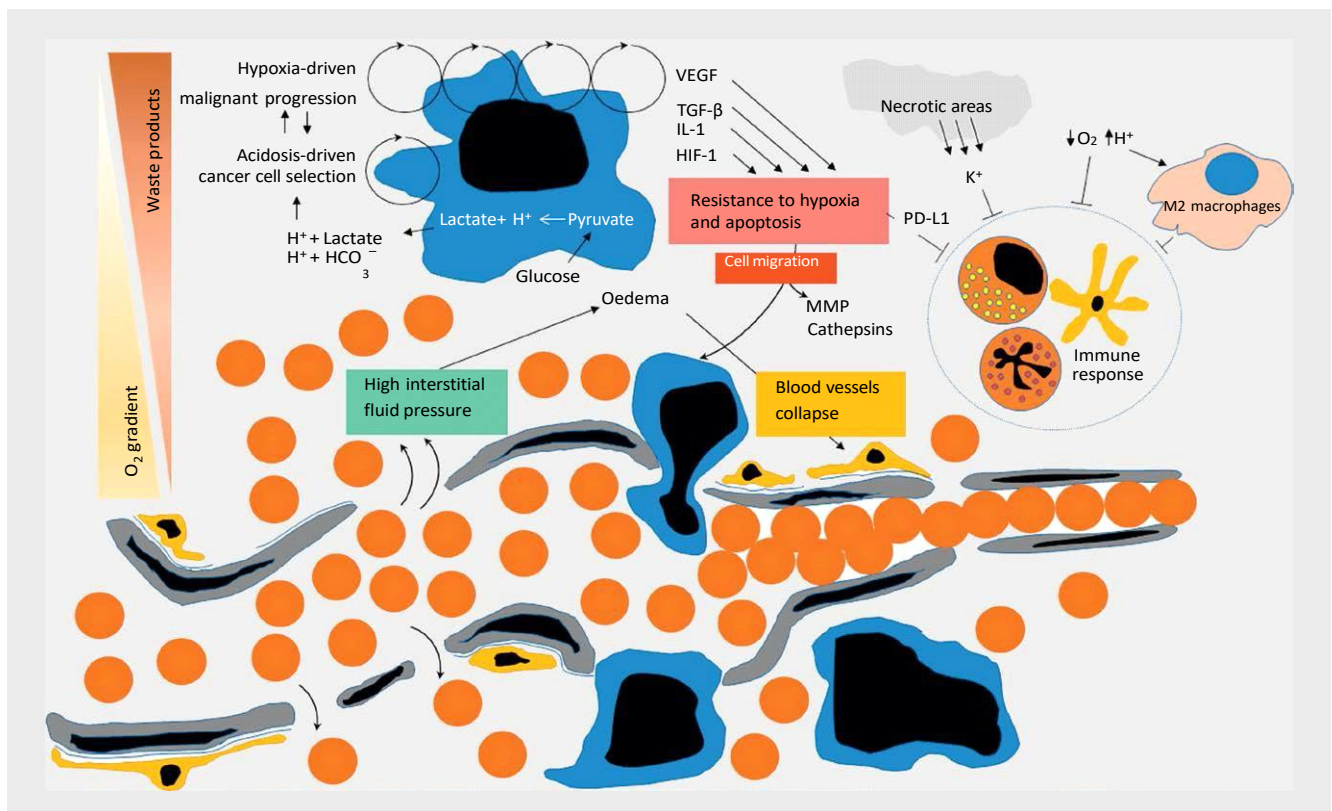


Fig. 1 Pathological features of tumour microenvironment. The tumor vessel network is chaotic, presenting low pericyte coverage, loose interendothelial cell junctions, and increased leakiness. The resultant high interstitial fluid pressure induces the collapse of blood vessels, leading to the establishment of hypoxic regions. In addition, the glycolytic nature of tumor cells promotes interstitial acidification, which together will impact the immune response, favouring tumor malignant progression. HIF-1, hypoxia inducible factor-1; IL-1, interleukin-1; MMP, metalloproteases; PD-L1, programmed death-ligand 1; TGF-β, transforming growth factor-β; VEGF, vascular endothelial growth factor.

of the abnormal structure and function of the tumor vasculature down to a level where the tumor vasculature might acquire features similar to the functional and quiescent blood vessels of the normal tissues [48]. Indeed, the presence of a temporary window during anti-angiogenic treatment was already demonstrated, wherein a rebalancing of pro- and antiangiogenic signals occurs to the point that vessels become more functional, improving blood flow and oxygenation in tumor regions that were previously poorly perfused [49]. Given the importance of a good vascularization to allow O₂ delivery and the penetration of immune cells into tumor tissue, this strategy aims to turn the abnormal tumor blood vessels into a more functional phenotype by increasing pericyte coverage and basement membrane [50]. Consequently, during the restoring window, tumor vessels become more stable and uniform, reducing the potential for metastasis [51–53]. Pre-clinical and clinical studies already identified this window, typically occurring within 1–2 days after the onset of therapy, followed by a closure coinciding with the loss of functional features (about 6 days) [54]. The window's closure may be related either to the excessively high or prolonged dosing of AIs or due to the resistance promoted by the activation of alternative pro-angiogenic pathways [54, 55]. Therefore, the transient nature of such therapy means that this approach may be difficult to predict and apply in the clinical setting, thus better strategies to modulate tumor vasculature are still needed [56].

Thus emerging data suggests that the regular practice of physical exercise has a positive impact on vascular remodeling, thus representing a potential non-pharmacological modality to repair tumor vasculature [57–60]. Physical exercise modifies the morphology of vessels along the arterial tree in the skeletal muscle and in the myocardium, increasing its number (angiogenesis) and its diameter (arteriogenesis) [61–63]. The cellular sources of vasodilator compounds during physical exercise include endothelial cells (ECs), erythrocytes, and skeletal muscle. The endothelium releases nitric oxide (NO), prostacyclin (PGI₂), and endothelium-derived hyperpolarizing factor (EDHF); skeletal muscles release NO, ATP, and adenosine; and red blood cells release ATP and NO [64]. Similarly, regular physical exercise may increase the number of visible tumor vessel lumens and decrease the average sprout length [10]. In a murine model of breast cancer, voluntary running significantly increased the density and maturity of tumor blood vessels, as assessed by CD31⁺ vessels and desmin-positive pericyte coverage of capillaries, respectively, resulting in decreased tumor growth [58]. Additional studies have corroborated these findings in mouse models of breast and prostate cancer [60, 65].

In addition to growth factor signaling, blood vessels are regulated by mechanical cues produced by changes in blood flow, velocity, and pressure [66]. Physical exercise can impact the physical forces acting on blood vessels including shear stress, transmural

pressure, and cyclic stretch [67]. Specifically, in skeletal muscle, each bout of physical exercise transiently increases shear stress due to higher blood flow, transmural pressure due to a higher blood pressure, and cyclic stretch of blood vessels due to the increased heart rate (HR) and the pulsatile nature of blood flow [68]. Of these, shear stress plays an essential role driving vascular adaptations in contracting muscles. Shear stress, the mechanical force on the endothelium that is critical for the establishment of mature and functional vasculature, is enhanced when mechanosensors recognize higher muscle perfusion and mediates arterial enlargement leading to an acute increase in vascular conductance [10, 69]. In response to chronic shear stress, ECs hypertrophy, the density of actin microfilaments and focal adhesions increases, and the number of focal adhesion-associated proteins is higher than in cells not exposed to shear stress [70]. Indeed, one of the many effects of aerobic exercise is a systemic increase in blood flow that stimulates the mechanical force on ECs in proportion to exercise intensity (Fig. 2) [71]. Accordingly, Schädler and co-workers observed a significant increase in blood flow due to aerobic exercise in mice with melanoma and pancreatic tumors that triggered the remodeling of tumor vessels to a more functional state, inhibiting tumor growth [10]. The authors identified a previously unknown shear stress-responsive pathway in ECs, calcineurin–nuclear factor of activated T cells (NFAT)–thrombospondin 1 (TSP-1) signaling, and demonstrated that TSP-1 expression is critical for exercise-induced tumor vascular repair. During increased shear, the activation of calcineurin–NFAT signaling in ECs upregulates the anti-angiogenic protein TSP-1, which promotes the cross-talk between ECs under shear stress and ECs in areas absent of shear, thus inhibiting the proliferation of naïve ECs and promoting vascular remodeling [10]. Moreover, due to their narrowness and low blood rate, tumor vessels have been reported to have cancer cells integrated into their walls

[72]. As physical exercise increases blood flow, it could potentially prevent the infiltration of tumor cells in vessel walls and their dissemination to surrounding tissues, thus preventing the development of pre-metastatic niches (Fig. 2) [73].

In tumors, there is also significant arterial–venous shunt perfusion and chaotic vascular organization without any regulation matched to the metabolic demands or functional status of the tissue [74]. Indeed, about 50% of tumor vessels are non-functional, with vessel walls presenting wide interendothelial junctions and transendothelial channels formed by vesicles and pores more than 100 times the size of those in healthy vessels [75, 76]. Some studies already showed that the pore size of tumor microvessels varies from 100 nm to 780 nm in diameter, while microvessels in normal tissues are less leaky, with tight junctions between ECs < 2 nm, whereas the pore size in postcapillary venules is larger at up to 6 nm [77]. In contrast to quiescent ECs, which adhere to the vessel wall, tumor ECs undergo endothelial-to-mesenchymal transition (EMT) and move away from their resident site, leaving behind empty sleeves of endothelial cell-devoid matrix channels, hindering blood flow [78]. Thus, whereas normal vessels are lined with a quiescent monolayer of interconnected adherent ECs that are disposed in the direction of blood flow for optimal perfusion, tumor ECs lose their polarity, detach from the basement membrane and stack upon each other [79, 80]. Other tumor ECs can die or slough off, establishing gateways for cancer cells that become exposed to the blood [81]. The phenomenon of vessel hyperpermeability is influenced by the activation of the sphingosine-1-phosphate receptors 1 and 2 (S1PR1 and S1PR2) on the surface of ECs, which act as shear stress responsive mechanoreceptors that regulate vessel integrity through their signaling pathways [82, 83]. Specifically, S1PR1 signaling promotes tight intercellular junction assembly to properly modulate vascular permeability and induce vessel matu-

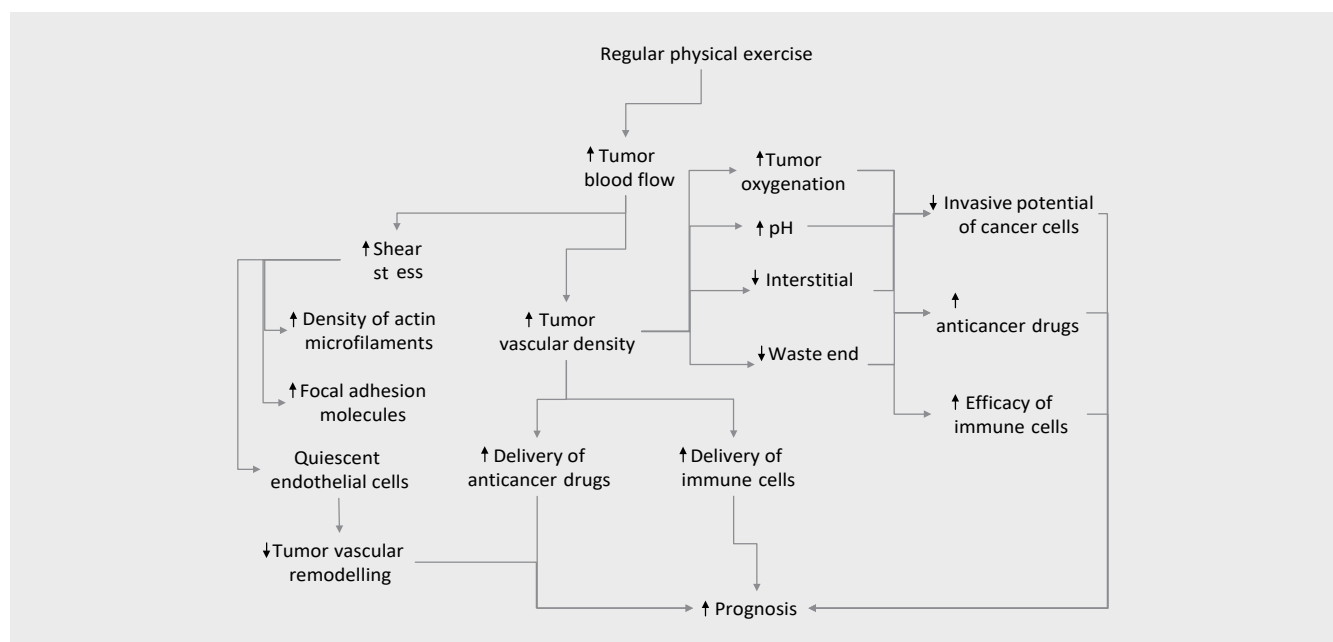


Fig. 2 Mechanisms linking regular physical exercise with improved cancer prognosis through vascular network changes.

riety, whereas S1PR2 expression is associated with pathologic endothelium and higher vascular permeability and is upregulated by the disturbed tumor blood flow [84, 85]. The elegant study by Morrel and colleagues showed that two weeks of treadmill running increased S1PR1 and decreased S1PR2 signaling in tumor-bearing mice, reducing tumor vascular permeability and improving tumor oxygenation [57]. These important studies clearly demonstrated that exercise training may have a unique role in tumor vascular repair, eliminating shunts that leave certain tumor regions deprived of blood supply (**Fig. 2**).

However, growing tumors continuously demand O₂, and because of this high metabolic demand, oncogene activation and exposure to a disturbed microenvironment, tumor cells produce excessive pro-angiogenic factors that render vessels even more abnormal [86, 87]. Compared to normal tissues, tumors show elevated levels of growth factors like VEGF and vasoactive factors like bradykinin and NO, which increase vascular permeability even more [88, 89]. The resultant TME is characterized by heterogeneous hypoperfusion, high IFP, hypoxia, and acidity that favor the shedding of cancer cells into circulation (**Fig. 1**) [90].

Abnormal Vasculature and Tumor Microenvironment: The Role of Exercise

Hypoperfusion

In vessels, blood flow is directly proportional to the pressure's difference between arteries and veins and inversely proportional to the viscous and geometric resistance [91]. However, due to their continuous remodeling, tumor vessels are leaky and result in higher IFP. Consequently there is an increase in vascular resistance, rendering blood flow not only chaotic but also stagnant in some places, hampering tumor perfusion [92]. Indeed, compared to normal tissues, tumors show greater blood viscosity due to the presence of tumor cells and large molecules like proteins and collagen drained from the extravascular space, and a larger vessel diameter with thin and ragged walls that culminates in increased blood flow resistance [93, 94]. Furthermore, in a given vessel, blood may travel in one direction and then in the opposite way. These flow patterns, together with the abnormal pressure gradients, hinder the uniform delivery of nutrients, O₂, and anticancer drugs [13, 95, 96]. Although disturbances of tumor microcirculation are apparent at early growth stages, blood flow rate tends to decrease with tumor growth [3, 4]. The pathogenic mechanisms directly responsible for this weight-adjusted flow decline, involve a progressive rarefaction of the vascular bed (decrease in the number of patent vessels/g of tumor), an escalation of the structural and functional abnormalities of the tumor vessel network, and the development of necrosis [3, 97]. Indeed, most of the blood vessels in the internal region of tumors are veins or venules, whereas peripheral regions have a reduced number of arteries and arterioles [98, 99]. Therefore, the arteriole-venule pressure difference as a driving force for blood flow is negligible in the central region of tumors and greater in the periphery and, consequently, blood flow is lower at the centre and higher at tumor periphery [100]. As a result, the average blood flow in tumors is lower than in normal tissues, with reduced delivery of O₂ and nutrients and decreased clearance of by-products of me-

tabolism, which leads to hypoxic and acidic regions, mainly in the central part of the tumor (**Fig. 1**) [101, 102].

Endurance exercise represents the most potent physiological stimulus for blood vessel remodeling in skeletal muscle [103]. Indeed, vasoactive factors in the muscle interstitium may have a direct effect on ECs and smooth muscle cells in capillaries, inducing conducted vasodilation [104]. Conducted vasodilation is a mechanism to redirect blood flow to the contracting muscles in which the signal for vasodilation travels along the vascular wall, up and downstream, through gap junctions [105]. This mechanism is critical to accurately modulate blood flow as it allows the occurrence of vasodilation in the distant larger vessels that do not necessarily experience the chemical or mechanical stimuli that is present in the working muscle fibers [106]. These adaptations in the blood vessel network elicit the improvement in the regional distribution of blood to assure proper flow in areas of muscle bed vasodilation [71]. Several studies have shown that regular exercise practice is associated with increased vascularization in tumors as well, which is correlated with enhanced blood perfusion and reduced tumor hypoxia (**Fig. 2**) [58–60, 107]. Recently, McCullough and colleagues demonstrated that an acute bout of treadmill running increased the prostate tumor blood flow in rats up to 200%, owing in part to the inability of the tumor vasculature to vasoconstrict; importantly, the increase in tumor blood flow resulted in a significant increase in O₂ delivery and a 50% reduction in tumor hypoxic area [60]. During physical exercise, there is a redistribution of cardiac output, with a shunting of blood flow from non-active tissues to the contracting muscles [108]. In part, this shunting is due to the enhancement of sympathetic system activity and the release of norepinephrine that binds to α -adrenergic receptors on the vascular smooth muscle, inducing vasoconstriction [109]. Indeed, following physical exercise onset there is an increase in HR driven by the vagal withdrawal and the β -adrenergic stimulation [110, 111]. Exercise hyperemia is a highly complex and well structured process that involves sympathetic vasoconstriction and the integration of many vasodilator signals. During physical exercise as the higher sympathetic activity promotes arteriolar constriction, an efficient amount of vasodilators needs to be activated in contracting muscles to promote accurate blood perfusion [112]. This reduced vasoconstriction during contraction (functional sympatholysis) allows adequate O₂ delivery to the active fibers. As a result, there is a decrease in α -adrenergic responsiveness on the smooth muscle cells, promoting a physical exercise-induced sympatholysis [113]. Accordingly, Heinonen and colleagues found that the inhibition of α -adrenergic tone disturbed the perfusion of blood flow to contracting muscles by directing it to the inactive muscle fibers as well [114]. Therefore, the diminished α -adrenergic vasoconstriction and the loss of myogenic tone at higher intraluminal pressures of tumor arterioles may inhibit the action of the sympathetic nervous system in tumor vessels, resulting in the enhancement of tumor blood flow during physical exercise [60]. Clinically, this suggests that physical exercise has the potential to improve tumor oxygenation and thus to be able to mitigate the hypoxic microenvironments that are associated with more aggressive tumor phenotypes (**Fig. 2**) [57, 58, 60].

There are several benefits to improved intratumoral vascularization and blood perfusion: first, there is an increase in tumor oxygenation, preventing the development of intra-tumoral hypoxia;

second, the accessibility of circulating immune cells is greater; and third, the delivery of anti-neoplastic medication to tumors is facilitated [115–117]. Indeed, tumor blood supply plays an important role in the delivery of therapeutic agents to solid tumors [118]. As tumor cells proliferate more quickly than capillary ECs, these cells force tumor vessels apart creating an unfavorable vascular network for drug delivery, which means that most anticancer drugs show modest effects, in part due to limited penetration into tumor cells [91]. Nacev and co-workers observed that tumor cells are often located 100 μm away from the nearby vessels, whereas in normal tissues, cells are closer to capillaries (50–100 μm) [119]. As drug molecules need to travel a longer distance and the commonly used cytotoxic agents like doxorubicin (DOX) are unable to penetrate more than 40–50 μm from blood vessels, there is an insufficient drug penetration rate to kill cancer cells [120].

Indeed, it has been shown that exercise training may also improve the penetration of chemotherapeutic agents into tumors (Fig. 2). The study of Schadler and collaborators demonstrated that exercised mice with pancreatic adenocarcinoma presented a 24% increase in functional vessels and improved the delivery of gemcitabine to tumors [10]. Further, to investigate the delivery of DOX to tumors, Morrel et al. submitted mice with Ewing's sarcoma to a moderate-intensity exercise training protocol and concluded that tumors from exercised mice had significantly higher DOX levels than tumors from sedentary mice [57]. As such, these studies additionally reinforce the importance of functional tumor vasculature to access TME, and physical exercise seems to be a viable option to achieve it.

High Interstitial Fluid Pressure

The interstitial space mediates the exchange of O_2 , nutrients, and waste products between the vascular system and cells, and the movement of fluid across the vessel wall is governed by the difference in hydrostatic and oncotic pressures (Starling's forces) [121]. In human capillaries, the oncotic pressure is about 28 mmHg, which tends to keep fluid in the capillaries [122]. Contrarily, the capillary hydrostatic pressure (20 mmHg), the interstitial-fluid osmotic pressure (8 mmHg), and the interstitial-fluid hydrostatic pressure (–1 to –3 mmHg) tend to move fluid out from the capillaries [123]. As such, normally there is a net outward filtration pressure from the capillaries into surrounding tissues of 1–3 mmHg [122]. This outward pressure guarantees a flow of fluid out from the vessels and through the interstitium, contributing to the transport of molecules to and from cells [123]. However, in tumors, the incomplete or even missing endothelial lining and discontinuous basement membranes increase vascular permeability with extravasation of blood to the interstitial space (Fig. 1) [75, 124]. Indeed, it was already shown in normal tissues that the physiological influx of water into the interstitial compartment varies between 0.5 and 1.0% of plasma flow, but in tumors this value reaches more than 15% [125]. Consequently, tumors are characterized by higher geometric and viscous resistance to blood flow, increased microvascular hydrostatic pressure, high resistance to interstitial fluid flow, and impaired lymphatic drainage, resulting in the drastic elevation of the IFP [126–129]. In normal tissues IFP is just above or below atmospheric values (–3 to +3 mmHg), but in tumors it is generally high

at the center, reaching values of 50–100 mmHg and drops steeply at the tumor periphery [130]. This interstitial hypertension causes edema, leading to sluggish blood flow due to the clogging of red blood cells and to the collapse of blood vessels, particularly at the centre of the tumor, triggering the proliferation of cancer cells (Fig. 1) [131]. Therefore, functional vessels are present mainly in the tumor periphery, where there is a drop of the IFP to a more physiological level [132, 133].

In many tumors the supportive stroma also contributes to the increase of IFP, through the release of cytokines, growth, and angiogenic factors, which in turn can compress intratumoral vessels, impairing their function [134]. The resultant edema associated with the tumor's high osmotic pressure forms a barrier to adequate infiltration of immune cells and delivery of anticancer drugs [135]. Since IFP was identified to be significantly higher in tumors compared to normal tissues, an enhanced IFP has been observed in several solid tumors and it has been considered a critical prognostic factor [136, 137]. Indeed, in the study of van der Voort van Zyp et al., an increased extracellular pressure of just 15 mmHg was enough to stimulate colon cancer cell adhesion to surgical wounds, leading to tumor recurrence [138]. Based on these data, it has been suggested that tumor IFP forms a link between tumor angiogenesis, tumor hypoxia, and metastasis in which dysfunctional angiogenesis led to the development of a vascular network with serious architectural abnormalities [139]. The result is a TME characterized by elevated IFP and hypoxic regions that promotes the upregulation of pro-angiogenic factors, forcing the interstitial fluid to flow from the center to the tumor periphery where stimulates tumor hemangiogenesis, peritumoral lymphangiogenesis, and consequently lymph node metastasis [139].

Therefore, strategies capable of lowering fluid accumulation in tumors by decreasing the leakiness of tumor vessels or by draining the interstitial fluid should lower the levels of tumor IFP [140]. In this way, by improving vascular maturity, physical exercise may lead to a decrease of non-functional sprouts and an increased hierarchical vasculature with reduced leakiness that may attenuate IFP [55]. Decreasing the level of IFP could potentiate macromolecular and nanoparticle drug transport (> 1000 MW), because the transport of these drugs is governed by the pressure gradient across the vessel wall [120]. Indeed, Betof and co-workers studied the effect of exercise training in mice with breast cancer under chemotherapy treatment and observed an increase in the amount of viable tumor area containing pericyte-covered vessels ($p = 0.006$), an increased vessel area covered by pericytes ($p = 0.024$), and a 3- to 4-fold higher rate of mature vessels when compared to sedentary animals; as a result, the rate of tumor growth was significantly lower [58]. Similarly, in the study of Morrel and collaborators, two weeks of treadmill running promoted the remodeling of tumor vasculature, significantly reducing vessel permeability; however the authors did not observe any impact on tumor growth [57].

In contractile muscles, fluid filtration from the blood to the interstitium markedly increases during acute physical exercise. Indeed, because the movement of fluid initially exceeds the capacity of the lymphatic system, interstitial fluid volume significantly increases at the onset of physical exercise [141]. The transient enhancement of IFP occurs because capillary hydrostatic pressure is higher due to the arteriolar vasodilation, the increased microvascu-

lar surface area, the increased muscle vessel density, and the higher osmotic concentration that results from the release of metabolites during skeletal muscle contraction [142]. However, with the continuation of physical exercise, transcapillary fluid movement slows to the point that lymphatic drainage and net filtration are in balance, blocking the accumulation of fluid [141]. As physical exercise-induced hypervolemia is associated with higher plasma albumin content, there is a reduced transcapillary escape rate (TER) of albumin and a redistribution from the interstitial to the intravascular compartment [143–145]. Accordingly, Mack and collaborators verified that in contracting muscles the decrease in hydrostatic pressure and increased oncotic pressure promoted a higher lymphatic return of fluid and protein to the vascular compartment [144].

Thus, by enhancing draining volume flow, the skeletal muscle contraction acts as an edema safety agent to limit the increase in IFP [146]. Indeed, the removal of tissue proteins produces a filtrate of plasma that becomes progressively protein-poor in concentration, which decreases the colloid osmotic pressure of interstitial fluid. Consequently, the transcapillary oncotic pressure increases, reducing fluid accumulation in tissues [147]. Physical exercise can prevent the elevation of IFP by different mechanisms. First, the contraction of muscle fibers acts on lymphatics by pumping lymph into the collecting vessels [146]. Second, physical exercise also boosts blood from contracting skeletal muscle by the action of muscle pump, which mitigates the higher capillary pressure induced by arteriolar vasodilation [71]. Finally, the decreased postcapillary resistance induced by muscle contraction also limits the accumulation of interstitial fluid during physical exercise [141].

Collectively, these data suggest that physical exercise could be a potential strategy to prevent high tumor IFP, but further research is still needed.

Oxygen Deprivation

Normally, tumor cells proliferate faster than ECs, reducing vascular density particularly at the center of the tumor, turning it very distant from the blood supply ($> 100\mu\text{m}$) [148]. Due to the poorly organized vascular architecture, irregular blood flow, and the compression of blood and lymphatic vessels, the O_2 consumption rate of neoplastic cells may outweigh an insufficient O_2 supply, resulting in the development of areas of very low oxygenation [149].

Because TME also depends on the cellular consumption of O_2 and essential nutrients, tumor cells closer to the vascular supply consume what they need for growth and survival, with less being available for those cells further away. Consequently, radial O_2 , nutrients, and pH gradients are established (Fig. 1) [97]. Indeed, it has been reported that the O_2 partial pressure (pO_2) of cells next to capillaries can be as low as 15 mmHg, which will reduce the O_2 diffusion distance [150]. All these factors will result in the development of regions of necrosis, which are commonly observed in advanced solid tumors, reflecting the consequences of prolonged periods at O_2 levels that are insufficient to maintain cell viability (Fig. 1) [151].

Primarily, hypoxia leads to the loss of proliferative capacity and finally to death of normal and most cancer cells [102]. On the other hand, some tumor cells can survive hypoxia-induced cell death by triggering alterations in the proteome and/or genome, favoring

tumor development. This phenomenon is called the “Janus face of hypoxia” [152]. Indeed, the critical pO_2 in tumors below which the harmful changes related to decreased O_2 consumption have been observed is 8–10 mmHg [153]. Specifically, the pioneering work of Kolstad shows that whereas pO_2 is about 36 mmHg in normal cervical mucosa, in cervical cancer it progressively drops to 5 mmHg with the development of the disease [154]. These data are indicative of inadequate tissue oxygenation, most probably due to a restriction of the microcirculation and thus a restriction of O_2 availability to cancer cells in vivo. This phenomenon gets significantly worse with tumor growth [3]. Evidence shows that 50–60% of locally advanced solid tumors may present hypoxic and/or anoxic regions that are heterogeneously distributed within the tumor mass [155]. Tissue hypoxia results in the stabilization of the hypoxia-inducible factor (HIF) because in normoxic conditions the HIF- α units are unstable and quickly degraded by the proteasome pathway through the ubiquitin E3 ligase complex, the recognition component of which is the von Hippel Lindau (VHL) [156]. However, since very low O_2 levels are available for hydroxylation under hypoxic conditions, the HIF-1 α subunit is stabilized, forming a heterodimer with HIF-1 β to functionally mediate an array of genes involved in angiogenesis, metabolism, tumor cell survival and proliferation, drug and radiation resistance, and immune evasion [157, 158]. As a result, during hypoxia-driven malignant progression, the capacity for local invasive growth and perifocal tumor cell spreading and dissemination is higher [159, 160]. Indeed, hypoxia enhances the invasive potential of tumors through the activation of pro-migratory proteins and pro-invasive extracellular matrix (ECM) molecules [161]. The first clinical study demonstrating a clear association between tumor hypoxia and the likelihood of distant metastasis was reported by Brizel and co-workers, who observed that the pO_2 of metastasizing tumors (7.5 mmHg) was significantly lower than the non-metastasizing tumors (20 mmHg) [162]. Additionally, hypoxia-induced inhibition of gene expression was observed for cell-surface integrins favoring tumor cell migration [163]. Indeed, the first study involving the exposure of murine fibrosarcoma cells to different O_2 concentrations showed that the more hypoxic the exposure, the greater number of metastases developed [164]. At the same time, recent evidence suggested that hypoxic cells are resistant to radiation therapy and can repopulate the tumor due to the presence of cancer stem cells in hypoxic regions [165, 166]. Finally, in the presence of O_2 , most anticancer drugs produce free radicals that damage DNA [167]. These drugs accept electrons from biological sources and then transfer them to O_2 [168]. However, at low O_2 concentrations the cytotoxicity of these drugs, whose activity is mediated by free radicals, is decreased and the elimination of the tumor is ineffective [169].

Collectively, these observations may explain why intratumoral hypoxia favors tumor growth and dissemination, correlating it with a poor prognosis in many human cancers [170]. Unfortunately, today there is no accepted standard of care for reducing tumor hypoxia. Nevertheless, to maintain homeostasis, O_2 supply and demand in skeletal muscle occurs in a dynamic balance and may be modified by changes in blood flow, O_2 tension and by the number of capillaries participating in gas exchange [171]. Indeed, α -adrenergic response to physical exercise causes vasoconstriction of inactive regions, redirecting blood flow to the working muscles

to match the increased demand for O_2 [103]. To this end, the splanchnic circulatory system that accounts for about 25% of cardiac output at rest, markedly decreases during physical exercise [172]. Similarly, renal flow also decreases proportionally with increasing exercise intensity. Endo and colleagues (2008) noted that even cycling ergometer exercise for 4 min at 40 W decreased blood flow to the kidneys by 20% [173]. Inversely, physical exercise-induced hyperemia increases the recruitment of non-perfused capillaries in contracting muscles, which not only improves the surface area available for gas exchange but also decreases diffusion distance for O_2 flux [174]. Indeed, the flux of O_2 in skeletal muscles during physical exercise can increase 100-fold over resting values [175]. At rest and during physical exercise, skeletal muscle oxygenation depends on the fine-tuning between O_2 delivery through the microcirculation and O_2 consumption by the skeletal myocytes [176]. The transport of O_2 through the microcirculation is accomplished by convection and diffusion [177]. Convection is an efficient way to move O_2 in blood over large distances, mostly in the larger arterial and venous vessels. In peripheral tissues O_2 moves short distances by diffusion from the terminal vascular sites to nearby parenchymal cells, where it is utilized by the mitochondria [178]. In this way, in contracting muscles, due to increased capillarization and a bigger exchange surface area, there is higher arterio-venous O_2 extraction, which improves O_2 diffusion to the cells [179]. Indeed, it was shown that during physical exercise, O_2 consumption can increase up to a point that 70–80% of the O_2 delivered to the active muscles may be extracted [180].

Also, the chronic adaptations of skeletal muscle to exercise training leads to better aerobic performance at higher intensity through an improvement in metabolic capacity and O_2 supply [181]. Supporting this notion, it was observed that after exercise training there is an increased mitochondrial enzymatic content, which is associated with larger diffusional O_2 conductance and increased O_2 extraction [182, 183]. In addition, two-legged acute exercise after 4 weeks of unilateral knee-extensor training, resulted in higher HIF-1 α concentration only in the untrained leg [184]. Similarly, 6 weeks of endurance training showed increased expression of HIF-1 α inhibitors that led to an attenuated PDK-1 expression, thus increasing the muscle capacity to utilize O_2 [185]. Therefore, it seems that exercise training can improve O_2 delivery and extraction, attenuating the acute HIF-1 α response and enhancing oxidative metabolism [181].

Accordingly, the physiological effects of physical exercise as an inhibitor of tumor hypoxia have already been shown (Fig. 2) [58–60]. Betof and colleagues reported that voluntary running throughout tumor development decreased the hypoxic fraction by 50% [58]. McCullough and collaborators also verified that the practice of chronic exercise significantly improved microvascular pO_2 and decreased the hypoxic fraction within the prostate tumors of rats compared with their sedentary counterparts [59]. Additionally, the same author studied the acute effects of exercise training on tumor hypoxia and verified that a single bout of treadmill running reduced the tumor hypoxic fraction by 15% [60]. This means that exercise training immediately promotes adaptations on TME that can be exploited to mitigate hypoxia and cancer progression.

Acidic pH

In tumors, the development of hypoxic regions promotes the activation of genes involved in the regulation of glycolysis, increasing glucose consumption and leading to the extrusion of lactic and carbonic acid outside the cell [186]. The result is ineffective clearance of by-products of metabolism that lowers the interstitial pH, leading to tumor acidosis (Fig. 1) [187]. Indeed, at perfusion rates < 0.2 ml/g/min tumor venous blood become acidic and pH falls below 7.0, which means that tumor tissue acidosis mainly develops because of inadequate drainage [188]. This was true for several tissue samples from cancer patients that were shown to be acidic, with an extracellular pH (pHe) of 5.6 to 6.8, whereas the pHe of normal tissues is significantly more alkaline, at 7.2–7.5 [189, 190]. In contrast, the cytosolic pH of tumor cells is commonly preserved at a slightly more alkaline level (7.2–7.5), acting as a pre-requisite for effective protein and DNA synthesis and cell survival [191]. In order to ensure this pattern, tumor cells secrete the excessive produced acids and protons to the extracellular space [192]. However, as quickly growing tumor cells exhibit high glycolytic rates and high lactate production together with insufficient drainage, there is an accumulation of H^+ in the respective tissue [193]. The result is an acid-outside pH gradient typically in opposition to that observed in normal tissues in which intracellular pH (pHi) is lower than pHe [194].

In addition to non-optimal vascularization and drainage, the development of tumor acidosis can also occur through the availability of glucose to hypoxic cancer cells, which usually occurs because the diffusion distance for glucose is larger than for O_2 [97]. This was first noted by Kallinowski et al., when the authors observed that the hypoxic tumor cells distant from the supplying vessel could still cleave glucose to lactic acid [195]. Therefore, it is not unusual to observe an inverse correlation between the lactic acid content and the interstitial pH in many tumors [196]. Although lactate was initially thought to be just a glycolytic waste product, it is now established that it can be consumed by aerobic tumor cells, thus reserving glucose for distant hypoxic tumor cells [197]. So, sharing of energy substrates between aerobic and hypoxic tumor cells is a key mechanism for the survival of hypoxic tumor cells [198]. Indeed, acidosis causes additional stress on cancer cells, leading to the selection of cells that are best prepared to withstand harsh conditions, likely owing to the acquisition of specific mutations, including the activation of VEGF, transforming growth factor (TGF)- β , interleukin (IL)-1 and HIF-1 [199, 200]. Together, these mutations turn tumor cells in the acidic regions more resistant to cell death than in normally perfused regions [192]. These observations led to the development of the “acid-mediated invasion hypothesis” in which tumor acidification favors tumor cell migration by enhancing tumor cell resistance and ECM degradation through the activity of matrix metalloproteases (MMP) and cathepsins, thus creating the pre-condition for tumor dissemination (Fig. 1) [186].

Low pH levels have also been shown to decrease the radiosensitivity of cells and to modulate the cytotoxicity of certain anticancer drugs [201]. Indeed, as in tumors pHe is low and the pHi is alkaline, weakly basic drugs with an acid dissociation constant of 7.5–9.5, such as DOX and mitoxantrone, are protonated, displaying decreased cellular uptake [202]. Interestingly, the acidic TME has also been shown to be partially responsible for unsuccessful anti-

angiogenic therapies. Faes and collaborators showed that exposure to an acidic milieu of pH 6.4 decreased the expression of VEGFR-2 in ECs, making AIAs inefficient [203]. Taken together, it seems that tumor cells have the ability to adapt to a low pH, activating mechanisms for cancer survival and proliferation [193]. Because this acidic milieu is so favorable for tumor migration, strategies to rebalance the pH levels of cancer cells are a viable option in complex anticancer treatment [204].

In resting skeletal muscles, the regulation of pH_i depends on passive membrane fluxes of hydrogen ions (H⁺) and bicarbonate, and membrane transport systems mediating a secondary active transport, thus balancing the acidic load [207]. However, muscular contraction induces several chemical, physical, and physiological challenges within and outside the cells. Therefore, as a consequence of intense physical exercise, the formation of lactate, carbon dioxide (CO₂), and H⁺ induces intracellular acidosis [208]. The physiological adaptation to these insults involves a quick and intense increase in ventilation to match the higher O₂ demand and to remove CO₂, and the increase of lactate-dependent and lactate-independent H⁺ transport [209, 210]. At low-intensity physical exercise, as lactate formation is low, H⁺ removal is managed mainly by the sodium (Na⁺)-dependent transport system and involves Na⁺/H⁺ exchange and Na⁺/bicarbonate co-transport, whereas lactate-dependent H⁺ removal plays a minor role [207]. Nevertheless, because after high-intensity physical exercise the lactate concentration may reach 40 mM with extensive H⁺ release from the contracting cells, the pH_i of skeletal muscle may decrease to 6.5 [211]. Therefore, the lactate/H⁺ and Na⁺/H⁺ carriers increase to mediate lactate and H⁺ efflux. Indeed, during high-intensity physical exercise both systems increase, but lactate-independent H⁺ removal quickly reaches its maximal capacity, whereas lactate-dependent H⁺ removal can increase by at least five times. Therefore, the lactate/H⁺ co-transporters seem to be the most important systems to remove H⁺ during high-intensity physical exercise [207].

Importantly, the regulation of pH in blood and skeletal muscle can adapt in response to exercise training and the beneficial effect of training has been described at the protein and functional level [207]. Indeed, exercise training can increase the density of the lactate/H⁺ co-transporter proteins MCT1 and MCT4, and the capacity to transport lactate and H⁺ [212, 213]. Also, the amount of the Na⁺/H⁺ exchanger protein NHE1 increased in humans after 8 weeks of submaximal exercise training [212]. Similarly, soleus Na⁺/bicarbonate transporters of Wistar rats were shown to increase after 5 weeks of high-intensity training [214]. On the other hand, the improved blood flow induced by physical exercise probably contributes to the increased release of H⁺ and lactate with training. Indeed, the involvement of increased blood flow is consistent with the finding that a better washout of lactate and H⁺ can occur with smaller muscle-to-arterial concentration gradients after exercise training [207]. Na⁺/potassium (K⁺) homeostasis also improved in trained subjects, representing another mechanism through which exercise training potentiates pH regulation in skeletal muscle [215].

Therefore, given that exercise training can decrease lactate and H⁺ levels, it is possible that acidic TME could also be impacted (Fig. 2). Currently, there are no reports on how physical exercise affects tumor pH. However, treadmill running in mice with mam-

mary carcinomas reduced tumor and circulating lactate concentrations by approximately 17% compared with sedentary counterparts [205]. Accordingly, Bacurau et al. also reported that a moderate-intensity treadmill running protocol significantly decreased glucose consumption and lactate production in rats with Walker-256 tumors [206]. Nonetheless, as evidence is lacking, future studies are required.

Immunosuppression

The impaired vascular supply of tumors and the consequent hostile TME characterized by hypoxia and acidosis hampers T-cell infiltration, function, and survival [216]. Physiologically, immune cells permanently patrol tissues to signal and remove pathogens, foreign antigens, and abnormal cells [217]. However, under hypoxic and acidic conditions there is an upregulation of growth factors and cytokines that inhibit the activity of T-cells and the ability of dendritic cells (DCs) to process and present tumor antigens (Fig. 1) [218–220]. Although poor lymphocyte infiltration due to an abnormal tumor vasculature represents the main reason for the immune failure in the elimination of cancer cells, the presence of immune suppressive agents in the TME is also important because it leads to T-cell dysfunction in the context of anergy, senescence, and exhaustion [221]. Consequently, the immune cells that successfully infiltrate in the TME are not fully effective [222].

A critical player of the metabolic reprogramming in the TME is hypoxia, and cells of both innate and adaptive immunity are very sensitive to these conditions, because it was shown that hypoxia can regulate aggregation, invasion, and motility of macrophages and neutrophils, favoring local inflammation and immunosuppression [223, 224]. Low O₂ levels can also impact T-cell differentiation by changing the balance toward a T helper (Th) 17/T regulatory (Treg) phenotype and impairing the cytotoxic properties of natural killer (NK) cells [225]. Additionally, hypoxia can inhibit T-cell function by exacerbating glucose deprivation, reducing cytosolic levels of calcium (Ca²⁺), which is essential for cytokine production and promoting excessive formation of reactive oxygen species (ROS) [226]. Indeed, it was proved that hypoxia can upregulate programmed death-ligand 1 (PD-L1) on tumor cells, which in turn dampens T-cell function by interacting with the inhibitory receptor programmed cell death protein 1 (PD-1) on the T-cell surface (Fig. 1) [227]. Hypoxia also upregulates indoleamine 2,3-dioxygenase (IDO) expression in DCs, which promotes Treg survival and function, inhibiting the generation of effector CD4⁺ and CD8⁺ T cells [228]. In addition, IDO leads to the upregulation of several cytokines like IL-6, IL-10, and TGF-β, further accentuating the immunosuppressive state [229].

Inefficient tumor vasculature and subsequent hypoxia lead to the development of necrotic foci, a very common feature of solid tumors [230]. Necrosis within TME results in increased concentrations of K⁺, which suppresses T-cell function (Fig. 1) [231]. Indeed, K⁺ released by necrotic cells, can be internalized by infiltrating T cells, inhibiting their effector functions due to the downregulation the Akt/mTOR signaling [232]. Besides, abnormal tumor vasculature not only deprives TME of O₂ and nutrients, but it also fills it with byproducts of metabolism, some of which display immune suppressive activities [233]. Indeed, the degradation of glu-

cose into lactate results in the acidification of the TME that further suppresses T-effector functions [220, 234]. At reduced pH, polymorphonuclear leukocytes (PMNs) display reduced chemotaxis and respiratory and bactericidal capacity, and lymphocyte cytotoxicity is also inhibited [235]. Further, low pH levels increase the suppressive activity of tumor-infiltrating myeloid derived suppressor cells (MDSCs) and tumor-associated macrophages (TAMs), reducing the proliferation of cytotoxic T-lymphocytes (CTLs) in the TME (Fig. 1) [236]. The inhibitory effects of acidic pH on effector T-cell response in the TME is associated with generalized T-cell anergy, impaired cytokine secretion, and decreased perforin degranulation [237]. In addition, Soriano and colleagues observed that tumors also escape CD8⁺ T-cell-induced cytotoxicity by overexpressing the inhibitors of perforin/granzyme pathway. In their study, the authors found that tumors showed an increased expression of protease inhibitor-9, a granzyme-B inhibitor [238]. Consequently, the capability of CTLs to kill tumor cells is reduced by one half [239]. Thus, similar to hypoxia, tumor acidity negatively regulates tumor-specific effector T cells, contributing to the dysfunction of antitumor immunity [240]. Therefore, alternative strategies to increase T-cell activation, infiltration, and cytotoxicity remain one of the main challenges today [241].

Immune recognition and elimination are important intrinsic mechanisms against tumors and their immunological profile is strongly associated with cancer prognosis [242]. A general model of exercise-driven modulation of immune cell distribution in tissues was proposed, describing the way that NK cells, T cells and B cells are mobilized to the blood [243]. This action seems to represent the recruitment of stored immune cells and not the formation of new ones [244]. Indeed, both local and systemic effects of physical exercise may potentiate T-cell incursion into tumors [245]. First, it increases circulating levels of IL-6, which can upregulate the adhesion molecules on tumor vascular endothelium, thus enhancing T-cell trafficking [246]. Second, exercise training causes redistribution of NK and CD8⁺ T cells that have cytotoxic activity against cancer cells, increasing their concentrations approximately 10-fold and 2.5-fold, respectively [247]. Physical exercise can also influence the immune system by regulating Th1 and Th2 responses. The increase in Th1-type cytokine (IL-12 and interferon gamma (IFN- γ)) production and T-box transcription factor (T-bet) expression after exercise training may contribute to the increased immune response against tumors [248]. T-bet is the main transcription factor involved in the differentiation of CD4⁺ T cells in Th1 cells that are able to inhibit tumor growth and metastasis [248]. However, several studies have shown that tumor cells produce molecules that inhibit DC maturation such as IL-10, prostaglandin E2 (PGE2), and TGF- β [249–251]. Nevertheless, it was shown that exercise training potentiates the synthesis of IFN- γ , tumor necrosis factor (TNF)- α and IL-12, inducing an increase and maturation of DCs [248]. Additionally, recent evidence indicates that the practice of regular physical exercise also tends to increase the infiltration of CTLs in murine tumors [252]. Indeed, exercise training may represent an effective strategy to improve immune response. A study comparing healthy and tumor-bearing animals concluded that physical exercise had a positive impact on macrophage function, which induced a reduction in metastasis [253]. On the contrary, sedentary tumor-bearing animals

showed an increase in Th2 and Treg profile, resulting in a lower antitumor immune response and, consequently, facilitating the development of external tumor masses [254]. Furthermore, the typical conversion of type 1 macrophages (M1) into type 2 (M2) seen in tumors produces higher levels of IL-10 and TGF- β , changing the differentiation of T cells away from a cytotoxic Th1 response [255, 256]. IL-10 downregulates the adaptive immune response, inhibiting the activation of pro-inflammatory cytokines, further compromising the ability of effector T cells to maintain the inflammatory response [257]. Indeed, tumor-bearing animals exposed to long-term physical exercise developed less infiltrative and more benign histological lesions compared to sedentary tumor-bearing rats [258, 259]. Exercise training may hinder the progression from non-invasive to invasive lesions by inhibiting uncontrolled cell proliferation and by increasing cell death, which partially explains the significant decrease in the total amount of malignant cells in exercised animals [259].

Similar to the remaining tissues, skeletal muscle contains resident immune cells to maintain homeostasis [260]. However, as pathological and physiological conditions like muscle contraction can induce chemical and mechanical stress threatening this condition, there is an additional infiltration of immune cells in skeletal muscles following physical exercise [261]. For immune cells to infiltrate skeletal muscle, the production of factors with attracting and activating properties is necessary [260]. Experimental evidence showed exercise-induced muscle production of several cytokines, including IL-6, IL-8, IL-10, IL-15, CC-chemokine ligand (CCL) 2, IL-1 receptor antagonist, and VEGF [262]. An important function of these cytokines in contracting muscles is to attract immune cells to control inflammatory processes and to support muscle regeneration following physical exercise [263]. Accordingly, Malm and colleagues observed that single bouts of eccentric exercise increased immune cell infiltration and inflammatory markers (CD11b, CD3 and HIF-1 β) in muscle epimysium [264]. Indeed, supporting the role of inflammatory response in muscles recovering from physical exercise, cDNA array analysis identified changes in gene products regulated by the NF- κ B pathway such as CCL2, IL-18, C-X-C motif chemokine ligand 1 (CXCL1), leukemia inhibitory factor (LIF), and TGF- β 1 [265]. Of these, CCL2 regulates migration and infiltration of monocytes and macrophages in skeletal muscle, which are indispensable for tissue repair [260]. The macrophages that infiltrate the tissue earlier after injury are activated M1. They generate inflammatory cytokines and sustain the activation and the proliferation of muscle stem cells [266]. Alternatively, activated M2 predominates during the resolution phase, regulating the ending of the inflammatory response [267]. Monocyte-derived macrophages undergo dynamic transitions between M1 and M2 and this timely transition is increasingly felt to be the key to muscle homeostasis [268]. Indeed, although PMNs, including neutrophils and eosinophils, are the first leukocytes to be recruited into the tissue, macrophages accumulate shortly thereafter and subsequently become the dominant leukocyte population [269]. In contrast, the accumulation of CD4⁺ or CD8⁺ T lymphocytes reflects a persistent inflammatory or immune-mediated injury, such as that taking place as a consequence of muscular dystrophy or inflammatory myopathies [270]. The importance of these cellular adaptations in the

health-promoting effects of physical exercise is further emphasized by the adverse effects of nonsteroidal anti-inflammatory drugs (NSAIDs) that inhibit the acute rise in skeletal muscle protein synthesis and the activation of satellite cells [271]. Therefore, these data support the role of physical exercise-induced immune response as a beneficial mechanism to a stress-inducing condition.

Taken together, these observations allow us to conclude that by contributing to the repair of tumor vasculature, physical exercise creates better conditions to improve antitumor immune response, systemically, by increasing Tbet gene and Th1 cytokines expression and, locally, by providing superior access and function of immune cells (Fig. 2).

Conclusion

The spatial and temporal heterogeneities in blood supply and vessel permeability associated with inefficient lymphatic drainage exacerbate the dysfunctional microenvironment that impairs a uniform distribution of O₂ and therapeutic agents in tumors [50, 90, 272]. In addition, pharmacological approaches to restore tumor vasculature present transient effects and, in some cases, may increase tumor hypoxia, which is a strong stimulus for tumor cells to metastasize [273]. Since tumor vascular repair in response to physical exercise has been described, new hopes were raised to mitigate tumor aggressiveness that is intimately associated with hypoxic microenvironments. Taken together, these data suggest that exercise training has direct antioncogenic effects [252, 259, 274]. The mechanistic insight into the immunological role of physical exercise indicates that it may be able to mediate intratumoral adaptations, by triggering alterations in the TME that lead to vascular repair, enhancing tumor perfusion, oxygenation, and infiltration of immune cells [275]. Thus, the practice of regular physical exercise as a non-pharmacologic therapy for remodeling tumor vasculature may overcome the limitations associated with AIs [10, 57]. In addition to the associated adverse side effects, pharmacologic AIs have a small therapeutic window with lower doses being ineffective and higher doses depleting too many vessels [45]. In contrast, it is unlikely that exercise training will over-prune tumor vasculature because capillaries adapt to chronic exercise by maturing and then becoming quiescent [276].

Globally, the available evidence suggests that exercise training may represent a valid therapeutic modality to the remodeling of tumor vasculature, improving TME and inhibiting tumor development. However, as the responses to physical exercise are complex and may be influenced by training parameters and by patients' socio-demographic variables, the results from pre-clinical investigations need to be replicated in the clinical setting, so additional studies are imperative.

