Universidade de Aveiro Departamento de Química
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Impacto do Exercício Físico na Caquexia Cardíaca Induzida pelo Cancro

Impact of Exercise Training in Cancer-Induced Cardiac Cachexia

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Bioquímica, realizada sob a orientação científica do Doutor Daniel Moreira-Gonçalves, Professor Auxiliar Convidado do Departamento de Cirurgia e Fisiologia da Faculdade de Medicina da Universidade do Porto, e da Doutora Rita Ferreira, Professora Auxiliar do Departamento de Química da Universidade de Aveiro.

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palavras-chave Exercício físico, remodelação cardíaca, cancro, terapia, prevenção

resumo Com o aumento da incidência e prevalência de cancro nos próximos anos, é expectável um aumento na incidência das síndromes paraneoplásicas que, por sua vez, têm um elevado impacto na qualidade de vida e sobrevida dos pacientes oncológicos. O exercício físico confere vários benefícios ao nível da função cardiovascular, tanto em condições fisiológicas como patológicas, pelo que se questionou a sua potencial utilização como medida preventiva e/ou terapêutica na caquexia cardíaca induzida pelo cancro. Assim, neste trabalho, reuniu-se o conhecimento atual sobre a caquexia cardíaca induzida pelo cancro, uma síndrome com impacto ao nível funcional, celular e molecular, discutindo-se as vias moleculares que poderão estar a ser moduladas no coração pelo exercício físico. Neste contexto, é de salientar a modulação da proliferação tumoral, do metabolismo e defesas antioxidantes, e da fibrose e regeneração cardíaca. Com base neste conhecimento, e tendo em consideração que o cancro da próstata (CaP) é um dos mais incidentes e prevalentes a nível mundial, foi avaliado o impacto do exercício físico de longa duração na função e reestruturação cardíaca de um modelo animal de CaP induzido por via química e hormonal. Apesar de apenas se terem verificado alterações subtis na função cardíaca dos animais pelo CaP, detetou-se um aumento de acilcarnitinas e aminoácidos ramificados no coração, em conjunto com diminuição da eficiência mitocondrial, indicativo de depleção das reservas energéticas do coração, do tecido adiposo e possivelmente de outros grupos musculares. Por sua vez, os resultados obtidos nos animais com CaP treinados evidenciaram um aumento mais subtil do metabolismo de ácidos gordos, não demonstrando qualquer alteração do metabolismo de aminoácidos ramificados, o que parece estar relacionado com um aumento da eficiência mitocondrial, através da modulação da expressão quer de mediadores moleculares, como o PGC-1α, quer de efetores, como a citrato sintase ou ATP sintase β. Ao mesmo tempo, um aumento da expressão de CITED4 foi detetado nestes animais, indicativo de ativação das vias de regeneração cardíaca. Estas alterações parecem relacionar-se com o aumento de expressão dos recetores de androgénio e estrogénio no coração.

keywords Physical activity, cardiac remodelling, cancer, therapy, prevention

abstract With the incidence and prevalence of cancer expected to increase in the coming years, an increase in the incidence of concomitant paraneoplastic syndromes with great impact in patient quality of life and survival can also be expected. Since exercise training provides several cardiovascular benefits in both physiologic and pathologic conditions, its use as a preventive and/or therapeutic tool for cancer-induced cardiac cachexia has been hypothesised. In this work, we report on the current knowledge on cancer-induced cardiac cachexia, a syndrome with impact at the functional, cellular and molecular levels, and discuss the molecular pathways that may be modulated by exercise training in this setting, particularly, modulation of tumoral activity, metabolism and antioxidant defences, and cardiac fibrosis and regeneration. Based on this knowledge, and given that prostate cancer (PCa) is one of the most incident and prevalent cancers, the impact of life-long exercise training on cardiac function and remodelling was studied in an animal model with chemically- and hormonally-induced PCa. Despite subtle changes in animals' cardiac function, PCa promoted an increase of acylcarnitines and branched-chain amino acids, together with diminished mitochondrial efficiency, which is indicative of energetic storages depletion in the heart, adipose tissue, and possibly other muscle groups. On the other hand, the heart of trained PCa animals was more reliant on fatty acid oxidation, without involvement of amino acid metabolism, which appeared to be related with increased mitochondrial efficiency, through the modulation of expression of molecular regulators, such as PGC-1α, and molecular effectors, such as ATP synthase β and citrate synthase. At the same time, the increased expression of CITED4, a marker of cardiac regenerative capacity, was detected in these animals. These changes were apparently associated with increased expression of androgen and oestrogen receptors in the heart.

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CHAPTER I

General Introduction

Cancer is the second leading cause of death worldwide, and was responsible for 9.56 million deaths in 2017 [1]. Even though this number represents an increase of 25.4% in the number of deaths in just one decade, the corresponding estimated death rate is diminishing, currently standing at 121.2 deaths per 100 000, a decrease of 4.4% in the same time period [1]. In part, this trend is explained by the increased number of years living with disability (YLD), with modern diagnostic and therapeutic interventions allowing for a significant improvement in life expectancy post-cancer diagnosis. This is a double edge-sword, with increased YLD leading to an increased cancer prevalence among worldwide population, which is currently estimated to be over 100 million persons [2]. This number is expected to increase mainly because the incidence of cancer is estimated to rise by as much as 75% in the next two decades [3]. This poses a dreadful problematic as cancer is one of the deadliest group of diseases and is also associated with a number of paraneoplastic syndromes that have been the subject of a great deal of scrutiny in recent years. Of particular interest is cancer cachexia, a condition that is present in 50–80 % of cancer patients (more prevalent within pancreatic, colon, or non-small-cell lung malignancies) and accounts for up to 20 % of cancer-associated deaths [4]. Cancer cachexia is an insidious multi-organ syndrome that not only has a dramatic impact on the patient's quality of life, but is also associated with poor responses to chemotherapy and decreased survival [5]. The heart is among the organs suffering from cancer-induced changes, with the first reports of such changes in patients dating back to 1968. In that seminal study, Burch and colleagues described EKG changes, cardiac atrophy, cardiomyocyte disarrangements and myocardial infiltration by leucocytes in patients that died from cancer [6]. Until very recently, these features were considered side-effects of cardiotoxic anti-cancer therapy, a notion that has been recently updated with the observation of cardiac cachexia in therapy-naïve cancer patients [7,8]. The clinical impact of these findings needs to be discussed, as the negative effects of the cancer-induced cardiac changes could eventually limit tolerance to anti-cancer therapy, postponing its initiation, and/or it may exacerbate cardiac dysfunction and failure secondary to cardiotoxic anti-cancer therapy, thus leading to dyspnoea, fatigue and reduced quality of life [9]. With this in mind, a new challenge is emerging in terms of developing strategies that could counteract the cardiac effects of cancer. Exercise training has a great potential for such a role, given its numerous benefits that have been extensively demonstrated over the past couple of years, not only in physiological states, but also in a myriad of pathological conditions [10]. The

mechanisms by which exercise training exerts its beneficial effects are still not fully understood, though a combination of factors, both locally and systemically, could be involved [11]. In recent years, the increasing number of studies reporting benefits in exercising patients suffering from cardiac diseases has mustered support for its use amongst clinical physicians [10]. This is in contrast with what was recommended not so long ago for these patients, where rest was mandatory [12]. While there are specific medical conditions that may limit or even contraindicate exercise [10], the truth is that exercise is safe, beneficial and should be recommended for stable and well medicated cardiac patients [12]. In accordance, it has been hypothesised that exercise training could potentially be used as an adjuvant therapy to prevent cancer-induced cardiac cachexia. This is, however, an underdeveloped area with only two pre-clinical studies published so far, in the setting of bladder and breast cancer, showing that exercise training positively modulated cardiac structural and metabolic remodelling and improved antioxidant defences in the myocardium [13,14]. Therefore, it is of utmost importance to explore the beneficial effects of exercise training in the specific setting of cancer-induced cardiac cachexia and to characterize the underlying molecular and cellular pathways. Pre-clinical studies might help not only to push for the use of exercise training in daily clinical practice, but could also lead to the discovery of therapeutic targets with potential to be used as an alternative to exercise [15], a critical need for patients whose mobility/physical condition is reduced or that exercise is contraindicated. To add new insights on this topic, the present work begins with a narrative review followed by an experimental work. In the first, we discuss what is known so far about cancer-induced cardiac cachexia, about the exercise-induced cardiac adaptations in physiological states and, by hypothesizing interplay between these two settings, we further discuss possible cellular and molecular pathways modulated by exercise in cancer-induced cardiac cachexia (Antunes *et al.*, 2018). On the second, we evaluated the impact of life-long exercise training on cardiac function and remodelling in an animal model of prostate cancer.

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CHAPTER II

Review. **Exercise Training as Therapy for Cancer-Induced Cardiac Cachexia**

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REVIEWS

Review

Exercise Training as Therapy for Cancer-Induced Cardiac Cachexia

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Cancer-induced cardiac cachexia is an insidious syndrome with a dramatic impact on a patient's quality of life and survival. Since exercise training provides several cardiovascular benefits in both physiological and pathological conditions (e.g., athletes and patients with heart failure, respectively), its use as a preventive and/or therapeutic tool for cancer-induced cardiac cachexia has been hypothesized. Existing evidence on the effects of exercise training in this particular setting is limited, but points towards a beneficial outcome. We report the current knowledge on cancer-induced cardiac cachexia and discuss the molecular pathways that may be modulated by exercise training in this setting, providing insights into possible future roads of study, namely in stem cell research and cardiac regeneration.

Highlights

Cancer-induced cardiac cachexia is an insidious syndrome that may occur independently of cardiotoxic chemotherapy.

Exercise training modulates several molecular pathways involved in cardiac hypertrophy, regeneration, and metabolism, in both physiological and pathological conditions.

Preclinical data support the notion that exercise training prevents and reverts the cardiac remodeling suffered during cancer-induced cardiac cachexia, potentially by modulating tumoral activity, inflammation, metabolism, and fibrosis.

