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Role of Physical Exercise and Metformin in *in vitro* models of tumor progression control

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Organization of the thesis

After the Introduction, the **First Chapter** will give a complete view of the possible mechanisms involved in exercise-induced control of triple-negative breast cancer, reviewing recent data about exercise modulation of mTOR pathway and the possible effects resulted from different exercise protocols.

In the **Second Chapter**, the optimization procedure of a three-dimensional growth assay to assess the effects of an intense session of exercise on triple-negative breast cancer clonogenic potential will be described.

The **Third Chapter** will focus on the central project of the present thesis, in which the evaluation of systemic responses to structured high-intensity endurance cycling sessions of exercise and to a high-intensity interval training period will be considered. The modulation of the proliferative and tumorigenic capacities of triple-negative breast cancer and prostate cancer cells stimulated with the exercise-conditioned human sera will be evaluated. Moreover, the possible relations between the *in vitro* results and the biological responses measured during the exercise will be assessed.

In the **Fourth Chapter**, a possible pharmacological approach to prevent cancer initiation will be taken into account, with the analysis of metformin effects in preventing cell tumorigenesis.

Finally, after the Conclusions, the **Appendix** will focus on a work related to a different research line, in comparison to that analyzed in the central Ph.D. project. In this last part, the possible chemopreventive activity of an *in vitro* culture derived from an apple will be considered in two colon cancer cell lines and in a pre-neoplastic cell line, in terms of antiproliferative and antitumorigenic activity, cell cycle blockade and molecular mechanisms involved.

CONTENT

Original Papers

The present thesis is based on the following original research articles

I. Agostini D*, Natalucci D*, Baldelli G*, De Santi M, Donati Zeppa S, Vallorani L, Annibalini G, Lucertini F, Federici A, Izzo R, Stocchi V and Barbieri E. **New Insights into the Role of Exercise in Inhibiting mTOR Signaling in Triple-Negative Breast Cancer**. Oxidative Medicine and Cellular Longevity, 2018 Sep 30; 2018:5896786. doi: 10.1155/2018/5896786.
**Authors contributed equally to this work.*

II. De Santi M, Baldelli G, Lucertini F, Natalucci V, Brandi G and Barbieri E. **A dataset on the effect of exercise-conditioned human sera in three-dimensional breast cancer cell culture**. Data in Brief. Published Online 2019 Oct 21; 27:104704. doi: 10.1016/j.dib.2019.104704. eCollection 2019 Dec.

III. Baldelli G. et al. **Effects of human sera conditioned by high-intensity endurance cycling sessions and a high-intensity interval training period on cancer cell proliferation and tumorigenesis**
In preparation

IV. De Santi M, Baldelli G, Diotallevi A, Galluzzi L, Schiavano GF, Brandi G. **Metformin prevents cell tumorigenesis through autophagy-related cell death**. Scientific Reports. 2019 Jan 11; 9(1):66. doi: 10.1038/s41598-018-37247-6.

V. **Appendix**. Baldelli G, De Santi M, Fraternale D, Brandi G, Fanelli M, Schiavano GF. **Chemopreventive Potential of Apple Pulp Callus Against Colorectal Cancer Cell Proliferation and Tumorigenesis**. Journal of Medicinal Food. 2019 Jun;22(6):614-622. doi: 10.1089/jmf.2018.0188.

INTRODUCTION

1.1 Cancer incidence and mortality

1.1.1 Worldwide Data

Noncommunicable diseases (NCDs) are responsible for most of the worldwide deaths and cancer is one of the most commonly diagnosed pathologies and one of the most common leading death diseases [1].

The estimation of total cancer cases worldwide and of the cancer incidence and mortality rates in 2018 have been described by the GLOBOCAN project [2], which showed the occurrence of 18.1 million new cancer cases and 9.6 million cancer deaths in 2018 worldwide; the 20% risk of getting cancer before 75 years old and a 10% risk of dying from it have been detected.

In men, prostate cancer was the most commonly diagnosed cancer type in 12 regions of the world, followed by lung cancer. The lung cancer was also the most frequent cause of death from cancer, followed by prostate and liver cancers (Figure 1).

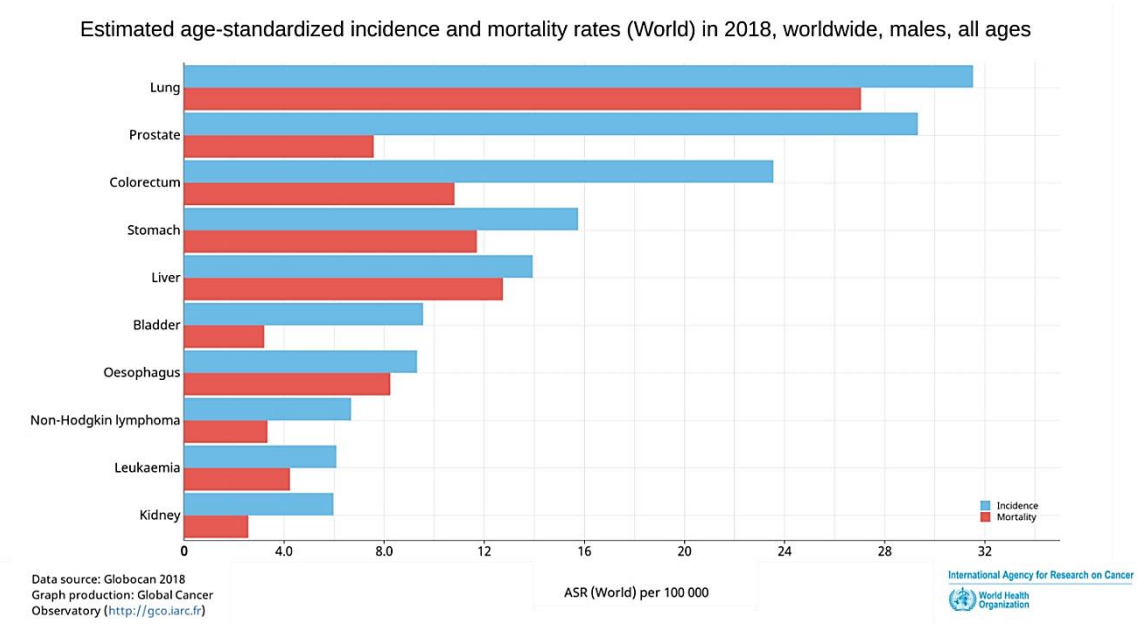

Figure 1: Estimated age-standardized incidence and mortality rate (Word) in 2018, in males, all ages. Slightly modified from Ferlay et al. [3].

In women, breast cancer was the most frequently diagnosed cancer and the most frequent cause of death from cancer, followed by lung cancer (Figure 2).

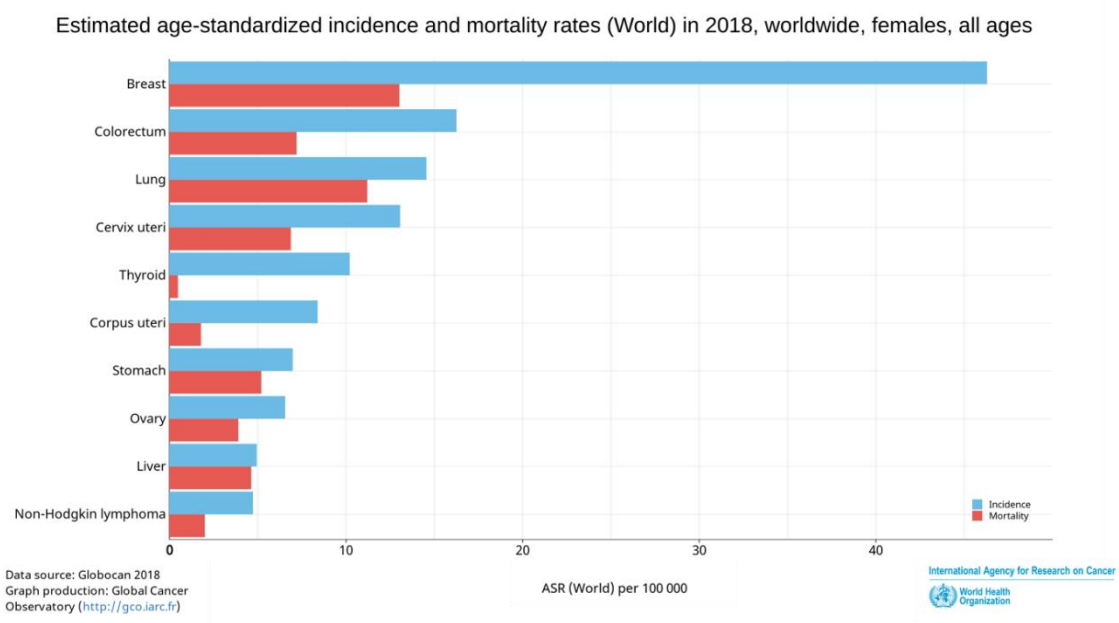

Figure 2: Estimated age-standardized incidence and mortality rate (Word) in 2018, in females, all ages. Slightly modified from Ferlay et al. [3].

About 25% of the new cancer cases (4,229,662 cases) and 20% of the cancer-related deaths (1,943,478 deaths) occurred in the European regions, even if they account for only 9% of the global population.

1.1.2 Cancer Incidence and Mortality: Italian Data

According to AIOM ("Associazione Italiana di Oncologia Medica", Italian Association of Medical Oncology), about 371,000 new cancer cases are diagnosed each year in Italy; about 53% of cancer cases are diagnosed in men (196,000 cases) and 47% in women (175,000 cases) [4].

The most frequent tumor sites assessed in Italy considering the entire population are breast (14%), colon-rectum (13%), lung (11%), prostate (10%) and bladder (8%). Excluding skin cancers, the most frequently diagnosed cancers in men are prostate (19%), lung (15%) and colorectal (14%), and in women breast (30%), colorectal (12%) and lung (12%) cancers.

In Italy 175,741 cancer-related deaths were estimated in 2018 (98,513 in men and 77,228 in women) [3]. Based on these data, on average, one out of three 3 men and one out of 6 women are likely to die from cancer every year, in Italy.

Last available data about cancer survival rate indicated that women have a 5-year survival rate of 63% and men of 54% and that the survival rate resulted better in young cancer patients than in older ones (in men: 79% in population between 15 and

44 years old and 44% in population older than 75; in women: 86% and 42%, in population between 15 and 44 years old and in those older than 75, respectively) [4].
AIOM estimated 3.5 million people as cancer-diagnosed patients in Italy in 2019, which corresponds to 6% of the entire Italian population.
Being the cancer pathology the second cause of deaths worldwide, and being the cancer incidence increasing every year, the study on prevention strategies, on the determination of possible subtypes and targeted therapies is essential, nowadays.

1.2 Breast cancer

1.2.1 Epidemiology

Breast cancer (BC) is the most common invasive tumor diagnosed worldwide in women; epidemiological data about cancer showed that 2.1 million women have been newly diagnosed BC worldwide, in 2018, and that about 627,000 women died for BC [5]. The BC incidence is increasing by about 3% every year [6]. The survival rate varies worldwide, according to the prevention strategies applied and the exposition to risk factors: the highest survival percentage has been showed in high-income countries, in which BC is often diagnosed in the early stages, thanks to the availability of prevention strategies and screening programs. On the other hand, the delayed diagnosis of BCs and the absence of effective treatments lead to a higher mortality rate in low- and middle-income countries, even if the BC incidence is lower than in high-income ones [7].

1.2.2 Breast cancer classification

According to the diagnosed subtype, the prognosis, therapies and survival rates for BCs are different. The tumor size and tumor grade at the diagnosis, the lymphovascular invasion of tumor cells and the analysis of the status of axillary lymph nodes are some of the factors which determine the prognosis of BC. Furthermore, the analysis of gene expression profile and the presence or absence of hormonal receptors in the tumor cell surface are other analyses to be considered to understand BC biology [8]. Different molecular subtypes of BC have been identified in the last 15 years, which have been associated with different treatment responses and outcomes. Basing on receptors expressed by cancer cells, BCs have been classified as Estrogen Receptor, Progesterone Receptor and Human Epidermal Receptor 2 (HER2) positive or negative BCs or Triple-Negative BCs.

The estrogen receptor (ER) is a nuclear hormone receptor that acts as a transcription regulator factor and which is one of the prognostic biomarkers for BC. The isoform alpha of ER (ERα) is the measured one during the immunohistochemical examination of tumor and patients whose tumor expresses ER (ER-positive tumor, ER+) are about 80% of the entire BC patients population; ER+ BC patients usually have a better prognosis than ER-negative ones, having a great response to endocrine therapies which targets ER (such as aromatase inhibitors or tamoxifen) [9]; moreover, this type of BC has a lower recurrence rate and cancer-related mortality risk than other BC subtypes [10]. Another receptor analyzed during the classification of BC is the progesterone receptor (PR). PR-positive (PR+) BC patients account for about 55-65% of BCs; in most cases, PR and ER expressions correlate and in PR+ BCs the outcome is usually well responsive to endocrine therapy and often leads to a greater outcome than ER- and PR-negative BC [9].

The human epidermal receptor 2 (HER2) has been found in 13-15% of BCs and it is linked to the HER2 pathway activation, which induces tumor cell proliferation and survival and metastatic process [11]. In HER2-positive (HER2+) BCs, the standard therapy is composed of chemotherapy associated with anti-HER2 blockade strategies. Importantly, HER2+ BCs show a high rate of radioresistance; for this reason, radiotherapy is not usually prescribed in BC patients which expresses high levels of HER2. HER2+ BC is associated with the highest death rate, followed by the triple-negative BC subtype [11].

The last BC subtype includes 15% of BC cases and is triple-negative breast cancer (TNBC). Epidemiological studies showed that TNBC patients usually have a higher waist to hip ratio, early menarche, short periods of breastfeeding, higher body mass index than other BC subtypes patients and that it is the most commonly diagnosed in pre-menopausal women [12]. TNBCs do not express ER, PR, and HER2 and, for this reason, hormonal treatment and anti-HER2 therapies are not effective for TNBC subtype. After the biopsy and immunohistochemical identification, TNBCs are treated with surgery, radiotherapy, and chemotherapy, alone or in combinations [13].

The evaluation of new therapies for TNBC is an evolving field and new approaches are emerging, such as phosphatidylinositol-3-kinase/Akt/mammalian target of rapamycin (PI3K-Akt-mTOR) signaling inhibitors [14].

TNBC is very aggressive and it shows a poor prognosis, high risk of lung and brain metastases and a high rate of recurrence; the highest recurrence peak for TNBC

occurs in the first three-five years after the diagnosis, with local or distant recurrence, and decreases to minimal levels after 8 years from the diagnosis [15].

Concluding, the immunohistochemical classification of receptors expressed by BC cells is essential and it is often performed before the surgery, enabling a rapid therapeutic plan establishment.

1.3 Prostate cancer

1.3.1 Epidemiology

Prostate cancer is the second most frequently diagnosed cancer type in men worldwide. In 2018, 1,276,106 new cases have been diagnosed and 358,989 prostate cancer-caused deaths have been counted; prostate cancer cases represent 7.1% of all cancer cases in men, and the number of prostate cancer-related deaths represent 3.8% of all deaths caused by cancer in men worldwide [5].

The incidence and the mortality rates correlate with age, which is a non-modifiable risk factor for prostate cancer, and the average age for the diagnosis is 66 years. Prostate cancer incidence rates are highly variable worldwide, and it is mainly due to genetic predisposition and preventive screening strategies [16].

Even if the incidence of prostate cancer is high in men worldwide, the 5-year survival rates are around 98% in the USA and 83% in Europe [17]; this is mainly because prostate cancer is often diagnosed at the beginning, in an early stage and effective therapeutic approaches are available.

About 20% of prostate cancer cases are diagnosed in patients which report a family history, not only because of the genetic predisposition acquired but also because of the same exposure to environmental risk factors and unhealthy lifestyles, such as a diet rich in fat and red meat [18] and physical inactivity [19].

1.4 Physical Exercise and cancer prevention

Epidemiological studies analyzed the correlation between physical exercise and the risk of different types of cancer and suggested a beneficial effect of physical exercise in primary prevention, decreasing the risk of different cancer types, like breast, colon and prostate cancer [20-23].

As reported by a review published in 2015, about 90% of cancer cases are also correlated to environmental risk factors and lifestyle-related risk factors, of which physical inactivity is part [24].

The World Health Organization (WHO) affirmed that more active individuals have a lower risk of death related to high blood pressure, stroke, metabolic syndrome, colon and breast cancer and other non-communicable diseases (NCDs); starting from this state, WHO strongly suggests at least 2.5 hours per week of moderate-intensity physical activity (PA), 1.25 hours per week of vigorous-intensity PA, or a combination, to reduce risks of NCDs [25].

In last decades, growing evidence showed an inverse association between PA level and cancer risk, especially considering breast cancer; moreover, a linear association between high PA intensity and reduced BC risk has been found, with a better beneficial effect of PA in case of vigorous PA than moderate one [26-29].

Furthermore, several articles highlighted the beneficial effects of post-diagnosis physical exercise in decreasing cancer recurrence risk and cancer-related mortality, considering different cancer types. Analyzing studies about breast, colorectal and prostate cancer patients, different works reviewed a 35-37% reduction in risk of cancer-caused mortality, comparing the patients most active versus the less active ones [30, 31].

The investigation about the possible correlation between different PA level (expressed in MET-hours/week) and reduced BC-related mortality risk in cancer patients have shown that high level of PA (higher than 10 MET-hours/week) is associated with 25% reduction in BC-related mortality risk, in comparison to the patients who do not perform that level of PA [32]. Moreover, Kenfield and colleagues showed that men more physically active have higher survival rates than men less active; prostate cancer patients who practice more than 3 hours per week of vigorous PA had a 61% lower risk of prostate cancer-caused death in comparison to patients who performed less PA [33].

Different systemic responses have been hypothesized for PA induced reduction in the risk of cancer and in cancer recurrence and mortality, including the effects of PA in reducing adiposity, sex hormones level increasing, inflammation, myokines production, and immune cell increased mobilization [21, 34].

A growing body of studies explored the correlation between PA and cancer progression, both *in vivo* or *in vitro* models, considering different types of cancers, like breast, prostate and lung cancer [35-40]; *in vivo* studies agreed on the reduction of tumor volume in exercised mice, in comparison to control group, with a reported

60% reduction in both tumor incidence and growth in five different tumor models [35]. Moreover, to better investigate the effects of PA on tumor growth inhibition, several groups evaluated the effects of exercise-conditioned sera on cancer cell proliferation, *in vitro*; also in that case, all the studies showed a great beneficial effects of sera obtained post PA, in decreasing the proliferative capacity of tumor cell, in comparison to sera taken at rest, resulting in about 10-15% of proliferative capacity decreasing [37-39].

Studies about the possible molecular mechanisms involved in the highlighted beneficial effects of PA on cancer prevention have been performed by several groups, resulting in the suggestion of different pathways that could be modulated by exercise.

One of the essential mechanisms for organ formation, and which is induced at high levels in tumor formation, is the Hippo signaling pathway, through which several pro-proliferation genes are transcribed [41]. In this pathway, if the protein YAP is de-phosphorylated, it translocates into the nucleus and acts as a transcriptional coactivator; on the other hand, if it is phosphorylated, it remains in the cytosol and it is degraded [42]. Recent evidence showed that exercise and exercise-conditioned human sera led to phosphorylation of YAP in BC cells, in an epinephrine-dependent manner, resulting in tumor formation blockade and cancer cell viability inhibition [43].

Another mechanism considered by several preclinical studies is the Akt/mTOR pathway, which has been found as deregulated in tumors, during PA sessions [44]. It is a signaling pathway involved in protein synthesis and endurance PA has been shown to deactivate it, in cancer murine models, being able to reduce cellular protein amount and cell proliferation.

The releasing of myokines by muscles during PA is another mechanism reviewed by Hojman and colleagues [45], involved in PA-induced decreased viability of cancer cells, *in vitro*. Currently, 600 potential myokines have been estimated and the best characterized one is the Interleukin-6 (IL-6) [46]. Preclinical studies demonstrated that some myokines inhibit BC cell viability, *in vitro*, and the tumorigenic process of colon cancer cells, in mice models [47]; furthermore, Pedersen et al. [35] showed that epinephrine and IL-6 are essential in BC responses to PA, because of the epinephrine-dependent mobilization of Natural Killer (NK) cells and the IL-6-dependent redistribution in tumor tissues. High levels of NK cells and cytotoxic T

cells in tumors have been associated with better prognosis [48], suggesting that PA could lead to a control of the tumor growth also inducing this specific mechanism.

1.5 Metformin and cancer

Metformin is a biguanide with a hypoglycemic effect, used as first-line treatment of Type 2 Diabetes Mellitus (T2DM), being approved by the Food and Drug Administration in 1957 [49]. Several studies showed a lower risk of cancer incidence [50-53], and cancer-related mortality [54, 55] in T2DM patients who were treated with metformin, showing a positive correlation with the dosage of metformin administered and leading to a great interest about the possible anticancer effects of metformin.

Even if the data about the possible antitumorigenic potential of metformin are a lot, the epidemiological literature relates only to diabetic individuals and the effects of metformin in non-diabetic subjects must be considered and demonstrated.

Moreover, the *in* vitro and *in vivo* experiments reported did not fully elucidate the possible mechanisms through which metformin could exert those beneficial effects on cancer control.

Some possible cellular and molecular mechanisms have been hypothesized, which comprise the induction of cell cycle arrest and apoptosis and the inhibition of the unfolded protein response, which is considered a mechanism able to protect cancer cells from stress conditions, as hypoxia or glucose deprivation [56]. Another possible mechanism by which metformin exerts its antitumor effects is the IGF1 receptor pathway, by which metformin could reduce insulin and IGF-1 levels in the blood and inactivate the downstream pathway, normally involved in cell proliferation [57]. Moreover, metformin is e well-known autophagic process inducer and autophagy has been suggested as a possible mechanism that could have a role in cancer prevention, eliminating damaged proteins and organelles and avoiding the accumulation of mutations and genetic instability [58]. All these mechanisms could explain the hypothesized chemopreventive properties of metformin, but further investigations in non-diabetic patients are needed.

AIMS OF THE THESIS

The aim of this thesis was to provide new evidence about the beneficial effects of exercise on cancer prevention and progression, with particular attention on triple-negative breast cancer and prostate cancer.

After reviewing the effects of exercise in mTOR pathway inhibition in Triple-Negative Breast Cancer (TNBC), the first goal of this work was to optimize the three-dimensional growth technique (soft agar assay) to assess the effects of an intense bout of physical exercise in modulating the clonogenic capacity of TNBC cells MDA-MB-231. The soft agar assay mimics the formation of the tumor in an anchorage-independent manner and is usually performed to assess the tumorigenic process of cancer cells in vitro, but it has never been used before to assess the exercise-conditioned human sera effects on cancer cell colonies growth.

After the optimization of the technique with a first pilot study, a second project has started, in collaboration with the Unit of Exercise and Health Sciences of the Department of Biomolecular Sciences of the University of Urbino "Carlo Bo", Italy, in which the primary outcome was to evaluate the systemic responses to structured high-intensity endurance cycling (HIEC) tests in decreasing cancer cell proliferative ability and tumorigenic capacity, considering TNBC cells and prostate cancer cells. Moreover, the results obtained by exercise-conditioned human sera taken after the HIEC tests were compared to those obtained from a structured high-intensity interval training (HIIT) period, to understand if the beneficial effects of physical exercise can be due to a HIEC test or to HIIT period, too.

Particular attention will be given to the possible mechanisms involved in the physical exercise beneficial effects in cancer prevention and control, and to the possible relation between the results obtained *in vitro* and the systemic factors usually measured during physical exercise to understand the amount of exercise performed and the individual response to it.

In the last part of the present study, a possible pharmacological approach to prevent cancer will be taken into account, with the analysis of metformin in preventing cell tumorigenesis.

Starting from the idea that autophagy could be one of the mechanisms involved in the physical exercise positive effects on cancer prevention and that metformin is an autophagic process inducer, a possible synergism between physical exercise and metformin would be considered in TNBC and prostate cancer prevention and modulation.

CHAPTER 1

Review Article

New Insights into the Role of Exercise in Inhibiting mTOR Signaling in Triple-Negative Breast Cancer

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Abstract

Triple-negative breast cancer (TNBC) does not express estrogen receptor, progesterone receptor, and human epidermal growth factor receptor 2 and is characterized by its aggressive nature, lack of targets for targeted therapies, and an early peak of recurrence. Due to these specific characteristics, chemotherapy does not usually yield substantial improvements and new target therapies and alternative strategies are needed. The beneficial responses of TNBC survivors to regular exercise, including a reduction in the rate of tumor growth, are becoming increasingly apparent. Physiological adaptations to exercise occur in skeletal muscle but have an impact on the entire body through systemic control of energy homeostasis and metabolism, which in turn influence the TNBC tumor microenvironment. Gaining insights into the causal mechanisms of the therapeutic cancer control properties of regular exercise is important to improve the prescription and implementation of exercise and training in TNBC survivors. Here, we provide new evidence of the effects of exercise on TNBC prevention, control, and outcomes, based on the inhibition of the phosphatidylinositol-3-kinase (PI3K)/protein kinase B (PKB also known as Akt)/mammalian target of rapamycin (mTOR) (PI3K-Akt-mTOR) signaling. These findings have wide-ranging clinical implications for cancer treatment, including recurrence and case management.

1. Introduction

Breast cancer (BC) is one of the most common carcinomas and one of the main causes of cancer-related death worldwide [1]. Among the various subtypes, triple-negative BC (TNBC) accounts for approximately 20% of BC cases. The absence of estrogen and progesterone receptors and human epidermal receptor 2 (HER2) in malignant cells reduces treatment options and increases the risk of recurrence and death, especially in the first 3–5 years of follow-up after surgery [2]. Thus, TNBC exhibits a more aggressive clinical course than non-TNBC. Most TNBC cases are diagnosed in women under the age of 60, and in 20% of diagnosed cases, there is a mutation of the germinal BC (BRCA) gene [3–7]. In patients with metastatic TNBC, there are currently no available targeted therapies and chemotherapy is the only possible treatment option. In addition to the biological-molecular aspects associated with prognosis and BC development, a growing body of evidence highlights the impact of lifestyle on disease-related outcomes. Unhealthy lifestyles with low levels

of physical activity (PA) result in overweight and obesity, which appear to have a negative impact on BC [8], increasing the risk of recurrence and death in all subtypes, including TNBC [9]. Conversely, proper diet, weight loss, and increased PA lead to more favorable outcomes in the short and long term [10, 11]. The mechanisms underlying the effects of exercise on breast carcinogenesis are not clear, but experimental evidence suggests that PA induces phosphatidylinositol-3-kinase (PI3K)/protein kinase B (PKB also known as Akt)/mammalian target of rapamycin (mTOR) (PI3K-Akt-mTOR) signaling inhibition and slows TNBC tumor cell growth [12–14]. Physiological adaptations to exercise occur primarily in skeletal muscle, but the effects of exercise and training also impact other tissues through systemic control of energy homeostasis and metabolism, thus influencing the TNBC tumor microenvironment and mTOR inhibition [15]. Given the scope of this review, we summarise recent discoveries related to the underlying biology of exercise-induced modulation of the mTOR pathway in TNBC, examining the benefits induced by different exercise and training protocols. We also consider how exercise affects the level of microRNAs (miRNAs) linked to the mTOR pathway involved in TNBC initiation and progression [16, 17], and how nutrients can influence mTOR signaling. Finally, we discuss how exercise induces beneficial adaptations and why it should be prescribed as a coadjuvant "medicine," which has the potential to improve TNBC outcomes.

2. mTOR Signaling

2.1. mTOR Pathway and mTOR Activation in BC. mTOR is a serine-threonine kinase that interacts with several proteins to form two distinct complexes, mTORC1 and mTORC2, which show different sensitivities to rapamycin [18]. mTORC1 is acutely sensitive to rapamycin and responds to growth factors, stress, amino acids, and energy, promoting protein translation and synthesis, cell growth, mass, division, and survival. mTORC1 comprises mTOR, the regulatory associated protein of mTOR (Raptor), the G-protein βsubunit-like protein (GβL), also known as mLST8, DEP domain-containing mTOR-interacting protein (Deptor), proline-rich Akt substrate of 40 kDa (PRAS40), and Tti1/ Tel2 complex. mTORC2 is insensitive to acute rapamycin treatment and contains mTOR, the rapamycin-insensitive companion of mTOR (Rictor), the mammalian stress-activated map kinase-interacting protein 1 (mSIN1), GβL, Deptor, protein observed with Rictor-1/2 (Protor

1/2), and Tti1/Tel2. Raptor and PRAS40 are unique to mTORC1, while Rictor, mSIN1, and Protor 1/2 are unique to mTORC2 [18]. The various components of mTORC1, which is the most widely studied complex, have several regulatory effects: Raptor, Tti1, and Tel2 are positive regulators, whereas PRAS40 and Deptor are negative regulators [19]. Several factors regulating mTORC1 activation converge in the tubular sclerosis complex (TSC), consisting of hamartin (TSC1), tuberin (TSC2), and TBC1 domain family member 7 (TBC1D7) [20]; the complex works via the Ras homolog enriched in brain (Rheb) GTPase, negatively regulating mTORC1 [21]. An upstream regulator of TSC is the PI3K/Akt pathway activated by growth factors such as insulin-like growth factor 1 (IGF-1) and insulin. PI3K phosphorylates phosphatidylinositol (3,4)-bis-phosphate (PIP2) lipid to phosphatidylinositol (3,4,5)-trisphosphate (PIP3), which recruits phosphoinositide-dependent kinase-1 (PDK1) and Akt. Akt phosphorylates TSC2 and PRAS40 inactivating them and inducing, in turn, mTORC1 activation [22]. TSC2 can also be phosphorylated and inactivated by the activated Ras/extracellular signal-regulated kinase (ERK)/mitogen-activated protein kinase (MAPK) signaling pathway [19]. Another critical regulator of mTORC1 is the adenosine monophosphate-activated protein kinase (AMPK), which is activated when the cellular energy level is low. AMP linking to AMPK allows its phosphorylation (while ATP availability prevents it) triggering repression of energy-consuming processes, also inhibiting mTOR, and enhancing energy-producing processes. AMPK phosphorylates TSC2 in different sites than Akt, activating rather than inactivating TSC2, and phosphorylates Raptor, thus achieving mTORC1 repression [23]. mTORC1 activation requires sufficient amino acid levels, though it is not clear how these levels are sensed. Amino acid regulation requires the formation of a Rag GTPase complex, which binds Raptor, in order to translocate mTORC1 to the lysosome allowing its association with Rheb, and thus its activation [24]. The activation of mTORC1 leads to several downstream effects, including protein synthesis promotion. Raptor binds to the eukaryotic translation initiation factor 4E- (eIF4E-) binding protein 1 (4E-BP1) and the ribosomal protein S6 kinase beta-1 (S6K1), recruiting them to the mTORC1 complex and allowing their phosphorylation [25, 26]. Hyperphosphorylation of 4E-BP1 by mTOR prevents the association of 4E-BP1 and eIF4E, allowing eIF4E to bind eIF4G to begin translation. Phosphorylation of S6Ks, including several S6K1 isoforms and S6K2, by mTOR promotes their activation and thus the phosphorylation of their targets involved in

mRNA translation. S6K1 is also involved in negative feedback on mTORC1 and mTORC2 [27]. The mTORC1 complex and AMPK also regulate the autophagic process, a cellular mechanism through which cells eliminate damaged components associated with a wide range of diseases, including cancer. After glucose deprivation, AMPK associates with, and directly phosphorylates, the serine/threonine Unc-51-like autophagy activating kinase (ULK1), an upstream component of the autophagy mechanism. By contrast, when nutrients are plentiful, mTORC1 phosphorylates ULK1, preventing its association with and activation by AMPK, inhibiting autophagy [28]. Aberrant activation of the PI3K/Akt/mTOR pathway is often found in human cancers and promotes cell proliferation [29]. Activation has been shown in the lung, head, and neck and breast, gynecologic, colorectal, and prostate cancers and glioblastoma multiforme [30] and also in B-lineage acute lymphoblastic leukemia [31]. PI3Ks are pivotal molecules in this pathway and possess eight isoforms grouped into class I, class II, and class III. Class I PI3Ks (PI3Kα, β, γ, and δ), stimulated by Tyr kinases, G protein-coupled receptors, and Ras, are currently the focus of research in drug development. Mutation of the PIK3CA gene, which encodes the catalytic subunit α (p110α), one of the class I PI3K isoforms, is found in several cancers [32]. The signaling and biological roles of class II and III PI3Ks are not clear, and they have not been implicated in oncogenesis [32]. In TNBC, the activation of the PI3K/Akt/mTOR pathway is induced by an overexpression of upstream regulators (i.e., growth hormone receptors), mutations of the PIK3CA gene, and by decreased activity of the phosphatase and tensin homolog (PTEN) and of the proline-rich inositol polyphosphatase, which are downregulators of PI3K [33–35]. By contrast, activation of downstream effectors of PI3K (e.g., Akt and mTOR) and activation of downstream effectors of parallel pathways (MAPK and Ras) are rare events in TNBC [36]. Furthermore, other oncogenic pathways (i.e., FGFR, cMET, and RAF) regulated by P53 inactivation converge to activate the PI3K pathway [37]. Due to the frequent activation of the PI3K/Akt/mTOR pathway in human cancers, more than 50 inhibiting drugs are in development, and several clinical trials are ongoing [38]. The first established therapeutic anticancer agents targeting this pathway are everolimus and temsirolimus, which abrogate mTOR signaling, and have been approved by the U.S. Food and Drug Administration. Based on the results obtained with everolimus in pancreatic neuroendocrine tumors [39], and temsirolimus for advanced renal cell cancer [40], these agents are now approved for

the treatment of these diseases. Therapies targeting other pathway members have been described. Monotherapy using pan-class I PI3K, which inhibits all class I PI3K isoforms, has effects at dose-limiting toxicity, leading to prolonged disease stabilization in some patients with advanced solid tumors (especially the lung) during phase I clinical trials [41]. Isoform-specific PI3K inhibitors have also been tested and have shown antitumor activity in tumors such as p110δ- (isoform δ-) driven hematologic malignancies [42] or PIK3CA-mutant HR-positive BC [43]. Akt inhibitors and mTORC1/2 inhibitors aimed to suppress not only mTORC1, but also the feedback activation of Akt by mTORC2 [44], are currently being investigated in clinical studies [45]. The use of PI3K/Akt/mTOR pathway inhibitors is often associated with MAPK inhibitors, growth factor receptor inhibitors, and endocrine therapy. Furthermore, they might sensitize tumors to chemotherapy synergistically inducing apoptosis, as shown in sarcomas [46]. These promising strategies are now under investigation for the treatment of several tumors, including nonsmall cell lung cancer [47], colorectal cancer [48], nonmedullary thyroid carcinoma [49], and B-lineage acute lymphoblastic leukemia [31]. Although these strategies have been shown to be effective, there is great variability in the duration and quality of their benefits and the long-term side effects for patients. Thus, the identification of protein and/or genetic biomarkers to recognize subjects that will benefit the most from these therapeutic strategies is essential [50]. In TNBC, the development of PI3K/Akt/mTOR-targeted therapies, taking into account the inhibitors of this pathway alone or in combination with other strategies, will provide new tools to control disease progression and improve outcomes [51]. In a recent phase 2 clinical trial, the efficacy of ipatasertib (an Akt inhibitor) in association with paclitaxel (an antineoplastic agent used in TNBC treatment) was shown [52].