Conflict of Interest

The authors declare, beyond the absence of any conflict of interest, that the manuscript complied with IJSM ethical standards [277].

References

- [1] Fan F, Schimming A, Jaeger D et al. Targeting the tumor microenvironment: Focus on angiogenesis. *J Oncol* 2012; 2012: 281261. doi:10.1155/2012/281261
- [2] Endrich B, Reinhold HS, Gross JF et al. Tissue perfusion in homogeneity during early tumor growth in rats. *J Natl Cancer Inst* 1979; 62: 387–395
- [3] Hendriksen EM, Span PN, Schuurin J et al. Angiogenesis, hypoxia and VEGF expression during tumour growth in a human xenograft tumour model. *Microvasc Res* 2009; 77: 96–103. doi:10.1016/j.mvr.2008.11.002
- [4] Jain RK. Delivery of novel therapeutic agents in tumors: Physiological barriers and strategies. *J Natl Cancer Inst* 1989; 81: 570–576. doi:10.1093/jnci/81.8.570
- [5] Schaaf MB, Garg AD, Agostinis P. Defining the role of the tumor vasculature in antitumor immunity and immunotherapy. *Cell Death Dis* 2018; 9: 115. doi:10.1038/s41419-017-0061-0
- [6] Li X, Li Y, Lu W et al. The tumor vessel targeting strategy: A double-edged sword in tumor metastasis. *Cells* 2019; 8: 1602. doi:10.3390/cells8121602
- [7] Zhang X, Ashcraft KA, Betof Warner A et al. Can exercise-induced modulation of the tumor physiologic microenvironment improve antitumor immunity? *Cancer Res* 2019; 79: 2447–2456. doi:10.1158/0008-5472.CAN-18-2468
- [8] Maj E, Papiernik D, Wietrzyk J. Antiangiogenic cancer treatment: The great discovery and greater complexity (Review). *Int J Oncol* 2016; 49: 1773–1784. doi:10.3892/ijo.2016.3709
- [9] Horsman MR, Siemann DW. Pathophysiologic effects of vascular-targeting agents and the implications for combination with conventional therapies. *Cancer Res* 2006; 66: 11520–11539. doi:10.1158/0008-5472.CAN-06-2848
- [10] Schadler KL, Thomas NJ, Galie PA et al. Tumor vessel normalization after aerobic exercise enhances chemotherapeutic efficacy. *Oncotarget* 2016; 7: 65429–65440. doi:10.18632/oncotarget.11748
- [11] Ashcraft KA, Warner AB, Jones LW et al. Exercise as adjunct therapy in cancer. *Semin Radiat Oncol* 2019; 29: 16–24. doi:10.1016/j.semradonc.2018.10.001
- [12] Gunnell AS, Joyce S, Tomlin S et al. Physical activity and survival among long-term cancer survivors and non-cancer cohorts. *Front Public Health* 2017; 5: 19. doi:10.3389/fpubh.2017.00019
- [13] Siemann DW, Horsman MR. Modulation of the tumor vasculature and oxygenation to improve therapy. *Pharmacol Ther* 2015; 153: 107–124. doi:10.1016/j.pharmthera.2015.06.006
- [14] Saman H, Raza SS, Uddin S et al. Inducing angiogenesis, a key step in cancer vascularization, and treatment approaches. *Cancers (Basel)* 2020; 12: 1172. doi:10.3390/cancers12051172
- [15] Lugano R, Ramachandran M, Dimberg A. Tumor angiogenesis: Causes, consequences, challenges and opportunities. *Cell Mol Life Sci* 2020; 77: 1745–1770. doi:10.1007/s00018-019-03351-7
- [16] Dvorak HF. Angiogenesis: Update 2005. *J Thromb Haemost* 2005; 3: 1835–1842. doi:10.1111/j.1538-7836.2005.01361.x
- [17] Nagy JA, Chang SH, Shih SC, et al. Heterogeneity of the tumor vasculature. *Semin Thromb Hemost* 2010; 36: 321–331. doi:10.1055/s-0030-1253454
- [18] Denekamp J. The tumour microcirculation as a target in cancer therapy: A clearer perspective. *Eur J Clin Invest* 1999; 29: 733–736. doi:10.1046/j.1365-2362.1999.00558.x
- [19] Denekamp J, Hill S. Angiogenic attack as a therapeutic strategy for cancer. *Radiother Oncol* 1991; 20: 103–112. doi:10.1016/0167-8140(91)90196-n
- [20] Hinnen P, Eskens FA. Vascular disrupting agents in clinical development. *Br J Cancer* 2007; 96: 1159–1165. doi:10.1038/sj.bjc.6603694

- [21] Bridges E, Harris AL. Vascular-promoting therapy reduced tumor growth and progression by improving chemotherapy efficacy. *Cancer Cell* 2015; 27: 7–9. doi:10.1016/j.ccell.2014.12.009
- [22] Rajendran P, Rengarajan T, Thangavel J et al. The vascular endothelium and human diseases. *Int J Biol Sci* 2013; 9: 1057–1069. doi:10.7150/ijbs.7502
- [23] Siemann DW. The unique characteristics of tumor vasculature and preclinical evidence for its selective disruption by tumor-vascular disrupting agents. *Cancer Treat Rev* 2011; 37: 63–74. doi:10.1016/j.ctrv.2010.05.001
- [24] Horsman MR, Murata R. Combination of vascular targeting agents with thermal or radiation therapy. *Int J Radiat Oncol Biol Phys* 2002; 54: 1518–1523. doi:10.1016/s0360-3016(02)03926-3
- [25] Seidi K, Jahanban-Esfahlan R, Zarghami N. Tumor rim cells: From resistance to vascular targeting agents to complete tumor ablation. *Tumour Biol* 2017; 39: 1010428317691001. doi:10.1177/1010428317691001
- [26] Rajabi M, Mousa SA. The role of angiogenesis in cancer treatment. *Biomedicines* 2017; 5: 34. doi:10.3390/biomedicines5020034
- [27] Teleanu RI, Chircov C, Grumezescu AM et al. Tumor angiogenesis and anti-angiogenic strategies for cancer treatment. *J Clin Med* 2019; 9: 84. doi:10.3390/jcm9010084
- [28] Guyot M, Hilmi C, Ambrosetti D et al. Targeting the pro-angiogenic forms of VEGF or inhibiting their expression as anti-cancer strategies. *Oncotarget* 2017; 8: 9174–9188. doi:10.18632/oncotarget.13942
- [29] Kunkel P, Ulbricht U, Bohlen P et al. Inhibition of glioma angiogenesis and growth in vivo by systemic treatment with a monoclonal antibody against vascular endothelial growth factor receptor-2. *Cancer Res* 2001; 61: 6624–6628
- [30] Shaheen RM, Tseng WW, Vellagas R et al. Effects of an antibody to vascular endothelial growth factor receptor-2 on survival, tumor vascularity, and apoptosis in a murine model of colon carcinomatosis. *Int J Oncol* 2001; 18: 221–226
- [31] Wedge SR, Ogilvie DJ, Dukes M et al. ZD6474 inhibits vascular endothelial growth factor signaling, angiogenesis, and tumor growth following oral administration. *Cancer Res* 2002; 62: 4645–4655
- [32] Beecken WD, Fernandez A, Jousen AM et al. Effect of antiangiogenic therapy on slowly growing, poorly vascularized tumors in mice. *J Natl Cancer Inst* 2001; 93: 382–387. doi:10.1093/jnci/93.5.382
- [33] Batchelor TT, Gerstner ER, Emblem KE et al. Improved tumor oxygenation and survival in glioblastoma patients who show increased blood perfusion after cediranib and chemoradiation. *Proc Natl Acad Sci USA* 2013; 110: 19059–19064. doi:10.1073/pnas.1318022110
- [34] Tong RT, Boucher Y, Kozin SV et al. Vascular normalization by vascular endothelial growth factor receptor 2 blockade induces a pressure gradient across the vasculature and improves drug penetration in tumors. *Cancer Res* 2004; 64: 3731–3736. doi:10.1158/0008-5472.CAN-04-0074
- [35] Dvorak HF. Vascular permeability factor/vascular endothelial growth factor: A critical cytokine in tumor angiogenesis and a potential target for diagnosis and therapy. *J Clin Oncol* 2002; 20: 4368–4380. doi:10.1200/JCO.2002.10.088
- [36] Siemann DW, Chaplin DJ, Horsman MR. Vascular-targeting therapies for treatment of malignant disease. *Cancer* 2004; 100: 2491–2499. doi:10.1002/cncr.20299
- [37] Ribatti D, Annese T, Ruggieri S et al. Limitations of anti-angiogenic treatment of tumors. *Transl Oncol* 2019; 12: 981–986. doi:10.1016/j.tranon.2019.04.022
- [38] Stebbing J, Waters L, Davies L et al. Incidence of cancer in individuals receiving chronic zopiclone or eszopiclone requires prospective study. *J Clin Oncol* 2005; 23: 8134–8136. doi:10.1200/JCO.2005.03.5881
- [39] Nakahara T, Norberg SM, Shalinsky DR et al. Effect of inhibition of vascular endothelial growth factor signaling on distribution of extravasated antibodies in tumors. *Cancer Res* 2006; 66: 1434–1445. doi:10.1158/0008-5472.CAN-05-0923
- [40] Morgan B, Thomas AL, Dreys J et al. Dynamic contrast-enhanced magnetic resonance imaging as a biomarker for the pharmacological response of PTK787/ZK 222584, an inhibitor of the vascular endothelial growth factor receptor tyrosine kinases, in patients with advanced colorectal cancer and liver metastases: Results from two phase I studies. *J Clin Oncol* 2003; 21: 3955–3964. doi:10.1200/JCO.2003.08.092
- [41] Ebos JM, Lee CR, Cruz-Munoz W et al. Accelerated metastasis after short-term treatment with a potent inhibitor of tumor angiogenesis. *Cancer Cell* 2009; 15: 232–239. doi:10.1016/j.ccr.2009.01.021
- [42] Paez-Ribes M, Allen E, Hudock J et al. Antiangiogenic therapy elicits malignant progression of tumors to increased local invasion and distant metastasis. *Cancer Cell* 2009; 15: 220–231. doi:10.1016/j.ccr.2009.01.027
- [43] Siemann DW, Shi W. Efficacy of combined antiangiogenic and vascular disrupting agents in treatment of solid tumors. *Int J Radiat Oncol Biol Phys* 2004; 60: 1233–1240. doi:10.1016/j.ijrobp.2004.08.002
- [44] Jahanban-Esfahlan R, Seidi K, Manjili MH et al. Tumor cell dormancy: Threat or opportunity in the fight against cancer. *Cancers (Basel)* 2019; 11: 1207. doi:10.3390/cancers11081207
- [45] Martin JD, Seano G, Jain RK. Normalizing function of tumor vessels: Progress, opportunities, and challenges. *Annu Rev Physiol* 2019; 81: 505–534. doi:10.1146/annurev-physiol-020518-114700
- [46] Roskoski R Jr.. Vascular endothelial growth factor (VEGF) signaling in tumor progression. *Crit Rev Oncol Hematol* 2007; 62: 179–213. doi:10.1016/j.critrevonc.2007.01.006
- [47] Fukumura D, Duda DG, Munn LL et al. Tumor microvasculature and microenvironment: Novel insights through intravital imaging in pre-clinical models. *Microcirculation* 2010; 17: 206–225. doi:10.1111/j.1549-8719.2010.00029.x
- [48] Ehling M, Mazzone M. Vessel normalization in the spot-LIGHT of cancer treatment. *Trends Mol Med* 2016; 22: 85–87. doi:10.1016/j.molmed.2015.12.009
- [49] Jain RK. Antiangiogenesis strategies revisited: From starving tumors to alleviating hypoxia. *Cancer Cell* 2014; 26: 605–622. doi:10.1016/j.ccell.2014.10.006
- [50] Jain RK, Martin JD, Stylianopoulos T. The role of mechanical forces in tumor growth and therapy. *Annu Rev Biomed Eng* 2014; 16: 321–346. doi:10.1146/annurev-bioeng-071813-105259
- [51] Goel S, Wong AH, Jain RK. Vascular normalization as a therapeutic strategy for malignant and nonmalignant disease. *Cold Spring Harb Perspect Med* 2012; 2: a006486. doi:10.1101/cshperspect.a006486
- [52] Leite de Oliveira R, Deschoemaeker S, Henze AT et al. Gene-targeting of Phd2 improves tumor response to chemotherapy and prevents side-toxicity. *Cancer Cell* 2012; 22: 263–277. doi:10.1016/j.ccr.2012.06.028
- [53] Li W, Quan YY, Li Y et al. Monitoring of tumor vascular normalization: The key points from basic research to clinical application. *Cancer Manag Res* 2018; 10: 4163–4172. doi:10.2147/CMAR.S174712
- [54] Goel S, Duda DG, Xu L et al. Normalization of the vasculature for treatment of cancer and other diseases. *Physiol Rev* 2011; 91: 1071–1121. doi:10.1152/physrev.00038.2010
- [55] Jain RK. Normalization of tumor vasculature: An emerging concept in antiangiogenic therapy. *Science* 2005; 307: 58–62. doi:10.1126/science.1104819
- [56] Cantelmo AR, Pircher A, Kalucka J et al. Vessel pruning or healing: Endothelial metabolism as a novel target? *Expert Opin Ther Targets* 2017; 21: 239–247. doi:10.1080/14728222.2017.1282465

- [57] Morrell MBG, Alvarez-Florez C, Zhang A et al. Vascular modulation through exercise improves chemotherapy efficacy in Ewing sarcoma. *Pediatr Blood Cancer* 2019; 66: e27835. doi:10.1002/pbc.27835
- [58] Betof AS, Lascola CD, Weitzel D et al. Modulation of murine breast tumor vascularity, hypoxia and chemotherapeutic response by exercise. *J Natl Cancer Inst* 2015; 107. doi:10.1093/jnci/djv040
- [59] McCullough DJ, Nguyen LM, Siemann DW et al. Effects of exercise training on tumor hypoxia and vascular function in the rodent preclinical orthotopic prostate cancer model. *J Appl Physiol* (1985) 2013; 115: 1846–1854. doi:10.1152/jappphysiol.00949.2013
- [60] McCullough DJ, Stabley JN, Siemann DW et al. Modulation of blood flow, hypoxia, and vascular function in orthotopic prostate tumors during exercise. *J Natl Cancer Inst* 2014; 106: dju036. doi:10.1093/jnci/dju036
- [61] Brown MD. Exercise and coronary vascular remodeling in the healthy heart. *Exp Physiol* 2003; 88: 645–658. doi:10.1113/eph8802618
- [62] Yang HT, Ren J, Laughlin MH et al. Prior exercise training produces NO-dependent increases in collateral blood flow after acute arterial occlusion. *Am J Physiol Heart Circ Physiol* 2002; 282: H301–H310. doi:10.1152/ajpheart.00160.2001
- [63] Schirmer SH, Millenaar DN, Werner C et al. Exercise promotes collateral artery growth mediated by monocytic nitric oxide. *Arterioscler Thromb Vasc Biol* 2015; 35: 1862–1871. doi:10.1161/ATVBAHA.115.305806
- [64] Hellsten Y, Nyberg M, Jensen LG et al. Vasodilator interactions in skeletal muscle blood flow regulation. *J Physiol* 2012; 590: 6297–6305. doi:10.1113/jphysiol.2012.240762
- [65] Jones LW, Viglianti BL, Tashjian JA et al. Effect of aerobic exercise on tumor physiology in an animal model of human breast cancer. *J Appl Physiol* (1985) 2010; 108: 343–348. doi:10.1152/jappphysiol.00424.2009
- [66] Qiu Y, Myers DR, Lam WA. The biophysics and mechanics of blood from a materials perspective. *Nat Rev Mater* 2019; 4: 294–311. doi:10.1038/s41578-019-0099-y
- [67] Atkinson CL, Carter HH, Naylor LH et al. Opposing effects of shear-mediated dilation and myogenic constriction on artery diameter in response to handgrip exercise in humans. *J Appl Physiol* (1985) 2015; 119: 858–864. doi:10.1152/jappphysiol.01086.2014
- [68] Kojda G, Hambrecht R. Molecular mechanisms of vascular adaptations to exercise. Physical activity as an effective antioxidant therapy? *Cardiovasc Res* 2005; 67: 187–197. doi:10.1016/j.cardiores.2005.04.032
- [69] Tzima E, Irani-Tehrani M, Kiosses WB et al. A mechanosensory complex that mediates the endothelial cell response to fluid shear stress. *Nature* 2005; 437: 426–431. doi:10.1038/nature03952
- [70] Ballermann BJ, Ott MJ. Adhesion and differentiation of endothelial cells by exposure to chronic shear stress: a vascular graft model. *Blood Purif* 1995; 13: 125–134. doi:10.1159/000170195
- [71] Green DJ, Hopman MT, Padilla J et al. Vascular adaptation to exercise in humans: Role of hemodynamic stimuli. *Physiol Rev* 2017; 97: 495–528. doi:10.1152/physrev.00014.2016
- [72] Chang YS, di Tomaso E, McDonald DM et al. Mosaic blood vessels in tumors: frequency of cancer cells in contact with flowing blood. *Proc Natl Acad Sci USA* 2000; 97: 14608–14613. doi:10.1073/pnas.97.26.14608
- [73] Goetz JG. Metastases go with the flow. *Science* 2018; 362: 999–1000. doi:10.1126/science.aat9100
- [74] Jain RK. Determinants of tumor blood flow: A review. *Cancer Res* 1988; 48: 2641–2658
- [75] Hashizume H, Baluk P, Morikawa S et al. Openings between defective endothelial cells explain tumor vessel leakiness. *Am J Pathol* 2000; 156: 1363–1380. doi:10.1016/S0002-9440(10)65006-7
- [76] Hobbs SK, Monsky WL, Yuan F et al. Regulation of transport pathways in tumor vessels: Role of tumor type and microenvironment. *Proc Natl Acad Sci USA* 1998; 95: 4607–4612. doi:10.1073/pnas.95.8.4607
- [77] Yuan F, Salehi HA, Boucher Y et al. Vascular permeability and microcirculation of gliomas and mammary carcinomas transplanted in rat and mouse cranial windows. *Cancer Res* 1994; 54: 4564–4568
- [78] Potenta S, Zeisberg E, Kalluri R. The role of endothelial-to-mesenchymal transition in cancer progression. *Br J Cancer* 2008; 99: 1375–1379. doi:10.1038/sj.bjc.6604662
- [79] Baluk P, Hashizume H, McDonald DM. Cellular abnormalities of blood vessels as targets in cancer. *Curr Opin Genet Dev* 2005; 15: 102–111. doi:10.1016/j.gde.2004.12.005
- [80] Ozawa MG, Yao VJ, Chanthery YH et al. Angiogenesis with pericyte abnormalities in a transgenic model of prostate carcinoma. *Cancer* 2005; 104: 2104–2115. doi:10.1002/cncr.21436
- [81] Baluk P, Morikawa S, Haskell A et al. Abnormalities of basement membrane on blood vessels and endothelial sprouts in tumors. *Am J Pathol* 2003; 163: 1801–1815. doi:10.1016/S0002-9440(10)63540-7
- [82] Jung B, Obinata H, Galvani S et al. Flow-regulated endothelial S1P receptor-1 signaling sustains vascular development. *Dev Cell* 2012; 23: 600–610. doi:10.1016/j.devcel.2012.07.015
- [83] Hughes SK, Wacker BK, Kaneda MM et al. Fluid shear stress modulates cell migration induced by sphingosine 1-phosphate and vascular endothelial growth factor. *Ann Biomed Eng* 2005; 33: 1003–1014. doi:10.1007/s10439-005-5756-1
- [84] Zhao J, Garcia D, Gartung A et al. Sphingosine-1-phosphate receptor subtype 2 signaling in endothelial senescence-associated functional impairments and inflammation. *Curr Atheroscler Rep* 2015; 17: 504. doi:10.1007/s11883-015-0504-y
- [85] Cattoretti G, Mandelbaum J, Lee N et al. Targeted disruption of the S1P2 sphingosine 1-phosphate receptor gene leads to diffuse large B-cell lymphoma formation. *Cancer Res* 2009; 69: 8686–8692. doi:10.1158/0008-5472.CAN-09-1110
- [86] Carmeliet P, Jain RK. Principles and mechanisms of vessel normalization for cancer and other angiogenic diseases. *Nat Rev Drug Discov* 2011; 10: 417–427. doi:10.1038/nrd3455
- [87] Jaszi J, Schmidt MHH. Trends and challenges in tumor anti-angiogenic therapies. *Cells* 2019; 8: 1102. doi:10.3390/cells8091102
- [88] Dellian M, Witwer BP, Salehi HA et al. Quantitation and physiological characterization of angiogenic vessels in mice: Effect of basic fibroblast growth factor, vascular endothelial growth factor/vascular permeability factor, and host microenvironment. *Am J Pathol* 1996; 149: 59–71
- [89] Zhou Y, Wang W, Wei R et al. Serum bradykinin levels as a diagnostic marker in cervical cancer with a potential mechanism to promote VEGF expression via BDKRB2. *Int J Oncol* 2019; 55: 131–141. doi:10.3892/ijo.2019.4792
- [90] Stylianopoulos T, Munn LL, Jain RK. Reengineering the physical microenvironment of tumors to improve drug delivery and efficacy: From mathematical modeling to bench to bedside. *Trends Cancer* 2018; 4: 292–319. doi:10.1016/j.trecan.2018.02.005
- [91] Tredan O, Galmarini CM, Patel K et al. Drug resistance and the solid tumor microenvironment. *J Natl Cancer Inst* 2007; 99: 1441–1454. doi:10.1093/jnci/djm135
- [92] Boucher Y, Jain RK. Microvascular pressure is the principal driving force for interstitial hypertension in solid tumors: Implications for vascular collapse. *Cancer Res* 1992; 52: 5110–5114
- [93] Gao Y, Shi Y, Fu M et al. Simulation study of the effects of interstitial fluid pressure and blood flow velocity on transvascular transport of nanoparticles in tumor microenvironment. *Comput Methods Programs Biomed* 2020; 193: 105493. doi:10.1016/j.cmpb.2020.105493

- [94] Jang SH, Wientjes MG, Lu D et al. Drug delivery and transport to solid tumors. *Pharm Res* 2003; 20: 1337–1350. doi:10.1023/a:1025785505977
- [95] Graham K, Unger E. Overcoming tumor hypoxia as a barrier to radiotherapy, chemotherapy and immunotherapy in cancer treatment. *Int J Nanomedicine* 2018; 13: 6049–6058. doi:10.2147/IJN.S140462
- [96] Jain RK, Stylianopoulos T. Delivering nanomedicine to solid tumors. *Nat Rev Clin Oncol* 2010; 7: 653–664. doi:10.1038/nr-clinonc.2010.139
- [97] Vaupel P, Kallinowski F, Okunieff P. Blood flow, oxygen and nutrient supply, and metabolic microenvironment of human tumors: A review. *Cancer Res* 1989; 49: 6449–6465
- [98] Hori K, Suzuki M, Tanda S et al. In vivo analysis of tumor vascularization in the rat. *Jpn J Cancer Res* 1990; 81: 279–288. doi:10.1111/j.1349-7006.1990.tb02562.x
- [99] Hori K, Suzuki M, Tanda S et al. Characterization of heterogeneous distribution of tumor blood flow in the rat. *Jpn J Cancer Res* 1991; 82: 109–117. doi:10.1111/j.1349-7006.1991.tb01753.x
- [100] Forster JC, Harriss-Phillips WM, Douglass MJ et al. A review of the development of tumor vasculature and its effects on the tumor microenvironment. *Hypoxia (Auckl)* 2017; 5: 21–32. doi:10.2147/HP.S133231
- [101] Petrova V, Annicchiarico-Petruzzelli M, Melino G et al. The hypoxic tumour microenvironment. *Oncogenesis* 2018; 7: 10. doi:10.1038/s41389-017-0011-9
- [102] Jing X, Yang F, Shao C et al. Role of hypoxia in cancer therapy by regulating the tumor microenvironment. *Mol Cancer* 2019; 18: 157. doi:10.1186/s12943-019-1089-9
- [103] Hellsten Y, Nyberg M. Cardiovascular adaptations to exercise training. *Compr Physiol* 2015; 6: 1–32. doi:10.1002/cphy.c140080
- [104] Sarelius I, Pohl U. Control of muscle blood flow during exercise: local factors and integrative mechanisms. *Acta Physiol (Oxf)* 2010; 199: 349–365. doi:10.1111/j.1748-1716.2010.02129.x
- [105] Bagher P, Segal SS. Regulation of blood flow in the microcirculation: role of conducted vasodilation. *Acta Physiol (Oxf)* 2011; 202: 271–284. doi:10.1111/j.1748-1716.2010.02244.x
- [106] Cohen KD, Berg BR, Sarelius IH. Remote arteriolar dilations in response to muscle contraction under capillaries. *Am J Physiol Heart Circ Physiol* 2000; 278: H1916–H1923. doi:10.1152/ajpheart.2000.278.6.H1916
- [107] Garcia E, Becker VG, McCullough DJ et al. Blood flow responses to mild-intensity exercise in ectopic vs. orthotopic prostate tumors; Dependence upon host tissue hemodynamics and vascular reactivity. *J Appl Physiol* (1985) 2016; 121: 15–24. doi:10.1152/japplphysiol.00266.2016
- [108] Rowell LB. Human cardiovascular adjustments to exercise and thermal stress. *Physiol Rev* 1974; 54: 75–159. doi:10.1152/physrev.1974.54.1.75
- [109] Mishra RC, Rahman MM, Davis MJ et al. Alpha1 -adrenergic stimulation selectively enhances endothelium-mediated vasodilation in rat cremaster arteries. *Physiol Rep* 2018; 6: e13703. doi:10.14814/phy2.13703
- [110] Matsukawa K. Central command: control of cardiac sympathetic and vagal efferent nerve activity and the arterial baroreflex during spontaneous motor behaviour in animals. *Exp Physiol* 2012; 97: 20–28. doi:10.1113/expphysiol.2011.057661
- [111] White DW, Raven PB. Autonomic neural control of heart rate during dynamic exercise: Revisited. *J Physiol* 2014; 592: 2491–2500. doi:10.1113/jphysiol.2014.271858
- [112] Tschakovsky ME, Sujirattanawimol K, Ruble SB et al. Is sympathetic neural vasoconstriction blunted in the vascular bed of exercising human muscle? *J Physiol* 2002; 541: 623–635. doi:10.1113/jphysiol.2001.014431
- [113] Mortensen SP, Nyberg M, Gliemann L et al. Exercise training modulates functional sympatholysis and alpha-adrenergic vasoconstrictor responsiveness in hypertensive and normotensive individuals. *J Physiol* 2014; 592: 3063–3073. doi:10.1113/jphysiol.2014.273722
- [114] Heinonen I, Wendelin-Saarenhovi M, Kaskinoro K et al. Inhibition of alpha-adrenergic tone disturbs the distribution of blood flow in the exercising human limb. *Am J Physiol Heart Circ Physiol* 2013; 305: H163–H172. doi:10.1152/ajpheart.00925.2012
- [115] McGee MC, Hamner JB, Williams RF et al. Improved intratumoral oxygenation through vascular normalization increases glioma sensitivity to ionizing radiation. *Int J Radiat Oncol Biol Phys* 2010; 76: 1537–1545. doi:10.1016/j.ijrobp.2009.12.010
- [116] Missiaen R, Mazzone M, Bergers G. The reciprocal function and regulation of tumor vessels and immune cells offers new therapeutic opportunities in cancer. *Semin Cancer Biol* 2018; 52: 107–116. doi:10.1016/j.semcancer.2018.06.002
- [117] Martin JD, Fukumura D, Duda DG et al. Reengineering the tumor microenvironment to alleviate hypoxia and overcome cancer heterogeneity. *Cold Spring Harb Perspect Med* 2016; 6: a027094. doi:10.1101/cshperspect.a027094
- [118] Seynhaeve ALB, Amin M, Haemmerich D et al. Hyperthermia and smart drug delivery systems for solid tumor therapy. *Adv Drug Deliv Rev* 2020. Online ahead of print. doi:10.1016/j.addr.2020.02.004
- [119] Nacev A, Kim SH, Rodriguez-Canales J et al. A dynamic magnetic shift method to increase nanoparticle concentration in cancer metastases: A feasibility study using simulations on autopsy specimens. *Int J Nanomedicine* 2011; 6: 2907–2923. doi:10.2147/IJN.S23724
- [120] Dewhirst MW, Secomb TW. Transport of drugs from blood vessels to tumour tissue. *Nat Rev Cancer* 2017; 17: 738–750. doi:10.1038/nrc.2017.93
- [121] Aukland K, Reed RK. Interstitial-lymphatic mechanisms in the control of extracellular fluid volume. *Physiol Rev* 1993; 73: 1–78. doi:10.1152/physrev.1993.73.1.1
- [122] Jacob M, Chappell D, Becker BF. Regulation of blood flow and volume exchange across the microcirculation. *Crit Care* 2016; 20: 319. doi:10.1186/s13054-016-1485-0
- [123] Heldin CH, Rubin K, Pietras K et al. High interstitial fluid pressure – an obstacle in cancer therapy. *Nat Rev Cancer* 2004; 4: 806–813. doi:10.1038/nrc1456
- [124] Carraway RE, Cochrane DE. Enhanced vascular permeability is hypothesized to promote inflammation-induced carcinogenesis and tumor development via extravasation of large molecular proteins into the tissue. *Med Hypotheses* 2012; 78: 738–743. doi:10.1016/j.mehy.2012.02.021
- [125] Horsman MR, Vaupel P. Pathophysiological basis for the formation of the tumor microenvironment. *Front Oncol* 2016; 6: 66. doi:10.3389/fonc.2016.00066
- [126] Garnier L, Gkoutidi AO, Hugues S. Tumor-associated lymphatic vessel features and immunomodulatory functions. *Front Immunol* 2019; 10: 720. doi:10.3389/fimmu.2019.00720
- [127] Simonsen TG, Gaustad JV, Leinaas MN et al. High interstitial fluid pressure is associated with tumor-line specific vascular abnormalities in human melanoma xenografts. *PLoS One* 2012; 7: e40006. doi:10.1371/journal.pone.0040006
- [128] Munson JM, Shieh AC. Interstitial fluid flow in cancer: Implications for disease progression and treatment. *Cancer Manag Res* 2014; 6: 317–328. doi:10.2147/CMAR.S65444
- [129] Wagner M, Wiig H. Tumor interstitial fluid formation, characterization, and clinical implications. *Front Oncol* 2015; 5: 115. doi:10.3389/fonc.2015.00115
- [130] Boucher Y, Baxter LT, Jain RK. Interstitial pressure gradients in tissue-isolated and subcutaneous tumors: Implications for therapy. *Cancer Res* 1990; 50: 4478–4484

- [131] Padera TP, Stoll BR, Tooredman JB et al. Pathology: cancer cells compress intratumour vessels. *Nature* 2004; 427: 695. doi:10.1038/427695a
- [132] Prabhakar U, Maeda H, Jain RK et al. Challenges and key considerations of the enhanced permeability and retention effect for nanomedicine drug delivery in oncology. *Cancer Res* 2013; 73: 2412–2417. doi:10.1158/0008-5472.CAN-12-4561
- [133] Padera TP, Kadambi A, di Tomaso E et al. Lymphatic metastasis in the absence of functional intratumor lymphatics. *Science* 2002; 296: 1883–1886. doi:10.1126/science.1071420
- [134] Wei R, Liu S, Zhang S et al. Cellular and extracellular components in tumor microenvironment and their application in early diagnosis of cancers. *Anal Cell Pathol (Amst)* 2020; 2020: 6283796. doi:10.1155/2020/6283796
- [135] Shang M, Soon RH, Lim CT et al. Microfluidic modelling of the tumor microenvironment for anti-cancer drug development. *Lab Chip* 2019; 19: 369–386. doi:10.1039/c8lc00970h
- [136] Mori T, Koga T, Shibata H et al. Interstitial fluid pressure correlates clinicopathological factors of lung cancer. *Ann Thorac Cardiovasc Surg* 2015; 21: 201–208. doi:10.5761/atcs.0a.14-00208
- [137] Milosevic M, Fyles A, Hedley D et al. Interstitial fluid pressure predicts survival in patients with cervix cancer independent of clinical prognostic factors and tumor oxygen measurements. *Cancer Res* 2001; 61: 6400–6405
- [138] van der Voort van Zyp J, Thamilselvan V, Walsh M et al. Extracellular pressure stimulates colon cancer cell adhesion in vitro and to surgical wounds by Src (sarcoma protein) activation. *Am J Surg* 2004; 188: 467–473. doi:10.1016/j.amjsurg.2004.07.005
- [139] Rofstad EK, Galappathi K, Mathiesen BS. Tumor interstitial fluid pressure—a link between tumor hypoxia, microvascular density, and lymph node metastasis. *Neoplasia* 2014; 16: 586–594. doi:10.1016/j.neo.2014.07.003
- [140] Jain RK, Tong RT, Munn LL. Effect of vascular normalization by antiangiogenic therapy on interstitial hypertension, peritumor edema, and lymphatic metastasis: Insights from a mathematical model. *Cancer Res* 2007; 67: 2729–2735. doi:10.1158/0008-5472.CAN-06-4102
- [141] Gillen CM, Lee R, Mack GW et al. Plasma volume expansion in humans after a single intense exercise protocol. *J Appl Physiol* (1985) 1991; 71: 1914–1920. doi:10.1152/jappl.1991.71.5.1914
- [142] Gillen CM, Nishiyasu T, Langhans G et al. Cardiovascular and renal function during exercise-induced blood volume expansion in men. *J Appl Physiol* (1985) 1994; 76: 2602–2610. doi:10.1152/jappl.1994.76.6.2602
- [143] Nagashima K, Cline GW, Mack GW et al. Intense exercise stimulates albumin synthesis in the upright posture. *J Appl Physiol* (1985) 2000; 88: 41–46. doi:10.1152/jappl.2000.88.1.41
- [144] Mack GW, Yang R, Hargens AR et al. Influence of hydrostatic pressure gradients on regulation of plasma volume after exercise. *J Appl Physiol* (1985) 1998; 85: 667–675. doi:10.1152/jappl.1998.85.2.667
- [145] Haskell A, Nadel ER, Stachenfeld NS et al. Transcapillary escape rate of albumin in humans during exercise-induced hypervolemia. *J Appl Physiol* (1985) 1997; 83: 407–413. doi:10.1152/jappl.1997.83.2.407
- [146] Zawieja DC. Contractile physiology of lymphatics. *Lymphat Res Biol* 2009; 7: 87–96. doi:10.1089/lrb.2009.0007
- [147] Reed RK, Rubin K. Transcapillary exchange: role and importance of the interstitial fluid pressure and the extracellular matrix. *Cardiovasc Res* 2010; 87: 211–217. doi:10.1093/cvr/cvq143
- [148] Denekamp J, Hobson B. Endothelial-cell proliferation in experimental tumors. *Br J Cancer* 1982; 46: 711–720. doi:10.1038/bjc.1982.263
- [149] Vaupel P, Hockel M. Blood supply, oxygenation status and metabolic micromilieu of breast cancers: characterization and therapeutic relevance. *Int J Oncol* 2000; 17: 869–879. doi:10.3892/ijo.17.5.869
- [150] Helmlinger G, Yuan F, Dellian M et al. Interstitial pH and pO₂ gradients in solid tumors in vivo: High-resolution measurements reveal a lack of correlation. *Nat Med* 1997; 3: 177–182. doi:10.1038/nm0297-177
- [151] Bertout JA, Patel SA, Simon MC. The impact of O₂ availability on human cancer. *Nat Rev Cancer* 2008; 8: 967–975. doi:10.1038/nrc2540
- [152] Bayer C, Shi K, Astner ST et al. Acute versus chronic hypoxia: why a simplified classification is simply not enough. *Int J Radiat Oncol Biol Phys* 2011; 80: 965–968. doi:10.1016/j.ijrobp.2011.02.049
- [153] Hockel M, Vaupel P. Tumor hypoxia: definitions and current clinical, biologic, and molecular aspects. *J Natl Cancer Inst* 2001; 93: 266–276. doi:10.1093/jnci/93.4.266
- [154] Kolstad P. Intercapillary distance, oxygen tension and local recurrence in cervix cancer. *Scand J Clin Lab Invest Suppl* 1968; 106: 145–157
- [155] Vaupel P, Mayer A, Hockel M. Tumor hypoxia and malignant progression. *Methods Enzymol* 2004; 381: 335–354. doi:10.1016/S0076-6879(04)81023-1
- [156] Kaelin WG Jr.. The VHL tumor suppressor gene: Insights into oxygen sensing and cancer. *Trans Am Clin Climatol Assoc* 2017; 128: 298–307
- [157] Nakayama K, Kataoka N. Regulation of gene expression under hypoxic conditions. *Int J Mol Sci* 2019; 20: 3278. doi:10.3390/ijms20133278
- [158] Semenza GL. Targeting HIF-1 for cancer therapy. *Nat Rev Cancer* 2003; 3: 721–732. doi:10.1038/nrc1187
- [159] Wang Y, Wang H, Li J et al. Direct visualization of the phenotype of hypoxic tumor cells at single cell resolution in vivo using a new hypoxia probe. *Intravital*. 2016; 5: e1187803. doi:10.1080/21659087.2016.1187803
- [160] Muz B, de la Puente P, Azab F et al. The role of hypoxia in cancer progression, angiogenesis, metastasis, and resistance to therapy. *Hypoxia (Auckl)* 2015; 3: 83–92. doi:10.2147/HP.S93413
- [161] Semenza GL. Oxygen sensing, hypoxia-inducible factors, and disease pathophysiology. *Annu Rev Pathol* 2014; 9: 47–71. doi:10.1146/annurev-pathol-012513-104720
- [162] Brizel DM, Scully SP, Harrelson JM et al. Tumor oxygenation predicts for the likelihood of distant metastases in human soft tissue sarcoma. *Cancer Res* 1996; 56: 941–943
- [163] Hasan NM, Adams GE, Joiner MC et al. Hypoxia facilitates tumour cell detachment by reducing expression of surface adhesion molecules and adhesion to extracellular matrices without loss of cell viability. *Br J Cancer* 1998; 77: 1799–1805. doi:10.1038/bjc.1998.299
- [164] Young SD, Marshall RS, Hill RP. Hypoxia induces DNA overreplication and enhances metastatic potential of murine tumor cells. *Proc Natl Acad Sci USA* 1988; 85: 9533–9537. doi:10.1073/pnas.85.24.9533
- [165] Dewhirst MW. A potential solution for eliminating hypoxia as a cause for radioresistance. *Proc Natl Acad Sci USA* 2018; 115: 10548–10550. doi:10.1073/pnas.1814212115
- [166] Sharma A, Arambula JF, Koo S et al. Hypoxia-targeted drug delivery. *Chem Soc Rev* 2019; 48: 771–813. doi:10.1039/c8cs00304a
- [167] Yokoyama C, Sueyoshi Y, Ema M et al. Induction of oxidative stress by anticancer drugs in the presence and absence of cells. *Oncol Lett* 2017; 14: 6066–6070. doi:10.3892/ol.2017.6931
- [168] Kim SJ, Kim HS, Seo YR. Understanding of ROS-inducing strategy in anticancer therapy. *Oxid Med Cell Longev* 2019; 2019: 5381692. doi:10.1155/2019/5381692
- [169] Wang H, Jiang H, Van De Gucht M et al. Hypoxic Radioresistance: Can ROS be the key to overcome it? *Cancers (Basel)* 2019; 11: 112. doi:10.3390/cancers11010112

- [170] Wilson WR, Hay MP. Targeting hypoxia in cancer therapy. *Nat Rev Cancer* 2011; 11: 393–410. doi:10.1038/nrc3064
- [171] Ferreira LF, Poole DC, Barstow TJ. Muscle blood flow-O₂ uptake interaction and their relation to on-exercise dynamics of O₂ exchange. *Respir Physiol Neurobiol* 2005; 147: 91–103. doi:10.1016/j.resp.2005.02.002
- [172] Perko MJ, Nielsen HB, Skak C et al. Mesenteric, coeliac and splanchnic blood flow in humans during exercise. *J Physiol* 1998; 513: 907–913. doi:10.1111/j.1469-7793.1998.907ba.x
- [173] Endo MY, Suzuki R, Nagahata N et al. Differential arterial blood flow response of splanchnic and renal organs during low-intensity cycling exercise in women. *Am J Physiol Heart Circ Physiol* 2008; 294: H2322–H2326. doi:10.1152/ajpheart.91491.2007
- [174] Fry BC, Roy TK, Secomb TW. Capillary recruitment in a theoretical model for blood flow regulation in heterogeneous microvessel networks. *Physiol Rep* 2013; 1: e00050. doi:10.1002/phy2.50
- [175] Radak Z, Zhao Z, Koltai E et al. Oxygen consumption and usage during physical exercise: the balance between oxidative stress and ROS-dependent adaptive signaling. *Antioxid Redox Signal* 2013; 18: 1208–1246. doi:10.1089/ars.2011.4498
- [176] Ortiz-Prado E, Dunn JF, Vasconez J et al. Partial pressure of oxygen in the human body: a general review. *Am J Blood Res* 2019; 9: 1–14
- [177] Pittman RN. Oxygen transport in the microcirculation and its regulation. *Microcirculation* 2013; 20: 117–137. doi:10.1111/micc.12017
- [178] Pittman RN. Oxygen supply to contracting skeletal muscle at the microcirculatory level: diffusion vs. convection. *Acta Physiol Scand* 2000; 168: 593–602. doi:10.1046/j.1365-201x.2000.00710.x
- [179] Cardinale DA, Larsen FJ, Jensen-Urstad M et al. Muscle mass and inspired oxygen influence oxygen extraction at maximal exercise: role of mitochondrial oxygen affinity. *Acta Physiol (Oxf)* 2019; 225: e13110. doi:10.1111/apha.13110
- [180] Joyner MJ, Casey DP. Regulation of increased blood flow (hyperemia) to muscles during exercise: a hierarchy of competing physiological needs. *Physiol Rev* 2015; 95: 549–601. doi:10.1152/physrev.00035.2013
- [181] Lindholm ME, Rundqvist H. Skeletal muscle hypoxia-inducible factor-1 and exercise. *Exp Physiol* 2016; 101: 28–32. doi:10.1113/EP085318
- [182] Skattebo O, Capelli C, Rud B et al. Increased oxygen extraction and mitochondrial protein expression after small muscle mass endurance training. *Scand J Med Sci Sports* 2020; 30: 1615–1631. doi:10.1111/sms.13707
- [183] Rud B, Foss O, Krstrup P et al. One-legged endurance training: leg blood flow and oxygen extraction during cycling exercise. *Acta Physiol (Oxf)* 2012; 205: 177–185. doi:10.1111/j.1748-1716.2011.02383.x
- [184] Lundby C, Gassmann M, Pilegaard H. Regular endurance training reduces the exercise induced HIF-1 α and HIF-2 α mRNA expression in human skeletal muscle in normoxic conditions. *Eur J Appl Physiol* 2006; 96: 363–369. doi:10.1007/s00421-005-0085-5
- [185] Lindholm ME, Fischer H, Poellinger L et al. Negative regulation of HIF in skeletal muscle of elite endurance athletes: a tentative mechanism promoting oxidative metabolism. *Am J Physiol Regul Integr Comp Physiol* 2014; 307: R248–R255. doi:10.1152/ajpregu.00036.2013
- [186] Gatenby RA, Gawlinski ET, Gmitro AF et al. Acid-mediated tumor invasion: a multidisciplinary study. *Cancer Res* 2006; 66: 5216–5223. doi:10.1158/0008-5472.CAN-05-4193
- [187] Gatenby RA, Gillies RJ. Why do cancers have high aerobic glycolysis? *Nat Rev Cancer* 2004; 4: 891–899. doi:10.1038/nrc1478
- [188] Kallinowski F, Vaupel P, Runkel S et al. Glucose uptake, lactate release, ketone body turnover, metabolic micromilieu, and pH distributions in human breast cancer xenografts in nude rats. *Cancer Res* 1988; 48: 7264–7272
- [189] Luo Z, Loja MN, Farwell DG et al. Widefield optical imaging of changes in uptake of glucose and tissue extracellular pH in head and neck cancer. *Cancer Prev Res (Phila)* 2014; 7: 1035–1044. doi:10.1158/1940-6207.CAPR-14-0097
- [190] Loja MN, Luo Z, Greg Farwell D et al. Optical molecular imaging detects changes in extracellular pH with the development of head and neck cancer. *Int J Cancer* 2013; 132: 1613–1623. doi:10.1002/ijc.27837
- [191] Griffiths JR. Are cancer cells acidic? *Br J Cancer* 1991; 64: 425–427. doi:10.1038/bjc.1991.326
- [192] Avnet S, Chano T, Massa A et al. Acid microenvironment promotes cell survival of human bone sarcoma through the activation of cIAP proteins and NF- κ B pathway. *Am J Cancer Res* 2019; 9: 1127–1144
- [193] de la Cruz-Lopez KG, Castro-Munoz LJ, Reyes-Hernandez DO et al. Lactate in the regulation of tumor microenvironment and therapeutic approaches. *Front Oncol* 2019; 9: 1143. doi:10.3389/fonc.2019.01143
- [194] Gerweck LE. Tumor pH: implications for treatment and novel drug design. *Semin Radiat Oncol* 1998; 8: 176–182. doi:10.1016/s1053-4296(98)80043-x
- [195] Kallinowski F, Runkel S, Fortmeyer HP et al. L-glutamine: A major substrate for tumor cells in vivo? *J Cancer Res Clin Oncol* 1987; 113: 209–215. doi:10.1007/BF00396375
- [196] Longo DL, Bartoli A, Consolino L et al. In vivo imaging of tumor metabolism and acidosis by combining PET and MRI-CEST pH imaging. *Cancer Res* 2016; 76: 6463–6470. doi:10.1158/0008-5472.CAN-16-0825
- [197] Jiang B. Aerobic glycolysis and high level of lactate in cancer metabolism and microenvironment. *Genes Dis* 2017; 4: 25–27. doi:10.1016/j.gendis.2017.02.003
- [198] Al Tameemi W, Dale TP, Al-Jumaily RMK et al. Hypoxia-modified cancer cell metabolism. *Front Cell Dev Biol* 2019; 7: 4. doi:10.3389/fcell.2019.00004
- [199] Gillies RJ, Gatenby RA. Metabolism and its sequelae in cancer evolution and therapy. *Cancer J* 2015; 21: 88–96. doi:10.1097/PPO.0000000000000102
- [200] Fais S, Venturi G, Gatenby B. Microenvironmental acidosis in carcinogenesis and metastases: new strategies in prevention and therapy. *Cancer Metastasis Rev* 2014; 33: 1095–1108. doi:10.1007/s10555-014-9531-3
- [201] Kato Y, Ozawa S, Miyamoto C et al. Acidic extracellular microenvironment and cancer. *Cancer Cell Int* 2013; 13: 89. doi:10.1186/1475-2867-13-89
- [202] Gerweck LE, Vijayappa S, Kozin S. Tumor pH controls the in vivo efficacy of weak acid and base chemotherapeutics. *Mol Cancer Ther* 2006; 5: 1275–1279. doi:10.1158/1535-7163.MCT-06-0024
- [203] Faes S, Uldry E, Planche A et al. Acidic pH reduces VEGF-mediated endothelial cell responses by downregulation of VEGFR-2; relevance for anti-angiogenic therapies. *Oncotarget* 2016; 7: 86026–86038. doi:10.18632/oncotarget.13323
- [204] Kolosenko I, Avnet S, Baldini N et al. Therapeutic implications of tumor interstitial acidification. *Semin Cancer Biol* 2017; 43: 119–133. doi:10.1016/j.semcancer.2017.01.008
- [205] Aveseh M, Nikoobie R, Aminaie M. Exercise-induced changes in tumour LDH-B and MCT1 expression are modulated by oestrogen-related receptor alpha in breast cancer-bearing BALB/c mice. *J Physiol* 2015; 593: 2635–2648. doi:10.1113/JP270463