Exercise training presents itself as a promising therapy and/or adjuvant in cancer-induced cardiac cachexia.

Exercise Training in Cancer-Induced Cardiac Cachexia: A Novel Approach

The incidence of cancer is expected to increase by up to 75% over the next two decades [1], posing a problem to healthcare systems worldwide. Cancer results in several comorbidities, including **cachexia** (see Glossary), which is of particular interest due to its high prevalence (present in 50–80% of patients with cancer) and high mortality (up to 20% of all cancer-related deaths) [2]. It is an insidious syndrome that not only has a high impact on a patient's quality of life, but is also associated with poor responses to chemotherapy and decreased survival [3]. Accumulating data depict cancer cachexia as a multiorgan and/or multi-tissue syndrome, affecting the brain, heart, liver, and gut, to name a few [4]. In fact, as early as 1968, Burch *et al.* identified a different cardiac phenotype in postmortem samples from several patients with cancer [5]. Of note, there is evidence supporting the occurrence of extensive cardiac remodeling in patients with cancer even before they start any anticancer therapy [6]; however, the mechanisms underlying these cancer-induced changes, the impact on patient prognosis, and whether it can be prevented remain poorly understood, making it a critical field of research.

Exercise training has emerged as a potential therapy against cardiovascular diseases (CVDs), suggesting it could have a promising role against cancer-induced cardiac cachexia [7,8]. Indeed, recent data from preclinical models support this notion [9,10]. In this review, we initially discuss the cardiac remodeling suffered by **therapy-naïve patients with cancer**, integrating it from a molecular and cellular perspective, focusing then on the potential molecular and cellular pathways modulated by exercise training in the setting of cancer-induced cardiac cachexia. We highlight the potential of exercise training in the treatment and/or prevention of cancer-induced cardiac cachexia, namely through the modulation of signaling pathways involved in inflammation, metabolism, hypertrophy and/or atrophy, regeneration, protein homeostasis, and further establish exercise as the true **polypill**.

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Cancer-Induced Cardiac Cachexia

Cardiac cachexia is a relatively well-known syndrome in the context of CVDs, particularly in advanced chronic heart failure. In the cancer setting, it is recognized that the risk of cardiac events and cardiac cachexia increases in patients with underlying risk factors (e.g., hypertension, myocardial ischemia, and heart failure) [8], or as an adverse effect of cancer therapy [11,12]. Recently, it was acknowledged that the cancer itself could directly affect the heart because therapy-naïve individuals exhibited characteristics of cardiac cachexia [6,11,13,14]. However, the few available studies regarding therapy-naïve patients with cancer-induced cardiac cachexia (Table 1) do not comprehensively examine the heart of the patients *a priori*, leaving the contribution of underlying cardiac disease to cardiac cachexia unresolved and potentially contributing to misleading results. Nevertheless, the concept of cancer-induced cardiac cachexia is also supported at the preclinical level (Table 2). These data highlight the need for research on cancer-induced cardiac cachexia, envisaging the design of new therapeutic approaches.

Functional Alterations

A trait common to all patients with advanced cancer is exercise intolerance. This can result from the combination of central and peripheral factors, with a significant proportion being attributed to skeletal muscle wasting, paralleled by reduced strength and endurance [7,15]. However, it was shown that therapy-naïve patients with cancer may present cardiac dysfunction, as illustrated by their reduced **left ventricular ejection fraction** (LVEF) during a **treadmill exercise test** [11]. Thus, a lower cardiac functional reserve may also contribute to the reduced exercise tolerance in these patients. Notably, in two recent clinical studies, despite therapy-naïve patients with solid tumors exhibiting preserved LVEF or right VEF (RVEF) in resting conditions, both left or right ventricle mechanics (strain analysis by echocardiography) were impaired compared with control patients, further supporting the hypothesis that cancer itself can impact the heart. These studies also highlighted the subtleness of cardiac function and/or structure changes because they occurred even in the presence of preserved traditional functional and/or structural parameters (e.g., ejection fraction, end-diastolic and end-systolic dimensions, interventricular septum thickness, and posterior wall thickness) [16,17]. Overall, such changes could significantly add to the burden of symptoms in affected patients, including breathlessness, lethargy, reduced exercise tolerance, and, on occasion, overt congestive cardiac failure [18]. Moreover, concentrations of circulating peptides related to cardiac dysfunction, such as NT-proBNP, MR-proANP, MR-proADM, CT-pro-ET-1, and hsTnT, were elevated in an unselected population of therapy-naïve patients with cancer compared with normal range values [6]. Further data showing cardiac functional impairment stem from preclinical studies (Table 2). Two different models, the CD2F1 mouse model inoculated with C26 adenocarcinoma and the Wistar–Han rat model inoculated with Ah-130 hepatoma cells, consistently showed reduced **left ventricular fractional shortening** (LVFS) and LVEF compared with the respective model without cancer [19–25]. Accordingly, *in vitro* analysis performed in isolated cardiomyocytes from CD2F1 mice with colon-26 (C26) adenocarcinoma revealed impaired contractile function (**time-to-90% shortening** and **time-to-90% relengthening** increased) compared with isolated cardiomyocytes from control mice [26]. Notably, in Wistar–Han rats inoculated with AH-130 hepatoma cells, changes in LVFS and LVEF were only detected after tumor burden had reached a certain threshold, suggesting that cardiac dysfunction is cancer stage dependent [14]. By contrast, reports from studies using CD2F1 mice with C26 adenocarcinoma [27,28], and C57BL/6J mice with Lewis lung carcinoma [29] found no changes in any of the analyzed cardiac function parameters, such as LVEF, LVFS, left ventricular end-diastolic diameter (LVEDD), or left ventricular end-systolic diameter (LVESD). The reasons behind these discrepant results are unknown but might be related to the

Glossary

Browning: the process by which white adipose tissue (WAT) undergoes transformation and acquires properties of brown adipose tissue (BAT). Under appropriate stimuli, such as exercise or prolonged cold exposure, WAT can acquire a series of biochemical and morphological features of BAT, such as increased mitochondrial density and metabolic efficiency.

Cachexia: syndrome characterized by an increased inflammatory state, negative protein and energy balance, and an involuntary loss of body weight (≥5% in 12 months), with or without wasting of adipose tissue. Importantly, cachexia is not fully reversed by increased nutritional support.

Endogenous cardiac stem cells (eCSCs): an internal cardiac pool of multipotent cells, positive for stem cells markers (c-Kit+, Sca-1+, and Isl-1+), that are capable of originating new cells of different lineages (e.g., endothelium and cardiomyocytes).

Hypercatabolic: biochemical state where an organism is excessively catabolic, breaking down complex substances, such as protein, at increased and oftentimes detrimental rates. It is often associated with serious conditions, such as cancer cachexia-induced muscle wasting.

Irisin: peptide hormone produced by post-translational cleavage of fibronectin type III domain-containing protein 5 (FNDC5) in myocytes. Its production is upregulated by exercise and it is thought irisin is responsible for some of the systemic benefits of exercise, such as WAT browning.

Left ventricular ejection fraction (LVEF): a measure of cardiac contractility; represents the fraction of blood ejected by the left ventricle in each contraction. It is obtained by dividing the volume ejected by the ventricle during systole by the volume collected in the ventricle at the end of diastole.

Left ventricular fractional shortening (LVFS): a measure of cardiac contractility; represents the percentage of reduction of the end-diastolic diameter during systole. It is obtained by subtracting the end systolic diameter to the end-diastolic

Table 1. Overview of Existing Clinical Studies on Cancer-Induced Cardiac Cachexia[a,b]

Sample	Objectives	Main Results	Refs
75 women with different ovarian masses	Investigate TnI in patients with cancer	• TnI and hsTnI ↑	[13]
• 50 patients with CRC (40.0% men) – 59.9 ± 12.0 y • 51 patients with CHF (82.3% men) – 63.5 ± 10.9 years	Determine whether similar patterns of cardiovascular impairment are present in patients with CHF and CRC	• HRV in therapy-naïve patients ↓ • hsCRP in therapy-naïve patients ↑ • In general, all studied parameters worsened by treatment commencement	[11]
Heart morphology • 12 with cancer (7 men) – 59 ± 2 u • 14 with cancer cachexia (6 men) – 60 ± 2 y *Plasma analysis* • 32 with cancer (18 men) – 59 ± 2 y • 20 with cancer cachexia (11 men) – 60 ± 2 y	Determine whether cardiac cachexia is present in patients with different types of cancer	• HW ↓ ↔ LVPWT and RVPWT ↓ (only in cancer cachexia) • Fibrosis ↑ (both settings) • BNP ↑ (steeper in cancer cachexia)	[14]
555 patients with cancer (197 men) – 62 ± 10 y	Study the correlation between circulating cardiovascular hormones and peptides, and cancer mortality	• NT-proBNP, MR-proADM, CT-pro-Et-1, copepetin, and hsTnT ↑ →; progressively increased with cancer stage • IL-6 and CRP ↑ ↔ MR-proADM, NT-proBNP, and MR-proANP ↑ • hsTnT ↑ ↔ CRP ↑	[6]
122 patients with cancer (63 men) – 56 ± 9 y	Investigate LV function and mechanics in patients with different types of cancer	• Preserved LVEF, LVEDD, LVESD, IVSWT, and LVPWT • LV global longitudinal, circumferential, and radial strains ↓ • Endocardial and mid-myocardial LV longitudinal strains ↓; epicardial LV longitudinal strain – • Endocardial, mid-myocardial and epicardial LV circumferential strains ↓	[16]
101 patients with cancer (49 men) – 55 ± 10 y	Investigate RV structure, function, and strain in patients with different types of cancer	• Preserved LVEF, LVEDD, LVESD, IVSWT, and LVPWT • RV global longitudinal strain ↓ • Endocardial and mid-myocardial RV longitudinal strains ↓; epicardial RV longitudinal strain –	[17]

[a]↑ increase; ↓ decrease; – no change; ↔ correlation.
[b]Abbreviations: BNP, brain natriuretic peptide; CHF, chronic heart failure; CRC, colorectal cancer; CRP, C-reactive protein; CT-pro-ET-1, C-terminal pro-endothelin-1; HRV, heart rate variability; hsCRP, high-sensitivity C-reactive protein; hsTnI, high-sensitivity troponin-I; hsTnT, high-sensitivity troponin-T; HW, heart weight; IL-6, interleukin-6; IVSWT, interventricular septum thickness; LV – left ventricle; LVEF – LV ejection fraction; LVEDD – LV end-diastolic diameter; LVESD, LV end-systolic diameter; LVPWT, LV posterior wall thickness; MR-proADM, mid-regional pro-adrenomedullin; MR-proANP, mid-regional pro-atrial natriuretic peptide; NT-proBNP, N-terminal pro BNP; RV, right ventricle; RVPWT, RV posterior wall thickness; TnI, troponin-I.

type and stage of cancer, the age and/or gender of the animals used in the experiments, as well as the model of cancer induction.