2.2. MicroRNAs and mTOR Signaling in BC. Several studies highlight the role of circulating microRNAs (miRNAs), in different tumors, including BC and the TNBC subtype [16]. In particular, recent evidence has shown that miR10a is downregulated in triple-negative BC cells [53]. Furthermore, overexpression of miR-10a decreases the proliferation and migration of TNBC cell lines via PI3K/Akt/mTOR signaling and through the mitochondrial apoptotic pathway [53]. Recently, Phua et al. [54] demonstrated that miR-184 is also downregulated in TNBC patients and that miR-184 overexpression in TNBC cells leads to a reduced expression of mTOR. The

decreased cancer cell proliferation, due to mTOR reduction, has been confirmed *in vivo*: mice injected with mir-184-transfected MDA-MB-231 cells showed a delayed primary tumor formation and reduced metastatic burden. Emerging evidence points to epigenetic silencing by hypermethylation as a possible mechanism through which these tumor suppressor/growth inhibitor miRNAs are downregulated in TNBC [55]. In metastatic breast tumors, miR-184 has been found to be hypermethylated compared to the methylation status of miR-184 in normal breast tissue, suggesting a selective pressure in silencing this miRNA during the metastatic process [54]. The upregulation of miR-21 was detected in TNBC tissues and in MDA-MB-468 cells by Fang et al. [56]. Inhibition of this miRNA resulted in decreased proliferation, viability, and invasiveness of TNBC cells and enhanced apoptosis. Experiments to identify miR-21 targets have shown that PTEN is downregulated, suggesting an activation of mTOR and the oncogenic properties of miR-21 in TNBC, with increased proliferation and invasion by TNBC cells. Another miRNA that has been found to be upregulated in TNBC tissues in comparison to non-TNBC or adjacent tissues is miR-146a. Indeed, it has been reported to be significantly related to tumor size and histological stage: patients with elevated miR-146a expression have lower survival rates and worse prognoses than low-expression individuals [57]. In addition, miR-146a has been shown to bind the 3′-UTR region of BRCA1, inhibiting its expression; the BRCA1 protein is absent or present at very low levels in about one third of sporadic BCs [58]. Evidence suggests that the downregulation of BRCA1 expression leads to Akt/mTOR oncogenic pathway activation [59]. Hence, strategies that could modify the deregulated status of these miRNAs in TNBC could have a pivotal role in inhibiting the Akt/mTOR pathway and could affect TNBC initiation and progression. It is not yet known how these miRNAs might be modulated by exercise and whether they can be associated positively or negatively with TNBC progression, for which there are no reliable prognostic factors.

2.3. Autophagy and mTOR Signaling. Autophagy is the cellular mechanism responsible for the degradation of cytoplasmic components. It is through this mechanism that cells maintain cellular homeostasis by eliminating damaged proteins and organelles and by providing substrates for energy generation and biosynthesis under stress conditions. The mTOR complex is a major negative regulator of autophagy. It suppresses autophagy in response to nutrients, growth factors, and

hormone availability, promoting protein synthesis, cell division, and metabolism. The mTOR signaling pathway is frequently activated in tumor cells, resulting in the activation of its growth-promoting functions and the inhibition of autophagy [60]. In cancer, the cytoprotective role of autophagy could prevent tumorigenic transformation by inhibiting chronic tissue damage. By contrast, once cancer occurs, cancerous cells could utilize autophagy to enhance fitness and survive in the hostile tumor microenvironment, providing energy via substrate degradation. Autophagy could, therefore, be tumor suppressive (for example, via elimination of damaged cellular components), as well as tumor-promoting in established cancers [61]. In addition, autophagy has recently been shown to play a role in necroptosis, and, together with apoptosis, autophagy also regulates other death pathways, including immunogenic cell death, entosis, and pyroptosis [62]. It has been demonstrated that suppression of autophagy in epidermal growth factor receptor- (EGFR-) driven nonsmall cell lung adenocarcinoma xenografts promotes cell proliferation, tumor growth, and dedifferentiation, as well as resistance to EGFR tyrosine kinase inhibitor therapy [63]. Moreover, autophagy suppresses early oncogenesis in lung adenocarcinoma through effects on regulatory T cells [64], and autophagy genes are often required for the cytotoxic effects of chemotherapy [65]. In view of the complex- and context-dependent role of autophagy in cancer progression and response to therapy, it could be hypothesized that the inhibition of the mTOR pathway and the consequent induction of autophagy may be useful in certain cancers through autophagy-dependent antitumor immunity, autophagy-dependent cytotoxic effects, or other tumor suppressor effects [66]. In addition to its effects on skeletal muscle, exercise has also been found to induce autophagy in the liver, pancreas, adipose tissue, and cerebral cortex in transgenic mouse models [67, 68]. Whether exercise-induced stress activates autophagy in healthy cells (or cells primed for malignant transformation), or cancer cells themselves, and whether such effects inhibit or potentiate tumorigenesis, is not known and needs further investigation [15].

3. Evidence of mTOR Modulation by Exercise in TNBC

3.1. mTOR and Exercise. PA reduces mortality for all diseases, including tumors [69], reducing the incidence of primary development and ameliorating the prognosis [15]. Hence, it should be prescribed like a medication indicating the correct typology, dose, and timing, i.e., the type, intensity, duration, and frequency of exercise as

described in Exercise Prescription in BC Survivors. Physiological adaptations to exercise occur not only in skeletal muscle but also systemically in other metabolically active tissues involved in the exercise response (such as the bone, heart, adipose, endothelium tissue, and brain) profoundly altering the systemic milieu, in turn influencing the tumor microenvironment and cancer hallmarks [15]. In order to understand the effect of PA on mTOR and BC, muscular, systemic, and microenvironment effects should be considered.

3.1.1. Aerobic Exercise and Muscular Effects. In skeletal muscle, aerobic exercise activates several adaptive pathways, including protein kinases, transcription, and coregulatory factors that, by gene expression modification, increase mitochondrial biogenesis and stimulate metabolic reprogramming [70]. Exercise induces a depletion of nutrients, energetic substrates, and nicotinamide adenine dinucleotide (NAD)H that elevate the ratios of AMP:ATP and NAD+ : NADH, directly activating AMPK and other metabolic sensors, including NAD-dependent protein deacetylase sirtuin 1 (SIRT1) and kinases, such as ERK1/ 2, p38 MAPK, and Jun N-terminal kinase (JNK) [71]. These energy sensors trigger the transcriptional regulator peroxisome proliferator-activated receptor-γ coactivator 1α (PGC1α), which regulates the expression of mitochondrial biogenesis, increases the expression of mitochondrial transcription factor A (TFAM), which, once transferred to the mitochondria, controls transcription of mitochondrial DNA [71]. Moreover, aerobic exercise, through PGC1α phosphorylation, influences other transcription factors, including peroxisome proliferator-activated receptor-γ (PPARγ), an important regulator of fatty acid oxidation and estrogen-related receptor-α (ERRα) and ERRγ, which directly regulate mitochondrial energy metabolism by oxidative phosphorylation, fatty acid oxidation, and the tricarboxylic acid (TCA) cycle [72, 73]. In this regard, the reactive oxygen species (ROS) and reactive nitrogen species produced by exercise also directly or indirectly regulate contraction-induced mitochondrial biogenesis [74] and skeletal muscle metabolic reprogramming via AMPK and PGC-1α [75]. AMPKmediated cell survival requires inhibition of mTOR. Therefore, AMPK and mTOR play antagonistic roles in cells and inhibition of mTOR is essential for AMPK-mediated metabolic homeostasis [76].

3.1.2. Resistance Exercise and Muscular Effects. In skeletal muscle, resistance exercise causes an increase in muscle size and strength via mTOR activation. In canonical growth factor signaling, mTOR is activated by PI3K/Akt, through IGF-1 and insulin signaling, but a considerable body of evidence suggests that mTORC1 is also likely activated by a growth factor-independent movement of proteins to and from the lysosome, via resistance exercise-induced phosphorylation of TSC2 [77]. Cellular trafficking of mTOR and its association with positive regulators that occur in human skeletal muscle leading to protein synthesis after resistance exercise, in fed condition, were recently confirmed by Song and colleagues [78].

3.1.3. Systemic and Microenvironment Effects of Exercise. Exercise stimulates the release of molecular signals such as muscle derived regulatory RNAs, metabolites, and myokines with autocrine, paracrine effect on energetic substrate oxidation, hypertrophy, angiogenesis, inflammation, and regulation of the extracellular matrix. To better evaluate the systemic response to PA, a distinction must be drawn between long term (training) and acute exercise. Training induces a reduction of the basal concentration of circulatory sex hormones and lowers adiposity, both recognized risk factors [79], while acute exercise causes a sharp increase in circulating hormones, cytokines, and immune cells [80–82]. Both the systemic adaptations to training and the strong response to acute exercise support plausible mechanisms that inhibit carcinogenesis by suppressing the activation of mTOR signaling network. Hence, exercise may improve BC outcomes [14] (Figure 1). Moreover, both long-term training and a single bout of exercise control energy availability and induce a hormetic response that accounts for the physiological cellular stress adaptation [83, 84].

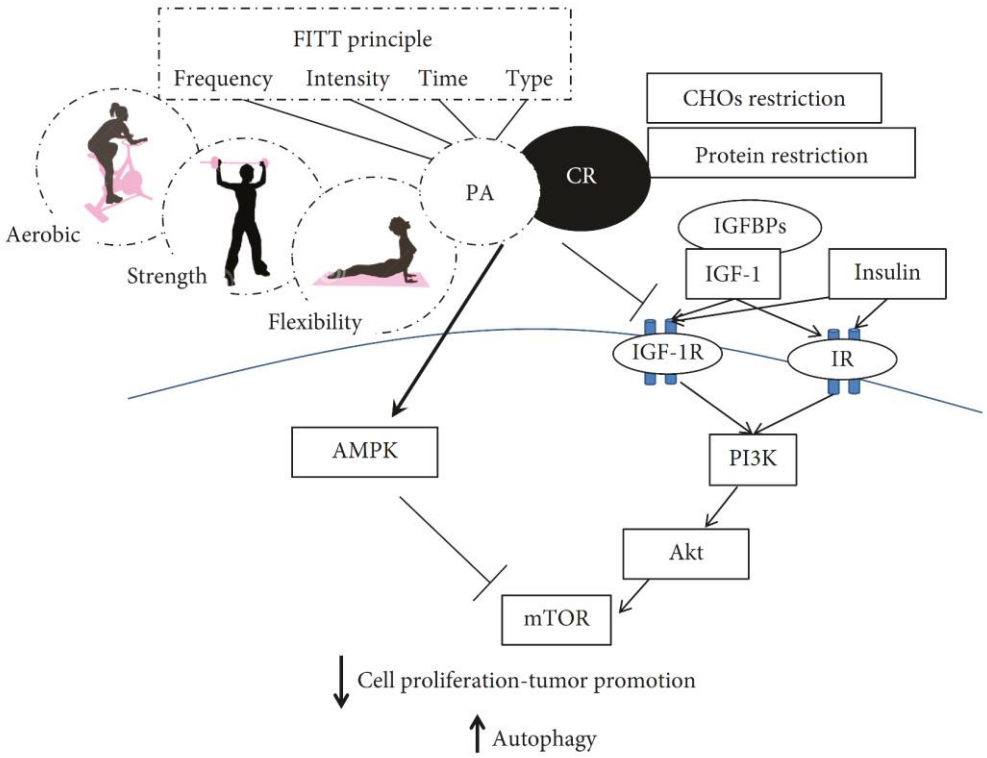

Figure 1: In this figure, we consider potential mechanisms regulated by physical activity and caloric restriction in inhibiting the mTOR pathway. Both refer to energy availability inhibiting carcinogenesis by suppressing the activation of the mTOR signaling network in this subtype of mammary carcinoma. The mTOR inhibition is mediated through the effects of vigorous PA or long-term exercise on systemic responses such as concentrations of the circulating growth factors and hormones (i.e., IGF-1 and insulin) that regulate the mTOR network. The network is controlled through the PI3K/Akt signaling pathway, the glycemia and glutamine levels, inducing apoptosis and reversing malignancy-associated metabolic programming. Moreover, the control of energy availability by both exercise and CR induces a mitohormetic response that accounts for a physiological cellular stress adaptation through AMPK activation inducing mTOR inhibition. In this context, exercise should be considered in terms of its four components: frequency, intensity, time, and type; however, dose-dependent effects of each component on cancer protection via mTOR inhibition have not yet been clarified. Most data indicate that vigorous PA, either long-term or in adulthood, may reduce a woman's risk of mammarian cancer, especially TNBC relapse. The inhibition of the mTOR complex and its cell growth-promoting functions leads to a reduction of cell proliferation, control of cancer progression, and consequent autophagy induction probably involved in tumorigenesis prevention. Thus, we hypothesized that exercise-induced inhibition of the mTOR pathway may be useful in the control of cancer progression, including TNBC. PA: physical activity; CR: caloric restriction; CHOs: carbohydrates; mTOR: mammalian target of rapamycin; IGF-1: insulin-like growth factor 1; IGF-1R: insulin-like growth factor receptor 1; IR: insulin receptor; IGFBPs: insulin-like growth factor binding proteins; PI3K: phosphatidylinositol-3-kinase; AMPK: adenosine monophosphate-activated protein kinase; TNBC: triple-negative breast cancer. FITT-VP principle, which reflects the frequency (F), intensity (I), time (T), and type (T) of exercise, and its volume (V) and progression (P) over time, in an individualized exercise training program.

Hormesis is a process whereby exposure to a low dose of potential stress favours adaptive changes in the cell that enables it to better tolerate subsequent stress [85, 86]. This type of stress is often related to reactive oxygen species (ROS) originating from the mitochondrial respiratory chain [87]. The accumulation of transient low doses of ROS through exercise influences signaling from the mitochondrial compartment to the cell [88]. Remarkably, this coordinated response to mild mitochondrial stress appears to induce mitochondrial metabolism, increase stress resistance, stimulate various long-lasting cytoprotective pathways, and favour the establishment of an oxidant-resistant phenotype, hence preventing oxidative damage and chronic diseases. Accordingly, low levels of ROS elicit positive effects on physiological cellular and systemic responses and ultimately increase lifespan [83, 88–93]. The hormetic nature of the exercise, which produces low levels of ROS, emerges as a key feature for cancer control. Indeed, in the tumor microenvironment, the activation of exercise-induced hormesis of the AMPK-p38- PGC1-α axis supports oxidative metabolism maintaining the cellular ATP pool and conserving cellular energy and viability during the metabolic stress condition: AMPK regulates metabolism and energy homeostasis [94, 95]. Exercise-induced mitochondrial biogenesis improves mitochondrial function in addition to the upregulation of antioxidant defenses that function as back regulators of intracellular ROS levels and leads to improved redox homeostasis [96, 97] as well as significantly improved insulin sensitivity. By contrast, high levels of ROS cause functional oxidative damage to proteins, lipids, nucleic acids, and cell components, induce a significant increase in intracellular Ca2+, and promote signaling cascades for apoptosis or autophagy via NFκB or forkhead box subgroup O (FoxO) pathways. High ROS levels are therefore reputed to act as etiological, or at least exacerbating factors in chronic/aging-related diseases. The typical hormetic response modulated by exercise involves kinases, deacetylases, and transcription factors; many of which have also been shown to be involved in the carcinogenic process [86]. The most studied are sirtuins (SIRT), which are histone deacetylases, and the FoxO family of transcription factors. The pathways in which NF-kappaB and the Nrf-2/ARE are components are also involved in hormetic responses and implicated in carcinogenesis and are modulated by exercise [86]. FoxO transcription factors play a critical role in cell cycle control and cellular stress responses. FoxOs are known to be regulated by the insulin signaling pathway; however, recently, the research group of Burnet

demonstrated that AMPK phosphorylates 6 specific residues on FoxO and opposes the phosphorylation of other FoxO sites by Akt [98]. Phosphorylation of FoxO by AMPK affects the conformation of the protein in such a way that sirtuin-mediated deacetylation is also modified [99]. The dependence of sirtuins on nicotinamide adenine dinucleotide (NAD(+)) links their activity to cellular metabolic status. Emerging evidence indicates that the deacetylation of FoxO by SIRT1 favours the expression of cell survival/stress resistance and the downregulation of proapoptotic genes [85, 100, 101]. Sirtuins, therefore protect against cancer development as they regulate the cellular stress responses and ensure that damaged DNA is not propagated and that mutations do not accumulate [99]. However, how FoxO activation is influenced by exercise remains unclear. In addition, cytokines such as those that we and others have found to be regulated by exercise and training [14, 102–104] have been reported to have direct and indirect effects on cellular stress responses modulated by acetylation/deacetylation reactions, and these effects can be further modified by cortical steroids, which exercise dramatically induces [105]. Similarly, various chemical mimetics of PA and caloric restriction (CR) such as AICAR, PPARδ agonist, resveratrol, and metformin can trigger a beneficial response by activation of key regulators of stress tolerance at the level of transcription, post-transcriptional modifications, and regulation of energy metabolism [92, 106]. Cross talk between major CR hormesis-induced pathways, especially AMPK/PPAR and antioxidant systems, IGF-1, and homeostatic energy balance, reveals the correlation between CR and exercise mimetics [107]. Likewise, depending on the exercise, the level/persistence could induce an adaptive response that might turn the same process from “physiologic” into “pathologic,” as in the case of inflammation. Careful titration of ROS levels within specific tumor microenvironments may lie at the crossroads between the prevention, protection, and/or initiation and progression of the disease, in particular, as regards the induction of mitochondrial functionality, cellular homeostasis, and more generally, cellular metabolic health. Considering the type of exercise, both aerobic and resistance training increase glucose uptake in skeletal muscle via insulin-independent mechanisms, with a subsequent decrease in circulating levels of insulin, IGF-1, and glucose [108]. In a model of mammary carcinogenesis, PA caused a delay in carcinogenesis with concomitant activation of AMPK and a reduction in Akt and mTOR activation and reduction in insulin and IGF-1 in circulation [12]. Reduction of insulin levels is an important aspect given

that hyperinsulinemia and insulin resistance are commonly observed in obesity with adipokine alterations, conditions associated with increased risk of BC and poor prognosis [8]. Insulin resistance is a condition in which the target tissues of insulin such as skeletal muscle, adipose tissue, and liver show a reduction in their response to physiological concentrations of the insulin hormone. As a consequence, the pancreatic β-cells produce more of the hormone to compensate for the defective response of target tissues, thus leading to hyperinsulinemia. BC cells express high levels of the insulin receptor (IR), and increased circulating insulin is associated with BC recurrence and death [109]. In contrast, PA has a fundamental role in reducing muscle insulin resistance and normalizing circulating insulin levels. Regular exercise in both healthy and oncological conditions ameliorates glycemic control including glycated hemoglobin (HbA1c) and insulin sensitivity in a "dose"-dependent manner according to duration and intensity [110, 111]. Skeletal muscle in virtue of its mass and high rate of insulin and exercise-stimulated glucose transport represents the most important tissue in glucose uptake. Exercise *per sè* increases the trafficking of glucose transporter 4 (GLUT4) to the plasma membrane through insulin-independent mechanisms [112]. Under normal physiological conditions, in skeletal muscle, insulin actions are mediated by the IR-catalyzed phosphorylation of the IR substrates 1 and 2 (IRS1 and IRS2). The tyrosine-phosphorylated IRS proteins then interact with and activate PI3K, a critical player in insulin signaling, particularly with regard to glucose homeostasis. Activation of PI3K generates PIP3 that induces membrane translocation of the serine/threonine kinase Akt. PIP3 activation of PDK1 and the Rictor/mTOR complex 2 leads to phosphorylation and subsequent activation of Akt [113]. Akt phosphorylates TBC1D4 (also known as Akt substrate of 160 kDa, AS160) and TBC1D1 promoting the translocation of GLUT4 vesicles from intracellular compartments to the membrane for glucose uptake [114]. Although recent findings help to better understand the effect of exercise on glycemic control, the specific exercise-induced signaling mechanisms leading to the acute and long-term adaptations favouring enhanced glycemic control are less clear [112, 115]. Endurance and, to a lesser extent, resistance exercise represents significant metabolic stress, activating AMPK and thus inhibiting mTOR also in nonmuscular tissue such as liver, fat, and tumor tissues. In order to better evaluate the impact of exercise on mTOR in the BC microenvironment, not only AMPK but also other circulating factors, should be considered. IGF-1, as well as insulin, activates the MAPK pathway

and the PI3K pathway, which are both involved in cancer development and progression. The importance of IGF-1 axis in the development and progression of BC has been clearly shown [116]. The overexpression of IGF-1R in BC has been reported and related to poorer survival rates [117]. The IGF signaling system is composed of IGF-1 and IGF-2, insulin-like growth factor binding proteins (IGFBPs), a family of binding proteins regulating IGF half-lives and available in circulation and extracellular fluids, IGF receptors, and insulin receptors. Furthermore, we recently evaluated the complexity of the IGF-1 gene [118] and the biological activity of IGF-1 isoforms in BC cell lines [119] showing that the IGF-1 isoforms induced cell proliferation via IGF1R phosphorylation. Some studies have reported conflicting results regarding the regulation of IGF-1. Such studies report an increase, no difference or a decrease in circulating IGF-1 levels associated with PA [120–123]. These results are not surprising because the IGF-1 levels are influenced by several clinical factors such as gender, age, body mass index (BMI), sex steroid concentrations, nutrition, stress, level of PA, and intervening illness. Thus, exercise prescription should take into consideration most of these variables. Another process through which exercise might regulate tumor metabolism is the autophagic machinery [15], as described in Autophagy and mTOR Signaling. It is clear that exercise can ameliorate the BC microenvironment and can be very important in reducing BC risk and tumor burden when canonical radiochemotherapy or chemical mTOR inhibitors are not working, as in TNBC. Exercise workouts for these subjects will be explained in Exercise Prescription in BC Survivors. Ex vivo experimental data, using TNBC cell lines stimulated with sera collected before and after a single aerobic exercise bout (pre- or post-exercise serum/a), are described in Experimental Evidence of mTOR Inhibition.

3.2. Experimental Evidence of mTOR Inhibition. As regards the mechanisms involved in the exercise-induced reduction of TNBC risk and tumorigenesis, few data are available. Ex vivo experiments, working with TNBC cells stimulated with sera collected before and after a single aerobic exercise bout (pre- or post-exercise serum/a), are a good starting point to understand how exercise could affect the progression and recrudescence of TNBC. The research group of Dethlefsen has demonstrated that incubation of MCF-7 estrogen responsive BC cells and MDA-MB-231 TNBC cells treated with post-exercise serum, from both healthy volunteers [124]

and operated cancer patients [14, 124], resulted in a reduction of BC cell viability in comparison with BC cells incubated with pre-exercise sera. In particular, it has been demonstrated that MCF-7 and MDA-MB-231 stimulation with sera leads to a viability reduction of 11% in MCF-7 cells and 9% in MDA-MB-231 cells in the case of supplementation with post-exercise serum from operated cancer patients receiving adjuvant chemotherapy compared to pre-exercise serum [124]. Furthermore, the viability of both BC cell lines supplemented with sera from healthy women was also significantly reduced by the exercise-conditioned sera, resulting in a 10% and 19% reduction in MCF-7 viability and a 14% and 13% reduction in MDA-MB-231 viability by 1 h and 2 h post-exercise sera, respectively. The reduced viability of MDA-MB-231 supplemented with 5% of healthy women 2-hour post-exercise serum has also been confirmed by a pilot study that we performed working with culture medium with a physiological concentration of glucose (80mg/dl), resulting in a statistically significant reduction in cell proliferation of about 10% compared to cells supplemented with pre-exercise human serum [103]. Promising data on the tumorigenic potential of cancer cells in mice are also available. As reported by Dethlefsen et al. in 2017 [124], different outcomes in incidence and growth of tumors were detected inoculating NMRI-Foxn1nu mice with MCF-7 or MDAMB-231 BC cells preincubated for 48 hours with pre or post-exercise sera from healthy volunteers. In particular, only 45% of the mice inoculated with MCF-7 supplemented with post-exercise human serum formed tumors compared with 90% of mice inoculated with MCF-7 preincubated with at rest sera, and the volume of tumors was reduced by 76%. Moreover, tumor incidence in mice inoculated with MDAMB-231 cells preincubated with post-exercise sera tended to be lower than it was in mice inoculated with MDA-MB-231 cells preincubated with rest sera, but no difference in tumor volume was observed between the two groups. These results show that exercise-stimulated changes suppress BC cell proliferation and reduce the tumorigenic potential of BC cells, also in the case of TNBC cells. Another important aspect to be considered is the fact that PA has been reported to lead to an increased level of the catecholamines epinephrine (EPI) and norepinephrine (NE) [82]; this result has also been confirmed in BC survivors two hours after a single exercise session [124]. Moreover, by blocking the β-adrenergic signaling pathway in BC cells, the effects of post-exercise sera in BC cell viability is completely blunted, indicating the crucial role of catecholamines in inhibiting BC cells viability and

tumor growth [124]. Their role in exercise-induced effects on BC cell viability has also been confirmed by MCF-7 and MDA-MB-231 treatment with different doses of EPI and NE, resulting in a dose-dependent growth inhibitory effect in both BC cell lines. Catecholamines have been shown to induce dose-dependent phosphorylation of yes-associated protein (YAP) in MDA-MB-231 cells [125]; YAP is the main downstream target of the mammalian Hippo pathway and, when phosphorylated, it is retained in the cytoplasm. Hippo pathway is a tumor suppressor signaling cascade that regulates cell growth, and it has been shown to be a dysregulated pathway in several types of cancers, including BC, in which there is an activation of YAP oncoproteins and transcriptional coactivators with the PDZ-binding motif (TAZ) associated with tumor formation, growth and progression, metastasis, and drug resistance [126]. Dethlefsen et al. showed that the Hippo pathway is regulated by exercise-conditioned sera: incubation of BC cells with postexercise sera led to a time-dependent phosphorylation of YAP in MCF-7 BC cells and to a decreased expression of YAP target genes, due to phosphorylated-YAP cytoplasmic retention, in both MCF-7 BC cells and MDA-MB-231 TNBC cells [124]. Studies performed by Tumaneng et al. demonstrated that the Hippo pathway is related to the mTOR signaling cascade: YAP mediates the effects of the Hippo pathway regulating target genes, including the miR-29; this miRNA family has been proven to inhibit PTEN, an upstream activator of mTOR [127]. In summary, the Hippo pathway can be activated by exercise through the production of the catecholamines EPI and NE and can inhibit BC cell growth through the action of YAP and miR-29, inactivating the mTOR pathway. As mentioned above, several miRNAs have been found to be deregulated in TNBC cells and patients; evidence suggests that different types of exercise can regulate these miRNAs in different ways. One of these miRNAs is miR-21, which has been found to be upregulated in TNBC patients; it has an oncogene activity and plays a crucial role in tumor cell proliferation and invasion, repressing PTEN [128]. Nielsen et al. [129] showed how miR-21 level significantly decreased 3–5 days after endurance training (60 min of cycle ergometer exercise at 65% of Pmax, 5 times a week for 12 weeks), at rest. However, levels of miR-21 were also found to be upregulated immediately after a single exhausting cycling exercise at a low heart rate, just as it was after a training period of 90 days [130]. Discrepancies between data obtained by these two studies could be explained by the different types of exercise considered, as confirmed by Wardle et al. [131]. The microRNA

precursor miR-146a has also been found to be an upregulated miRNA in TNBC tissues, and its level is related to tumor size and survival rate. Nielsen et al. [129] showed that miR-146a levels significantly decreased immediately after a single session of pedalling exercise performed at 65% of the maximal power output. In this case, depending on the different exercises considered, miR-146 levels can be dysregulated: after a single exhausting cycling exercise at a low heart rate, it has been found to be upregulated [130]. Variations in miR-146 levels when comparing strength or endurance exercise groups to controls were observed; levels increase in the endurance group, while they decrease in the strength group [131]. The downregulation of miR-146a after strength exercise was also confirmed by a study that involved a single strength exercise session performed at 70% of one-repetition maximum [132] in which the miR-146a level was found to have decreased 3 days after exercise. In short, a subset of circulating miRNAs, including miR-21 and miR-146a, are associated with the whole-body adaptive response to differential forms of exercise and training. These miRNAs have been found to be upregulated in TNBC patients and related to the repression of PTEN or BRCA1 with consequent mTOR pathway activation. Hence, their downregulation with specific types of exercises could be a very promising approach to control TNBC initiation and progression.