- [206] Bacurau RF, Belmonte MA, Seelaender MC et al. Effect of a moderate intensity exercise training protocol on the metabolism of macrophages and lymphocytes of tumour-bearing rats. *Cell Biochem Funct* 2000; 18: 249–258. doi:10.1002/1099-0844(200012)18:4 < 249::AID-CBF879 > 3.0.CO;2-2
- [207] Juel C. Regulation of pH in human skeletal muscle: adaptations to physical activity. *Acta Physiol (Oxf)* 2008; 193: 17–24. doi:10.1111/j.1748-1716.2008.01840.x
- [208] Stickland MK, Lindinger MI, Olfert IM et al. Pulmonary gas exchange and acid-base balance during exercise. *Compr Physiol* 2013; 3: 693–739. doi:10.1002/cphy.c110048
- [209] Wasserman K, Cox TA, Sietsema KE. Ventilatory regulation of arterial H(+) (pH) during exercise. *Respir Physiol Neurobiol* 2014; 190: 142–148. doi:10.1016/j.resp.2013.10.009
- [210] Pilegaard H, Domino K, Noland T et al. Effect of high-intensity exercise training on lactate/H⁺ transport capacity in human skeletal muscle. *Am J Physiol* 1999; 276: E255–E261. doi:10.1152/ajpendo.1999.276.2.E255
- [211] Juel C. Regulation of cellular pH in skeletal muscle fiber types, studied with sarcolemmal giant vesicles obtained from rat muscles. *Biochim Biophys Acta* 1995; 1265: 127–132. doi:10.1016/0167-4889(94)00209-w
- [212] Juel C, Holten MK, Dela F. Effects of strength training on muscle lactate release and MCT1 and MCT4 content in healthy and type 2 diabetic humans. *J Physiol* 2004; 556: 297–304. doi:10.1113/jphysiol.2003.058222
- [213] Pilegaard H, Bangsbo J, Richter EA et al. Lactate transport studied in sarcolemmal giant vesicles from human muscle biopsies: relation to training status. *J Appl Physiol* (1985) 1994; 77: 1858–1862. doi:10.1152/jappl.1994.77.4.1858
- [214] Thomas C, Bishop D, Moore-Morris T et al. Effects of high-intensity training on MCT1, MCT4, and NBC expressions in rat skeletal muscles: influence of chronic metabolic alkalosis. *Am J Physiol Endocrinol Metab* 2007; 293: E916–E922. doi:10.1152/ajpendo.00164.2007
- [215] Nielsen JJ, Mohr M, Klarskov C et al. Effects of high-intensity intermittent training on potassium kinetics and performance in human skeletal muscle. *J Physiol* 2004; 554: 857–870. doi:10.1113/jphysiol.2003.050658
- [216] Anderson KG, Stromnes IM, Greenberg PD. Obstacles posed by the tumor microenvironment to T cell activity: a case for synergistic therapies. *Cancer Cell* 2017; 31: 311–325. doi:10.1016/j.ccell.2017.02.008
- [217] Park SL, Gebhardt T, Mackay LK. Tissue-resident memory T cells in cancer immunosurveillance. *Trends Immunol* 2019; 40: 735–747. doi:10.1016/j.it.2019.06.002
- [218] Paardekooper LM, Vos W, van den Bogaart G. Oxygen in the tumor microenvironment: effects on dendritic cell function. *Oncotarget* 2019; 10: 883–896. doi:10.18632/oncotarget.26608
- [219] Vito A, El-Sayes N, Mossman K. Hypoxia-driven immune escape in the tumor microenvironment. *Cells*. 2020; 9: 992. doi:10.3390/cells9040992
- [220] Huber V, Camisaschi C, Berzi A et al. Cancer acidity: an ultimate frontier of tumor immune escape and a novel target of immunomodulation. *Semin Cancer Biol* 2017; 43: 74–89. doi:10.1016/j.semcancer.2017.03.001
- [221] Gajewski TF, Fuertes M, Spaepen R et al. Molecular profiling to identify relevant immune resistance mechanisms in the tumor microenvironment. *Curr Opin Immunol* 2011; 23: 286–292. doi:10.1016/j.coi.2010.11.013
- [222] Davoodzadeh Gholami M, Kardar GA, Saeedi Y et al. Exhaustion of T lymphocytes in the tumor microenvironment: significance and effective mechanisms. *Cell Immunol* 2017; 322: 1–14. doi:10.1016/j.cellimm.2017.10.002
- [223] Krzywinska E, Stockmann C. Hypoxia, metabolism and immune cell function. *Biomedicines* 2018; 6: 56. doi:10.3390/biomedicines6020056
- [224] Lin Y, Xu J, Lan H. Tumor-associated macrophages in tumor metastasis: biological roles and clinical therapeutic applications. *J Hematol Oncol* 2019; 12: 76. doi:10.1186/s13045-019-0760-3
- [225] Labiano S, Palazon A, Melero I. Immune response regulation in the tumor microenvironment by hypoxia. *Semin Oncol* 2015; 42: 378–386. doi:10.1053/j.seminoncol.2015.02.009
- [226] Kondoh M, Ohga N, Akiyama K et al. Hypoxia-induced reactive oxygen species cause chromosomal abnormalities in endothelial cells in the tumor microenvironment. *PLoS One* 2013; 8: e80349. doi:10.1371/journal.pone.0080349
- [227] Xu-Monette ZY, Zhang M, Li J et al. PD-1/PD-L1 Blockade: Have we found the key to unleash the antitumor immune response? *Front Immunol* 2017; 8: 1597. doi:10.3389/fimmu.2017.01597
- [228] Song X, Zhang Y, Zhang L et al. Hypoxia enhances indoleamine 2,3-dioxygenase production in dendritic cells. *Oncotarget* 2018; 9: 11572–11580. doi:10.18632/oncotarget.24098
- [229] Prendergast GC, Smith C, Thomas S et al. Indoleamine 2,3-dioxygenase pathways of pathogenic inflammation and immune escape in cancer. *Cancer Immunol Immunother* 2014; 63: 721–735. doi:10.1007/s00262-014-1549-4
- [230] Kiraga L, Cheda L, Taciak B et al. Changes in hypoxia level of CT26 tumors during various stages of development and comparing different methods of hypoxia determination. *PLoS One* 2018; 13: e0206706. doi:10.1371/journal.pone.0206706
- [231] Conforti L. Potassium channels of T lymphocytes take center stage in the fight against cancer. *J Immunother Cancer*. 2017; 5: 2. doi:10.1186/s40425-016-0202-5
- [232] Eil R, Vodnala SK, Clever D et al. Ionic immune suppression within the tumour microenvironment limits T cell effector function. *Nature* 2016; 537: 539–543. doi:10.1038/nature19364
- [233] Renner K, Singer K, Koehl GE et al. Metabolic hallmarks of tumor and immune cells in the tumor microenvironment. *Front Immunol* 2017; 8: 248. doi:10.3389/fimmu.2017.00248
- [234] Brand A, Singer K, Koehl GE et al. LDHA-associated lactic acid production blunts tumor immunosurveillance by T and NK cells. *Cell Metab* 2016; 24: 657–671. doi:10.1016/j.cmet.2016.08.011
- [235] Lardner A. The effects of extracellular pH on immune function. *J Leukoc Biol* 2001; 69: 522–530
- [236] Gkretsi V, Stylianou A, Papageorgis P et al. Remodeling components of the tumor microenvironment to enhance cancer therapy. *Front Oncol* 2015; 5: 214. doi:10.3389/fonc.2015.00214
- [237] Calcinotto A, Filipazzi P, Grieco M et al. Modulation of microenvironment acidity reverses anergy in human and murine tumor-infiltrating T lymphocytes. *Cancer Res* 2012; 72: 2746–2756. doi:10.1158/0008-5472.CAN-11-1272
- [238] Soriano C, Mukaro V, Hodge G et al. Increased proteinase inhibitor-9 (PI-9) and reduced granzyme B in lung cancer: mechanism for immune evasion? *Lung Cancer* 2012; 77: 38–45. doi:10.1016/j.lungcan.2012.01.017
- [239] Gottfried E, Kunz-Schughart LA, Ebner S et al. Tumor-derived lactic acid modulates dendritic cell activation and antigen expression. *Blood* 2006; 107: 2013–2021. doi:10.1182/blood-2005-05-1795
- [240] Bellone M, Calcinotto A, Filipazzi P et al. The acidity of the tumor microenvironment is a mechanism of immune escape that can be overcome by proton pump inhibitors. *Oncoimmunology* 2013; 2: e22058. doi:10.4161/onci.22058
- [241] Caruana I, Simula L, Locatelli F et al. T lymphocytes against solid malignancies: winning ways to defeat tumors. *Cell Stress* 2018; 2: 200–212. doi:10.15698/cst2018.07.148

- [242] Hojman P, Gehl J, Christensen JF et al. Molecular mechanisms linking exercise to cancer prevention and treatment. *Cell Metab* 2018; 27: 10–21. doi:10.1016/j.cmet.2017.09.015
- [243] Nielsen HB, Secher NH, Kappel M et al. Lymphocyte, NK and LAK cell responses to maximal exercise. *Int J Sports Med* 1996; 17: 60–65. doi:10.1055/s-2007-972809
- [244] Walsh NP, Gleeson M, Pyne DB et al. Position statement. Part two: Maintaining immune health. *Exerc Immunol Rev* 2011; 17: 64–103
- [245] Koelwyn GJ, Wennerberg E, Demaria S et al. Exercise in regulation of inflammation-immune axis function in cancer initiation and progression. *Oncology (Williston Park)* 2015; 29: 908–920
- [246] Stromberg A, Rullman E, Jansson E et al. Exercise-induced upregulation of endothelial adhesion molecules in human skeletal muscle and number of circulating cells with remodeling properties. *J Appl Physiol (1985)* 2017; 122: 1145–1154. doi:10.1152/jappphysiol.00956.2016
- [247] Campbell JP, Riddell NE, Burns VE et al. Acute exercise mobilises CD8+ T lymphocytes exhibiting an effector-memory phenotype. *Brain Behav Immun* 2009; 23: 767–775. doi:10.1016/j.bbi.2009.02.011
- [248] Bianco TM, Abdalla DR, Desiderio CS et al. The influence of physical activity in the anti-tumor immune response in experimental breast tumor. *Immunol Lett* 2017; 190: 148–158. doi:10.1016/j.imlet.2017.08.007
- [249] DeVito NC, Plebanek MP, Theivanthiran B et al. Role of tumor-mediated dendritic cell tolerization in immune evasion. *Front Immunol* 2019; 10: 2876. doi:10.3389/fimmu.2019.02876
- [250] Kim R, Emi M, Tanabe K. Cancer cell immune escape and tumor progression by exploitation of anti-inflammatory and pro-inflammatory responses. *Cancer Biol Ther* 2005; 4: 924–933. doi:10.4161/cbt.4.9.2101
- [251] Kusmartsev S, Gabrilovich DI. Effect of tumor-derived cytokines and growth factors on differentiation and immune suppressive features of myeloid cells in cancer. *Cancer Metastasis Rev* 2006; 25: 323–331. doi:10.1007/s10555-006-9002-6
- [252] Pedersen L, Idorn M, Olofsson GH et al. Voluntary running suppresses tumor growth through epinephrine- and IL-6-dependent NK cell mobilization and redistribution. *Cell Metab* 2016; 23: 554–562. doi:10.1016/j.cmet.2016.01.011
- [253] Ashcraft KA, Peace RM, Betof AS et al. Efficacy and mechanisms of aerobic exercise on cancer initiation, progression, and metastasis: a critical systematic review of in vivo preclinical data. *Cancer Res* 2016; 76: 4032–4050. doi:10.1158/0008-5472.CAN-16-0887
- [254] Abdalla DR, Murta EF, Michelin MA. The influence of physical activity on the profile of immune response cells and cytokine synthesis in mice with experimental breast tumors induced by 7,12-dimethylbenzanthracene. *Eur J Cancer Prev* 2013; 22: 251–258. doi:10.1097/CEJ.0b013e3283592cbb
- [255] Qi L, Yu H, Zhang Y et al. IL-10 secreted by M2 macrophage promoted tumorigenesis through interaction with JAK2 in glioma. *Oncotarget* 2016; 7: 71673–71685. doi:10.18632/oncotarget.12317
- [256] Kwasniak K, Czarnik-Kwasniak J, Maziarz A et al. Scientific reports concerning the impact of interleukin 4, interleukin 10 and transforming growth factor beta on cancer cells. *Cent Eur J Immunol* 2019; 44: 190–200. doi:10.5114/ceji.2018.76273
- [257] Maynard CL, Weaver CT. Diversity in the contribution of interleukin-10 to T-cell-mediated immune regulation. *Immunol Rev* 2008; 226: 219–233. doi:10.1111/j.1600-065X.2008.00711.x
- [258] Faustino-Rocha AI, Silva A, Gabriel J et al. Long-term exercise training as a modulator of mammary cancer vascularization. *Biomed Pharmacother* 2016; 81: 273–280. doi:10.1016/j.biopha.2016.04.030
- [259] Figueira ACC, Figueira MC, Silva C et al. Exercise training-induced modulation in microenvironment of rat mammary neoplasms. *Int J Sports Med* 2018; 39: 885–892. doi:10.1055/a-0660-0198
- [260] Pillion NJ, Bilan PJ, Fink LN et al. Cross-talk between skeletal muscle and immune cells: muscle-derived mediators and metabolic implications. *Am J Physiol Endocrinol Metab* 2013; 304: E453–E465. doi:10.1152/ajpendo.00553.2012
- [261] Yang W, Hu P. Skeletal muscle regeneration is modulated by inflammation. *J Orthop Translat* 2018; 13: 25–32. doi:10.1016/j.jot.2018.01.002
- [262] Peake JM, Della Gatta P, Suzuki K et al. Cytokine expression and secretion by skeletal muscle cells: regulatory mechanisms and exercise effects. *Exerc Immunol Rev* 2015; 21: 8–25
- [263] Hoffmann C, Weigert C. Skeletal muscle as an endocrine organ: the role of myokines in exercise adaptations. *Cold Spring Harb Perspect Med* 2017; 7: a029793. doi:10.1101/cshperspect.a029793
- [264] Malm C, Sjodin TL, Sjoberg B et al. Leukocytes, cytokines, growth factors and hormones in human skeletal muscle and blood after uphill or downhill running. *J Physiol* 2004; 556: 983–1000. doi:10.1113/jphysiol.2003.056598
- [265] Hyldahl RD, Xin L, Hubal MJ et al. Activation of nuclear factor-kappaB following muscle eccentric contractions in humans is localized primarily to skeletal muscle-residing pericytes. *FASEB J* 2011; 25: 2956–2966. doi:10.1096/fj.10-177105
- [266] Arnold L, Henry A, Poron F et al. Inflammatory monocytes recruited after skeletal muscle injury switch into anti-inflammatory macrophages to support myogenesis. *J Exp Med* 2007; 204: 1057–1069. doi:10.1084/jem.20070075
- [267] Ruffell D, Mourkioti F, Gambardella A et al. A CREB-C/EBPbeta cascade induces M2 macrophage-specific gene expression and promotes muscle injury repair. *Proc Natl Acad Sci USA* 2009; 106: 17475–17480. doi:10.1073/pnas.0908641106
- [268] Mosser DM, Edwards JP. Exploring the full spectrum of macrophage activation. *Nat Rev Immunol* 2008; 8: 958–969. doi:10.1038/nri2448
- [269] Chazaud B. Macrophages: supportive cells for tissue repair and regeneration. *Immunobiology* 2014; 219: 172–178. doi:10.1016/j.imbio.2013.09.001
- [270] Villalta SA, Rosenthal W, Martinez L et al. Regulatory T cells suppress muscle inflammation and injury in muscular dystrophy. *Sci Transl Med* 2014; 6: 258ra142. doi:10.1126/scitranslmed.3009925
- [271] Trappe TA, Carroll CC, Dickinson JM et al. Influence of acetaminophen and ibuprofen on skeletal muscle adaptations to resistance exercise in older adults. *Am J Physiol Regul Integr Comp Physiol* 2011; 300: R655–R662. doi:10.1152/ajpregu.00611.2010
- [272] Zhang B, Hu Y, Pang Z. Modulating the tumor microenvironment to enhance tumor nanomedicine delivery. *Front Pharmacol* 2017; 8: 952. doi:10.3389/fphar.2017.00952
- [273] De Bock K, Mazzone M, Carmeliet P. Antiangiogenic therapy, hypoxia, and metastasis: risky liaisons, or not? *Nat Rev Clin Oncol* 2011; 8: 393–404. doi:10.1038/nrclinonc.2011.83
- [274] Hvid T, Lindegaard B, Winding K et al. Effect of a 2-year home-based endurance training intervention on physiological function and PSA doubling time in prostate cancer patients. *Cancer Causes Control* 2016; 27: 165–174. doi:10.1007/s10552-015-0694-1
- [275] Idorn M, Hojman P. Exercise-dependent regulation of NK cells in cancer protection. *Trends Mol Med* 2016; 22: 565–577. doi:10.1016/j.molmed.2016.05.007
- [276] Di Francescomarino S, Sciartilli A, Di Valerio V et al. The effect of physical exercise on endothelial function. *Sports Med* 2009; 39: 797–812. doi:10.2165/11317750-000000000-00000
- [277] Harriss DJ, MacSween A, Atkinson G. Ethical standards in sport and exercise science research: 2020 update. *Int J Sports Med* 2019; 40: 813–817. doi:10.1055/a-1015-3123

CHAPTER III

EMPIRICAL STUDIES

3.1. Study III

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Regular Voluntary Running Inhibits Androgen-Independent Prostate Cancer Growth in Mice

Mário Esteves, Carina Silva, Sofia S. Pereira, Tiago Morais, Ângela Moreira, Madalena M. Costa, Mariana P. Monteiro, and José A. Duarte

Introduction: Benefits of regular physical exercise were demonstrated as preventive and coadjuvant nonpharmacological anticancer therapy. However, the role of exercise in modulating prostate cancer behavior has yet to be established. **Methods:** Prostate tumors were induced in C57BL/6 male mice ($n=28$) by subcutaneous inoculation of a suspension of murine androgen-independent RM1 cells (1.5×10^5 cells/500 μ L phosphate-buffered saline) in the dorsal region. Mice were randomly allocated into 2 study groups: sedentary tumor-induced ($n=14$) and exercised tumor-induced ($n=14$). Exercise consisted of voluntary running in wheeled cages. Mice ($n=7$ per group) were sacrificed either 14 or 28 days after cell inoculation to evaluate tumor weight and percentage of area occupied by immunohistochemistry stained cells for Ki-67 and TdT-mediated dUTP-biotin nick end labeling, used as surrogate markers of cell proliferation and apoptosis, respectively. **Results:** Compared with sedentary tumor-induced mice, the tumors developed by exercised tumor-induced mice were significantly smaller at 14 days (0.17 [0.12] g vs 0.48 [0.24] g, $P < .05$) and at 28 days (0.92 [0.73] g vs 2.09 [1.31] g, $P < .05$), with smaller Ki-67 and greater TdT-mediated dUTP-biotin nick end-labeling stained areas ($P < .05$). **Conclusion:** These results suggest that regular voluntary running inhibits prostate cancer cell growth by reducing cell proliferation and enhancing apoptosis.

Keywords: tumor, physical exercise, cellular proliferation, apoptosis

Prostate cancer (PCa) is the second most frequent cancer diagnosed in men worldwide, representing 7.1% of all cancers in males.¹ The highest incidence rate occurs in developed countries, and the cancer becomes more prevalent with increasing age.² In the European Union alone, the predicted number of deaths by PCa in 2020 is about 78,800, which represents a mortality rate of 10.0/100,000.³ Frequently, PCa cells are initially androgen dependent but can become androgen independent during the progression of the disease, leading to a worse prognosis.⁴ According to disease stage, treatment modalities can include active surveillance, radical prostatectomy, radiation therapy, androgen deprivation therapy, and, occasionally, chemotherapy.⁵ PCa tends to be a slow-growing cancer, providing an opportunity for interventions capable of delaying its progression to improve life expectancy.^{6,7}

In this regard, physical exercise has been recognized as a safe and efficacious strategy for preventing and attenuating physiological and psychological sequelae of anticancer therapy.⁸ Interestingly, epidemiological studies have shown that the regular practice of physical exercise following PCa diagnosis inhibited disease progression and reduced mortality rate.^{9–11} In addition, two other studies observed that voluntary wheel¹² and treadmill¹³ running were able to inhibit the growth rate of prostate tumor cells in animal models.

Therefore, it was proposed that physical exercise may directly impact tumor physiology by inhibiting tumor growth and metastasis, highlighting its potential for delaying disease progression.¹⁴ A recent meta-analysis of preclinical studies showed that the regular practice of physical exercise was able to inhibit tumor progression, mainly in breast cancer models.¹⁵ Thus, it appears that physical exercise, as a multimodal intervention, may have tumor-mitigating properties that encompass a favorable regulation at cellular, organ, and systemic levels.¹⁶ Nevertheless, because the majority of studies involved patients with breast cancer or animal models, the results cannot be generalized to other types of cancer.¹⁷ Significant comparisons and conclusions from the current PCa research are limited due to heterogeneity in the methodologies applied and to inconclusive results obtained from some studies.^{18–20} Consequently, an improved understanding of the effects of physical exercise on tumor biology is necessary, and well-controlled preclinical studies are essential in order to fully elucidate the mechanisms through which physical exercise modulates cancer prognosis.²¹ Therefore, the present study aimed to investigate the effect of regular voluntary running on the ectopic tumor growth of mice inoculated with RM1 androgen-independent prostate carcinoma cells. We hypothesized that regular physical exercise would inhibit tumor growth by favorably modulating the tumor microenvironment (TME), that is, by reducing the tumor proliferative capacity and/or by improving its ability to undergo programmed cell death.

Materials and Methods

Animals and Procedures

Twenty-eight C57BL/6 male mice (9 wk old) were obtained from Charles River (Barcelona) and randomly assigned into 2 groups: sedentary tumor-induced (SED+T, $n=14$) and voluntary exercise tumor-induced (EX+T, $n=14$). All mice were individually

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housed with corncob for bedding under controlled conditions of temperature and humidity, on a 12-hour light–dark cycle. Tap water and a standard laboratory chow were supplied ad libitum throughout the study. Cages were cleaned and water was changed once per week. On the day of tumor cell inoculation, the mice from the EX + T group were individually housed in cages equipped with free access to an activity wheel with coupled counter to record daily running distances. The mice from the SED + T group were individually housed in cages without any activity wheel in order to restrict physical activity to the cage area, preventing the mice from moving/running long distances or achieving high running velocities such as would occur in a wild environment. Both cages had identical floor areas (800 cm²; Tecniplast, Buguggiate, Italy). Food intake and running distance were evaluated daily and body weight (BW) was evaluated weekly. At the end of the study, accurate BW was calculated by subtracting tumor weight from total BW. The study was approved by the local ethics committee, and all procedures were carried out in accordance with European and national legislation (European Directive 2010/ 63/EU and National Decree-law 113/2013).

Tumor Model

SED + T and EX + T mice were inoculated with a suspension of RM1 androgen-independent murine prostate carcinoma cells (1.5 × 10⁵ cells/500 µL PBS) that was administered by subcutaneous injection into the dorsal region according to Ribeiro et al.²²

Necropsy

In each group, mice were sacrificed either on the 14th day or on the 28th day after tumor cell inoculation. Mice were deeply anesthetized with an intraperitoneal injection of ketamine/xylazine (0.09 and 0.01 mg/g, respectively) and then sacrificed by cervical dislocation. Tumors were removed by surgical dissection and weighed and sectioned in pieces of approximately 0.5 cm³ for routine histology processing and paraffin embedding before being sectioned for immunohistochemistry analysis.

Histology and Immunohistochemistry Analysis

After tissue processing and embedding for light microscopy, 4-µm-thick sections of tumors were performed. After deparaffinization, hydration, antigen retrieval, and blockade of endogenous peroxidase activity, sections were incubated overnight at 4°C with a primary antibody for Ki-67 (NCL-L-Ki67-MM1; Novocastra, Newcastle, United Kingdom) at a dilution of 1:100. Then, sections were incubated with a secondary antibody (polyclonal swine anti-rabbit; Dako, Glostrup, Denmark) for 30 minutes at a dilution of 1:200 and exposed to avidin–biotin complex (Vector Laboratories, Burlingame, CA) at a dilution of 1:100. DAB (diaminobenzidine tetrahydrochloride; Dako) was used as chromogen, and hematoxylin was used as nuclear counterstaining. For negative control, the sections were incubated with 5% bovine serum albumin (Sigma-Aldrich, St. Louis, MO) instead of the primary antibody. To assess apoptosis, a TdT-mediated dUTP-biotin nick end-labeling (TUNEL) assay (TACS[®] 2 TdT DAB; Trevigen, Gaithersburg, MD) was performed according to manufacturer instructions. Sections were exposed to DAB for 7 minutes and counterstained with 1% methyl green. Ki-67 and TUNEL immune stained sections were digitally scanned (Olympus VS110 Virtual Microscopy System, Center Valley, PA) to quantify the percentage of tumor stained area using a computerized morphometric analysis tool

(DAB area/tumor area; Fiji software, Madison, WI), as previously described.²³

Statistical Analysis

Normality and homogeneity of variances were verified by the Shapiro–Wilk and Levene tests. If there was a normal distribution, the outcomes are presented as mean and SD, and the statistical inference was done through the parametric test 1-way analysis of variance with Tukey post hoc test. Without a normal distribution, the outcomes are expressed by median and interquartile range (25%–75%). In this case, statistical inference was done through the nonparametric Kruskal–Wallis test. Statistical correlations were measured using Pearson or Spearman correlation coefficients. Homogeneity and correlations were calculated with SPSS (version 20; IBM Corp, Chicago, IL), and the remaining data were statistically analyzed with GraphPad Prism 6.00 (San Diego, CA). A *P* value below .05 was considered statistically significant.

Results

General Findings

Cumulative food intake at 28 days was significantly higher in the group of EX + T mice, but no significant differences in BW were identified between the 2 study groups (Table 1). In addition, EX + T mice ran an average of 4.72 (1.55) km/d during the first 14 days and 4.71 (0.93) km/d until the end of the intervention (28 days), without reaching a significant difference between the 2 moments (Figure 1).

Prostate Tumors

EX + T mice presented lower weight tumors compared to the SED + T mice at both time points, that is, at 14 days (0.17 [0.12] g vs 0.48 [0.24] g, *P* < .05) and at 28 days (0.92 [0.73] g vs 2.09 [1.31] g, *P* < .05) (Figure 2).

Tumor Cell Proliferation and Apoptosis

The percentage of tumor area occupied by Ki-67 stained cells was significantly different between the groups at 28 days, being significantly lower in the EX + T group (EX + T = 5.58% [1.08%–6.61%] vs SED + T = 9.65% [5.92%–11.28%], *P* < .05; Figures 3 and 4). Tumors from the EX + T mice showed a higher percentage of TUNEL stained area at 28 days compared to what was observed at 14 days (3.65% [2.77%–5.04%] vs 0.89% [0.46%–2.31%], *P* < .01; Figure 5). Furthermore, at 28 days, the TUNEL stained area

Table 1 BW and Food Intake

	SED + T	EX + T
BW, g		
Baseline	22.64 (1.47)	22.08 (0.72)
14 d	23.78 (1.23)	21.94 (1.21)
28 d	27.27 (2.39)	25.22 (1.52)
Food intake, g		
14 d	3.60 (0.26)	3.95 (0.25)
28 d	3.47 (0.33)	4.19 (0.46)*

Abbreviations: BW, body weight; EX + T indicates exercised tumor bearing; SED + T indicates sedentary tumor bearing.
**P* < .05 versus SED + T.

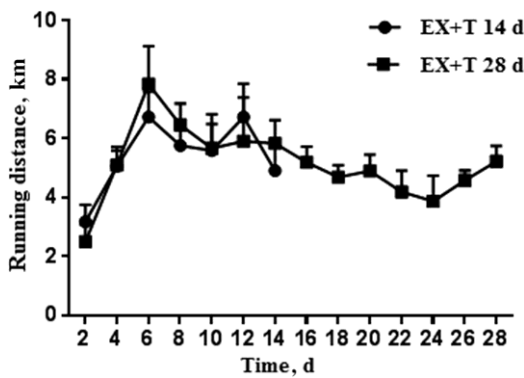


Figure 1 — Daily running distance (km) of EX + T mice. EX + T indicates exercised tumor bearing.

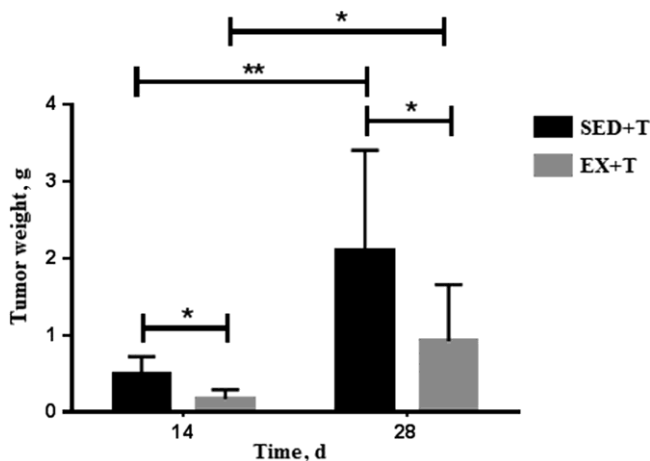


Figure 2 — Tumor weight (g) from SED + T and EX + T mice at 14 and 28 days. EX + T indicates exercised tumor bearing; SED + T, sedentary tumor bearing. * $P < .05$. ** $P < .01$.

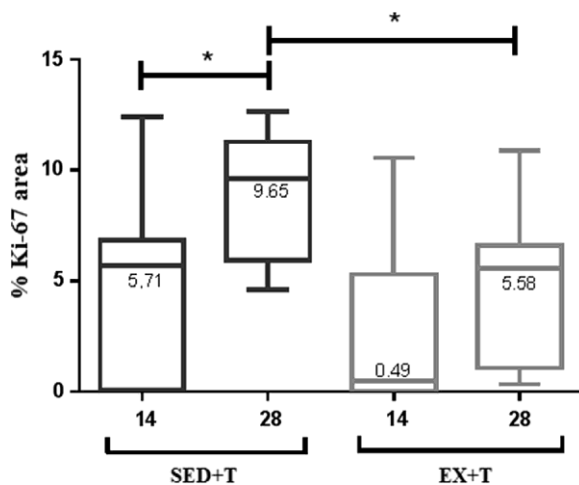


Figure 3 — Box plot analysis of tumor proliferative area (%) from SED + T and EX + T mice at 14 and 28 days. EX + T indicates exercised tumor bearing; SED + T, sedentary tumor bearing. * $P < .05$.

was superior in the EX + T mice compared to the SED + T mice (3.65% [2.77%–5.04%] vs 2.37% [1.85%–3.46%], $P < .05$; Figure 6).

Correlation Analysis

Running distance positively correlated with TUNEL stained area ($r = .523$; $P < .01$). However, there was not a significant correlation between running distance and tumor weight ($r = -.214$; $P > .05$) or Ki-67 stained area ($r = -.234$; $P > .05$).

Discussion

Consistent with our hypothesis, the results of the present study provided strong evidence for the protective effect of regular voluntary running on androgen-independent prostate tumor growth by inducing a higher apoptotic rate and decreasing cellular proliferation. These findings were in line with other reports that have showed the effectiveness of physical exercise in inhibiting cancer progression.^{12,13,24} Zheng et al¹² showed that voluntary running inhibited the growth of androgen-independent prostate PC-3 xenograft tumors in severe combined immunodeficient mice. Similar to our results, the authors found an inferior percentage of mitotic cells and a higher percentage of apoptotic cells in exercised mice compared with the control group. In addition, regular voluntary running decreased the ratio of mitotic cells/caspase-3 positive cells by 32%, which represented a shift toward programmed cell death and, consequently, toward decreased tumor growth.¹²

Opoku-Acheampong et al¹³ also observed that 11 weeks of treadmill running significantly reduced AT-1 cell viability in conditioned media from the prostate and serum of Copenhagen rats. Exercise training significantly increased the expression of caspase-3 within the prostate, impacting the supportive TME and consequently inducing higher apoptosis. Indeed, caspases play an important role in apoptotic cell death, and caspase-3 is the main active caspase in eliminating cells with increased potential for malignancy (cells with damaged DNA or aberrant cell cycling).^{25,26} This is of particular clinical importance, because decreased caspase-3 expression in PCa compared with benign prostatic hyperplasia in humans has already been shown.²⁷

In our study, we used an androgen-independent prostate carcinoma cell line in order to prevent the potential influence of testosterone concentration variations occurring with exercise training. Indeed, while acute physical exercise increases the levels of serum testosterone,²⁸ a reduction in circulating levels of testosterone occurs as a chronic adaptation to exercise training,²⁹ resulting in the upregulation of caspase-3 expression.²⁵ Thus, the inhibitory effect of physical exercise on tumor growth rate found in the present study does not seem to be a consequence of altered systemic androgen concentrations. A C3(1)Tag mouse model confirmed these data by showing that physical exercise delayed PCa progression in a dose-dependent manner.²⁴ The authors reported that 10 weeks of voluntary running inhibited tumor growth, particularly in animals that ran more than 5 km/d. Voluntary wheel running is a model of endurance exercise for rodents, and mice normally have a high activity on wheels (3–7 h and 4–20 km per night),^{30–32} depending on strain, sex, and age.²⁴ The average running distance in the present study was in accordance with the reported standard values, which means that despite the clinical condition, the exercised mice maintained a relatively high activity level throughout the experiment. In addition, a prospective study of physical activity among PCa patients also revealed a

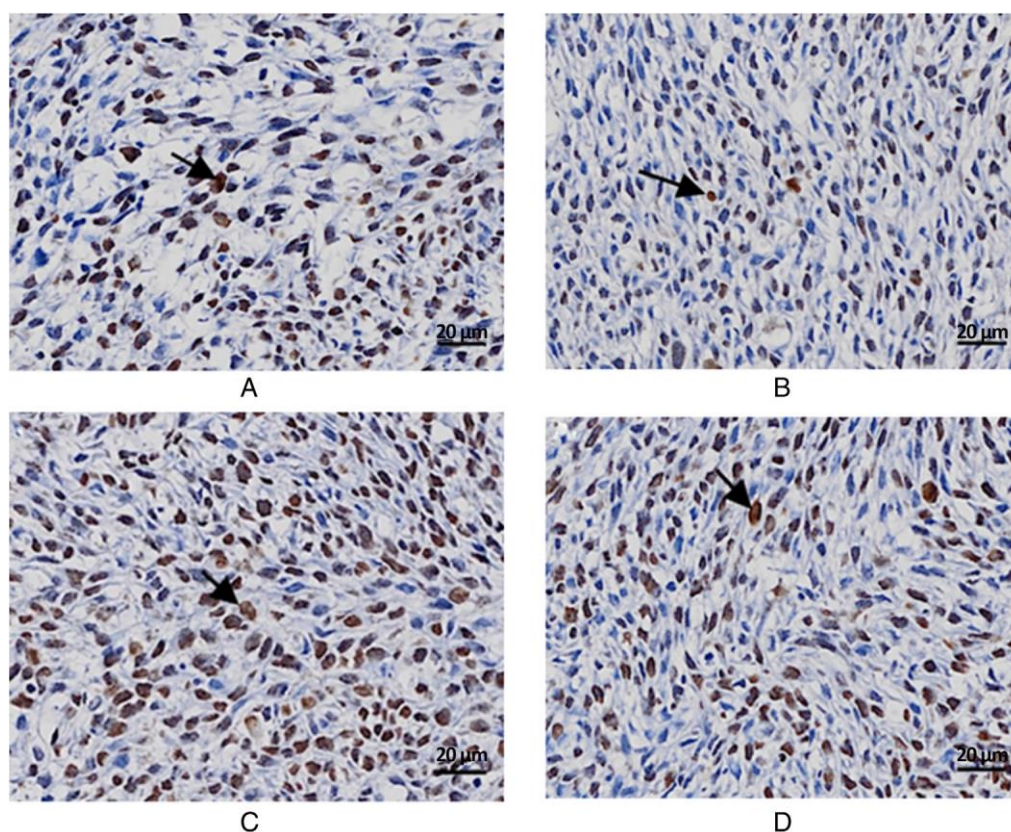


Figure 4 — Immunohistochemistry images of tumor cells stained with Ki-67 antibody (arrow) showing cellular proliferation of SED + T mice at 14 (A) and 28 days (C); and EX + T mice at 14 (B) and 28 days (D). There was higher staining in SED + T mice (C) compared to EX + T mice (D). EX + T indicates exercised tumor bearing; SED + T, sedentary tumor bearing.

strong inverse relationship between walking distance after diagnosis and tumor progression. Indeed, men who walked 3 or more hours per week at a brisk pace significantly reduced the rate of PCa progression by 57% compared with men who walked less than 3 hours per week ($P = .03$).¹⁰ The physical activity guidelines for adults with chronic conditions already recommend at least 150 minutes per week of aerobic activity³³; therefore, it seems that there may be an inverse relationship between time spent walking and tumor progression.

The systemic mechanisms underlying the potential benefits of physical exercise on limiting cancer cell growth comprise the modulation of host circulating levels of metabolic and sex steroid hormones, increased immune surveillance, and reduced oxidative damage and systemic inflammation.³⁴ Indeed, on a systemic level, insulin-like growth factor-1 and its binding proteins seem to play a major role in cell growth regulation.³⁵ After binding to its receptor tyrosine kinase, insulin-like growth factor-1 activates several signaling pathways that result in inhibition of apoptosis and induction of tumor cell proliferation.^{36–38} Indeed, it is well-recognized that induction of apoptosis is important in inhibiting cancer progression and, by demonstrating a strong positive correlation between the running distance and tumor apoptotic area, our study shows that regular voluntary running could be a proper strategy to achieve this. Additional support for this mechanism emerged from another study showing decreased cell growth and increased apoptosis of PCa cells cultured in serum from healthy men who practiced regular aerobic exercise.³⁹

However, contrary to the results of the present study, using an orthotopic model of murine PCa, Jones et al²⁰ demonstrated that

after 8 weeks of voluntary running, tumor growth rate was comparable between exercised and control animals. Nonetheless, the levels of CD31 were 2.8-fold higher in the exercise group ($P = .014$), suggesting that physical exercise may have impacted endothelial vessel density. Similarly, McCullough et al¹⁹ analyzed the effect of 7 weeks of treadmill running on PCa growth in Copenhagen rats and also found no differences between exercised and nonexercised animals. Even so, exercise training improved tumor microvascular partial pressure of oxygen and decreased tumor hypoxic levels ($P < .05$).

These results show that aside from the modulation of systemic mechanisms, physical exercise may also locally impact the TME.^{40–42} Indeed, because neoplastic cells are tremendously influenced by the surrounding microenvironment, such crosstalk plays a major role during cancer progression.⁴³ Therefore, by reducing tumor vascular resistance and increasing tumor vascular conductance, it seems that physical exercise has the potential to enhance tumor blood flow and, consequently, tumor perfusion.⁴⁴ First, improving intratumoral vascularization and blood perfusion leads to higher levels of tumor oxygenation, preventing the development of intratumoral hypoxia that is associated with tumor aggressiveness; second, the access for circulating immune cells to infiltrate within the TME is facilitated, avoiding an immunosuppressive state; and third, the delivery of antineoplastic medication to tumors is improved, enhancing its cytotoxic effects.^{45,46}

Collectively, these studies suggest that regular voluntary running may induce systemic changes and modulate the supporting

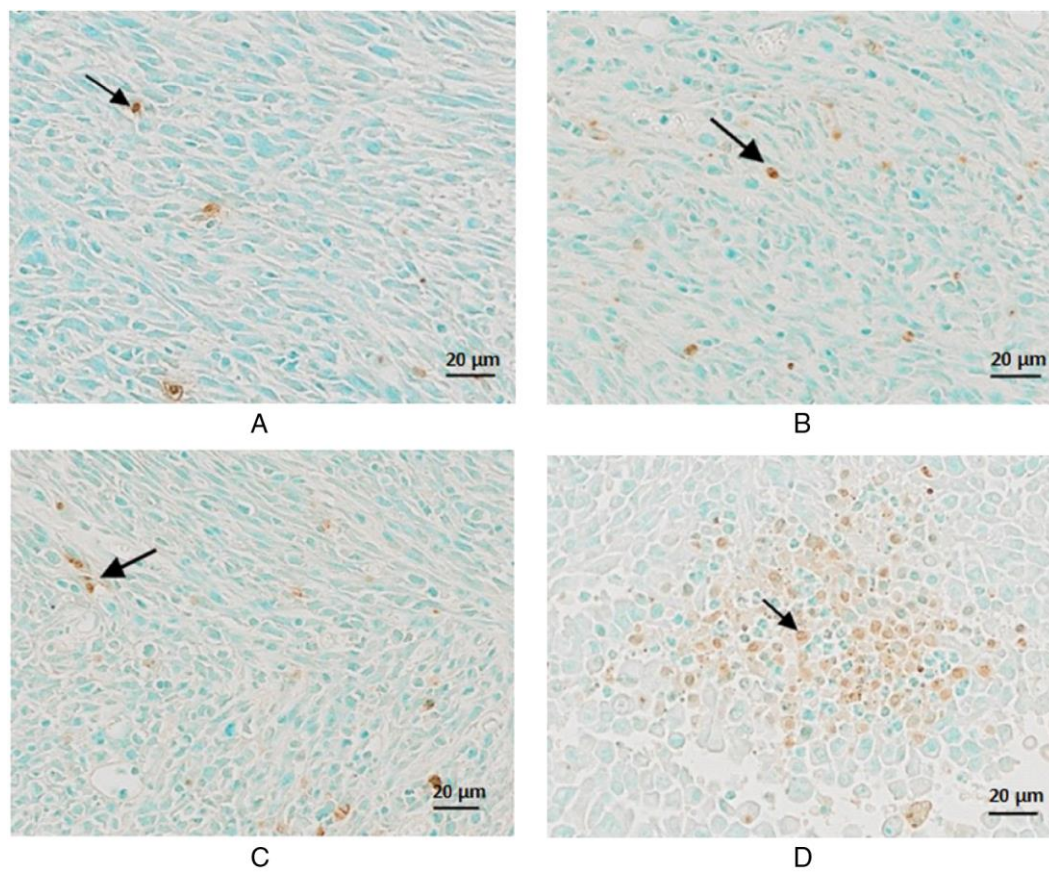


Figure 5 — Immunohistochemistry images of tumor cells stained with TUNEL assay (arrow), from SED + T mice at 14 (A) and 28 days (C); and EX + T mice at 14 (B) and 28 days (D). Counterstaining with 1% methyl green. There was higher staining in EX + T mice (D) than in SED + T mice (C). EX + T indicates exercised tumor bearing; SED + T, sedentary tumor bearing; TUNEL, TdT-mediated dUTP-biotin nick end labeling.

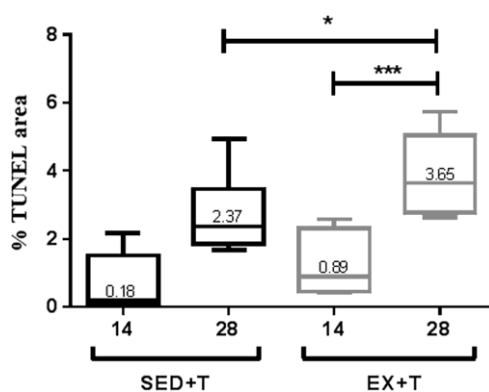


Figure 6 — Box plot analysis of tumor apoptotic area (%) from SED + T and EX + T mice at 14 and 28 days. EX + T indicates exercised tumor bearing; SED + T, sedentary tumor bearing; TUNEL, TdT-mediated dUTP-biotin nick end labeling. * $P < .05$. *** $P < .001$.

TME to reduce cancer cell viability and replication, slowing cancer progression. Accordingly, the present study showed that the regular practice of voluntary running enhanced tumor apoptosis and reduced the proliferation of neoplastic cells, providing one potential mechanism for the increased survivorship of cancer patients that engage in exercise training.⁹ However, there are a few study limitations that must be acknowledged, such as the

observation period being restricted to 28 days, which could have limited the evaluation of long-term effects of regular voluntary running as a coadjuvant anticancer therapy. In addition, a group of mice mimicking normal physical activity—rather than using a constrained environment that is not consistent with typical daily activity patterns—was not included in the study design, which could have provided additional and far reaching insights into how physical activity impacts PCa cell biology. In addition, given the pleiotropic nature of physical exercise on the host systems and the complexity of tumor biology, further studies are clearly warranted in order to unravel the relationship between physical exercise and androgen-independent prostate carcinoma progression.

Conclusion

The main finding of the present study was that regular voluntary running delayed androgen-independent prostate carcinoma cell progression by decreasing cellular proliferation and by inducing programmed cell death. Therefore, given the dependence of tumor cells on the surrounding microenvironment, it is necessary to keep looking for the best strategy to effectively impact the TME in order to inhibit tumor progression.

Acknowledgments

The authors declare that they have no conflicts of interest.

References

- Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin.* 2018;68(6):394–424. PubMed ID: [30207593](#) doi:[10.3322/caac.21492](#)
- Teoh JYC, Hirai HW, Ho JMW, Chan FCH, Tsoi KKF, Ng CF. Global incidence of prostate cancer in developing and developed countries with changing age structures. *PLoS One.* 2019;14(10):e0221775. PubMed ID: [31647819](#) doi:[10.1371/journal.pone.0221775](#)
- Carioli G, Bertuccio P, Boffetta P, et al. European cancer mortality predictions for the year 2020 with a focus on prostate cancer. *Ann Oncol.* 2020;31(5):650–658. PubMed ID: [32321669](#) doi:[10.1016/j.annonc.2020.02.009](#)
- Feldman BJ, Feldman D. The development of androgen-independent prostate cancer. *Nat Rev Cancer.* 2001;1(1):34–45. PubMed ID: [11900250](#) doi:[10.1038/35094009](#)
- Heidenreich A, Bastian PJ, Bellmunt J, et al. EAU guidelines on prostate cancer. Part II: treatment of advanced, relapsing, and castration-resistant prostate cancer. *Eur Urol.* 2014;65(2):467–479. PubMed ID: [24321502](#) doi:[10.1016/j.eururo.2013.11.002](#)
- Han M, Partin AW, Zahurak M, Piantadosi S, Epstein JI, Walsh PC. Biochemical (prostate specific antigen) recurrence probability following radical prostatectomy for clinically localized prostate cancer. *J Urol.* 2003;169(2):517–523. PubMed ID: [12544300](#) doi:[10.1016/S0022-5347\(05\)63946-8](#)
- Taylor RA, Farrelly SG, Clark AK, Watt MJ. Early intervention exercise training does not delay prostate cancer progression in Pten(-/-) mice. *Prostate.* 2020;80(11):906–914. PubMed ID: [32519789](#) doi:[10.1002/pros.24024](#)
- Schmitz KH, Courneya KS, Matthews C, et al. American college of sports medicine roundtable on exercise guidelines for cancer survivors. *Med Sci Sports Exerc.* 2010;42(7):1409–1426. PubMed ID: [20559064](#) doi:[10.1249/MSS.0b013e3181e0c112](#)
- Kenfield SA, Stampfer MJ, Giovannucci E, Chan JM. Physical activity and survival after prostate cancer diagnosis in the health professionals follow-up study. *J Clin Oncol.* 2011;29(6):726–732. PubMed ID: [21205749](#) doi:[10.1200/JCO.2010.31.5226](#)
- Richman EL, Kenfield SA, Stampfer MJ, Paoletti A, Carroll PR, Chan JM. Physical activity after diagnosis and risk of prostate cancer progression: data from the cancer of the prostate strategic urologic research endeavor. *Cancer Res.* 2011;71(11):3889–3895. PubMed ID: [21610110](#) doi:[10.1158/0008-5472.CAN-10-3932](#)
- Betof AS, Dewhirst MW, Jones LW. Effects and potential mechanisms of exercise training on cancer progression: a translational perspective. *Brain Behav Immun.* 2013;30:S75–S87. doi:[10.1016/j.bbi.2012.05.001](#)
- Zheng X, Cui XX, Huang MT, et al. Inhibitory effect of voluntary running wheel exercise on the growth of human pancreatic Panc-1 and prostate PC-3 xenograft tumors in immunodeficient mice. *Oncol Rep.* 2008;19(6):1583–1588. PubMed ID: [18497969](#) doi:[10.3892/or.19.6.1583](#)
- Opoku-Acheampong AB, Baumfalk DR, Horn AG, et al. Prostate cancer cell growth characteristics in serum and prostate-conditioned media from moderate-intensity exercise-trained healthy and tumor-bearing rats. *Am J Cancer Res.* 2019;9(4):650–667. PubMed ID: [31105994](#)
- Hojman P, Gehl J, Christensen JF, Pedersen BK. Molecular mechanisms linking exercise to cancer prevention and treatment. *Cell Metab.* 2018;27(1):10–21. PubMed ID: [29056514](#) doi:[10.1016/j.cmet.2017.09.015](#)
- Eschke RK, Lampit A, Schenk A, et al. Impact of physical exercise on growth and progression of cancer in rodents—a systematic review and meta-analysis. *Front Oncol.* 2019;9:35. PubMed ID: [30805305](#) doi:[10.3389/fonc.2019.00035](#)
- McTiernan A. Mechanisms linking physical activity with cancer. *Nat Rev Cancer.* 2008;8(3):205–211. PubMed ID: [18235448](#) doi:[10.1038/nrc2325](#)
- Bourke L, Smith D, Steed L, et al. Exercise for men with prostate cancer: a systematic review and meta-analysis. *Eur Urol.* 2016;69(4):693–703. PubMed ID: [26632144](#) doi:[10.1016/j.eururo.2015.10.047](#)
- Gueritaz J, Lefevre-Orfila L, Vincent S, et al. Exercise training combined with antioxidant supplementation prevents the antiproliferative activity of their single treatment in prostate cancer through inhibition of redox adaptation. *Free Radic Biol Med.* 2014;77: 95–105. PubMed ID: [25236740](#) doi:[10.1016/j.freeradbiomed.2014.09.009](#)
- McCullough DJ, Nguyen LM, Siemann DW, Behnke BJ. Effects of exercise training on tumor hypoxia and vascular function in the rodent preclinical orthotopic prostate cancer model. *J Appl Physiol.* 2013;115(12):1846–1854. doi:[10.1152/japplphysiol.00949.2013](#)
- Jones LW, Antonelli J, Masko EM, et al. Exercise modulation of the host-tumor interaction in an orthotopic model of murine prostate cancer. *J Appl Physiol.* 2012;113(2):263–272. doi:[10.1152/japplphysiol.01575.2011](#)
- Pedersen L, Christensen JF, Hojman P. Effects of exercise on tumor physiology and metabolism. *Cancer J.* 2015;21(2):111–116. PubMed ID: [25815851](#) doi:[10.1097/PPO.0000000000000096](#)
- Ribeiro AM, Andrade S, Pinho F, et al. Prostate cancer cell proliferation and angiogenesis in different obese mice models. *Int J Exp Pathol.* 2010;91(4):374–386. PubMed ID: [20666851](#) doi:[10.1111/j.1365-2613.2010.00726.x](#)
- Pereira SS, Pereira R, Santos AP, et al. Higher IL-6 peri-tumoural expression is associated with gastro-intestinal neuroendocrine tumour progression. *Pathology.* 2019;51(6):593–599. PubMed ID: [31466863](#) doi:[10.1016/j.pathol.2019.07.001](#)
- Esser KA, Harpole CE, Prins GS, Diamond AM. Physical activity reduces prostate carcinogenesis in a transgenic model. *Prostate.* 2009;69(13):1372–1377. PubMed ID: [19489028](#) doi:[10.1002/pros.20987](#)
- Smitherman AB, Gregory CW, Mohler JL. Apoptosis levels increase after castration in the CWR22 human prostate cancer xenograft. *Prostate.* 2003;57(1):24–31. PubMed ID: [12886520](#) doi:[10.1002/pros.10271](#)
- Logue SE, Martin SJ. Caspase activation cascades in apoptosis. *Biochem Soc Trans.* 2008;36(1):1–9. PubMed ID: [18208375](#) doi:[10.1042/BST0360001](#)
- O'Neill AJ, Boran SA, O'Keane C, et al. Caspase 3 expression in benign prostatic hyperplasia and prostate carcinoma. *Prostate.* 2001;47(3):183–188. PubMed ID: [11351347](#) doi:[10.1002/pros.1061](#)
- D'Andrea S, Spaggiari G, Barbonetti A, Santi D. Endogenous transient doping: physical exercise acutely increases testosterone levels—results from a meta-analysis. *J Endocrinol Invest.* 2020;43(10): 1349–1371. PubMed ID: [32297287](#) doi:[10.1007/s40618-020-01251-3](#)
- Hackney AC. Endurance exercise training and reproductive endocrine dysfunction in men: alterations in the hypothalamic-pituitary-testicular axis. *Curr Pharm Des.* 2001;7(4):261–273. PubMed ID: [11254889](#) doi:[10.2174/1381612013398103](#)
- De Bono JP, Adlam D, Paterson DJ, Channon KM. Novel quantitative phenotypes of exercise training in mouse models. *Am J Physiol Regul Integr Comp Physiol.* 2006;290(4):R926–R934. PubMed ID: [16339385](#) doi:[10.1152/ajpregu.00694.2005](#)

31. Allen DL, Harrison BC, Maass A, Bell ML, Byrnes WC, Leinwand LA. Cardiac and skeletal muscle adaptations to voluntary wheel running in the mouse. *J Appl Physiol*. 2001;90(5):1900–1908. doi:[10.1152/jappl.2001.90.5.1900](https://doi.org/10.1152/jappl.2001.90.5.1900)
32. Creer DJ, Romberg C, Saksida LM, van Praag H, Bussey TJ. Running enhances spatial pattern separation in mice. *Proc Natl Acad Sci U S A*. 2010;107(5):2367–2372. PubMed ID: [20133882](https://pubmed.ncbi.nlm.nih.gov/20133882/) doi:[10.1073/pnas.0911725107](https://doi.org/10.1073/pnas.0911725107)
33. Campbell KL, Winters-Stone KM, Wiskemann J, et al. Exercise guidelines for cancer survivors: consensus statement from international multidisciplinary roundtable. *Med Sci Sports Exerc*. 2019; 51(11):2375–2390. PubMed ID: [31626055](https://pubmed.ncbi.nlm.nih.gov/31626055/) doi:[10.1249/MSS.0000000000002116](https://doi.org/10.1249/MSS.0000000000002116)
34. Hojman P. Exercise protects from cancer through regulation of immune function and inflammation. *Biochem Soc Trans*. 2017;45(4): 905–911. PubMed ID: [28673937](https://pubmed.ncbi.nlm.nih.gov/28673937/) doi:[10.1042/BST20160466](https://doi.org/10.1042/BST20160466)
35. Brahmkhatri VP, Prasanna C, Atreya HS. Insulin-like growth factor system in cancer: novel targeted therapies. *Biomed Res Int*. 2015; 2015:538019. doi:[10.1155/2015/538019](https://doi.org/10.1155/2015/538019)
36. Yu H, Rohan T. Role of the insulin-like growth factor family in cancer development and progression. *J Natl Cancer Inst*. 2000;92(18):1472–1489. PubMed ID: [10995803](https://pubmed.ncbi.nlm.nih.gov/10995803/) doi:[10.1093/jnci/92.18.1472](https://doi.org/10.1093/jnci/92.18.1472)
37. Lubik AA, Gunter JH, Hollier BG, et al. IGF2 increases de novo steroidogenesis in prostate cancer cells. *Endocr Relat Cancer*. 2013;20(2):173–186. PubMed ID: [23319492](https://pubmed.ncbi.nlm.nih.gov/23319492/) doi:[10.1530/ERC-12-0250](https://doi.org/10.1530/ERC-12-0250)
38. Pollak M. Insulin and insulin-like growth factor signalling in neoplasia. *Nat Rev Cancer*. 2008;8(12):915–928. PubMed ID: [19029956](https://pubmed.ncbi.nlm.nih.gov/19029956/) doi:[10.1038/nrc2536](https://doi.org/10.1038/nrc2536)
39. Ngo TH, Barnard RJ, Leung PS, Cohen P, Aronson WJ. Insulin-like growth factor I (IGF-I) and IGF binding protein-1 modulate prostate cancer cell growth and apoptosis: possible mediators for the effects of diet and exercise on cancer cell survival. *Endocrinology*. 2003; 144(6):2319–2324. PubMed ID: [12746292](https://pubmed.ncbi.nlm.nih.gov/12746292/) doi:[10.1210/en.2003-221028](https://doi.org/10.1210/en.2003-221028)
40. Betof AS, Lascola CD, Weitzel D, et al. Modulation of murine breast tumor vascularity, hypoxia and chemotherapeutic response by exercise. *J Natl Cancer Inst*. 2015;107(5). doi:[10.1093/jnci/djv040](https://doi.org/10.1093/jnci/djv040)
41. Morrell MBG, Alvarez-Florez C, Zhang A, et al. Vascular modulation through exercise improves chemotherapy efficacy in Ewing sarcoma. *Pediatr Blood Cancer*. 2019;66(9):e27835. PubMed ID: [31136074](https://pubmed.ncbi.nlm.nih.gov/31136074/) doi:[10.1002/pbc.27835](https://doi.org/10.1002/pbc.27835)
42. Schadler KL, Thomas NJ, Galie PA, et al. Tumor vessel normalization after aerobic exercise enhances chemotherapeutic efficacy. *Oncotarget*. 2016;7(40):65429–65440. PubMed ID: [27589843](https://pubmed.ncbi.nlm.nih.gov/27589843/) doi:[10.18632/oncotarget.11748](https://doi.org/10.18632/oncotarget.11748)
43. Stylianopoulos T, Munn LL, Jain RK. Reengineering the physical microenvironment of tumors to improve drug delivery and efficacy: from mathematical modeling to bench to bedside. *Trends Cancer*. 2018;4(4):292–319. PubMed ID: [29606314](https://pubmed.ncbi.nlm.nih.gov/29606314/) doi:[10.1016/j.trecan.2018.02.005](https://doi.org/10.1016/j.trecan.2018.02.005)
44. McCullough DJ, Stabley JN, Siemann DW, Behnke BJ. Modulation of blood flow, hypoxia, and vascular function in orthotopic prostate tumors during exercise. *J Natl Cancer Inst*. 2014;106(4):dju036. doi:[10.1093/jnci/dju036](https://doi.org/10.1093/jnci/dju036)
45. McGee MC, Hamner JB, Williams RF, et al. Improved intratumoral oxygenation through vascular normalization increases glioma sensitivity to ionizing radiation. *Int J Radiat Oncol Biol Phys*. 2010;76(5): 1537–1545. PubMed ID: [20338480](https://pubmed.ncbi.nlm.nih.gov/20338480/) doi:[10.1016/j.ijrobp.2009.12.010](https://doi.org/10.1016/j.ijrobp.2009.12.010)
46. Missiaen R, Mazzone M, Bergers G. The reciprocal function and regulation of tumor vessels and immune cells offers new therapeutic opportunities in cancer. *Semin Cancer Biol*. 2018;52(2):107–116. PubMed ID: [29935312](https://pubmed.ncbi.nlm.nih.gov/29935312/) doi:[10.1016/j.semcancer.2018.06.002](https://doi.org/10.1016/j.semcancer.2018.06.002)

3.2. Study IV

Esteves M, Monteiro MP, Duarte JA. The effects of physical exercise on tumor vasculature: systematic review and meta-analysis. *Int J Sports Med* 2021; 42 (14): 1237-1249. DOI: 10.1055/a-1533-1876.