Structural and Cellular Modifications

Cardiac atrophy has been reported in at least one study, in which patients with cancer who died from cancer cachexia had a significant reduction in heart weight (−25.6%) and LV wall thickness (−12.1%) compared with control patients who died from noncancer-related pathologies [14]. This has been systematically observed in several preclinical models of cancer [14,19,21–25,27,28,30–35], together with thinning of septal, interventricular, and posterior

diameter, followed by dividing by the latter.
miRNAs: a novel class of noncoding RNAs that are involved in the regulation of a multitude of cellular processes.
Natriuretic peptides: small peptide hormones, such as brain natriuretic peptide (BNP) and atrial natriuretic peptide (ANP), that are released under specific circumstances (e.g., stretching of ventricular wall by increased blood flow, or dilated cardiomyopathy) and induce natriuresis (i.e., the excretion of sodium by the kidneys). ANP and BNP are considered powerful predictors of heart failure.
Polypill: the combination of different active pharmaceutical ingredients in just one pill to treat different aspects of a pathology (e.g., cardiovascular disease).
Time-to-90% relengthening: a measure of diastolic function. It represents the time a cell takes to achieve 90% of baseline relaxation.
Time-to-90% shortening: a measure of systolic function. It represents the time a cell takes to achieve 90% of peak shortening.
Treadmill exercise test: an exercise test performed on a treadmill according to a specific predetermined protocol adequate to the desired population of study and/or results needed, often to evaluate the exercise capacity of the subjects.
Therapy-naïve patients with cancer: patients who have not been submitted to any type of anticancer therapy.

Table 2. Overview of Existing Preclinical Studies on Cancer-Induced Cardiac Cachexia[a,b]

Animal Model/Sample	Objectives	Main Results	Refs
Wistar–Han rats (male) AH-130 hepatoma (intraperitoneal injection of 10^8 cells) Age: unknown Sacrifice: 2, 4, and 6 days post tumor inoculation Sample: whole heart	Determine whether the proteolytic Ca^{2+}-dependent Calpain system is active in heart muscle in cancer cachexia	• Cardiac atrophy • Activity of calpastatin and 130-kDa Ca^{2+}-ATPase ↓	[30]
Wistar–Han rats (male) AH-130 hepatoma (intraperitoneal injection of 10^8 cells) Age: 6 weeks Sacrifice: 7 days post tumor inoculation Sample: whole heart	Determine whether redox imbalance exists in heart muscle in cancer cachexia. If so, determine the nature of oxidized proteins	• Cardiac atrophy • MDA- and HNE-protein adducts ↑, with predominance of MDA-protein adducts • Mn-SOD and catalase protein expression ↑	[31]
CD2F1 mice (male) C26 adenocarcinoma (subcutaneous injection of 10^6 cells) Age: 5 weeks Sacrifice: 17 days post tumor inoculation Sample: both ventricles	Evaluate cardiac function *in vivo*. Explore whether functional modifications are caused by cellular abnormalities	• Cardiac atrophy and ↑ fibrosis • LVFS and HR ↓, and LVIDs ↑ • Sarcomere structure disruption • Troponin I ↓; MHCβ ↑ and MHCα ↓	[19]
CD2F1 mice (female) C26 adenocarcinoma (subcutaneous injection of 5×10^5 cells) Age: adult Sacrifice: 19 days post tumor inoculation Sample: whole heart	Determine whether muscle wasting occurs in the heart of cachectic mice and whether it is related to functional changes	• No cardiac atrophy • LVFS and LVPWTd ↓; LVIDs ↑; HR – • TS90% and TR90% ↑ *in vitro* • mRNA of Atrogin-1 and Bnip3 ↑	[26]
CD2F1 mice (male + female) C26 adenocarcinoma (subcutaneous injection of 5×10^5 cells) Age: 8 weeks Sacrifice: unknown Sample: left ventricle	Characterize differences in tumor development between genders. Determine which proteolytic pathway is active	• Cardiac atrophy and ↑ fibrosis (more pronounced in males) • Aortic pressure and velocity ↓ (male only); LVEF and LVFS – (both genders) • Cellular size decrease – no apoptosis; sarcomere destruction • Total protein ↓; MHCβ ↑ and MHCα ↓ • Autophagy ↑; UPS –	[27]
BALB/c mice (male) C26 adenocarcinoma (subcutaneous injection of 5×10^5 cells) Age: 6–9 weeks Sacrifice: 17 days post tumor inoculation Sample: both ventricles	Evaluate Compound A and NBD efficacy against cancer cachexia-induced cardiac atrophy and dysfunction	• Cardiac atrophy; LVPWTd and LVAWTd ↓; cardiomyocyte area ↓; LVFS and LVEF ↓ • NF-κB (p65) activity ↑ • mRNA of MuRF-1, Atrogin-1, ANF and MHCβ ↑;	[32]
C57BL/6J mice (female) Lewis lung carcinoma (subcutaneous injection of 2×10^6 cells) Age: 8–9 weeks Sacrifice: 21 days post tumor inoculation Sample: whole heart	Determine whether cachexia is related to structural modifications of nervous system of heart	• No cardiac atrophy or fibrosis • No cardiomyocyte loss or morphological modifications; sarcoplasm ↑; axon length ↓ • Apoptosis –; oxidative stress –	[29]
CD2F1 mice (female) C26 adenocarcinoma (subcutaneous injection of 7.5×10^5 cells) Age: 6–10 weeks Sacrifice: unknown Sample: whole heart	Evaluate resveratrol efficacy against cancer cachexia-induced cardiac atrophy and dysfunction	• Cardiac atrophy; LVPWTd and LVAWTd ↓ • No systolic dysfunction • NF-κB (p65) activity ↑ • mRNA of MuRF-1 ↑	[28]
CD2F1 mice (male) C26 adenocarcinoma (subcutaneous injection of 10^6 cells) Age: 5 weeks Sacrifice: 17 days post tumor inoculation Sample: both ventricles	Elucidate cardiac tissue alterations	• LVFS and LVEF ↓; LVPWTs, IVSWTs, IVSWTd ↓ • mRNA of MHCβ ↑ and MHCα ↓; GLUT4 ↓ and GLUT1 ↑ • MHC and troponin I ↓ • MuRF-1 and polyubiquitinated proteins ↑	[20]

Table 2. (continued)