4. Energy Intake in TNBC and mTOR Modulation

mTORC1 is a key regulator of cell growth and proliferation, and at the same time, it is also at the center of nutrient regulation and utilization. In this regard, a large number of studies have demonstrated the role of excessive energy intake on cancer development, and by contrast, the protective effects of CR [133]. While the antitumorigenic effects of CR are well established, the mechanism behind this relationship is not completely clear, though it is believed that the tumor-suppressive effects are mediated, as they are for exercise, by enhanced apoptosis, modulation of systemic signals such as IGF-1, insulin, metabolic, and inflammatory pathways, as well as by reduced angiogenesis [134]. Specifically, a large quantity of data points to the role of mTOR activation in cancer development through protein-induced IGF-1 signaling and to the beneficial effects of caloric and protein restriction not only on aging-associated diseases such as cancer but also on life span [135, 136] (Figure 1). CR increases the level of the circulating adiponectin, which can exert anticancer effects through mechanisms that include an increase in insulin sensitivity, a decrease

in insulin/IGF-1 and mTOR signaling via AMPK activation as well as a reduction in the proinflammatory cytokine expression via inhibition of the nuclear factor κ-light-chain-enhancer of activated B-cells (NF-κB) [136, 137]. AMPK, as mentioned above, is an important mediator in the maintenance of cellular energy homeostasis, and recently, it has gained attention for its possible role as a metabolic tumor suppressor and in cancer prevention and control. Since AMPK phosphorylation is regulated by energy availability (AMP:ATP ratio), AMPK activators, such as metformin, CR, and aerobic exercise, reduce the incidence of cancer. Leptin is a peptide hormone produced by white adipose tissue. It affects several tissues and acts on the hypothalamus to regulate appetite and energy expenditure. It also impacts carcinogenesis, angiogenesis, immune responses, cytokine production, and other biological processes [138, 139]. Intermittent CR is associated with the suppression of murine mammary tumor incidence and a decrease in the leptin-to-adiponectin ratio [139]. This ratio, when elevated, is related to metabolic syndrome and some cancers [140, 141]. In TNBC metastases, CR decreases proliferation, increases apoptosis, and downregulates the IGF1-1R pathway, coadiuvating canonical therapies [142]. Taken together, these findings show that dietary interventions can ameliorate systemic milieu and tumor microenvironment. Chronic CR is not suitable for cancer patients at risk for weight loss, cachexia, and immunosuppression, but it can be substituted with intermittent CR, fasting-mimicking diets, low carbohydrate/ketogenic diets, or CR mimetic drugs. Fasting and low carbohydrate diets have been shown to reduce side effects and to enhance the effectiveness of chemotherapy and radiation therapy in animal models, and there is a great deal of interest in the potential clinical value of these interventions. Protein consumption has different effects on cancer mortality, which vary according to age, with an increased risk in middle age and a reduction in the elderly [143]. Protein restriction (PC) for the middle-aged followed by moderate protein intake in elderly subjects may increase longevity and health span since protein restriction is sufficient to reduce growth hormone receptor (GHR)-IGF1 activity and can reduce cancer incidence in model organisms regardless of energy intake [144]. Moreover, L-type amino acid transporter 1 (LAT1), which transports large quantities of neutral amino acids, was found highly expressed in human BC tissues. The upregulation of LAT1 plays an important role in BC progression because more amino acids are required for protein synthesis and cellular proliferation [145]. The activation of the mTOR/S6K1 signaling pathway depends on the availability of

amino acids (AA), particularly branched-chain AA, such as leucine, and also glucose [106]. Growth factor signals, which usually activate mTORC1 signaling, have little or no impact in the absence of AA. Leucine deprivation causes an upregulation of insulin-like growth factor binding protein 1 through transcriptional activation and mRNA stabilization, probably decreasing the effects of IGF1 and thus lowering cell proliferation [146]. However, in most BC cell lines with constitutively activated Akt/mTOR signaling, leucine restriction is not efficient in inhibiting mTOR signaling since it is associated with activation of survival molecule Akt, making leucine deprivation an undesirable approach for BC therapy [146]. Glutamine is another AA involved in the regulation of the mTOR pathway inducing the uptake of leucine [147]. Tumor cells are more sensitive to amino acid deprivation than normal cells; thus, glutamine restriction and/or transporter inhibition decrease mTOR activity [147]. A novel therapeutic approach based on whey protein concentrate (WPC) supplementation for BC treatment has been suggested by Cheng et al. [148]. WPC is rich in bioavailable cysteine, which can be used for glutathione synthesis, and contains all nine essential AAs. WPC promotes muscle protein synthesis [149] and can be used as a nutritional supplement during chemotherapy [150]. WPC has also been shown to enhance rapamycin sensitivity in MDA-MB-231 TNBC cells, a cell line resistant to rapamycin and other mTOR inhibitors [148]. The combination of conventional therapies and n-3 polyunsaturated fatty acid (PUFA) supplementation (nutritional interventions) increases the sensitivity of tumor cells to conventional therapies, possibly improving their efficacy especially against cancers resistant to treatment, as suggested by D'Eliseo and Velotti [151]. Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) have anticancer effects on different cancer types by inducing apoptotic cell death in human cancer cells either alone or in combination with canonical therapies. EPA and/or DHA also have proapoptotic effects in both triple-negative [152] and ER+ BC subtypes [153], although when compared at the same dose, DHA appears to be more effective. This might be due to the structural differences between DHA and EPA. The proapoptotic effects occur with increases in plasma membrane incorporation and decreases in cell viability [152–154], PI3K/Akt pathway [155], and pEGFR activation [152]. In agreement, CR and other nutritional interventions could play an important role in the support of conventional therapies to improve TNBC outcomes.

5. Exercise Prescription in BC Survivors

In general, reviews and meta-analyses tend to group PA and exercise interventions into general categories and rarely examine the specific exercise protocols employed in the studies. Therefore, which characteristics make an exercise protocol safe and effective for BC survivors and, particularly, for TNBC patients? Since the 2009 roundtable consensus statement on exercise guidelines for cancer survivors [156], which outlined the situations in which deviations from the 2008 US Physical Activity Guidelines for Americans (PAGA) were appropriate and included relevant implementation strategies [157], exercise recommendations from several internationally recognized institutions, such as the American Cancer Society [158] and the National Comprehensive Cancer Network [159], have been published for BC survivors. Fortunately, all of the abovementioned publications have recently been reviewed within the framework for exercise prescription of the American College of Sports Medicine (ACSM) [160], along with others providing practical guidance for exercise prescription in these patients [161, 162]. ACSM's framework for exercise prescription employs the so-called FITT-VP principle [160], which considers the frequency (F), intensity (I), time (T), and type (T) of exercise and its volume (V) and progression (P) overtime in an individualized exercise training program. A detailed description of the FITT-VP principle for each type of exercise - i.e., aerobic, resistance, and flexibility - adapted to BCS needs is provided in Tables 1, 2, and 3. Note that the following guidelines should not be regarded as specific for BC patients because no studies, to date [163], have adopted (and/or reported) the proper application of the principles of specificity, progression, overload, initial values, and adherence, within their exercise interventions. Therefore, although specific exercise guidelines for cancer survivors still need to be outlined, particularly for TNBC survivors, the following information represents the most up-to-date adaptations of the PAGA to BCS, including TBNC patients. Improving the reporting of exercise prescriptions will also allow for more specific recommendations regarding types and doses of exercise for BCS (and, hopefully, for the TNBC subgroup), in order to identify effective exercise interventions to be delivered to this growing community.

Table 1: Aerobic (cardiorespiratory endurance) exercise recommendations.

Intensity (I)	Frequency (F)	Time (T) (duration)	Type (T) (mode) (examples)	Volume (V) (quantity)	Progression (P) (rate of)	Specific notes
Light: 30–39% VO_2R/HRR; 57–63% HR_{max}; 9–11 RPE.	At least 5 d wk^{-1}.	30 to 60 min each session (i.e., at least 150 min wk^{-1}).	Continuous and rhythmic exercises that involve major muscle groups (walking, cycling, slow dancing, jogging, running, rowing, stepping, fast dancing, etc.).	≥500–1,000 MET min wk^{-1}.	Increase gradually any of the FITT components as tolerated by the patient (gradual progression is required to minimize the risks of muscular soreness, injury, undue fatigue, and the long-term risk of overtraining). Initiate increasing exercise duration (as tolerated): an example for healthy people is adding 5–10 min every 1–2 wk over the first 4–6 wk and adjusting upward over the next 4–8 months to meet the recommended FITT components, but slower progression may be needed for BCS.	If tolerated without adverse effects of symptoms or side effects, moderate to vigorous intensity and 3–5 d wk^{-1} frequency are recommended, but lower (light) intensities and frequencies are still beneficial when the current physical activity level is low. Avoid prescribing and monitoring intensity using %HRR (using $\%HR_{max}$ or RPE is recommended in BCS). Be aware of fracture risk, because bone is a common site of metastases in breast cancer: BCS with metastatic disease to the bone will require modification of their exercise program (e.g., reduced impact, intensity, and volume) given the increased risk of bone fragility and fractures.
Moderate: 40–59% VO_2R/HRR; 64–75% HR_{max}; 12-13 RPE.	At least 5 d wk^{-1}.	30 to 60 min each session (i.e., at least 150 min wk^{-1}).				
Vigorous: 60–89% VO_2R/HRR; 76–95% HR_{max}; 14–17 RPE.	At least 3 d wk^{-1}.	20 to 60 min each session (i.e., at least 75 min wk^{-1}).				

Modified from [160]. VO2R: oxygen uptake reserve, calculated as the difference between maximal oxygen uptake and resting oxygen uptake; HRR: heart rate reserve, calculated as the difference between maximal heart rate and resting heart rate; HR_{max}: maximal heart rate; RPE: rate of perceived exertion on the 6–20 scale; MET-min: metabolic equivalents (MET) of energy expenditure for a physical activity performed for a given number of minutes (min), calculated as MET × min; FITT: frequency, intensity, time, and type of exercise.

Table 2: Resistance (strength) exercise recommendations.

Intensity (I)	Frequency (F)	Time (T) (duration)	Type (T) (mode) (examples)	Volume (V) (quantity)	Progression (P) (rate of)	Specific notes
Light: 30–49% 1-RM.	2-3 d wk^{-1}.	Depends on exercise volume (number of sets, repetitions for each set, and rest intervals in-between) and is not associated with effectiveness.	Any form of movement designed to improve muscular fitness by exercising a muscle or a muscle group against external resistance: exercise and breathing techniques are of paramount importance and symptom-limited ROMs should be adopted according to BCS responses to exercise (free weights, resistance machines, weight-bearing functional tasks, etc.).	2–4 sets of 8–15 repetitions (at least 1 set of 8–12 repetitions can be effective in BCS) with 2-3 min rest between sets.	BCS should start with a supervised program of at least 16 sessions and very low resistance (<30% 1-RM), and progress with smallest increment possible (e.g., 2–10% 1-RM, depending on muscular size and involvement, is recommended for healthy adults). If a break is taken, lower the level of resistance by 2 wk worth for every week of no exercise.	No upper limit on the account of weight to which BCS can progress. Individuals with lymphedema should wear a compression sleeve during resistance training activity. Watch for arm/shoulder symptoms including lymphedema and reduce resistance or stop specific exercises according to symptom response. Be aware of risk of fracture (see aerobic exercise for details).
Moderate: 50–69% 1-RM.	2-3 d wk^{-1}.					
Vigorous: 70–84% 1-RM.	2-3 d wk^{-1}.					

Modified from [160]. 1-RM: one-repetition maximum, i.e., the load that can be lifted one time only; ROM: range of motion; BCS: breast cancer survivors.

Table 3: Flexibility (stretching) exercise recommendations.

Intensity (I)	Frequency (F)	Time (T) (duration)	Type (T) (mode) (examples)	Volume (V) (quantity)	Progression (P) (rate of)	Specific notes
Stretch to the point of feeling tightness or slight discomfort.	≥2-3 d wk^{-1} (stretching on a daily basis is most effective).	Hold a static stretch for at least 10–30 s (30–60 s may confer greater benefit). Accumulate a total of 60 s of stretching for each flexibility exercise by adjusting time/duration and repetitions (see volume) according to individual needs.	Stretching exercise that increases the ability to move a joint through its complete ROM (provided individual specific conditions are accounted for) (static active flexibility, static passive flexibility, dynamic flexibility, ballistic flexibility, proprioceptive neuromuscular facilitation, etc.).	Repeat each exercise 2–4 times in order to attain the goal of 60 s stretch time (e.g., two 30 s stretches or four 15 s stretches). A stretching routine can be completed approximately in ≤10 min.	Optimal progression is still unknown.	BCS should focus on joints in which a loss of ROM occurred because of surgery, corticosteroid use, and/or radiation therapy. Flexibility exercises are most effective when the muscles are warm.

Modified from [160]. ROM: range of motion; BCS: breast cancer survivors.

6. Benefits of Exercise Pre- and Post-diagnoses

Humans have not been "designed" for a sedentary lifestyle. The absence of an adequate level of PA puts us at an increased risk of developing cancer. This has been highlighted by the European Breast Cancer Conference [164], issued an important statement: regular PA reduces the risk of BC for women of any age and body weight by 12%. PA as a nonpharmacological treatment to combat the collateral effects associated with BC is under considerable scientific attention [160, 165, 166]. To allow physicians to prescribe PA to patients before and after treatment, scientific clarity and evidence supporting the thesis that PA programs reduce the damaging effects of cancer and its treatment are needed. Very little is known about the effect of exercise on TNBC outcomes, but data suggest that pre- and postdiagnosis PA may be one of the factors, which, if appropriately prescribed, could bring benefits to patients. Generally, TNBC has poor treatment outcomes because of a lack of receptor targets for conventional drugs to act upon. However, there is irrefutable evidence of the effectiveness of regular PA in primary and secondary prevention of premature death from any cause, including BC. Thus, different types of exercise can influence the prevention and progression of the disease through several common mechanisms such as reduction of insulin resistance and improvement in immunity and cardiovascular function. Research in humans shows that exercise can regulate inflammation [13, 167], oxidation [168, 169], and gene expression [170]. Together with the potential mechanisms underlying the effects of exercise on breast carcinogenesis, Thompson [12] proposed three interesting hypotheses: (i) the hormesis hypothesis: oncological response to exercise is antithetical to a physiological cellular stress response; (ii) the metabolic reprogramming hypothesis: exercise reduces the glucose and glutamine available to mammary carcinomas, inducing apoptosis and reversing tumor-associated metabolic program; and (iii) the mTOR network hypothesis: exercise inhibits carcinogenesis by suppressing the activation of the mTOR signaling network in mammary carcinomas. Recent investigations have revealed that the most active women had, on average, a 25-30% lower BC risk than women in the lowest category of recreational PA [171]. Data from the California Teachers Study (CTS) suggest that PA has a protective role in prediagnoses and may reduce a woman's risk of BC, especially the TNBC subtype. An analysis of the risk index (HR) associated with variations in the amount of PA hours among TNBC women yielded significant results. The HR results show significant associations when moderate-intense activity

is considered as the only variable. When they are considered as separate variables, there are no statistically significant associations between moderate activity and TNBC, whereas intense activity is inversely associated with TNBC [172]. The reduced risk associated with baseline strenuous recreational PA was statistically significant among overweight or obese pre- or postmenopausal women, but not among their leaner counterparts. In patients with BC postdiagnosis, acute and chronic symptoms, such as muscle mass loss, fatigue, weight gain, hormone alterations, bone loss, cachexia, and adverse psychological effects, may all be favourably influenced by regular exercise. A prospective cohort study analyzed modifiable lifestyle factors, including exercise, associated with total mortality and recurrence/disease-specific mortality in patients with TNBC [173]. The association between TNBC prognoses and exercise postdiagnosis yielded important results: women who engaged in exercise regularly during the first 6 months postdiagnosis had a lower risk of total mortality and recurrence/disease-specific mortality, with adjusted HRs of 0.58 and 0.54, respectively. In addition, those who engaged in PA for a long time (2.5 h/wk) or women who exercised ≥7.6 metabolic equivalent hours/wk had a reduced risk of all causes and recurrence/disease-specific mortality compared with non-exercisers. Survivors who maintain a healthy weight and stay physically active have a better response to treatment and better survival outcomes. Thus, it is necessary to identify an appropriate promotion and prescription of regular PA for BC survivors in order to improve their prognosis, response to therapy, and quality of life. As previously described, the mTOR signaling pathway is differentially regulated by different exercise modalities, and it represents one of the main key regulators of the protective effects of exercise.

7. Conclusions

In this review, we presented new insights into the downregulation of mTOR signaling in TNBC by exercise and CR. It has been shown that mTOR network inhibition is mostly mediated through the effects of CR and vigorous PA as well as long-term exercise, which decreases the level of circulating growth factors and hormones. During exercise, the body is exposed to different types of stressors, including temperature, metabolism, hypoxic, oxidative, and mechanical stress. These stressors initiate biochemical targets, which in turn actuate different signaling pathways that regulate gene expression and adaptive responses. Beneficial adaptation

likely depends on the basal state of oxidative stress and inflammation at the beginning of exercise training. In turn, this basal state may depend on the periodization of training and recovery, together with age, health status, and diet. Exercise, as a hormetic agent, has the potential for beneficial energy upregulation. The dose-response effects are complex and reflect the activation of major defensive pathways in both systemic and local environments. A mitohormetic stimulus that occurs through a physiological cellular stress adaptation and AMPK activation across hormetic control circuits, such as the increase of oxidative metabolism, mitochondrial biogenesis, angiogenesis, immune regulation and a decrease in BMI, and insulin secretion, are induced by exercise. Moreover, PA increases glucagon, catecholamines, and other hormones and influences miRNAs involved in cancer. Exercise as well as CR limit glycemia and glutamine availability to mammary carcinomas, inducing apoptosis and reversing malignancy-associated metabolic programming. It is also known that intratumoral metabolism is regulated by exercise, but how this affects tumor growth and the metastatic rate is not clearly understood. Although the signal for these hormonal and autonomic changes has been partially described in ex vivo experiments, such changes are difficult to transfer *in vivo*. Currently, there is an agreement in the literature that there is a role for exercise as a coadjuvant "medicine" in canonical therapies and that it has an increasingly protective tumorigenic effect. In this context, PA needs to be broken down into its main components: frequency, intensity, time, and type; however, the dose-dependent effects of each of these components on cancer protection via mTOR inhibition are still unclear. Most data suggest that both vigorous and long-term PA in adulthood may reduce a woman's risk of mammalian cancer, especially the TNBC subtype. Finally, we can assert that there is sufficient evidence showing that sedentary behaviour and nutritional risk factors for TNBC are modifiable. Hence, the suggestions regarding the modification of such risk factors highlighted in this review could have wide-ranging implications for society and may improve public healthcare cancer management. Accordingly, we would like to emphasize the importance of promoting physically active lifestyles to reduce the risk of relapse in TNBC. Fostering active lifestyles can provide important support during conventional cancer treatment, preventing the potential negative impacts on patients' physical condition, as well as their emotional and social well-being.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this article.

Authors' Contributions

Deborah Agostini, Valentina Natalucci, and Giulia Baldelli contributed equally to this work.

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CHAPTER 2

In Press Article. Journal Pre-Proof.

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A dataset on the effect of exercise-conditioned human sera in three-dimensional breast cancer cell culture

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Abstract

Epidemiological evidence shows that physical activity lowers the risk of developing breast cancer and decreases the risk of disease recurrence [1; 2]. The main hypothesis on the positive effects of exercise-oncology has focused on lowering the basal systemic levels of cancer risk factors with exercise training. Recently, the effects of cancer progression control by components released after acute exercise bouts have gained attention [3; 4]. However, the evaluation of the antiproliferative potential of a single exercise bout needs technical improvement. Here, we present data of a pilot study showing how to evaluate the anti-cancer potential of single exercise bouts with an *in vitro* three-dimensional cell growth assay, using a triple-negative breast cancer cell line cultured with exercise-conditioned serum.

Keywords Exercise; triple-negative breast cancer; three-dimensional *in vitro* culture; cell proliferation.

Specifications Table

Subject	Sport Sciences, Therapy and Medicine
Specific subject area	Evaluation of the effects of exercise using cancer cell growth assays
Type of data	Graph Figure
How data were acquired	MTS assay and soft-agar assay. Statistical analysis has been performed with Prism5 software, using 1-way ANOVA followed by Bonferroni-corrected multiple comparisons.
Data format	Raw and Analyzed

Experimental Factors	Cells were cultured in standard conditions. During the experiments, the standard culture medium was replaced by Dulbecco's Modified Eagle's Medium without red phenol, with 0.8/1.2 mg/mL of glucose, supplemented with 5% of human pre- or post-exercise serum. Cells were cultured in an anchorage-dependent manner or in anchorage-independent conditions (soft agar assay) in a 0.3% soft-agar layer.
Experimental features	The cell viability was evaluated by the CellTiter 96® Aqueous Non-Radioactive Cell Proliferation Assay (Promega, Madison, WI, USA) after 72h of incubation with pre- or post-exercise sera. The cancer progression control potential was evaluated in anchorage-independent culture conditions, counting the colonies composed by more than 20 cells, formed in each well after 18 days of cell incubation with pre- or post-exercise sera.
Data source location	Institution: University of Urbino Carlo Bo – Department of Biomolecular Sciences, Hygiene Unit and Division of Exercise and Health Sciences City/Town/Region: Urbino Country: Italy
Data accessibility	All data are presented within this article

Value of the Data

- These data show that exercise-conditioned serum could be used in three-dimensional *in vitro* culture to evaluate the potential of exercise on cancer progression control.
- The application of three-dimensional cell growth in soft agar offers the possibility to quantify cancer cell growth in response to exercise-conditioned sera avoiding *in vivo* models.
- This model could be useful to compare the cancer progression control with different exercise protocols to personalize the prescription of exercise in terms of FITT principle (Frequency, Intensity, Time and Type).
- Future experiments will be aimed to evaluate the effect of a single exercise bout during adjuvant treatment in breast cancer patients and in the follow-up.

Data

This Data in Brief presents the optimization of methods for the evaluation of triple-negative breast cancer (TNBC) cell MDA-MB-231 responses induced by acute exercise-conditioned sera, considering also the capacity of exercise-conditioned sera to modulate three-dimensional (3D) anchorage-independent cancer cell growth. The time schedule of the aerobic exercise session performed by the three subjects is presented in Fig. 1.

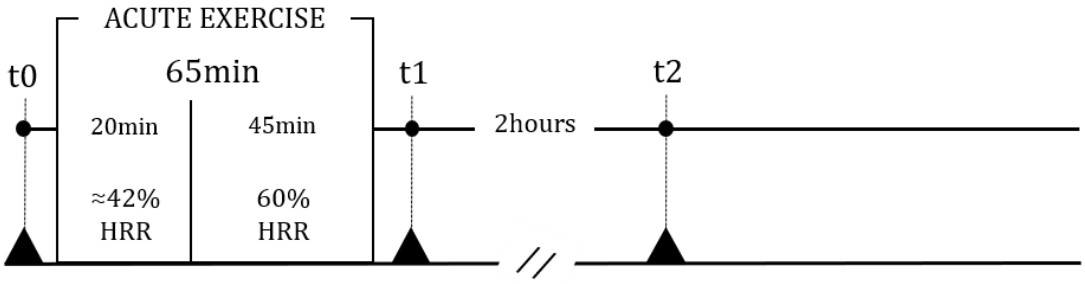

Fig. 1 Details of the acute exercise interventions. The time schedule of pre- (t0) and post-exercise (t1; t2) sampling is shown in the timeline. Blood samples for *in vitro* assays were drawn according to protocol exercise at t0, t1 and t2. //, rest period; HRR, heart rate reserve.

The effects of the sera sampled pre- or post-exercise on the capacity of TNBC cell to proliferate were monitored by exposing MDA-MB-231 cells to 5% of human sera for 72 hours, after which the cell viability was evaluated by MTS assay (Fig. 2). Data of

supplementation of the culture medium with a physiological or hyperglycemic concentration of glucose (0.80 mg/mL or 1.20 mg/mL) showed a lower ability of the exercise-conditioned sera (t1 and t2) to induce TNBC cell proliferation than the sera collected pre-exercise (at rest sera, t0). Interestingly, the inhibition obtained was higher with a physiological concentration of glucose (0.80 mg/mL), leading to a statistically significant reduction in the ability of cells to proliferate in two out of three subjects considered (s-02 and s-03). In particular, in these two subjects, t1 and t2 sera led to a reduction of 5% and 9%, respectively (n.s. and $p<0.05$, respectively). Analyzing the highest concentration of glucose tested (1.2 mg/mL), t1 and t2 sera led to a reduction of cell proliferation of only 2% and 6% (n.s.), considering the sera of subjects s-02 and s-03 (Raw data are presented as supplementary file “RAW DATA FIG.2”).

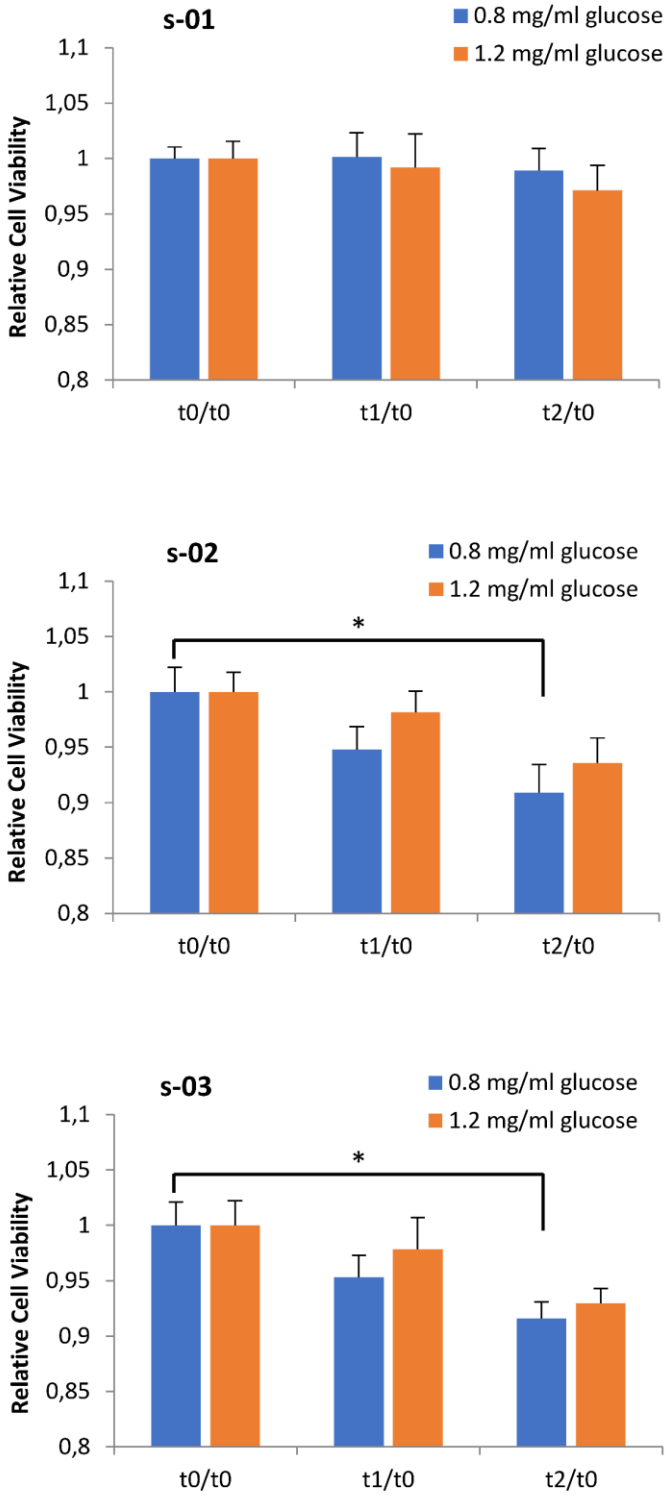

Fig. 2. MTS assay. Evaluation of cell viability after 72 hours of incubation with 5% of pre- or post-exercise sera and with culture medium supplemented with 0.8 or 1.2 mg/ml of glucose. t0: pre-exercise serum; t1: immediately after exercise serum; t3: 2 hours post-exercise serum. s-01, subject 01; s-02, subject 02; s-03, subject 03. Data are expressed as mean ± SEM of five experiments; *$p<0.05$.

The effects of exercise-conditioned sera on the tumorigenic potential of MDA-MB-231 cells was monitored performing the anchorage-independent three-dimensional growth assay (soft-agar), which is considered one of the most reliable tests to assess the malignant transformation process *in vitro* [5]. In this technique, TNBC cells were dispersed in the central layer, composed by 0.3% agar and exposed to 0.80 mg/ml of glucose and 5% of pre-exercise (t0) or post-exercise sera (t1 or t2). Data of 3D anchorage-independent cancer cell growth are presented in Fig. 3, expressed as the total number of colonies formed by more than 20 cells, counted in each well after 18 days of incubation. Data show that all exercise-conditioned sera (t1 and t2 sera) reduced in a statistically significant manner the ability of TNBC cells to form colonies in soft agar, in comparison to pre-exercise sera (t0) ($p<0.01$; $p<0.001$). In particular, exercise-conditioned sera of the subject s-01 induced a reduction of colony number of 10% and 14% (t1 and t2, respectively); analysing the subject s-02, the post-exercise sera induced reduction was of 6% and 8% (t1 and t2, respectively) and evaluating the subject s-03, the reduction induced by t1 and t2 sera was of 10% and 15%, respectively (Raw data are presented as supplementary file "RAW DATA FIG.3").

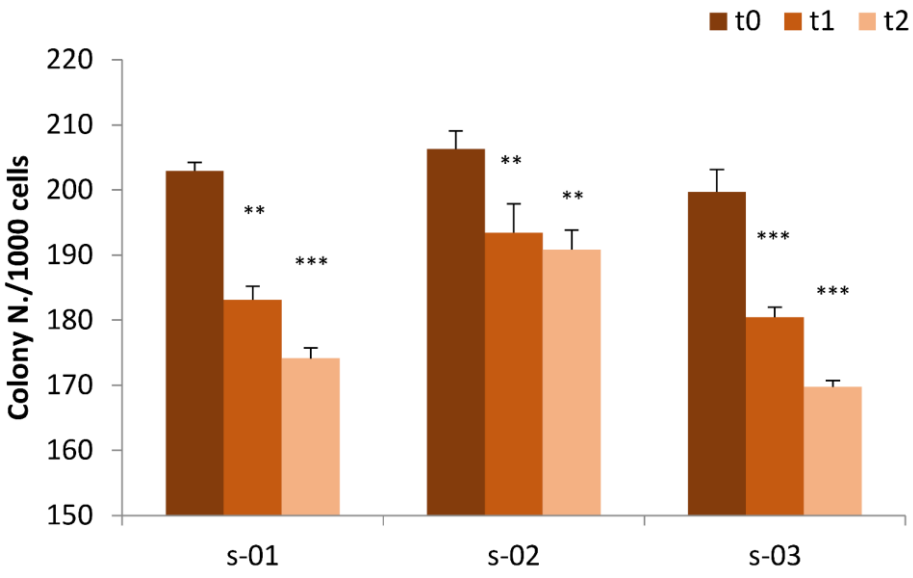

Fig. 3. Soft-agar assay. Evaluation of MDA-MB-231 colony formation after 18 days of incubation with 5% of pre- or post-exercise human sera. s-01, subject 01; s-02, subject 02; s-03, subject 03. Data are expressed as mean ± SEM of four experiments; **p<0.01, ***p<0.001, respect to t0.

Experimental Design, Materials, and Methods

Subjects

Three healthy and sedentary pre-menopausal women were included in the study. Their median (range) age, height and weight were 43.3±9.8 yrs, 164.4±4.7 cm and 60.2±5.8 kg, respectively. The study was carried out according to the Helsinki Declaration for research with human volunteers and all signed an informed consent form to participate.

Acute Exercise Session

On the experimental day, participants performed a single bout of exercise. In particular, the participants performed 65 min of moderate to baseline vigorous intensity aerobic exercise on a treadmill. In the first 20 minutes, subjects ran at a heart rate reserve (HRR; ≈42%) corresponding to 50% of their own estimated VO_{2max}, then exercise intensity was increased to 65% of VO_{2max} (*i.e.*, 60% HRR) and maintained for 45 min [6].

Blood samples, cell line and cell cultures

Blood samples were collected in venous blood collection tubes (BD Vacutainer, 10mL, no additives) just before exercise, immediately after and 2 hours post-exercise (Fig.1). Serum was obtained centrifuging blood samples at $1x10^3$ x g for 15 minutes at 4°C, after an incubation of 15-30 minutes at room temperature. Sera were aliquoted and stored at -80 °C; before the experiments, sera were heat-inactivated at 56°C for 30 minutes, centrifuged at 12.000 rpm at 4°C for 10 minutes and transferred to new sterile tubes.

TNBC cell line MDA-MB-231 was purchased from the American Type Cell Culture Collection (ATCC, Rockville, MD, USA) and cultured in Dulbecco's Modified Eagle Medium (DMEM) high glucose, supplemented with 10% of heat-inactivated Fetal Bovine Serum (FBS), 2 mM glutamine, 0.1 g/L streptomycin, 100 units/ml penicillin, 1 mM Na-pyruvate and 1× MEM Non-essential Amino Acid Solution. During the experiments, DMEM high-glucose was replaced by DMEM without red phenol (DMEM-RPF), supplemented with 5% of heat-inactivated human sera (HS) pre- or post-exercise, 2 mM glutamine, 0.1 g/L streptomycin, 100 units/ml penicillin, 1 mM Na-pyruvate, 1× MEM Non-essential Amino Acid Solution and 0.80 or

1.2 mg/mL glucose. All cell culture materials were purchased from Sigma-Aldrich (St. Louis, MO, USA). Cells were maintained for a maximum of fifteen passages, in a humidified incubator with 5% of CO_2, at 37 °C.