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The Effects of Physical Exercise on Tumor Vasculature: Systematic Review and Meta-analysis

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cancer, physical activity, vascularization, perfusion, hypoxia, angiogenesis

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Abstract

A wealth of evidence supports an association between physical exercise, decreased tumor growth rate, and reduced risk of cancer mortality. In this context, the tumor vascular microenvironment may play a key role in modulating tumor biologic behavior. The present systematic review and meta-analysis aimed to summarize the evidence regarding the effects of physical exercise on tumor vasculature in pre-clinical studies. We performed a computerized research on the PubMed, Scopus, and EBSCO databases to identify pre-clinical studies that evaluated the effect of physical exercise on tumor vascular outcomes. Mean differences were calculated through a random effects model. The present systematic review included 13 studies involving 373 animals. From these, 11 studies evaluated chronic intratumoral vascular adaptations and 2 studies assessed the acute intratumoral vascular adaptations to physical exercise. The chronic intratumoral vascular adaptations resulted in higher tumor microvessel density in 4 studies, increased tumor perfusion in 2 studies, and reduced intratumoral hypoxia in 3 studies. Quantitatively, regular physical exercise induced an increased tumor vascularization of 2.13 [1.07, 3.20] ($p < 0.0001$). The acute intratumoral vascular adaptations included increased vascular conductance and reduced vascular resistance, which improved tumor perfusion and attenuated intratumoral hypoxia. In pre-clinical studies, physical exercise seems to improve tumor vascularization.

Introduction

Despite the advances in diagnostic and therapeutic approaches, cancer remains one of the leading causes of death worldwide [1]. Although understudied for a long time, the immunosuppressive features of the tumor microenvironment (TME) are now recognized as key players in the development of the disease [2]. Specifically, the supportive blood vessel network has a critical role, since the presence of a dysfunctional tumor vasculature characterized by im-

mature, tortuous, and leaky vessels has a profound impact on the TME, leading to intratumoral hypoxia and acidosis, reducing immunocell infiltration and activity, and increasing the risk of metastasis [3]. Indeed, a poorly vascularized TME is associated with treatment resistance as well as disease progression and recurrence [4, 5]. Therefore, as cancer treatment in solid tumors is highly dependent on the TME and on local hemodynamics in and around the tumor

[6, 7], the identification of strategies able to induce a permissive and less hostile tumor milieu that favors an appropriate immune response is of major importance [8]. In this way, modulation of tumor vasculature emerged as a potential way to improve vascular function, tumor perfusion, and therapeutic outcomes [9, 10]. However, the impact of such pharmacological interventions failed to accomplish the initial expectations [11–13]. Indeed, long-term anti-angiogenic therapy seems to cause excessive pruning of blood vessels and to impair tumor perfusion, leading to the exacerbation of hypoxia [14, 15]. Additionally, it was shown that angiogenesis inhibitors are not suitable for all cancers, failing in more desmoplastic tumors with an abundant number of compressed vessels [16, 17].

On the other hand, during the last decade, the interest in exercise oncology has increased, driven by consistent epidemiological evidence proving that regular physical activity is associated with a reduced incidence of cancer [18–20]. More recently, preliminary evidence showed that regular physical exercise after a cancer diagnosis decreased the risk of recurrence and mortality and should be considered an important co-adjuvant anticancer therapy [21]. Additionally, it was shown in murine models that the regular practice of physical exercise resulted not only in the inhibition of tumor growth but also in increased life span [22, 23]. A recent meta-analysis of pre-clinical studies suggested that although regular physical exercise impacts tumor size, the underlying mechanisms are complex and multifactorial [22]. Indeed, while the regulation of systemic factors such as sex or metabolic hormones, cytokines, and immune function has been suggested as candidate mechanisms linking exercise training to cancer protection [24], recent studies suggested that it may exert direct effects on tumor biology as well [25, 26]. It was postulated that physical exercise may increase tumor perfusion through a structural remodeling of tumor vasculature, thus preventing cancer cells to adopt an aggressive phenotype associated with the hypoxic milieu [27, 28]. However, to date, no consensus was established, with some studies showing controversial results [29–31]. Therefore, the present review aimed to systematize and quantify the existing evidence in pre-clinical studies regarding the effects of physical exercise on tumor vasculature.

Materials and Methods

Search strategy

An extensive literature search was performed during December of 2020 in the following electronic databases: Pubmed, Scopus, and EBSCO. The search terms were based on a combination of the following key words: (cancer OR tumor) AND (vascularization OR perfusion OR oxygenation OR angiogenesis) AND (physical exercise OR physical activity). No limits were set for the year of publication, the observation period, or the type of physical exercise. Reference lists of the identified studies were also searched to find further papers related to the topic of the review. The present review was reported according to preferred reporting items to systematic reviews and meta-analyses (PRISMA) guidelines [32].

Selection criteria

All studies retrieved from our search needed to meet the following inclusion criteria: full papers published as original articles written in English, studies involving solid tumors, animal models, evaluated tumor microvessel density (MVD) or other tumor vascular parameters, and randomized controlled trials (RCTs). Studies were excluded if they were letters or editorial articles, review papers, involved interventions other than physical exercise or physical activity, or included only a single group of animals with cancer for comparisons.

Study selection

Duplicates were first removed. Then the title, abstract, and keywords were analyzed to assess the type and relevance of the publication for the scope of the review. If the publication was potentially relevant, the full text was screened for content to decide its inclusion. **Figure 1** shows the process of the study identification and selection.

Data extraction

Data from the included studies were extracted in a structured table format and included the author's last name and year of publication, sample, tumor model, intervention, vascular outcomes, results and findings.

Risk of bias

The SYRCLE's risk of bias tool was used to assess the risk of bias of all included studies. This tool has been adjusted for aspects of bias that play a specific role in animal intervention studies. It is composed by 10 domains, related to 6 types of bias: selection bias, performance bias, detection bias, attrition bias, reporting bias, and other bias. These 10 domains are organized in the form of questions that support a "Yes" (low bias), "No" (high bias), or "Unclear answer" (uncertain risk of bias). The "Other sources of bias" domain referred to the inappropriate influence of funders and potential conflict of interests [33].

Statistical analysis

We performed the statistical analysis using Review Manager 5.3 software. Mean differences are shown in both groups due to the existence of continuous data, which were grouped through a random effects model in which the weighting given to individual comparisons depends on the variance within those comparisons and on the overall heterogeneity [34]. It is a more conservative technique than fixed-effects meta-analysis, because the risk of overestimating the effect size is smaller [35]. *p* values lower than 0.05 were considered statistically significant.

Results

Study selection

A total of 244 studies were identified in the electronic databases search. After reviewing the title and abstract, 12 papers were identified as duplicates retrieved from two different databases and excluded. After screening the title, keywords, and abstract, 204 arti-

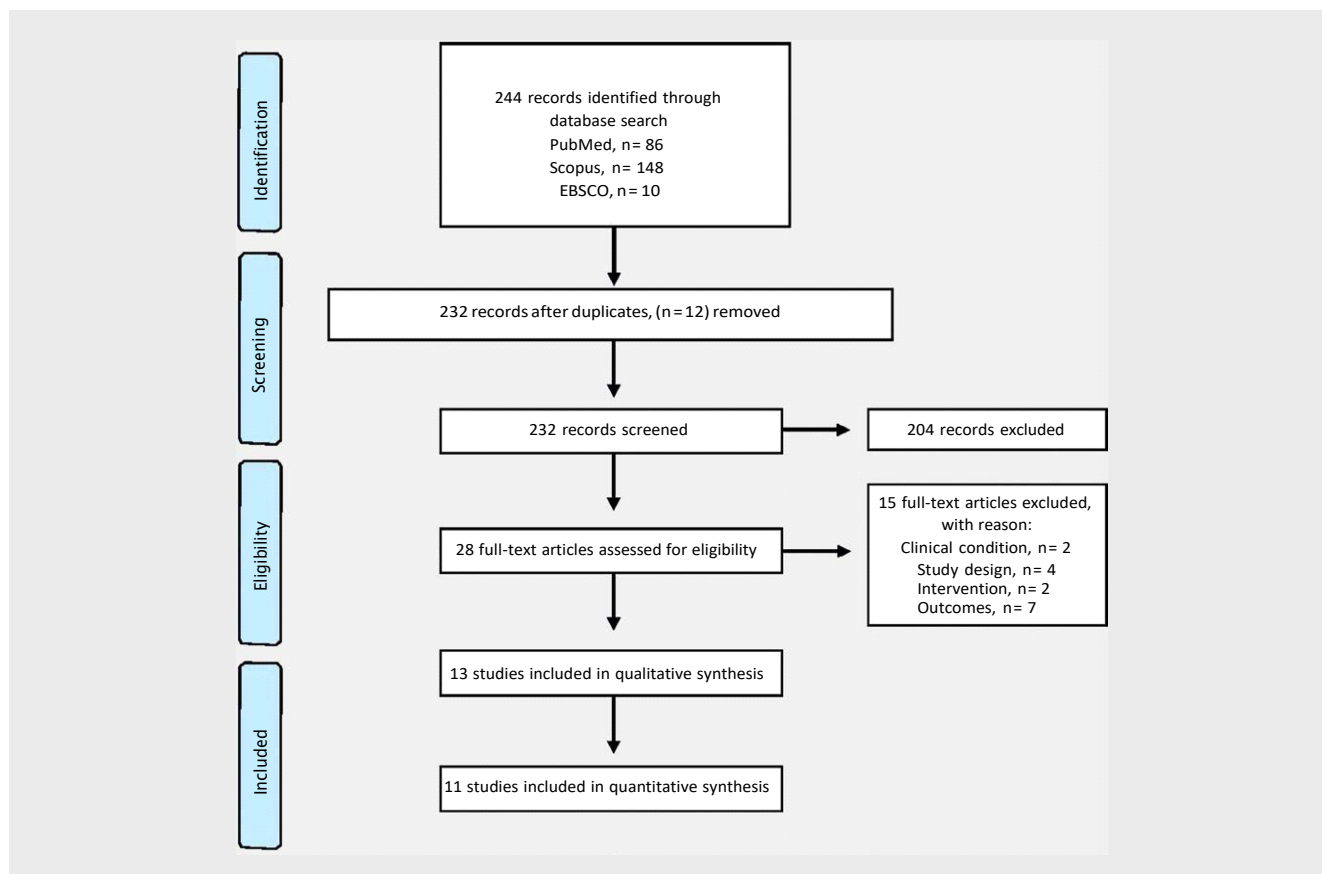


Fig. 1 Study flow diagram.

cles were discarded for not fulfilling the inclusion criteria. The full text of 28 potentially relevant studies was assessed and 15 papers were further excluded for the following reasons: non-solid tumor as clinical condition (n = 2), no RCTs (n = 4), intervention involving therapies other than physical exercise (n = 2), outcomes did not include vascularization markers (n = 5), or the method of evaluation used was inadequate for inclusion (n = 2). Therefore, 13 original studies were included in the analysis (**Fig. 1**).

Description of studies

The 13 original studies included in the present review enrolled a total of 373 animals, with a sample size ranging from 10 [36] to 59 [37]. Of these, 9 studies used an immune-competent mouse model [29, 30, 37–43], and 4 studies involved immune-compromised mice [31, 36, 44, 45] ranging from 3 weeks [45] to 6 months [39, 43]. Regarding the type of cancer, 5 studies involved breast cancer [29, 30, 38, 41, 45], 5 studies evaluated prostate cancer [31, 37, 39, 42, 43], 2 studies focused on pancreatic ductal adenocarcinoma [36, 40], and the remaining study assessed Ewing Sarcoma [44]. Of the total selected studies, 7 used an allograft model [29, 30, 39–43], 4 studies used xenograft models [31, 36, 44, 45], 1 study used a transgenic cancer model [37], and 1 used the carcinogen N-methyl-N-nitrosourea (MNU) [46]. Additionally, considering the studies involving tumor cells' inoculation, 7 applied it orthotopically [30, 37, 39, 41–43, 45] and 5 subcutaneously [29, 31, 36, 40, 44].

Regarding intervention modality, 9 studies involved physical exercise through a forced treadmill-running protocol [29, 31, 36, 38–40, 42–44], and 4 studies by applying a voluntary wheel-running model [30, 37, 41, 45], which were compared to a sedentary tumor-bearing group. With the exception of 2 studies [39, 43] that evaluated the acute effects of physical exercise on tumor perfusion and for this reason were included only in the qualitative analysis, the remaining 11 studies assessed the chronic tumor vascular adaptations to exercise training with an observation period varying between 2 [31, 44] to 35 weeks [38] and were included in the quantitative analysis.

Concerning the vascular outcomes, 10 studies evaluated tumor MVD through the CD31 [29–31, 36, 37, 40, 41, 44, 45] or the VEGF [38] markers. MVD was defined by counting the number of vessels [29, 30, 36, 38, 41], by measuring the tumor area covered by vessels [31] or by quantifying the levels [37, 45] or structures [40, 44] of CD31. Moreover, 6 studies measured tumor perfusion with Hoechst 33342 [42, 43, 45] or with Lectin [36, 40], 2 studies assessed pericyte coverage by using Desmin [44] or NG2 [41], and Morrel and colleagues analyzed the level of tumor vessel maturity through the vessel maturation index (α -SMA⁺/CD31) [44]. Finally, tumor hypoxia was evaluated by 4 studies using the markers Pimonidazole [30] or EF5 [41–43], and McCullough and co-workers also measured the partial pressure of oxygen (PO₂) in tumors [42].



Fig. 2 Risk of bias of all included studies.

Risk of bias

All included studies show low risk of bias to the domains “Allocation concealment,” “Random housing,” and “Selective outcome reporting.” No study detailed the random generation process, therefore the risk of bias in this domain is uncertain. In addition, because most of these studies did not use all animals to analyze the outcomes, the risk of attrition bias is high [29, 30, 37, 40, 42–45]. Overall, three studies present a low risk of bias in 7 domains [37, 38, 45], 6 studies in 6 domains [30, 31, 36, 39, 41, 42], 2 studies in 5 domains [40, 44], and the remaining 2 studies in 4 domains [29, 43] (**Fig. 2**).

Synthesis of the results

Most of the studies quantified the tumor MVD. Of these, 4 studies found an increased tumor MVD in response to physical exercise [36–38, 41], 1 study observed an inverse relationship between exercise training and tumor vascularization [29] and 5 studies did not show any differences between exercised and sedentary mice [30, 31, 40, 44, 45]. Two studies analyzed the tumor acute vascular adaptations to treadmill-running and found that tumor vascular conductance increased more than 2 times [39], and tumor vascular resistance decreased approximately 3 times [43]. Indeed, the reduction of vascular resistance resulted in increased tumor perfusion and the reduction of intratumoral hypoxia [43]. Similarly, 2 studies also showed that tumors from exercised mice were more perfused than sedentary animals [40, 45]. However, these findings were not observed in 3 other studies [30, 36, 42]. Besides the study of McCullough and collaborators [43], 3 additional studies also demonstrated a significant reduction of tumor hypoxia in physically active animals [41, 42, 44], and 1 study found that exercise training improved intratumoral PO₂ in rats [42]. These observations may have resulted from tumor vascular remodeling induced by physical

exercise, which was observed in 3 studies by showing a higher amount of pericyte-covered vessels [41, 44], improved blood vessel maturation [44], increased sphingosine-1-phosphate receptors (S1PR1) [44] and more vessels with open lumens within the tumor [40]. Consequently, 3 studies found that treadmill [29, 31] or voluntary [41] running promoted a significant inhibition of tumor growth and 1 study concluded that long-term tumor-bearing exercised rats showed a higher proportion of benign lesions [38]. However, most studies did not observe any changes in tumor growth rate [30, 36–38, 40, 42, 44, 45]. Detailed information about each study is provided in **table 1**.

Results of the meta-analysis

Eleven studies evaluating the chronic vascular adaptations of tumors to regular physical exercise were included in the meta-analysis, involving a total of 279 animals. The experimental group (EG) included physically active animals and the control group (CG) was composed of sedentary animals. The mean intervention period was 49.36 days (14–245). Regular physical exercise improved tumor vascularization by 2.13 [1.07, 3.20] ($p < 0.0001$) (**Fig. 3**).

Discussion

The overall results of the current meta-analysis suggest that physical exercise significantly improved tumor vascularization in rodents. Indeed, the studies of Faustino-Rocha et al. [38] and Bedoya et al. [36], which used a forced treadmill-running paradigm, and the studies of Betof et al. [41] and Jones et al. [37], which used a free running-wheel intervention, showed that physical exercise increased tumor MVD. Interestingly, in the study of Betof et al. [41], the improved MVD of physically active mice with breast cancer was accompanied by a higher percentage of pericyte-covered vessels.

Table. 1 Studies of the effects of physical exercise on biochemical markers of tumor vascularization.

Author, year	Animals	Tumor	Intervention		Vascular outcomes	Results	Findings
			Type	Follow-up			
Chronic Vascular Adaptations							
Buss et al., 2020 [28]	24 C57BL/6 mice (6–10 weeks old)	Breast cancer (EO771) Allograft model Orthotopic inoculation	Voluntary wheel- running	3 weeks	MVD (CD31) Perfusion (Hoechst 33342) Hypoxia (Pimonidazole)	MVD(vessels/field):CG=25±7vs. EG=26 ±6,p=ns Perfusion(vessels):CG=9±5vs. EG=8 ±2,p=ns Hypoxia(%):CG=15±7vs.EG=17±7, p= ns	Tumor volume: No differences
Bedoya et al., 2019 [34]	10 female nude mice (age not stated)	Pancreatic ductal adenocarcinoma PDX Xenograft model Subcutaneous inoculation	Treadmill-running, 12 m/ min, 45 min/day, 5 days/ week	4 weeks	MVD (CD31) Perfusion (Lectin)	MVD (vessels/field): CG=5±1.5 vs. EG=25 ±6,p=0.001 Perfusion (vessels): CG=10±12 vs. EG=25 ±15,p=ns	Tumor volume: No differences
Dufresne et al., 2019 [29]	17 male nude mice (5–7 weeks old)	Human PCa PPC-1 cells Xenograft model Subcutaneous inoculation	Treadmill-running, 18 m/ min, 25 min (1 st session) to 60 min (since 5 th session)/ day, 5 days/ week	2 weeks	MVD (CD31)	MVD (CD31 ⁺ / tumor area (%)): CG= 1.5 ± 0.1 vs. EG = 1.2 ± 0.1 , p= ns	Tumor volume (mm ³): CG=286.9±31.67 vs. EG=192.6±30.38, p < 0.01
Morrel et al., 2019 [42]	14 male nude mice (4–6 weeks old)	Human Ewing sarcoma (A673) Xenograft model Subcutaneous inoculation	Treadmill-running: 12 m/ min, 45 min/day, 5 days/ week	2 weeks	MVD (CD31) Vessel maturation index (α–SMA ⁺ /CD31) Pericytes (Desmin) Hypoxia (CA-IX) Shear stress receptors (S1PR1:CD31)	MVD(CD31 ⁺ structures):CG=12±3vs. EG= 15±1,p=ns Vessel maturation index: CG=0.6±0.005 vs. EG=1.0± 0.005,p<0.001 Pericytes:CG=0.4±0.1vs. EG=0.8±0.1, p< 0.001 Hypoxia (relative fold change): CG=1.25±0.25vs. EG=0.5±0.20,p=0.03 Shear stress: EG=0.84±0.02vs. CG=0.7± 0.05,p=0.04	Tumor volume: No differences
Faustino-Rocha et al., 2016 [36]	21 female Sprague-Dawley rats (4–5 weeks old)	N-methyl-N-nitrosourea (MNU) carcinogen	Treadmill-running: 20 m/ min, 60 min/day, 5 days/ week	35 weeks	MVD (VEGF)	MVD (vessels/field): CG=11.82±1.09 vs. EG=18.35±2.93, p<0.05	Tumor volume: No differences Benign lesions: CG = 1 vs. EG = 7, p = 0.034
Isanejad et al., 2016 [27]	16 Inbred female BALB/c mice (6–8 weeks old)	Mammary adenocarcinoma (MC4-L2) Allograft model Subcutaneous inoculation	Treadmill-running: 16–18 m/min, 10–14 min/ day, 5 days/week	5 weeks	MVD (CD31)	MVD (semi-quantitative score, grade of vessels/field: CD31: CG=2.4±0.4 vs. EG=1.3 ±0.2, p<0.05	Tumor volume (mm ³): CG = 1430 ± 110 vs. EG = 380 ± 50, p < 0.05
Schadler et al., 2016 [38]	12 C57Bl/6J mice (6–8 weeks old)	Pancreatic ductal adenocarcinoma (PDAC-4662) Allograft model; Subcutaneous inoculation	Treadmill-running: 12 m/ min, 45 min/day, 5 days/ week	3 weeks	MVD (CD31) Perfusion (Lectin) Open lumens	MVD (CD31 ⁺ structures):CG=62±8vs. EG= 60±4,p=ns Perfusion(%):CG=0.5±0.05vs. EG= 0.7±0.025,p<0.05 Openlumens:CG=1.5±0.5vs. EG=3.5 ±0.4,p<0.05	Tumor volume: No differences

Table 1 Continued

Author, year	Animals	tumor	Intervention		Vascular outcomes	Results	Findings
			Type	Follow-up			
Chronic Vascular Adaptations							
Betof et al., 2015 [39]	44 BALB/c female mice (age not stated)	Breast cancer (4T1) Allograft model Orthotopic inoculation	Voluntary wheel-running	18 days	MVD (CD31) Pericytes (NG2) Hypoxia (EF5)	MVD (vessels/mm ²): CG = 30.3 ± 10.42 vs. EG = 42.2 ± 18.94, p = 0.04 Pericytes (%): CG = 0.8 [0.2–1.4] vs. EG = 3.3[2.1–4.5], p < 0.001 Hypoxia (%): CG = 48.8 [35.5–62.1] vs. EG = 25.5 [12.7–38.4], p = 0.012	Tumor volume (mm ³): CG=570±100 vs. EG=450±50, p = 0.004
McCullough et al., 2013 [40]	32 Copenhagen rats (5 months old) 12 Nude rats (6 months old)	Prostate adenocarcinoma (Dunning R3327-AT-1) Allograft model Orthotopic inoculation	Treadmill-running: 15 m/ min, 60 min/day, 5 days/ week	7 weeks	Perfusion (Hoechst 33342) PO ₂ Hypoxia (EF5)	Perfusion (vessels): CG = 11 ± 1 vs. EG = 10 ± 1, p = ns αPO ₂ (mmHg): CG = 6.0 ± 0.3 vs. EG = 12.2 ± 1.0, p ≤ 0.05 Hypoxia (%): CG = 39 ± 12 vs. EG = 4 ± 1, p ≤ 0.05	Tumor weight: No differences
Jones et al., 2012 [35]	59 C57BL/6 male mice (6–8 weeks old)	Prostate adenocarcinoma (TRAMP C-1) Transgenic model Orthotopic inoculation	Voluntary wheel-running	8 weeks	MVD (CD31)	MVD (mRNA levels of CD31): CG = 1 ± 0.25 vs. EG = 2.5 ± 0.5, p < 0.05	Tumor weight: No differences
Jones et al., 2010 [43]	50 athymic homozygous female mice (3–4 weeks old)	Mammary adenocarcinoma (MDA-MB-231) Xenograft model Orthotopic inoculation	Voluntary wheel-running	6 weeks	MVD (CD31) Perfusion (Hoechst 33342)	MVD (protein levels of CD31): CG = 18.9 ± 3.0 vs. EG = 22.9 ± 4.0, p = ns Perfusion (vessels): CG = 80 ± 20 vs. EG = 200 ± 50, p = 0.03	Tumor volume: No differences
Acute Vascular Adaptations							
Garcia et al., 2016 [37]	20 Copenhagen rats (6 months of age)	Prostate adenocarcinoma (Dunning R3327-MLL) Allograft model Orthotopic inoculation	Treadmill-running: 15 m/ min, 5 min	1 session	Tumor vascular conductance	Tumor vascular conductance (mL/100 g/ min/mmHg): Rest=0.14±0.01 vs. Exercise=0.33±0.05, p≤0.05	Tumor blood flow: Rest=18±1 mL/ min/100 g vs. Exercise=45±5 mL/ min/100 g, p < 0.05
McCullough et al., 2014 [41]	42 male Copenhagen rats (4–6 months old)	Prostate adenocarcinoma (Dunning R3327-MLL) Allograft model Orthotopic inoculation	Treadmill-running: 5 min, 15 m/min	1 session	Tumor vascular resistance Perfusion (Hoechst 33342) Hypoxia (EF5)	Tumor vascular resistance (mmHg/mL/ min/100 g): Rest = 7.24 vs. Exer- cise = 2.65, p < 0.001 Perfusion (mL/min/100 g): Rest=12.7±1.3 vs. Exercise=14.3±0.6, p = 0.02 Hypoxia (%): Rest=35.5±10.4 vs. Exercise= 15.4±8.1, p<0.001	Tumor blood flow: Rest=18±4 vs. Exercise=54±8 mL/ min/100 g, p < 0.001
CA–IX, carbonic anhydrase 9; CD31, cluster of differentiation 31; CG, control group; EF5, 2–nitroimidazole; EG, exercise group; MNU, N-methyl-N-nitrosourea; MVD, microvessel density; NG2, neuron–glial antigen 2; ns, not significant; PCa, prostate cancer; PDX, patient–derived xenograft; PO ₂ , partial pressure of oxygen; S1PR1, sphingosine-1-phosphate receptors 1; TRAMP, transgenic adenocarcinoma of mouse prostate; VEGF, vascular endothelial growth factor; αSMA, α–smooth muscle actin.							

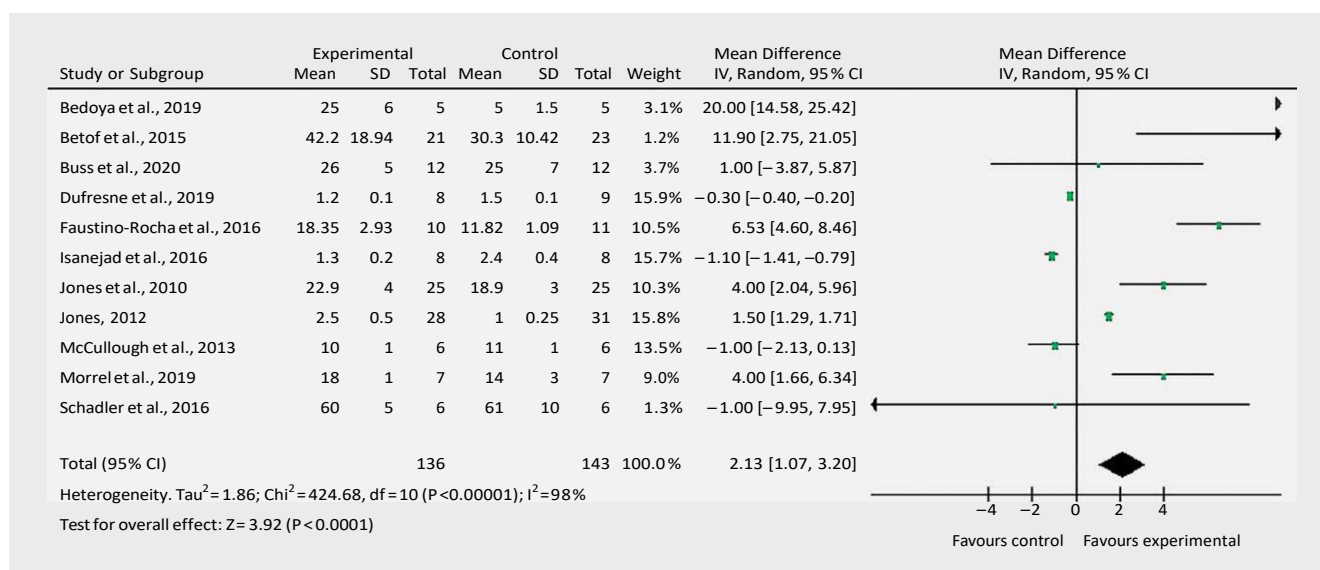


Fig.3 Forest plot of the meta-analysis describing the influence of regular physical exercise on tumor vascularization.

Similarly, although Morrel et al. [44] did not observe changes in MVD, the authors demonstrated that exercise training increased pericyte coverage and induced a higher expression of α -smooth muscle actin proteins, promoting the maturation of tumor capillaries. This phenomenon was due to the activation of calcineurin-nuclear factor of activated T cells (NFAT)-Thrombospondin 1 (TSP-1) signaling in endothelial cells (ECs) triggered by the increased shear stress, the mechanical stimuli exerted on ECs by blood flow [40]. Higher shear stress promotes an influx of calcium in ECs, leading to the activation of calcineurin and the dephosphorylation of the NFAT transcription factors, which result in NFAT nuclear import [47, 48]. Consequently, after exposure to shear stress, NFAT up-regulates TSP-1, which allows cross-talk between ECs experiencing shear stress and ECs in regions of disrupted shear, to prevent the proliferation of naive ECs, thus re-establishing vascular differentiation [40, 49]. In addition, the aberrant tumor vasculature negatively regulates endothelial adhesion molecules, which causes vessel hyperpermeability, impairing tumor perfusion [50, 51]. However, it seems that physical exercise may regulate S1PR1 and S1PR2 expression on the surface of ECs to suppress the excessive tumor vessel leakage [52, 53]. These receptors act as shear stress responsive receptors that regulate vessel barrier integrity [54]. S1PR1 prevents excessive permeability through the stabilization of vascular endothelial cadherin in response to laminar flow [55], and S1PR2, which is associated with pathological endothelium and increased vascular permeability, is up-regulated by disturbed blood flow, such as found in tumors, and is inhibited by shear stress [56]. Morrel et al. [44] confirmed these expectations by demonstrating that two weeks of treadmill-running increased tumor vessel S1PR1 signaling in mice with Ewing sarcoma and reduced the levels of tumor hypoxia, which means that the modulation of tumor vasculature induced by physical exercise was able to increase the delivery of O_2 to cells. Therefore, while angiogenesis-inhibiting agents commonly used in oncology therapies such as Bevacizumab act by blocking the angiogenic stimuli in order to inhibit new vessel formation,

physical exercise does not seem to involve tumor vessel pruning but instead, by inducing significant vascular maturation, it renders the vessels more stable [9]. As a result, considering that vessel leakiness is associated with tumor progression and dissemination, the blood vessel modulation induced by physical exercise seems to make the tumor vasculature more functional, increasing tumor perfusion and inhibiting its proliferation potential [57].

In addition, Jones et al. [45] also verified that although the MVD of breast tumors from female mice (4 weeks old) with access to a running wheel was not different when compared to their sedentary counterparts, physical activity significantly improved the tumor perfusion grade. Since the number of tumor vessels in the two groups was identical, this observation suggests that the activity on the wheel induced an improved function of pre-existent capillaries that ensured the greater perfusion observed [40]. McCullough et al. [42] reinforced this notion by showing that after 7 weeks of treadmill-running, prostate adenocarcinomas of exercised rats (5–6 months old) presented increased tumor microvascular oxygenation and consequently reduced tumor hypoxia, which was found to be associated with a better prognosis [58, 59]. Therefore, one possible method to improve the outcomes of patients with cancer is to remodel the vasculature within the tumor [36]. However, it should be mentioned that a large number of studies have reported the occurrence of some age-related behavioral dysfunctions in mice [60–63]. Particularly, the mean running distance per day declines steadily after 8 weeks of age, which has been linked to a decreased running duration and velocity with longer break times [64]. Additionally, a network of immune suppression manifestations commonly occurs with aging [65], including decreased number of T cells, decline of NK cell function, inefficient action of monocytes and dendritic cells in the uptake and presentation of antigens and chronic inflammation [66]. Therefore, the potential effects of physical exercise on TME in elderly mice may be attenuated owing to the decreased level of activity. Interestingly, it seems that the daily running distance of young females is about 30% higher than

the maximum values of male mice because of higher velocity and shorter breaks [67, 68]. Thus, in addition to age, voluntary running activity is also strongly dependent on gender [64].

Moreover, it seems that females have a greater capacity to overcome cellular stress and to mount stronger immune responses than males [69–71]. In this line, epidemiological studies have pointed out sexual dimorphism as a relevant factor for cancer incidence and survival [72–74]. The major genetic changes involved in the differences existing between males and females reside in the X chromosome, which possesses several genes involved in innate and adaptive immunity (TLR7, TLR8, IRAK1, IL2RG, FOXP3, and CD40L), conferring a higher responsiveness to the female immune system [75]. In the adaptive immune system, T cells also express numerous genes carrying the estrogen response element (IFN- γ , IFI6, CX3CL1, CX3CL2, IL-1, IL-5, and IL-16), leading to stronger inflammatory and cytotoxic T cell responses in TME of females [76]. Females also have a higher percentage of T lymphocytes within the total lymphocyte pool [77] and have more active circulating neutrophils and macrophages [78]. Similarly, females present more efficient antigen-presenting cells (APCs) than males owing to the higher expression of MHCII and co-stimulatory molecules [79]. Importantly, by activating the sympathetic nervous system and increasing the mobilization of IL-6 sensitive NK cells to the site of tumors, voluntary running induced a > 60 % reduction in tumor incidence and growth across five different cancer models [80]. Intriguingly, after 1 hour of physical exercise, Edwards and colleagues [81] found female IL-6 values greater than in male mice, thus suggesting greater NK cells mobilization to tumors in females. These data indicate that because of sex hormones and X-linked genes, the more robust B and T cell response in females may improve their antitumor immunity, inducing a more favorable modulation of the TME [82].

A multitude of rodent studies have shown that physical exercise in the form of voluntary wheel-running, forced treadmill-running, or swimming can reduce the incidence, growth, and metastatic potential of several cancer types, such as breast, prostate, and colon [83, 84]. However, as the diversity of cancers exceeds 200 distinct entities, the expression of some heterogenic degree regarding the influence of local TME may exist [85]. Indeed, although current evidence suggests that the tissue of tumor growth can influence its molecular composition including RNA, protein, and metabolites [86], comparisons between orthotopic and ectopic tumors have found contradictory results. Although some studies suggest that the TME of orthotopic tumors seem to be more immune-suppressive and pro-tumorigenic because of its higher levels of adenosine and cancer-associated genes [87–89], other studies argued that orthotopic tumors have a stronger overall antitumor immune response by showing a higher infiltration of cytotoxic immune cells and less myeloid-derived suppressive cells than ectopic tumors [90, 91]. However, these studies referred only to certain cell types, reflecting a reduced dimension from the total immune system. Most importantly, the choice of immune-competent versus immune-compromised models may influence the effect of physical exercise on TME. As immune-compromised animals do not produce mature T cells and exhibit a reduced number of circulating lymphocytes, these are unable to mount an efficient immune response due to a lack of antibody production [92, 93]. Consequently, the concentration of immune cells within the TME will be reduced, impacting the anti-tumor im-

mune response and the modulation of TME by physical exercise [94]. Generally, exercised immune-compromised mice have larger tumors than immune-competent mice, indicating that T cells play an important role in the inhibition of tumor growth [80]. However, in contrast to the lymphoid lineage, the development, differentiation, and function of granulocytes, monocytes/macrophages, and NK-cells of immune-compromised mice remains intact [95]. In this line, regular running induced a 66 % reduction in B16 tumor volume from immune-compromised mice, proving that even in the absence of functional T cells, physical exercise can inhibit tumor growth through an NK cells-mediated response [80]. Taken together, these data support the idea that TME heterogeneity is an important functional determinant that drives tissue-specific tumorigenesis and may influence the overall effect of physical exercise on cancer progression [3].

Nevertheless, strong evidence was found to support the ability of physical exercise to limit the development of different tumors, including colon, breast, endometrial, kidney, bladder, esophagus, and stomach [96]. It was demonstrated that regular running reduces cancer cell proliferation and induces apoptosis in several studies, essentially through caspase activation and down-regulation of Bcl-2 expression [97, 98]. Follow-up analysis revealed that exercise training leads to increased catecholamine and myokine release that improve tumor perfusion and O₂ supply, reducing intratumoral metabolic stress [25]. The dynamic changes in blood pressure, shear force, and epinephrine-mediated stimulation of β_2 -adrenergic receptors on the surface of lymphocytes induce its demargination and circulation [99–101], with NK cells and CD8⁺ T cells responding more strongly than B cells and CD4⁺ T cells [102–104]. This leukocytosis promoted by physical exercise can mobilize vascular, pulmonary, hepatic, and splenic white blood cells into peripheral circulation, thus increasing NK cell and CD8⁺ T cell concentrations 10- and 2.5-fold, respectively [102, 105, 106]. Importantly, higher vascular conductance increases the TME accessibility and receptivity for circulating immune cells to delay tumor growth and inhibit the development of aggressive phenotypes [107]. Thus, considering that the installation of hypoxia modifies the composition and key signaling components of the TME to support tumor growth and dissemination [108], physical exercise may assume a distinguished role as an inhibitory agent of intratumoral hypoxia [66]. Moreover, Schadler et al. [40] observed that 3 weeks of physical exercise increased the amount of open lumens in pancreatic ductal adenocarcinomas. Indeed, in addition to a systemic increase in blood flow during physical exercise, there is also a re-distribution of cardiac output, with a shunting of blood flow from non-active tissues to the contracting muscles [109]. In part, this shunting is provided by the stimulation of the sympathetic nerve activity and release of norepinephrine, which binds to α -adrenergic receptors on the vascular smooth muscle, inducing vasoconstriction. However, the diminished α -adrenergic vasoconstriction and the loss of myogenic tone at higher intra-luminal pressures of tumor arterioles hinder the action of the sympathetic nervous system, promoting the increase of tumor blood flow during physical exercise [43]. Yet, it should be highlighted that the modality of physical exercise chosen may induce different adaptations because voluntary wheel running is considerably less stressful to animals than forced exercise paradigms [110]. Indeed, it was shown that treadmill-running is very often performed with the use of negative stimuli and inter-

feres with the normal nocturnal-diurnal rhythmicity of the animals [111], promoting a stressful reaction characterized by altered body, spleen, and adrenal weights with the up-regulation of stress hormones [112]. The major pathways activated in such conditions are the hypothalamic-pituitary-adrenal (HPA) axis and the autonomic nervous system (ANS) [113]. In response to these stressors, the hypothalamus secretes corticotropin-releasing hormone (CRH), which increases the sympathetic activity through the activation of α 1-adrenoceptors on pre-ganglion sympathetic neurons [114] and decreases the parasympathetic signal through the activation of α 2- adrenoceptors on pre-ganglion parasympathetic neurons [115]. As a result, there is an increase in cardiac output and higher blood pressure, particularly systolic, that may impact peripheral vascular conductance and resistance [116].

McCullough et al. [43] found that during treadmill-running there was a 200 % increase in the number of perfused vessels and a 50 % decrease in tumor hypoxia of rats with prostate adenocarcinoma. These findings were corroborated later by Garcia et al. [39], which also analyzed the acute effects of treadmill-running and concluded that there was a higher tumor vascular conductance during physical exercise that resulted in higher intratumoral blood flow, augmented cardiac output and systemic blood pressure that improved the levels of tumor perfusion. Consequently, in response to physical exercise, the enhanced blood flow and higher arterial pressure, combined with the inability of tumor vasculature to increase resistance due to dysfunctional vasoconstriction, likely improved the perfusion of vessels that were not supplied in a resting state [43], thus suggesting a more homogenous spatial distribution of blood flow within the tumor [39].

Therefore, considering that the regular practice of physical exercise promotes the maturation of tumor vasculature, increasing perfusion and reducing the hypoxic fraction, it could represent a viable co-adjuvant strategy to modulate the TME and to enhance the delivery of chemotherapeutic drugs and the efficacy of radiotherapy [117]. Nonetheless, similar to most pre-clinical studies investigating the impact of physical exercise on a specific outcome, control animals from all included studies did not receive any intervention, which means that despite the housing conditions, these animals did not have the opportunity to conduct their habitual physical activity behavior, as would occur in the wild while searching for food and water or engaging in territorial defense [118]. Consequently, the observed modulation of tumor vasculature induced by physical exercise may be influenced by the physical inactivity of control animals. By defining inclusion criteria and by assessing participants' activity behavior during the intervention, clinical trials can register the potential changes in leisure time physical activity when applying physical exercise as an intervention [119–121]. In contrast, in pre-clinical studies, if both exercised and control animals show impaired activity levels, the conclusions should be considered with caution because the exercise-associated effects may partially result just from the mitigation of the mechanisms of degeneration [118].

Study limitations

In general there are some limitations to this study that should be mentioned. First, the included studies involved a significant variety of cancer types, implantation processes, and rodent models,

demonstrating that the available evidence about the effect of physical exercise on vascularization of a specific type is very limited. Second, as the present meta-analysis involved studies with two exercise modalities, treadmill and voluntary running, it is difficult to interpret the impact of each intervention on intratumoral hemodynamic response individually. Finally, the presence of distinct observation periods between studies and the heterogeneity of training intensity may also have limited the expressiveness of the present conclusions.

Future directions

The current meta-analysis supports the notion of improved intratumoral vascularization induced by physical exercise. Clinically, these findings raise new perspectives in the oncology field because a higher quality of tumor vasculature may potentiate the efficacy of other therapies. However, additional pre-clinical and clinical studies are required in order to: 1) define the appropriate training parameters to modulate tumor vasculature, 2) evaluate the temporary window of the tumor vascular remodelling, and 3) verify if the vascular improvement induced by physical exercise takes place in high desmoplastic tumors.

Conclusion

To the best of our knowledge, this meta-analysis is the first attempt to quantify the impact of physical exercise on tumor vascularization. Data from the studies included suggests that both short- and long-term physical exercise in the form of forced treadmill- or voluntary wheel-running appear to improve intratumoral vasculature in animal studies. This finding suggests that physical exercise could be a promising strategy for inducing vascular modulation within tumors.

Conflict of Interest

The authors declare, beyond the absence of any conflict of interest, that the manuscript was built based on the IJSM ethical standards [122].