Animal Model/Sample	Objectives	Main Results	Refs
$Apc^{Min/+}$ mice (male) Colorectal cancer (genetically induced carcinogenesis) Age: 12 and 20 weeks (sacrificed after acclimatization) Sample: whole heart	Evaluate impact of PI2K/Akt/mTOR axis and AMPK on cancer cachexia-induced cardiac atrophy	• Cardiac atrophy; protein synthesis ↓ (20 weeks) • pAkt, pmTOR, pAMPK ↑ (12/20 weeks); pS6rp and 4EBP1 ↓ (20 weeks); 4EBP1 ↑ (12 weeks) • Autophagy ↑; UPS –; apoptosis –	[33]
Wistar–Han rats (unknown) AH-130 hepatoma (intraperitoneal injection of 10^8 cells) Age: juvenile Sacrifice: 14 days post tumor inoculation	Evaluate simvastatin efficacy against cancer cachexia-induced cardiac atrophy and dysfunction	• Cardiac atrophy • LVEF, LVFS, LVEDD, HR, LVSV, LVCO, aortic diameter and velocity ↓ (day 11)	[21]
Wistar–Han rats (male) AH-130 hepatoma (intraperitoneal injection of 10^8 cells) Age: Unknown Sacrifice: 16 days post tumor inoculation	Evaluate xanthine oxidase inhibition efficacy against cancer cachexia-induced cardiac atrophy and dysfunction	• Cardiac atrophy • LVEF, LVFS, LVEDD, LVEDV, LVPWTs, HR, LVSV, LVCO ↓ (day 11); LVESD ↑ (day 11)	[22]
BALB/c nu/nu mice (unknown) MAC16 (location of injection unknown) Age: 6 weeks Sacrifice: 30 days post tumor inoculation Sample: both ventricles	Evaluate the role of oxidative stress in activation of UPS	• Cardiac atrophy • XO mRNA ↑; SOD activity ↓ • Atrogin-1 and MuRF-1 mRNA ↑	[34]
Wistar–Han rats (male) AH-130 hepatoma (intraperitoneal injection of 10^8 cells) Age: 7–8 weeks Sacrifice: 14 days post tumor inoculation	Evaluate rosiglitazone efficacy against cancer cachexia	• Cardiac atrophy • LVEF, LVFS, LVEDD, LVEDV, LVPWTs, IVSWTs, HR, LVSV, LVCO ↓ (day 11); LVESD ↑ (day 11)	[23]
Wistar–Han rats (male) Walker-256 tumor (intramuscular injection of 8×10^7 cells) Age: 8–10 weeks Sacrifice: 5 or 10 days post tumor inoculation Sample: RA and RV (RH)/LA and LV (LH)	Determine the influence of oxidative stress on activation of main proteolytic pathways; right and left heart differences	• RH atrophy/LH normal: RVWT ↓↓/LVWT ↓ (5/10 days) • RH antioxidant defenses ↑ (5/10 days)/LH antioxidant defenses ↓ (5/10 days): carbonylated proteins RH/LH ↑ (5 days) • LH calpain-like activity, chymotrypsin-like activity, caspase-like activity ↓ (5 and 10 days); RH calpain-like activity ↑ (5 days)	[45]
Wistar–Han rats (unknown) AH-130 hepatoma (intraperitoneal injection of 10^8 cells) Age: juvenile Sacrifice: 7, 11 and 13 days post tumor inoculation Sample: unknown	Evaluate cardiac functional impairment in cancer cachexia	• Cardiac atrophy and ↑ fibrosis (day 7) • LVEF and LVFS ↓ (day 9); LVSV and LVCO ↓ (day 7) • Troponin-I and –T, TIMP-1, fibrinogen and MCP-1 ↑ in plasma; IGF-1R, IR, pAkt, pGSK-3 and p4EBP1 ↓ in heart • Predominant loss of MHC • Autophagy ↑; UPS ↑	[14]
Sprague–Dawley rats (female) Mammary cancer (NMU-induced carcinogenesis) Age: 5 weeks Sacrifice: at end of exercise protocol Sample: whole heart Exercise: during 35 weeks postcancer induction	Evaluate the impact of endurance training on heart with special focus on signaling pathways modulated by proinflammatory and wasting cytokines	• Fibrosis ↑ (only sedentary); heart weight ↑ (both sedentary and exercise) • Cardiomyocyte enlargement and myofibrillar disarray (more pronounced in sedentary rats) • Akt, mTOR and p70S6K phosphorylation ↑ (only sedentary) • Exercise prevented p70S6K phosphorylation, but no changes in Akt and mTOR phosphorylation • Different Akt localization (cytoplasm – exercise and extracellular matrix – sedentary) • p50, TRAF6, Atrogin-1 ↑ (only sedentary)	[9]
CD2F1 mice (female) C26 adenocarcinoma (subcutaneous injection of 5×10^5 cells) Age: 10 weeks Sacrifice: 21 days post tumor inoculation Sample: left ventricle	Investigate the role of MMPs and TIMPs in cardiac wasting	• Fibrosis ↑ (collagen deposition and plasma hydroxyproline ↑) • Left ventricle protein expression of MMP-2,-3,-9 and –14, and TIMP-2 ↑	[36]

Table 2. (continued)

Animal Model/Sample	Objectives	Main Results	Refs
Wistar–Han rats (male) AH-130 hepatoma (intraperitoneal injection of 10^8 cells) Age: unknown Sacrifice: 16 days post tumor inoculation	Evaluate the effect of erythropoietin on cancer cachexia progression, cardiac wasting, and physical performance status	• Cardiac atrophy; LV atrophy • LVEF, LVFS, LVEDD, LVSV, LVCO ↓ (day 11); LVESD ↑ (day 11)	[25]
BALB/c mice (male) C26 adenocarcinoma (subcutaneous injection of 1.5×10^6 cells) Age: 9–10 weeks Sacrifice: 21 days post tumor inoculation	Identify tumor-borne signals and their impact on specific peripheral organs, particularly heart	• Cardiac atrophy; no fibrosis • LVFS and cardiomyocyte area ↓ • ↑ Gene expression of FAO-associated genes; ↑ fatty acid uptake; ↑ maximal mitochondrial respiratory capacity • ↓ Lipid storage capacity	[35]
Wistar–Han rats (male) AH-130 hepatoma (intraperitoneal injection of 10^8 cells) Age: 8 weeks Sacrifice: 16 days post tumor inoculation Sample: whole heart	Evaluate the effect of megestrol acetate on cardiac function and modulation of autophagy	• Cardiac atrophy • LVEF, LVFS, LVPWTs, LVSV, LVEDV and LVIDd ↓; LVESV and LVIDs ↑ • Autophagy ↑	[24]
Wistar rats (male) Urothelial carcinoma (BBN-induced carcinogenesis – 20 weeks) Age: 5 weeks Sacrifice: at end of exercise protocol Sample: heart apex Exercise: during 13 weeks after stoppage of BBN administration	Provide insights into the effects of exercise training initiation on advanced stages of cancer	• HW –; cardiomyocyte atrophy and ↑ fibrosis → exercise counteracted these modifications • TWEAK –; NF-κB p65 –; ↑ NF-κB p50 → counteracted by exercise • ↓ Citrate synthase activity → counteracted by exercise • ↑ GAPDH and ATP synthase with exercise (BBN-treated only); ↓ ETFDH with exercise • ↓ Mn-SOD → counteracted by exercise • ↑ PGC-1α and mtTFA with exercise (mtTFA only in BBN-treated animals) • Connexin 43 – and c-kit ↓ → exercise increased both	[10]

[a]↑ increase; ↓ decrease; – no change.
[b]Abbreviations: 4EBP1, eukaryotic initiation factor 4E-binding protein 1; Akt, protein kinase B; AMPK, AMP-activated protein kinase; ANF, atrial natriuretic factor; BBN, N-butyl-N-(4-hydroxybutyl)-nitrosamine; C26 adenocarcinoma, colon-26 adenocarcinoma; ETFDH, electron-transferring-flavoprotein dehydrogenase; FAO, fatty acid oxidation; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GLUT, glucose transporter; GSK3, glycogen synthase kinase-3; HNE, hydroxynonenal; HR, heart rate; HW, heart weight; IGF-1R, insulin-like growth factor-1 receptor; IR, insulin receptor; IVSWT(s/d), interventricular septal thickness (at systole/diastole); LA, left atrium; LH, left heart; LV, left ventricle; LVAWT(s/d), LV anterior wall thickness (at systole/diastole); LVCO, LV cardiac output; LVEDD, LV end-diastolic diameter; LVEDV, LV end-diastolic volume; LVEF, LV ejection fraction; LVESD, LV end-systolic diameter; LVFS, left ventricle fractional shortening; LVID(s/d), LV internal diameter (at systole/diastole); LVPWT(s/d), LV posterior wall thickness (at systole/diastole); LVSV, LV stroke volume; LVWT, LV wall thickness; MCP, monocyte chemoattractant protein; MDA, malondialdehyde; MHC, myosin heavy chain; MMP, matrix metalloproteinase; Mn-SOD, manganese superoxide dismutase; mTOR, mammalian target of rapamycin; mtTFA, mitochondrial transcription factor A; MuRF-1, muscle RING-finger protein-1; NF-κB, nuclear factor kappa-B; NMU, N-Methyl-N-nitrosourea; p70S6K, ribosomal protein S6 kinase; PGC-1α, peroxisome proliferator-activated receptor γ coactivator-1α; RA, right atrium; RH, right heart; RV, right ventricle; RVWT, RV wall thickness; S6rp - S6 ribosomal protein; TIMP, tissue inhibitors of metalloproteinases; TRAF6, tumor necrosis factor receptor-associated factor 6; TWEAK, tumor necrosis factor-related weak inducer of apoptosis; UPS, ubiquitin-proteasome system; XO, xanthine oxidase

walls [20,22,23,26,28,32], chamber dilation [19,21–24,26], and cardiomyocyte atrophy [10,27,32,35]. Increased disorganization of myofibrillar proteins, with sarcomere structure disruption or even destruction, has also been observed in preclinical models of cancer [9,19,27]. This feature has implications in cardiac contractility and is one of the main structural changes underlying cardiac dysfunction. Atrophy of the heart appears to occur in parallel with an increase in interstitial fibrosis at the preclinical level, with implications mainly associated with decreased contractility and impaired diastolic function [9,10,14,19,27,36]. These changes appear to be associated with the modulation of metalloproteinase (MMP) activity [36] and the activation of the protein kinase B (Akt)/mammalian target of rapamycin (mTOR) pathway at the extracellular matrix level [9]. Furthermore, augmented fibrosis has also been detected in patients with cancer who have died with and without cancer cachexia [14]. Moreover, the impairment in cardiac function appears to be paralleled by a switch from the adult myosin heavy chain (MHC)-α to the fetal MHCβ isoform (Figure 1) [19,20,27,32]. In a mouse model of mechanical and pharmacological cardiac stress, the switch in MHC isoforms was connected