Anchorage-dependent growth assay

MDA-MB-231 cells were seeded at a density of $2.5x10^3$ cells/well in 96-well plates. After overnight incubation, the medium was replaced with DMEM-RPF and cells were exposed to 5% of pre-exercise (t0), post-exercise (t1) or 2-hours post-exercise (t2) HS supplementation. Two different concentrations of glucose (0.8 or 1.2 mg/mL) were tested. After 72 h of incubation, cell viability was assessed by the CellTiter 96® Aqueous Non-Radioactive Cell Proliferation Assay (Promega, Madison, WI, USA). This method is a colorimetric assay based on the ability of viable cells to convert soluble tetrazolium salt (MTS) into a formazan product. Data are expressed as relative viable cells (mean ± SEM of five experiments) compared to cells supplemented with at rest serum (t0).

Anchorage-independent transformation assay (soft-agar assay)

Soft agar assay was performed in 12-well plates as reported previously [7]. Briefly, $1x10^3$ MDA-MB-231 cells were considered for each well and cultured in the central layer of agar, composed by a 0.5mL of 0.3% agar and 0.8 mg/mL glucose-DMEM-RPF solution, supplemented with 5% of t0, t1 or t2 HS. The bottom layer was composed by a 0.5mL solution of 0.6% agar and 0.80 mg/mL glucose-DMEM-RPF, supplemented with 5% of t0, t1 or t2 HS and the top layer was composed of 0.8 mg/mL glucose-DMEM-RPF added by 5% of t0, t1 or t2 HS. After 18 days of incubation, cells were stained with 0.01% crystal violet and only colonies formed by more than 20 cells were considered and counted (Supplementary information). Data are expressed as the total number of colonies (mean ± SEM of four experiments) counted in each well.

Statistical analysis

Data are expressed as means ± SEM of separate experiments. Data were analyzed with Prism5 software, using 1-way ANOVA followed by Bonferroni's multiple comparison test. Differences were considered significant at $p < 0.05$.

Acknowledgments

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The authors would like to thank Dr. S. Maggio and Dr. P. Ceccaroli for their technical contributions in this project.

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CHAPTER 3

Original Article

Effects of human sera conditioned by high-intensity endurance cycling sessions and a high-intensity interval training period on cancer cell proliferation and tumorigenesis.

Baldelli G. et al.

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In preparation, 2019

Abstract

A growing body of evidence is emerging about the lowered risk of cancer and cancer recurrence associated with physical exercise. It has been hypothesized that these beneficial effects could be related to the systemic responses to high-intensity exercise bouts or to a training period, and there is a growing interest to evaluate the systemic factors involved in different exercise protocols. In the present study, we assessed the effects of exercise-conditioned human sera on the proliferative capacity and tumorigenic ability of triple-negative breast cancer cells and prostate cancer cells, considering both high-intensity endurance cycling (HIEC) sessions and a high-intensity interval training period. Moreover, the possible relation between the results obtained by the clonogenic capacity of cells stimulated with exercise-conditioned sera and the systemic factors measured in all subjects after the exercise has been considered. The present study showed that human sera conditioned by HIEC-sessions impact the proliferative and tumorigenic capacities of triple-negative breast cancer and prostate cancer cells; furthermore, possible relationships between the results obtained in the tumorigenic potential of prostate cancer cells and some exercise parameter predictors have been shown.

INTRODUCTION

Cancer is the second leading cause of death worldwide [1] and according to the report GLOBOCAN 2018 [2], 18.1 million new cases and 9.6 million cancer deaths have been estimated in 2018. Considering the gender, breast cancer and prostate cancer are the most commonly diagnosed cancer in women and men, respectively. Moreover, breast cancer is the most common leading cause of cancer-related death in women, and prostate cancer is the second one in men.

Based on histological features, 15-20% of breast cancer diagnosed worldwide each year is represented by the triple-negative breast cancer (TNBC) subtype, which is the most prevalent and highly aggressive breast cancer among patients younger than 50 years old and which is characterized by the absence of estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) genes [3]. The lack of these receptors makes TNBC very aggressive and unresponsive to hormonal and targeted therapies. Furthermore, TNBC exhibits a very

high risk of recurrence within 3-5 years of treatment and has a high probability to metastasize [4].

Prostate cancer cases represent 7.1% of all cancer cases in men and 3.8% of all deaths caused by cancer in men worldwide [2]. Moreover, about 20% of prostate cancer cases are diagnosed in patients with a family history for the disease, which risk is associated not only to the genetic predisposition but also to the exposure to the same environmental risk factors and unhealthy lifestyles, such as physical inactivity [5].

"World Health Organization's global recommendations on physical activity for health" and "2018 Physical Activity Guidelines Advisory Committee Report" suggested 150 minutes of moderate-intensity activity per week for adults to avoid physical inactivity, which has been demonstrated as one of the major risk factors for premature death from non-communicable diseases. Conversely, regular physical exercise has been associated with reduced risks of these pathologies, including different types of cancer [6, 7].

As reported by a pooled analysis of data from twelve different prospective studies, published by Moore et al. [8], the risk of 13 types of cancer decreases in case of a high level of leisure-time physical activity. Moreover, a large group of epidemiological studies outlined the correlation between physical activity and reduced risk of breast and prostate cancer and showed the tight link between physical exercise and reduction in the risk of cancer mortality and recurrence in cancer survivors [9-14].

Recent growing interest aims to understand how physical exercise can induce those beneficial effects in cancer recurrence and in controlling cancer progression. As reviewed by Friedenreich and colleagues, more than 20 different studies reported an average 37% reduction in cancer-caused mortality in breast, colorectal and prostate cancer patients who were more active, in comparison to the less active ones; moreover, a decreased risk of recurrence was assessed, too [15].

Several systemic risk factors to BC and prostate cancer have been identified: elevated sex hormones levels are associated to a high risk of BC in premenopausal and postmenopausal women [16, 17]; high plasmatic insulin and IGF-1 levels increase incidence of postmenopausal BC and of prostate cancer risk and are associated with high rates of recurrence in BC and prostate cancer survivors, stimulating cancer cell proliferation [18-22]; elevated levels of inflammatory

molecules, like C-reactive protein (CRP), are linked to increased risk of BC and with worse prognosis after BC diagnosis [23].

Considering the systemic responses to physical exercise that could explain the induced beneficial effects on BC risk and BC progression control, Dethlefsen and Hojman described the differences between responses to training and intense bouts of physical exercise [24, 25], highlighting the reduction of sex hormones, insulin, and inflammatory molecules in response to a training period [26], and describing the increased level of catecholamines and myokines after intense exercise bouts [27]. Importantly, the effects shown after the training period on BC progression control seem to be highly related to the training-induced weight loss and do not seem to be directly induced by the exercise training itself.

The mechanisms involved in the effects of physical exercise in prostate cancer prevention are not yet fully understood, but it has been suggested that physical exercise could be able to decrease prostate cancer cell growth, with a linked decreased circulating IGF-1 levels and increased apoptosis [28, 29].

Importantly, most of the studies that take into account the effects of physical exercise on BC, do not consider the difference between the subtypes of BC, and little is known about the possible systemic responses in prostate cancer after physical exercise.

Starting from this evidence and hypotheses, the aim of this study was to understand if the systemic response to a high-intensity endurance (HIEC) session can induce beneficial effects on TNBC and prostate cancer cell proliferation and progression control, *in vitro*. Specifically, human sera obtained before and after the HIEC session were used to stimulate TNBC and prostate cancer cells. Moreover, the response to a nine-week high-intensity interval training (HIIT) period was evaluated, considering the effects induced by the HIEC tests, before and after the HIIT period, and by pre-HIEC sera, taken before and after HIIT period.

MATERIALS AND METHODS

The participants included in the study were from the University of Urbino, Marche Region, Italy. The study was performed in 2017, between September and December, at the Department of Biomolecular Sciences of the University of Urbino; the study followed the guidelines of the Helsinki Declaration for research with human volunteers (1975) and it has been approved by the Ethics Committee of the

University of Urbino Carlo Bo on July 10th, 2017. All the participants signed the written informed consent before participating in the study.

Subjects

Thirty healthy sedentary volunteers (12 women: age: 21.0 ± 0.8 years, body height: 160.0 ± 4.8 cm, body mass: 53.0 ± 5.4 kg, BMI: 21.0 ± 1.3 kg/m^2, and 18 men: age: 21.0 ± 1.4 years, body height: 176.0 ± 6.8 cm, body mass: 68.0 ± 10.2 kg, BMI: 22.0 ± 2.8 kg/m^2) were recruited to participate in the study, after a medical assessment, according to the following exclusion criteria: musculoskeletal injuries, cardiovascular disease risks, upper respiratory infections, any medical treatment in the 3 months preceding the beginning of the study. Moreover, another inclusion criterion was the physical inactivity for at least six months before the start of the study. The subjects were asked to maintain their routines in dietary lifestyles and to avoid all physical activities, except the sessions included in the study.

Preliminary testing

Anthropometric examinations

Before the first HIEC test, an anthropometric examination day have been performed with all the subjects recruited, to measure the body height (cm), the body mass (kg), the BMI (kg/m^2), the circumferences of legs, arms, waist and thighs (the main muscular groups, in cm), and the Bio-Electrical Impedance Analysis (BIA).

Incremental Test: evaluation of VO_{2max}, Pmax, and Lactate Thresholds

To assess the individual maximum oxygen consumption (VO_{2max}), lactate thresholds and the maximal power (Pmax), subjects were asked to perform a maximal incremental test to exhaustion with a SRM cycle ergometer (SRM Italia, Lucca, Italy).

After the baseline step at 50 W, the power output was incremented by 20 W every 3 minutes, until volitional exhaustion or until the cadence fell below 60 rpm. Oxygen consumption was monitored during all the trial, breath-by-breath, with a Cosmed K4b2 metabolimeter (COSMED, Rome, Italy). VO_{2max} was identified as the maximum value derived from the 15-breath moving average of oxygen consumption of the entire test, as suggested by Robergs et al. [30]. The blood lactate level was evaluated before the test and within the 30 seconds preceding the end of all stages,

with the Lactate Pro portable blood lactate meter (Arkray, Kyoto, Japan), puncturing with the lancing device the index finger of participants; blood lactate measurements were useful to define the individual training intensity zones, based on the following concentration values: zone 1, if the lactate concentrations were lower than 2.0 mM; zone 2, if the lactate concentrations were comprised between 2.0 and 4.0mM; zone 3, if the lactate concentrations were higher than 4.0mM. The heart rate (HR) was recorded with the Polar RS-800 heart rate monitor (POLAR, Kempele, Finland). The HR was monitored to determine values corresponding to LT1 and LT2 and maximal heart rate (HRmax) [31]. The percentages of the Pmax were used to determine the workloads to be undertaken during the experimental trials (e.g., power output (W) at a given % Pmax).

Furthermore, the same incremental test was repeated three days after the end of the nine-week high-intensity interval training (HIIT) period, to assess the training effects on the individual VO_{2max}, lactate thresholds and Pmax.

Study Design Protocol (Figure 1)

High-Intensity Endurance Cycling (HIEC) Test

On the experimental day, subjects were asked to arrive in the laboratory in a fasted state, two hours before the beginning of the test. All the participants had a standardized breakfast.

The HIEC test was performed on a "Technogym Group CycleTM Connect" equipped with a power-meter (Technogym S.p.A., Cesena, Italy).

During the test, the power output was checked by the power meter display, with a pre-defined cadence for the participants [32]. After a 2-minute warm-up, four 5-minute incremental stages of cycling were performed, at a workload corresponding to 50%, 55%, 60% and 70% individual Pmax. The last step was followed by ten 90-second sprints (SPR) at a workload corresponding to 90% individual Pmax, interspersed by a 180-second recovery stage at a workload corresponding to 55% individual Pmax. If the participant completed all the ten SPR, after the last 180-second recovery step, a time-to-exhaustion step was performed, at a workload corresponding to 90% individual Pmax. During the ten SPR, the exhaustion was considered as the inability to maintain the power output within 5 W of the expected 90% individual Pmax and the inability to restore it in a maximum of 15 seconds,

despite verbal encouragement; the same parameters were followed to calculate the time to exhaustion (TTE) in the last step, after the SPR.

Blood samples were collected just before the beginning of the HIEC test (t0), immediately after (t1), 4 hours (t2) and 24 hours (t3) after the HIEC test.

Moreover, the HIEC test was repeated after the HIIT period, performing also the incremental test three days before.

Training Protocol: HIIT period

The HIIT period was composed by 36 indoor cycling training sessions, performed in a 9-week period and structured in: 3 sessions per week in the first three weeks, of 53.1 ± 1.3 minutes; 4 sessions per week from the fourth to the sixth week, of 59.1 ± 1.2 minutes; 5 sessions per week from the seventh to the ninth week, of 68.2 ± 1.4 minutes.

All the sessions were taught and performed by an expert instructor, too, who verified that the same training program was performed by all the participants. The sessions followed the conventional principles highly used in the indoor cycling community, composed by warm-up, systematic interval exercise, and cool-down [33]. The training session program was elaborated according to the intensity distribution of a polarized model: 69 ± 3% of the session time in zone 1 (< LT2); 11 ± 2% in zone 2 (between LT1 and LT2); 20 ± 2% in zone 3 (> LT2), as described by Seiler et al. [31].

The HR of each participant was monitored with a Polar Team Pack 2 (POLAR, Kempele, Finland) and showed to the subjects being projected into the wall; the subjects have to maintain the same HR intensity zone which was maintained by the instructor.

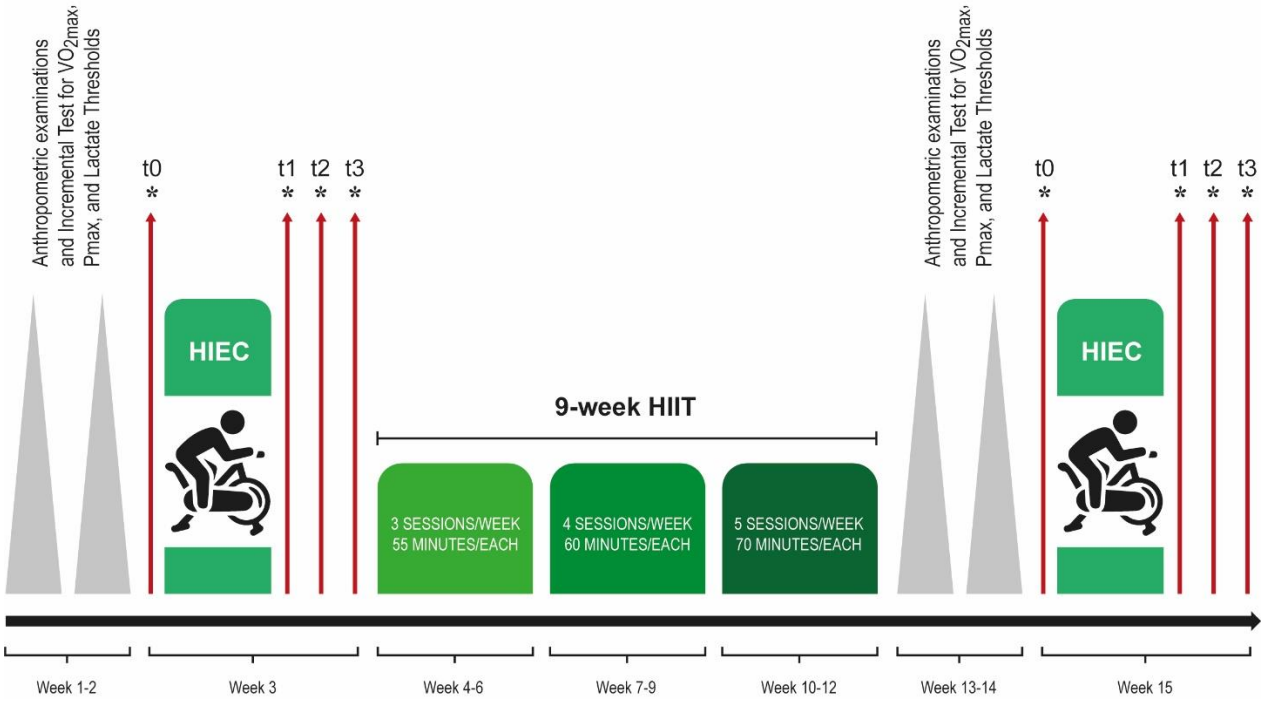

Figure 1: Study protocol design. High-Intensity Endurance Cycling sessions were performed before and after a 9-week training period. During each HIEC session, four blood samples were obtained. HIEC: High-Intensity Endurance Cycling session; HIIT: High-Intensity Interval Training period. *: blood samples. t0: pre-exercise sera; t1: immediately post-exercise sera; t2: 4-hours post-exercise sera; t3: 24-hours post-exercise sera.

ELISA Assays

Commercially available ELISA kits were used to determine the concentration of Creatine Kinase (CK) (Cloud-Clone Corp SEA109Hu; Aurogene S.r.l., Roma, RM, Italy). All samples were assayed in duplicate. The intra-assay coefficients of variation of the ELISA kits were: CK <10%.

Cell culture incubation experiments

Cancer cell lines

The triple-negative breast cancer (TNBC) cell line MDA-MB-231 and the prostate cancer cell line LNCaP were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). Cells were maintained in culture in Dulbecco's Modified Eagle Medium-high glucose, containing 4500 mg/L of glucose and supplemented with 10% Fetal Bovine Serum (FBS), 1x MEM Non-essential Amino Acid solution, 2 mmol/L L-glutamine, 1 mM Na-Pyruvate, 0.1 U/L penicillin, and 0.1 mg/ml streptomycin. Cells were maintained in a humidified incubator at 37 °C

and at 5% CO_2, during at maximum of fifteen passages. During experiments, the culture medium was replaced by Dulbecco's Modified Eagle's Medium without red phenol and without glucose, then supplemented with a physiological concentration of glucose (0.8 mg/ml); moreover, 10% FBS was replaced by with 5% of human serum obtained before or after the HIEC sessions. All cell culture materials were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Viability assay - cell proliferation assay

To study the effects of HIEC sessions and of HIIT period on cancer cell proliferative ability, cells were cultured with serum obtained at rest (t0 sera) or with exercise-conditioned sera (t1, t2 or t3). MDA-MB-231 and LNCaP cells were seeded at a density of 2×10^4 and 5×10^4 cells/well in 24-well plates, respectively, and after being attached to the bottom of the well, the culture medium was replaced by a serum-starvation medium for 18 hours. After overnight starvation, cells were incubated with the 0.8 mg/ml glucose culture media and stimulated with 5% v/v human sera, obtained at rest or after the HIEC session. Cell viability was assessed after 48 hours of incubation, trypsinizing and counting viable cells by hemocytometer and trypan blue exclusion test. The results are expressed as the total viable cells counted and derived from three different experiments.

Anchorage-independent assay (soft agar)

To evaluate the effects of HIEC sessions and of HIIT period on cancer cell ability to form three-dimensional colonies in anchorage-independent conditions, TNBC MDA-MB-231 and LNCaP prostate cancer cells were cultured in soft agar with 5% v/v serum obtained at rest (t0 sera) or with exercise-conditioned sera (t1, t2 or t3), as previously reported [34]. Briefly, 1×10^3 MDA-MB-231 cells and 0.5×10^3 LNCaP cells were resuspended in a 0.3% v/v agar layer and with 0.8 mg/ml glucose culture medium and stimulated with human sera and incubated for 18 days. After that period of time, cells were coloured with 0.01% crystal violet and only colonies formed by more than 20 cells were counted with a stereoscope.

Western blotting

To evaluate the possible mechanisms involved in the exercise-induced beneficial effects on TNBC cell proliferation and tumorigenesis modulation, MDA-MB-231 cells were seeded in 12-well plates at a density of 3 x 10^5 per well. After stimulation with human sera pre-HIEC or exercise-conditioned sera for 1 hour, cellular protein levels were analyzed as previously reported [35]. Moreover, cells of one well per each experiment were exposed to a culture medium without human sera, to have negative control. Briefly, after being lysed for 20 minutes on ice, cells were frozen and thawed twice and centrifuged at 4°C, at 12.000 rpm for 10 minutes. Lysis buffer was composed by 25 % v/v glycerol, 20 mmol/L HEPES, 0.42 mol/L NaCl, 1.5 mmol/L MgCl2, 0.2 mmol/L EDTA, 1 mmol/L NaF, 1 mmol/L Na3VO4, 0.5%v/v Nonidet P-40, and 1× complete protease inhibitor cocktail (Roche Diagnostics Ltd., Mannheim, Germany). The concentration of protein content (ug/mL) in all samples was measured by Bradford Reagent (Sigma-Aldrich, St. Louis, MO, USA), according to the manufacturer's instructions. Total cell normalized lysates were fractionated by SDS-PAGE and transferred to nitrocellulose membranes (0.2 μm pore size) (Bio-Rad Laboratories Inc., Hercules, CA, USA), to be incubated overnight with the selected primary antibody. The following antibodies were utilized: phospho-YAP (#4911), p-GSK3β (#8566 or #9322), GSK3β (#12456), phospho-p44/42 MAPK (ERK1/2) (Thr202/Tyr204) (#9101), p44/42 MAPK (ERK1/2) (#9102), β-actin (#8457), and GAPDH (#2118), purchased from Cell Signaling Technology (CST; Beverly, MA, USA). To detect protein bands, a horseradish peroxidase-conjugated secondary antibody has been used (Bio-Rad Laboratories Inc). The obtained blots were incubated with enhanced chemiluminescence reagents (AmershamHyperfilm ECL; GE Healthcare, Little Chalfont, UK) and immunoreactive bands were detected with ChemiDoc Imager (Bio-Rad Laboratories Inc). Resulting images were exported and analyzed with Image Lab Software (Bio-Rad Laboratories Inc).

Animal model tissues analysis

Thanks to a collaboration with Dr. Hojman and Professor Pedersen's working group (Centre for Physical Activity Research - CFAS, Rigshospitalet, University of Copenhagen), tissues from mice injected with MDA-MB-231 cells used for the project published by Dethlefsen et al in 2017 [27] were analyzed. In brief, mice were

randomized in control or exercise group, dividing them in cages with or without a running wheel, respectively, and the distance run by exercise-mice was monitored by an automated system connected with the running wheels.

The samples were analyzed by Western Blotting (as reported in "Western Blotting" section) to assess the phosphorylation level of GSK3β, to study the possible involvement of Wnt/β-catenin pathway in the exercise-induced reduction of tumor volume. Moreover, the possible modulation of the p44/42 MAPK ERK was evaluated. Furthermore, the effects of exercise on the expression levels of GSK3β mRNA and its target genes were evaluated by qPCR, as reported by "RNA isolation, cDNA synthesis, and qPCR" session.

RNA isolation, cDNA synthesis, and qPCR

Total RNA was isolated by Trizol reagent method (Invitrogen Life Technologies), and the concentration of RNA samples was determined by a nanodrop spectrophotometer (ThermoFisher Scientific). Reverse transcription reaction was performed with Reverse Transcriptase kit and random primers (Applied Biosystem), according to the manufacturer's protocol. All qPCRs were performed in with ViiA 7 Real-Time PCR System (ThermoFisher Scientific), in 384-well plates. All the samples were analyzed in triplicate, using SYBR Green PowerUp MasterMix (ThermoFisher Scientific) and the specific primers listed in Table 1. The relative expressions of the measured genes were calculated after normalization to the reference endogenous control gene Glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Differences in mRNA expression between the control and exercise group were determined using the $2^{-\Delta\Delta Ct}$ method [36].

Table 1. qPCR primers used to monitor the modulation of GSK3β-related target genes

Target mRNA	Accession number	Forward primer (5′-3′)	Reverse primer (5′-3′)
BCL2	NM_000633.2	GGACTGGCTAGTTAAACAAAGAGG	CTTATTAGATATGCCAAACCAGCTC
c-Myc	NM_002467.6	CACCAGCAGCGACTC	GATCCAGACTCTGAC
GSK3β	NM_002093.4	GTTAGTCGGGCAGTTGGTGT	GACATTTCACCTCAGGAGTGC
VEGF-B	NM_001243733.2	ATGGCCTGGAGTGTGTGC	GGGTACCGGATCATGAGGA
GAPDH	NM_002046.7	CTGACTTCAACAGCGACACC	TGCTGTAGCCAAATTCGTTGT

Statistical analyses

The effects of human sera used in this study are expressed as means of a minimum of three experiments.

Inferential statistics were evaluated by 2-way ANOVA with repeated measures (within-subject factors): the first factor considered (two levels) was the pre-post training timing; the second factor (four levels) was pre-, immediately post-, 4 hours post- and 24 hours post-HIEC test.

Mauchly's test (> 0.05) has been used to evaluate the sphericity assumption. In case of a lack of sphericity, Huynh-Feldt correction has been applied. Post-HIEC colony number was compared to the pre-HIEC colony number using simple contrast.

Multiple linear regression with backward stepwise elimination has been conducted using relative colony number weighted to the pre-HIEC colony number as the dependent variable. Predictors were TTE, LT1, LT2, VO_{2max}, VO_{2max}/kg, and CK levels.

Differences between western blot quantification have been evaluated by 1-way ANOVA with repeated measures followed by Dunnett's Multiple Comparison Test.

All statistical analyses were performed using SPSS software.

RESULTS

Effects of HIEC test-conditioned and HIIT period-conditioned human sera on triple-negative breast cancer and prostate cancer cell proliferation

TNBC cells MDA-MB-231 and prostate cancer cells LNCaP were stimulated with human sera pre (t0), post (t1), 4-hour post (t2) or 24-hour post (t3) HIEC session, considering both sessions, before and after the HIIT period.

Considering the effects due to the HIEC session before the training period, results revealed that all the exercised-conditioned sera led to a reduction in the cellular proliferative ability in comparison to rest serum (t1: -12.1%; t2: -24.0%; t3: -24.9% for TNBC MDA-MB-231 cells, and t1: -13.8%; t2: -21.8%; t3: -22.8% for LNCaP prostate cancer cells. $p < 0.05$ and $p < 0.001$) (Figure 2A); the same beneficial effects on reducing cancer cell proliferation were shown by the exercise-conditioned sera obtained by the HIEC session after the training period (t1: -15.7%; t2: -30.6%; t3: -35.3% for TNBC MDA-MB-231 cells, and t1: -14.0%; t2: -22.9%; t3: -27.2% for LNCaP prostate cancer cells ($p < 0.05$ and $p < 0.001$) (Figure 2B). To understand if the exercise-induced effects were linked to the HIEC sessions or also to the HIIT

period, the effects on cell proliferative ability of human sera taken at rest before and after the HIIT period were analyzed. Results showed that there is no statistically significant difference between the cellular responses after stimulation with sera taken at rest, before or after the training period (Figure 2C).

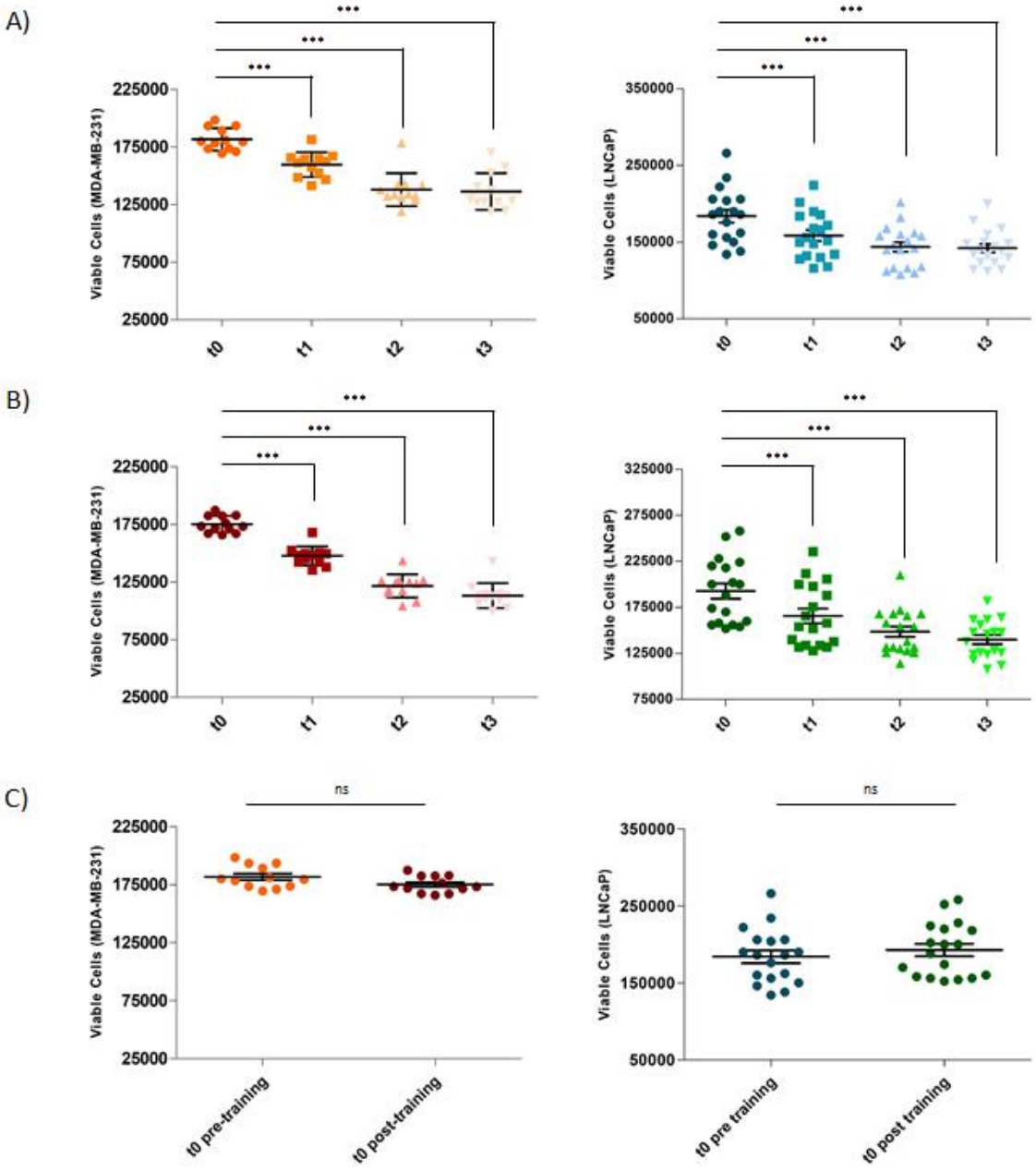

Figure 2: Anchorage-dependent growth assay. Assessment of the proliferative capacity of triple-negative breast cancer cells (MDA-MB-231 cells) and prostate cancer cells (LNCaP cells) after stimulation with sera obtained at rest or exercise-conditioned sera, before the training period (A) and after the training period (B). Comparison of viable cells after stimulation with sera obtained at rest, before and after the training period (C). t0: pre-exercise sera; t1: immediately-after exercise sera; t2: 4 hours post-exercise sera; t3: 24 hours post-exercise sera. ***: $p < 0.001$; n.s.: non-significant results.