References

- [1] Bray F, Ferlay J, Soerjomataram I et al. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin* 2018; 68: 394–424. doi:10.3322/caac.21492
- [2] Labani-Motlagh A, Ashja-Mahdavi M, Loskog A. The tumor microenvironment: a milieu hindering and obstructing antitumor immune responses. *Front Immunol* 2020; 11: 940. doi:10.3389/fimmu.2020.00940
- [3] Esteves M, Monteiro MP, Duarte JA. Role of regular physical exercise in tumor vasculature: favorable modulator of tumor milieu. *Int J Sports Med* 2021; 42: 389–406. doi:10.1055/a-1308-3476
- [4] Jing X, Yang F, Shao C et al. Role of hypoxia in cancer therapy by regulating the tumor microenvironment. *Mol Cancer* 2019; 18: 157. doi:10.1186/s12943-019-1089-9

- [5] Petrova V, Annicchiarico-Petruzzelli M, Melino G et al. The hypoxic tumour microenvironment. *Oncogenesis* 2018; 7: 10. doi:10.1038/s41389-017-0011-9
- [6] Siemann DW, Horsman MR. Modulation of the tumor vasculature and oxygenation to improve therapy. *Pharmacol Ther* 2015; 153: 107–124. doi:10.1016/j.pharmthera.2015.06.006
- [7] Jo Y, Choi N, Kim K et al. Chemoresistance of cancer cells: Requirements of tumor microenvironment-mimicking in vitro models in anti-cancer drug development. *Theranostics* 2018; 8: 5259–5275. doi:10.7150/thno.29098
- [8] Noman MZ, Hasmim M, Messai Y et al. Hypoxia: A key player in antitumor immune response. A review in the theme: Cellular responses to hypoxia. *Am J Physiol Cell Physiol* 2015; 309: C569–C579. doi:10.1152/ajpcell.00207.2015
- [9] Martin JD, Seano G, Jain RK. Normalizing function of tumor vessels: progress, opportunities, and challenges. *Annu Rev Physiol* 2019; 81: 505–534. doi:10.1146/annurev-physiol-020518-114700
- [10] Goel S, Wong AH, Jain RK. Vascular normalization as a therapeutic strategy for malignant and nonmalignant disease. *Cold Spring Harb Perspect Med* 2012; 2: a006486. doi:10.1101/cshperspect.a006486
- [11] Mittal K, Ebos J, Rini B. Angiogenesis and the tumor microenvironment: vascular endothelial growth factor and beyond. *Semin Oncol* 2014; 41: 235–251. doi:10.1053/j.seminoncol.2014.02.007
- [12] Ebos JM, Lee CR, Kerbel RS. Tumor and host-mediated pathways of resistance and disease progression in response to antiangiogenic therapy. *Clin Cancer Res* 2009; 15: 5020–5025. doi:10.1158/1078-0432.CCR-09-0095
- [13] Paez-Ribes M, Allen E, Hudock J et al. Antiangiogenic therapy elicits malignant progression of tumors to increased local invasion and distant metastasis. *Cancer Cell* 2009; 15: 220–231. doi:10.1016/j.ccr.2009.01.027
- [14] Multhoff G, Radons J, Vaupel P. Critical role of aberrant angiogenesis in the development of tumor hypoxia and associated radioresistance. *Cancers (Basel)* 2014; 6: 813–828. doi:10.3390/cancers6020813
- [15] Moser L, Jimenez-Valerio G, Casanovas O. Antiangiogenic therapies: Going beyond their limits. *Cancer Discov* 2014; 4: 31–41. doi:10.1158/2159-8290.CD-13-0199
- [16] Philip PA. Targeting angiogenesis in pancreatic cancer. *Lancet* 2008; 371: 2062–2064. doi:10.1016/S0140-6736(08)60770-9
- [17] Betrian S, Bergeron C, Blay JY et al. Antiangiogenic effects in patients with progressive desmoplastic small round cell tumor: Data from the French national registry dedicated to the use of off-labeled targeted therapy in sarcoma (OUTC's). *Clin Sarcoma Res* 2017; 7: 10. doi:10.1186/s13569-017-0076-4
- [18] McTiernan A, Friedenreich CM, Katzmarzyk PT et al. Physical activity in cancer prevention and survival: A systematic review. *Med Sci Sports Exerc* 2019; 51: 1252–1261. doi:10.1249/MSS.0000000000001937
- [19] Rezende LFM, Sa TH, Markozannes G et al. Physical activity and cancer: an umbrella review of the literature including 22 major anatomical sites and 770 000 cancer cases. *Br J Sports Med* 2018; 52: 826–833. doi:10.1136/bjsports-2017-098391
- [20] Moore SC, Lee IM, Weiderpass E et al. Association of leisure-time physical activity with risk of 26 types of cancer in 1.44 million adults. *JAMA Intern Med* 2016; 176: 816–825. doi:10.1001/jamaintern-med.2016.1548
- [21] Patel AV, Friedenreich CM, Moore SC et al. American College of Sports Medicine Roundtable Report on Physical Activity, Sedentary Behavior, and Cancer Prevention and Control. *Med Sci Sports Exerc* 2019; 51: 2391–2402. doi:10.1249/MSS.00000000000002117
- [22] Eschke RK, Lampit A, Schenk A et al. Impact of physical exercise on growth and progression of cancer in rodents – a systematic review and meta-analysis. *Front Oncol* 2019; 9: 35. doi:10.3389/fonc.2019.00035
- [23] Esteves M, Silva C, Pereira SS et al. Regular voluntary running inhibits androgen-independent prostate cancer growth in mice. *J Phys Act Health* 2021. Online ahead of print. doi:10.1123/jpah.2020-0761
- [24] Hojman P. Exercise protects from cancer through regulation of immune function and inflammation. *Biochem Soc Trans* 2017; 45: 905–911. doi:10.1042/BST20160466
- [25] Hojman P, Gehl J, Christensen JF et al. Molecular mechanisms linking exercise to cancer prevention and treatment. *Cell Metab* 2018; 27: 10–21. doi:10.1016/j.cmet.2017.09.015
- [26] Kesting S, Weeber P, Schonfelder M et al. Exercise as a potential intervention to modulate cancer outcomes in children and adults? *Front Oncol* 2020; 10: 196. doi:10.3389/fonc.2020.00196
- [27] Ashcraft KA, Warner AB, Jones LW et al. Exercise as adjunct therapy in cancer. *Semin Radiat Oncol* 2019; 29: 16–24. doi:10.1016/j.semradonc.2018.10.001
- [28] Koelwyn GJ, Quail DF, Zhang X et al. Exercise-dependent regulation of the tumour microenvironment. *Nat Rev Cancer* 2017; 17: 620–632. doi:10.1038/nrc.2017.78
- [29] Isanejad A, Alizadeh AM, Amani Shalamzari S et al. MicroRNA-206, let-7a and microRNA-21 pathways involved in the anti-angiogenesis effects of the interval exercise training and hormone therapy in breast cancer. *Life Sci* 2016; 151: 30–40. doi:10.1016/j.lfs.2016.02.090
- [30] Buss LA, Ang AD, Hock B et al. Effect of post-implant exercise on tumour growth rate, perfusion and hypoxia in mice. *PLoS One* 2020; 15: e0229290. doi:10.1371/journal.pone.0229290
- [31] Dufresne S, Guerit J, Chiavassa S et al. Exercise training improves radiotherapy efficiency in a murine model of prostate cancer. *FASEB J* 2020; 34: 4984–4996. doi:10.1096/fj.201901728R
- [32] Moher D, Liberati A, Tetzlaff J et al. Preferred reporting items for systematic reviews and meta-analyses: the PRISMA statement. *PLoS Med* 2009; 6: e1000097. doi:10.1371/journal.pmed.1000097
- [33] Hooijmans CR, Rovers MM, de Vries RB et al. SYRCL's risk of bias tool for animal studies. *BMC Med Res Methodol* 2014; 14: 43. doi:10.1186/1471-2288-14-43
- [34] Schmidt FL, Oh IS, Hayes TL. Fixed- versus random-effects models in meta-analysis: model properties and an empirical comparison of differences in results. *Br J Math Stat Psychol* 2009; 62: 97–128. doi:10.1348/000711007X255327
- [35] Macleod MR, O'Collins T, Howells DW et al. Pooling of animal experimental data reveals influence of study design and publication bias. *Stroke* 2004; 35: 1203–1208. doi:10.1161/01.STR.0000125719.25853.20
- [36] Florez Bedoya CA, Cardoso ACF, Parker N et al. Exercise during preoperative therapy increases tumor vascularity in pancreatic tumor patients. *Sci Rep* 2019; 9: 13966. doi:10.1038/s41598-019-49582-3
- [37] Jones LW, Antonelli J, Masko EM et al. Exercise modulation of the host-tumor interaction in an orthotopic model of murine prostate cancer. *J Appl Physiol* (1985) 2012; 113: 263–272. doi:10.1152/jappphysiol.01575.2011
- [38] Faustino-Rocha AI, Silva A, Gabriel J et al. Long-term exercise training as a modulator of mammary cancer vascularization. *Biomed Pharmacother* 2016; 81: 273–280. doi:10.1016/j.biopha.2016.04.030
- [39] Garcia E, Becker VG, McCullough DJ et al. Blood flow responses to mild-intensity exercise in ectopic vs. orthotopic prostate tumors; dependence upon host tissue hemodynamics and vascular reactivity. *J Appl Physiol* (1985) 2016; 121: 15–24. doi:10.1152/jappphysiol.00266.2016
- [40] Schadler KL, Thomas NJ, Galie PA et al. Tumor vessel normalization after aerobic exercise enhances chemotherapeutic efficacy. *Oncotarget* 2016; 7: 65429–65440. doi:10.18632/oncotarget.11748

- [41] Betof AS, Lascola CD, Weitzel D et al. Modulation of murine breast tumor vascularity, hypoxia and chemotherapeutic response by exercise. *J Natl Cancer Inst* 2015; 107: djv040. doi:10.1093/jnci/djv040
- [42] McCullough DJ, Nguyen LM, Siemann DW et al. Effects of exercise training on tumor hypoxia and vascular function in the rodent preclinical orthotopic prostate cancer model. *J Appl Physiol* (1985) 2013; 115: 1846–1854. doi:10.1152/jappphysiol.00949.2013
- [43] McCullough DJ, Stabley JN, Siemann DW et al. Modulation of blood flow, hypoxia, and vascular function in orthotopic prostate tumors during exercise. *J Natl Cancer Inst* 2014; 106: dju036. doi:10.1093/jnci/dju036
- [44] Morrell MBG, Alvarez-Florez C, Zhang A et al. Vascular modulation through exercise improves chemotherapy efficacy in Ewing sarcoma. *Pediatr Blood Cancer* 2019; 66: e27835. doi:10.1002/pbc.27835
- [45] Jones LW, Viglianti BL, Tashjian JA et al. Effect of aerobic exercise on tumor physiology in an animal model of human breast cancer. *J Appl Physiol* (1985) 2010; 108: 343–348. doi:10.1152/jappphysiol.00424.2009
- [46] Alvarado A, Gil da Costa RM, Faustino-Rocha AI et al. Effects of exercise training on breast cancer metastasis in a rat model. *Int J Exp Pathol* 2017; 98: 40–46. doi:10.1111/iep.12225
- [47] Mancini M, Toker A. NFAT proteins: emerging roles in cancer progression. *Nat Rev Cancer* 2009; 9: 810–820. doi:10.1038/nrc2735
- [48] Rinne A, Banach K, Blatter LA. Regulation of nuclear factor of activated T cells (NFAT) in vascular endothelial cells. *J Mol Cell Cardiol* 2009; 47: 400–410. doi:10.1016/j.yjmcc.2009.06.010
- [49] Lawler PR, Lawler J. Molecular basis for the regulation of angiogenesis by thrombospondin-1 and -2. *Cold Spring Harb Perspect Med* 2012; 2: a006627. doi:10.1101/cshperspect.a006627
- [50] Klein D. The tumor vascular endothelium as decision maker in cancer therapy. *Front Oncol* 2018; 8: 367. doi:10.3389/fonc.2018.00367
- [51] Stylianopoulos T, Jain RK. Combining two strategies to improve perfusion and drug delivery in solid tumors. *Proc Natl Acad Sci USA* 2013; 110: 18632–18637. doi:10.1073/pnas.1318415110
- [52] Cartier A, Leigh T, Liu CH et al. Endothelial sphingosine 1-phosphate receptors promote vascular normalization and antitumor therapy. *Proc Natl Acad Sci USA* 2020; 117: 3157–3166. doi:10.1073/pnas.1906246117
- [53] Hughes SK, Wacker BK, Kaneda MM et al. Fluid shear stress modulates cell migration induced by sphingosine 1-phosphate and vascular endothelial growth factor. *Ann Biomed Eng* 2005; 33: 1003–1014. doi:10.1007/s10439-005-5756-1
- [54] Zhao J, Garcia D, Gartung A et al. Sphingosine-1-phosphate receptor subtype 2 signaling in endothelial senescence-associated functional impairments and inflammation. *Curr Atheroscler Rep* 2015; 17: 504. doi:10.1007/s11883-015-0504-y
- [55] Jung B, Obinata H, Galvani S et al. Flow-regulated endothelial S1P receptor-1 signaling sustains vascular development. *Dev Cell* 2012; 23: 600–610. doi:10.1016/j.devcel.2012.07.015
- [56] Blankenbach KV, Schwalm S, Pfeilschifter J et al. Sphingosine-1-phosphate receptor-2 antagonists: Therapeutic potential and potential risks. *Front Pharmacol* 2016; 7: 167. doi:10.3389/fphar.2016.00167
- [57] Schumacher O, Galvao DA, Taaffe DR et al. Exercise modulation of tumour perfusion and hypoxia to improve radiotherapy response in prostate cancer. *Prostate Cancer Prostatic Dis* 2021; 24: 1–14. doi:10.1038/s41391-020-0245-z
- [58] van Kuijk SJ, Yaromina A, Houben R et al. Prognostic significance of carbonic anhydrase IX expression in cancer patients: a meta-analysis. *Front Oncol* 2016; 6: 69. doi:10.3389/fonc.2016.00069
- [59] Wilson WR, Hay MP. Targeting hypoxia in cancer therapy. *Nat Rev Cancer* 2011; 11: 393–410. doi:10.1038/nrc3064
- [60] Forster MJ, Dubey A, Dawson KM et al. Age-related losses of cognitive function and motor skills in mice are associated with oxidative protein damage in the brain. *Proc Natl Acad Sci USA* 1996; 93: 4765–4769. doi:10.1073/pnas.93.10.4765
- [61] Justice JN, Carter CS, Beck HJ et al. Battery of behavioral tests in mice that models age-associated changes in human motor function. *Age (Dordr)* 2014; 36: 583–592. doi:10.1007/s11357-013-9589-9
- [62] Shoji H, Takao K, Hattori S et al. Age-related changes in behavior in C57BL/6J mice from young adulthood to middle age. *Mol Brain* 2016; 9: 11. doi:10.1186/s13041-016-0191-9
- [63] Singhal G, Morgan J, Jawahar MC et al. Effects of aging on the motor, cognitive and affective behaviors, neuroimmune responses and hippocampal gene expression. *Behav Brain Res* 2020; 383: 112501. doi:10.1016/j.bbr.2020.112501
- [64] Bartling B, Al-Robaity S, Lehnich H et al. Sex-related differences in the wheel-running activity of mice decline with increasing age. *Exp Gerontol* 2017; 87: 139–147. doi:10.1016/j.exger.2016.04.011
- [65] Fulop T, Kotb R, Fortin CF et al. Potential role of immunosenescence in cancer development. *Ann N Y Acad Sci* 2010; 1197: 158–165. doi:10.1111/j.1749-6632.2009.05370.x
- [66] Wang Q, Zhou W. Roles and molecular mechanisms of physical exercise in cancer prevention and treatment. *J Sport Health Sci* 2021; 10: 201–210. doi:10.1016/j.jshs.2020.07.008
- [67] Koteja P, Swallow JG, Carter PA et al. Energy cost of wheel running in house mice: Implications for coadaptation of locomotion and energy budgets. *Physiol Biochem Zool* 1999; 72: 238–249. doi:10.1086/316653
- [68] Lightfoot JT, Turner MJ, Daves M et al. Genetic influence on daily wheel running activity level. *Physiol Genomics* 2004; 19: 270–276. doi:10.1152/physiolgenomics.00125.2004
- [69] Lista P, Straface E, Brunelleschi S et al. On the role of autophagy in human diseases: A gender perspective. *J Cell Mol Med* 2011; 15: 1443–1457. doi:10.1111/j.1582-4934.2011.01293.x
- [70] Klein SL, Flanagan KL. Sex differences in immune responses. *Nat Rev Immunol* 2016; 16: 626–638. doi:10.1038/nri.2016.90
- [71] Cook MB, Dawsey SM, Freedman ND et al. Sex disparities in cancer incidence by period and age. *Cancer Epidemiol Biomarkers Prev* 2009; 18: 1174–1182. doi:10.1158/1055-9965.EPI-08-1118
- [72] Siegel RL, Miller KD, Jemal A. Cancer statistics, 2019. *CA Cancer J Clin* 2019; 69: 7–34. doi:10.3322/caac.21551
- [73] Torre LA, Bray F, Siegel RL et al. Global cancer statistics, 2012. *CA Cancer J Clin* 2015; 65: 87–108. doi:10.3322/caac.21262
- [74] Siegel RL, Miller KD, Jemal A. Cancer statistics, 2015. *CA Cancer J Clin* 2015; 65: 5–29. doi:10.3322/caac.21254
- [75] Libert C, Dejager L, Pinheiro I. The X chromosome in immune functions: when a chromosome makes the difference. *Nat Rev Immunol* 2010; 10: 594–604. doi:10.1038/nri2815
- [76] Hewagama A, Patel D, Yarlagadda S et al. Stronger inflammatory/cytotoxic T-cell response in women identified by microarray analysis. *Genes Immun* 2009; 10: 509–516. doi:10.1038/gene.2009.12
- [77] Bouman A, Schipper M, Heineman MJ et al. Gender difference in the non-specific and specific immune response in humans. *Am J Reprod Immunol* 2004; 52: 19–26. doi:10.1111/j.1600-0897.2004.00177.x
- [78] Gillum TL, Kuennen MR, Schneider S et al. A review of sex differences in immune function after aerobic exercise. *Exerc Immunol Rev* 2011; 17: 104–121
- [79] Gubbels Bupp MR. Sex, the aging immune system, and chronic disease. *Cell Immunol* 2015; 294: 102–110. doi:10.1016/j.cellimm.2015.02.002
- [80] Pedersen L, Idorn M, Olofsson GH et al. Voluntary running suppresses tumor growth through epinephrine- and IL-6-dependent NK cell mobilization and redistribution. *Cell Metab* 2016; 23: 554–562. doi:10.1016/j.cmet.2016.01.011

- [81] Edwards KM, Burns VE, Ring C et al. Individual differences in the interleukin-6 response to maximal and submaximal exercise tasks. *J Sports Sci* 2006; 24: 855–862 doi:10.1080/02640410500245645
- [82] Rubin JB, Lagas JS, Broestl L et al. Sex differences in cancer mechanisms. *Biol Sex Differ* 2020; 11: 17. doi:10.1186/s13293-020-00291-x
- [83] Ashcraft KA, Peace RM, Betof AS et al. Efficacy and mechanisms of aerobic exercise on cancer initiation, progression, and metastasis: A critical systematic review of in vivo preclinical data. *Cancer Res* 2016; 76: 4032–4050. doi:10.1158/0008-5472.CAN-16-0887
- [84] Pedersen L, Christensen JF, Hojman P. Effects of exercise on tumor physiology and metabolism. *Cancer J* 2015; 21: 111–116. doi:10.1097/PPO.0000000000000096
- [85] Lambert AW, Pattabiraman DR, Weinberg RA. Emerging biological principles of metastasis. *Cell* 2017; 168: 670–691. doi:10.1016/j.cell.2016.11.037
- [86] Oliver AJ, Lau PKH, Unsworth AS et al. Tissue-dependent tumor microenvironments and their impact on immunotherapy responses. *Front Immunol* 2018; 9: 70 doi:10.3389/fimmu.2018.00070
- [87] Choudhury A, Moniaux N, Ulrich AB et al. MUC4 mucin expression in human pancreatic tumours is affected by organ environment: the possible role of TGFβ2. *Br J Cancer* 2004; 90: 657–664. doi:10.1038/sj.bjc.6601604
- [88] Hoover M, Adamian Y, Brown M et al. A novel method for RNA extraction from FFPE samples reveals significant differences in biomarker expression between orthotopic and subcutaneous pancreatic cancer patient-derived xenografts. *Oncotarget* 2017; 8: 5885–5894. doi:10.18632/oncotarget.11809
- [89] Zhan B, Wen S, Lu J et al. Identification and causes of metabonomic difference between orthotopic and subcutaneous xenograft of pancreatic cancer. *Oncotarget* 2017; 8: 61264–61281 doi:10.18632/oncotarget.18057
- [90] Zhao X, Li L, Starr TK et al. Tumor location impacts immune response in mouse models of colon cancer. *Oncotarget* 2017; 8: 54775–54787. doi:10.18632/oncotarget.18423
- [91] Brand M, Laban S, Theodoraki MN et al. Characterization and differentiation of the tumor microenvironment (TME) of orthotopic and subcutaneously grown head and neck squamous cell carcinoma (HNSCC) in immunocompetent mice. *Int J Mol Sci.* 2020; 22: 247. doi:10.3390/ijms22010247
- [92] Wettersten HI, Ganti S, Weiss RH. Metabolomic profiling of tumor-bearing mice. *Methods Enzymol* 2014; 543: 275–296. doi:10.1016/B978-0-12-801329-8.00014-3
- [93] Tian H, Lyu Y, Yang YG et al. Humanized rodent models for cancer research. *Front Oncol* 2020; 10: 1696. doi:10.3389/fonc.2020.01696
- [94] Shultz LD, Ishikawa F, Greiner DL. Humanized mice in translational biomedical research. *Nat Rev Immunol* 2007; 7: 118–130 doi:10.1038/nri2017
- [95] Bosma MJ, Carroll AM. The SCID mouse mutant: definition, characterization, and potential uses. *Annu Rev Immunol* 1991; 9: 323–350. doi:10.1146/annurev.iy.09.040191.001543
- [96] Piercy KL, Troiano RP, Ballard RM et al. The physical activity guidelines for Americans. *JAMA* 2018; 320: 2020–2028. doi:10.1001/jama.2018.14854
- [97] Pollan M, Casla-Barrio S, Alfaro J et al. Exercise and cancer: a position statement from the Spanish Society of Medical Oncology. *Clin Transl Oncol* 2020; 22: 1710–1729. doi:10.1007/s12094-020-02312-y
- [98] Cormie P, Zopf EM, Zhang X et al. The impact of exercise on cancer mortality, recurrence, and treatment-related adverse effects. *Epidemiol Rev* 2017; 39: 71–92. doi:10.1093/epirev/mxx007
- [99] Kruger K, Lechtermann A, Fobker M et al. Exercise-induced redistribution of T lymphocytes is regulated by adrenergic mechanisms. *Brain Behav Immun* 2008; 22: 324–338. doi:10.1016/j.bbi.2007.08.008
- [100] Benschop RJ, Nijkamp FP, Ballieux RE et al. The effects of beta-adrenoceptor stimulation on adhesion of human natural killer cells to cultured endothelium. *Br J Pharmacol* 1994; 113: 1311–1316. doi:10.1111/j.1476-5381.1994.tb17141.x
- [101] Dimitrov S, Lange T, Born J. Selective mobilization of cytotoxic leukocytes by epinephrine. *J Immunol* 2010; 184: 503–511. doi:10.4049/jimmunol.0902189
- [102] Campbell JP, Riddell NE, Burns VE et al. Acute exercise mobilises CD8+ T lymphocytes exhibiting an effector-memory phenotype. *Brain Behav Immun* 2009; 23: 767–775. doi:10.1016/j.bbi.2009.02.011
- [103] Turner JE, Spielmann G, Wadley AJ et al. Exercise-induced B cell mobilisation: preliminary evidence for an influx of immature cells into the bloodstream. *Physiol Behav* 2016; 164: 376–382. doi:10.1016/j.physbeh.2016.06.023
- [104] Kruger K, Alack K, Ringseis R et al. Apoptosis of T-cell subsets after acute high-intensity interval exercise. *Med Sci Sports Exerc* 2016; 48: 2021–2029. doi:10.1249/MSS.0000000000000979
- [105] Adams GR, Zaldivar FP, Nance DM et al. Exercise and leukocyte interchange among central circulation, lung, spleen, and muscle. *Brain Behav Immun* 2011; 25: 658–666. doi:10.1016/j.bbi.2011.01.002
- [106] Sitlinger A, Brander DM, Bartlett DB. Impact of exercise on the immune system and outcomes in hematologic malignancies. *Blood Adv* 2020; 4: 1801–1811. doi:10.1182/bloodadvances.2019001317
- [107] Idorn M, Hojman P. Exercise-dependent regulation of NK cells in cancer protection. *Trends Mol Med* 2016; 22: 565–577. doi:10.1016/j.molmed.2016.05.007
- [108] Vaupel P. Hypoxia and aggressive tumor phenotype: implications for therapy and prognosis. *Oncologist* 2008; 13: 21–26. doi:10.1634/theoncologist.13-S3-21
- [109] Rowell LB. Human cardiovascular adjustments to exercise and thermal stress. *Physiol Rev* 1974; 54: 75–159. doi:10.1152/physrev.1974.54.1.75
- [110] Smeda M, Przyborowski K, Proniewski B et al. Breast cancer pulmonary metastasis is increased in mice undertaking spontaneous physical training in the running wheel; a call for revising beneficial effects of exercise on cancer progression. *Am J Cancer Res* 2017; 7: 1926–1936
- [111] Goh J, Ladiges W. Voluntary wheel running in mice. *Curr Protoc Mouse Biol* 2015; 5: 283–290. doi:10.1002/9780470942390.mo140295
- [112] Brown DA, Johnson MS, Armstrong CJ et al. Short-term treadmill running in the rat: what kind of stressor is it? *J Appl Physiol* (1985) 2007; 103: 1979–1985. doi:10.1152/japplphysiol.00706.2007
- [113] Ader R, Cohen N, Felten D. Psychoneuroimmunology: Interactions between the nervous system and the immune system. *Lancet* 1995; 345: 99–103. doi:10.1016/S0140-6736(95)90066-7
- [114] Lewis DI, Coote JH. Excitation and inhibition of rat sympathetic preganglionic neurones by catecholamines. *Brain Res* 1990; 530: 229–234. doi:10.1016/0006-8993(90)91287-q
- [115] Unnerstall JR, Kopajtic TA, Kuhar MJ. Distribution of alpha 2 agonist binding sites in the rat and human central nervous system: Analysis of some functional, anatomic correlates of the pharmacologic effects of clonidine and related adrenergic agents. *Brain Res* 1984; 319: 69–101. doi:10.1016/0165-0173(84)90030-4
- [116] Won E, Kim YK. Stress, the autonomic nervous system, and the immune-kynurenine pathway in the etiology of depression. *Curr Neuropharmacol* 2016; 14: 665–673. doi:10.2174/1570159x14666151208113006
- [117] Goel S, Duda DG, Xu L et al. Normalization of the vasculature for treatment of cancer and other diseases. *Physiol Rev* 2011; 91: 1071–1121. doi:10.1152/physrev.00038.2010

- [118] Joisten N, Schenk A, Zimmer P. Talking about physical "activity" or "inactivity"? The need of accurate activity controlling in exercise studies in rodents. *Front Physiol* 2020; 11: 611193. doi:10.3389/fphys.2020.611193
- [119] Cox KL, Cyarto EV, Etherton-Beer C et al. A randomized controlled trial of physical activity with individual goal-setting and volunteer mentors to overcome sedentary lifestyle in older adults at risk of cognitive decline: the INDIGO trial protocol. *BMC Geriatr* 2017; 17: 215. doi:10.1186/s12877-017-0617-y
- [120] Arikawa AY, O'Dougherty M, Kaufman BC et al. Women in Steady Exercise Research (WISER): study design and methods. *Contemp Clin Trials* 2010; 31: 457–465. doi:10.1016/j.cct.2010.05.008
- [121] Amaro-Gahete FJ, De-la OA, Jurado-Fasoli L et al. Metabolic rate in sedentary adults, following different exercise training interventions: The FIT-AGEING randomized controlled trial. *Clin Nutr* 2020; 39: 3230–3240. doi:10.1016/j.clnu.2020.02.001
- [122] Harriss DJ, MacSween A, Atkinson G. Ethical standards in sport and exercise science research: 2020 update. *Int J Sports Med* 2019; 40: 813–817. doi:10.1055/a-1015-3123

3.3. Study V

Esteves M, Silva C, Bovolini A, Pereira SS, Morais T, Moreira A, Costa MM, Monteiro MP, Duarte JA. Regular voluntary running enhances intratumoral vascularization and immune cells infiltration decreasing tumor growth in mice. Submitted to International Journal of Sports Medicine (under review).

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Abstract

Tumors present limited perfusion that hinders immune cells delivery. Preclinical studies suggested that regular physical exercise could improve tumor vascular function, however whether this effect is accompanied by enhanced immune cell infiltration and reduced tumor growth has not been demonstrated. We aimed to investigate if regular voluntary running promotes tumor vascular remodelling, improves the intratumoral immune cells infiltration and inhibits tumor growth. Tumors were induced in C57BL/6 male mice (n=28) by subcutaneous inoculation in the dorsal region with a suspension of RM1 cells (1.5×10^5 cells/500 μ L PBS) and randomly allocated into two groups: sedentary (n=14) and exercised (n=14). Seven mice from each group were sacrificed 14 and 28 days after cells' inoculation to evaluate tumor weight, microvessel density, vessels' lumen regularity and the intratumoral quantity of NKG2D receptors, CD4⁺ and CD8⁺ T cells, by immunohistochemistry. Exercised mice developed smaller tumors at 14 and 28 days after inoculation ($p < 0.05$), with higher microvessel density ($p < 0.05$), more regular tumor vessels' lumen ($p < 0.05$), and higher CD8⁺ T cells infiltration ($p < 0.01$) after 28 days. NKG2D receptors expression was higher in exercised mice at 14 ($p < 0.05$) and 28 ($p < 0.001$) days. Regular voluntary running modulates tumor vasculature, increases immune cells infiltration and inhibits tumor growth, in mice.

Keywords: Cancer, physical exercise, physical activity, vasculature, immunity.

1 Introduction

Cancer remains one of the leading causes of mortality in the world, responsible for approximately 10 million deaths in 2020 [1]. During tumor development and progression, a specific immune system response is mounted as an attempt to arrest the entire oncogenic process [2]. However, immune response often fails to control the disease, since tumor cells develop several mechanisms of escape [3]. Among these mechanisms are altered expression of apoptotic molecules that allow to avoid the cytotoxic effects of immune cells, as well as secretion of immunosuppressive factors that inhibit effector T-cells activation [4]. In addition to these intrinsic mechanisms, tumor cells are able to create an inhospitable microenvironment that has been considered one of the major contributors for the ineffective immune response [5]. In particular, the dysfunctional blood vessel bed hinders effector immune cells delivery into tumors [6] and induces hypoxia and acidosis that further attenuate the immune cells killing potential [7, 8], thus rendering the tumor microenvironment (TME) immunosuppressive [9]. Indeed, due to the continuous pro-angiogenic stimuli, tumor endothelial cells (ECs) proliferate very quickly but are heterogeneous in size and shape, and depict fewer pericytes than normal endothelium [10]. Furthermore, tumor-associated ECs express lower levels of E-selectin, intercellular adhesion molecule (ICAM) 1/2 and vascular adhesion molecule 1 (VCAM1), which result in the loss of tight junctions within the tumor vessels endothelium [11]. Altogether, these structural abnormalities lead to an impaired regulation of vascular tone, increased interstitial fluid pressure (IFP) and heterogeneous blood flow pattern [12], therefore reducing tumor perfusion [13]. Consequently, the tumor vasculature establishes an active barrier that tumor-infiltrative lymphocytes (TILs) must cross [14], however immune cells extravasation is limited in result of an higher IFP [15], thus hindering the recognition and elimination of their targets [16]. Indeed, mounting evidence shows that although cytotoxic T cells can accumulate at the tumor peripheral margin, these often fail to penetrate deeply into the tumor core [17]. Hence, considering that a good vascular structure and function are necessary for a proper delivery of drugs, oxygen (O₂) and nutrients, for waste removal and for T cell trafficking [18], re-programming the TME through tumor vasculature modulation provides an attractive opportunity to improve anti-cancer immunity [19, 20]. Nonetheless, although the deregulation of angiogenesis and resulting abnormal vasculature have been understood as features that characterize malignant tumors [21], the so far available pharmacological therapies that target TME vascular remodelling did not demonstrate to achieve clinically meaningful effects [22, 23].

As such, the interest in physical exercise as an alternative approach to modulate the tumor vasculature arise from pre-clinical findings showing that regular practice of physical activity or exercise was able to increase tumor perfusion [24], to improve drug delivery [25] and to reduce tumor hypoxia [26]. It is well established that during physical exercise there is a combination of greater arterial pressure and a redistribution of cardiac output, with shunting of blood flow from non-active tissues towards contracting muscles [27], driven by the enhanced sympathetic system activity with release of noradrenaline that binds vascular smooth muscle α -adrenergic receptors to induce vasoconstriction [28]. However, given that the tumor vascular resistance cannot be augmented due to impaired α -adrenergic vasoconstriction, during physical exercise, a portion of the increased cardiac output is directed towards lower resistance tumor arterioles, increasing transmural pressure and re-opening previously collapsed vessels, thus prompting a

more homogenous blood distribution within the tumor [29]. Moreover, it is long known that a single bout of dynamic physical exercise, elicits a fast activation and mobilization of different immune cells with phenotypes associated with enhanced effector function, tissue migration, catecholamine sensitivity and antigen specificity to peripheral tissues that require enhanced immune surveillance, such as malignant tumors [30]. However, whether immune system stimulation and improved tumor vascularization associated with the regular practice of physical activity or exercise can contribute to its antitumor properties has not yet been thoroughly examined [31, 32].

Therefore, having in mind that the nature of the TME is of paramount importance on the malignant tumors progression [33], the present study aimed to investigate the effects of regular voluntary running on the vascularization and immune cells content of ectopic tumors in mice inoculated with a murine androgen-independent prostate carcinoma cell line. We hypothesized that regular practice of voluntary running would favourably modulate TME, through an improvement of its vascular pattern and enhancement of immune cell infiltration, thus ultimately inhibiting tumor growth.

2 Materials and methods

2.1 Animals and procedures

Mice were obtained from Charles River laboratories (Barcelona) with 9 weeks old and maintained under controlled conditions of temperature ($23\pm 2^{\circ}\text{C}$) and humidity ($50\pm 10\%$), on 12:12-h light-dark cycle. All cages had identical floor areas (800 cm^2 ; Tecniplast, Buguggiate, Italy) and tap water and a standard laboratory chow were supplied *ad libitum* throughout the study. Briefly, after one week of quarantine, C57BL/6 male mice ($n=28$) were subcutaneously inoculated in the dorsal region with a suspension of RM1 androgen-independent murine prostate carcinoma cells (1.5×10^5 cells/500 μl PBS), as previously described [34]. Mice were then randomly assigned into two groups: sedentary tumor induced (SED+T, $n=14$) and voluntarily exercised tumor induced (EX+T, $n=14$). EX+T mice were individually housed in cages equipped with free access to an activity wheel, while SED+T mice did not have access to a wheel, in order to restrict voluntary physical activity to the cage area. Food intake and running distance were recorded on a daily basis and body weight (BW) once a week. Seven mice per group were sacrificed on the 14th day and on the 28th day after tumor cells' inoculation. Mice were sacrificed by cervical dislocation and tumors were removed by surgical dissection, weighted and routinely processed for immunohistochemistry staining. At the end of the study, the accurate BW was recalculated by subtracting the tumors' weight to the total BW. The study was approved by the local Ethical Committee and all procedures were conducted in accordance with European and National Legislation (European Directive 2010/63/EU and National Decree-law 113/2013).

2.2 Immunohistochemistry staining and image analysis

After tissue processing and paraffin embedding for light microscopy, 4 μm -thick tumor sections were mounted on Silane coated glass slides. The tissue sections were then subjected to deparaffinization, hydration and antigen retrieval with 0.01M sodium citrate buffer (pH 6.0), using a pressure cooker, for 10 minutes. After cooling, the endogenous peroxidase activity was blocked with a 3% hydrogen peroxide in

methanol, for 30 minutes, and the non specific binding sites were blocked with 5% bovine serum albumin (BSA; Sigma-Aldrich), also for 30 minutes. Following this step, tissue samples were incubated overnight at 4°C with a primary rabbit antibody for CD31 (ab28364, Abcam), NKG2D (ab203353, Abcam), CD8 (ab209775, Abcam), and CD4 (ab203034, Abcam), at a dilution of 1:100 (CD31, NKG2D) or 1:200 (CD8, CD4). Sections were then incubated with a secondary antibody (Polyclonal goat anti-rabbit, Merck) for 2h at 37°C, at a dilution of 1:200 for samples primarily stained with CD31 and NKG2D, or 1:400, for samples first stained with CD4 and CD8. DAB (Diaminobenzidine tetrahydrochloride, ENZO) was used as chromogen and hematoxylin as nuclear counterstaining. Negative controls were performed with the omission of the of the primary antibody incubation.

Samples were analysed with a light microscope (Axio Imager A1, Carl Zeiss, Germany), with a 40x magnification objective and images recorded with a coupled digital camera (Leica DM4000D, Nussloch, Germany). Images were then examined with ImageJ software (NIH, Bethesda, MD) to calculate the NKG2D-, CD8-, and CD4- positive cells per mm². The microvessel density (MVD) was measured as the number of CD31-positive structures per microscopic field, whereas the regularity of the vessels' lumen was calculated as $\text{perimeter}^2/4\pi \cdot \text{area}$, with a ratio of 1.0 indicating a perfect circle and values greater than 1.0 indicating higher irregularity [35].

2.5 Statistical analysis

Normality and homogeneity of variances were verified by the Shapiro Wilk and Levene's test. For variables with a normal distribution, data is presented as mean and standard deviation and the statistical inference was done through the parametric test one-way ANOVA, with Tukey's post hoc test. For those variables without a normal distribution, data is presented as median and interquartile range (25-75%). In this case, the statistical inference was done through the non-parametric Kruskal Wallis test. All procedures were performed with SPSS, version 27 (Chicago, IL, USA). A p value below 0.05 was considered statistically significant.

3 Results

3.1 General findings

EX+T mice ran an average distance of 4.72±1.55 Km/day in the first 14 days and 4.71±0.93 Km/day until the end of intervention (28 days), without significant difference between the two time periods. EX+T mice presented a significantly higher cumulative food intake 28 days after tumor inoculation, but no significant difference in BW was identified between the two study groups (Table 1).

Table 1

3.2 Syngeneic ectopic tumors

Tumors of EX+T mice were significantly lower weight than those of SED+T mice, both at 14 days (0.17±0.12 g vs. 0.48±0.24 g, $p < 0.05$) and at 28 days (0.92±0.73 g vs. 2.09±1.31 g, $p < 0.05$) of observation.

3.3 Microvessel density and vessels' lumen regularity

At 28 days after inoculation, tumors pertaining to EX+T mice presented a higher intratumoral MVD than those of SED+T mice (21.20 ± 3.22 vs. 15.86 ± 4.03 vessels/ field, $p < 0.05$) (Figure 1). Moreover, at the same time point, the vessels' lumen was more regular in tumors pertaining to EX+T mice when compared to those of SED+T mice (1.06 ± 0.20 vs. 1.43 ± 0.20 , $p < 0.05$) (Table 2).

Figure 1

Table 2

3.4 CD4⁺ and CD8⁺ T cell infiltration

CD4⁺ T cells infiltration within the tumor was significantly higher at 28 days after inoculation when compared to 14 days in both groups, namely in tumors of SED+T mice (270.23 ± 31.17 vs. 166.90 ± 26.11 cells/mm², respectively; $p < 0.01$) and in tumors of EX+T mice (279.36 ± 46.10 vs. 206.77 ± 26.96 cells/mm², respectively; $p < 0.05$) (Figure 2). Furthermore, intratumoral CD8⁺ T cells infiltration was significantly higher in tumors of EX+T mice at 28 days as compared to 14 days (464.95 ± 47.98 vs. 336.91 ± 61.71 cells/mm², $p < 0.01$), and when compared to tumors of SED+T mice at 28 days (364.70 ± 49.42 cells/mm², $p < 0.05$). The number of CD8⁺ T cells in tumors of SED+T mice did not change significantly between the two time periods (Figure 3). The CD4⁺/CD8⁺ T cell ratio was not significantly different between groups (Table 2).

Figure 2

Figure 3

3.5 NKG2D expression

Tumors pertaining to EX+T mice had a higher NKG2D receptor expression at 28 days when compared to 14 days (454.72 ± 49.79 vs. 357.87 ± 37.93 cells/mm², $p < 0.05$). The NKG2D receptor expression was also significantly higher in tumors of EX+T mice as compared to those of SED+T mice at 14 days (263.27 ± 25.77 cells/mm², $p < 0.05$) and at 28 days (295.06 ± 56.22 cells/mm², $p < 0.001$) (Figure 4).

Figure 4

4 Discussion

Consistent with our hypothesis, the results of the present study show that regular voluntary running increased MVD and improved vessels' lumen regularity within the tumors. Such vascular remodelling was accompanied by a higher CD8⁺ T cell infiltration and up-regulation of intratumoral NKG2D receptors expression that contributed to decreased tumor growth. These results are in agreement with previous research conducted in different types of cancer including breast, pancreas, and prostate cancer, which demonstrated the improvement of tumor vascularization in response to regular physical exercise

[36-39]. Similar to our study, Betof and collaborators showed that 18 days of regular voluntary running increased the MVD and the percentage of pericyte covered vessels at breast tumors in BALB/c mice [36]. Accordingly, Bedoya and colleagues also found that treadmill running increased the MVD and improved the intratumoral perfusion of pancreatic tumors in mice [37]. Overall, data suggests that the mechanisms responsible for tumor vascular remodelling involve a higher vessel wall shear stress associated with physical exercise-enhanced blood flow, which activate the calcineurin-nuclear factor of activated T cells (NFAT)-Thrombospondin 1 (TSP-1) signalling [40], thus limiting the bioavailability of vascular endothelial growth factor (VEGF) and preventing the proliferation of naive ECs [18]. Consequently, the reduction of VEGF activity combined with the continuous increase in shear stress, turn the tumor vasculature of exercised animals more stable and regularly shaped [36, 40, 41], as observed in the present study. Indeed, Schadler and collaborators observed that treadmill running allowed the modification of tumor vasculature morphology in mice from disconnected small clumps of CD31⁺ ECs into elongated structures [40]. Consequently, the average vessel length and the number of visible lumens were significantly higher in tumors from exercised mice, increasing the fraction of perfused vessels. The improvement of intratumoral perfusion has many advantages including the increased delivery of O₂ and anti-cancer drugs, the prevention of local hypoxia and improved immune action [42]. Morrell and colleagues further corroborated this hypothesis by showing that exercise training improved the vessels' maturity in Ewing sarcomas, reflected by an enhanced expression of α -smooth muscle actin (SMA) and higher concentration of pericytes covering vessels, which culminated in improved O₂ and doxorubicin (DOX) delivery to tumors [41]. In addition, Jones and co-workers found that, although the breast tumor MVD of exercised mice was not significantly different when compared to its sedentary counterparts, regular voluntary running significantly increased the degree of intratumoral perfusion [43]. Therefore, it seems that the quality of blood vessels can have a major impact on tumor perfusion [44], because the presence of collapsed tumor vessels or vessels with irregular lumen cannot support an adequate blood flow into cells, leading to the installation of tumor hypoxia [45]. Interestingly, the present study demonstrated that regular voluntary running was an effective intervention to improve the regularity of tumor vasculature. This is in accordance with the findings of Van Blarigan and co-workers showing that brisk walking rendered the lumen of prostate carcinoma vessels more regular and reduced the risk of lethal disease [46].

Not surprisingly, as immunity is dependent on leukocyte-endothelium interaction for a proper intratumoral infiltration of immune cells [47], and the abnormal vasculature seen in tumors does not support the trans-vessel migration of leukocytes, the anti-tumor immune response is not fully effective, thus increasing the risk of malignant progression [48]. Nonetheless, the improvement of tumor vascularization induced by regular voluntary running in our study, allowed an increased intratumoral infiltration of CD8⁺ T cells that are likely to have contributed to inhibit its growth. Corroborating our findings, other studies already demonstrated that regular practice of physical exercise can facilitate the entry of immune cells into the tumor, reducing the immunosuppressive features of the TME [49-52]. Particularly, while Zielinski and collaborators showed that exercised mice have an increased number of intratumoral lymphocytes and a delayed tumor growth [50], Rundqvist and co-workers found that trained animals presented a higher density of CD8⁺ T cells with enhanced cytolytic capacity within the tumor

[51]. Therefore, by being associated with a better clinical outcome [53], a strong T cell infiltration plays a crucial role in mediating the host immune response to the tumor [54]. Given this, previous reports have demonstrated the requirement of both CD4⁺ and CD8⁺ T cells for a systemic tumor rejection to occur, because while CD4⁺ T cells provide conditioning signals to CD8⁺ T cells during its initial activation [55], CD8⁺ T cells are responsible for the lysis of antigen-bearing target cells [56]. Specifically, the intratumoral density of CD8⁺ T cells has become an independent marker associated with improved overall survival in several cancers and higher tumor immunogenicity [57]. Therefore, when there is a pronounced intratumoral lymphoid infiltration, a decreased CD4⁺/CD8⁺ T cells ratio has been associated with better prognosis [58]. However, in our study, although we observed a significant increase in the intratumoral infiltration of CD8⁺ T cells in exercised mice, it did not have significant impact in CD4⁺/CD8⁺ T cell ratio when compared to sedentary mice.

Nevertheless, as wheel running promoted the up-regulation of NKG2D receptors in tumors, it suggests that exercised mice are likely to possess an improved immune cytolytic function when compared to the sedentary animals. Indeed, considering that NKG2D is a stimulatory immune receptor expressed on all NK cells and several T cell subsets, including activated CD8⁺ T cells and some activated CD4⁺ T cells, it represents a major mediator of cancer cells immune recognition and a hallmark of cell-mediated cytotoxicity [59]. Accordingly, the pioneer study of Zimmer and colleagues found that half marathon running, considered an intense physical exercise, remarkably increased the NKG2D expression on circulating NK cells of cancer patients, for at least 24 h after the run [60], suggesting that physical exercise could be used as an adjuvant intervention to improve the NKG2D axis, and, consequently, the cytotoxic activity of the immune cells.

5 Conclusion

Altogether, our study results support the hypothesis that regular voluntary running can reduce tumor growth, by increasing tumor MVD, vessels' lumen regularity and CD8⁺ T cell density.

6 Compliance with Ethical Standards

6.1 Funding

Not applicable.

6.2 Conflict of Interest

The authors declare, beyond the absence of any conflict of interest, that the manuscript was built based on the IJSM ethical standards [61].

7 References

1. Sung H, Ferlay J, Siegel RL et al. Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. *CA Cancer J Clin* 2021; 71: 209-249. doi:10.3322/caac.21660

2. Gonzalez H, Hagerling C, Werb Z. Roles of the immune system in cancer: from tumor initiation to metastatic progression. *Genes Dev* 2018; 32: 1267-1284. doi:10.1101/gad.314617.118
3. Vinay DS, Ryan EP, Pawelec G et al. Immune evasion in cancer: Mechanistic basis and therapeutic strategies. *Semin Cancer Biol* 2015; 35 Suppl: S185-S198. doi:10.1016/j.semcancer.2015.03.004
4. Nurieva R, Wang J, Sahoo A. T-cell tolerance in cancer. *Immunotherapy* 2013; 5: 513-531. doi:10.2217/imt.13.33
5. Wang M, Zhao J, Zhang L et al. Role of tumor microenvironment in tumorigenesis. *J Cancer* 2017; 8: 761-773. doi:10.7150/jca.17648
6. Sackstein R, Schatton T, Barthel SR. T-lymphocyte homing: an underappreciated yet critical hurdle for successful cancer immunotherapy. *Lab Invest* 2017; 97: 669-697. doi:10.1038/labinvest.2017.25
7. Damgaci S, Ibrahim-Hashim A, Enriquez-Navas PM et al. Hypoxia and acidosis: immune suppressors and therapeutic targets. *Immunology* 2018; 154: 354-362. doi:10.1111/imm.12917
8. Vaupel P, Multhoff G. Accomplices of the Hypoxic Tumor Microenvironment Compromising Antitumor Immunity: Adenosine, Lactate, Acidosis, Vascular Endothelial Growth Factor, Potassium Ions, and Phosphatidylserine. *Front Immunol* 2017; 8: 1887. doi:10.3389/fimmu.2017.01887
9. Munn LL, Jain RK. Vascular regulation of antitumor immunity. *Science* 2019; 365: 544-545. doi:10.1126/science.aaw7875
10. Ribatti D, Nico B, Crivellato E et al. The structure of the vascular network of tumors. *Cancer Lett* 2007; 248: 18-23. doi:10.1016/j.canlet.2006.06.007
11. Cheng YQ, Wang SB, Liu JH et al. Modifying the tumour microenvironment and reverting tumour cells: New strategies for treating malignant tumours. *Cell Prolif* 2020; 53: e12865. doi:10.1111/cpr.12865
12. Nagy JA, Chang SH, Dvorak AM et al. Why are tumour blood vessels abnormal and why is it important to know? *Br J Cancer* 2009; 100: 865-869. doi:10.1038/sj.bjc.6604929
13. Siemann DW, Horsman MR. Modulation of the tumor vasculature and oxygenation to improve therapy. *Pharmacol Ther* 2015; 153: 107-124. doi:10.1016/j.pharmthera.2015.06.006
14. Hendry SA, Farnsworth RH, Solomon B et al. The Role of the Tumor Vasculature in the Host Immune Response: Implications for Therapeutic Strategies Targeting the Tumor Microenvironment. *Front Immunol* 2016; 7: 621. doi:10.3389/fimmu.2016.00621
15. Chen SC, Wu PC, Wang CY et al. Evaluation of cytotoxic T lymphocyte-mediated anticancer response against tumor interstitium-simulating physical barriers. *Sci Rep* 2020; 10: 13662. doi:10.1038/s41598-020-70694-8
16. Labani-Motlagh A, Ashja-Mahdavi M, Loskog A. The Tumor Microenvironment: A Milieu Hindering and Obstructing Antitumor Immune Responses. *Front Immunol* 2020; 11: 940. doi:10.3389/fimmu.2020.00940
17. Motz GT, Coukos G. Deciphering and reversing tumor immune suppression. *Immunity* 2013; 39: 61-73. doi:10.1016/j.immuni.2013.07.005
18. Dewhirst MW, Secomb TW. Transport of drugs from blood vessels to tumour tissue. *Nat Rev Cancer* 2017; 17: 738-750. doi:10.1038/nrc.2017.93
19. Zhao Y, Yu X, Li J. Manipulation of immunevascular crosstalk: new strategies towards cancer treatment. *Acta Pharm Sin B* 2020; 10: 2018-2036. doi:10.1016/j.apsb.2020.09.014
20. Ganss R. Tumour vessel remodelling: new opportunities in cancer treatment. *Vasc Biol* 2020; 2: R35-R43. doi:10.1530/VB-19-0032
21. Saman H, Raza SS, Uddin S et al. Inducing Angiogenesis, a Key Step in Cancer Vascularization, and Treatment Approaches. *Cancers (Basel)* 2020; 12. doi:10.3390/cancers12051172
22. Magnussen AL, Mills IG. Vascular normalisation as the stepping stone into tumour microenvironment transformation. *Br J Cancer* 2021; 125: 324-336. doi:10.1038/s41416-021-01330-z
23. Li S, Zhang Q, Hong Y. Tumor Vessel Normalization: A Window to Enhancing Cancer Immunotherapy. *Technol Cancer Res Treat* 2020; 19: 1533033820980116. doi:10.1177/1533033820980116
24. Esteves M, Monteiro MP, Duarte JA. The Effects of Physical Exercise on Tumor Vasculature: Systematic Review and Meta-analysis. *Int J Sports Med* 2021. doi:10.1055/a-1533-1876. doi:10.1055/a-1533-1876
25. Ashcraft KA, Warner AB, Jones LW et al. Exercise as Adjunct Therapy in Cancer. *Semin Radiat Oncol* 2019; 29: 16-24. doi:10.1016/j.semradi.2018.10.001

26. Schumacher O, Galvao DA, Taaffe DR et al. Exercise modulation of tumour perfusion and hypoxia to improve radiotherapy response in prostate cancer. *Prostate Cancer Prostatic Dis* 2021; 24: 1-14. doi:10.1038/s41391-020-0245-z
27. Rowell LB. Human cardiovascular adjustments to exercise and thermal stress. *Physiol Rev* 1974; 54: 75-159. doi:10.1152/physrev.1974.54.1.75
28. Sheng Y, Zhu L. The crosstalk between autonomic nervous system and blood vessels. *Int J Physiol Pathophysiol Pharmacol* 2018; 10: 17-28
29. Esteves M, Monteiro MP, Duarte JA. Role of Regular Physical Exercise in Tumor Vasculature: Favorable Modulator of Tumor Milieu. *Int J Sports Med* 2021; 42: 389-406. doi:10.1055/a-1308-3476
30. Pedersen BK, Hoffman-Goetz L. Exercise and the immune system: regulation, integration, and adaptation. *Physiol Rev* 2000; 80: 1055-1081
31. Koelwyn GJ, Quail DF, Zhang X et al. Exercise-dependent regulation of the tumour microenvironment. *Nat Rev Cancer* 2017; 17: 620-632. doi:10.1038/nrc.2017.78
32. Koelwyn GJ, Wennerberg E, Demaria S et al. Exercise in Regulation of Inflammation-Immune Axis Function in Cancer Initiation and Progression. *Oncology (Williston Park)* 2015; 29: 908-920, 922
33. Yuan Y, Jiang YC, Sun CK et al. Role of the tumor microenvironment in tumor progression and the clinical applications (Review). *Oncol Rep* 2016; 35: 2499-2515. doi:10.3892/or.2016.4660
34. Ribeiro AM, Andrade S, Pinho F et al. Prostate cancer cell proliferation and angiogenesis in different obese mice models. *Int J Exp Pathol* 2010; 91: 374-386. doi:10.1111/j.1365-2613.2010.00726.x
35. Mucci LA, Powolny A, Giovannucci E et al. Prospective study of prostate tumor angiogenesis and cancer-specific mortality in the health professionals follow-up study. *J Clin Oncol* 2009; 27: 5627-5633. doi:10.1200/JCO.2008.20.8876
36. Betof AS, Lascola CD, Weitzel D et al. Modulation of murine breast tumor vascularity, hypoxia and chemotherapeutic response by exercise. *J Natl Cancer Inst* 2015; 107. doi:10.1093/jnci/djv040
37. Florez Bedoya CA, Cardoso ACF, Parker N et al. Exercise during preoperative therapy increases tumor vascularity in pancreatic tumor patients. *Sci Rep* 2019; 9: 13966. doi:10.1038/s41598-019-49582-3
38. Faustino-Rocha AI, Silva A, Gabriel J et al. Long-term exercise training as a modulator of mammary cancer vascularization. *Biomed Pharmacother* 2016; 81: 273-280. doi:10.1016/j.biopha.2016.04.030
39. Jones LW, Antonelli J, Masko EM et al. Exercise modulation of the host-tumor interaction in an orthotopic model of murine prostate cancer. *J Appl Physiol* (1985) 2012; 113: 263-272. doi:10.1152/japplphysiol.01575.2011
40. Schadler KL, Thomas NJ, Galie PA et al. Tumor vessel normalization after aerobic exercise enhances chemotherapeutic efficacy. *Oncotarget* 2016; 7: 65429-65440. doi:10.18632/oncotarget.11748
41. Morrell MBG, Alvarez-Florez C, Zhang A et al. Vascular modulation through exercise improves chemotherapy efficacy in Ewing sarcoma. *Pediatr Blood Cancer* 2019; 66: e27835. doi:10.1002/pbc.27835
42. Jordan BF, Sonveaux P. Targeting tumor perfusion and oxygenation to improve the outcome of anticancer therapy. *Front Pharmacol* 2012; 3: 94. doi:10.3389/fphar.2012.00094
43. Jones LW, Viglianti BL, Tashjian JA et al. Effect of aerobic exercise on tumor physiology in an animal model of human breast cancer. *J Appl Physiol* (1985) 2010; 108: 343-348. doi:10.1152/japplphysiol.00424.2009
44. Tretiakova M, Antic T, Binder D et al. Microvessel density is not increased in prostate cancer: digital imaging of routine sections and tissue microarrays. *Hum Pathol* 2013; 44: 495-502. doi:10.1016/j.humpath.2012.06.009
45. Barth PJ, Weingartner K, Kohler HH et al. Assessment of the vascularization in prostatic carcinoma: a morphometric investigation. *Hum Pathol* 1996; 27: 1306-1310. doi:10.1016/s0046-8177(96)90342-1
46. Van Blarigan EL, Gerstenberger JP, Kenfield SA et al. Physical Activity and Prostate Tumor Vessel Morphology: Data from the Health Professionals Follow-up Study. *Cancer Prev Res (Phila)* 2015; 8: 962-967. doi:10.1158/1940-6207.CAPR-15-0132
47. Sato Y. Persistent vascular normalization as an alternative goal of anti-angiogenic cancer therapy. *Cancer Sci* 2011; 102: 1253-1256. doi:10.1111/j.1349-7006.2011.01929.x

48. Castermans K, Griffioen AW. Tumor blood vessels, a difficult hurdle for infiltrating leukocytes. *Biochim Biophys Acta* 2007; 1776: 160-174. doi:10.1016/j.bbcan.2007.07.005
49. Hagar A, Wang Z, Koyama S et al. Endurance training slows breast tumor growth in mice by suppressing Treg cells recruitment to tumors. *BMC Cancer* 2019; 19: 536. doi:10.1186/s12885-019-5745-7
50. Zielinski MR, Muenchow M, Wallig MA et al. Exercise delays allogeneic tumor growth and reduces intratumoral inflammation and vascularization. *J Appl Physiol* (1985) 2004; 96: 2249-2256. doi:10.1152/japplphysiol.01210.2003
51. Rundqvist H, Velica P, Barbieri L et al. Cytotoxic T-cells mediate exercise-induced reductions in tumor growth. *Elife* 2020; 9. doi:10.7554/eLife.59996
52. McClellan JL, Steiner JL, Day SD et al. Exercise effects on polyp burden and immune markers in the ApcMin/+ mouse model of intestinal tumorigenesis. *Int J Oncol* 2014; 45: 861-868. doi:10.3892/ijo.2014.2457
53. Fridman WH, Pages F, Sautes-Fridman C et al. The immune contexture in human tumours: impact on clinical outcome. *Nat Rev Cancer* 2012; 12: 298-306. doi:10.1038/nrc3245
54. Gajewski TF, Schreiber H, Fu YX. Innate and adaptive immune cells in the tumor microenvironment. *Nat Immunol* 2013; 14: 1014-1022. doi:10.1038/ni.2703
55. Kennedy R, Celis E. Multiple roles for CD4+ T cells in anti-tumor immune responses. *Immunol Rev* 2008; 222: 129-144. doi:10.1111/j.1600-065X.2008.00616.x
56. Philip M, Schietinger A. CD8(+) T cell differentiation and dysfunction in cancer. *Nat Rev Immunol* 2021. doi:10.1038/s41577-021-00574-3. doi:10.1038/s41577-021-00574-3
57. van der Leun AM, Thommen DS, Schumacher TN. CD8(+) T cell states in human cancer: insights from single-cell analysis. *Nat Rev Cancer* 2020; 20: 218-232. doi:10.1038/s41568-019-0235-4
58. Diederichsen AC, Hjelmberg J, Christensen PB et al. Prognostic value of the CD4+/CD8+ ratio of tumour infiltrating lymphocytes in colorectal cancer and HLA-DR expression on tumour cells. *Cancer Immunol Immunother* 2003; 52: 423-428. doi:10.1007/s00262-003-0388-5
59. Wensveen FM, Jelencic V, Polic B. NKG2D: A Master Regulator of Immune Cell Responsiveness. *Front Immunol* 2018; 9: 441. doi:10.3389/fimmu.2018.00441
60. Zimmer P, Bloch W, Schenk A et al. Exercise-induced Natural Killer Cell Activation is Driven by Epigenetic Modifications. *Int J Sports Med* 2015; 36: 510-515. doi:10.1055/s-0034-1398531
61. Harriss DJ, MacSween A, Atkinson G. Ethical Standards in Sport and Exercise Science Research: 2020 Update. *Int J Sports Med* 2019; 40: 813-817. doi:10.1055/a-1015-3123

Figure captions:

Figure 1: Representative microphotographs of CD31 immunostained RM1 tumors of sedentary mice (SED+T) at 14 (A) and 28 (B) days, and of exercised mice (EX+T) mice, at 14 (C) and 28 (D) days. At 28 days, intratumoral microvessel density of EX+T mice was significantly higher vs SED+T (*p< 0.05) (E).