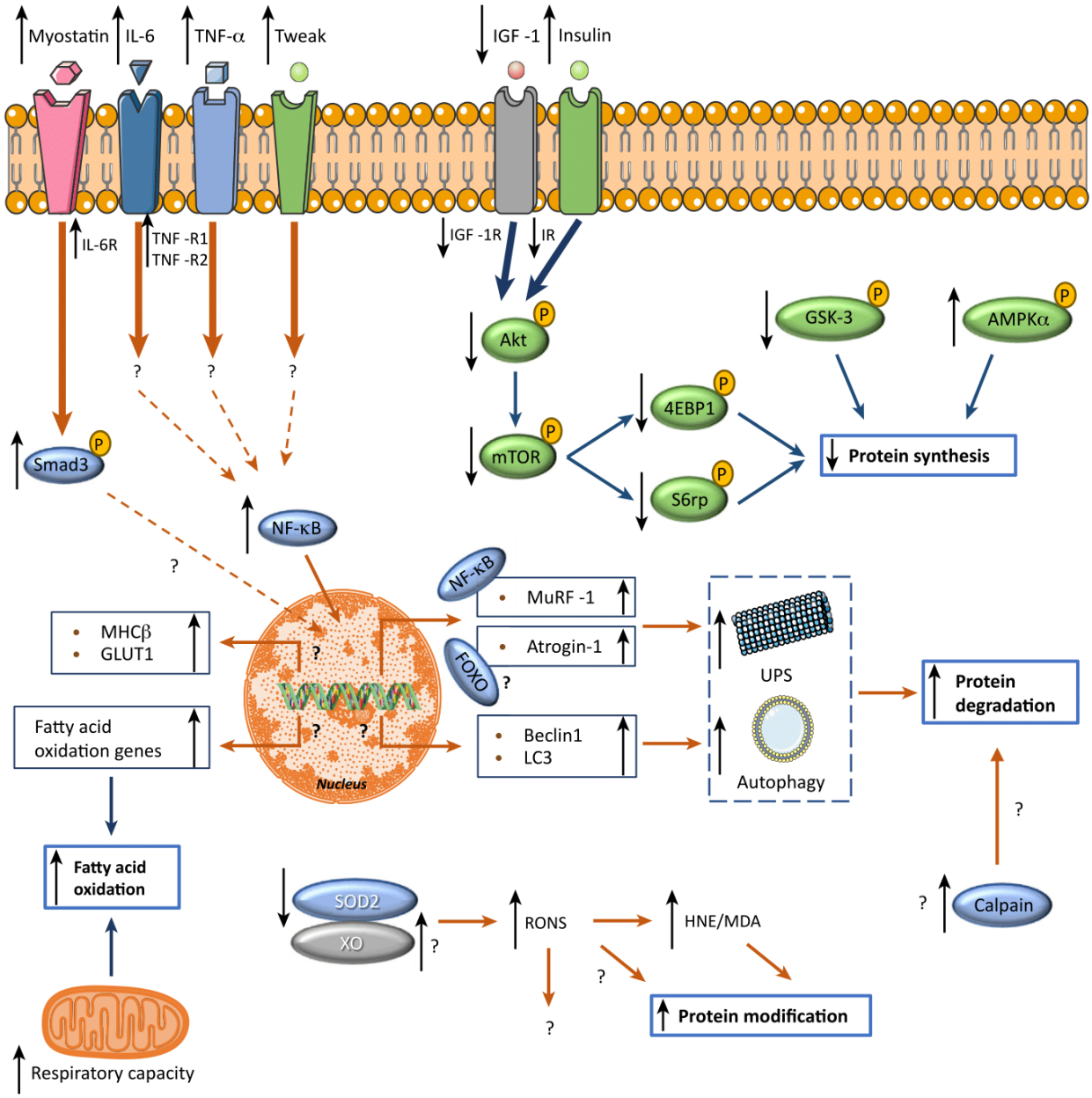

Figure 1. Integration of the Molecular Pathways Modulated by Cancer-Induced Cardiac Cachexia in Cardiomyocytes. Cancer impairs protein synthesis through downregulation of essential targets, such as insulin-like growth factor-1 receptor (IGF-1R) and insulin receptor (IR), with consequent downregulation of protein kinase B (Akt) activity. Concomitantly, an upregulation of protein synthesis inhibitory molecules, namely AMP-activated protein kinase (AMPK)-α and glycogen synthase kinase (GSK)-3, has been verified. Together with the upregulation of the proteolytic pathways, autophagy and the ubiquitin-proteasome system (UPS), activated by upregulation of nuclear factor-kappa B (NF-κB) and other transcription factors by cytokine receptors, it leads to protein metabolism imbalance. At the same time, antioxidant systems are impaired, leading to an increased production of reactive oxygen and nitrogen species (RONS), and consequently lipid peroxidation products, such as malondialdehyde (MDA) and hydroxynonenal (HNE), which leads to increased protein oxidation. Furthermore, fatty acid oxidation (FAO)-associated genes are overexpressed, which, aided by increased mitochondrial respiratory capacity, leads to FAO enhancement and, thus, FA depletion. Lastly, an increased expression of fetal gene products, such as myosin heavy chain (MHC)-β and glucose transporter (GLUT)-1, has been reported. Figure made using Servier Medical Art by Servier

(Figure legend continued on the bottom of the next page.)

with depressed cardiac function [37]. Thus, it is also possible that the cardiac functional impairment observed in models of cancer-induced cardiac cachexia could also be due, at least in part, to cardiac MHC isoforms changes.

Metabolic Remodeling and Redox Imbalance

Under normal conditions, fatty acid oxidation (FAO) is the main source of energy in the heart, accounting for up to 90% of all cardiac ATP production [38]. FAO disturbances have been reported in several CVDs, namely heart failure (reviewed in [38]). However, little is known about this aspect in the particular setting of cancer-induced cardiac cachexia. In fact, to our knowledge, only one study has approached metabolism adaptations in this setting, reporting the dysregulation of the expression of several genes associated with FAO in cardiomyocytes from the hearts of BALB/c mice inoculated with C26 adenocarcinoma cells compared with littermates that had not been inoculated (Figure 1). Consistently, *in vitro* testing in neonatal rat cardiomyocytes cultured in C26-conditioned medium showed reduced heart lipid storage capacity, enhanced fatty acid uptake, and increased maximal mitochondrial respiratory capacity compared with neonatal rat cardiomyocytes cultured in standard medium [35].

These metabolic adaptations of the heart in cancer cachexia appear to be associated with the reported redox imbalance. Under physiological conditions, the human cells have the capability to adequately handle reactive oxygen/nitrogen species (RONS) [39]; however, when the concentration of RONS overcomes the buffering capacity of the antioxidant systems, the functionality of the cells may be compromised [39]. This appears to happen in cancer-induced cardiac cachexia, resulting in protein oxidation either by radical or adduct formation [31]. In Wistar rats bearing AH-130 hepatoma, the heart contained protein oxidative modifications mainly in the form of adducts, both with hydroxynonenal (HNE) and malondialdehyde (MDA), two aldehydes resulting from lipid peroxidation and biomarkers of oxidative stress [40,41]. These modifications can disable enzymes essential for the physiological functions of the cell, namely ATP synthase and ubiquinone-oxidoreductase, and proteins essential for the correct functioning of the tissue, such as myosin and tropomyosin [31]. A concomitant increase in catalase and manganese-dependent superoxide dismutase (Mn-SOD) protein expression was also reported in these rats compared with nontumor-bearing rats [31]. Notwithstanding, a recent study detected decreased protein expression of Mn-SOD in a rat model of urothelial carcinoma compared with control rats [10]. Moreover, in a mouse model of murine adenocarcinoma, although no differences in mitochondrial or cytoplasmic SOD mRNA expression were detected, total SOD activity was decreased 1.5 times in mice with cachexia compared with mice without cachexia or cancer. In this same model, increased mRNA expression of xanthine oxidase was detected in cachectic mice compared both with control and noncachectic mice (Figure 1) [34]. Altogether, these data suggest that cancer-induced cardiac cachexia involves a switch to an energy-wasting state with increased RONS production and inactivation of antioxidant defenses, aggravating cardiac function.

Imbalance between Protein Synthesis and Proteolysis

The increased protein loss that leads to cardiac atrophy is possibly a result of an imbalance between proteolysis and protein synthesis (Figure 1) [42]. In fact, several studies using two different models of cancer (CD2F1 mice inoculated with C26 adenocarcinoma cells and

(https://smart.servier.com/), modified by the authors under the Creative Commons Attribution 3.0 Unported License (CC BY 3.0). Abbreviations: 4EBP1, eukaryotic initiation factor 4E-binding protein 1; FOXO, forkhead box O; IL-6 (R), Interleukin-6 (receptor); LC3, rat microtubule-associated protein 1 light chain 3; mTOR, mammalian target of rapamycin; MuRF-1, muscle RING-finger protein-1; S6rp, 6 ribosomal protein; TNF-α (R1 or R2), tumor necrosis factor-α (receptor 1 or receptor 2); TWEAK, tumor necrosis factor-related weak inducer of apoptosis.

Wistar–Han rats inoculated with AH-130 hepatoma cells) reported a decrease in the expression of essential cardiac muscle proteins, such as troponin-I, MHC, or actin, both at the mRNA and protein levels compared with control animals [14,19,20,27]. Regardless, whether proteolysis is proportional [19,20,27], or specific [14] remains at odds, with one study in Wistar–Han rats inoculated with AH-130 hepatoma cells reporting decreased protein expression of MHC, but no change in troponin-T or tropomyosin in tumor-bearing rats compared with controls [14]. Cell death has been consistently discarded as an alternative since most studies in this setting have not reported changes in caspase activity [27,29,33] and there is no loss of cells at the microscopic level [29]. Despite this, one study in Wistar–Han rats inoculated with AH-130 hepatoma cells detected increase caspase-3 activity in tumor bearing rats compared to controls, suggesting the possible participation of apoptosis in cardiac atrophy [14]. Notwithstanding, protein metabolism imbalance has been considered the main reason for cardiac atrophy. The mechanisms by which a cell becomes **hypercatabolic** have yet to be completely elucidated, but it is suspected that they are conserved throughout the human body (Box 1).

Few studies have approached the contribution of alterations in the protein synthesis machinery (Box 1) for cancer-induced cardiac cachexia [9,14,33]. In fact, only one study directly assessed the rate of protein synthesis. In this study, the rate of synthesis of heart myofibrillar proteins in 20-week-old male $APC^{min/+}$ mice that had developed colorectal cancer was measured using tandem mass spectrometry, finding a decrease in the rate of protein synthesis in 20-week-old male $APC^{min/+}$ mice compared with age-matched C57BL/6 mice [33]. The decrease was attributed to increased phosphorylation of AMP-activated protein kinase (AMPK)-α, a known molecular trigger for energy-saving shutdown of cellular processes, and to decreased phosphorylation of mTOR, despite an increase in the phosphorylation of Akt [43]. The reduced phosphorylation of mTOR was associated with reduced phosphorylation of both S6 ribosomal protein (S6rp; part of the 40S ribosomal subunit) and eukaryotic initiation factor 4E-binding protein 1 (4EBP1), further indicating the role of mTOR in the decreased rate of protein synthesis.