Effects of HIEC test-conditioned and HIIT period-conditioned human sera on triple-negative breast cancer and prostate cancer cell tumorigenic potential

To study the tumorigenic potential of TNBC cells stimulated with human sera pre (t0), post (t1), 4-hour post (t2) or 24-hour post (t3) HIEC sessions, cells were cultured in soft agar for 18 days. Soft agar technique is a reliable method to study the capacity of cells to growth in anchorage-independent conditions and to form three-dimensional colonies, which are characteristics and ability of cancer cells, only [37]. After 18 days of incubation, MDA-MB-231 and LNCaP cell tumorigenic capacity were reduced by the two HIEC sessions-conditioned sera in similar ways. Particularly, considering the effects of human sera obtained after the HIEC session before the training period, all the exercised-conditioned sera (t1, t2, and t3) led to a significant reduction in the cellular tumorigenic ability in comparison to that obtained by at rest serum (t1: -17.6%. $p < 0.01$; t2: -33.7%. $p < 0.001$; t3: -30.0% for MDA-MB-231 cells, and t1: -15.95%; t2: -21.45%; t3: -25.94% for LNCaP prostate cancer cells. $p < 0.01$ and $p < 0.001$) (Figure 3A); the same effect on reducing cancer cell tumorigenic potential was obtained with the exercise-conditioned sera taken in the HIEC session after the training period (t1: -10.1%. $p < 0.01$; t2: -18.2%. $p < 0.001$; t3: -15.6% for MDA-MB-231 cells, and t1: -14.44%; t2: -20.31%; t3: -25.79% for LNCaP prostate cancer cells. $p < 0.01$ and $p < 0.001$) (Figure 3B). The effects on cell tumorigenic ability of human sera taken at rest, before and after the HIIT period, were considered, to understand if the exercise-induced effects were caused by the HIEC sessions or also by the HIIT period. Results showed that there is no significant difference between the cellular responses after stimulation with sera taken at rest, before or after the training period (Figure 3C). On the other hand, statistical analysis revealed an interaction between the variables time and HIEC session ($p = 0.013$). Particularly, the effects of the HIEC session' sera collected before the training period on MDA-MB-231 was higher than the effects obtained with sera collected after the training period.

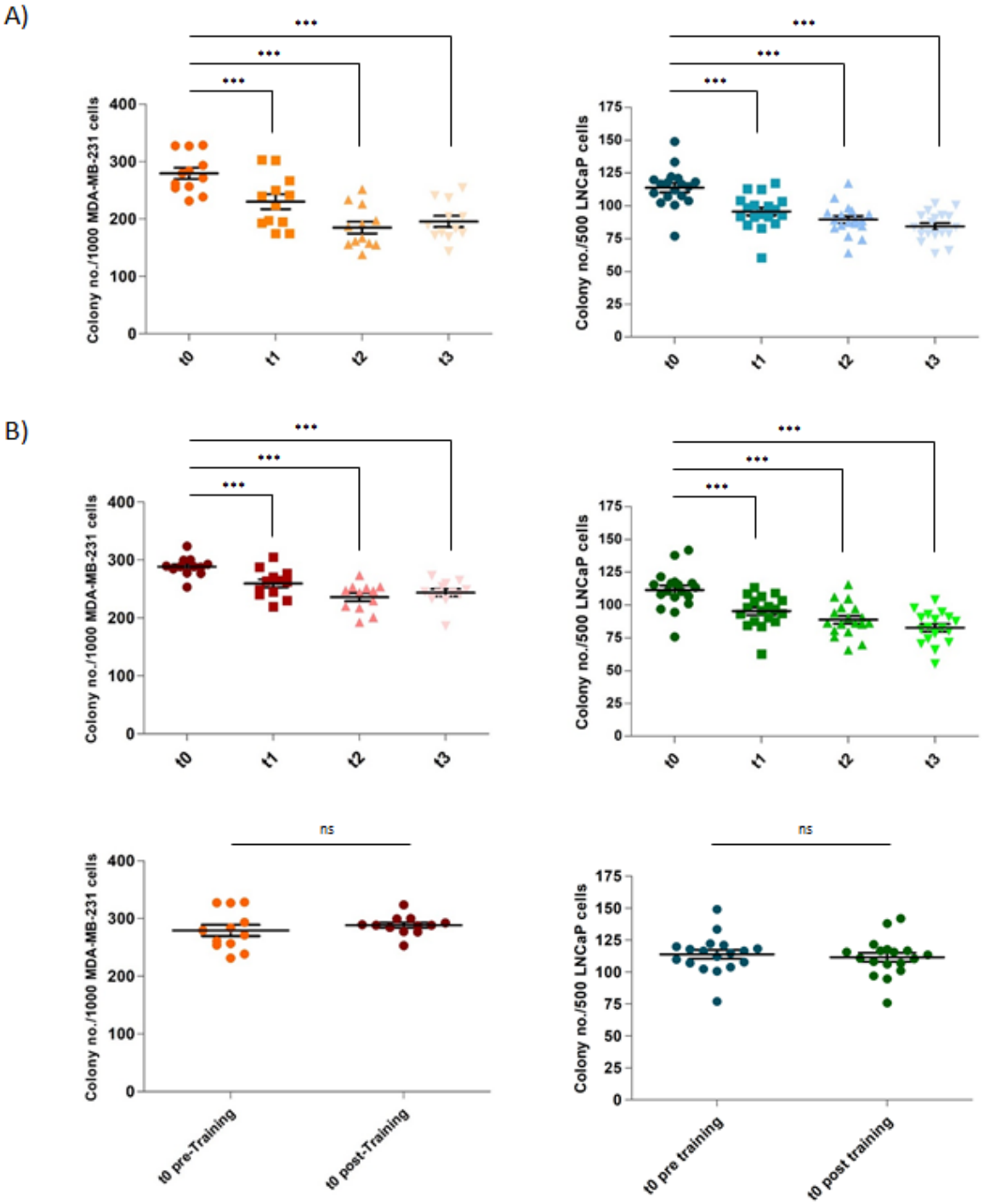

Figure 3: Anchorage-independent growth assay. Assessment of the tumorigenic capacity of triple-negative breast cancer cells (MDA-MB-231 cells) and prostate cancer cells (LNCaP cells) after stimulation with sera obtained at rest or exercise-conditioned sera, before the training period (A) and after the training period (B). Comparison of colony number after stimulation with sera obtained at rest, before and after the training period (C). t0: pre-exercise sera; t1: immediately-after exercise sera; t2: 4 hours post-exercise sera; t3: 24 hours post-exercise sera. ***: p <0.001; n.s.: non-significant results.

Relations between exercise parameters assessed during HIEC session and modulation of the tumorigenic capacity of cancer cells

To evaluate the possible relations between the effects of human sera collected during HIEC session on the colony number formation and exercise parameters (i.e. TTE, LT1, LT2, VO_{2max}, VO_{2max}/kg, and CK levels) a multiple linear regression analysis was performed. The analysis was performed using the LNCaP relative colony number weighted to pre-HIEC colony number (obtained in the pre-training HIEC session): t1/t0, t2/t0, t3/t0. Multiple adjusted R^2 showed a remarkable explanation of predictors set (t1/t0: $R^2 = 0.614$; t2/t0: $R^2 = 0.556$; t3/t0: $R^2 = 0.597$). The analysis revealed that the LNCaP relative colony number was directly related to LT1, and negatively related to VO_{2max}, suggesting that an increase of exercise intensity and anaerobic exercise are associated with a reduction of cellular tumorigenic potential. Even if the effect of human sera on MDA-MB-231 was higher than those obtained in LNCaP (e.g. -33.7% vs -21.45% at t2, respectively), multiple regression analysis did not reveal any significant effect of predictors, probably due to the different sample size of subjects stratified by gender.

Regulation of the Hippo pathway and Wnt/catenin pathway by HIEC session-conditioned sera in triple-negative breast cancer cells

Next, we evaluated if the Hippo pathway and Wnt/β-catenin pathway could be regulated by HIEC session-conditioned human sera. Stimulation of MDA-MB-231 cells with exercise-conditioned human sera obtained after the HIEC before the training period led to an average induction in phosphorylation of YAP protein, even if the results are not statistically significant, due to the intra-individual variability (Figure 4A). Moreover, the human sera obtained post-HIEC before the training period, do not change the phosphorylation levels of GSK3β protein in MDA-MB-231 cells, after stimulation with the exercise-conditioned sera for 1 hour (Figure 4B). Assessing the involvement of Hippo pathway and Wnt/β-Catenin pathway in exercise-induced effects in post-training HIEC session, we found that Hippo pathway does not seem to be involved in the reduced proliferation and tumorigenesis of TNBC cells obtained by post-training period exercise-conditioned sera, resulting in an unchanged levels of phosphorylation of YAP protein in cells stimulated by pre- or post-HIEC session (Figure 4C); on the other hand, an average statistically significant reduction of phosphorylated GSK3β resulted from t2 HS (sera obtained 4 hours after

HIEC; p <0.05) and from t3 HS (human sera obtained 24 hours after the HIEC session; p <0.01) stimulated cells with HS for 1 hour (Figure 4D).

Finally, in all protein samples from cells exposed to the culture medium without human sera, the phosphorylation of YAP protein increased and the phosphorylation of GSK3β protein decreased.

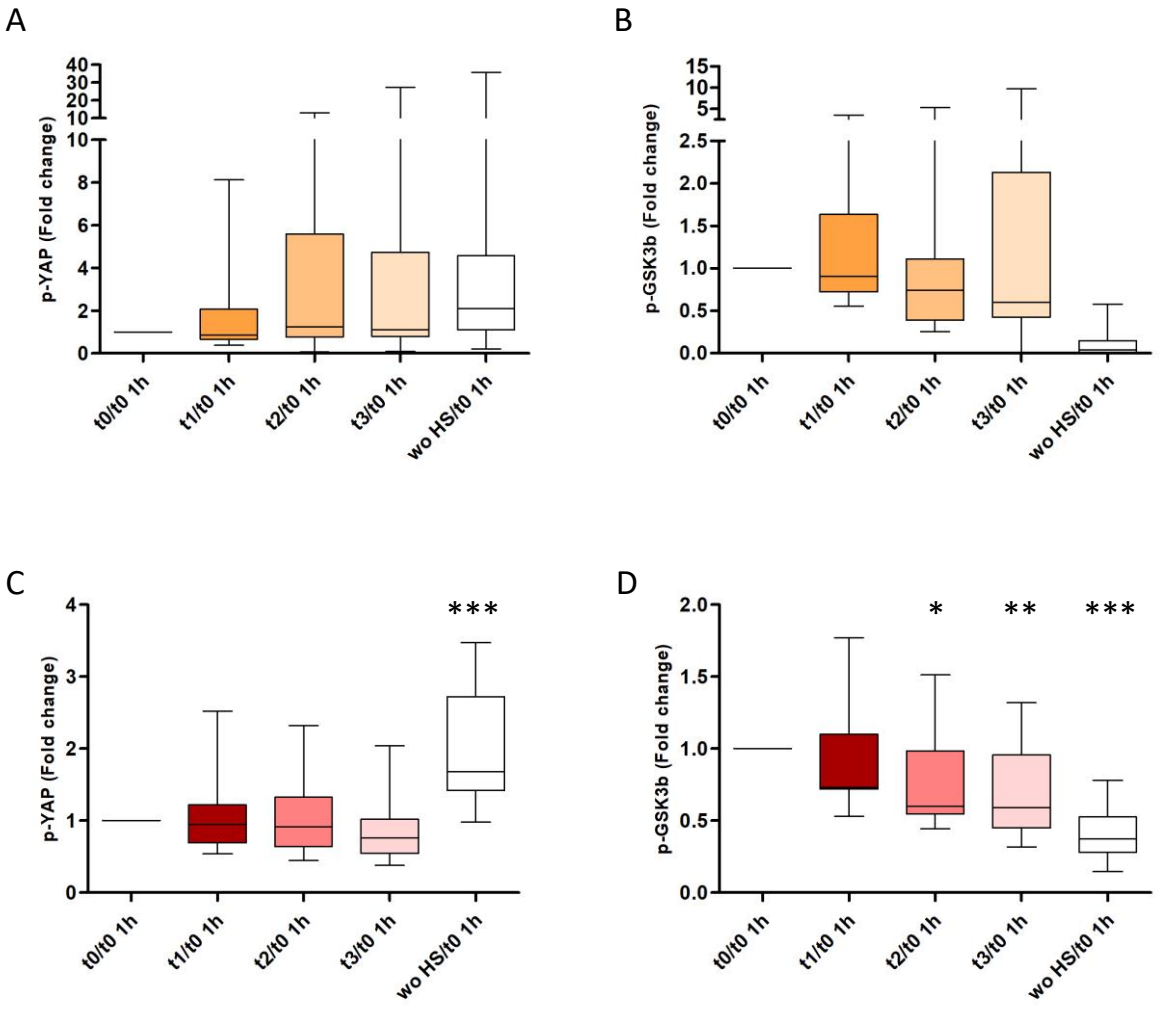

Figure 4: Phosphorylation levels of YAP and GSK3β proteins in MDA-MB-231 cells stimulated with sera collected pre- or post-session of exercise, before the training period (A and B, respectively) and after the training period (C and D, respectively). Results are normalized to β-Actin protein levels. Box and whiskers: 10–90 percentiles; medians are indicated by the black lines in the boxes. Results are expressed as relative data, versus the results obtained by the sera pre-exercise. t0/t0: pre-exercise sera versus pre-exercise sera; t1/t0: immediately post-exercise sera versus pre-exercise sera; t2/t0: 4-hours post-exercise sera versus pre-exercise sera; t3/t0: 24-hours post-exercise sera versus pre-exercise sera; w/o HS/t0: without human sera versus pre- exercise sera. *: p< 0.05; **: p< 0.01; ***: p< 0.001.

Involvement of the Hippo pathway and Wnt/catenin pathway in the voluntary wheel running-induced growth regulation of triple-negative breast cancer in mice

Female mice which were inoculated with MDA-MB-231 cells and which had access to the running wheels showed reduced tumor growth in comparison to the control group [27]. The authors found a 66% reduction in tumor volume induced by the voluntary wheel running, in comparison to the control group; moreover, evaluating if the Hippo pathway could be involved in the exercise-induced effects on tumor reduced growth, they did not find a significant difference between control and exercise group, in both Western Blotting analysis of phosphorylation of YAP protein and mRNA expression level of some YAP target genes by qPCR, suggesting that these results could be linked to the fact that the tumor tissues were excited when mice were at rest. We evaluated Wnt/β-catenin and p44/42 MAPK ERK pathways involvement in the exercise-induced effects (Figure 5A); a decreased level of GSK3β protein was found in the exercise group, in comparison to the control group and the phosphorylation level of GSK3β resulted partially reduced by the exercise ($p = 0.0848$). Furthermore, the modulation of the p-ERK pathway was evaluated, but no changes in its phosphorylation level were found between the exercise and the control group (Figure 5B).

Additionally, a down-regulation of GSK3β downstream genes was found in MDA-MB-231 tumors excised from the exercise group. In particular, the decreased expressions of GSK3β ($p = 0.0699$), BCL-2 ($p < 0.01$), c-Myc ($p = 0.0665$) and of VEGF ($p = 0.0833$) in the exercise group were shown (Figure 5C).

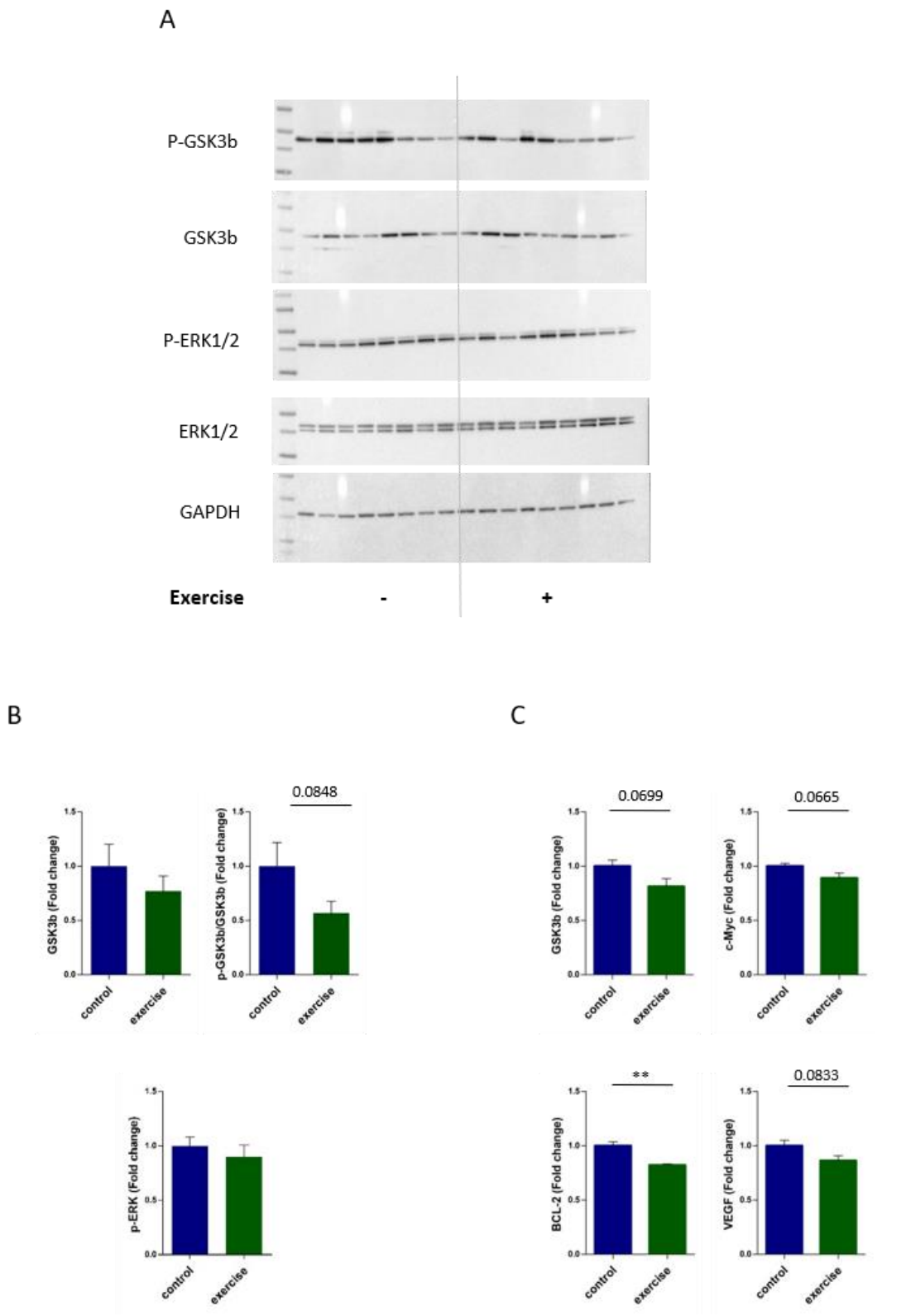

Figure 5: Phosphorylation levels of GSK3β protein and p44/42 ERK in MDA-MB-231 tumor tissues derived from mice of the control group or of the exercise group (A). Quantification of proteins level, normalized to GAPDH protein, in the control and exercise groups of mice (B). GSK3β mRNA and its target genes expression level in control or exercise group of mice, normalized to the reference endogenous control gene GAPDH (C). **: $p < 0.01$.

DISCUSSION

The risk of 13 different types of cancer decreases in the case of a high-level of leisure-time physical exercise, as shown by a pooled analysis of data from twelve different prospective studies [8]. Recent growing interest aims to understand how post-diagnosis physical exercise can induce those beneficial effects in cancer recurrence and in controlling cancer progression. As reviewed by Friedenreich and colleagues, an average 37% reduction in cancer-caused mortality in breast, colorectal and prostate cancer patients who were more active, in comparison to the less active ones has been reported by more than 20 different studies, lots of which assessed a decreased risk of recurrence, too [15].

Despite the great interest in physical exercise and the inhibition of cancer progression and recurrence of some types of cancer, little is known about the amount of exercise to be administered and the relationship between the physiological responses to the exercise bouts and cancer progression control.

In this study, we evaluated the effects of high-intensity endurance cycling (HIEC) sessions and of a high-intensity interval training (HIIT) period on triple-negative breast cancer (TNBC) subtype and in prostate cancer. Particularly, the present study aims to evaluate if both HIEC session and HIIT period can have a beneficial effects on TNBC and prostate cancer progression *in vitro*, and to study which are the possible mechanisms involved in those effects, also evaluating the possible relations between the effects of physical exercise on the control of cancer progression and the systemic factors measured during exercise bouts to understand the individual amount of exercise performed.

A large amount of epidemiological data showed an association between physical exercise and the lowered risk of different cancers [8]; a previous study demonstrated 9.4% reduction in breast cancer cell viability after being stimulated by sera obtained from healthy subjects or cancer patients, taken immediately after an exercise session, in comparison to cell viability resulted by the stimulation with at rest sera [26]. In line with these results, we obtained a reduction of TNBC cell viability by all sera taken after the HIEC session (t1: immediately-after HIEC session; t2: 4 hours after HIEC session; t3: 24 hours after HIEC session). Particularly, effects obtained by t1 sera were totally comparable to the one obtained by Dethlefsen and colleagues [26], leading to a 12.1% reduction in comparison to at rest sera.

Considering the effect of HIEC session on modulating the proliferative ability of prostate cancer LNCaP cells, our results agreed with those obtained by Rundqvist et al. [29], showing a decreased proliferative capacity of LNCaP cells after the high-intensity bout of physical exercise, in comparison to the proliferative ability given by at rest sera (t1: -13.8%). Moreover, the effects led by sera obtained 4 and 24 hours after the HIEC session (t2 and t3, respectively) have been considered for the first time. Interestingly, the resulting reduction in cell proliferative capacity given by t2 sera was maintained by the t3 sera, too, in both TNBC and prostate cancer cell lines.

After the first HIEC session, subjects have been trained for 9 weeks in a high-intensity interval training (HIIT) period, after which they performed a second HIEC session. This protocol allowed us to study the effects obtained by sera taken before and after the HIEC session, and to evaluate if the HIEC session performed after the HIIT period could modulate differently the proliferative capacity of cancer cell or if our results confirmed the previously obtained ones [26], which hypothesized that the exercise-induced effects on cell proliferation are mainly due to high-intensity exercise session and not by a training period.

Our results showed that the beneficial effects obtained by the HIEC session before the HIIT period are comparable to those obtained by the exercise-conditioned sera obtained by the HIEC session after the training period and that, also in this case, sera obtained 24 hours from the HIEC session induced a reduction in proliferative stimulation equivalent to the one obtained by t2 sera, suggesting that the beneficial effects of the intense session of exercise are maintained over time.

We have previously shown how to evaluate the antitumorigenic potential of physical exercise bouts in three-dimensional cell growth conditions (soft-agar assay) [34]; this technique allows the evaluation of the tumorigenic potential of cells *in vitro*, under different treatment or stimulating factors, mimicking the formation of three-dimensional tumor masses [37].

In the previously reported results, exercise-conditioned sera of healthy subjects decreased the ability of TNBC cells to form colonies, in comparison to sera taken at rest [34]; particularly, an average 8.7% reduction resulted by sera obtained immediately after exercise bout, and 12.3% reduction by sera obtained 2 hours after the exercise bout [34].

Starting from our preliminary results, in the present study we evaluated the capacity of TNBC, and prostate cancer cells stimulated with pre- or post-HIEC human sera to form three-dimensional colonies in soft agar.

Interestingly, the tumorigenic capacity of both cell lines resulted affected by all the exercise-conditioned sera, both before and after the training period. Moreover, maintenance of exercise-induced effects over time has been found: the reduction in the tumorigenic capacity revealed in cells treated with sera obtained after 24 hours of the exercise session is totally comparable to that obtained with post-4-hours sera.

The present approach could be useful also to assess if different exercise protocols could have different beneficial effects on cancer prevention and control in patients. To understand if the training period affected the results, the proliferative stimulatory capacity and tumorigenic capacity of sera obtained at rest (t0 sera) from the two HIEC sessions (before and after the training period) have been compared and no changes have been found, suggesting that the beneficial effects are mainly due to systemic responses to the high-intensity session of exercise. Given that the beneficial effects on cancer progression control of a training period have been suggested to be highly related to the weight loss usually induced by the training period [24], the weights of the subjects at the beginning and at the end of the study have been considered, resulting in no weight loss during the study (data not shown).

However, comparing the effects of HIEC sessions before and after the training period, we found that the reduction of MDA-MB-231 colony number obtained using the HIEC-conditioned sera before the training period was significantly higher with respect to colony number obtained using HIEC-conditioned sera after the training period. This could suggest that in women more trained the HIEC session could have a lower beneficial effect over time than in sedentary women.

To evaluate which molecular mechanisms could be involved in the mentioned exercise-induced effects on TNBC cell, the modulation of the Hippo signaling pathway has been evaluated. The Hippo signaling pathway is dysregulated in different cancer types, as also in breast cancer, in which the protein YAP is upregulated, leading to overexpression of the target genes, related to uncontrolled proliferation and associated with poor prognosis [38]. Previous data showed that exercise-conditioned sera were able to induce an increased level of phosphorylation of YAP protein and its further cytoplasmic retention and degradation in estrogen-responsive breast cancer cells [27]. Our results showed the possible modulation of

the Hippo pathway, assessing a partial increase in the average phosphorylation of YAP, induced by all the exercised-conditioned sera taken in the HIEC before the training period. However, the involvement of Hippo pathway was not shown by the exercise-conditioned sera taken after the HIEC post-training period, in which the phosphorylation of YAP seems to be constant in all samples.

Moreover, the possible modulation of Wnt/β-catenin pathway by physical exercise has been considered; this pathway plays a crucial role in tissue development and, if over-activated, it has been implicated in driving the formation of several human cancers [39]. Furthermore, the Hippo pathway and Wnt/β-catenin pathway seem to be linked: the phosphorylation of YAP with its further cytoplasmic retainment resulted in inhibition of β-catenin nuclear translocation and consequent suppression of Wnt/β-catenin pathway [40]. We found a significant decrease of GSK3β protein phosphorylation in cells stimulated with the exercise-conditioned human sera obtained after post-training HIEC session, suggesting a possible involvement of the inhibition of Wnt/β-catenin pathway in the effects of the intense exercise. The involvement of Wnt/β-catenin pathway has not been shown by the exercise-conditioned sera obtained after the HIEC session before the training period, suggesting that the same beneficial effects on TCNB and prostate cancer proliferation and progression given by the HIEC sessions before and after the training period could be maybe due to different biological mechanisms.

Finally, the possible involvement of Wnt/β-catenin pathway in exercise-induced tumor growth inhibition in mice shown by Dethlefsen et al. [27] was considered. The evaluation of GSK3β protein showed its decreased phosphorylation level in mice of the exercise group in comparison to the control group of mice and it has been confirmed by the downregulation of GSK3β mRNA and different GSK3β target genes.

The obtained results would suggest that Hippo pathway and Wnt/β-catenin pathway are both involved in the physical exercise effects on breast cancer control, and, considering the protocol design used in the present study, Hippo pathway seems to be modulated by HIEC before a training period, instead of Wnt/β-catenin pathway, which seems to be modulated by HIEC after the training period. However, the obtained data are preliminary results to be confirmed, because of the difficulty to study the transient phosphorylation of the proteins involved in the mechanisms that

were evaluated. Moreover, the given hypotheses have to be assessed also considering the molecular mechanisms modulated by HIEC sessions in prostate cancer cells.

Another fundamental aspect that was considered in the present study is the presence of possible relations between the modulation induced by physical exercise in the number of three-dimensional colonies formed in soft-agar assay, and the variations on the exercise parameters (i.e. TTE, LT1, LT2, VO_{2max}, VO_{2max}/kg, and CK levels) measured.

We found a positive relation between pre-training tumorigenic potential of LNCaP cells and LT1 and a negative relation with VO_{2max}; interestingly, these predictors (LT1 and $VO2_{max}$) resulted in explanatory variables for the results of colony number obtained by all the exercise-conditioned human sera, suggesting a strong relationship in every time-point considered. These results would suggest that the subjects who can reach a higher VO_{2max} and intensity of exercise, and who have a lower Lactate Threshold (LT1), with further use of the anaerobic metabolism during the exercise, show a better response in colony number decreasing after HIEC sessions.

However, the lower number of female subjects in the project did not allow the evaluation of the possible relations between breast cancer colony number and the predictors with the multiple regression method. Further investigations are needed to validate this method, with a bigger group of subjects.

This novel approach of performing multiple regression to find relations between the clonogenic potential of cancer cells and exercise parameters could identify some strong predictors to evaluate on each patient the responses to different protocols of exercise, allowing the customization of the exercise protocol to have the optimal response to exercise for cancer progression control.

In conclusion, we demonstrated the efficacy of HIEC bouts in cancer progression control, optimizing an experimental approach useful to quantify the effects of physiological changes induced by intense exercise on cancer cell biology.

Future directions will be aimed to apply this approach in breast and prostate cancer survivors, to find the optimal amount of exercise to be administered for recurrence and mortality risk reduction.

Acknowledgments

This paper is dedicated to the memory of Doctor Pernille Hojman, who was my supervisor during my experience abroad during the Ph.D. project, and who recently sadly passed away.

Moreover, the authors gratefully thank all the volunteers who participated in the study.

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CHAPTER 4

Original Article

Metformin prevents cell tumorigenesis through autophagy-related cell death

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Abstract

Autophagy is a cellular mechanism by which cells degrade intracellular components in lysosomes, maintaining cellular homeostasis. It has been hypothesized that autophagy could have a role in cancer prevention through the elimination of damaged proteins and organelles; this could explain epidemiological evidence showing the chemopreventive properties of the autophagy-inducer metformin. In this study, we analyzed the autophagy-related effect of metformin in both cancer initiation and progression in non-tumorigenic cells. We also analyzed the induction of tumorigenesis in autophagy-deficient cells, and its correlation with the ER stress. Our results showed that metformin induced massive cell death in preneoplastic JB6 Cl 41-5a cells treated with tumor promoter (phorbol) and in NIH/3T3 treated with H_2O_2. Inhibiting autophagy with wortmannin or ATG7 silencing, the effect of metformin decreased, indicating an autophagy-related cytotoxic activity under stress conditions. We also found induction of tumorigenesis in ATG7-silenced NIH/3T3 cell clone (3T3-619C3 cells), but not in wild-type and in scrambled transfected cells, and upregulation of unfolded protein response (UPR) markers in 3T3-619C3 cells treated with H_2O_2. These findings suggest that autophagic cell death could be considered as a new mechanism by which eliminate damaged cells, representing an attractive strategy to eliminate potential tumorigenic cells.

Introduction

Tumorigenesis is a complex and multistage process characterized by an accumulation of cellular damage promoted by chronic inflammation and exposure to carcinogens. Cancer prevention strategies could be addressed to different steps of the tumorigenic process, making the organism more resistant to mutagens/carcinogens and/or to inhibit disease progression by administering chemopreventive agents, inhibiting the initiation and/or progression of cell transformation [1].

Autophagy is the cellular mechanism appointed to the degradation of cytoplasmic components, maintaining cellular homeostasis through the elimination of damaged proteins and organelles. Despite autophagy is considered a survival mechanism for cancerous cells in the hostile tumor microenvironment, it could prevent chronic tissue stress that can induce cellular damage to proteins, organelles and DNA, inhibiting cancer initiation and progression [2-6].

Metformin, one of the most widely prescribed oral hypoglycemic agents, has recently received increased attention because of its potential antitumorigenic effects and because of the appealing strategy to repurpose drugs with well-described safety profiles [7-11]. Several epidemiological studies have documented a correlation between metformin and reduced cancer incidence and mortality; however, both animal and epidemiological studies have shown somewhat mixed effects and the epidemiological literature relates preferentially to individuals with diabetes [12]. The chemopreventive effect of metformin in non-diabetic subjects is still to be demonstrated, and the related cellular and molecular mechanisms are largely unknown. It has been hypothesized that metformin may have anticancer properties through different mechanisms, independent of its hypoglycemic effect; its main proposed anticancer molecular action is associated with the inhibition of mTORC1 - which is involved in metabolism, growth and differentiation of cancer cells [13] - mediated by AMPK activation or in an AMPK-independent manner. Other proposed mechanisms through which metformin could exert its anticancer effects include the induction of cell cycle arrest and/or apoptosis and the inhibition of the unfolded protein response (UPR) [14].

The UPR includes signal transduction pathways activated to overcome the perturbations of the endoplasmic reticulum (ER) homeostasis, known as “ER stress” [15], which is induced by an accumulation of unfolded/misfolded proteins, caused by depletion of Ca^{2+} levels, oxidative stress, low oxygen levels (hypoxia) or glucose deprivation [16]. Since the nutrient requirement of solid tumors can exceed the capacity of the cells’ microenvironment, hypoxia and glucose deprivation can occur, activating the UPR; this process is thought to be able to protect tumor cells from the stressful conditions of glucose deprivation and hypoxia as well as from immune surveillance [17].

The crosstalk between autophagy and ER stress is well known, and these two systems are dynamically interconnected, either stimulating or inhibiting each other. Moreover, the concurrence between ER stress and autophagy is common in several human pathologies, including neurodegenerative disorders, diabetes and cancer [18].