Figure 2: Representative microphotographs of CD4 immunostained tumors of sedentary mice (SED+T) at 14 (A) and 28 (B) days, and of exercised mice (EX+T) mice, at 14 (C) and 28 (D) days. A significantly higher CD4+ T cells density was observed in tumors of SED+T mice (*p< 0.01) and EX+T mice (#p< 0.05) at 28days vs 14 days (E).

Figure 3: Representative microphotographs of CD8 immunostained tumors of sedentary mice (SED+T) at 14 (A) and 28 (B) days, and of exercised mice (EX+T) mice, at 14 (C) and 28 (D) days. EX+T mice presented a significantly higher intratumoral CD8+ T cells density at 28 days than at 14 days (#p< 0.01), as well as significantly higher than observed in tumors of SED+T mice, at 28 days (*p< 0.05) (E).

Figure 4: Representative microphotographs of NKG2D immunostained tumors of sedentary mice (SED+T) at 14 (A) and 28 (B) days, and of exercised mice (EX+T) mice, at 14 (C) and 28 (D) days. Tumors of EX+T mice presented a significantly higher NKG2D expression as compared to those

of SED+T mice at both time points ($p < 0.05$; $p < 0.001$); NKG2D expression in tumors of EX+T mice at 28 days was also higher as compared to 14 days ($p < 0.05$) (E).

Table captions:

Table 1: Body weight and food intake (mean $\pm$ standard deviation).

Table 2: Vessels' lumen regularity and CD4⁺/CD8⁺ T cells ratio (mean $\pm$ standard deviation).

Table 1: Body weight and food intake (mean± standard deviation).

	SED+T	EX+T
Body weight		
Baseline (g)	22.64± 1.47	22.08± 0.72
14 days (g)	23.78±1.23	21.94±1.21
28 days (g)	27.27±2.39	25.22±1.52
Food intake		
14 days (g)	3.60±0.26	3.95±0.25
28 days (g)	3.47±0.33	4.19±0.46*
Daily running distance		
14 days (Km)	-	4.72±1.55
28 days (Km)	-	4.71±0.93
*p<0.05 vs SED+T		

SED+T: sedentary tumor induced; EX+T: voluntarily exercised tumor induced

Table 2: Vessels’ lumen regularity and CD4⁺/ CD8⁺ T cells ratio (mean± standard deviation).

	SED+T	EX+T
Vessels’ lumen regularity		
14 days	1.35±0.32	1.28±0.19
28 days	1.43±0.20	1.06±0.20*
CD4 ⁺ / CD8 ⁺ T cells ratio		
14 days	0.53±0.12	0.62±0.08
28 days	0.76±0.16	0.61±0.12
*p<0.05 vs SED+T		

SED+T: sedentary tumor induced; EX+T: voluntarily exercised tumor induced

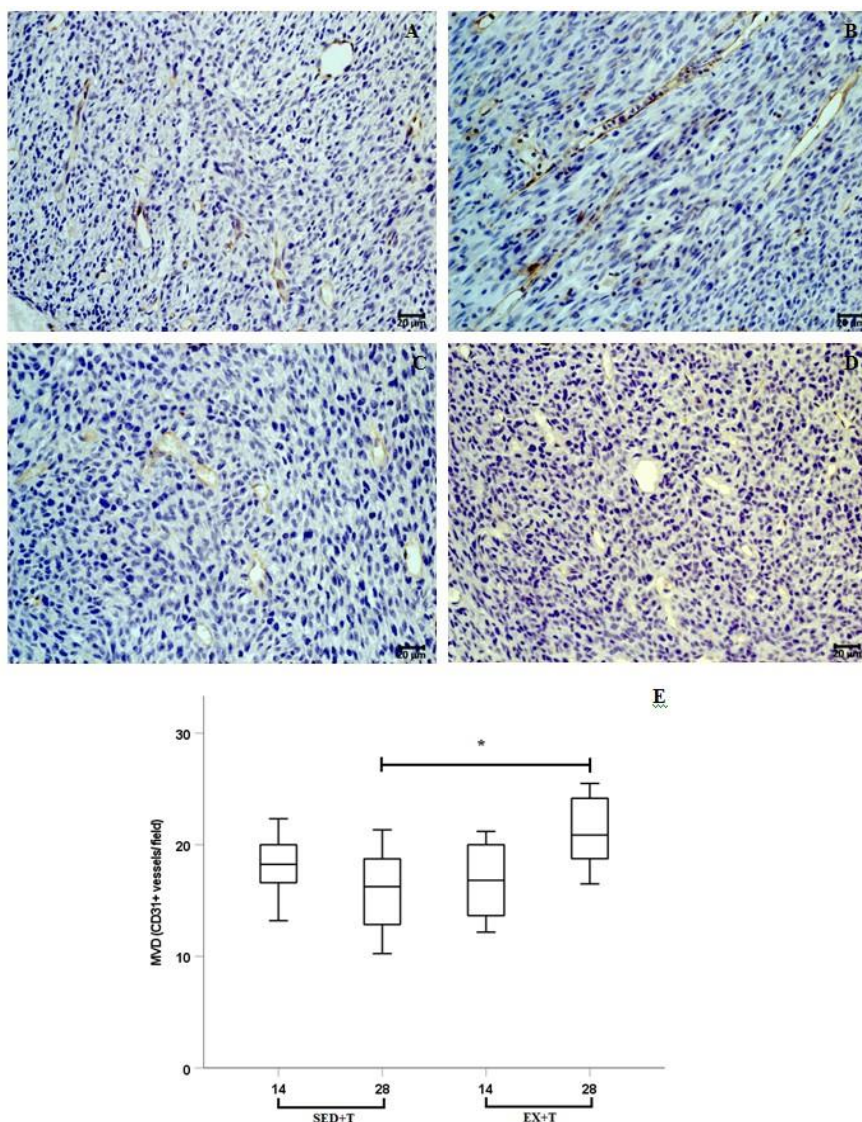


Figure 1: Representative microphotographs of CD31 immunostained tumors of sedentary mice (SED+T) at 14 (A) and 28 (B) days, and of exercised mice (EX+T) mice, at 14 (C) and 28 (D) days. At 28 days, intratumoral microvessel density of EX+T mice was significantly higher vs SED+T (* $p < 0.05$) (E).

185x237mm (96 x 96 DPI)

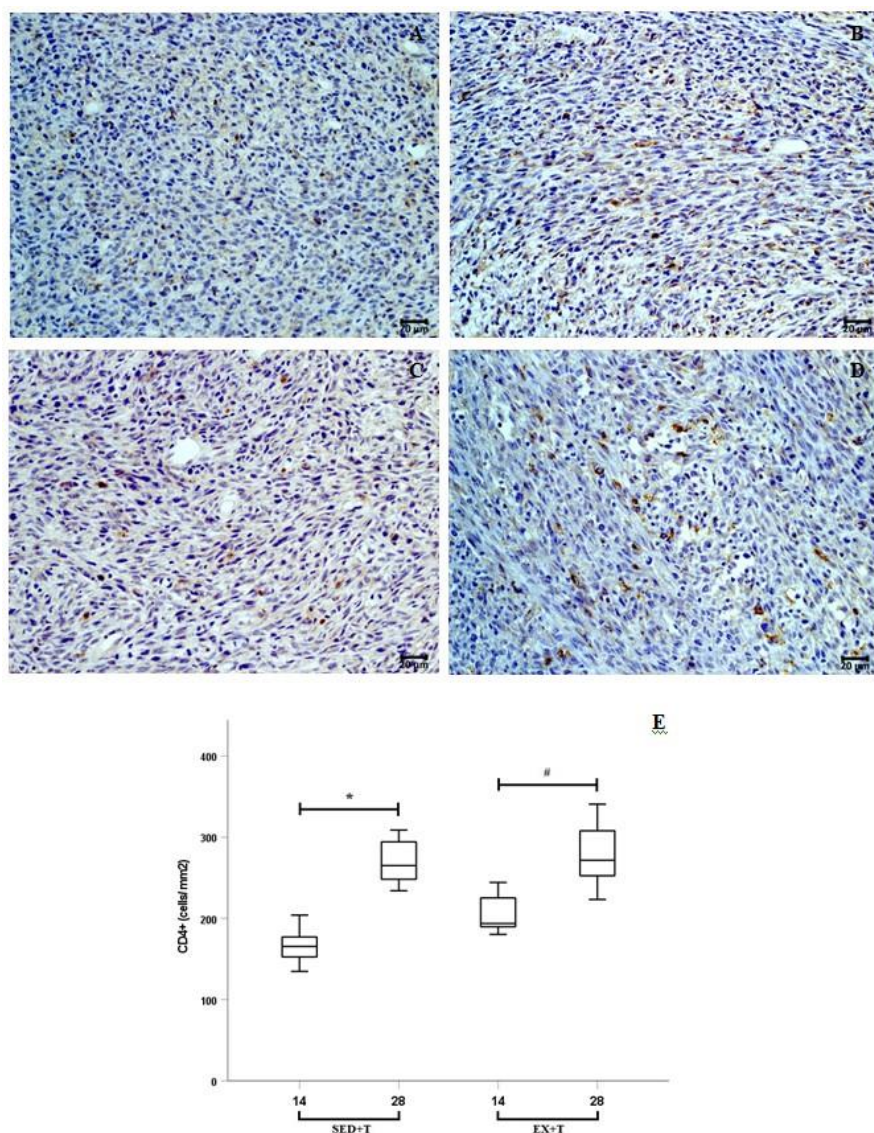


Figure 2: Representative microphotographs of CD4 immunostained tumors of sedentary mice (SED+T) at 14 (A) and 28 (B) days, and of exercised mice (EX+T) mice, at 14 (C) and 28 (D) days. A significantly higher CD4+ T cells density was observed in tumors of SED+T mice (* $p < 0.01$) and EX+T mice (# $p < 0.05$) at 28 days vs 14 days (E).

178x229mm (96 x 96 DPI)

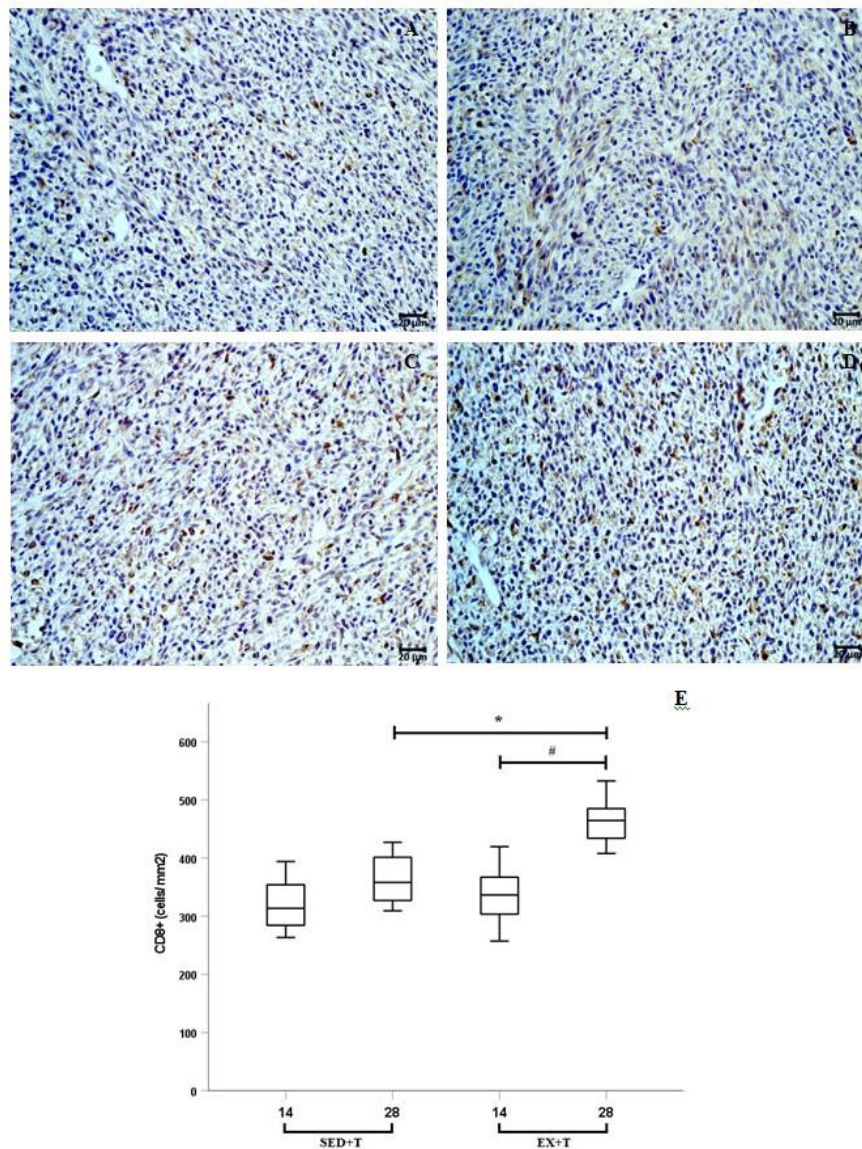


Figure 3: Representative microphotographs of CD8 immunostained tumors of sedentary mice (SED+T) at 14 (A) and 28 (B) days, and of exercised mice (EX+T) mice, at 14 (C) and 28 (D) days. EX+T mice presented a significantly higher intratumoral CD8+ T cells density at 28 days than at 14 days (# $p < 0.01$), as well as significantly higher than observed in tumors of SED+T mice, at 28 days (* $p < 0.05$) (E).

179x234mm (96 x 96 DPI)

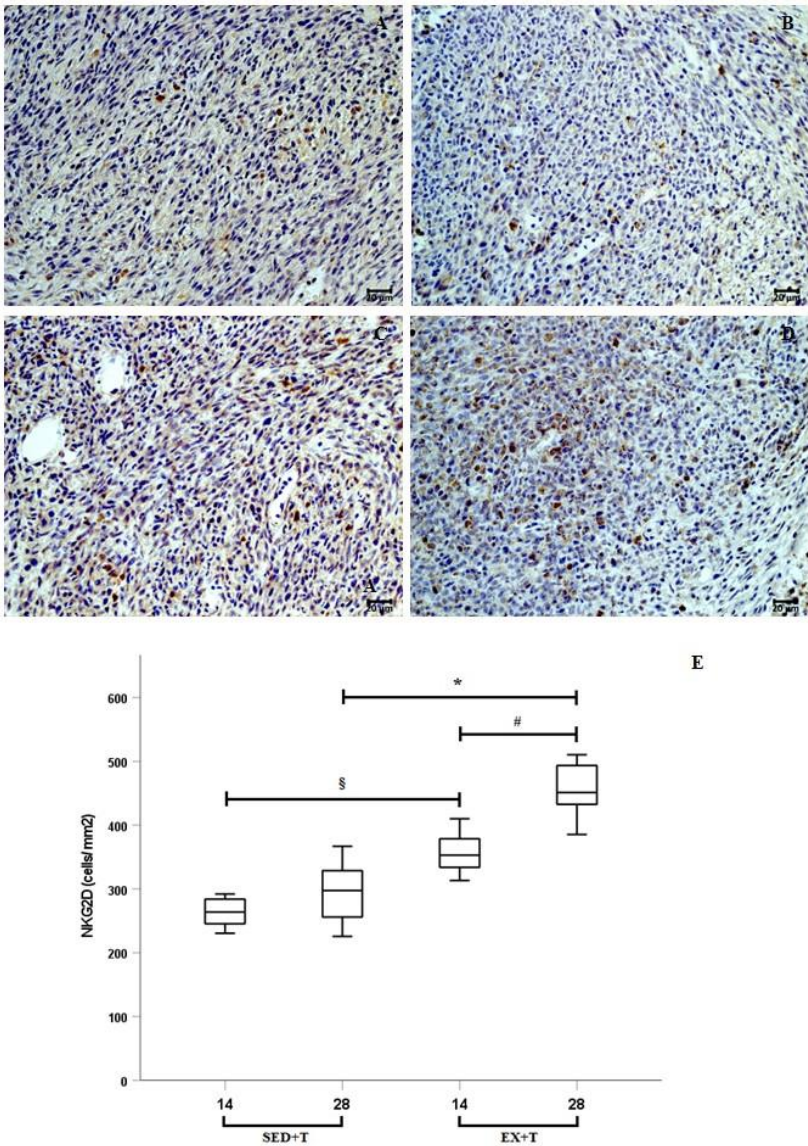


Figure 4: Representative microphotographs of NKG2D immunostained tumors of sedentary mice (SED+T) at 14 (A) and 28 (B) days, and of exercised mice (EX+T) mice, at 14 (C) and 28 (D) days. Tumors of EX+T mice presented a significantly higher NKG2D expression as compared to those of SED+T mice at both time points ($\S p < 0.05$; $*p < 0.001$); NKG2D expression in tumors of EX+T mice at 28 days was also higher as compared to 14 days ($\#p < 0.05$) (E).

178x251mm (96 x 96 DPI)

3.4. Study VI

Esteves M, Duarte M, Oliveira PA, Gil da Costa, RM, Monteiro MP, Duarte JA.
Skeletal muscle's sensitivity to wasting induced by urothelial carcinoma.
Submitted to Experimental Oncology (under review).

Title: Skeletal muscle's sensitivity to wasting induced by urothelial carcinoma

ABSTRACT

Background: Skeletal muscle wasting is a common phenotypic feature of several types of cancer and it is associated with functional impairment, respiratory complications and fatigue. However, equivocal evidence remains regarding the impact of cancer-induced muscle wasting on the different fiber types. **Aim:** The aim of this study was to investigate, in a cancer mouse model, the impact of urothelial carcinoma on the histomorphometric features and collagen deposition in different skeletal muscles. **Material and Methods:** Thirteen ICR (CD1) male mice were randomly assigned into two groups: exposed to 0.05% N-butyl-N-(4-hydroxybutyl) nitrosamine in the drinking water for 12 weeks, plus 8 weeks of tap water (BBN, n=8) or with access to tap water for 20 weeks (CONT, n=5). Tibialis anterior, soleus, and diaphragm muscles were collected from all animals. For cross-sectional area and myonuclear domain analysis, muscle sections were stained with Hematoxylin and Eosin and for collagen deposition assessment, muscle sections were stained with Picrosirius Red. **Results:** All animals from BBN group developed urothelial preneoplastic and neoplastic lesions, and the tibialis anterior from these animals presented a reduced cross-sectional area ($p<0.001$), with a decreased proportion of fibers with higher cross-sectional area, an increased collagen deposition ($p=0.017$) and higher myonuclear domain ($p=0.031$). BBN mice also showed a higher myonuclear domain in diaphragm ($p=0.015$). **Conclusion:** Urothelial carcinoma induced muscle wasting of tibialis anterior, expressed by a decreased cross-sectional area, higher infiltration of fibrotic tissue and increased myonuclear domain, which also increased in diaphragm, suggesting that fast glycolytic muscle fibers are more susceptible to be affected by cancer development.

Keywords: Cancer, histology, cross-sectional area, myonuclear domain, collagen deposition

INTRODUCTION

Cancer is considered one of the most debilitating diseases and its incidence continues to increase, reflecting the growth and aging of the population, as well as the exposition to the main risk factors [1]. In recent years, although the introduction of neoadjuvant chemotherapy and the development of new therapies like antibodies and immune checkpoint inhibitors improved the overall survival (OS), cancer remains a common cause of death worldwide [2]. Indeed, about 20% of all cancer-related deaths have been attributed to cachexia, a complex multi-organ syndrome that affects up to 80% of cancer patients [3]. Skeletal muscle loss with structural changes, the most significant events in cancer cachexia, are the main causes of functional impairment and are associated with an increased mortality rate [4]. Indeed, muscle wasting associated with cancer can range from 20% to 70% of muscle loss, and is mainly caused by an increase in myofibrillar protein degradation, particularly myosin heavy-chain (MHC), a decrease in protein synthesis or an imbalance between the two [5]. Consequently, tumor-bearing animals and cancer patients show clinical signs of muscle wasting, with a reduced muscle force production and an earlier onset of fatigue [6, 7].

For a long time chronic inflammation was considered the major mediator of cancer cachexia, involving the up-regulation of pro-inflammatory cytokines, acute phase proteins and increased insulin resistance that directly activate the ubiquitin–proteasome system (UPS) and autophagy, while inhibiting the synthesis of myofibrillar proteins [8]. However, it seems that the microenvironment of the skeletal muscle may influence its susceptibility to wasting [9]. Indeed, an increased production of reactive oxygen species (ROS) and the reduced expression of antioxidant-related genes have been functionally linked to the fast glycolytic muscles catabolism associated with cancer [9]. ROS can act as signalling molecules to regulate cellular viability, but excessive production can activate proteasome-dependent protein degradation by inducing the expression of muscle-specific E3 ligases, muscle atrophy F-box protein (Atrogin-1/MAFbx) and muscle ring-finger protein 1 (MuRF1) [10]. Consequently, there is an increased oxidation of proteins, lipids, and DNA promoting oxidative stress and cellular damage [11]. Thus, considering that the antioxidant capacity is lower in type II than in type I fibers, due to a heterogeneous mitochondria population and an increased ROS production [12], glycolytic muscles may experience higher oxidative burden than chronically active oxidative muscles, in response to cancer-associated wasting [9].

However, conflicting evidence remains as to whether there is a selective loss of myofibers or if all skeletal muscles are equally affected by cancer-induced wasting [13]. Interestingly, while some studies observed a selective atrophy of fast glycolytic muscles compared to slow oxidative muscles [14, 15], other authors found that type I fibers were more affected by cancer-induced wasting [16] or that cancer significantly decreased the cross-sectional area (CSA) of all fibers, independently of the MHC isoform expressed [17]. Although such differences may be partially due to variations in tumor phenotype [18], methodological discrepancies regarding the assessment of muscle wasting between studies could also contributed to these contradictory results because some authors only evaluated the mean weight of muscles [14, 15], while others only measured the CSA of myofibers without a proper analysis of a potential selective atrophy pattern [19]. Additionally, although the morphologic changes of skeletal muscles that represent the initial pathological change of wasting are often an occult condition [20], many studies tried to evaluate the potential selectively impact of cancer on skeletal muscles using severely cachectic models, which may bias an appropriate assessment because with the

installation of clinical signs of cachexia, it is likely that the occurrence of its manifestations became exacerbated to a point that all tissues are affected, hindering an accurate analysis [21].

Therefore, the present study aimed to compare, in a mouse model without clinical signs of cachexia, the impact of urothelial carcinoma (UCC) on the histomorphometric features, specifically the CSA and the myonuclear domain (MND), and on the fibrotic tissue deposition of three skeletal muscles with different phenotypic characteristics, i.e., tibialis anterior (TA) that is composed by type IIb (50%), type IIc (18%), type IIa (17%), type I (2%) and hybrid (12%) fibers [22]; soleus (SOL), which shows 70% of fibers expressing only type I MHC isoform, 5% to 10% expressing type IIa isoform and the remaining co-expressing type I and IIa MHC isoforms [23]; and diaphragm, formed by type I (60%), type IIc (27%) and type IIa (13%) fibers [22]. We hypothesized that type II fibers and, consequently, TA would be more susceptible to UCC-associated wasting than SOL or diaphragm, thus showing a reduced CSA, higher collagen deposition and increased MND.

MATERIALS AND METHODS

Animals and procedures

Thirteen ICR (CD1) male mice were obtained at the age of 4 weeks from Harlan (Barcelona, Spain) and were individually housed in cages with identical floor areas (800 cm²; Tecniplast, Buguggiate, Italy), under controlled conditions of 23± 2°C, 50± 10% humidity, on 12:12-h light-dark cycle. After a week's quarantine, mice were randomly assigned into two groups: exposed to 0.05% N-butyl-N-(4-hydroxybutyl) nitrosamine (BBN) in the drinking water, in light impermeable bottles due to its photosensitivity, over the course of 12 weeks, plus 8 weeks of tap water without any chemical supplementation (BBN, n=8) or with access to tap water, without any chemical supplementation over the course of 20 weeks (CONT, n=5). Due to the increased risk of mortality in BBN mice, the n in this group was superior to CONT group in order to face some hypothetical dropouts, guarantying with this procedure a sufficient sample in BBN group for a proper data analysis. According to previous studies with this model, tumor burden is expected after 12 weeks of oral exposition to BBN [24, 25]. The animals were maintained on a standard diet during the experiment and body weight (BW) was evaluated weekly. All mice concluded the protocol which was approved by the Portuguese Ethics Committee for Animal Experimentation, Direção Geral de Alimentação e Veterinária (licence number 020157; 2014-09-24) and was performed in accordance with European and National Legislation (European Directive 2010/63/EU and National Decree-law 113/2013).

Necropsy

All mice were euthanized 20 weeks after the initial exposure to BBN. The sacrifice was performed by exsanguination through cardiac puncture, under deeply anaesthesia. TA, SOL and diaphragm muscles were collected and processed for histology. Their urinary bladders were inflated in situ and collected as previously described [26].

Histological analysis

TA, SOL and diaphragm muscles were fixed (4% (v/v) buffered paraformaldehyde) by diffusion during 24h and subsequently dehydrated with graded ethanol, cleared in xylene and embedded in paraffin blocks. Cross-

sectional 4 μm -thick sections of paraffin blocks were cut on a Leica 2125 rotary microtome (Leica Microsystems, Germany) and used for histomorphometric analysis and collagen deposition assessment. After fixation during twelve hours, the bladders were longitudinally sectioned and further embedded in paraffin. Bladders' sections of 2 μm of thickness were stained with haematoxylin and eosin (H&E) and the mucosal surface was carefully examined for the existence of papillary or nodular lesions. At least one section per animal was evaluated under a light microscope by a pathologist (R.M.G.C.) to determine the existence of UCC and other urothelial lesions [27]. All the slides were examined in a blind fashion, without prior knowledge of the treatment given to the animals. The lesions found were classified and staged according to the World Health Organization/ International Society of Urological Pathology consensus classification of urothelial (transitional cell) neoplasms of the urinary bladder [28]. For CSA and MND analysis, muscle sections were stained with H&E and for collagen deposition assessment, muscle sections were stained with Picrosirius Red (SR), according to the method of Sweet and colleagues (for references see [29]). Briefly, slides were incubated on 0.1% SR picric acid for 90 minutes, rinsed in 0.5% acetic acid, dehydrated in ethanol and cleared in xylene. This technique stains collagen tissue bright red and muscle tissue yellow.

Samples were analysed with a light microscope (Axio Imager A1, Carl Zeiss, Germany), with a 40x magnification objective and images recorded with a coupled digital camera (Leica DM4000D, Nussloch, Germany). Images were then examined with ImageJ software (NIH, Bethesda, MD) to measure the CSA of myofibers, collagen deposition and the MND. The myofiber CSA was defined by its individual cross-sectional size (μm^2), the collagen deposition was evaluated by calculating the percentage of red and yellow area and the MND was determined by dividing the myofiber CSA by the number of its myonuclei. For consistency, multiple single frame images (10-15 per section) were acquired from the same muscle section (9 different sections per muscle). In BBN mice the number of fibers analysed was 4326 (1497 from TA, 1347 from SOL and 1482 from diaphragm), while in CONT mice we assessed 2378 fibers (904 from TA, 816 from SOL and 658 from diaphragm).

Statistical analysis

Normality and homogeneity of variances were verified by the Shapiro Wilk and Levene's test. For normal distributed variables, the outcomes were presented as mean and standard deviation and the statistical inference was done through the parametric independent samples T-test. Without a normal distribution, the outcomes were expressed by median and interquartile range (25-75%) and the statistical analysis was done through the non-parametric Mann-Whitney test. All procedures were performed with SPSS, version 24 (Chicago, IL, USA). A p value below 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

All animals from BBN group developed urothelial preneoplastic and neoplastic lesions. No signs of cachexia were evident throughout the entire experimental protocol, as corroborated by the absence of a weight loss $\leq 5\%$ [30] after 12 weeks of BBN exposure in the drinking water followed by 8 weeks of water consumption (Table 1). TA from BBN mice presented a lower mean fiber CSA compared to CONT (Table 2) that resulted from a decrease in the proportion of fibers with higher CSA ($> 1500 \mu\text{m}^2$) (Figure 1). Inversely, there were no

significant differences regarding mean fiber CSA of SOL (Figure 2) and diaphragm (Figure 3) muscles between groups (Table 2). The percentage of collagen deposition was significantly higher in TA muscle from BBN mice compared to CONT (Table 3; Figure 4). The percentage of collagen deposition in SOL (Figure 5) and diaphragm (Figure 6) muscles was not statistically different between groups (Table 3). TA and diaphragm from BBN mice showed a higher MND compared to CONT. In contrast, SOL muscle did not present differences in the MND between the two groups (Table 4).

Table 1

Table 2

Figure 1

Figure 2

Figure 3

Table 3

Figure 4

Figure 5

Figure 6

Table 4

The results of the present study showed that a mouse model of UCC significantly induced wasting of TA with a decreased myofiber CSA, increased collagen deposition and higher MND. Moreover, we found that, although there were no changes in the diaphragmatic myofiber CSA and fibrotic tissue infiltration, UCC promoted an important increase of the MND in this muscle. Contrarily, SOL muscle did not show any signs of wasting associated with UCC. Interestingly, by distributing the myofibers according to CSA categories, we observed in TA that fibers with higher CSA were more affected by UCC, indicating its particular susceptibility to wasting. This observation is in agreement with previous studies reporting that fast-glycolytic muscles seem to be more prone to cancer-induced wasting than slow-oxidative muscles [14, 15]. Indeed, the study of Julliene and co-workers, focusing on different hindlimb muscles found that SOL was the only skeletal muscle unaffected by peritoneal carcinosis [14]. The remaining muscles, including extensor digitorum longus (EDL), gastrocnemius (GAST) and TA had a significant weight reduction, ranging from 7% to 10% loss. Similarly, in a C-26 tumor model, Acharyya and colleagues observed a significant wasting of TA, with minimal change in SOL muscle mass [15]. However, although the assessment of muscle weight might be considered an operative variable to evaluate the occurrence of muscle atrophy, as this method do not differentiate the muscle fibers/ connective tissue contributions, it is not adequate for the evaluation of a potential myofiber-selective targeting [31]. Therefore, as the skeletal muscle is not exclusively composed by just one type of fibers, by not having identified the myofibers and not measured their CSA with a histogram to assess the most atrophied ones, it remains inconclusive if the weight reduction of the skeletal muscles observed in that studies was in fact attributed to a specific wasting of the type II fibers.

Aulino and co-workers also evaluated the impact of a C-26 tumor model in the histomorphometric properties of EDL, TA, and SOL from BALB/c mice and, contrary to our results, the authors found that all muscles showed signs of wasting [17]. Nonetheless, since the tumor-bearing mice assessed were severely cachectic, with a 30%

reduction in their BW, this means that the animals presented an advance pro-catabolic state that was not suitable for this type of comparison. Corroborating this notion, another two studies involving severely cachectic C-26 mice, showed a similar reduction in muscle mass of fast-glycolytic (EDL and GAST) and slow-oxidative (SOL) muscles, highlighting the development of a systemic wasting pattern in this later stage of the disease [16, 32].

Moreover, two studies recognized the occurrence of diaphragmatic dysfunction in cachectic C-26 mice [33, 34]. Indeed, Roberts and colleagues showed that the mean CSA of all diaphragm fibers was decreased by 26% [33], while Murphy and collaborators reported a 10% lower maximum specific force and lower absolute force production of diaphragm from severely cachectic mice [34]. The diaphragm is a chronically active muscle and, although being composed mainly by type I fibers, it also expresses type IIa and IIc fibers, where the high amount of oxidative fibers provide sustained respiration at rest and type IIc fibers allows an increased respiratory rate observed during physical exercise or during high-power output that occurs in sneezing and coughing [35]. Thus, according to our results, it seems that before the initiation of clinical signs of muscle wasting, the continuous activity of diaphragm for ventilation and also of SOL for postural control during standing and walking may provide an additional protection from the catabolic systemic environment. However, as the progression to a more severe cachectic phenotype is characterized by an intense systemic catabolism, increased energy expenditure, insulin resistance, anemia, and hypogonadism [30], these disturbing factors favour the installation of a general wasting pattern, hindering a rigorous evaluation about the susceptibility across different muscles and/or different fibers [36].

Additionally, in our study, the development of UCC also induced an increased collagen deposition in TA, but not in diaphragm or in SOL. Indeed, skeletal muscle fibrosis is a common hallmark of chronic muscle wasting disorders and, considering that an excessive accumulation of extracellular matrix (ECM) components, particularly collagen, in skeletal muscle results in reduced contractility, besides a decreased CSA [37], clinical guidelines highlighted the increased fibrotic tissue area as an important co-factor to define a pathological condition [38]. Interestingly, in a recent study, Judge and collaborators saw that the rectus abdominis from cachectic patients with pancreatic ductal adenocarcinoma (PDAC) had higher levels of collagen deposition, compared to non-cancer control subjects, which was correlated with lymph node metastasis and decreased OS [39]. Therefore, as tissue fibrosis is the end result of a cascade of events occurring from tissue injury to necrosis, via inflammation, that results in permanent scar formation [40], the replacement of muscle fibers by connective tissue represents a relevant outcome that must be assessed in order to predict cancer progression [39]. Furthermore, the replacement of muscle tissue by connective tissue is a known consequence of impaired muscle regeneration following muscle injury [40]. Indeed, the heterogeneity of satellite cells (SCs) can promote important differences in the regenerative capacity of slow oxidative and fast glycolytic skeletal muscles, because as a higher quantity of SCs is associated with the capillary density [41] and myonuclear number [42], it is assumed that type I fibers have a five to six greater concentration of SCs compared to type II fibers [43]. Indeed, the MND has been found to be different according to the oxidative capacity of the fibers and the MHC expressed, with type I fibers having smaller MND than type II fibers [44]. Curiously, several studies demonstrated that the reduction of myonuclear number during muscle atrophy was not always proportional to the decrease of myofiber CSA, resulting in significant changes in MND size [45-47]. The results of the present study corroborated this idea by showing an increased MND in TA and in diaphragm from BBN mice, but not in SOL. Therefore, considering that each myonucleus controls the transcription and consequently the protein synthesis of a limited amount of cytoplasm,

an increased MND means that the same myonucleus is responsible for a higher cytoplasmic volume, suggesting that the regenerative potential of TA and diaphragm muscles may have been compromised [49].

The present study provides fundamental knowledge regarding how UCC selectively impacts different skeletal muscles and their fibers, which should be considered in the clinical setting when addressing the prevention and management of cancer-associated muscle wasting and cachexia. In the future, studies addressing potential interventions that may prevent or overcome these findings and evaluating the long term effects of UCC in the respiratory muscles are welcomed. However, as muscle samples were fixed and further imbedding in paraffin blocks, it was not possible to process fresh cuts for identification of the different muscle fiber types. Nonetheless, considering that the fiber type composition of each muscle is well described in the literature [22, 23], the differences found between the muscles in the present study point to a higher susceptibility of fast glycolytic TA muscle, particularly the fibers with higher CSA, to the UCC-induced muscle atrophy.

In conclusion, the results indicate that under the condition of UCC, local skeletal muscle's features exerted influence over the installation of wasting. The fast-glycolytic TA muscle, particularly the fibers with higher CSA, seems to be more prone to the atrophic signal, when compared with the smaller CSA fibers and with chronically active muscles like SOL and diaphragm, reinforcing the notion of a heterogenic response to cancer-induced muscle wasting. Although TA presented an increased MND and a higher deposition of fibrotic tissue, indicating a more demanding muscle's regeneration process, the MND of diaphragm was also superior, suggesting that its regenerative capacity may be compromised, thus raising some concerns about the long-term respiratory impact of UCC.

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REFERENCES

1. **Bray F, Ferlay J, Soerjomataram I et al.** Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin* 2018; **68**: 394-424. doi:10.3322/caac.21492
2. **Arnold M, Rutherford MJ, Bardot A et al.** Progress in cancer survival, mortality, and incidence in seven high-income countries 1995-2014 (ICBP SURVMARK-2): a population-based study. *Lancet Oncol* 2019; **20**: 1493-1505. doi:10.1016/S1470-2045(19)30456-5
3. **Fearon KC.** Cancer cachexia: developing multimodal therapy for a multidimensional problem. *Eur J Cancer* 2008; **44**: 1124-1132. doi:10.1016/j.ejca.2008.02.033
4. **Argiles JM, Busquets S, Stemmler B et al.** Cancer cachexia: understanding the molecular basis. *Nat Rev Cancer* 2014; **14**: 754-762. doi:10.1038/nrc3829
5. **Eley HL, Tisdale MJ.** Skeletal muscle atrophy, a link between depression of protein synthesis and increase in degradation. *J Biol Chem* 2007; **282**: 7087-7097. doi:10.1074/jbc.M610378200
6. **Toledo M, Busquets S, Sirisi S et al.** Cancer cachexia: physical activity and muscle force in tumour-bearing rats. *Oncol Rep* 2011; **25**: 189-193
7. **Bower JE.** Cancer-related fatigue--mechanisms, risk factors, and treatments. *Nat Rev Clin Oncol* 2014; **11**: 597-609. doi:10.1038/nrclinonc.2014.127

8. **Biolo G, Cederholm T, Muscaritoli M.** Muscle contractile and metabolic dysfunction is a common feature of sarcopenia of aging and chronic diseases: from sarcopenic obesity to cachexia. *Clin Nutr* 2014; **33**: 737-748. doi:10.1016/j.clnu.2014.03.007
9. **Hardee JP, Montalvo RN, Carson JA.** Linking Cancer Cachexia-Induced Anabolic Resistance to Skeletal Muscle Oxidative Metabolism. *Oxid Med Cell Longev* 2017; **2017**: 8018197. doi:10.1155/2017/8018197
10. **Li YP, Chen Y, Li AS et al.** Hydrogen peroxide stimulates ubiquitin-conjugating activity and expression of genes for specific E2 and E3 proteins in skeletal muscle myotubes. *Am J Physiol Cell Physiol* 2003; **285**: C806-812. doi:10.1152/ajpcell.00129.2003
11. **Ott M, Gogvadze V, Orrenius S et al.** Mitochondria, oxidative stress and cell death. *Apoptosis* 2007; **12**: 913-922. doi:10.1007/s10495-007-0756-2
12. **Yu Z, Li P, Zhang M et al.** Fiber type-specific nitric oxide protects oxidative myofibers against cachectic stimuli. *PLoS One* 2008; **3**: e2086. doi:10.1371/journal.pone.0002086
13. **Johns N, Hatakeyama S, Stephens NA et al.** Clinical classification of cancer cachexia: phenotypic correlates in human skeletal muscle. *PLoS One* 2014; **9**: e83618. doi:10.1371/journal.pone.0083618
14. **Julienne CM, Dumas JF, Goupille C et al.** Cancer cachexia is associated with a decrease in skeletal muscle mitochondrial oxidative capacities without alteration of ATP production efficiency. *J Cachexia Sarcopenia Muscle* 2012; **3**: 265-275. doi:10.1007/s13539-012-0071-9
15. **Acharyya S, Ladner KJ, Nelsen LL et al.** Cancer cachexia is regulated by selective targeting of skeletal muscle gene products. *J Clin Invest* 2004; **114**: 370-378. doi:10.1172/JCI20174
16. **Hiroux C, Dalle S, Koppo K et al.** Voluntary exercise does not improve muscular properties or functional capacity during C26-induced cancer cachexia in mice. *J Muscle Res Cell Motil* 2021; **42**: 169-181. doi:10.1007/s10974-021-09599-6
17. **Aulino P, Berardi E, Cardillo VM et al.** Molecular, cellular and physiological characterization of the cancer cachexia-inducing C26 colon carcinoma in mouse. *BMC Cancer* 2010; **10**: 363. doi:10.1186/1471-2407-10-363
18. **Monitto CL, Berkowitz D, Lee KM et al.** Differential gene expression in a murine model of cancer cachexia. *Am J Physiol Endocrinol Metab* 2001; **281**: E289-297. doi:10.1152/ajpendo.2001.281.2.E289
19. **Marin-Corral J, Fontes CC, Pascual-Guardia S et al.** Redox balance and carbonylated proteins in limb and heart muscles of cachectic rats. *Antioxid Redox Signal* 2010; **12**: 365-380. doi:10.1089/ars.2009.2818
20. **Fearon KC, Glass DJ, Guttridge DC.** Cancer cachexia: mediators, signaling, and metabolic pathways. *Cell Metab* 2012; **16**: 153-166. doi:10.1016/j.cmet.2012.06.011
21. **Li P, Waters RE, Redfern SI et al.** Oxidative phenotype protects myofibers from pathological insults induced by chronic heart failure in mice. *Am J Pathol* 2007; **170**: 599-608. doi:10.2353/ajpath.2007.060505
22. **Talmadge RJ, Roy RR.** Electrophoretic separation of rat skeletal muscle myosin heavy-chain isoforms. *J Appl Physiol* (1985) 1993; **75**: 2337-2340
23. **Caiozzo VJ, Baker MJ, Baldwin KM.** Novel transitions in MHC isoforms: separate and combined effects of thyroid hormone and mechanical unloading. *J Appl Physiol* (1985) 1998; **85**: 2237-2248. doi:10.1152/jappl.1998.85.6.2237
24. **Vasconcelos-Nobrega C, Pinto-Leite R, Arantes-Rodrigues R et al.** In vivo and in vitro effects of RAD001 on bladder cancer. *Urol Oncol* 2013; **31**: 1212-1221. doi:10.1016/j.urolonc.2011.11.002
25. **Ferreira R, Neuparth MJ, Nogueira-Ferreira R et al.** Exercise Training Impacts Cardiac Mitochondrial Proteome Remodeling in Murine Urothelial Carcinoma. *Int J Mol Sci* 2018; **20**. doi:10.3390/ijms20010127
26. **Oliveira PA, Vasconcelos-Nobrega C, Gil da Costa RM et al.** The N-butyl-N-4-hydroxybutyl Nitrosamine Mouse Urinary Bladder Cancer Model. *Methods Mol Biol* 2018; **1655**: 155-167. doi:10.1007/978-1-4939-7234-0_13
27. **Oliveira PA, Gil da Costa RM, Vasconcelos-Nobrega C et al.** Challenges with in vitro and in vivo experimental models of urinary bladder cancer for novel drug discovery. *Expert Opin Drug Discov* 2016; **11**: 599-607. doi:10.1080/17460441.2016.1174690
28. **Epstein JI, Amin MB, Reuter VR et al.** The World Health Organization/International Society of Urological Pathology consensus classification of urothelial (transitional cell) neoplasms of the urinary bladder. *Bladder Consensus Conference Committee. Am J Surg Pathol* 1998; **22**: 1435-1448. doi:10.1097/00000478-199812000-00001
29. **Bovolini A, Garcia J, Andrade MA et al.** Relative contribution of fat diet and physical inactivity to the development of metabolic syndrome and non-alcoholic fat liver disease in Wistar rats. *Physiol Behav* 2020; **225**: 113040. doi:10.1016/j.physbeh.2020.113040

30. **Fearon K, Strasser F, Anker SD et al.** Definition and classification of cancer cachexia: an international consensus. *Lancet Oncol* 2011; **12**: 489-495. doi:10.1016/S1470-2045(10)70218-7
31. **McGregor RA, Cameron-Smith D, Poppitt SD.** It is not just muscle mass: a review of muscle quality, composition and metabolism during ageing as determinants of muscle function and mobility in later life. *Longev Healthspan* 2014; **3**: 9. doi:10.1186/2046-2395-3-9
32. **Roberts BM, Frye GS, Ahn B et al.** Cancer cachexia decreases specific force and accelerates fatigue in limb muscle. *Biochem Biophys Res Commun* 2013; **435**: 488-492. doi:10.1016/j.bbrc.2013.05.018
33. **Roberts BM, Ahn B, Smuder AJ et al.** Diaphragm and ventilatory dysfunction during cancer cachexia. *FASEB J* 2013; **27**: 2600-2610. doi:10.1096/fj.12-222844
34. **Murphy KT, Chee A, Trieu J et al.** Importance of functional and metabolic impairments in the characterization of the C-26 murine model of cancer cachexia. *Dis Model Mech* 2012; **5**: 533-545. doi:10.1242/dmm.008839
35. **Gransee HM, Mantilla CB, Sieck GC.** Respiratory muscle plasticity. *Compr Physiol* 2012; **2**: 1441-1462. doi:10.1002/cphy.c110050
36. **Aversa Z, Costelli P, Muscaritoli M.** Cancer-induced muscle wasting: latest findings in prevention and treatment. *Ther Adv Med Oncol* 2017; **9**: 369-382. doi:10.1177/1758834017698643
37. **Delaney K, Kasprzycka P, Ciemerych MA et al.** The role of TGF-beta1 during skeletal muscle regeneration. *Cell Biol Int* 2017; **41**: 706-715. doi:10.1002/cbin.10725
38. **Lieber RL, Ward SR.** Cellular mechanisms of tissue fibrosis. 4. Structural and functional consequences of skeletal muscle fibrosis. *Am J Physiol Cell Physiol* 2013; **305**: C241-252. doi:10.1152/ajpcell.00173.2013
39. **Judge SM, Nosacka RL, Delitto D et al.** Skeletal Muscle Fibrosis in Pancreatic Cancer Patients with Respect to Survival. *JNCI Cancer Spectr* 2018; **2**: pky043. doi:10.1093/jncics/pky043
40. **Mann CJ, Perdiguerro E, Kharraz Y et al.** Aberrant repair and fibrosis development in skeletal muscle. *Skelet Muscle* 2011; **1**: 21. doi:10.1186/2044-5040-1-21
41. **Christov C, Chretien F, Abou-Khalil R et al.** Muscle satellite cells and endothelial cells: close neighbors and privileged partners. *Mol Biol Cell* 2007; **18**: 1397-1409. doi:10.1091/mbc.e06-08-0693
42. **Pallafacchina G, Blaauw B, Schiaffino S.** Role of satellite cells in muscle growth and maintenance of muscle mass. *Nutr Metab Cardiovasc Dis* 2013; **23** Suppl 1: S12-18. doi:10.1016/j.numecd.2012.02.002
43. **Gibson MC, Schultz E.** The distribution of satellite cells and their relationship to specific fiber types in soleus and extensor digitorum longus muscles. *Anat Rec* 1982; **202**: 329-337. doi:10.1002/ar.1092020305
44. **Liu JX, Hoglund AS, Karlsson P et al.** Myonuclear domain size and myosin isoform expression in muscle fibres from mammals representing a 100,000-fold difference in body size. *Exp Physiol* 2009; **94**: 117-129. doi:10.1113/expphysiol.2008.043877
45. **Dupont-Versteegden EE, Murphy RJ, Houle JD et al.** Activated satellite cells fail to restore myonuclear number in spinal cord transected and exercised rats. *Am J Physiol* 1999; **277**: C589-597. doi:10.1152/ajpcell.1999.277.3.C589
46. **Zhong H, Roy RR, Siengthai B et al.** Effects of inactivity on fiber size and myonuclear number in rat soleus muscle. *J Appl Physiol* (1985) 2005; **99**: 1494-1499. doi:10.1152/japplphysiol.00394.2005
47. **Bruusgaard JC, Gundersen K.** In vivo time-lapse microscopy reveals no loss of murine myonuclei during weeks of muscle atrophy. *J Clin Invest* 2008; **118**: 1450-1457. doi:10.1172/JCI34022
48. **van Wessel T, de Haan A, van der Laarse WJ et al.** The muscle fiber type-fiber size paradox: hypertrophy or oxidative metabolism? *Eur J Appl Physiol* 2010; **110**: 665-694. doi:10.1007/s00421-010-1545-0
49. **Wang Y, Pessin JE.** Mechanisms for fiber-type specificity of skeletal muscle atrophy. *Curr Opin Clin Nutr Metab Care* 2013; **16**: 243-250. doi:10.1097/MCO.0b013e328360272d

Table 1. Body weight (mean± standard deviation).