Box 1. The Fine-Tuned Machinery of Protein Synthesis and Proteolysis

The maintenance of protein homeostasis by cellular machinery is dependent on a delicate balance between protein synthesis and proteolysis, with just a small deviance from this balance being enough for a cell to become hypercatabolic. To this point, it is thought that the dysregulation of several molecules normally involved in this process and strictly regulated in physiological situations is at the origin of hypercatabolism. The signaling pathways commanding the process of mRNA translation in humans appear to have mTOR as an essential molecule in the process [78,79]. After activation by external stimuli, such as growth hormones, mTOR phosphorylates other molecules, such as 4EBP1 and p70S6K. In its normal state, 4EBP1 acts as an inhibitory molecule of the eukaryotic initiation factor 4E (eIF4E), which is an initiation factor of translation. When 4EBP1 is phosphorylated, it becomes inactive, thus leading to the activation of eIF4E. p70S6K is normally expressed in its inactive form and becomes active when phosphorylated. Upon activation, it activates S6rp and several initiation and elongation factors through phosphorylation [78,79]. As such, mTOR and its downstream targets have become hallmarks of protein synthesis [80–82].

By contrast, proteolysis is a process with functions ranging from protein recycling to regulation of cell death and cell cycle [83,84]. Two main mechanisms are known to mediate protein recycling: proteasome-associated proteolysis (UPS), and lysosome-associated proteolysis (autophagy) [83,84]. The UPS is a selective three-step enzymatically controlled process in which ubiquitin ligases (E3 ligases) ubiquitinate proteins. E3 ligases have merited particular attention, since they confer specificity to the reaction (reviewed in [85,86]). The E3 ligases muscle atrophy F-box (MAFbx)/atrogin-1 and MuRF-1 have received particular interest since their expression is specifically enhanced in striated muscle over a varied set of atrophic settings [87]. By contrast, autophagy is a relatively unselective process of proteolysis that makes use of an ubiquitously present organelle in eukaryotic cells, the lysosome [84]. When autophagy is activated, an autophagosome is formed through the recruitment of several molecules, from which beclin1 [88], LC3-II [89], and bnip3 [90] are of particular importance, because they are working regulators and/or initiators of this process.

In addition, in Wistar–Han rats inoculated with AH-130 hepatoma cells, 4EBP1 and ribosomal protein S6 kinase (p70S6K) phosphorylation was also diminished, while heart mass was decreased by approximately 35% by the end of the protocol compared with control rats [14]. This was attributed to diminished expression of insulin-like growth factor-1 receptor (IGF-1R) and insulin receptor (IR) [14]. Reduced phosphorylation of glycogen synthase kinase 3 (GSK-3) was also associated with the decreased heart mass in this study. In another study, using Sprague–Dawley rats with *N*-methyl-*N*-nitrosourea (NMU)-induced mammary tumorigenesis, overexpression of Akt protein was reported in tumor-bearing rats compared with controls, but appeared to occur at the extracellular matrix level (and not in cardiomyocytes), potentially justifying the observed fibrosis and the increase in heart weight by the time the rats were sacrificed [9]. This contradictory result might be due to cancer type and stage, or age and gender of the animal. Overall, cancer-induced cardiac cachexia appears to involve the negative modulation of the Akt/mTOR pathway, leading to decreased rates of protein synthesis.

At the same time that an apparent diminished protein rate of synthesis was verified, several studies in different animal models of cancer reported an increase of proteolysis in the cardiac muscle [9,14,20,24,26–28,32–34]. These studies were based on the expression of common mediators of proteolytic pathways, the expression of common biomarkers of proteolysis, or even expression of transcription factors involved in the activation of the proteolytic pathways (Box 1). Regarding expression of transcription factors, increased activity of nuclear factor kappa-B (NF-κB) was associated with proteolysis in two different animal models of cancer cachexia: the BALB/c and CD2F1 mouse models inoculated with C26 adenocarcinoma cells [28,32]. In both models, the increased activity of NF-κB was correlated with an increased expression of muscle RING-finger protein-1 (MuRF-1), providing evidence that the *MuRF1* gene is a target of NF-κB in cardiac muscle. More findings on the contribution of these proteolytic mechanisms were reported: increased protein expression of beclin1 and rat microtubule-associated protein 1 light chain 3-II (LC3-II) and mRNA expression of bnip3, suggestive of autophagy activation [14,24,26,27,33], and increased protein expression of tumor necrosis factor receptor-associated factor 6 (TRAF6), and protein and mRNA expression of atrogin-1 and MuRF-1, indicative of ubiquitin-proteasome system (UPS) activation [9,14,20,26,34] were detected in the heart of distinct rat and mouse models of cancer cachexia. This effect of cancer on heart mass was suggested to be gender dependent, with female mice being affected to a lesser extent than male mice, possibly due to a protective effect of estrogen [27]. However, this is a field of study plagued by inconsistencies. A common limitation to these studies is the lack of concomitant analysis of UPS and autophagy, resulting in misleading results. In addition, it has been established that different cancer models, age, and even tumor site inoculation can influence the expression of several biomarkers. A recent study determined that the location of inoculation (e.g., subcutaneous versus intraperitoneal) can lead to 'unreal' tumor microenvironments that do not correspond to those of naturally occurring tumors [44]. This, in turn, might lead to different patterns of circulating cytokines, which can influence muscle wasting [4]. Furthermore, studies vary in their analysis of whole-heart lysates, both left and right ventricle lysates or just left ventricle lysates, which can interfere with results, since different sections of the heart have different characteristics, both morphologically and metabolically [45]. This latter evidence can help explain the different results obtained even within the same cancer model. Overall, data suggest that both pathways are involved in cancer-induced cardiac wasting (Figure 1).

Exercise Training, Cancer, and Cardiac Remodeling

The discovery of cancer-induced cardiac cachexia has changed the panorama regarding treatment of patients with cancer. Not only have cancer therapies evolved, focusing on even

more personalized treatment plans to potentially limit the cardiotoxic effect of cancer therapies, but pharmacological ways to counter cancer-induced cardiac cachexia have also been explored [14,21,24,28,32]. However, these have only been explored at the preclinical level and no approved pharmacological therapies currently exist for cancer-induced cardiac cachexia. More recently, exercise training has emerged as a promising complement to pharmacological therapies, even though information about the effects of exercise in this particular setting is still scarce; to the best of our knowledge, only two preclinical studies have specifically addressed its role in cancer-induced cardiac cachexia [9,10]. Nevertheless, exercise training is considered a useful therapeutic adjuvant in the field of CVDs and cancer [7,8,15]. Some preclinical studies have also tried to mimic the positive effects of exercise training through pharmacological therapies, with moderate to good results (reviewed in [46]). The cardiovascular benefits of exercise training, both in normal and disease states, have been well reported over the past few years, ranging from structural (Box 2) to molecular changes in the heart and even modifications to the vasculature of the body, ultimately improving heart function [47–49]. Herein, we hypothesize the putative mechanisms behind the benefits of exercise training in the heart, with an emphasis on cancer-induced cardiac cachexia.

Cellular Adaptations to Exercise Training

For a long time, heart growth was entirely attributed to the hypertrophy of cardiomyocytes. However, with the recent discovery of **endogenous cardiac stem cells** (eCSCs) [50] and the capability of differentiated cardiomyocytes to proliferate [51,52], the notion that cardiomyocyte proliferation and renewal occur during physiological growth has gained traction [53]. Moreover, the discovery of newly formed cardiomyocytes in mice and rats with exercise-induced cardiac hypertrophy [54–56] challenged the claim that hypertrophy was solely a consequence of cardiomyocyte growth. The activation of eCSCs was reportedly dependent on the downregulation of CCAAT-enhancer binding protein β (C/EBPβ) and stimulation of CBP/p300-interacting transactivator with ED-rich carboxy-terminal domain-4 (CITED4) in C57BL/6 mice [52,54]. The proliferation of already-differentiated cardiomyocytes was found to be connected with activation of the neuregulin-1 (NRG1)/ErbB2/ErbB4 axis [51]. In a transgenic mouse model of constitutively active ErbB2, NRG1 promoted cardiomyocyte proliferation, albeit leading to pathological hypertrophy.

Box 2. Structural and Functional Alterations of the Trained Heart

Exercise training is known to stimulate physiological growth in the form of adaptive hypertrophy, which results in enhancement of cardiac function [91]. Cardiac hypertrophy can be defined as eccentric (wider cardiac cavity dimensions) or concentric (thicker ventricular walls), and is dependent on the type of exercise (e.g., endurance training versus resistance and/or strength training) [91]. Endurance exercise training, such as running, cycling, or swimming, is commonly associated with eccentric growth; during endurance exercise training, the heart is challenged with a volume overload. That is, the heart is required to pump greater quantities of blood during extended periods of time, and it adapts to do so by developing a larger structure to support the higher volume of blood flow. By contrast, during resistance and/or strength exercise training, such as weightlifting, the heart is submitted to short and sudden peaks of pressure overload that, over time, lead to concentric growth [91].

The different types of overload lead to different functional adaptations, with endurance exercise training being associated with decreased resting and submaximal heart rate, increased strength of contraction, and increased peak cardiac output [91]. By contrast, while resistance exercise training was not shown to decrease resting heart rate, it was shown to decrease resting blood pressure. Interestingly, static resistance training was more effective than dynamic resistance training [92]. Despite these changes, most sports involve a combination of both types of overload, leading to a mixed-type growth and, thus, to mixed-type functional adaptations [91]. However, in pathological settings, hypertrophy is only transitorily beneficial because ultimately heart failure ensues [49]. The reason behind this different outcome is still unknown. It has been proposed that the features of the cardiac overload, such as the duration and magnitude of the stimuli, may have a role in the development of an adaptive or maladaptive phenotype [93]. Moreover, a conjunction of factors appears to prevail, from which cellular and structural adaptations and activation of different molecular pathways are of particular interest [47,49].