The aim of this study was to corroborate the role of autophagy in cancer initiation and progression, and to analyze the molecular pathways related to ER stress. Tumorigenesis was analyzed in the preneoplastic JB6 Cl 41-5a cells after autophagy inhibition with wortmannin, and in ATG7-silenced cell clones generated from non-

tumorigenic NIH/3T3 cells. The autophagy-related activity of metformin in these cell models was also evaluated.

Results

Metformin inhibits tumor promotion through autophagy-related cell death

To analyze the role of autophagy in cancer promotion, the pre-neoplastic JB6 P+ cell line has been used. These cells are sensitive to the growth induction by 12-O-Tetradecanoylphorbol 13-acetate (TPA) in both anchorage-dependent and -independent culture conditions, as shown in Fig. 1. Cell count of adherent cells after 5 days of incubation with the tumor promoter TPA increased of about 2.5-fold, whereas the anchorage-independent colony formation in soft agar, a hallmark of malignant transformation, after 21 days of incubation with TPA increased of 1.6-fold. To induce autophagy during tumor promotion we treated cells with 10 mM of metformin, an hypoglycemic drug with a well note ability to induce autophagy [14]. We found that metformin neither inhibits cell viability of non-proliferating pre-neoplastic cells plated at confluence, nor colony formation in soft agar. On the other hand, in TPA-treated JB6 P+ cells, metformin showed a marked cytotoxic effect, significantly reducing the number of viable cells and increasing the number of dead cells in anchorage-dependent conditions (Fig. 1A); moreover, in presence of TPA, metformin completely inhibited the colony formation in soft agar (Fig. 1B). To evaluate the role of autophagy in the cytotoxic activity of metformin, JB6 P+ cells were treated with the autophagy inhibitor wortmannin followed by TPA, metformin and combination treatments. Wortmannin alone did not significantly change cell proliferation or colony formation (not shown). Interestingly, wortmannin reversed the effect of metformin in adherent cells inhibiting its cytotoxic effect in presence of TPA (Fig. 1A), suggesting that metformin could induce autophagic cell death during tumor promotion. The effect of wortmannin was only partially confirmed in soft agar culture (Fig. 1B). To evaluate if apoptosis is involved in metformin-induced cell death, cells were treated with the pancaspase inhibitor zVAD-fmk; as shown in Fig. 1A, the cytotoxic activity of metformin in TPA-stimulated cells did not change after zVAD-fmk treatment, suggesting that apoptosis is not involved in JB6 P+ cell death.

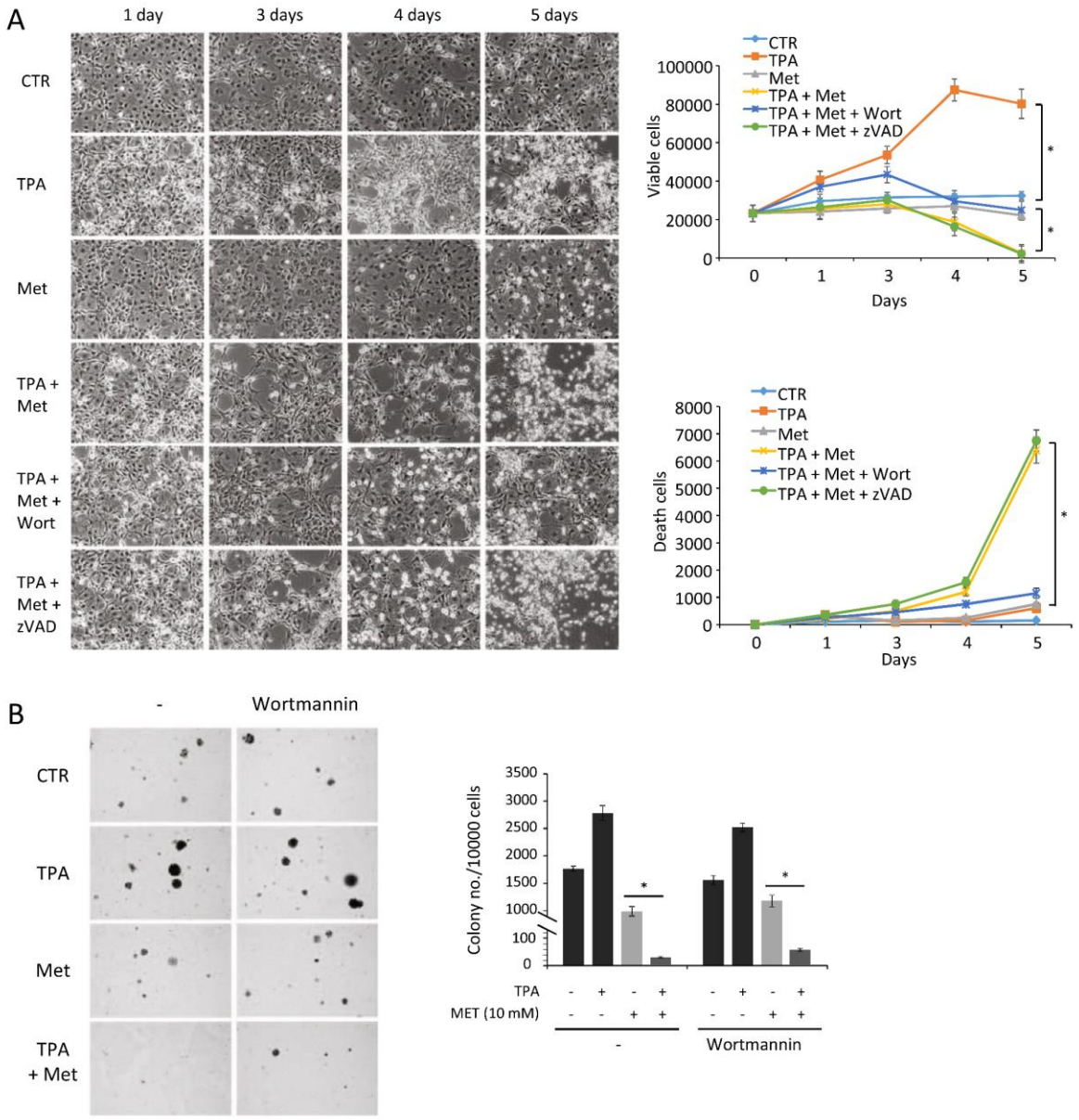

Figure 1. Effect of metformin on tumor promotion in JB6 P+ cells. Tumor promotion was induced with TPA and analyzed by (A) anchorage-dependent (adherent cells) and (B) –independent (soft agar) growth assays. JB6 cells were treated with TPA (10 ng/ml) and metformin (10 mM) for 5 days (adherent cells) or 3 weeks (soft agar); autophagy was inhibited with wortmannin (2 μM). Cell count of adherent cells and colony count of soft agar assays are shown as means ± SEM; $N = 3$, $P < 0.001$.

Next, we analyzed the molecular pathways involved in metformin activity finding an inhibition of phospho-ERK1/2 in cells treated with TPA and metformin, without wortmannin; phospho-JNK was found to be stimulated in TPA-treated cells but not in presence of metformin without wortmannin (Fig. 2A). These results suggest an autophagy-related inhibition of ERK1/2 and JNK signaling by metformin. The analysis of the autophagic markers LC3 and p62 confirmed that metformin is able to induce autophagy; in fact, p62 is upregulated after 8 h and decreases after 24 hours after metformin treatment; the analysis of LC3 also showed an increase of autophagic flux induced by metformin, that is converted in wortmannin-treated cells after 24 hours (Fig. 2B). Our results also show that metformin counteracts the activity of chloroquine, an inhibitor of the autophagic flux able to block the autophagosome fusion with lysosome; as shown in Fig. 2C, metformin reduces the accumulation of p62 and LC3-II induced by chloroquine, indicating induction of the autophagic flux. Again, the western blotting results did not reveal caspase-3 cleavage (Fig. 2B), suggesting that the apoptotic process was not involved in the cytotoxic activity of metformin. As positive control of apoptosis, JB6 P+ cells were treated with cisplatin for 48 hours showing a dose-dependent caspase-3 cleavage (Fig. 2D); the cleavage of caspase-3 was inhibited treating cells with zVAD-fmk (Fig. 2D), confirming the efficiency of zVAD-fmk in inhibiting apoptosis in our cell model (Fig. 1A).

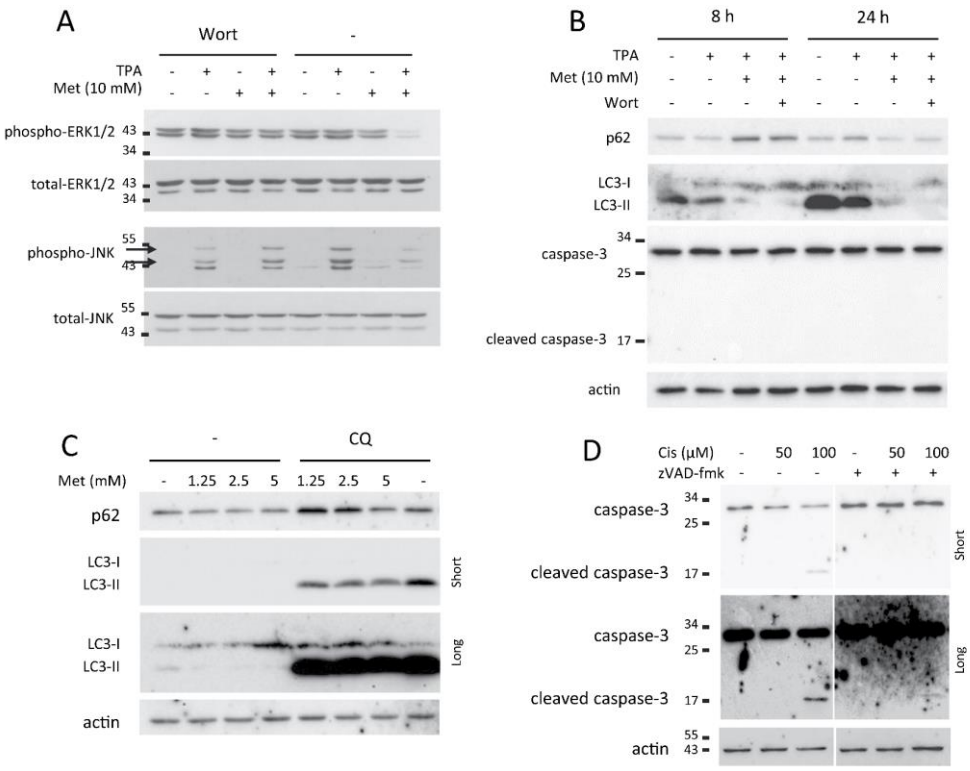

Figure 2. Western blotting analysis in JB6 P+ cells. Cells were cultured with TPA (10 ng/ml) for 24 hours and treated with metformin (10 mM) for an additional 8 (B) and 24 (A, B) hours. Autophagy was inhibited with wortmannin (2 μM) (A, B) or chloroquine (100 μM) (C); apoptosis was induced with cisplatin (50 and 100 μM) and inhibited with zVAD-fmk (10 μM) (D). Phosphorylation of ERK1/2 and JNK (A), p62 and LC3 (B, C), and caspase-3 cleavage (B, D) were analyzed. Total ERK1/2, total JNK and actin were used as a loading control. $N = 3$. Cropped blots are shown. Uncropped blots are presented in Supplementary Fig. S1.

Induction of tumorigenesis in shATG7 NIH/3T3 cells

To evaluate the role of autophagy in the tumorigenic cell transformation process, NIH/3T3 *Atg7*-silenced single-cell clones have been generated. Cells were transfected with shRNA plasmids containing four different sequences targeted to the *Atg7* gene, that encodes an E1-like activating enzyme essential for autophagosome formation (Supplementary Materials, Table S1). Single-cell clones were selected and analyzed for ATG7 protein expression as described in the material and methods section. The ATG7 expression and basal lipidated LC3 amount were analyzed by western blotting to evaluate the stability of transfection (not shown). The NIH/3T3 shATG7 clone 619-C3 (3T3-619C3 hereafter) was selected for further experiments. As transfection control, the clone 3T3-SCRD3 transfected with non-effective scrambled shRNA was selected. To evaluate whether the *Atg7* silencing

inhibits the autophagic process, NIH/3T3, 3T3-619C3 and 3T3-SCRD3 cells were starved in serum-deprived culture medium for 4 hours and autophagosome formation was analyzed by immunofluorescence of LC3. The results showed LC3 puncta in wild type and scrambled-transfected 3T3-SCRD3 cells, but not in ATG7-silenced 3T3-619C3 cells (Fig. 3A).

The two-stage transformation assay was carried out using MNU as tumor initiator and H_2O_2 as tumor initiator/promoter. First, the ATG7 expression and LC3-I/II amount were evaluated in cells treated with sub-lethal doses of H_2O_2. Our results showed that the ATG7 protein was not expressed in 3T3-619C3 cells, and that the level of protein did not change after H_2O_2 treatment (Fig. 3B). Likewise, 3T3-619C3 cells showed low amount of LC3-II protein (the lipidated form of LC3) (Fig. 3B) in both control cells and H_2O_2-treated cells, demonstrating that the ATG7 protein is required for the formation of autophagosomes, and sub-lethal doses of H_2O_2 did not induce autophagy. Next, MNU/H_2O_2 two-stage cell transformation was carried out and, after two weeks of treatment, cell transformation was monitored by colony formation in soft agar. Results showed that the capability of anchorage-independent proliferation significantly increased only in ATG7-silenced cells (Fig. 3C; $p < 0.01$). Moreover, morphological cell transformation was revealed by foci formation of MNU/H_2O_2-treated shATG7 cells in anchorage-dependent culture conditions (Fig. 3C).

There is growing evidence that the endoplasmic reticulum stress and the related UPR could have a role in cancer [19]. The UPR was investigated in NIH/3T3, 3T3-619C3 and 3T3-SCRD3 treated with H_2O_2 in order to evaluate the crosstalk between autophagy and the response to the endoplasmic reticulum stress. The stress marker phospho-p38 and UPR marker CHOP were analyzed by western blotting in cells treated with H_2O_2 (20 μM) for 4 and 24 hours (Fig. 3D). The UPR markers CHOP, ATF3, ATF4, GRP78/BiP, CHAC1 and CEBPB were also analyzed by qPCR in cells treated with H_2O_2 (20 μM) for 4 hours (Fig. 3E). Results showed that H_2O_2 treatment induced the stress marker phospho-p38 in all tested cells; instead, UPR markers were significantly upregulated more in shATG7 3T3-619C3 cells than in the wild type and/or scrambled-transfected cells treated with H_2O_2 (Fig. 3D, E).

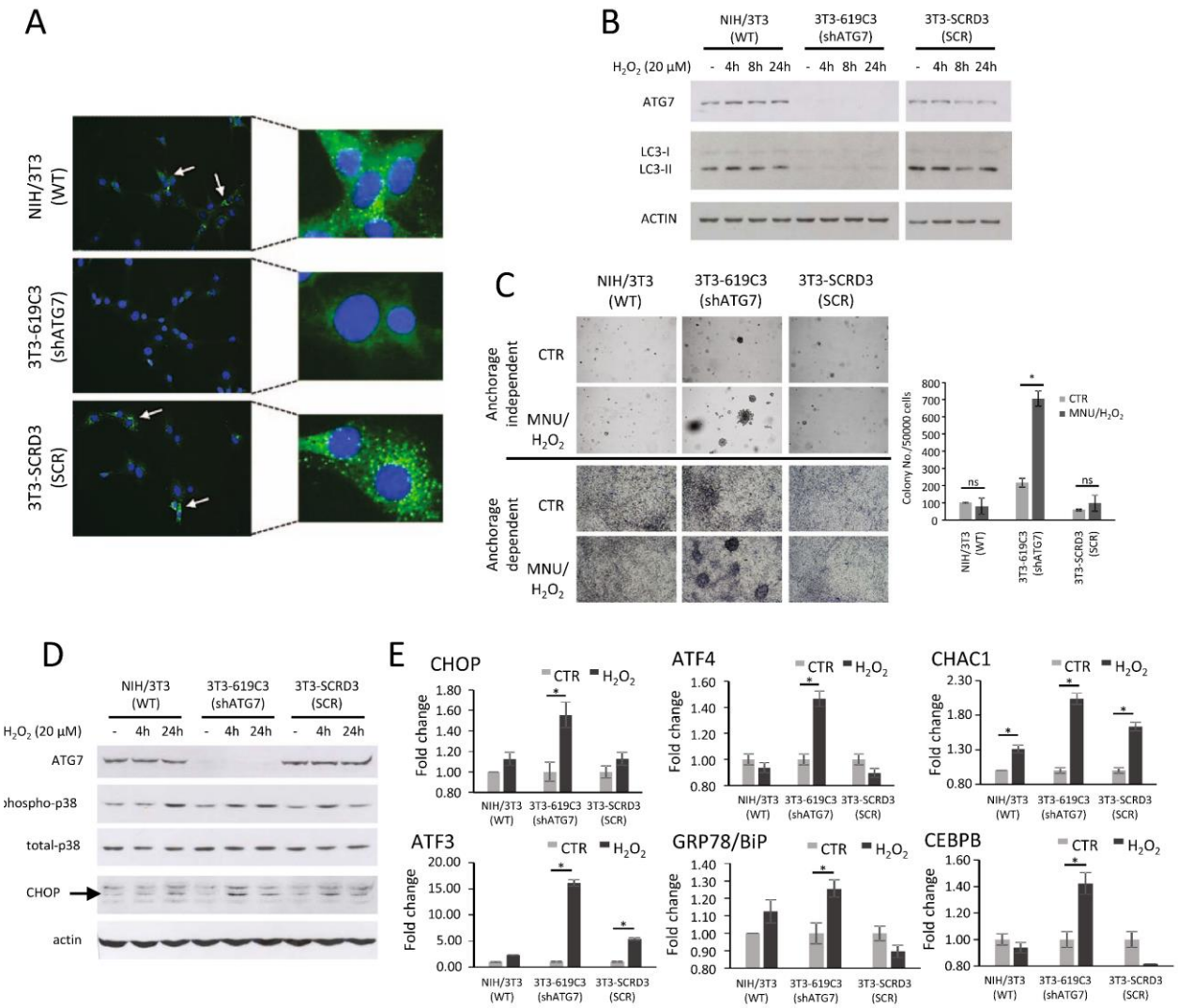

Figure 3. Induction of tumorigenesis in wild type, shATG7 and scrambled transfected NIH/3T3 cells, and unfolded protein response analysis. (A) Immunofluorescence analysis of LC3A/B protein in cells starved with the serum-deprived medium for 4 hours. LC3A/B fluorescent puncta indicate autophagosome formation. (B) Western blotting analysis of ATG7 and LC3B-I/II in cells treated with H_2O_2 20 μM for 4, 8 and 24 hours. Actin was used as a loading control. $N = 3$. Cropped blots are shown. Uncropped blots are presented in Supplementary Fig. S2. (C) Two-stage tumorigenesis induction. Cells were treated for 1 hour with MNU (50 μg/ml) and cultured up to three weeks in medium containing H_2O_2 (10 μM). Cell transformation was analyzed by anchorage-dependent (adherent cells) and –independent (soft agar) growth assays. Colonies were counted after 21 days. Data are means ± SEM; $N = 3$, $P < 0.001$. (D) Western blotting analysis of ATG7, phospho-p38 and CHOP in cells treated with H_2O_2 (20 μM) for 4 and 24 hours. Actin and total p38 were used as a loading control. $N = 3$. Cropped blots are shown. Uncropped blots are presented in Supplementary Fig. S2. (E) Real-time PCR gene expression analysis of CHOP, ATF3, ATF4, GRP78/BiP, CHAC1 and CEBPB in cells treated with H_2O_2 (20 μM) for 4 hours. Data are means ± SD. $N = 3$, $P < 0.01$.

Metformin induces autophagy-related cell death in tumor-initiated cells

Cell damage accumulation could predispose cells to the tumorigenic transformation. To evaluate the potential role of autophagy in inhibiting cell viability of damaged cells, NIH/3T3, 3T3-619C3, 3T3-619D3, 3T3-621B1, 3T3-621B6 and 3T3-SCRD3 cells were treated with 50 µg/ml of MNU for 1 hour and cultured for one week with 10 µM H_2O_2 - to induce tumorigenesis - and 10 mM metformin to induce autophagy. Cell survival was evaluated by cell count, revealing that metformin induced cytostatic effect in all cell lines; on the other hand, in MNU/H_2O_2 treated cells, metformin inhibited cell viability in NIH/3T3 and 3T3-SCRD3 but not in shATG7 cells (Fig. 4A), suggesting an induction of autophagy-related cell death in damaged cells.

To evaluate if apoptosis is involved in metformin-treated 3T3-SCRD3 cell death, cells were treated with metformin and MNU/H_2O_2 with or without the pan-caspase inhibitor zVAD-fmk. Our results show that neither cell viability nor cell death induced by metformin significantly changed in 3T3-SCRD3 cells treated with zVAD-fmk (Fig. 4B).

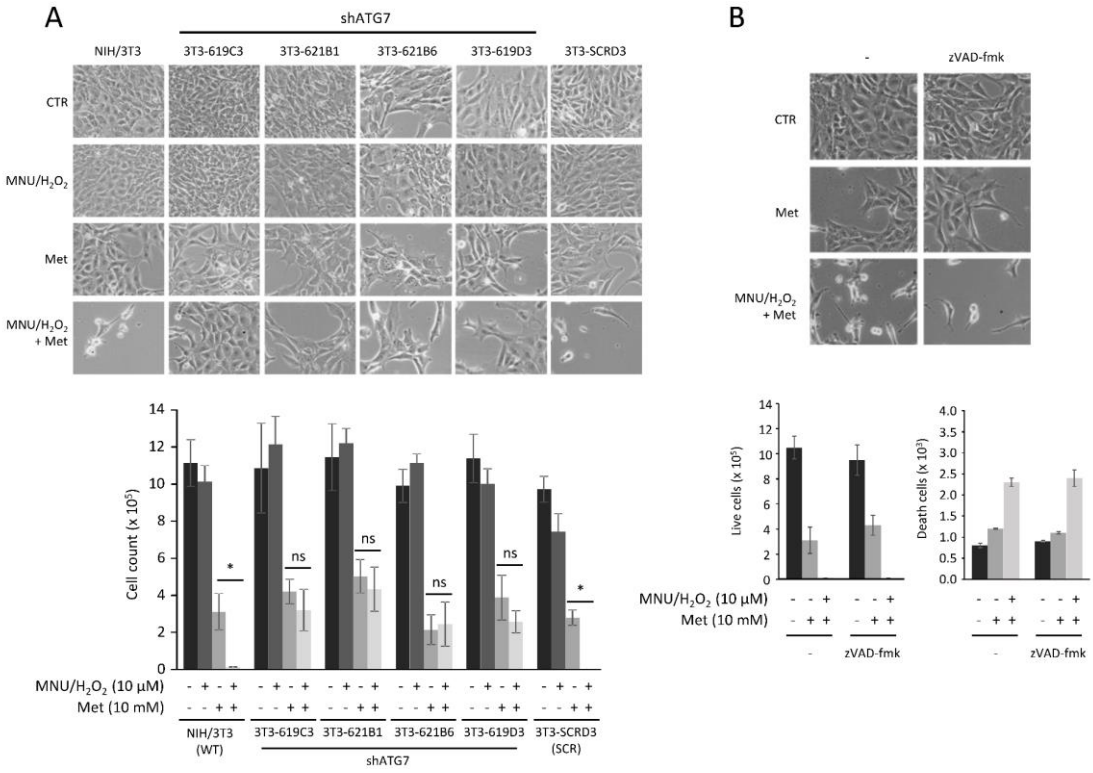

Figure 4. Metformin activity in wild type (A), shATG7 (A) and scrambled transfected (A, B) NIH/3T3 cells. Cell viability was evaluated after 1-hour treatment with MNU (50 µg/ml) and 1-week culture with H_2O_2 (10 µM) and metformin (10 mM) (A, B), with or without zVAD-fmk (B). After treatments, cells were counted and photographed at 10× magnification. Data are means ± SEM; $N = 3$, $P < 0.001$.

To evaluate the molecular mechanisms involved in metformin-induced autophagic cell death, apoptosis markers and MAP kinases activation were analyzed by western blotting analysis. 3T3-619C3 and 3T3-SCRD3 cells were treated with 10 μM H_2O_2 for 24 hours to induce cell damage, and then treated with metformin for an additional 24 hours. Metformin was able to inhibit ERK1/2 phosphorylation in 3T3-SCRD3 cells, but not in shATG7 3T3-619C3 cells (Fig. 5A). To evaluate the induction of the autophagic flux by metformin, p62 accumulation was evaluated. The results showed that metformin induced an increase in p62 expression after 24 hours, and a decrease after 48 hours in 3T3-SCRD3 cells; this effect was not revealed in shATG7 3T3-619C3 cells (Fig. 5B), indicating that autophagy was efficiently inhibited in the ATG7-silenced cells.

On the contrary, results did not reveal caspase-3 cleavage (Fig. 5A), suggesting that metformin did not inhibit cell viability via apoptosis. Again, as a positive control of apoptosis, 3T3-SCRD3 cells were treated with cisplatin and zVAD-fmk. Our results show that cisplatin induced a dose-dependent caspase-3 cleavage after 48 hours, that was found to be inhibited in zVAD-fmk-treated cells (Fig. 5C). These results suggest that metformin could induce autophagic cell death inhibiting molecular pathways involved in cell proliferation and/or survival.

We also analyzed the UPR markers in cells treated with H_2O_2, metformin or combinations by qPCR and western blotting. The 3T3-619C3 and 3T3-SCRD3 were treated with 10 μM H_2O_2 for 24 hours, and then treated with metformin for an additional 4 hours. Our results showed that metformin significantly induced the overexpression of *CHOP*, *ATF4*, *ATF3*, *CHAC1* and *CEBPB* genes in 3T3-619C3, while only *ATF3* and *CHAC1* were found to be significantly upregulated in 3T3-SCRD3, but to a lower extent than in 3T3-619C3 cells (Fig. 5D). In H_2O_2-pretreated 3T3-619C3 cells, *CHOP*, *ATF4*, *ATF3* and *CHAC1* genes were found to be upregulated by metformin (Fig. 5D). However, the expression levels of both spliced and unspliced XBP-1 and PDI mRNAs did not increase neither in 3T3-619C3 nor in 3T3-SCRD3 cells (Fig. 5D). Western blotting analysis confirmed the overexpression of CHOP in 3T3-619C3 treated with metformin after H_2O_2 pretreatment, and, interestingly, also revealed overexpression of GRP78/Bip in metformin-treated cells, differently to the gene expression analysis in which GRP78/Bip was found significantly upregulated in H_2O_2-treated cells only (Fig. 5A).

Taken together, our results showed that the UPR was preferentially induced by metformin in damaged, autophagy-defective cells.

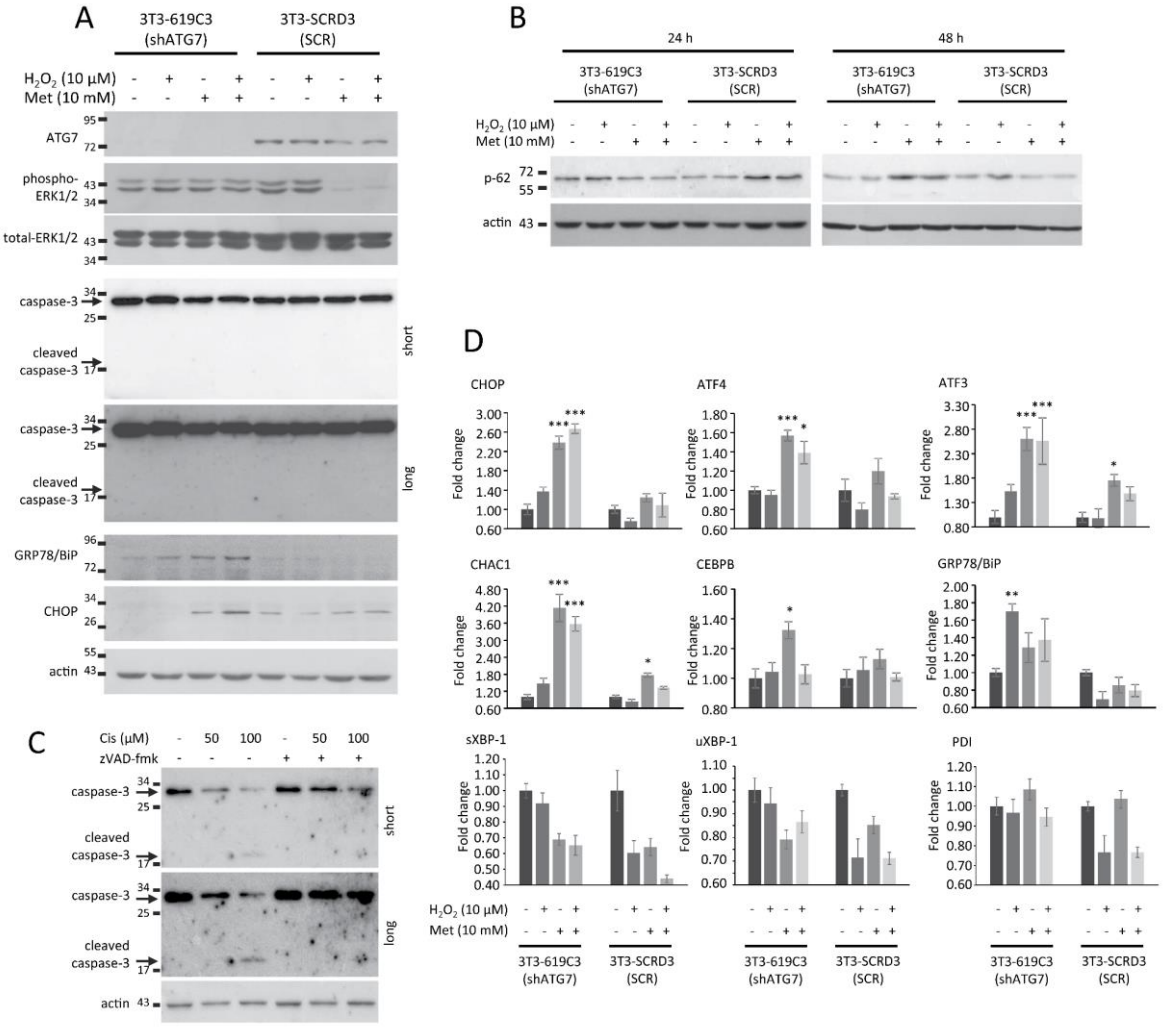

Figure 5. Evaluation of molecular changes in 3T3-619C3 and 3T3-SCRD3 cells. Western blotting analysis of ATG7, phospho-ERK1/2, caspase-3 cleavage, GRP78/Bip, CHOP (A) and p62 (B) in cells cultured overnight with H_2O_2 (10 μM) and treated with metformin (10 mM) for 24 (A, B) and 48 (B) hours. (C) Western blotting analysis of caspase-3 cleavage in 3T3-SCRD3 cells treated with cisplatin and/or zVAD-fmk for 48 hours. Total ERK1/2 (A) and actin (A, B, C) were used as a loading control. $N = 3$; Cropped blots are shown; Uncropped blots are presented in Supplementary Fig. S3 (D) Gene expression analysis of shATG7 and scrambled transfected NIH/3T3 cells cultured for 24 hours with H_2O_2 (10 μM) and treated with metformin (10 mM) for 4 hours. Data are means ± SD. $N = 3$; $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

Discussion

Given that cancer is a complex multistage process and that autophagy performs its effects in different ways, the role of autophagy in tumorigenesis can be evaluated in a context-dependent manner. Once cancer occurs, autophagy is upregulated to make the survival of cancer cells in the hostile tumor microenvironment. On the other hand, it can be considered as a cytoprotective pathway, that prevents chronic tissue damage which could lead to cancer initiation and progression; in this case, autophagy stimulation or restoration could be useful for chemoprevention.

Metformin, a biguanide anti-diabetic drug, is able to trigger autophagy by AMPK activation and subsequent inhibition of mTOR, which is one of the major inhibitors of the autophagic flux [14]. Epidemiological studies showed that the use of metformin in diabetic patients is associated with a decrease in various types of cancer incidence, most significantly in pancreatic cancer, hepatocellular carcinoma, and colon cancer [12, 20, 21]. However, whether metformin has activity against cancer in non-diabetics still has to be demonstrated. It has been hypothesized that metformin could act with both direct and indirect mechanisms, primarily decreasing glucose, IGF-1 and insulin signaling, thereby creating an unfavourable environment for tumor growth that is similar to that created by caloric restriction [22, 23]. Through AMPK activation and mTORC signaling inhibition, metformin suppresses protein synthesis and cell proliferation [24, 25].