	Body weight (g)	
	CONT	BBN
Baseline	24.14± 2.04	25.80± 0.85
Final	28.07± 3.83	27.69± 0.98

Table 2. Cross-sectional area

	Cross-sectional area (μm^2)				
	CONT		BBN		p
	Mean $\pm$ SD	95% CI	Mean $\pm$ SD	95% CI	
Tibialis Anterior	1518.23 $\pm$ 122.82	1365.74 $\pm$ 1670.73	1166.93 $\pm$ 99.99	1083.34, 1250.53	0.0001*
Diaphragm	696.24 $\pm$ 54.39	628.71 $\pm$ 763.78	676.88 $\pm$ 28.77	652.83, 700.94	0.414
Soleus	664.20 $\pm$ 42.94	610.88 $\pm$ 717.52	640.02 $\pm$ 43.31	603.81, 676.22	0.347

CI: confidence interval; SD: standard deviation.

Table 3. Collagen deposition

	Collagen deposition (%)				p
	CONT		BBN		
	Mean±SD	95% CI	Mean± SD	95% CI	
Tibialis Anterior	4.47± 0.41	3.96± 4.99	5.08± 0.36	4.78, 5.37	0.017*
Diaphragm	3.89± 0.54	3.21± 4.57	4.32± 0.44	3.95, 4.70	0.143
Soleus	3.76± 0.57	3.05± 4.47	3.98± 0.54	3.53, 4.43	0.508

CI: confidence interval; SD: standard deviation.

Table 4. Myonuclear domain

	Myonuclear domain (μm^2)				p
	CONT		BBN		
	Mean $\pm$ SD	95% CI	Mean $\pm$ SD	95% CI	
Tibialis Anterior	569.79 $\pm$ 116.36	425.31 $\pm$ 714.26	729.45 $\pm$ 111.67	636.09, 822.81	0.031*
Diaphragm	316.77 $\pm$ 82.28	214.60 $\pm$ 41.93	445.05 $\pm$ 75.86	381.63, 508.46	0.015*
Soleus	334.35 $\pm$ 103.80	205.46 $\pm$ 463.24	419.67 $\pm$ 65.22	365.15, 474.19	0.093

CI: confidence interval; SD: standard deviation.

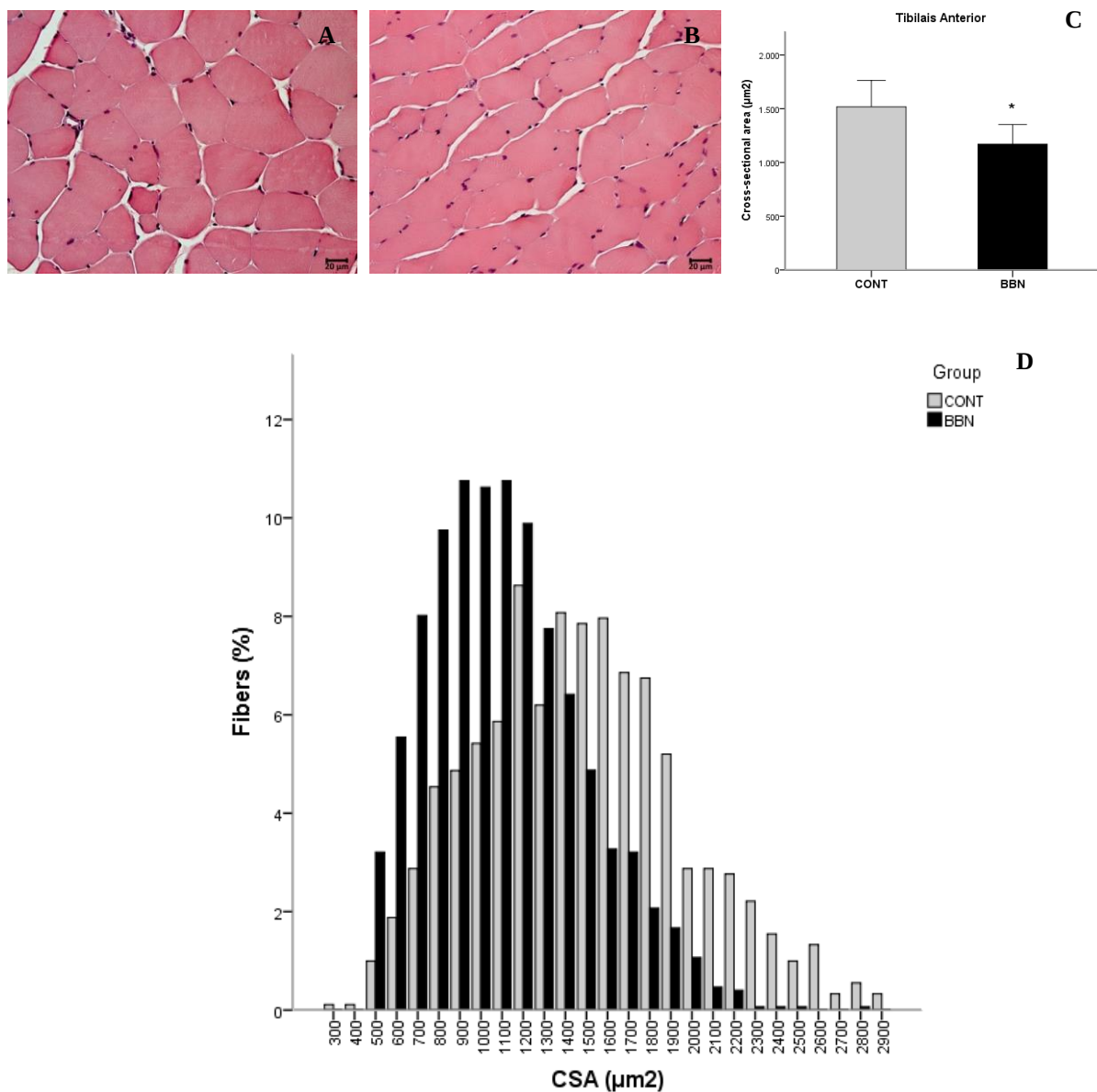


Figure 1. Representative images of tibialis anterior muscle stained with haematoxylin-eosin of CONT (A) and BBN (B) mice. It is clear a reduction of mean myofiber cross-sectional area (* $p < 0.001$) (C), and a decrease in the proportion of fibers with higher CSA ($> 1500 \mu\text{m}^2$) (D). The absolute number of fibers studied was 904 and 1497 for CONT and BBN mice, respectively.

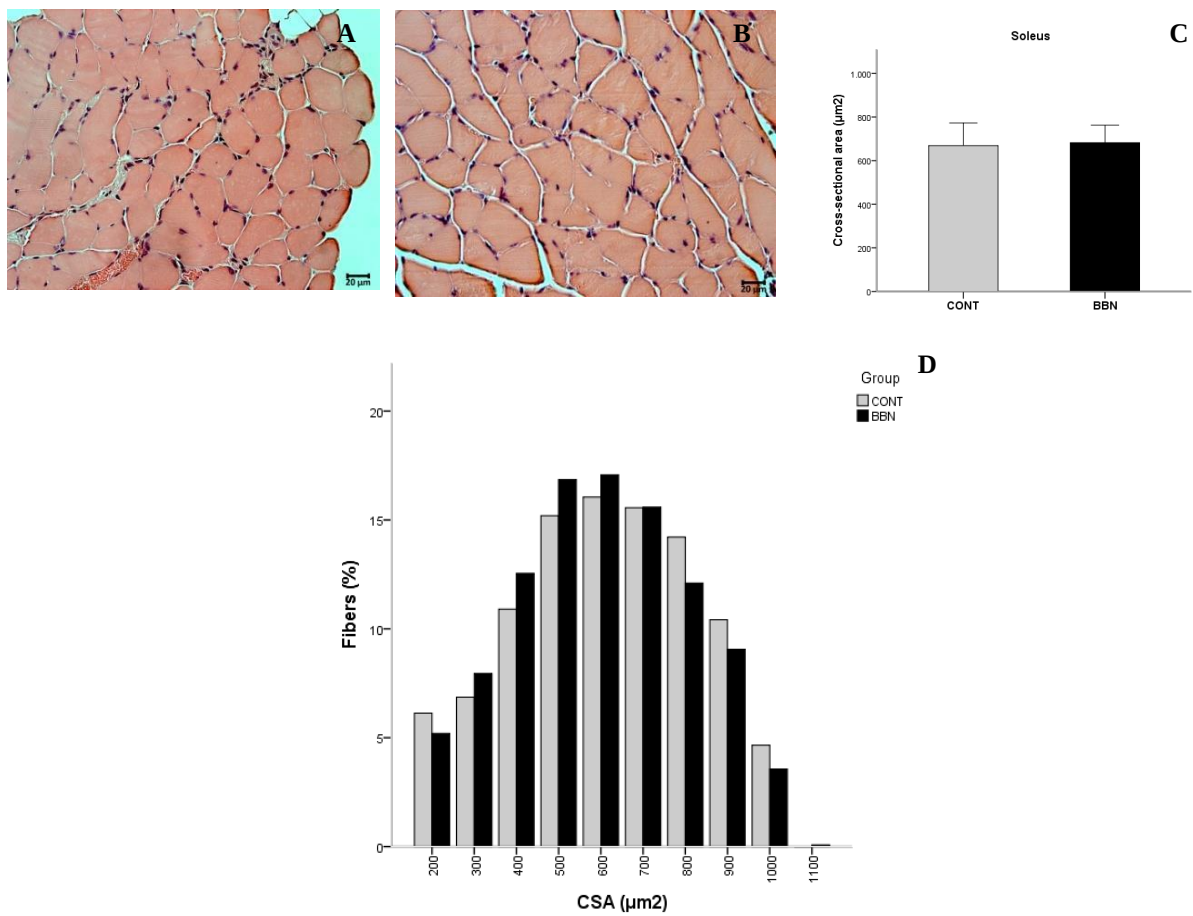


Figure 2. Representative images of soleus muscle stained with haematoxylin-eosin of CONT (A) and BBN (B) mice. There were no differences regarding mean fiber CSA between groups (C and D). The absolute number of fibers studied was 816 and 1347 for CONT and BBN mice, respectively.

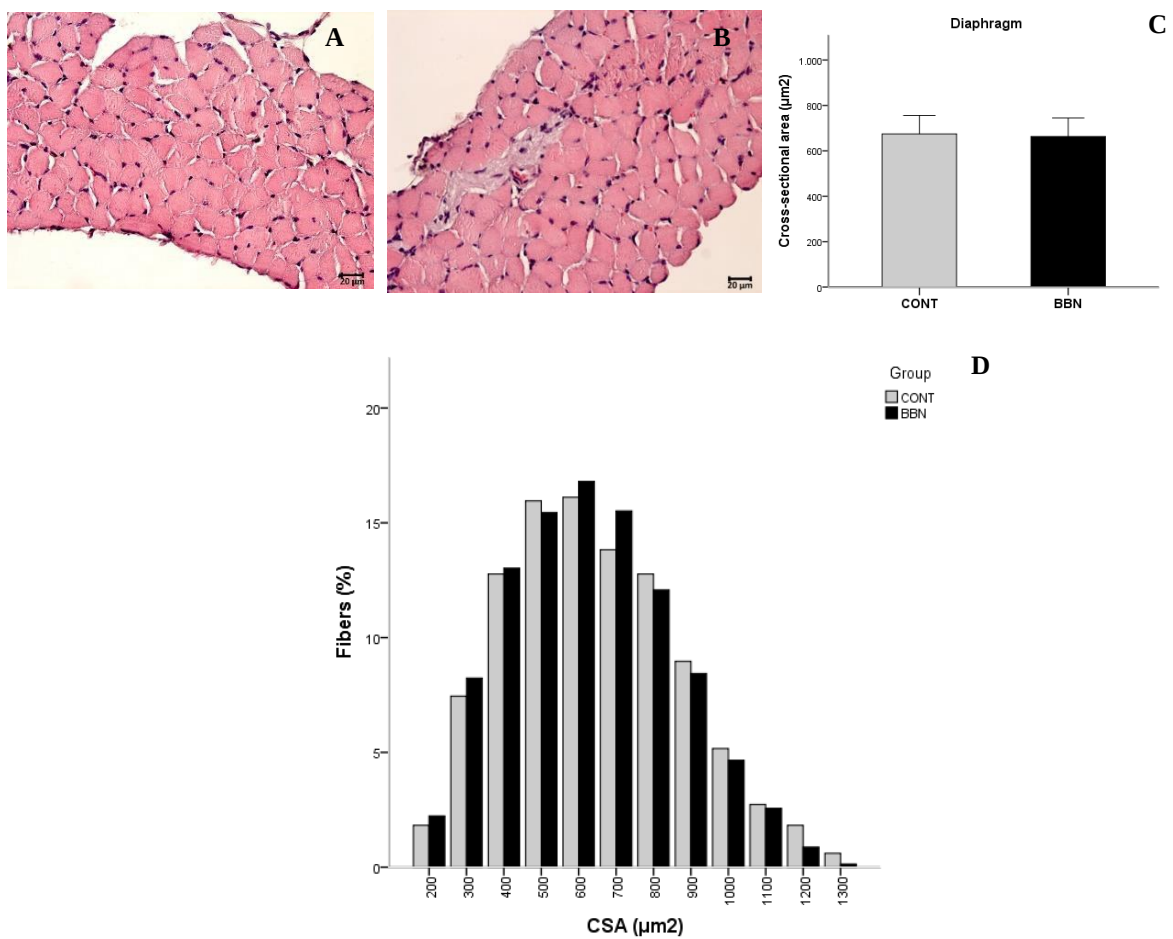


Figure 3. Representative images of diaphragm muscle stained with haematoxylin-eosin of CONT (A) and BBN (B) mice. There were no differences regarding mean fiber CSA between groups (C and D). The absolute number of fibers studied was 658 and 1482 for CONT and BBN mice, respectively.

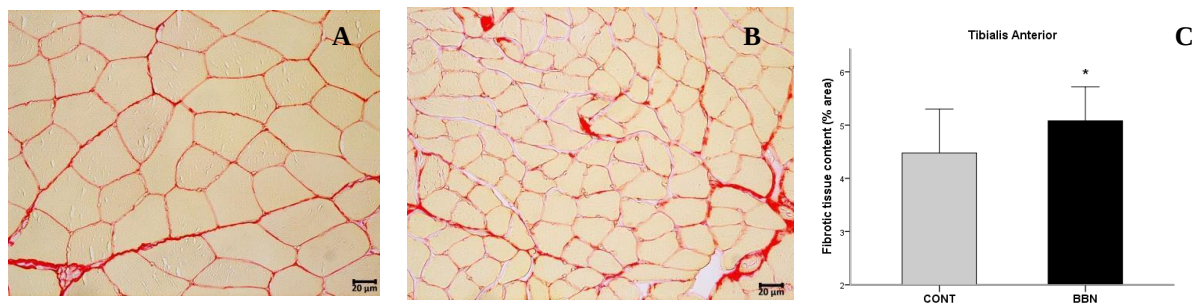


Figure 4. Representative images of tibialis anterior muscle stained with picosirius red of CONT (A) and BBN (B) mice. The mean percentage of collagen deposition is higher in BBN mice (*p= 0.017) (C).

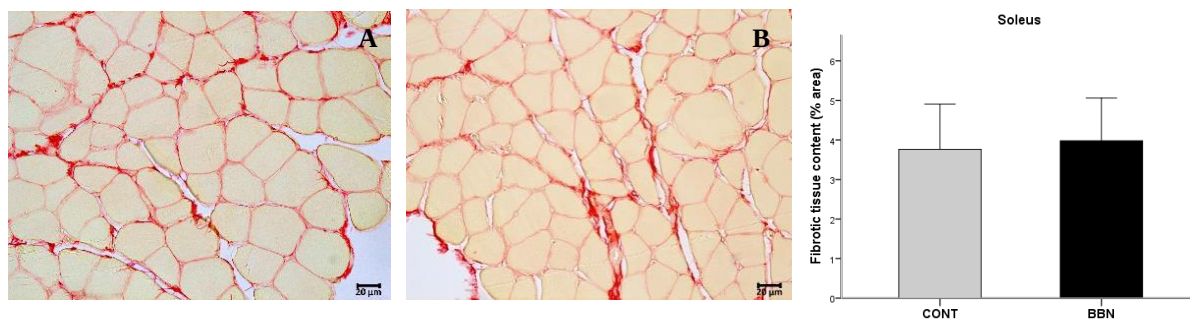


Figure 5. Representative images of soleus muscle stained with picrosirius red of CONT (A) and BBN (B) mice. The percentage of collagen deposition was not different between groups (C).

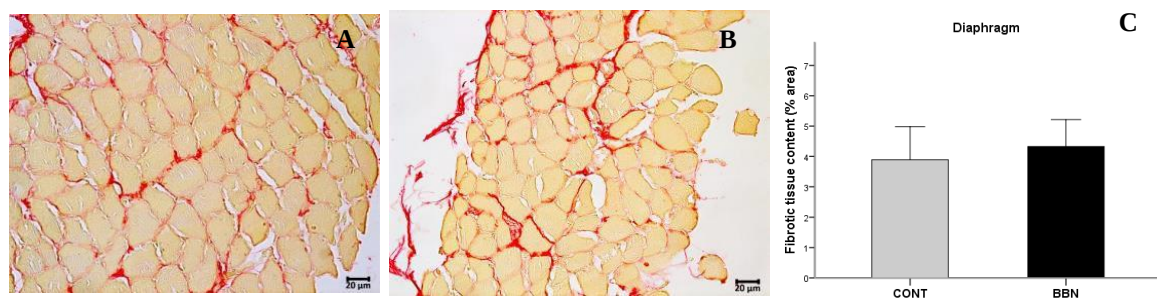


Figure 6. Representative images of diaphragm muscle stained with picosirius red of CONT (A) and BBN (B) mice. The percentage of collagen deposition was not different between groups (C).

CHAPTER IV
GENERAL DISCUSSION

4. General discussion

This chapter is organized in three sections that encompass a discussion about the methodological approaches involved and regarding the results obtained, and the clinical applicability.

4.1. Discussion of the methodology

Cancer has become one of the major diseases that seriously endanger human health [76], so the development and research of new diagnostic methods and treatment options are essential to reduce its global incidence and mortality [77]. However, as ethical principles limit the study of specific hypothesis in humans, pre-clinical research allows to elucidate, under controlled conditions, the cellular mechanisms involved in its occurrence, development and metastasis, as well as the potential therapeutic targets [78]. Indeed, while 99% of human genes have synonyms in the mice genome, mice and humans also have the same organs and systemic physiology, sharing metabolic homogeneity and showing great similarities in disease pathogenesis [79]. Particularly, as the tumors of mice present histological features comparable to human tumors [80], rodent models represent for a long a time an important bridge between cell experiments and clinical trials, in order to study gene and protein function *in vivo* [81], and to develop innovative approaches across numerous types of cancer [82]. Recently, with the continuous demand of personalized therapeutic interventions, pre-clinical research was also used to study the effects of physical exercise in cancer prevention, treatment, recovery, and survivorship [83]. However, previous studies often involved distinct physical exercise paradigms, tumor models and outcomes, limiting the strength of the available evidence regarding its role in cancer treatment and prognosis [84]. As such, after we systematized and quantified the existing evidence about the effects of tumor vascularization on its growth, we proposed to summarize the state-of-the-art regarding, specifically, the effects of physical exercise on tumor microenvironment (TME), thus gathering comprehensive knowledge and providing a solid background for

the experimental research. Then, we evaluated the effects of physical exercise on tumor vascularization and, to do that, we performed a second systematic review with meta-analysis to quantify its impact on the microvessel density (MVD) of tumors. Meta-analysis is a quantitative, formal and epidemiological study design used to systematically assess the results of previous research and to withdraw conclusions about that specific issue [85]. It is a valuable tool for determining the degree to which research supports theoretically based hypotheses and provides more statistical power to detect potential effects than individual studies [86]. Using meta-analysis, it is also possible to solve inconsistencies in research findings, because it has demonstrable benefits in addressing the limitations of sample size and in including diverse populations, which gives the opportunity to evaluate new hypothesis [85].

Afterwards, to investigate the effects of regular voluntary running on tumor growth and its impact on TME, we used a syngeneic model where mice were subcutaneously inoculated with a suspension of RM1 androgen-independent murine prostate carcinoma cells. Indeed, although orthotopic models seem to simulate better clinical cancer development and progression, by applying a heterotopic implantation, we obtained a prompt tumor engraftment that could be observed during the first week after cancer cell transplantation, as a local nodule [87]. In addition, with a subcutaneous inoculation, regular monitoring of tumor size could be done by using a digital calliper [88], making it an attractive model to study tumor growth [89]. Moreover, the subcutaneous injection of this type of cells allows the study of tumors *in vivo*, showing 100% of prevalence with a lower concentration of tumor cells inoculated [90]. Nevertheless, to determine the most appropriated concentration of RM1 cells, we previously studied the effect of three different dosages (1.5×10^3 , 3×10^3 , 4.5×10^3) in 12 C57BL/6 mice, for 18 days. The animals were divided into 3 groups and each group were inoculated with a specific concentration. Since inoculation with 1.5×10^3 RM1 cell showed a 100% tumor incidence with a lower growth rate, this dosage was selected to evaluate the potential effects of regular running on tumor progression with a low risk of mortality during the experimental time span.

Mice were then randomly assigned into two groups: sedentary tumor-induced (SED+T) and exercised tumor-induced (EX+T). EX+T mice were individually housed in cages equipped with free access to an activity wheel, while SED+T mice did not have access to a wheel. Although this type of behaviour is not consistent with typical daily activity of the animals due to the limited cage area, the activity of SED+T mice was conveniently restricted to the cage area and used as control of regular running of EX+T mice. Additionally, patients with cancer are reported to have lower levels of physical activity when compared not only to individuals without cancer but also to the levels observed before cancer diagnosis [91]. By restricting the activity levels of SED+T mice, we also simulated the increased sedentary time and the decline in daily living activities of patients diagnosed with cancer.

Moreover, we defined wheel running as a modality of physical activity, to allow mice to run voluntarily, at any period of the day or night, without a pre-established duration and intensity [92]. Pre-clinical studies with rodents normally involve voluntary wheel running or forced treadmill running, with both methods showing advantages and disadvantages [93]. On the wheel, as mice run spontaneously in several bouts of intense activity with periods of light running or no running, almost entirely during the active dark cycle and rarely during the inactive light cycle [94], there is general agreement that voluntary running is the one that better reflects the natural activity pattern of the animals [95]. Indeed, although this method precludes the control of the total amount and intensity of the activity performed [94], it minimizes the stress-related conditions of the animal because does not deregulates its circadian cycles [96]. Consequently, wheel running prevents some confounding effects on the molecular mechanisms induced by the fluctuation of stress-associated hormones [92]. Specifically, previous studies have demonstrated that the exposure to a stressful habitat promotes the activation of sympathetic pathways via β -adrenoreceptors [97, 98]. The result is the up-regulation of neuroendocrine stress hormones (epinephrine, norepinephrine, and cortisol) that enhances vascular endothelial growth factor (VEGF)-dependent angiogenesis [99, 100]. Thus, as we aimed to investigate the effects of regular running on tumor

vasculature, considering the natural running pattern of mice and the confounding effects of higher stress levels on tumor angiogenesis, we opted for a voluntary activity model to reduce the potential bias induced by the forced exercise paradigms [101].

Concerning the outcome measures, to evaluate the impact of regular voluntary running on tumor growth we assessed tumor weight and quantified the proliferative and apoptotic levels of tumor cells through immunohistochemical assessment of Ki-67 antigen and Tdt-mediated dUTP-biotin Nick-End Labeling (TUNEL) fluorescence assay, respectively. Uncontrolled proliferation is a hallmark of cancer and the quantification of Ki-67 antigen is a widely used molecular marker because it is associated with all cell cycle phases with the exception of G0 [102], thus providing information about the proliferative capacity of cancer cells [103]. In turn, the TUNEL assay is a well-established technique that exploits the unique ability of terminal deoxynucleotidyl transferase (TdT), a DNA polymerase, to catalyze the addition of nucleotides to the free 3'-hydroxyl terminus of DNA fragments, in a template-independent manner [104]. The presence of these groups is considered a marker of apoptosis, providing a good representation of the number and location of apoptotic tumor cells [105].

Furthermore, to understand if the changes in proliferation and apoptosis of tumor cells, and the consequent reduction of tumor weight induced by regular running involved a favourable modulation of the TME, we evaluated the intratumoral MVD with a primary antibody for CD31 and quantified the lymphocytes within the tumor through an antibody for NKG2D, CD8⁺ and CD4⁺ T cells. Tumor vessels can be observed using immunohistochemical methods to identify endothelial cells (ECs), which can be stained with several antibodies, including those targeting CD31, CD34 and von Willebrand factor (vWF) [106]. Of these, CD31, also known as platelet-endothelial cell adhesion molecule (PECAM-1), is a cluster of differentiation factor that occurs mainly on the membranes of ECs, staining pre-existing and newly formed vasculature [107]. Although CD31 may co-stain inflammatory cells like macrophages or platelets, it is more specific than the other endothelial markers [108], because it is less affected during the fixation procedure, thus showing a superior staining on

paraffin sections [109]. Moreover, the vWF stains large vessels with high sensitivity, but capillaries and angiogenic microvessels with a decreased intensity [106], while CD34 is not a specific vascular marker because it is expressed by a variety of mesenchymal cells, being difficult to interpret [110]. Therefore, anti-CD31 antibodies are preferred over anti-vWF, because of a greater specificity for the vascular endothelium, and over anti-CD34, to prevent an abundant stromal background staining [111, 112].

The assessment of MVD is achieved by counting any stained ECs or cell clusters clearly detached from adjacent microvessels, tumor cells and other connective tissue elements, as an individual vessel [113], and can be performed by two distinct approaches: (1) the analysis of “hot spots”, containing the maximal vascular density and (2) the selection of random representative areas or whole tissue count [114]. Although, the first approach is more popular due to its relative simplicity, there is no agreement regarding the ideal number of “hot spots” to be quantified, which are not always representative of the entire tumor [115, 116]. Consequently, vessel counting is significantly biased by the tumor size, personal experience and preferences of the observer [117, 118], limiting the applicability of this procedure [119]. On the contrary, the second approach is more objective, but involves a cumbersome examination [120]. Additionally, other commonly used vessel size- and shape-related parameters, including major and minor axis length, compactness ($\text{perimeter}^2/\text{area}$), and lumen regularity ($\text{perimeter}^2/4\pi.\text{area}$) give information about the quality of the vasculature [121, 122]. Therefore, for involving a more rigorous assessment, we investigated the MVD by counting the number of blood vessels per microscopic field, in the entire tumor, and we also evaluated vessel’s lumen regularity to assess the quality of the tumor vasculature.

Indeed, more mature and stable intratumoral blood vessels are associated with progression-free survival [123], due to an improved O_2 delivery, a higher lymphocytic infiltration and, consequently, to a successful immunity [124, 125]. However, the hypoperfusion induced by the dysfunctional tumor vasculature and by the dense TME represents a barrier to T cell trafficking into the tumor [126]. In particular, low CD4^+ and CD8^+ T cell migration is a rate-limiting step

during the effector phase of antitumor immunity, because while CD8⁺ T cells, when activated, are crucial in mediating the control of tumor growth, CD4⁺ T cells provide help during priming, to achieve a full activation and effector function [127]. In addition, NKG2D has been shown to be important in the surveillance of tumorigenesis by activating NK and T cells [128], and by increasing the sensitivity of tumor cells to cytolysis [129]. Therefore, given that intratumoral infiltration of CD4⁺ and CD8⁺ T cells is linked to a more favourable prognosis in many cancers [130], and NKG2D is considered a hallmark of cell-mediated cytotoxicity [131], we proceeded to explore, by immunohistochemistry, the quantity of CD4⁺ and CD8⁺ T cells, and the expression of NKG2D within the tumor. The immunohistochemistry technique enables the observation of intratumoral cell's location and staining patterns [132], thus a greater range of information is provided for a timely and accurate diagnosis and treatment response's evaluation [133].

Unfortunately, despite all the benefits provided by the inoculated tumor model used, as cancer incidence and development are the result of an interplay between external and internal factors [134], the subcutaneous injection of cancer cells is not capable to entirely replicate the disease background found in humans [135]. On the contrary, chemical carcinogens can overcome this limitation by simulating the original installation and evolution of cancer development from the early initiation, representing an ideal approach to investigate the aetiology, prevention, diagnosis, and treatment of cancer in all stages [136]. Specifically, when compared with transplanted and genetically engineered tumors, carcinogen-induced models carry a high mutation burden, replicating some of the cardinal genetic features of human cancers and providing routes to understand the general mechanisms of cellular changes and tissue-specific features [82]. Therefore, to understand how cancer development impacts on musculoskeletal system, we opted to use the N-butyl-N-(4-hydroxybutyl) nitrosamine (BBN)-induced tumor model. BBN is considered one of the most appropriate carcinogens for the induction of orthotopic urinary bladder cancers, because 12 weeks of oral exposure selectively leads to the development of urothelial carcinoma (UCC) in laboratory animals, with

morphological characteristics resembling human urothelial lesions [137, 138]. Consequently, the urothelial lesions induced by BBN promote a marked inflammatory response that allows to study the repercussions in other organs, including the skeletal muscle [139, 140]. Following this notion, we intended to compare the impact of UCC, induced by 12 weeks of oral exposure to BBN, on three skeletal muscles with different phenotypic characteristics, to verify if UCC affects all skeletal muscles in a similar fashion, or if the local properties of each muscle may influence its susceptibility to wasting. Accordingly, although it was not possible to perform the identification of the different fibre types, because muscle samples were fixed and further imbedding in paraffin blocks, as the composition of each muscle is already very well described in the literature [141, 142], we selected the tibialis anterior (TA) for being a fast-glycolytic muscle, the soleus (SOL) for being a slow-oxidative postural muscle and the diaphragm as a chronically active inspiratory muscle that although providing sustained ventilation at rest, must ensure a high-power output and an increased respiratory rate during more demanding events [143, 144].

To achieve this, light microscopy represents an indispensable tool, by allowing us to gather general information about the different shapes, sizes and arrangements of the cells within the muscle tissue and, consequently, by adding detailed information to our understanding about the effects of UCC development on the musculoskeletal system [145]. However, it is important to consider the existence of some restrictions in biological imaging, namely, its resolving power, the limited resolution and the finitude of the optical field that narrows the observation of the entire preparation [146]. Nonetheless, these limitations were restrained by acquiring several consecutive images in order to exploit and analyze all the preparation under study. Therefore, after staining the muscle sections with haematoxylin and eosin (H&E), the cross-sectional area (CSA) of individual fibers was determined as the measurement of muscle plasticity, because it represents a valid and established outcome to evaluate the changes occurring in skeletal muscles [147]. Indeed, as the mass and the function of skeletal muscles are directly related to the CSA, it is a more accurate measure of the muscle's health and potential strength than muscle weight [148].

Besides, we opted to show the values of CSA with a histogram, according to the range of frequency distribution. The histogram is a useful data analysis representation that provides an easy observation and comparison of the different myofibers, according to their CSA range, to find the fibers that were more affected by UCC and, consequently, to understand if there was a selective pattern of wasting [149]. Additionally, to ascertain if the malignant process compromised the regenerative potential of skeletal muscles, the myonuclear domain (MND) and the content of collagen deposition were assessed. The MND was calculated by dividing the myofiber CSA by the number of its myonuclei to determine the cytoplasmic volume regulated by one specific myonucleus, while to determine the content of intramuscular collagen deposition, sections were stained with Picrosirius Red (SR) and the percentage of red area was quantified. Therefore, by standardizing all the previous methodological procedures as reported, although outcome assessments were not blinded, we are convinced that we successfully minimized the risk of bias involved in each study.

Studies I and IV were conducted according to the 2009 preferred reporting items to systematic reviews and meta-analyses (PRISMA) guidelines [150]. For this reason, we did not performed a public registration for the systematic reviews because under the umbrella of 2009 PRISMA guidelines, public registration, although desirable, is not mandatory [150]. In study I, the quality of the included studies was assessed using the modified Collaborative Approach to Meta-Analysis and Review of Animal Data from Experimental Studies (CAMARADES) [151], while in study IV the risk of bias were evaluated through the Systematic Review Center for Laboratory animal Experiments risk of bias (SYRCLE's RoB) tool [152]. In these studies, data analysis was performed with Review Manager 5.3 software. Mean differences were presented in experimental and control groups due to the existence of continuous data, which were grouped through a random effects model because the risk to overestimate the effect size is smaller. Additionally, we opted to use mean difference as effect measure instead of standardized mean difference (SMD) for two reasons. First, the obtained measurements from different staining vary equally, directly

and linearly, with the actual parameter under study. Second, as the SMD indicates the difference before and after the intervention in terms of standard deviations instead of actual scores, it assumes that different outcome scales are linear transformation of each other, which not always make comparisons meaningful [153]. On the other hand, in studies III, V and VI the choice to use parametric or non-parametric tests was based on the existence or not of normal data distribution, respectively.

4.2. Discussion of the results

The major findings of the studies included in the present scientific work point to the notion that: (1) regular voluntary running may be a potential strategy to attenuate tumor growth, by favourably modulating TME, and (2) there is a skeletal muscle's heterogenic response to cancer-induced wasting, where the fast-glycolytic TA, particularly the fibers with higher CSA, seem to be more prone to the atrophic signal, which involves profound functional consequences, including decreased muscle strength, power and ability to safely perform daily living activities.

Although study I demonstrated that reduced tumor vascularization induced by antiangiogenic therapy contributed to a decrease in the tumor growth rate, as the mean duration of intervention was 25 days, it hindered a complete observation of the full effects. Indeed, it was already established that the anti-angiogenic therapy with DC101, an agent used in 4 of the 6 included studies, can decrease the vascular density and attenuate tumor growth only during the first month of therapy, after which tumors re-grow with an increased vascularization [154]. Therefore, as effective long-term strategies to manage abnormal tumor vasculature and to attenuate tumor growth are still missing, we evaluated the effect of regular physical activity and we found in study III that regular running inhibited androgen-independent prostate carcinoma progression, by reducing its proliferation and increasing its apoptosis. In accordance with our results, evidence suggests that in tumor-bearing mice, the proliferation of cancer cells decreased by 60% due to regular wheel running [155]. Moreover, recent research indicates that, moderate and high-intensity physical exercise can inhibit cancer cell proliferation and activate apoptosis in mice, preventing the malignant transformation of carcinoma *in situ* [156]. Specifically, physical exercise can up-regulate apoptosis in tumors by increasing the expression of p53 protein and pro-apoptotic proteins such as Bax, Bak and caspase-3, while decreasing Bcl-2 expression [157]. Accordingly, Higgins and co-workers showed a delayed tumor growth in exercising mice and the levels of p53 were significantly higher when compared to sedentary mice,

suggesting an increased occurrence of p53-driven apoptosis with regular voluntary running [158]. Similarly, Zheng and collaborators demonstrated that voluntary running was able to suppress prostate and pancreatic tumors growth by stimulating apoptosis, as suggested by the increase in caspase-3 expression [159].

However, during tumor growth, the diminished availability of O₂ protects cancer cells against apoptosis, increasing the potential to proliferate and disseminate to other organs [160]. As such, it has been hypothesized that an improved tumor vasculature may inhibit the proliferation and dissemination of cancer cells, while allowing a more efficient cytotoxic immune function to reduce tumor cell survival [161]. The results of study IV support this hypothesis by demonstrating that physical exercise and activity improved the intratumoral vasculature in animal models, by increasing the MVD and the quality of tumor blood vessels. As such, contrary to occasional pre-clinical studies that found no impact of physical exercise on tumor vascularization [162-164], our meta-analytic approach allowed us to weight the strength and direction of all the previous research on this specific topic and, consequently, to demonstrate the beneficial effect of both short and long-term physical exercise and activity, in the form of forced treadmill or voluntary wheel running, on tumor vascularization. Accordingly, in addition to an increased tumor MVD, Betof and co-workers observed that tumors of physically active mice were smaller and presented more pericyte covered vessels than tumors of sedentary animals [165]. Similarly, Morrell and collaborators found that exercise training increased pericyte coverage and induced a higher expression of α -smooth muscle actin proteins in tumor capillaries, which are rendered more stable and mature [166]. Considering that cell-cell communication is essential for vessels to act as a synchronized unit along its longitudinal axis [167], these results suggest that regular physical exercise and activity can induce the remodelling of tumor vessels by increasing pericyte-ECs interactions, which contributes to a higher intratumoral perfusion. Corroborating this notion, Jones and associates observed a higher intratumoral perfusion in physical active mice [164], while Schadler and colleagues found

that exercise training increased the number of open vessels, also improving tumor perfusion [168].

Collectively, these findings suggest that besides the modulation of systemic mechanisms, such as the circulating levels of metabolic and sex-steroid hormones, the prevention of oxidative damage and the reduction of inflammation [169], regular physical exercise and activity can modulate the supportive TME, particularly the tumor vascular network [165, 166, 168]. Consequently, as neoplastic cells are influenced by the surrounding milieu, such crosstalk plays an essential role in the regulation of cancer cells' viability and growth [170]. Indeed, the results of study V further support this hypothesis by showing a greater MVD and more regular vessel lumens in physically active tumor-bearing mice, which were accompanied by a higher tumor infiltration of CD8⁺ T cells and a higher expression of intratumoral NKG2D receptors. Our results are in accordance with some previous pre-clinical findings showing that regular physical exercise induced a marked increase in tumor MVD [171-173]. Commonly, due to increased proliferation of ECs during tumor growth, vessels present irregular courses and elongated cross-sectional shapes that can turn the blood flow fluctuant and turbulent, resulting in intratumoral hypoperfusion and in the formation of necrotic regions [13]. However, as tumor vessels show an impaired α -adrenergic vasoconstriction, during physical exercise and activity a portion of the increased cardiac output is re-directed into the lower resistance tumor arterioles, increasing transmural pressure and re-opening previously collapsed blood vessels [174]. Consequently, by assuming more regular shaped lumens, the blood flow within tumor capillaries increases, thus improving tumor perfusion [175]. Indeed, the pioneer study of McCullough and co-workers demonstrated that during treadmill running there was a 200% increase in the number of perfused vessels that resulted in higher intratumoral blood flow [174]. Consequently, the continuous increase in laminar tumor blood flow induces shear stress on the endothelial surface [176], which promotes ECs' quiescence and alignment, turning the vessels more stable and regularly shaped [177]. Morrell and colleagues confirmed this phenomenon in tumor vessels, by showing that exercise training increased the proportion of shear stress

receptors in tumor vessels of mice, thus improving tumor vessels' maturity [166].

Moreover, as ECs play an essential role in controlling immune cells entry into healthy and pathological tissues [178], tumor endothelium can directly suppress T cell trafficking and function [179]. Indeed, most cancer patients present lack of spontaneous antitumor immunity, due to an absent or minimal intratumoral T-cell infiltration [180]. Therefore, we proceeded to explore whether the remodelling of tumor vasculature induced by regular voluntary running improved the infiltration of T cells and we found a significant increase in intratumoral CD8⁺ T cells and NKG2D receptors. Similar to our results, Zielinski and collaborators showed a greater number of intratumoral lymphocytes in tumors of exercised mice when compared to sedentary mice [181], and more recently Rundqvist and co-workers observed that treadmill running increased the quantity of CD8⁺ T cells within the tumors [182]. Albeit the immune system can rely on a variety of cells to fight cancer, preclinical and clinical evidence points to CD8⁺ T cells as key player in immune-mediated tumor rejection [183-185]. Accordingly, Camus and colleagues assessed through immunohistochemistry a large cohort of 435 cancer patients and found that a higher quantity of CD8⁺ T cells in tumors correlated with the absence of metastasis and protection against disease recurrence [186]. Not surprisingly, the intratumoral infiltration of CD8⁺ T cells became an independent positive prognostic marker in most solid cancers [187]. Furthermore, as wheel running promoted the up-regulation of NKG2D receptors in tumors, it suggests that physically active mice are likely to possess an improved immune cytolytic function when compared to the sedentary animals [188]. NKG2D receptors are considered a central player in immune surveillance by detecting abnormal transformed cells, activating NK and T cells and directly mediating a killing effect [189]. Although the seminal study of Zimmer and colleagues found that physical exercise, remarkably increased the NKG2D expression on circulating NK cells of cancer patients [190], to our knowledge this is the first observation of an up-regulation of intratumoral NKG2D receptors induced by regular running, in mice.

Finally, the results of study VI demonstrated that the development of UUC induced wasting of the fast-glycolytic TA, which was expressed by a decreased CSA, higher infiltration of fibrotic tissue and increased MND. Specifically, when compared with the smaller CSA fibers and with chronically active muscles like SOL and diaphragm, the fibers with higher CSA were more prone to the atrophic signal, thus reinforcing the notion of a heterogenic response to cancer-induced muscle wasting. This observation is in agreement with previous studies reporting that fast-glycolytic muscles seem to be more susceptible to cancer-associated wasting than slow-oxidative muscles [74, 75]. Accordingly, Julliene and collaborators observed that peritoneal carcinomatosis induced wasting of extensor digitorum longus, gastrocnemius and TA, but not of SOL, in rats [74], while Acharyya and co-workers found that although colon-26 tumors did not impact the SOL in mice, led to the installation of muscle wasting of TA [75]. Indeed, at the muscle level, the activation of the inflammatory response, a hallmark of cancer development and progression, by pro-inflammatory cytokines, acute phase proteins and tumor secreted proteolysis-inducing factor [48], promotes mitochondrial dysfunction and increases reactive oxygen species (ROS) levels, thus causing oxidative stress in cells [191].

The presence of dysfunctional mitochondria, characterized by a loss of efficiency in the electron transport chain and reduced synthesis of high-energy molecules, such as adenosine-5'-triphosphate (ATP), increases ROS production [192], thus contributing to the muscle protein hypercatabolism seen in cancer patients [193, 194]. Although ROS can act as signalling molecules during regeneration and repair in skeletal muscles [195], local sustained high ROS levels may cause a plethora of irreversible damaging effects that induce tissue injury [196, 197]. Particularly, while low levels of hydrogen peroxide (H₂O₂) are associated with positive effects on muscle physiology, by mediating the genetic expression of antioxidant enzymes like catalase, manganese superoxide dismutase, and glutathione peroxidase, thus protecting the cell from DNA damage [198], its excessive production accelerates skeletal muscle proteolysis [199]. However, it is established that muscle activity increases peroxisome proliferator-activated receptor-gamma co-activator (PGC)-1 α gene expression,

which protects mitochondrial function and content [200]. As several lines of evidence are consistent with the hypothesis that PGC-1 α is more abundant in type I than in type II myofibers [201], chronic muscle activity has been established as a potent regulator of mitophagy, possessing the potential to attenuate mitochondrial dysfunction and to facilitate ROS removal in slow oxidative skeletal muscles [202, 203]. Moreover, the skeletal muscle activity level can also impact its endocrine regulation and metabolism [204]. Hormones are key regulators of human muscle metabolism in both health and disease [205], and glucocorticoids have direct catabolic effects in skeletal muscle by increasing protein turnover and by favouring net efflux of amino acids from muscle intracellular pools [206]. Interestingly, the glucocorticoids-driven imbalance between muscle protein synthesis and breakdown is further exacerbated by muscle inactivity [207]. Accordingly, the seminal study of Goldberg and Goodman found that the catabolic effects of cortisone are more pronounced in the less active plantaris muscle, while the hormone action spares the physiologically more active SOL muscle [208]. Similarly, Ferrando and collaborators showed that prolonged inactivity sensitized skeletal muscles to the catabolic effects of cortisol [209], which up-regulated the expression of the forkhead box O (FOXO) transcription factors and stimulated the degradation of myoblast determination protein 1 (MyoD) [210]. Interestingly, the previous study of Hughes and co-workers reported that contrary to type I fibers, the satellite cells (SCs) in type II fibers contain a high MyoD:myogenin ratio [211]. Therefore, considering that MyoD plays a key role in myogenic stem cell function and differentiation, and is necessary to maintain the differentiated phenotype of fast-twitch fibres, the marked decrease in MyoD expression is consistent with the hypothesis that cancer-induced wasting may affect more severely glycolytic than chronically active oxidative muscles [212].

Accordingly, in study VI, contrary to TA, the continuous contractile activity of diaphragm for ventilation and SOL for postural control may have conferred an additional protection to oxidative stress and, consequently, to cancer-induced muscle wasting. Similar to a decreased CSA, TA of tumor-bearing mice also presented an increased collagen deposition, a common hallmark of muscle

wasting disorders. The replacement of muscle fibers by connective tissue is a known consequence of impaired muscle regeneration that is also worsen by a prolonged inflammatory state [213]. Corroborating this idea, Judge and collaborators observed that the rectus abdominis of cachectic patients with pancreatic ductal adenocarcinoma (PDAC) had higher levels of collagen deposition when compared to non-cancer subjects, which was correlated with decreased overall survival (OS) [214]. Thus, as tissue fibrosis is the end result of a cascade of events occurring from tissue injury to necrosis, the replacement of muscle fibers by connective tissue should be appreciated in order to monitor cancer progression and to predict patients' OS [214]. Additionally, an increased MND was seen in TA and in diaphragm of BBN mice, but not in SOL. The MND has been found to be different according to the oxidative capacity of the fibers, with type I fibers having smaller MND than type II fibers, which can influence the regenerative capacity of fast glycolytic skeletal muscles [215]. Indeed, although the diaphragm is a chronically active muscle and, therefore, composed mainly by type I fibers, contrary to SOL, it also expresses type II fibers, to provide the increased respiratory rate observed during physical exercise and the high-power output that occurs in sneezing and coughing [144]. Taking into account that each myonucleus controls the transcription and consequently the protein synthesis of a limited amount of cytoplasm, an increased MND means that the same myonucleus is responsible for a higher cytoplasmic volume, suggesting that the regenerative potential of TA and diaphragm muscles may have been compromised [217]. Therefore, the relevance and contribution of potential local skeletal muscle mechanisms induced by regular contractile activity seem to protect type I myofibers and, consequently, oxidative muscles from the installation of systemic atrophic stimuli induced by cancer development [51].

4.3. Clinical applicability

Although the present research work was conducted in a pre-clinical context, and for that reason appropriate caution is needed in extrapolating all the findings collected to the clinical setting, it is possible to understand the importance of physical activity, specifically, regular voluntary running, as more than a simple component of the cancer rehabilitation program.

Indeed, it is established that regular physical activity can reduce the risk of primary cancer by contributing to improve the BMI, the modulation of circulating metabolic and sex steroid hormones and the immune function [218, 219], while after cancer diagnosis, high levels of physical activity can reduce the risk of recurrence and mortality [220].

In study III we showed that voluntary running was an effective strategy to attenuate cancer growth by directly impacting tumor cells' proliferation and by inducing apoptosis. Additionally, in study V we observed that regular wheel running was able to modulate the TME, which means that it can be applied in the rehabilitation program throughout the continuum of care as a co-adjuvant anti-cancer treatment. Particularly, by remodelling the tumor vasculature and increasing the quantity of intratumoral CD8⁺ T cells and NKG2D receptors, physiotherapists can adopt regular voluntary running as a strategy to directly inhibit tumor growth and to indirectly enhance the efficacy of chemotherapy, by improving the access and delivery of anti-cancer drugs to tumor cells, but also the success of immunotherapy, by improving T cells tumor homing. Therefore, the concept of physiotherapy in the oncology setting as we know today must evolve to encompass not only the body function and quality of life, but also the tumor biology as therapeutic targets. Moreover, considering the impact of these dimensions on surgery success and prevention of postoperative complications, the intervention of the physiotherapist should be initiated at the prehabilitation phase, in order to potentiate the patient's recovery [221].

In turn, study VI provides fundamental knowledge regarding the selective impact of cancer development on fast glycolytic muscles. Therefore, as physical exercise may attenuate the adverse effects of cancer-induced muscle wasting by suppressing the inflammatory burden and enhancing insulin sensitivity,

protein synthesis, and antioxidant enzymes [222], it is of paramount importance that physiotherapists in the oncology field consider the higher susceptibility of type II myofibers to the atrophic signal when planning the rehabilitation intervention, to preserve the patients' capacity to perform high intensity daily activities and their fine motor skills.

CHAPTER V
CONCLUSIONS AND FUTURE RESEARCH DIRECTIONS

5.1. Conclusions

The results of this thesis support our two working hypothesis, thus emphasizing the following conclusions:

- 1- As our meta-analytic approach indicated, regular physical exercise and activity promoted an increased tumor vascularization in pre-clinical studies.
- 2- Regular voluntary running attenuated androgen-independent prostate carcinoma growth.
- 3- Regular voluntary running induced beneficial changes in the levels of proliferation and apoptotic biomarkers in androgen-independent prostate carcinoma.
- 4- Regular voluntary running increased tumor MVD, vessels' lumen regularity, CD8⁺ T cell density and NKG2D expression in androgen-independent prostate carcinoma.
- 5- Regular voluntary running did not impact CD4⁺ T cell density in androgen-independent prostate carcinoma.
- 6- The fast-glycolytic TA, particularly the fibers with higher CSA, were more prone to cancer induced-muscle wasting, when compared with the smaller CSA fibers and with chronically active muscles like SOL and diaphragm.

5.2. Future research directions

From the present scientific work, some implications for future research can be highlighted:

1. There is a need to expand the current knowledge on the effects of regular physical exercise and activity on TME. Future studies should aim to evaluate whether physical exercise and activity can: 1) modulate intratumoral lactate concentration, pO_2 and pH; 2) impact the dissemination of tumor cells and metastasis formation; 3) induce a favourable vascular remodelling in more desmoplastic cancer models.
2. It is important to identify additional mechanisms that could explain the attenuation of tumor growth observed such as the endocrine and metabolic responses to regular voluntary running.
3. It should be investigated if regular physical exercise and activity can protect type II myofibers and, consequently, glycolytic muscles from cancer-associated wasting.