Nevertheless, transient induction of ErbB2 expression after myocardial infarction led to improved cardiac regeneration [51]. NRG1 is upregulated in the trained heart, but whether its signaling pathway is activated remains undetermined [54]. Whether exercise training counteracts cancer-induced atrophy by promoting stem cell proliferation also remains unknown.

Fibroblast activity and/or proliferation also appears to increase in both types of growth, although leading to distinct outcomes. Fibrosis has severe consequences for heart function, leading to a stiffening of the heart and favoring arrhythmias [57]. In rats with cancer-induced cachexia, exercise counteracted cardiac fibrosis [9,10].

Molecular Adaptations to Exercise Training

Exercise training is capable of reversing the expression of the fetal gene set commonly associated with pathological stimuli, which includes **natriuretic peptides** and fetal isoforms of essential proteins [58,59]. Moreover, in Wistar rats, exercise training is capable of reversing the induction of gene expression associated with glycolysis and repression of genes associated with FAO, commonly associated with pathological stimuli, leading to an improvement in FAO [59]. This improvement appears to be related to mitochondrial biogenesis, with increased mitochondrial (mt)DNA and expression of genes associated with FAO enzymes (Figure 2) [59,60]. The molecule behind the regulation of gene expression is the transcription regulator peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α), which has been associated with fuel metabolism and mitochondrial biogenesis in response to exercise training (Figure 2). However, its role might be more broad than was previously thought, with the recent discovery of a novel function: PGC-1α is responsible for the transcription of irisin in skeletal muscle (Figure 2) [61]. Irisin is a newly identified exercise-induced peptide hormone, responsible for white adipose tissue **browning,** and recent evidence suggests that it has a cardioprotective role [62,63]. However, whether it is transcribed in the heart remains undetermined. Other myokines might also be involved in exercise-induced cardiac remodeling in the cancer setting (Box 3).

The transcription regulator C/EBPβ is also responsible for mediating exercise-induced adaptations (Figure 2) [47,49]. In C57BL/6 mice submitted to swimming, C/EBPβ was found to be downregulated compared with nonexercised mice, and knockdown of C/EBPβ resulted in cardiomyocyte growth, with expression of genes associated with exercise, including *PGC1a* [52]. The effects of C/EBPβ are mediated, at least partially, by CITED4. Upon C/EBPβ downregulation, CITED4 is upregulated, leading to a proliferation response by cardiomyocytes (Figure 2) [52]. Moreover, in a preclinical model of transgenic mice with overexpression of CITED4 in the heart, cardiac growth was characterized by preserved function, and without activation of fetal genes. These effects were mediated by interactions with mTOR, without activation of Akt1 (Figure 2) [64]. Taking into account that cancer-induced cardiac cachexia involves the negative modulation of the Akt/mTOR pathway, induction of fetal gene programs, and metabolic inefficiency, it will be important to test the potential of exercise training to revert cancer-induced cardiac cachexia through the modulations of these processes.

Another important set of molecules that appears to mediate the cardiac exercise-induced adaptations through gene regulation are **miRNAs** [65]. Numerous miRNAs have been reported as critical regulators in cardiac physiology and pathology [66]. Inside the vast host of miRNAs described, two are deserving of particular attention for being particularly overexpressed in exercised hearts: miR-29 and miR-222 [65]. miR-29 was associated with decreased cardiac fibrosis in a rat model of exercise [67]. This effect was achieved through the downregulation of the expression of various collagen isoforms by miR-29 (Figure 2), which was upregulated with

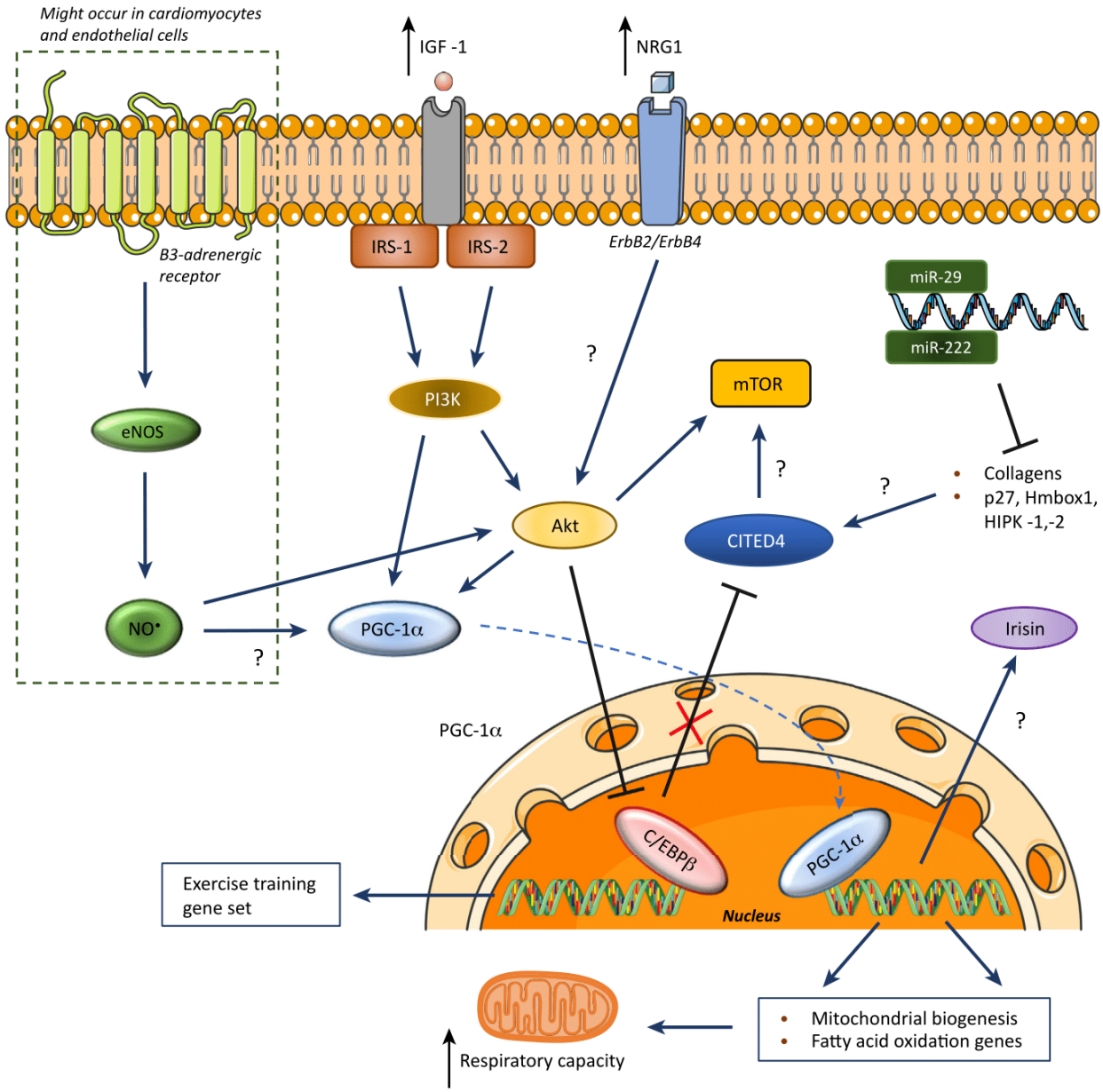

Figure 2. Integration of the Exercise-Induced Molecular Adaptations in the Heart. Exercise training upregulates insulin-like growth factor-1 (IGF-1). In turn, IGF-1 initiates a cascade of signals in cardiomyocytes, in which the phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt) axis takes a central role. Through Akt-dependent and -independent pathways, PI3K activates peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α), which is responsible for the transcription of genes associated with mitochondrial biogenesis and fatty acid oxidation, ultimately leading to increased respiratory capacity. Activation of PGC-1α and Akt can also happen through the endothelial nitric oxide synthase (eNOS)/NO axis. Akt is also responsible for inhibiting CCAAT-enhancer binding protein β (C/EBPβ), which activates the transcription of an exercise training gene set and activates CBP/p300-interacting transactivator with ED-rich carboxy-terminal domain-4 (CITED4), which is thought to interact with mammalian target of rapamycin (mTOR) and lead to cardiomyocyte growth. Newly discovered miRs, namely miR-29 and miR-222, have an impact across all the network, with special emphasis on activation of CITED4 by miR-222 through inhibition of cyclin-dependent kinase inhibitor 1B (p27), homeobox containing 1 (Hmbox1) and homeodomain-interacting protein kinase-1,-2 (HIPK-1,-2). Figure made using Servier Medical Art by Servier (https://smart.servier.com/), and modified by the authors under the Creative Commons Attribution 3.0 Unported License (CC BY 3.0). Abbreviations: IRS-1,-2, insulin receptor substrate-1,-2; NRG1, neuregulin-1.

Box 3. The Systemic Effects of Exercise Training: Myokines

Over the past few decades, evidence has accumulated regarding the powerful systemic effects of exercise training, motivating scientists to search for an 'exercise factor' [94]. This search has proven fruitful, with the discovery of several 'exercise factors', the so-called 'myokines'. Myokines are skeletal muscle-derived molecules the synthesis and secretion of which can be stimulated by exercise, mediating crosstalk between different organs in an autocrine, paracrine, or endocrine fashion (Figure I) [94]. One such molecule is interleukin-6 (IL-6), the first myokine to be described. This classical proinflammatory cytokine has taken on a new role in the context of exercise training, with studies reporting that skeletal muscle-derived IL-6 underlies part of the beneficial effects of exercise on inflammation or even tumorigenesis and tumor progression [75]. Following IL-6, myriad novel myokines were discovered, namely IL-8, IL-15, decorin, irisin, meteorin-like protein, and fibroblast growth factor 21 (FGF21) [95]. Recently, even molecules that were once thought to be adipokines were 'rebranded' as myokines, such as apelin [96]. With functions ranging from stimulation of angiogenesis to WAT browning, and even neuronal development, myokines are involved in an inexhaustible list of cellular processes [95].