In this study, we evaluated the effect of metformin on tumor cell progression; for this purpose, the preneoplastic cell line JB6 cl 41-5a P+ has been used. The JB6 P+ cells are sensitive to TPA stimulation, which induces growth in soft agar and foci formation in adherent culture conditions, two hallmarks of tumorigenesis. Our results show a cytostatic effect of metformin in JB6 P+ cells. However, in presence of TPA, metformin induces massive cell death in both anchorage-dependent and -independent culture conditions. In adherent cells, the cytotoxic effect of metformin was inhibited by wortmannin but did not change using the pan-caspase inhibitor z-VAD-fmk, indicating an autophagy-related effect and excluding apoptosis. The autophagic process may be essential for cell death in certain settings, in which excessive or uncontrolled levels of autophagy are able to induce autophagy-dependent cell death [26]. These results underline the potential of metformin as antitumor agent: we can suppose a selective effect of this molecule, that eliminate only preneoplastic cells stimulated to form a tumor mass. At molecular levels, we found an inhibition of

ERK1/2 and JNK phosphorylation only in cells treated with metformin and TPA, but not in presence of wortmannin, indicating that metformin could act via autophagy in the inhibition of proliferative-related signaling.

Autophagy is a process that involves several proteins, starting from phagophores formation and multimerization of Atg proteins, to LC3 processing and insertion in extending phagophores membrane, targets capturing for degradation and autophagosomes – lysosomes fusion [27]. ATG7 is a protein with a pivotal role for the initial step of autophagy, being essential during the formation of phagophores: it acts like an E1 ubiquitin-activating enzyme, activating ATG5-ATG12 conjugation, which is involved in phagophore extension step [27]. Loss of autophagy through *Atg7* knockdown causes severe physiological dysfunction; for instance, *Atg7*-defective mice showed neurodegeneration [28] and undergone to atrophy, myopathy and loss of muscle mass [29,30].

Our results show that the silencing of the *Atg7* gene leads to an increased cell transformation of the non-tumorigenic NIH/3T3 cells induced by tumor promoters. In fact, after two-stage cell transformation with MNU and H_2O_2, only shATG7 cells (3T3-619C3 cells) showed a significant increase of anchorage-independent growth and foci formation in anchorage-dependent culture conditions.

Despite it has been shown that loss of *Atg7* is not sufficient to trigger a tumor-prone phenotype [4], our results suggest that ATG7 could have a role in the maintenance of cell integrity under stress conditions, eliminating damaged proteins and organelles that could induce tumorigenesis, and supporting the role of autophagy induction for cancer prevention.

Autophagic pathways are strictly related to ER stress. The ER is responsible for post-translational modifications and appropriate folding of proteins. Because this process is complex and error-prone, the capacity of ER can be saturated under some physiological or pathological conditions, such as glucose deprivation, hypoxia, oxidative injury or increased protein synthesis. In these cases, accumulation of unfolded proteins occurs and leads to a signaling response, the UPR, which is activated to recovery ER functions, through translation attenuation, upregulation of the folding machinery, and ER-associated degradation mediated by ubiquitin-proteasome complex and autophagy [18]. Therefore, autophagy represents a fundamental mechanism that targets both partially processed proteins and protein aggregates [31].

In this context, we found a persistent ER stress condition in autophagy-deficient cells, revealed by UPR markers upregulation (CHOP, ATF3, ATF4, GRP78/BiP, CHAC1 and CEBPB) in 3T3-619C3 cells treated with H_2O_2, suggesting an accumulation of damaged proteins and corroborating the autophagy-UPR crosstalk. These results underline the importance to inhibit damaged protein accumulation for carcinogenesis prevention.

Despite autophagy plays a crucial pro-survival role in cell homeostasis, it has been shown that in extreme conditions of starvation or stress, autophagy could induce a programmed cell death [32, 33].

We analyzed the effect of metformin in tumor initiation using the NIH/3T3 cells and the ATG7-silenced NIH/3T3 clones. This cell line is characterized by a high proliferation rate, making difficult to distinguish between antiproliferative or cytotoxic effect. However, we analyzed the effect of autophagy induction using metformin in our cell models. Our results show that under stress conditions induced by MNU/H_2O_2, the effect of metformin in NIH/3T3 cells seems to be cytotoxic. Interestingly, this effect was not revealed in 3T3-619C3 cells, suggesting that metformin is able to induce autophagy-related cell death in damaged cells, eliminating potential tumorigenic cells. This evidence needs to be deeply analyzed, improving cell damage accumulation in order to treat cells for shorter time, reducing cell proliferation.

The mitogen-activated protein kinases (MAPKs) signaling pathway is a key regulator of cell growth and survival in physiological and pathological processes and has a critical role in driving tumor initiation and progression [34]. We revealed a correlation between metformin effect and the inhibition of ERK1/2 phosphorylation, which was found to be inhibited only in 3T3-SCRD3 cells. This suggests that metformin reduces cell proliferation and survival through autophagy, inhibiting ERK1/2 signaling.

UPR inhibition is another mechanism that has been proposed for the potential antitumorigenic effect of metformin [14]. Under stress conditions such as in solid malignancies, cells increase the protein folding capacity of the ER; if this is inhibited, cells activate signaling pathways leading to cell death, as demonstrated in human cancer cells treated with metformin [17]. In addition, the activation of UPR branches has been widely reported in a variety of human tumors including glioblastoma, lymphoma, myeloma and carcinoma of the cervix and breast [19]. The

UPR is mediated by 3 transmembrane ER stress sensors IRE1, PERK and ATF6, which activate 3 signaling cascades to restore ER homeostasis. The 3 UPR branches and autophagy are strictly interconnected [18]. In fact, the IRE1 signaling can regulate autophagy through induction of sXBP1; ATF6, which induces XBP1 expression, can cooperate with IRE1; the PERK signaling can promote autophagy through induction of SESN2 and DDIT4/REDD1, two inhibitors of mTOR [18].

In autophagy-deficient cells (3T3-619C3), the treatment with metformin significantly activated the PERK/eIF2α/ATF4 branch of UPR, compared to 3T3-SCRD3 cells, as revealed by the upregulation of PERK signaling UPR downstream markers (CHOP, ATF3, ATF4, GRP78/BiP, CHAC1 and CEBPB).

On the other hand, the IRE1 pathway appeared not to be activated in both cell lines, since the splicing induction of XBP-1 was not detected. Furthermore, the activation of the ATF-6 was not observed since the expression levels of XBP-1 [35] and PDI mRNAs (two ATF6 target genes) did not increase in metformin-treated cells. Our results revealed an increase of UPR markers in 3T3-619C3 cells treated with metformin, especially with the addition of H_2O_2, suggesting that metformin could mitigate ER stress by inducing autophagy. In this view, the accumulation of unfolded/damaged proteins, that cannot be eliminated by the autophagic flux, results in an upregulation of UPR.

In conclusion (Fig. 6), the autophagy-UPR crosstalk could protect cells exposed to stress or tumor promoter, preventing the accumulation of damaged proteins and organelles and maintaining cell homeostasis; if autophagy is inhibited, cell damage could accumulate inducing tumorigenesis; metformin could inhibit this process inducing a selective autophagic cell death, *de facto* preventing tumor initiation. Although preliminary, these findings support the epidemiological evidence of metformin chemopreventive properties and provide the rationale to deeply analyze the role of autophagy inducers in foods [36] and physical exercise-induced autophagy [37] for cancer prevention.

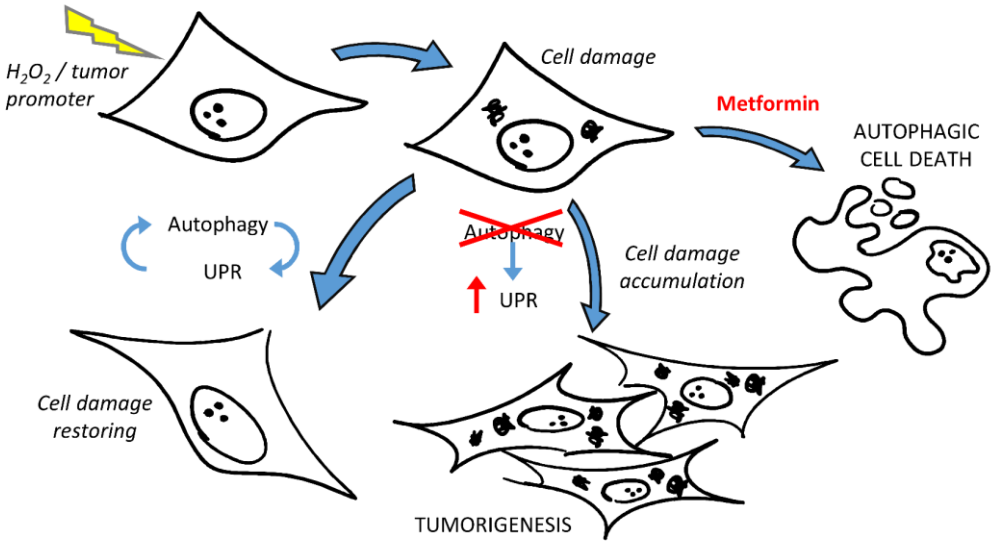

Figure 6. Representative scheme of results. Cells exposed to stressor or tumor promoter undergo cell damage; the autophagy-UPR crosstalk restores damaged protein and organelle maintaining cell homeostasis; inhibiting autophagy, the UPR increased indicating an accumulation of damaged proteins, which could induce tumorigenesis; metformin could trigger autophagic cell death in damaged cells eliminating potentially tumorigenic cells.

Materials and Methods

Cell lines and cell culture

The JB6 Cl 41-5a promotion-sensitive (JB6 P+) cell line was obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA); the NIH/3T3 cell line was kindly provided by Dr. Stefano Amatori (European Institute of Oncology, Milan - Italy) and verified in our laboratory. Cells were cultured in DMEM (NIH/3T3) or EMEM (JB6 P+) media supplemented with 10% or 5% fetal bovine serum (FBS) for NIH/3T3 or JB6 P+ cells respectively, and 2 mmol/L L-glutamine, 1 × MEM Non-essential Amino Acid Solution, 0.1 mg/ml streptomycin, 0.1 U/L penicillin and 1 mM Na-pyruvate (JB6 P+ only). Cells were maintained in a humidified incubator (5% CO_2) at 37 °C during at maximum of fifteen passages. All cell culture materials were purchased from Sigma-Aldrich (St. Louis, MO, USA).

ATG7 silencing and clones isolation

Stable transfection in NIH/3T3 cells was carried out using TransIT®-X2 (Mirus Bio, Madison, WI, USA) as transfection reagent according to the manufacturer's instructions. Cells were plated in 12-well plates at a density of 1×10^5 cells/dish in antibiotics free culture media, incubated overnight and transfected with shRNA

plasmid panels (OriGene Technologies, Inc., Rockville, MD, USA) containing four ATG7-specific shRNA sequences (Supplementary Materials, Table S1) and one non-effective scrambled shRNA sequence. After 72 hours, cells were harvested and plated in 60 mm dishes at a density of 1×10^5 cells/dish with 1 μg/ml puromycin for the antibiotic selection step. After 1 week, cells were harvested and plated in 96-well plates at a density of 1 cell/well with 1 μg/ml puromycin. Next, single-cell clones were expanded in 24-well plates and analyzed in western blotting for ATG7 expression. ATG7-silenced clones were stored in liquid nitrogen vapor phase for further experiments.

Anchorage-independent transformation assay (soft-agar assay)

Wild type NIH/3T3 cells, ATG7-silenced clone 3T3-619C3 and scrambled transfected 3T3-SCRD3 were seeded on 35 mm cell culture dishes at a density of 2×10^4 cells/dish. After overnight incubation, cells were treated for 1 hour with *N*-methyl-*N*-nitrosourea (MNU), washed with fresh medium and cultured in growth medium containing 10 μM H_2O_2. Weekly, cells were harvested and passaged at a density of 2×10^4 cells/dish in growth medium containing 10 μM H_2O_2. The passages were repeated up to three times. At passage two and three, 5×10^4 cells/35-mm dish of each condition were seeded in soft agar as described previously [38]. Briefly, cells were suspended in 1 ml of 0.3% w/v agar in complete medium and layered over the bottom layer consisted of 1 ml of 0.6% w/v agar in complete medium.

JB6 P+ cells were directly suspended in 0.3% agar in complete medium with 10 ng/ml of 12-O-Tetradecanoylphorbol 13-acetate (TPA), 10 mM metformin, 2 μM wortmannin or combinations, and layered over the bottom layer.

Weekly, 500 μl of complete medium was added to maintain humidity. Cells were incubated at 37 °C and 5% of CO_2 for 21 days. The colonies were then stained with crystal violet (0.01% w/v) and counted with a stereoscope. Only clusters containing more than 20 cells were counted as colonies.

Cell viability assay

Wild type NIH/3T3 cells, ATG7-silenced clones 3T3-619C3, 3T3-619D3, 3T3-621B1, 3T3-621B6, 3T3-430A1 and scrambled transfected 3T3-SCRD3 cells were seeded at a density of 2×10^4 cells/dish in 35 mm dishes and treated as indicated in the figures. After 6 days, cells were trypsinized, plated at a density of

2×10^4 cells/dish in 35 mm dishes, and treated for additional 6 days. Cell viability was evaluated by trypsinization and cell counting by trypan blue exclusion assay using a hemocytometer.

Western blotting

After treatments, cellular protein expression and phosphorylation were analyzed by western blotting as previously reported [39]. Briefly, cells were lysed for 20 min on ice with 20 mmol/L HEPES (pH 7.9), 25% v/v glycerol, 0.42 mol/L NaCl, 0.2 mmol/L EDTA, 1.5 mmol/L MgCl2, 0.5%v/v Nonidet P-40, 1 mmol/L NaF, 1 mmol/L Na3VO4, and 1× complete protease inhibitor cocktail (Roche Diagnostics Ltd., Mannheim, Germany). The cell lysates were frozen and thawed twice and clarified by centrifugation at 12,000 rpm for 10 min at 4 °C. Total cell lysates were fractionated by SDS-PAGE and transferred to a nitrocellulose membranes (0.2 μm pore size) (Bio-Rad Laboratories Inc., Hercules, CA, USA). The following primary antibodies have been used: ATG7 (#2631), LC3B (#2775), phospho-p38 MAPK (Thr180/Tyr182) (#9211), p38 MAPK (#9212), phospho-SAPK/JNK (Thr183/Tyr185) (#9251), SAPK/JNK (#9252), phospho-p44/42 MAPK (ERK1/2) (Thr202/Tyr204) (#9101), p44/42 MAPK (ERK1/2) (9102), BiP (C50B12) (#3177), CHOP (L63F7) (#2895) and Caspase-3 (#9662) purchased from Cell Signaling Technology (Beverly, MA, USA); Actin (#A2066) purchased from Sigma-Aldrich. Protein bands were detected using a horseradish peroxidase-conjugated secondary antibody (Bio-Rad Laboratories Inc). Blots were treated with enhanced chemiluminescence reagents and the immunoreactive bands were detected with chemiluminescence film (Amersham Hyperfilm ECL; GE Healthcare, Little Chalfont, UK).

Immunofluorescence

The NIH/3T3, 3T3-619C3 and 3T3-SCRD3 cells were seeded in 4-well chamber slide at a density of 5×10^4 cells/well, incubated overnight and serum-starved for 4 hours. Next, cells were fixed with 4% paraformaldehyde for 15 minutes, permeabilized with 0.2% TritonX-100, blocked with 1% of goat serum and incubated overnight at 4 °C with LC3A/B (D3U4C) (Alexa Fluor 488 Conjugate; #12082; Cell Signaling). After DAPI staining, cells were mounted with Fluoreshield (Sigma) and photographed with a fluorescence microscope.

Quantitative real-time PCR (qPCR)

The NIH/3T3, 3T3-619C3 and 3T3-SCRD3 cells were lysed with 700 µl QIAzol Lysis Reagent (Qiagen, Hilden, Germany) and total RNA was purified using the miRNeasy Mini kit (Qiagen), following manufacturer's instructions. The RNA was quantified using a NanoVue Plus™ spectrophotometer (GE Healthcare Life Sciences, Piscataway, NJ, USA) and 500 ng were used for the cDNA synthesis with the PrimeScript™ RT Master Mix (Perfect Real Time) (Takara Bio Inc., Otsu, Shiga, Japan).

The expression of selected ER stress marker genes was monitored by qPCR as previously described [40], using primers listed in Table 1. B2M (beta-2-microglobulin) and GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) were used as reference genes. Differences in mRNA expression were determined using the $2^{-\Delta\Delta Ct}$ method [41]. All RT-qPCR expression values were the result of 3 independent biological replicates.

Target mRNA	Accession number	Forward primer (5′-3′)	Reverse primer (5′-3′)
CHOP	NM_007837	GAGTCCCTGCCTTTCACCTT	TTCCTCTTCGTTTCCTGGGG
HSPA5	NM_001163434	TCCGGCGTGAGGTAGAAAAG	GGCTTCATGGTAGAGCGGAA
ATF3	NM_007498	CTCTCACCTCCTGGGTCACT	TCTGGATGGCGAATCTCAGC
ATF4	NM_001287180	GCAGTGTTGCTGTAACGGAC	ATCTCGGTCATGTTGTGGGG
CEBPB	NM_009883	ACCGGGTTTCGGGACTTGA	TTGCGTCAGTCCCGTGTCCA
CHAC1	NM_026929	TATAGTGACAGCCGTGTGGG	GCTCCCCTCGAACTTGGTAT
SXBP1	NM_001271730	CTGAGTCCGCAGCAGGT	TGTCCAGAATGCCCAAAAGG
UXBP1	NM_013842	CCGCAGCACTCAGACTATG	TGTCCAGAATGCCCAAAAGG
PDI	NM_011032.3	GATCAAGCCCCACCTGATGA	ACCTCTTCAAAGTTCGCCCC
B2M	NM_009735	TGCTATCCAGAAAACCCCTCAA	GGATTTCAATGTGAGGCGGG
GAPDH	NM_001289726	TGCCCCCATGTTTGTGATG	TGTGGTCATGAGCCCTTCC

Table 1. qPCR primers used to monitor the expression of ER stress-related genes.

CHOP, Mus musculus C/EBP homologous protein; HSPA5, Mus musculus heat shock protein 5; ATF3, Mus musculus activating transcription factor 3; ATF4, Mus musculus activating transcription factor 4; CEBPB, Mus musculus CCAAT/enhancer-binding protein (C/EBP), beta; CHAC1, Mus musculus ChaC, cation transport regulator 1; sXBP1, Mus musculus X-Box Binding Protein 1, transcript variant 2 (spliced); uXBP1, Mus musculus X-Box Binding Protein 1, transcript variant 1 (unspliced); PDI, Mus musculus Protein disulfide-isomerase; B2M, Mus musculus Beta-2-Microglobulin; GAPDH, Mus musculus glyceraldehyde-3-phosphate dehydrogenase.

Statistical analysis

Statistical analyses were performed using the unpaired student's t test, and one-way or two-way ANOVA as appropriate, followed by Bonferroni's multiple comparison post hoc tests (GraphPad Software, Inc., La Jolla, CA, USA).

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Author Contributions

M.D.S. and G.Ba. conceived and designed the experiments; M.D.S., G.Ba. and G.F.S. performed cell culture, western blotting and immunofluorescence experiments; A.D. performed gene expression experiments; M.D.S., G.Ba., A.D. and L.G. performed data analysis; G.Br. and G.F.S. contributed reagents/materials/analysis tools; L.G. and G.Br. contributed intellectually to the interpretation and discussion of results; M.D.S. wrote the manuscript; All authors reviewed the manuscript.

Notes

Competing Interests

The authors declare no competing interests.

Footnotes

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Electronic supplementary material

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CONCLUSIONS

The present thesis gave rise to new evidence about the beneficial effects of physical exercise on triple-negative breast cancer (TNBC) and prostate cancer prevention and progression; a first crucial goal of the present work was the optimization of the three-dimensional soft agar assay as a possible technique to assess the modulation performed by physical exercise on TNBC and prostate cancer tumorigenic capacity, based on an *in vitro* model that mimics the formation of the tumor [1]. This technique is widely used for the evaluation of the clonogenic capacity of cancer cells under different pharmacological treatments [2], but it has never been used before in the assessment of exercise effects in modulating cancer cell tumorigenic capacity.

Moreover, this study allowed the evaluation of beneficial effects given by intense sessions of exercise (High-Intensity Endurance Cycling tests, HIEC) or by a training period (High-Intensity Interval Training, HIIT) in decreasing the proliferative activity and tumorigenic potential of TNBC and prostate cancer cells. Our results were in accordance with previously reported ones, which showed that the decreased capacity of breast cancer cells to proliferate after exercise could be mainly due to the intense session and not to the training period [3; 4]. Furthermore, these results of the beneficial effects of HIEC sessions were confirmed in prostate cancer cells, considering the proliferative and tumorigenic capacities.

The results obtained by the evaluation of the possible molecular mechanisms involved in exercise-induced effects on TNBC cell proliferation and tumorigenic abilities would suggest a possible impairment of two different pathways: the Hippo pathway and the Wnt/β-catenin pathway, with a partial increased phosphorylation level of YAP protein given by the exercise-conditioned pre-training human sera and a significant decreased phosphorylation level of GSK3β protein resulted by the exercise-conditioned post-training human sera. However, given that the assessment of the phosphorylation level of the proteins can be difficult because of the transient characteristic of the process and because of the variations between the subjects, further studies will be aimed to confirm these preliminary data.

The method proposed in this thesis could be applied to evaluate the possible relations between the decreased clonogenic capacity given by exercise-conditioned human sera and the variations of exercise parameters usually measured during physical exercise, to understand the amount of exercise performed and the individual's response to it (i.e. TTE, LT1, LT2, VO_{2max}, VO_{2max}/kg, and CK levels). Interestingly, our results showed the positive relation between the decreasing in prostate cancer

cells colony number after stimulation with pre-training exercise-conditioned sera and the level of individual VO_{2max}, and a negative relationship with the Lactate threshold (LT1); these results could give rise to a novel approach to evaluate which are the exercise parameters that could be used as strongly related predictors of the beneficial effects of exercise on cancer progression control and to find the optimal exercise protocol in a customized way, for every cancer patient.

Finally, given the uncertainty of the molecular mechanisms involved in the physical exercise-induced beneficial effects found in this project, and given that the autophagic process has been hypothesized as one of the mechanisms involved in cancer prevention [5], we decided to assess the effects of a pharmacological approach in cancer prevention and progression. For this reason, metformin has been used in evaluating the autophagy-related effects in cancer initiation and progression in non-tumorigenic cells, and in assessing the tumorigenic process induction in autophagy-deficient cells, under H_2O_2 stress, too [6]. The results showed the crucial role of autophagy as an inducer of massive cell death in pre-neoplastic cells in the presence of tumor promoter phorbol, and in H_2O_2-stressed cells. This was confirmed by the fact that metformin did not lead to cell death in autophagy-deficient cells and it would suggest that autophagic cell death can be considered as a mechanism involved in eliminating damaged cells and potential tumorigenic cells, inhibiting the cancer initiation process.

Starting from the obtained results, a possible synergism between physical exercise and metformin will be considered in future studies, to study its possible role in TNBC and prostate cancer prevention and modulation.

Conclusions and Future Perspectives for Intervention Programs

- The data of the first pilot study (Chapter 2) of this thesis showed that exercise-conditioned serum could be used in three-dimensional *in vitro* culture to evaluate the potential of exercise on cancer progression control, offering the possibility to quantify cancer cell growth in response to exercise-conditioned sera and avoiding *in vivo* models.
- The results of the central project of the present thesis (Chapter 3) showed that human sera conditioned by structured high-intensity endurance cycling sessions, and not by a training period, impact the proliferative and tumorigenic capacities of triple-negative breast cancer and prostate cancer cells; furthermore, possible relationships between the results obtained in the tumorigenic potential of prostate cancer cells and some exercise parameter predictors have been shown.
- Starting from literature data about the positive effects of structured exercise in the outcome of cancer therapies, and of the beneficial effects of exercise in decreasing the risk of cancer recurrence, future projects will be aimed to evaluate the role of administered structured exercise in tertiary cancer prevention. Furthermore, the three-dimensional *in vitro* culture optimized in this thesis will be used to evaluate the potential of different exercise protocols on cancer progression control, being able to personalize the prescription of exercise for each patient, in terms of FITT principle (Frequency, Intensity, Time and Type). Moreover, the method proposed in this thesis could be applied to evaluate the possible relations between the decreased clonogenic capacity given by exercise-conditioned human sera and the variations of exercise parameters, to understand the amount of exercise performed and the individual's response to it (i.e. TTE, LT1, LT2, VO_{2max}, VO_{2max}/kg, and CK levels).
- Finally, the results obtained in Chapter 4 showed that in normal conditions the autophagy-UPR (unfolded protein response) crosstalk could protect cells exposed to stress or tumor promoter, preventing the accumulation of damaged proteins and organelles. However, when UPR increases, cell damages accumulate and could induce tumorigenesis; our results showed that metformin could inhibit this process, by inducing a selective autophagic cell death and preventing tumor initiation. Given that the autophagic process has been hypothesized as one of the mechanisms involved in cancer prevention, and that exercise is able to induce autophagy in muscles and other tissues, future projects will consider a possible synergism between structured physical intense exercise bouts and metformin, to understand if they could have possible cumulative beneficial effects in cancer prevention.

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APPENDIX

Original Article

Chemopreventive Potential of Apple Pulp Callus Against Colorectal Cancer Cell Proliferation and Tumorigenesis

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ABSTRACT

This study focused on the evaluation of the chemopreventive potential of tissue *in vitro* culture of the ‘‘Mela Rosa Marchigiana’’ apple (MRM callus) that allows the amplification of secondary metabolites. The MRM pulp and MRM callus chemopreventive potential were evaluated in terms of antiproliferative activity, inhibition of tumorigenesis in soft agar cultures, cell cycle and western blotting analyses in CaCo2 and LoVo colon cancer cell lines and in JB6 promotion sensitive (JB6 P+) cells. MRM callus induced a strong concentration-dependent inhibition of colon cancer cell proliferation and suppressed 12-o-tetra-decanoyl-phorbol-13-acetate-induced tumorigenesis of JB6 P+ cells in soft agar cultures. MRM callus inhibited the phosphorylation of JNK, p38, and eIF2alpha. Our data indicate that the MRM callus exerts a good antiproliferative and antitumorigenic potential through the MAP kinase inhibition and could provide natural compounds with chemopreventive properties. KEYWORDS: apple chemoprevention triterpenic acids tumorigenesis

KEYWORDS: apple, chemoprevention, triterpenic acids, tumorigenesis

INTRODUCTION

Cancer is a complex disease in which genetic and environmental exposure and lifestyle serve to initiate tumor development and progression [1]. For this reason, researchers focused on chemoprevention, the use of synthetic or natural agents to inhibit, prevent, or slow induction and progression of cancer [2]. Colon cancer is the third most prevalent cause of cancer death in the world; due to the long precancerous stage of colorectal cancer and to the direct contact between cancer cells and chemopreventive dietary agents, chemoprevention could be a very promising strategy. Several studies considered the role of fruit and vegetable nonnutrient compounds in chemoprevention, to test their abilities to interfere with tumorigenesis [3]. Apple consumption counteracts various degenerative disorders, such as ischemic heart disease, inflammation, and cancer [4, 5]. The preventive effects of bioactive phytochemicals contained in apple juice hve been demonstrated in colon cancer [6, 7]. Apples or apple products (apple juice, extracts, dehydrated apples) could exert biological effects to prevent or reduce the proliferation of colorectal cancer, both in *in vitro* and *in vivo* models and in case-control studies, by which the risk of

colorectal cancer risk resulted inversely correlated with the number of consumed apples [8–13]. Those beneficial effects have been attributed to the apple's bioactive molecule mixture, which is more effective than a single bioactive molecule in exerting their biological activities [14–16]. We have demonstrated that a specific apple is particularly rich in polyphenols and that its juice leads to the strongest antiproliferative activity against breast cancer cells, in comparison to the five other apple varieties, suggesting that polyphenols play an important role [17]. Although access to fresh fruits is determined by seasonal availability, drying processing techniques and *in vitro* culture (callus) induction allow for the extension of the shelf life of fruits. Callus production is used to amplify fruit and vegetable secondary metabolites that have beneficial effects for human health; several studies focused on the optimization of this technique to obtain bioactive components from a variety of natural foods [18–24]. We have previously demonstrated that the callus derived from the pulp of the Italian ''Mela Rosa Marchigiana'' apple (MRM apple) (Fig. 1) produces a higher amount of triterpenic acids than those contained in the apple itself, in particular maslinic acid (MA) and tormentic acid (TA) [25].

Triterpenoids exert antiproliferative activity against several human cancer cell lines [26, 27] and MA has a strong antiproliferative activity against human colon cancer cells, HT29 and CaCo2, activating apoptosis [28, 29]. The aim of this study was to evaluate MRM callus and MRM pulp antiproliferative effects in CaCo2 and LoVo human colon cancer cell lines and in preneoplastic JB6 Cl 41–5a promotion-sensitive cells (JB6 P+ cells) and to investigate MRM callus antitumorigenic potential in JB6 P+ cells.

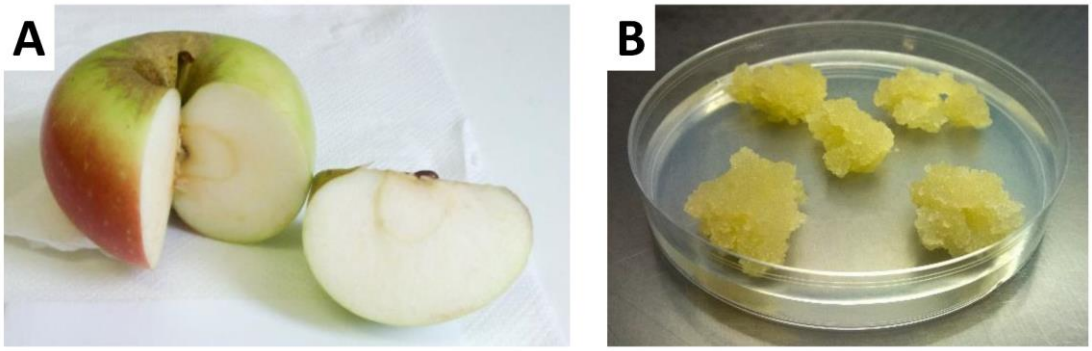

FIG. 1. (A) MRM apple and (B) MRM callus. MRM, Mela Rosa Marchigiana.

MATERIALS AND METHODS

Cell cultures and chemical reagents

Human colorectal adenocarcinoma cell lines, CaCo2 and LoVo, and murine skin epidermal JB6 Cl 41–5a promotion-sensitive (JB6 P+) cells were purchased from the American Type Cell Culture Collection (ATCC, Rockville, MD, USA). Cells were cultured in Dulbecco's Modified Eagle Medium (CaCo2 and LoVo cells) or Eagle's Minimum Essential Medium (EMEM) (JB6 P+ cells) media, supplemented with 10% or 5% of heat-inactivated fetal bovine serum, respectively, and 2 mM L-glutamine, 1 x MEM Nonessential Amino Acid Solution, 0.1 mg/mL streptomycin, 0.1 U/L penicillin, and 1 mM Na pyruvate. The 12-o-tetradecanoylphorbol-13-acetate (TPA) was dissolved in dimethyl sulfoxide. All cell culture materials and Maslinic Acid ((2a,3b)-Dihydroxyolean-12-en-28-oic acid, MA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The TA, obtained from the MRM callus, has been kindly provided by ABR (Active Botanicals Research) (Brendola, VI, Italy).

Plant material and tissue culture

MRM apples were collected from an orchard in the Marche region of Italy. After sterilization with ethanol 90%, apples were opened under the laminar flow cabinet with a sterile blade and cultures were conducted as previously described [25]. Cultures were incubated in the dark at 25 °C ± 2 °C and subcultures were obtained after 28 days in the same media. The callus obtained by subcultures (Fig. 1B) was stored at -80 °C and lyophilized before the extraction.

Extraction of MRM callus and MRM pulp

Lyophilized MRM pulp and MRM callus were ground into powder and extracted in sterilized Milli-Q H_2O (1.94 g in 8.95 mL and 1.2 g in 18.4 mL, respectively) under magnetic stirring, followed by centrifugation at 3600 g for 15 min at 4 °C. The supernatants were filtered with a 0.45 µm membrane, aliquoted, and stored at -20 °C.