CHAPTER VI

REFERENCES

6. References

1. Bray F, Ferlay J, Soerjomataram I et al. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin* 2018; 68: 394-424. doi:10.3322/caac.21492
2. Siegel RL, Miller KD, Fuchs HE et al. Cancer Statistics, 2021. *CA Cancer J Clin* 2021; 71: 7-33. doi:10.3322/caac.21654
3. Barrett JC. Mechanisms of multistep carcinogenesis and carcinogen risk assessment. *Environ Health Perspect* 1993; 100: 9-20. doi:10.1289/ehp.931009
4. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell* 2011; 144: 646-674. doi:10.1016/j.cell.2011.02.013
5. Fouad YA, Aanei C. Revisiting the hallmarks of cancer. *Am J Cancer Res* 2017; 7: 1016-1036
6. Anderson AR, Weaver AM, Cummings PT et al. Tumor morphology and phenotypic evolution driven by selective pressure from the microenvironment. *Cell* 2006; 127: 905-915. doi:10.1016/j.cell.2006.09.042
7. Patel A. Benign vs Malignant Tumors. *JAMA Oncol* 2020; 6: 1488. doi:10.1001/jamaoncol.2020.2592
8. Balkwill FR, Capasso M, Hagemann T. The tumor microenvironment at a glance. *J Cell Sci* 2012; 125: 5591-5596. doi:10.1242/jcs.116392
9. McAllister SS, Weinberg RA. Tumor-host interactions: a far-reaching relationship. *J Clin Oncol* 2010; 28: 4022-4028. doi:10.1200/JCO.2010.28.4257
10. Wu T, Dai Y. Tumor microenvironment and therapeutic response. *Cancer Lett* 2017; 387: 61-68. doi:10.1016/j.canlet.2016.01.043
11. Nagy JA, Chang SH, Dvorak AM et al. Why are tumour blood vessels abnormal and why is it important to know? *Br J Cancer* 2009; 100: 865-869. doi:10.1038/sj.bjc.6604929

12. Siemann DW. The unique characteristics of tumor vasculature and preclinical evidence for its selective disruption by Tumor-Vascular Disrupting Agents. *Cancer Treat Rev* 2011; 37: 63-74. doi:10.1016/j.ctrv.2010.05.001
13. Forster JC, Harriss-Phillips WM, Douglass MJ et al. A review of the development of tumor vasculature and its effects on the tumor microenvironment. *Hypoxia (Auckl)* 2017; 5: 21-32. doi:10.2147/HP.S133231
14. Vaupel P, Hockel M. Blood supply, oxygenation status and metabolic micromilieu of breast cancers: characterization and therapeutic relevance. *Int J Oncol* 2000; 17: 869-879. doi:10.3892/ijo.17.5.869
15. Labiano S, Palazon A, Melero I. Immune response regulation in the tumor microenvironment by hypoxia. *Semin Oncol* 2015; 42: 378-386. doi:10.1053/j.seminoncol.2015.02.009
16. Buckanovich RJ, Facciabene A, Kim S et al. Endothelin B receptor mediates the endothelial barrier to T cell homing to tumors and disables immune therapy. *Nat Med* 2008; 14: 28-36. doi:10.1038/nm1699
17. Bouzin C, Brouet A, De Vriese J et al. Effects of vascular endothelial growth factor on the lymphocyte-endothelium interactions: identification of caveolin-1 and nitric oxide as control points of endothelial cell anergy. *J Immunol* 2007; 178: 1505-1511. doi:10.4049/jimmunol.178.3.1505
18. Gajewski TF, Schreiber H, Fu YX. Innate and adaptive immune cells in the tumor microenvironment. *Nat Immunol* 2013; 14: 1014-1022. doi:10.1038/ni.2703
19. Boon T, Coulie PG, Van den Eynde BJ et al. Human T cell responses against melanoma. *Annu Rev Immunol* 2006; 24: 175-208. doi:10.1146/annurev.immunol.24.021605.090733
20. Anderson KG, Stromnes IM, Greenberg PD. Obstacles Posed by the Tumor Microenvironment to T cell Activity: A Case for Synergistic Therapies. *Cancer Cell* 2017; 31: 311-325. doi:10.1016/j.ccell.2017.02.008

21. Motz GT, Santoro SP, Wang LP et al. Tumor endothelium FasL establishes a selective immune barrier promoting tolerance in tumors. *Nat Med* 2014; 20: 607-615. doi:10.1038/nm.3541
22. Idos GE, Kwok J, Bonthala N et al. The Prognostic Implications of Tumor Infiltrating Lymphocytes in Colorectal Cancer: A Systematic Review and Meta-Analysis. *Sci Rep* 2020; 10: 3360. doi:10.1038/s41598-020-60255-4
23. Zhang L, Conejo-Garcia JR, Katsaros D et al. Intratumoral T cells, recurrence, and survival in epithelial ovarian cancer. *N Engl J Med* 2003; 348: 203-213. doi:10.1056/NEJMoa020177
24. Cipponi A, Wieers G, van Baren N et al. Tumor-infiltrating lymphocytes: apparently good for melanoma patients. But why? *Cancer Immunol Immunother* 2011; 60: 1153-1160. doi:10.1007/s00262-011-1026-2
25. Vasudev NS, Reynolds AR. Anti-angiogenic therapy for cancer: current progress, unresolved questions and future directions. *Angiogenesis* 2014; 17: 471-494. doi:10.1007/s10456-014-9420-y
26. Franco M, Man S, Chen L et al. Targeted anti-vascular endothelial growth factor receptor-2 therapy leads to short-term and long-term impairment of vascular function and increase in tumor hypoxia. *Cancer Res* 2006; 66: 3639-3648. doi:10.1158/0008-5472.CAN-05-3295
27. Kunkel P, Ulbricht U, Bohlen P et al. Inhibition of glioma angiogenesis and growth in vivo by systemic treatment with a monoclonal antibody against vascular endothelial growth factor receptor-2. *Cancer Res* 2001; 61: 6624-6628
28. Shaheen RM, Ahmad SA, Liu W et al. Inhibited growth of colon cancer carcinomatosis by antibodies to vascular endothelial and epidermal growth factor receptors. *Br J Cancer* 2001; 85: 584-589. doi:10.1054/bjoc.2001.1936
29. Paez-Ribes M, Allen E, Hudock J et al. Antiangiogenic therapy elicits malignant progression of tumors to increased local invasion and distant metastasis. *Cancer Cell* 2009; 15: 220-231. doi:10.1016/j.ccr.2009.01.027

30. Ramadan WS, Zaher DM, Altaie AM et al. Potential Therapeutic Strategies for Lung and Breast Cancers through Understanding the Anti-Angiogenesis Resistance Mechanisms. *Int J Mol Sci* 2020; 21. doi:10.3390/ijms21020565
31. Nakayama K, Kataoka N. Regulation of Gene Expression under Hypoxic Conditions. *Int J Mol Sci* 2019; 20. doi:10.3390/ijms20133278
32. Zhu X, Stergiopoulos K, Wu S. Risk of hypertension and renal dysfunction with an angiogenesis inhibitor sunitinib: systematic review and meta-analysis. *Acta Oncol* 2009; 48: 9-17. doi:10.1080/02841860802314720
33. Wu S, Chen JJ, Kudelka A et al. Incidence and risk of hypertension with sorafenib in patients with cancer: a systematic review and meta-analysis. *Lancet Oncol* 2008; 9: 117-123. doi:10.1016/S1470-2045(08)70003-2
34. An MM, Zou Z, Shen H et al. Incidence and risk of significantly raised blood pressure in cancer patients treated with bevacizumab: an updated meta-analysis. *Eur J Clin Pharmacol* 2010; 66: 813-821. doi:10.1007/s00228-010-0815-4
35. Sanidas E, Papadopoulos DP, Velliou M et al. The Role of Angiogenesis Inhibitors in Hypertension: Following "Ariadne's Thread". *Am J Hypertens* 2018; 31: 961-969. doi:10.1093/ajh/hpy087
36. Noman MZ, Hasmim M, Messai Y et al. Hypoxia: a key player in antitumor immune response. A Review in the Theme: Cellular Responses to Hypoxia. *Am J Physiol Cell Physiol* 2015; 309: C569-579. doi:10.1152/ajpcell.00207.2015
37. Ashcraft KA, Warner AB, Jones LW et al. Exercise as Adjunct Therapy in Cancer. *Semin Radiat Oncol* 2019; 29: 16-24. doi:10.1016/j.semradonc.2018.10.001
38. McNeely ML, Dolgoy N, Onazi M et al. The Interdisciplinary Rehabilitation Care Team and the Role of Physical Therapy in Survivor Exercise. *Clin J Oncol Nurs* 2016; 20: S8-S16. doi:10.1188/16.CJON.S2.8-16

39. Stout NL, Silver JK, Raj VS et al. Toward a National Initiative in Cancer Rehabilitation: Recommendations From a Subject Matter Expert Group. *Arch Phys Med Rehabil* 2016; 97: 2006-2015. doi:10.1016/j.apmr.2016.05.002
40. Patel AV, Friedenreich CM, Moore SC et al. American College of Sports Medicine Roundtable Report on Physical Activity, Sedentary Behavior, and Cancer Prevention and Control. *Med Sci Sports Exerc* 2019; 51: 2391-2402. doi:10.1249/MSS.0000000000002117
41. McTiernan A, Friedenreich CM, Katzmarzyk PT et al. Physical Activity in Cancer Prevention and Survival: A Systematic Review. *Med Sci Sports Exerc* 2019; 51: 1252-1261. doi:10.1249/MSS.0000000000001937
42. Hong BS, Lee KP. A systematic review of the biological mechanisms linking physical activity and breast cancer. *Phys Act Nutr* 2020; 24: 25-31. doi:10.20463/pan.2020.0018
43. Wang Q, Zhou W. Roles and molecular mechanisms of physical exercise in cancer prevention and treatment. *J Sport Health Sci* 2021; 10: 201-210. doi:10.1016/j.jshs.2020.07.008
44. Schumacher O, Galvao DA, Taaffe DR et al. Exercise modulation of tumour perfusion and hypoxia to improve radiotherapy response in prostate cancer. *Prostate Cancer Prostatic Dis* 2021; 24: 1-14. doi:10.1038/s41391-020-0245-z
45. Zhang X, Ashcraft KA, Betof Warner A et al. Can Exercise-Induced Modulation of the Tumor Physiologic Microenvironment Improve Antitumor Immunity? *Cancer Res* 2019; 79: 2447-2456. doi:10.1158/0008-5472.CAN-18-2468
46. Ruiz-Casado A, Martin-Ruiz A, Perez LM et al. Exercise and the Hallmarks of Cancer. *Trends Cancer* 2017; 3: 423-441. doi:10.1016/j.trecan.2017.04.007
47. Dvorak HF. Tumors: wounds that do not heal-redux. *Cancer Immunol Res* 2015; 3: 1-11. doi:10.1158/2326-6066.CIR-14-0209
48. Tisdale MJ. Mechanisms of cancer cachexia. *Physiol Rev* 2009; 89: 381-410. doi:10.1152/physrev.00016.2008

49. Fearon KC, Glass DJ, Guttridge DC. Cancer cachexia: mediators, signaling, and metabolic pathways. *Cell Metab* 2012; 16: 153-166. doi:10.1016/j.cmet.2012.06.011
50. Fearon K, Strasser F, Anker SD et al. Definition and classification of cancer cachexia: an international consensus. *Lancet Oncol* 2011; 12: 489-495. doi:10.1016/S1470-2045(10)70218-7
51. Hardee JP, Montalvo RN, Carson JA. Linking Cancer Cachexia-Induced Anabolic Resistance to Skeletal Muscle Oxidative Metabolism. *Oxid Med Cell Longev* 2017; 2017: 8018197. doi:10.1155/2017/8018197
52. Carson JA, Hardee JP, VanderVeen BN. The emerging role of skeletal muscle oxidative metabolism as a biological target and cellular regulator of cancer-induced muscle wasting. *Semin Cell Dev Biol* 2016; 54: 53-67. doi:10.1016/j.semcdb.2015.11.005
53. Fearon KC. Cancer cachexia: developing multimodal therapy for a multidimensional problem. *Eur J Cancer* 2008; 44: 1124-1132. doi:10.1016/j.ejca.2008.02.033
54. Sanver MF, Yagli NV, Saglam M et al. Comparison of respiratory muscle strength and endurance, maximal oxygen consumption, and fatigue in colorectal cancer survivors with healthy adults. *Support Care Cancer* 2021. doi:10.1007/s00520-020-05963-3. doi:10.1007/s00520-020-05963-3
55. Bulbul Y, Eris B, Orem A et al. Pulmonary atelectasis and survival in advanced non-small cell lung carcinoma. *Ups J Med Sci* 2010; 115: 176-180. doi:10.3109/03009731003695624
56. Pin F, Couch ME, Bonetto A. Preservation of muscle mass as a strategy to reduce the toxic effects of cancer chemotherapy on body composition. *Curr Opin Support Palliat Care* 2018; 12: 420-426. doi:10.1097/SPC.0000000000000382
57. Vaughan VC, Martin P, Lewandowski PA. Cancer cachexia: impact, mechanisms and emerging treatments. *J Cachexia Sarcopenia Muscle* 2013; 4: 95-109. doi:10.1007/s13539-012-0087-1

58. Prado CM, Lima IS, Baracos VE et al. An exploratory study of body composition as a determinant of epirubicin pharmacokinetics and toxicity. *Cancer Chemother Pharmacol* 2011; 67: 93-101. doi:10.1007/s00280-010-1288-y
59. Hopkins JJ, Sawyer MB. A review of body composition and pharmacokinetics in oncology. *Expert Rev Clin Pharmacol* 2017; 10: 947-956. doi:10.1080/17512433.2017.1347503
60. Sealy MJ, Dechaphunkul T, van der Schans CP et al. Low muscle mass is associated with early termination of chemotherapy related to toxicity in patients with head and neck cancer. *Clin Nutr* 2020; 39: 501-509. doi:10.1016/j.clnu.2019.02.029
61. Bonetto A, Penna F, Aversa Z et al. Early changes of muscle insulin-like growth factor-1 and myostatin gene expression in gastric cancer patients. *Muscle Nerve* 2013; 48: 387-392. doi:10.1002/mus.23798
62. Pedersen BK. Exercise-induced myokines and their role in chronic diseases. *Brain Behav Immun* 2011; 25: 811-816. doi:10.1016/j.bbi.2011.02.010
63. Pedersen BK, Febbraio MA. Muscle as an endocrine organ: focus on muscle-derived interleukin-6. *Physiol Rev* 2008; 88: 1379-1406. doi:10.1152/physrev.90100.2007
64. Biolo G, Cederholm T, Muscaritoli M. Muscle contractile and metabolic dysfunction is a common feature of sarcopenia of aging and chronic diseases: from sarcopenic obesity to cachexia. *Clin Nutr* 2014; 33: 737-748. doi:10.1016/j.clnu.2014.03.007
65. Hiroux C, Dalle S, Koppo K et al. Voluntary exercise does not improve muscular properties or functional capacity during C26-induced cancer cachexia in mice. *J Muscle Res Cell Motil* 2021; 42: 169-181. doi:10.1007/s10974-021-09599-6
66. Aulino P, Berardi E, Cardillo VM et al. Molecular, cellular and physiological characterization of the cancer cachexia-inducing C26 colon carcinoma in mouse. *BMC Cancer* 2010; 10: 363. doi:10.1186/1471-2407-10-363

67. Roberts BM, Frye GS, Ahn B et al. Cancer cachexia decreases specific force and accelerates fatigue in limb muscle. *Biochem Biophys Res Commun* 2013; 435: 488-492. doi:10.1016/j.bbrc.2013.05.018
68. Murphy KT, Chee A, Trieu J et al. Importance of functional and metabolic impairments in the characterization of the C-26 murine model of cancer cachexia. *Dis Model Mech* 2012; 5: 533-545. doi:10.1242/dmm.008839
69. Roberts BM, Ahn B, Smuder AJ et al. Diaphragm and ventilatory dysfunction during cancer cachexia. *FASEB J* 2013; 27: 2600-2610. doi:10.1096/fj.12-222844
70. Lim S, Brown JL, Washington TA et al. Development and progression of cancer cachexia: Perspectives from bench to bedside. *Sports Med Health Sci* 2020; 2: 177-185. doi:10.1016/j.smhs.2020.10.003
71. Tan BH, Fearon KC. Cachexia: prevalence and impact in medicine. *Curr Opin Clin Nutr Metab Care* 2008; 11: 400-407. doi:10.1097/MCO.0b013e328300ecc1
72. Christensen JF, Simonsen C, Hojman P. Exercise Training in Cancer Control and Treatment. *Compr Physiol* 2018; 9: 165-205. doi:10.1002/cphy.c180016
73. Johns N, Hatakeyama S, Stephens NA et al. Clinical classification of cancer cachexia: phenotypic correlates in human skeletal muscle. *PLoS One* 2014; 9: e83618. doi:10.1371/journal.pone.0083618
74. Julienne CM, Dumas JF, Goupille C et al. Cancer cachexia is associated with a decrease in skeletal muscle mitochondrial oxidative capacities without alteration of ATP production efficiency. *J Cachexia Sarcopenia Muscle* 2012; 3: 265-275. doi:10.1007/s13539-012-0071-9
75. Acharyya S, Ladner KJ, Nelsen LL et al. Cancer cachexia is regulated by selective targeting of skeletal muscle gene products. *J Clin Invest* 2004; 114: 370-378. doi:10.1172/JCI20174
76. Cao B, Bray F, Beltran-Sanchez H et al. Benchmarking life expectancy and cancer mortality: global comparison with cardiovascular disease 1981-2010. *BMJ* 2017; 357: j2765. doi:10.1136/bmj.j2765

77. Pucci C, Martinelli C, Ciofani G. Innovative approaches for cancer treatment: current perspectives and new challenges. *Ecancermedicalscience* 2019; 13: 961. doi:10.3332/ecancer.2019.961
78. Cekanova M, Rathore K. Animal models and therapeutic molecular targets of cancer: utility and limitations. *Drug Des Devel Ther* 2014; 8: 1911-1921. doi:10.2147/DDDT.S49584
79. Demetrius L. Of mice and men. When it comes to studying ageing and the means to slow it down, mice are not just small humans. *EMBO Rep* 2005; 6 Spec No: S39-44. doi:10.1038/sj.embor.7400422
80. Balmain A, Harris CC. Carcinogenesis in mouse and human cells: parallels and paradoxes. *Carcinogenesis* 2000; 21: 371-377. doi:10.1093/carcin/21.3.371
81. Klausner RD. Studying cancer in the mouse. *Oncogene* 1999; 18: 5249-5252. doi:10.1038/sj.onc.1203089
82. Onaciu A, Munteanu R, Munteanu VC et al. Spontaneous and Induced Animal Models for Cancer Research. *Diagnostics (Basel)* 2020; 10. doi:10.3390/diagnostics10090660
83. Lugo D, Pulido AL, Mihos CG et al. The effects of physical activity on cancer prevention, treatment and prognosis: A review of the literature. *Complement Ther Med* 2019; 44: 9-13. doi:10.1016/j.ctim.2019.03.013
84. Yang L, Morielli AR, Heer E et al. Effects of Exercise on Cancer Treatment Efficacy: A Systematic Review of Preclinical and Clinical Studies. *Cancer Res* 2021; 81: 4889-4895. doi:10.1158/0008-5472.CAN-21-1258
85. Haidich AB. Meta-analysis in medical research. *Hippokratia* 2010; 14: 29-37
86. Mikolajewicz N, Komarova SV. Meta-Analytic Methodology for Basic Research: A Practical Guide. *Front Physiol* 2019; 10: 203. doi:10.3389/fphys.2019.00203
87. Zhu Y, Hu Y, Cheng M et al. Establishment and Characterization of a Nude Mouse Model of Subcutaneously Implanted Tumors and

- Abdominal Metastasis in Gastric Cancer. *Gastroenterol Res Pract* 2017; 2017: 6856107. doi:10.1155/2017/6856107
88. Faustino-Rocha A, Oliveira PA, Pinho-Oliveira J et al. Estimation of rat mammary tumor volume using caliper and ultrasonography measurements. *Lab Anim (NY)* 2013; 42: 217-224. doi:10.1038/labani.254
 89. Schmidt KM, Geissler EK, Lang SA. Subcutaneous Murine Xenograft Models: A Critical Tool for Studying Human Tumor Growth and Angiogenesis In Vivo. *Methods Mol Biol* 2016; 1464: 129-137. doi:10.1007/978-1-4939-3999-2_12
 90. Voeks DJ, Martiniello-Wilks R, Russell PJ. Derivation of MPR and TRAMP models of prostate cancer and prostate cancer metastasis for evaluation of therapeutic strategies. *Urol Oncol* 2002; 7: 111-118. doi:10.1016/s1078-1439(01)00180-6
 91. Bower JE. Cancer-related fatigue--mechanisms, risk factors, and treatments. *Nat Rev Clin Oncol* 2014; 11: 597-609. doi:10.1038/nrclinonc.2014.127
 92. Goh J, Ladiges W. Voluntary Wheel Running in Mice. *Curr Protoc Mouse Biol* 2015; 5: 283-290. doi:10.1002/9780470942390.mo140295
 93. Ashcraft KA, Peace RM, Betof AS et al. Efficacy and Mechanisms of Aerobic Exercise on Cancer Initiation, Progression, and Metastasis: A Critical Systematic Review of In Vivo Preclinical Data. *Cancer Res* 2016; 76: 4032-4050. doi:10.1158/0008-5472.CAN-16-0887
 94. Manzanares G, Brito-da-Silva G, Gandra PG. Voluntary wheel running: patterns and physiological effects in mice. *Braz J Med Biol Res* 2018; 52: e7830. doi:10.1590/1414-431X20187830
 95. De Bono JP, Adlam D, Paterson DJ et al. Novel quantitative phenotypes of exercise training in mouse models. *Am J Physiol Regul Integr Comp Physiol* 2006; 290: R926-934. doi:10.1152/ajpregu.00694.2005
 96. Moraska A, Fleshner M. Voluntary physical activity prevents stress-induced behavioral depression and anti-KLH antibody suppression. *Am J*

- Physiol Regul Integr Comp Physiol 2001; 281: R484-489. doi:10.1152/ajpregu.2001.281.2.R484
97. Thaker PH, Han LY, Kamat AA et al. Chronic stress promotes tumor growth and angiogenesis in a mouse model of ovarian carcinoma. *Nat Med* 2006; 12: 939-944. doi:10.1038/nm1447
 98. Landen CN, Jr., Lin YG, Armaiz Pena GN et al. Neuroendocrine modulation of signal transducer and activator of transcription-3 in ovarian cancer. *Cancer Res* 2007; 67: 10389-10396. doi:10.1158/0008-5472.CAN-07-0858
 99. Garg J, Feng YX, Jansen SR et al. Catecholamines facilitate VEGF-dependent angiogenesis via beta2-adrenoceptor-induced Epac1 and PKA activation. *Oncotarget* 2017; 8: 44732-44748. doi:10.18632/oncotarget.17267
 100. Goldstein DS. Stress-induced activation of the sympathetic nervous system. *Baillieres Clin Endocrinol Metab* 1987; 1: 253-278. doi:10.1016/s0950-351x(87)80063-0
 101. Khataei T, Romig-Martin SA, Harding AMS et al. Comparison of murine behavioural and physiological responses after forced exercise by electrical shock versus manual prodding. *Exp Physiol* 2021; 106: 812-819. doi:10.1113/EP089117
 102. Gerdes J, Lemke H, Baisch H et al. Cell cycle analysis of a cell proliferation-associated human nuclear antigen defined by the monoclonal antibody Ki-67. *J Immunol* 1984; 133: 1710-1715
 103. Scholzen T, Gerdes J. The Ki-67 protein: from the known and the unknown. *J Cell Physiol* 2000; 182: 311-322. doi:10.1002/(SICI)1097-4652(200003)182:3<311::AID-JCP1>3.0.CO;2-9
 104. Mirzayans R, Murray D. Do TUNEL and Other Apoptosis Assays Detect Cell Death in Preclinical Studies? *Int J Mol Sci* 2020; 21. doi:10.3390/ijms21239090
 105. Liu S, Edgerton SM, Moore DH, 2nd et al. Measures of cell turnover (proliferation and apoptosis) and their association with survival in breast cancer. *Clin Cancer Res* 2001; 7: 1716-1723

106. Pusztaszeri MP, Seelentag W, Bosman FT. Immunohistochemical expression of endothelial markers CD31, CD34, von Willebrand factor, and Fli-1 in normal human tissues. *J Histochem Cytochem* 2006; 54: 385-395. doi:10.1369/jhc.4A6514.2005
107. Ilan N, Cheung L, Pinter E et al. Platelet-endothelial cell adhesion molecule-1 (CD31), a scaffolding molecule for selected catenin family members whose binding is mediated by different tyrosine and serine/threonine phosphorylation. *J Biol Chem* 2000; 275: 21435-21443. doi:10.1074/jbc.M001857200
108. Newman PJ. The biology of PECAM-1. *J Clin Invest* 1997; 99: 3-8. doi:10.1172/JCI119129
109. Wang D, Stockard CR, Harkins L et al. Immunohistochemistry in the evaluation of neovascularization in tumor xenografts. *Biotech Histochem* 2008; 83: 179-189. doi:10.1080/10520290802451085
110. Sidney LE, Branch MJ, Dunphy SE et al. Concise review: evidence for CD34 as a common marker for diverse progenitors. *Stem Cells* 2014; 32: 1380-1389. doi:10.1002/stem.1661
111. Lindenmayer AE, Miettinen M. Immunophenotypic features of uterine stromal cells. CD34 expression in endocervical stroma. *Virchows Arch* 1995; 426: 457-460. doi:10.1007/BF00193168
112. da Silva BB, Lopes-Costa PV, dos Santos AR et al. Comparison of three vascular endothelial markers in the evaluation of microvessel density in breast cancer. *Eur J Gynaecol Oncol* 2009; 30: 285-288
113. Weidner N, Semple JP, Welch WR et al. Tumor angiogenesis and metastasis--correlation in invasive breast carcinoma. *N Engl J Med* 1991; 324: 1-8. doi:10.1056/NEJM199101033240101
114. Tretiakova M, Antic T, Binder D et al. Microvessel density is not increased in prostate cancer: digital imaging of routine sections and tissue microarrays. *Hum Pathol* 2013; 44: 495-502. doi:10.1016/j.humpath.2012.06.009
115. de Jong JS, van Diest PJ, Baak JP. Heterogeneity and reproducibility of microvessel counts in breast cancer. *Lab Invest* 1995; 73: 922-926

116. Martin L, Green B, Renshaw C et al. Examining the technique of angiogenesis assessment in invasive breast cancer. *Br J Cancer* 1997; 76: 1046-1054. doi:10.1038/bjc.1997.506
117. Rubin MA, Buyyounouski M, Bagiella E et al. Microvessel density in prostate cancer: lack of correlation with tumor grade, pathologic stage, and clinical outcome. *Urology* 1999; 53: 542-547. doi:10.1016/s0090-4295(98)00561-5
118. Bono AV, Celato N, Cova V et al. Microvessel density in prostate carcinoma. *Prostate Cancer Prostatic Dis* 2002; 5: 123-127. doi:10.1038/sj.pcan.4500572
119. van der Laak JA, Westphal JR, Schalkwijk LJ et al. An improved procedure to quantify tumour vascularity using true colour image analysis. Comparison with the manual hot-spot procedure in a human melanoma xenograft model. *J Pathol* 1998; 184: 136-143. doi:10.1002/(SICI)1096-9896(199802)184:2<136::AID-PATH970>3.0.CO;2-9
120. Kim NT, Elie N, Plancoulaine B et al. An original approach for quantification of blood vessels on the whole tumour section. *Anal Cell Pathol* 2003; 25: 63-75. doi:10.1155/2003/473902
121. Korkolopoulou P, Viniou N, Kavantzias N et al. Clinicopathologic correlations of bone marrow angiogenesis in chronic myeloid leukemia: a morphometric study. *Leukemia* 2003; 17: 89-97. doi:10.1038/sj.leu.2402769
122. Korkolopoulou P, Gribabis DA, Kavantzias N et al. A morphometric study of bone marrow angiogenesis in hairy cell leukaemia with clinicopathological correlations. *Br J Haematol* 2003; 122: 900-910. doi:10.1046/j.1365-2141.2003.04527.x
123. Korkolopoulou P, Apostolidou E, Pavlopoulos PM et al. Prognostic evaluation of the microvascular network in myelodysplastic syndromes. *Leukemia* 2001; 15: 1369-1376. doi:10.1038/sj.leu.2402220

124. Jain RK, Martin JD, Stylianopoulos T. The role of mechanical forces in tumor growth and therapy. *Annu Rev Biomed Eng* 2014; 16: 321-346. doi:10.1146/annurev-bioeng-071813-105259
125. Lanitis E, Irving M, Coukos G. Targeting the tumor vasculature to enhance T cell activity. *Curr Opin Immunol* 2015; 33: 55-63. doi:10.1016/j.coi.2015.01.011
126. Lanitis E, Dangaj D, Irving M et al. Mechanisms regulating T-cell infiltration and activity in solid tumors. *Ann Oncol* 2017; 28: xii18-xii32. doi:10.1093/annonc/mdx238
127. Ostroumov D, Fekete-Drimusz N, Saborowski M et al. CD4 and CD8 T lymphocyte interplay in controlling tumor growth. *Cell Mol Life Sci* 2018; 75: 689-713. doi:10.1007/s00018-017-2686-7
128. Jamieson AM, Diefenbach A, McMahon CW et al. The role of the NKG2D immunoreceptor in immune cell activation and natural killing. *Immunity* 2002; 17: 19-29. doi:10.1016/s1074-7613(02)00333-3
129. Lopez-Soto A, Huergo-Zapico L, Acebes-Huerta A et al. NKG2D signaling in cancer immunosurveillance. *Int J Cancer* 2015; 136: 1741-1750. doi:10.1002/ijc.28775
130. Liu JY, Yang GF, Chen FF et al. Evaluating the prognostic significance of tumor-infiltrating lymphocytes in solid tumor: practice of a standardized method from the International Immuno-Oncology Biomarkers Working Group. *Cancer Manag Res* 2019; 11: 6815-6827. doi:10.2147/CMAR.S201538
131. Wensveen FM, Jelencic V, Polic B. NKG2D: A Master Regulator of Immune Cell Responsiveness. *Front Immunol* 2018; 9: 441. doi:10.3389/fimmu.2018.00441
132. Zaha DC. Significance of immunohistochemistry in breast cancer. *World J Clin Oncol* 2014; 5: 382-392. doi:10.5306/wjco.v5.i3.382
133. Inamura K. Update on Immunohistochemistry for the Diagnosis of Lung Cancer. *Cancers (Basel)* 2018; 10. doi:10.3390/cancers10030072

134. Wu S, Zhu W, Thompson P et al. Evaluating intrinsic and non-intrinsic cancer risk factors. *Nat Commun* 2018; 9: 3490. doi:10.1038/s41467-018-05467-z
135. Rashid OM, Nagahashi M, Ramachandran S et al. Is tail vein injection a relevant breast cancer lung metastasis model? *J Thorac Dis* 2013; 5: 385-392. doi:10.3978/j.issn.2072-1439.2013.06.17
136. Liu Y, Yin T, Feng Y et al. Mammalian models of chemically induced primary malignancies exploitable for imaging-based preclinical theragnostic research. *Quant Imaging Med Surg* 2015; 5: 708-729. doi:10.3978/j.issn.2223-4292.2015.06.01
137. Lu Y, Liu P, Wen W et al. Cross-species comparison of orthologous gene expression in human bladder cancer and carcinogen-induced rodent models. *Am J Transl Res* 2010; 3: 8-27
138. Cohen SM. Urinary bladder carcinogenesis. *Toxicol Pathol* 1998; 26: 121-127. doi:10.1177/019262339802600114
139. Oliveira MM, Teixeira JC, Vasconcelos-Nobrega C et al. Mitochondrial and liver oxidative stress alterations induced by N-butyl-N-(4-hydroxybutyl)nitrosamine: relevance for hepatotoxicity. *J Appl Toxicol* 2013; 33: 434-443. doi:10.1002/jat.1763
140. Vasconcelos-Nobrega C, Colaco A, Lopes C et al. Review: BBN as an urothelial carcinogen. *In Vivo* 2012; 26: 727-739
141. Talmadge RJ, Roy RR. Electrophoretic separation of rat skeletal muscle myosin heavy-chain isoforms. *J Appl Physiol* (1985) 1993; 75: 2337-2340
142. Caiozzo VJ, Baker MJ, Baldwin KM. Novel transitions in MHC isoforms: separate and combined effects of thyroid hormone and mechanical unloading. *J Appl Physiol* (1985) 1998; 85: 2237-2248. doi:10.1152/jappl.1998.85.6.2237
143. Crupi AN, Nunnelee JS, Taylor DJ et al. Oxidative muscles have better mitochondrial homeostasis than glycolytic muscles throughout life and maintain mitochondrial function during aging. *Aging (Albany NY)* 2018; 10: 3327-3352. doi:10.18632/aging.101643

144. Gransee HM, Mantilla CB, Sieck GC. Respiratory muscle plasticity. *Compr Physiol* 2012; 2: 1441-1462. doi:10.1002/cphy.c110050
145. Stephens DJ, Allan VJ. Light microscopy techniques for live cell imaging. *Science* 2003; 300: 82-86. doi:10.1126/science.1082160
146. Antony PM, Trefois C, Stojanovic A et al. Light microscopy applications in systems biology: opportunities and challenges. *Cell Commun Signal* 2013; 11: 24. doi:10.1186/1478-811X-11-24
147. Nygren AT, Greitz D, Kaijser L. Changes in cross-sectional area in human exercising and non-exercising skeletal muscles. *Eur J Appl Physiol* 2000; 81: 210-213. doi:10.1007/s004210050032
148. Jones EJ, Bishop PA, Woods AK et al. Cross-sectional area and muscular strength: a brief review. *Sports Med* 2008; 38: 987-994. doi:10.2165/00007256-200838120-00003
149. Nuzzo RL. Histograms: A Useful Data Analysis Visualization. *PM R* 2019; 11: 309-312. doi:10.1002/pmrj.12145
150. Moher D, Liberati A, Tetzlaff J et al. Preferred reporting items for systematic reviews and meta-analyses: the PRISMA statement. *PLoS Med* 2009; 6: e1000097. doi:10.1371/journal.pmed.1000097
151. Vesterinen HM, Sena ES, Egan KJ et al. Meta-analysis of data from animal studies: a practical guide. *J Neurosci Methods* 2014; 221: 92-102. doi:10.1016/j.jneumeth.2013.09.010
152. Hooijmans CR, Rovers MM, de Vries RB et al. SYRCLE's risk of bias tool for animal studies. *BMC Med Res Methodol* 2014; 14: 43. doi:10.1186/1471-2288-14-43
153. Cummings P. Arguments for and against standardized mean differences (effect sizes). *Arch Pediatr Adolesc Med* 2011; 165: 592-596. doi:10.1001/archpediatrics.2011.97
154. Casanovas O, Hicklin DJ, Bergers G et al. Drug resistance by evasion of antiangiogenic targeting of VEGF signaling in late-stage pancreatic islet tumors. *Cancer Cell* 2005; 8: 299-309. doi:10.1016/j.ccr.2005.09.005
155. Pedersen L, Idorn M, Olofsson GH et al. Voluntary Running Suppresses Tumor Growth through Epinephrine- and IL-6-Dependent NK Cell

- Mobilization and Redistribution. *Cell Metab* 2016; 23: 554-562. doi:10.1016/j.cmet.2016.01.011
156. Lu J, Yin X, Jiang J. Sports-induced blood sugar utilization prevents development of pancreatic ductal adenocarcinoma. *Tumour Biol* 2015; 36: 663-667. doi:10.1007/s13277-014-2684-4
 157. Irmawati A, Jasmin N, Sidarningsih. The effect of moderate exercise on the elevation of Bax/Bcl-2 ratio in oral squamous epithelial cells induced by benzopyrene. *Vet World* 2018; 11: 177-180. doi:10.14202/vetworld.2018.177-180
 158. Higgins KA, Park D, Lee GY et al. Exercise-induced lung cancer regression: mechanistic findings from a mouse model. *Cancer* 2014; 120: 3302-3310. doi:10.1002/cncr.28878
 159. Zheng X, Cui XX, Huang MT et al. Inhibitory effect of voluntary running wheel exercise on the growth of human pancreatic Panc-1 and prostate PC-3 xenograft tumors in immunodeficient mice. *Oncol Rep* 2008; 19: 1583-1588
 160. Jing X, Yang F, Shao C et al. Role of hypoxia in cancer therapy by regulating the tumor microenvironment. *Mol Cancer* 2019; 18: 157. doi:10.1186/s12943-019-1089-9
 161. Hojman P, Gehl J, Christensen JF et al. Molecular Mechanisms Linking Exercise to Cancer Prevention and Treatment. *Cell Metab* 2018; 27: 10-21. doi:10.1016/j.cmet.2017.09.015
 162. Buss LA, Ang AD, Hock B et al. Effect of post-implant exercise on tumour growth rate, perfusion and hypoxia in mice. *PLoS One* 2020; 15: e0229290. doi:10.1371/journal.pone.0229290
 163. Dufresne S, Gueritat J, Chiavassa S et al. Exercise training improves radiotherapy efficiency in a murine model of prostate cancer. *FASEB J* 2020; 34: 4984-4996. doi:10.1096/fj.201901728R
 164. Jones LW, Viglianti BL, Tashjian JA et al. Effect of aerobic exercise on tumor physiology in an animal model of human breast cancer. *J Appl Physiol* (1985) 2010; 108: 343-348. doi:10.1152/japplphysiol.00424.2009

165. Betof AS, Lascola CD, Weitzel D et al. Modulation of murine breast tumor vascularity, hypoxia and chemotherapeutic response by exercise. *J Natl Cancer Inst* 2015; 107. doi:10.1093/jnci/djv040
166. Morrell MBG, Alvarez-Florez C, Zhang A et al. Vascular modulation through exercise improves chemotherapy efficacy in Ewing sarcoma. *Pediatr Blood Cancer* 2019; 66: e27835. doi:10.1002/pbc.27835
167. Pries AR, Hopfner M, le Noble F et al. The shunt problem: control of functional shunting in normal and tumour vasculature. *Nat Rev Cancer* 2010; 10: 587-593. doi:10.1038/nrc2895
168. Schadler KL, Thomas NJ, Galie PA et al. Tumor vessel normalization after aerobic exercise enhances chemotherapeutic efficacy. *Oncotarget* 2016; 7: 65429-65440. doi:10.18632/oncotarget.11748
169. Hojman P. Exercise protects from cancer through regulation of immune function and inflammation. *Biochem Soc Trans* 2017; 45: 905-911. doi:10.1042/BST20160466
170. Stylianopoulos T, Munn LL, Jain RK. Reengineering the Physical Microenvironment of Tumors to Improve Drug Delivery and Efficacy: From Mathematical Modeling to Bench to Bedside. *Trends Cancer* 2018; 4: 292-319. doi:10.1016/j.trecan.2018.02.005
171. Florez Bedoya CA, Cardoso ACF, Parker N et al. Exercise during preoperative therapy increases tumor vascularity in pancreatic tumor patients. *Sci Rep* 2019; 9: 13966. doi:10.1038/s41598-019-49582-3
172. Faustino-Rocha AI, Silva A, Gabriel J et al. Long-term exercise training as a modulator of mammary cancer vascularization. *Biomed Pharmacother* 2016; 81: 273-280. doi:10.1016/j.biopha.2016.04.030
173. Jones LW, Antonelli J, Masko EM et al. Exercise modulation of the host-tumor interaction in an orthotopic model of murine prostate cancer. *J Appl Physiol* (1985) 2012; 113: 263-272. doi:10.1152/japplphysiol.01575.2011
174. McCullough DJ, Stabley JN, Siemann DW et al. Modulation of blood flow, hypoxia, and vascular function in orthotopic prostate tumors during exercise. *J Natl Cancer Inst* 2014; 106: dju036. doi:10.1093/jnci/dju036

175. Stylianopoulos T, Jain RK. Combining two strategies to improve perfusion and drug delivery in solid tumors. *Proc Natl Acad Sci U S A* 2013; 110: 18632-18637. doi:10.1073/pnas.1318415110
176. Traub O, Berk BC. Laminar shear stress: mechanisms by which endothelial cells transduce an atheroprotective force. *Arterioscler Thromb Vasc Biol* 1998; 18: 677-685. doi:10.1161/01.atv.18.5.677
177. Chistiakov DA, Orekhov AN, Bobryshev YV. Effects of shear stress on endothelial cells: go with the flow. *Acta Physiol (Oxf)* 2017; 219: 382-408. doi:10.1111/apha.12725
178. Vestweber D. How leukocytes cross the vascular endothelium. *Nat Rev Immunol* 2015; 15: 692-704. doi:10.1038/nri3908
179. Huang X, Bai X, Cao Y et al. Lymphoma endothelium preferentially expresses Tim-3 and facilitates the progression of lymphoma by mediating immune evasion. *J Exp Med* 2010; 207: 505-520. doi:10.1084/jem.20090397
180. Koelwyn GJ, Wennerberg E, Demaria S et al. Exercise in Regulation of Inflammation-Immune Axis Function in Cancer Initiation and Progression. *Oncology (Williston Park)* 2015; 29: 908-920, 922
181. Zielinski MR, Muenchow M, Wallig MA et al. Exercise delays allogeneic tumor growth and reduces intratumoral inflammation and vascularization. *J Appl Physiol* (1985) 2004; 96: 2249-2256. doi:10.1152/japplphysiol.01210.2003
182. Rundqvist H, Velica P, Barbieri L et al. Cytotoxic T-cells mediate exercise-induced reductions in tumor growth. *Elife* 2020; 9. doi:10.7554/eLife.59996
183. Schuler T, Blankenstein T. Cutting edge: CD8⁺ effector T cells reject tumors by direct antigen recognition but indirect action on host cells. *J Immunol* 2003; 170: 4427-4431. doi:10.4049/jimmunol.170.9.4427
184. Stringhini M, Spadafora I, Catalano M et al. Cancer therapy in mice using a pure population of CD8(+) T cell specific to the AH1 tumor rejection antigen. *Cancer Immunol Immunother* 2021; 70: 3183-3197. doi:10.1007/s00262-021-02912-9

185. Piersma SJ, Jordanova ES, van Poelgeest MI et al. High number of intraepithelial CD8+ tumor-infiltrating lymphocytes is associated with the absence of lymph node metastases in patients with large early-stage cervical cancer. *Cancer Res* 2007; 67: 354-361. doi:10.1158/0008-5472.CAN-06-3388
186. Camus M, Tosolini M, Mlecnik B et al. Coordination of intratumoral immune reaction and human colorectal cancer recurrence. *Cancer Res* 2009; 69: 2685-2693. doi:10.1158/0008-5472.CAN-08-2654
187. Huber V, Camisaschi C, Berzi A et al. Cancer acidity: An ultimate frontier of tumor immune escape and a novel target of immunomodulation. *Semin Cancer Biol* 2017; 43: 74-89. doi:10.1016/j.semcancer.2017.03.001
188. Coudert JD, Held W. The role of the NKG2D receptor for tumor immunity. *Semin Cancer Biol* 2006; 16: 333-343. doi:10.1016/j.semcancer.2006.07.008
189. Liu H, Wang S, Xin J et al. Role of NKG2D and its ligands in cancer immunotherapy. *Am J Cancer Res* 2019; 9: 2064-2078
190. Zimmer P, Bloch W, Schenk A et al. Exercise-induced Natural Killer Cell Activation is Driven by Epigenetic Modifications. *Int J Sports Med* 2015; 36: 510-515. doi:10.1055/s-0034-1398531
191. Laviano A, Meguid MM, Preziosa I et al. Oxidative stress and wasting in cancer. *Curr Opin Clin Nutr Metab Care* 2007; 10: 449-456. doi:10.1097/MCO.0b013e328122db94
192. Nicolson GL. Mitochondrial Dysfunction and Chronic Disease: Treatment With Natural Supplements. *Integr Med (Encinitas)* 2014; 13: 35-43
193. Pin F, Barreto R, Couch ME et al. Cachexia induced by cancer and chemotherapy yield distinct perturbations to energy metabolism. *J Cachexia Sarcopenia Muscle* 2019; 10: 140-154. doi:10.1002/jcsm.12360
194. Penna F, Ballaro R, Costelli P. The Redox Balance: A Target for Interventions Against Muscle Wasting in Cancer Cachexia? *Antioxid Redox Signal* 2020; 33: 542-558. doi:10.1089/ars.2020.8041

195. Tidball JG. Inflammatory processes in muscle injury and repair. *Am J Physiol Regul Integr Comp Physiol* 2005; 288: R345-353. doi:10.1152/ajpregu.00454.2004
196. Mittal M, Siddiqui MR, Tran K et al. Reactive oxygen species in inflammation and tissue injury. *Antioxid Redox Signal* 2014; 20: 1126-1167. doi:10.1089/ars.2012.5149
197. Wang J, Sun D, Huang L et al. Targeting Reactive Oxygen Species Capacity of Tumor Cells with Repurposed Drug as an Anticancer Therapy. *Oxid Med Cell Longev* 2021; 2021: 8532940. doi:10.1155/2021/8532940
198. Veal EA, Day AM, Morgan BA. Hydrogen peroxide sensing and signaling. *Mol Cell* 2007; 26: 1-14. doi:10.1016/j.molcel.2007.03.016
199. Powers SK, Ji LL, Kavazis AN et al. Reactive oxygen species: impact on skeletal muscle. *Compr Physiol* 2011; 1: 941-969. doi:10.1002/cphy.c100054
200. Wenz T, Rossi SG, Rotundo RL et al. Increased muscle PGC-1alpha expression protects from sarcopenia and metabolic disease during aging. *Proc Natl Acad Sci U S A* 2009; 106: 20405-20410. doi:10.1073/pnas.0911570106
201. Gouspillou G, Sgarioto N, Norris B et al. The relationship between muscle fiber type-specific PGC-1alpha content and mitochondrial content varies between rodent models and humans. *PLoS One* 2014; 9: e103044. doi:10.1371/journal.pone.0103044
202. Vainshtein A, Grumati P, Sandri M et al. Skeletal muscle, autophagy, and physical activity: the menage a trois of metabolic regulation in health and disease. *J Mol Med (Berl)* 2014; 92: 127-137. doi:10.1007/s00109-013-1096-z
203. Yan Z, Lira VA, Greene NP. Exercise training-induced regulation of mitochondrial quality. *Exerc Sport Sci Rev* 2012; 40: 159-164. doi:10.1097/JES.0b013e3182575599

204. Ball D. Metabolic and endocrine response to exercise: sympathoadrenal integration with skeletal muscle. *J Endocrinol* 2015; 224: R79-95. doi:10.1530/JOE-14-0408
205. Martin AI, Priego T, Lopez-Calderon A. Hormones and Muscle Atrophy. *Adv Exp Med Biol* 2018; 1088: 207-233. doi:10.1007/978-981-13-1435-3_9
206. Kuo T, Harris CA, Wang JC. Metabolic functions of glucocorticoid receptor in skeletal muscle. *Mol Cell Endocrinol* 2013; 380: 79-88. doi:10.1016/j.mce.2013.03.003
207. Paddon-Jones D, Sheffield-Moore M, Urban RJ et al. The catabolic effects of prolonged inactivity and acute hypercortisolemia are offset by dietary supplementation. *J Clin Endocrinol Metab* 2005; 90: 1453-1459. doi:10.1210/jc.2004-1702
208. Goldberg AL, Goodman HM. Relationship between cortisone and muscle work in determining muscle size. *J Physiol* 1969; 200: 667-675. doi:10.1113/jphysiol.1969.sp008715
209. Ferrando AA, Stuart CA, Sheffield-Moore M et al. Inactivity amplifies the catabolic response of skeletal muscle to cortisol. *J Clin Endocrinol Metab* 1999; 84: 3515-3521. doi:10.1210/jcem.84.10.6046
210. Sun L, Trausch-Azar JS, Muglia LJ et al. Glucocorticoids differentially regulate degradation of MyoD and Id1 by N-terminal ubiquitination to promote muscle protein catabolism. *Proc Natl Acad Sci U S A* 2008; 105: 3339-3344. doi:10.1073/pnas.0800165105
211. Hughes SM, Taylor JM, Tapscott SJ et al. Selective accumulation of MyoD and myogenin mRNAs in fast and slow adult skeletal muscle is controlled by innervation and hormones. *Development* 1993; 118: 1137-1147
212. Costelli P, Muscaritoli M, Bossola M et al. Skeletal muscle wasting in tumor-bearing rats is associated with MyoD down-regulation. *Int J Oncol* 2005; 26: 1663-1668. doi:10.3892/ijo.26.6.1663

213. Mann CJ, Perdiguero E, Kharraz Y et al. Aberrant repair and fibrosis development in skeletal muscle. *Skelet Muscle* 2011; 1: 21. doi:10.1186/2044-5040-1-21
214. Judge SM, Nosacka RL, Delitto D et al. Skeletal Muscle Fibrosis in Pancreatic Cancer Patients with Respect to Survival. *JNCI Cancer Spectr* 2018; 2: pky043. doi:10.1093/jncics/pky043
215. Liu JX, Hoglund AS, Karlsson P et al. Myonuclear domain size and myosin isoform expression in muscle fibres from mammals representing a 100,000-fold difference in body size. *Exp Physiol* 2009; 94: 117-129. doi:10.1113/expphysiol.2008.043877
216. van Wessel T, de Haan A, van der Laarse WJ et al. The muscle fiber type-fiber size paradox: hypertrophy or oxidative metabolism? *Eur J Appl Physiol* 2010; 110: 665-694. doi:10.1007/s00421-010-1545-0
217. Wang Y, Pessin JE. Mechanisms for fiber-type specificity of skeletal muscle atrophy. *Curr Opin Clin Nutr Metab Care* 2013; 16: 243-250. doi:10.1097/MCO.0b013e328360272d
218. Schmitz KH, Holtzman J, Courneya KS et al. Controlled physical activity trials in cancer survivors: a systematic review and meta-analysis. *Cancer Epidemiol Biomarkers Prev* 2005; 14: 1588-1595. doi:10.1158/1055-9965.EPI-04-0703
219. Speck RM, Courneya KS, Masse LC et al. An update of controlled physical activity trials in cancer survivors: a systematic review and meta-analysis. *J Cancer Surviv* 2010; 4: 87-100. doi:10.1007/s11764-009-0110-5
220. Brown JC, Gilmore LA. Physical Activity Reduces the Risk of Recurrence and Mortality in Cancer Patients. *Exerc Sport Sci Rev* 2020; 48: 67-73. doi:10.1249/JES.0000000000000214
221. Kendall F, Oliveira J, Peleteiro B et al. Inspiratory muscle training is effective to reduce postoperative pulmonary complications and length of hospital stay: a systematic review and meta-analysis. *Disabil Rehabil* 2018; 40: 864-882. doi:10.1080/09638288.2016.1277396

222. Gould DW, Lahart I, Carmichael AR et al. Cancer cachexia prevention via physical exercise: molecular mechanisms. *J Cachexia Sarcopenia Muscle* 2013; 4: 111-124. doi:10.1007/s13539-012-0096-0

Appendices

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The effects of vascularization on tumor development: A systematic review and meta-analysis of pre-clinical studies

Author: Mário Esteves, Mariana P. Monteiro, José Alberto Duarte

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