Anchorage-dependent growth assay

CaCo2 and LoVo cells (1 x 10^4 cells/well) and JB6 P+ cells (2 x 10^4 cells/well) were seeded in 96-well plate; after overnight incubation, cells were treated in triplicate with MRM pulp, MRM callus, MA, and TA (only CaCo2 and LoVo cells) at final concentrations of 0–5% v/v of MRM pulp and MRM callus, 0–50 µg/mL of MA and

0–100 µg/mL of TA. JB6 P+ cells were stimulated with 10 ng/mL TPA. After 72 h of treatment, cell viability was evaluated using the CellTiter 96 Aqueous NonRadioactive Cell Proliferation Assay (Promega, Madison, WI, USA), as previously reported [17].

Anchorage-independent transformation assay

JB6 P+ cells were cultured in 35-mm dishes (1 x 10^4 cells/dish) to investigate MRM pulp, MRM callus, and MA effects on TPA-induced cell transformation. The intermediate agar layer was composed of 0.3% agar–10% FBS-EMEM and cells, with different concentrations of MRM pulp, MRM callus (0%, 0.10%, 0.5%, 5% v/v), and MA (0, 0.3, 1.5, 15 µg/mL), with a bottom layer of 0.6% agar–10% FBS-EMEM. Cells were maintained in an incubator for 21 days, stained with 0.1% Crystal Violet, counted, and photographed with an inverted microscope.

Flow cytometric analysis

CaCo2 cells were seeded in six-well plates (5 x 10^5 cells/well) and then treated with 2.5% and 5% v/v of MRM pulp, 2.5 and 5% v/v MRM callus, or with 12.5 and 25 µg/mL MA, for 24 h. After treatment, cell cycle analysis was performed as previously reported [17]. Cytofluorimetric acquisitions and sample analysis were performed with a Partec PAS flow cytometer (Partec, Mu¨nster, Germany) and FlowJo 8.6.3 software (TreeStar, Inc., Ashland, OR, USA), respectively.

Immunoblot analysis

CaCo2 cells were seeded in six-well plates (5 x 10^5 cells/well) and treated for 4 and 8 h with MRM pulp, MRM callus, or MA. JB6 P+ cells were seeded in six-well plates (2.5 x 10^5 cells/well), starved in 0.1% FBS-EMEM for 24 h, and then subjected to a 1-h pretreatment with MRM pulp, MRM callus, or MA, after which were stimulated with TPA 10 ng/mL for 30 min. After treatments, cells were lysed for western blotting analysis as previously reported [30]. The following primary antibodies have been used: phospho-p38 MAPK, p38 MAPK, phospho-SAPK/JNK, SAPK/JNK, phospho-p44/42 MAPK (ERK1/2), p44/42 MAPK (ERK1/2), phospho-eIF2alpha, and Cleaved Caspase-3 (Cell Signaling Technology, Beverly, MA, USA). Protein bands were detected incubating blots with horseradish peroxidase-conjugated secondary antibody and then with Clarity Western ECL Substrate (Bio-Rad

Laboratories, Inc., Hercules, CA, USA); the immunoreactive bands were detected with chemiluminescence film (Amersham Hyperfilm ECL; GE Health Care, Little Chalfont, United Kingdom). Equal proteins loaded were confirmed using anti Actin antibody (Sigma-Aldrich, St. Louis, MO, USA).

Statistical analysis

All the data are expressed as mean ± SEM of at least three different experiments. Significant differences between the mean values were analyzed using Prism5 software, with 1- or 2-way ANOVA followed by Dunnett's or Bonferroni *post-hoc* tests. P-values minor than .05 were considered as significant.

RESULTS

MRM pulp and MRM callus antiproliferative effects in human colon cancer cells

Anchorage-dependent growth inhibition MTS assay was performed in CaCo2 and LoVo cells. As shown in Figure 2A, in both cancer cell lines MRM pulp and MRM callus led to a concentration-dependent reduction of cellular proliferation; MRM callus significantly reduced the proliferation of CaCo2 and LoVo cells starting from a concentration of 0.625% v/v ($P < .001$) and from 1.25% v/v ($P < .05$), respectively. Moreover, the MRM callus was more effective than MRM pulp ($P < .001$), leading to a IC_{50} of 3.7 ± 0.5 or 6.6% ± 0.6% v/v in CaCo2 and LoVo, respectively (Table 1). Based on previously reported chemical analysis,25 the amounts of MA and TA – the most representative MRM callus triterpenic acids – contained in 3.7% and 6.6% v/v of MRM callus were 11.3 and 20 µg/mL of MA and 33.6 and 59.6 µg/mL of TA, respectively. To understand whether MA and TA were responsible for MRM callus antiproliferative effects in the colon cancer cell lines tested, different concentrations of MA and TA were considered (Fig. 2B). MA resulted in a concentration-dependent reduction of cell proliferation: the obtained MA IC_{50} was of 20.9 ± 0.8 and 24.9 ± 0.9 µg/mL (Table 1), 1.85- fold and 1.25-fold higher than the MA contained in the IC_{50} concentration of MRM callus, in CaCo2 and LoVo cells, respectively. The TA showed lower antiproliferative activity than MA, inhibiting 23.8% and 29.0% of cell proliferation at the highest concentration tested (100 µg/mL) in CaCo2 and LoVo cells, respectively. For this reason, only MA was considered for further experiments.

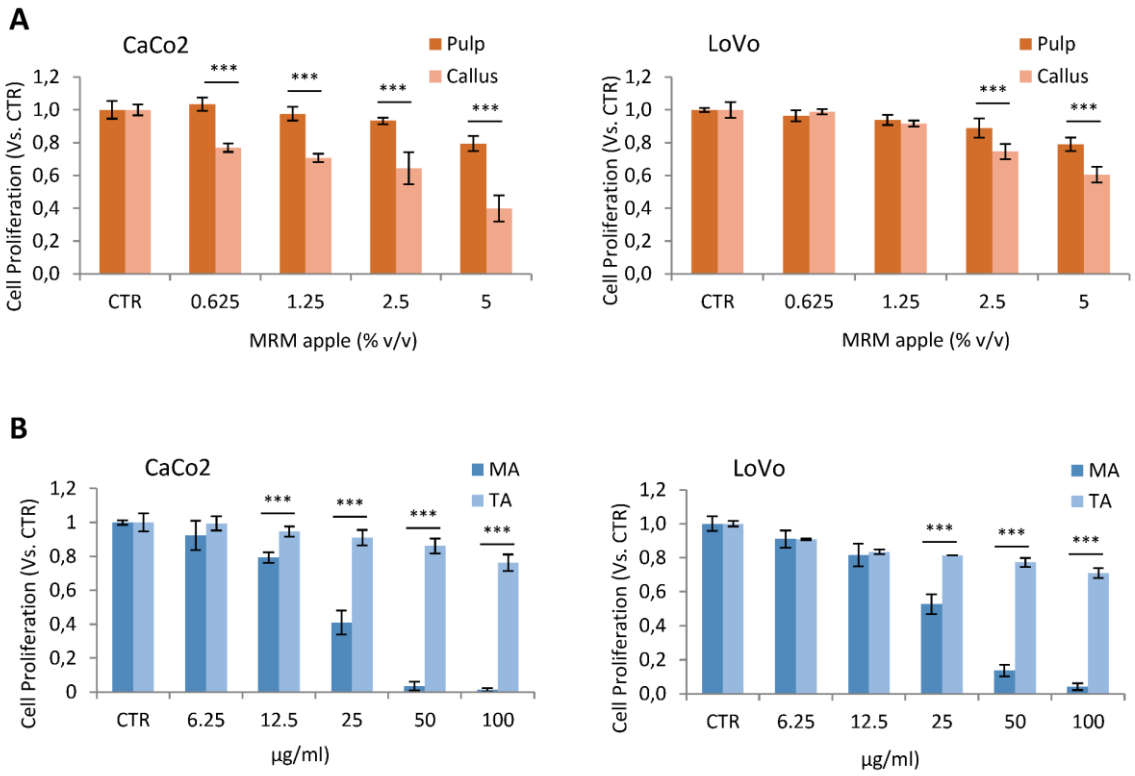

FIG. 2. Effect of MRM pulp, MRM callus, MA, and TA on CaCo2 and LoVo cell proliferation. CaCo2 and LoVo cells were treated with MRM pulp, MRM callus (A) or MA and TA (B) for 72 h and cell proliferation was evaluated by MTS assay. Data show results of at least three separate experiments and are expressed as mean of relative cell proliferation (vs. untreated cells) – SEM. ***P < .001; two-way ANOVA followed by Bonferroni post-hoc test. MA, maslinic acid; TA, tormentic acid.

Table 1. IC_{50} Values. Half maximal inhibitory concentrations of MRM pulp, MRM callus (% v/v) MA and TA (µg/mL) obtained in CaCo2 and LoVo cells.

	Pulp (% v/v ± SD)	**Callus**(% v/v± SD)	**MA** (µg/mL± SD)	**TA** (µg/mL± SD)
CaCo2	9.9 ± 1.9 (R^2 = 0.80)*	3.7 ± 0.5 (R^2 = 0.90)	20.9 ± 0.8 (R^2 = 0.98)	n.c.
LoVo	18.9 ± 5.5 (R^2 = 0.82)	6.6 ± 0.6 (R^2 = 0.93)	24.9 ± 0.9 (R^2 = 0.97)	n.c.

* IC50 were calculated by nonlinear regression analysis (see "Anchorage-dependent growth assay" section)

n.c.: not calculable.

MRM, Mela Rosa Marchigiana; MA, maslinic acid; TA, tormentic acid.

Analysis of cell cycle perturbations

The analysis of cell cycle was performed in CaCo2 cells. Results showed that only MA at a concentration of 25 µg/mL led to a perturbation of cell cycle, with an increased cellular population in the G1 phase, from 45.87% ± 2.19% of the control to 61.23% ± 0.81%, and a decreased cellular population in the S phase, from 33.67% ± 1.27% of the control to 18.63% ± 0.64% ($P < .001$) (Fig. 3A). These results showed that only MA produces significant changes in cell cycle profile; the link between MA-induced cell growth inhibition and cell cycle arrest in G1 phase was confirmed by the western blotting analysis of phosphorylated ERK 1/2, which resulted to be downregulated only by MA (Fig. 3B). The apoptotic pathway involvement was assessed by hypodiploidy analysis, which revealed that MRM pulp and MRM callus did not induce alterations; these data were confirmed by the analysis of cleaved caspase-3 through western blotting, in which only MA activated the apoptotic pathway (data not shown).

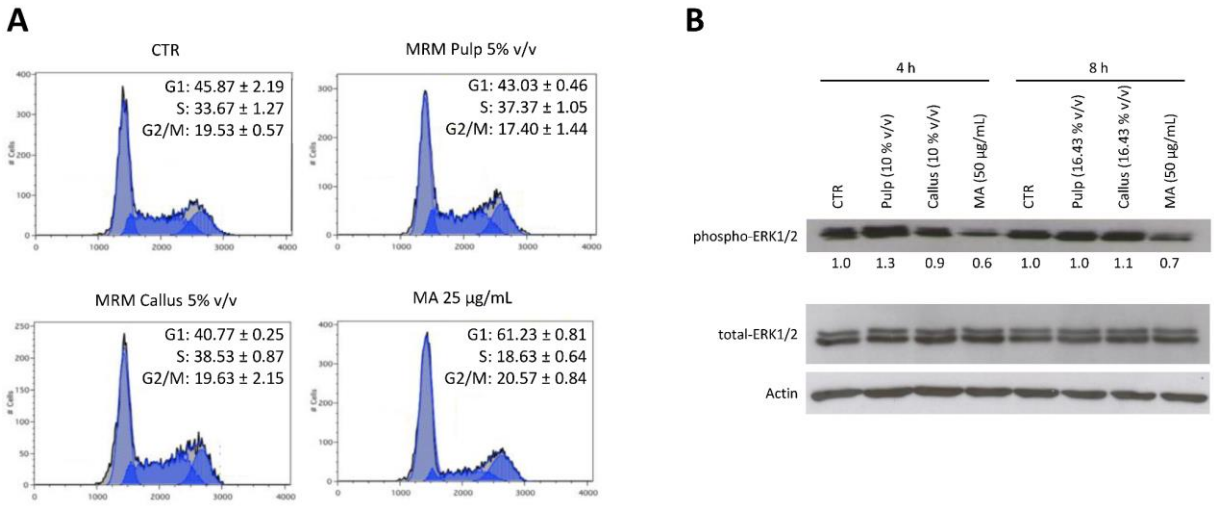

FIG. 3. (A) Cell cycle perturbation analysis in CaCo2 cells treated with MRM pulp, MRM callus, or MA. Cells were treated with MRM pulp, MRM callus (2.5–5% v/v), or MA (12.5–25 µg/mL) for 24 h and stained with Propidium Iodide and analyzed with flow cytometry. Data are expressed as mean– SD. (B) Western blot analysis of ERK1/2 phosphorylation. Representative image of three experiments giving similar results of total cell extracts of CaCo2 cells treated with MRM pulp, MRM callus, or MA for 4 or 8 h. Total ERK1/2 and actin were used as loading controls. Densitometry values for specific proteins relative to untreated cells (set as one-fold) are included below the lanes. Color images are available online.

MRM pulp and MRM callus antiproliferative effects in TPA-stimulated preneoplastic JB6 P+ cells

JB6 P+ cells were stimulated with 10 ng/mL TPA and simultaneously treated with different concentrations of MRM pulp or MRM callus for 72 h. Morphological examination of cells resulted in significant changes only in cotreated TPA-MRM callus cells, starting from a concentration of 1.25% v/v and being more evident at a concentration of 5% v/v (Fig. 4A). In non-TPA-stimulated cells, we found no significant proliferation reduction after treatment with MRM callus. Notably, MRM callus induced a reduction of 100% of TPA-stimulated proliferation at a concentration of 5% v/v ($P < .001$). On the other hand, MRM pulp only partially reduced JB6 P+ cell proliferation and TPA-induced proliferation (Fig. 4B).

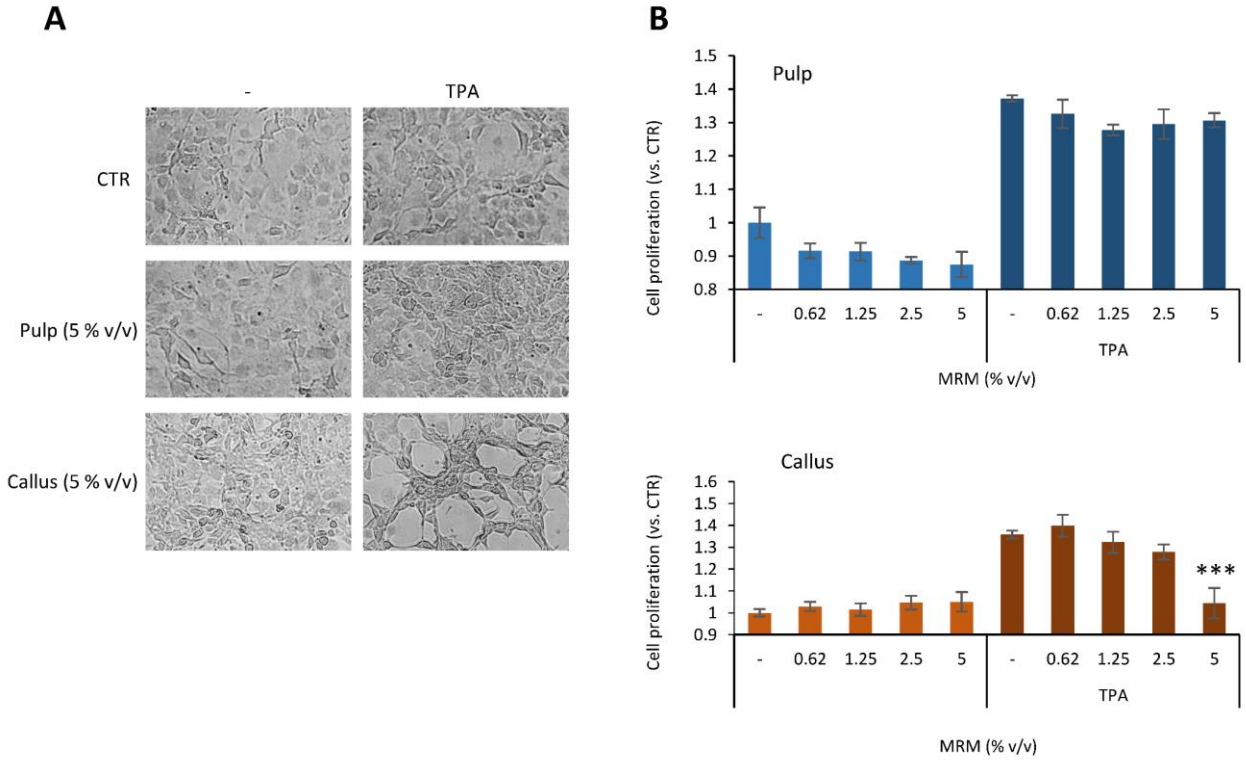

FIG. 4. Effect of MRM pulp, MRM callus, and MA on JB6 P+ cell proliferation. Morphological examination (A) and cell viability assay (B) of JB6 P+ cells treated with MRM pulp or MRM callus (0–5% v/v) and stimulated with TPA (10 ng/mL) for 72 h. Relative cell proliferation to nonstimulated control was evaluated by MTS assay and data were expressed as mean of at least three separate experiments – SEM. ***$P < .001$; one-way ANOVA followed by Dunnett's multiple comparison test.

MRM pulp and MRM callus effects on TPA-induced JB6 P+ cell transformation

The ability of JB6 P+ cells to form colonies in anchorage-independent cultures under different treatments was monitored (Fig. 5A). Results showed that both MRM pulp and MRM callus significantly decreased JB6 P+ cell's colony formation ability in TPA presence or absence, starting from a concentration of 0.10% v/v. Interestingly, MRM callus was more effective than MRM pulp: MRM pulp resulted in a reduction of colony number of about 7.5% in non-stimulated cells and 34.8% in TPA-stimulated JB6 P+ cells at 0.1% v/v; moreover, to inhibit more than 90% of colony formation, a concentration of 5% v/v is necessary ($P < .01$ and $P < .001$, respectively); MRM callus led to a reduction of about 81.9% in non-stimulated cells and completely inhibits colony formation in TPA-stimulated JB6 P+ cells at 0.1% v/v. Furthermore, to understand whether the MA was responsible for the effect of MRM callus in the suppression of TPA-induced colony formation, different concentrations of MA were tested in soft agar cultures. MA did not lead to statistically significant differences in colony number in non-stimulated cells (Fig. 5B); in TPA-treated cells, a final concentration of 0.3 µg/mL MA, corresponding to 0.1% v/v of MRM apple callus, did not have the same effect observed in MRM callus-treated cells, leading to a small reduction in colony number ($P < .05$) (Fig. 5B).

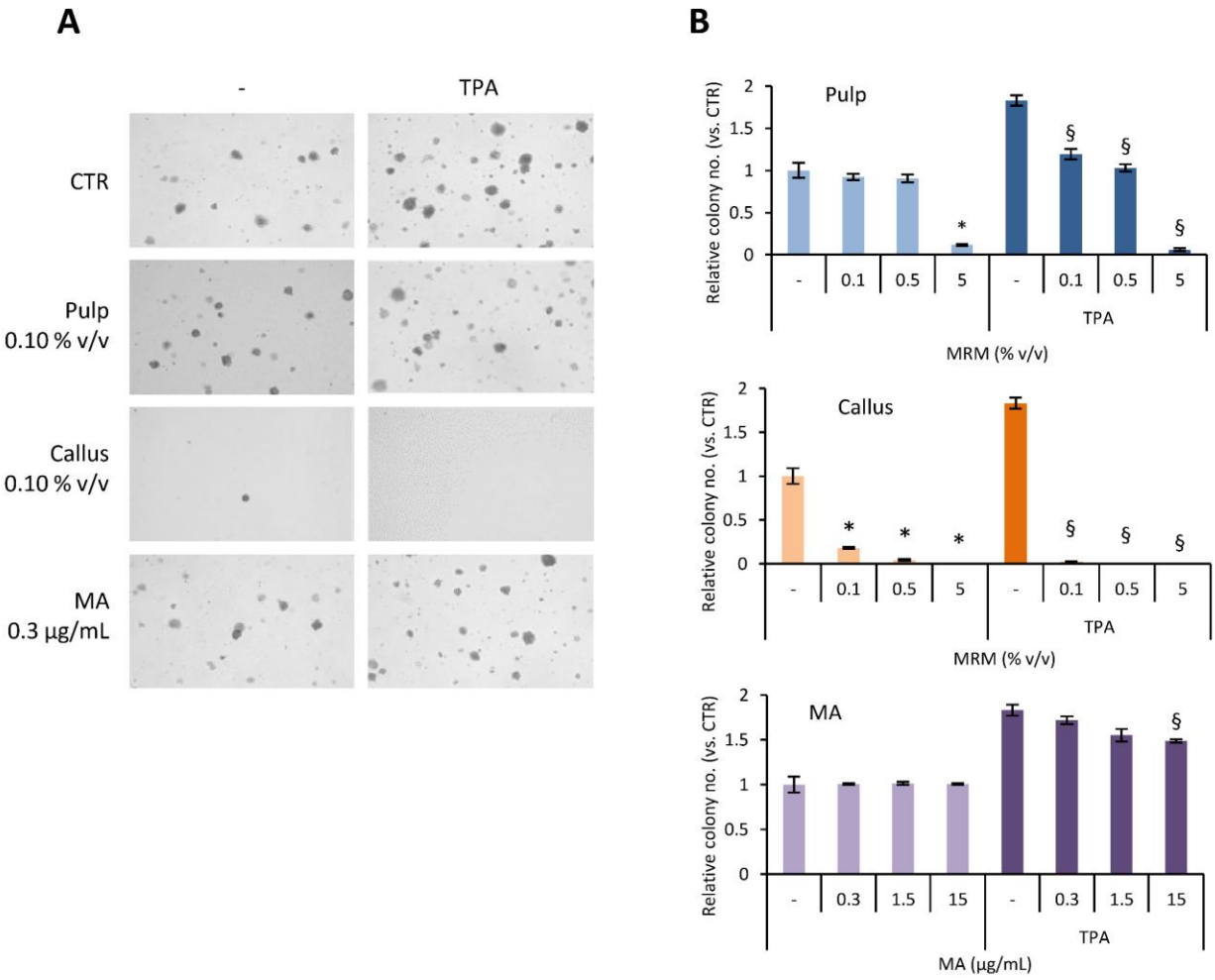

FIG. 5. Effect of MRM pulp, MRM callus, and MA on *in vitro* tumorigenesis of JB6 P+ cells. Anchorage-independent cell growth assay. Cells were treated with MRM pulp, MRM callus (0–5% v/v), or MA (0–15 µg/mL), stimulated with TPA (10 ng/mL), cultured in soft agar for 21 days, and colonies containing more than 20 cells were counted. (A) Representative images of soft agar results (B) Data are expressed as mean of relative colony numbers with respect to the untreated cells –SEM of at least three different experiments. * Statistically different with respect to untreated cells; § statistically different with respect to TPA-stimulated cells; P <.001; one-way ANOVA followed by Dunnett's multiple comparison test.

MRM pulp and MRM callus' effects on TPA-induced phosphorylation of MAPK pathways in JB6 P+ cells

MAP kinase phosphorylation was evaluated by western blotting analysis. Figure 6 shows that only MRM callus inhibited TPA-induced JNK phosphorylation and avoided the accumulation of phosphorylated p38. However, ERK phosphorylation level did not change in MRM pulp, MRM callus, or MA treatment. To better

understand the involvement of p38 in MRM callus effects in JB6 P+ cells' growth inhibition in soft agar conditions, the phosphorylation of eIF2alpha was considered. Our results showed that TPA-induced phosphorylation of eIF2alpha was inhibited only by MRM callus, confirming the involvement of p38.

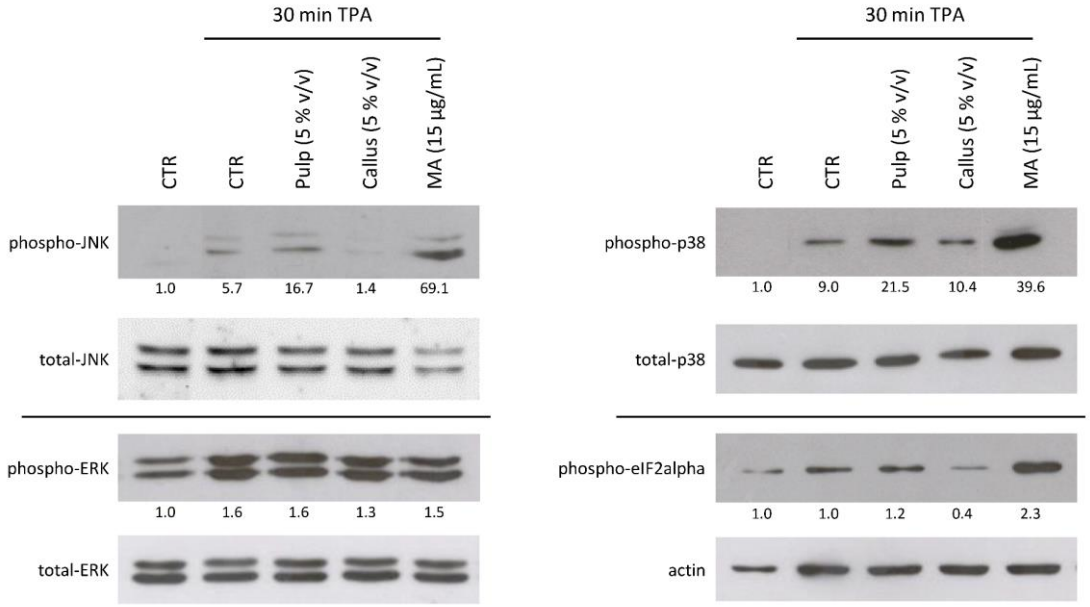

FIG. 6. Western blot analysis of JB6 P+ pretreated with MRM pulp, MRM callus (5% v/v), or MA (15 µg/mL) for 1 h and stimulated with TPA (10 ng/mL) for 30 min. Phosphorylation of SAPK/JNK, ERK1/2, p38 MAPK (Thr180/Tyr182), and eif2alpha was analyzed. Total SAPK/JNK, p38, ERK1/2, and actin were used as loading controls for phospho-JNK, phospho-p38, phospho-ERK, and phospho-eIF2alpha, respectively. Densitometry values for specific proteins relative to unstimulated cells (set as one-fold) are included below the lanes.

DISCUSSION

Several epidemiological studies suggested that increasing consumption of plant-based foods could be a strategy to prevent some chronic diseases, such as cardiovascular diseases, age-related function decline, and cancer [31]. Biologically active components in fruits and vegetables, particularly phytochemicals, non-nutrient plant compounds, such as flavonoids, phenolic acids, and carotenoids, can modulate many processes in the development of diseases, reducing their incidence. For these reasons, the scientific community has focused attention on micronutrients as potential cancer-preventive agents, studying diet-derived antioxidants in chemoprevention [32]. Apples represent a significant part of the European diet and,

in Italy, are the most highly consumed fruit. Apples are rich in antioxidants and phenolic compounds and are a great source of polyphenols, flavonoids, and catechins, as well as triterpenoids and anthocyanins [4].

Given that fruits and vegetables contain biologically active components that can modulate processes of different diseases, several studies aimed to produce secondary metabolites useful to promote health, using *in vitro* cultures (callus) of fruit and plant tissues [18–20, 22, 23]. We have previously demonstrated that *in vitro* cultures of the pulp of ‘‘Mela Rosa Marchigiana’’ apple (MRM callus) produce more triterpenic acids than those found in its pulp, resulting in an amplification of important secondary metabolites with antioxidant properties [25]. In this study we evaluated the antiproliferative activities of MRM callus in two human colon cancer cell lines (CaCo2 and LoVo cells), comparing them with the effects of MRM pulp. Our results demonstrated that MRM callus significantly inhibits CaCo2 and LoVo cell proliferation and that it is more effective than the pulp of the same apple. These results suggest that MRM pulp *in vitro* culture is a key to amplify the micronutrient content. Apples’ total triterpenoids have potent antiproliferative effects against human cancer cells [33]; however, TA possesses lower CaCo2 permeability than MA [34]. Our results show low antiproliferative activity of TA in human colon cancer cells, maybe due to the low permeability of this compound. On the other hand, MA exerts an antiproliferative effect against human colon cancer cells [28, 29]; our results confirm this antiproliferative effects in CaCo2 and LoVo cells. Considering the antiproliferative effect given by the same amount of MA contained in MRM callus, MRM callus shows greater antiproliferative activity than MA alone, suggesting that the efficacy of MRM callus is not exerted only by MA, but that the whole MRM callus content contributes to the effect. Moreover, MRM pulp and MRM callus effects on cell cycle perturbation were evaluated in colon cancer cell line CaCo2, showing that neither MRM pulp nor MRM callus impact cell cycle progression; on the contrary, MA leads to strong G1 phase cell accumulation and to a caspase-3 activation, confirming that MA is not the main compound exerting biological activity of MRM callus. Furthermore, MRM callus and MRM pulp antiproliferative activity was evaluated in the preneoplastic JB6 Cl 41–5a promotion sensitive (JB6 P+) cells, demonstrating that both MRM pulp and MRM callus are not toxic in preneoplastic non-stimulated JB6 P+ cells and that, interestingly, MRM callus completely inhibits TPA-induced JB6 P+ cell proliferation; MRM pulp,

however, does not exert this effect. To evaluate MRM pulp and MRM callus antitumorigenic potential, we considered their ability to inhibit JB6 P+ cell colony formation in anchorage-independent culture conditions (soft agar). Our results show that MRM callus blocks TPA-induced cell transformation, inhibiting colony formation in soft agar cultures; on the contrary, MRM pulp is less effective, and MA does not lead to statistically significant inhibition of TPA-induced colony formation. These divergent results would suggest a synergistic effect between MA and other MRM callus secondary metabolites, which are not produced at the same level by MRM pulp. This result is in accordance with several epidemiological studies [14–16], suggesting that the whole phytocomplex MRM callus is involved in the resulting effects, representing the optimal environment for the produced metabolites to exert their biological activities. To evaluate the molecular mechanisms involved in the ability of MRM callus to inhibit colony formation in soft agar cultures, we analyzed MAP kinase pathways, because of the positive correlation between p38, JNK, and ERK1/2 phosphorylation induced by TPA or Epidermal Growth Factor and colony formation in soft agar culture [35–39]. Our results excluded ERK1/2 involvement, whereas inhibition of p38 and JNK phosphorylation was exerted only by MRM callus in TPA-stimulated JB6 P+ cells, suggesting the involvement of p38 and JNK pathways in the ability of MRM callus to inhibit tumorigenic process *in vitro*. Moreover, the involvement of p38 was also considered through the analysis of a related protein, eIF2alpha; eIF2alpha is phosphorylated at a higher percentage in stomach, colon, and rectal tumors than in normal tissues [40]. eIF2alpha phosphorylation is an adaptive response to stresses and facilitates tumor cell survival and growth [41]. Zykova et al. showed the induction of eIF2alpha phosphorylation in stimulated JB6 P+ cells and demonstrated that p38 is required for this result [42]. Our results showed that phosphorylation of eIF2alpha is inhibited only in TPAstimulated JB6 P+ cells treated with MRM callus, confirming the involvement of p38 in MRM callus efficacy. Notably, MRM callus and MA mechanisms of action are markedly different, underlying the importance of multitarget inhibitors; phytochemicals are known to inhibit several targets and their combination contained in the whole extracts provides synergistic or additive effects, increasing efficacy and chemopreventive potential [43]. Results from this study could clarify how MRM callus can modulate cancer initiation and proliferation, serving as a starting point for further research about the use of MRM callus in diet-derived antioxidant

chemoprevention. Moreover, the prospect of using MRM callus in comparison with single compounds or the fruit itself must be considered; the opportunity to produce a variety of secondary metabolites from MRM apple *in vitro* cultures without depending on seasonal maturation or storage problems is highly advantageous. However, further experiments would be necessary to assess the effect of the other compounds found in high amount in the MRM callus. Further studies will be conducted to evaluate whether the employed plant material could be used for the development of a nutraceutical product capable of contributing to the protection of people's health.

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AUTHOR DISCLOSURE STATEMENT

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