Investigations supporting the role of myokines in the cardiovascular system have greatly increased in recent years, with emphasis on some myokines, such as follistatin-like 1 (Fstl1), vascular endothelium growth factor (VEGF), and IL-8 [97,98]. In addition, in a recent study, skeletal muscle-derived musclin, which was upregulated with exercise, was shown to interact with cardiac-derived ANP [99], further supporting the crosstalk between organs. However, there is still a lack of studies on the putative role of heart in the production of myokines (named cardiomyokines). Comprehensibly, clinical studies of the heart to the same extent of skeletal muscle are troublesome due to the invasive techniques utilized (biopsy). Notwithstanding, it is plausible that the heart is involved to some extent in the production of some cardiomyokines; the heart already produces factors that are not considered cytokines but exert effects in an auto- and endocrine fashion, the natriuretic peptides ANP and BNP. In fact, at least one cytokine has been reported to be produced and secreted by the heart, the mesencephalic astrocyte-derived neurotrophic factor (MANF) [100], opening the door for others to be discovered.

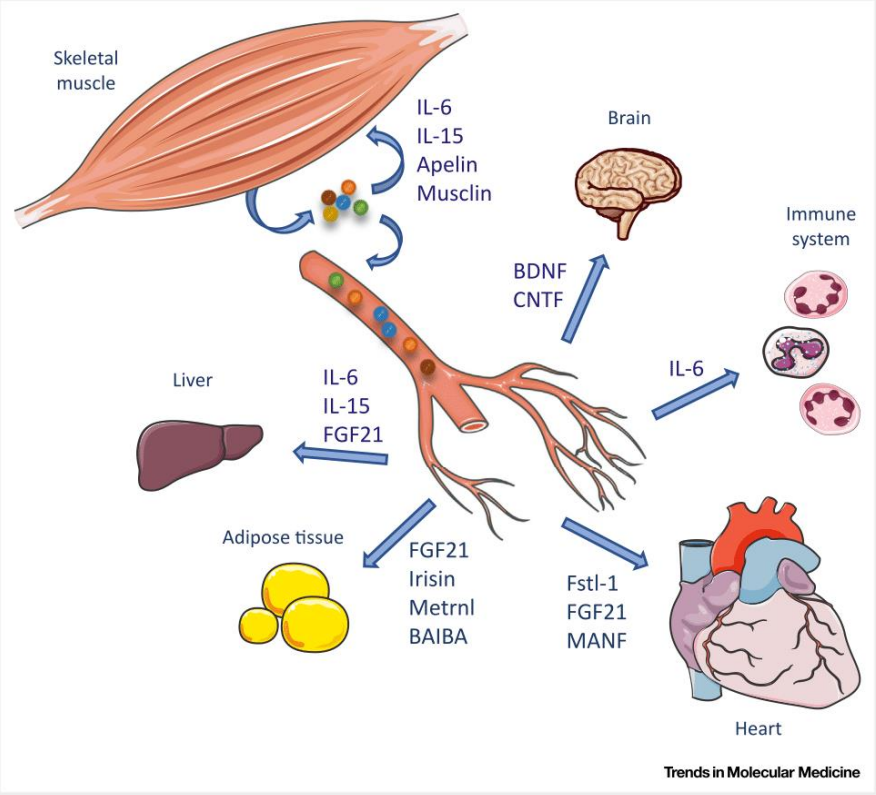

Figure I. [{Figure I}] Skeletal Muscle as an Endocrine Organ. Skeletal muscle produces and releases specific molecules into the bloodstream, the myokines. These molecules exert autocrine, paracrine, and endocrine functions in

different organs, namely the brain and heart. Figure made using the Servier Medical Art by Servier (https://smart.servier.com/), and modified by the authors under the Creative Commons Attribution 3.0 Unported License (CC BY 3.0). Abbreviations: BAIBA, β-aminoisobutyric acid; BDNF, brain-derived neurotrophic factor; CNTF, ciliary neurotrophic factor; FGF21, fibroblast growth factor 21; Fstl-1, follistatin-like 1; IL-6, interleukin-6; IL-15, interleukin-15; MANF, mesencephalic astrocyte-derived neurotrophic factor; Metrnl, meterorin-like.

exercise. However, histological analyses were missing to validate the morphological alterations promoted by exercise training [67]. miR-222 has been associated with a proproliferative and hypertrophic phenotype both *in vitro* (miR-222 silencing) in primary neonatal rat ventricular cardiomyocytes and *in vivo* in C57BL/6 mice subjected to a swimming protocol [68]. The effects of this miRNA appear to be mediated by the downregulation of four targets: p27, HIPK-1 and -2, and Hmbox1 (Figure 2). Notably, the combinatory interaction of these effectors appeared to induce CITED4, which, as mentioned, has an important role in cardiomyocyte growth [68]. While miRNA activation and function are still underexplored areas in the setting of exercise, it would be interesting to evaluate the role of these miRNA in the protection against fibrosis observed in cancer-induced cardiac cachexia.

The phosphoinositide 3-kinase (PI3K)/Akt axis appears to be the dominant signaling pathway in the activation of the aforementioned transcription pathways (Figure 2). Interestingly, PI3K(110α) had an essential role in physiological (exercise), but not in the pathological (aortic banding) growth of heart in a mouse model [58]. Activation of PI3K is mainly the result of growth factor stimulation, namely insulin and IGF-1, through their respective receptors (IR and ICG-1R), which are upregulated in the heart of exercised mice [69]. However, for the hypertrophic adaptation verified in response to exercise training, IGF-1 appears to be the only required factor (Figure 2), with IR-knockout mice still showing a hypertrophic response to exercise training [69]. Moreover, IGF-1R-knockout mice exhibited enhanced AMPK activation in response to exercise training compared with exercised control mice, despite having phosphorylation of Akt, highlighting the importance of the IGF-1 pathway in maintaining the hypertrophic response to exercise training [69]. Still, both receptors appear to have common effectors: insulin receptor substrate-1 (IRS-1) and -2 (IRS-2) (Figure 2) [60]. In a recent preclinical study, cardiomyocyte-specific deletion of IRS-1 or IRS-2 in mice prevented PI3K and PGC-1α activation through Akt-dependent and -independent mechanisms, ultimately leading to reduced mitochondrial capacity following exercise [60]. The mediator of the effect of PI3K is Akt, which is essential for physiological growth (Figure 2) [70]. In Akt-deficient mice, not only did the heart show impaired growth in response to exercise, but it also demonstrated an exacerbated response to pressure overload [70]. The molecular targets of Akt appear to be vast. It leads not only to PGC-1α activation, but also to C/EBPβ inhibition, with subsequent activation of CITED4 (Figure 2) [52]. Nevertheless, Akt activation can be stimulated by other pathways [69], namely endothelial nitric oxide synthase (eNOS)/NO (Figure 2) [71]. This pathway is upregulated in the trained heart and is associated with improved metabolic efficiency [71]. Another pathway for Akt activation is the NRG1/ErbB2/ErbB4 axis, which is active in proliferating and differentiated cardiomyocytes, as mentioned above [51]. This effect appears to be partially mediated by Akt, but its direct targets are still undetermined (Figure 2) [51]. Since cancer-induced cardiac cachexia involves the negative modulation of the Akt/mTOR pathway, the positive modulation of this pathway by exercise is an interesting field of research.

Another putative pathway that leads to PGC-1α activation is the eNOS/NO axis (Figure 2). While eNOS production of NO and other metabolites appears to be cardioprotective, with effects lasting for some time after stoppage of exercise training and persisting even after the Akt/mTOR axis has lost effect [72], genetic deletion of eNOS in a mouse model was shown to be

associated with abrogation of the positive exercise-induced effects [71]. Nevertheless, the interaction between eNOS and PGC-1α is still not fully understood, and the relative contribution of each cell lineage to the production of cardiac NO remains undetermined (Figure 2) [71].

A possible role for exercise training in the modulation of the heart antioxidant defenses was recently explored in a model of aging rats. In this model, exercise training led to increased activity of enzymes associated with the redox balance, a decreased concentration of lipid peroxidation products, and an increased activity of sirtuin 1 (Sirt1), which was thought to promote these changes through association with the forkhead box O3a (FOXO3a) [73]. Given that cancer-induced cardiac cachexia induces a state of redox imbalance in the heart, the capability of exercise training to improve antioxidant defenses should be explored.

Molecular Adaptations to Exercise Training in Cancer-Induced Cardiac Cachexia

The question that is left unanswered is how exercise training impacts cancer-induced cardiac cachexia. Recent studies have suggested that the benefits of exercise training are related to the modulation of tumoral activity (reviewed in [74–76]); exercise training has an impact across several hallmarks of cancer, ultimately leading to tumor size reduction. This reduction has obvious implications, most importantly the reduction of release of tumor-borne signals into the blood stream, which have been implicated in the cardiac remodeling induced by cancer cachexia [35]. However, the field of exercise oncology is still plagued by a lack of standardization of methodologies and cancer versus exercise models used, which can lead to contradictory results (reviewed in [74]). Contrary to the impact of exercise training on cancer physiology, only two studies have been published regarding the effects of exercise training in cancer-induced cardiac cachexia, both at the preclinical level [9,10]. In the first study, using Sprague–Dawley rats, 35 weeks of moderate (i.e., 60 min/day at 20 m/min 5 days/week) endurance training post cancer induction exerted a preventive effect in the cardiac remodeling induced cancer cachexia [9]. Exercise training not only had effects at the molecular level, by affecting heart protein expression and serum concentration of tumor necrosis factor-related weak inducer of apoptosis (TWEAK), but also impacted the structure of the heart, by reducing cardiomyocyte disorganization and fibrosis compared with sedentary tumor-bearing rats [9]. By contrast, in the second study, using Wistar rats, the effects of initiating exercise training in the later stages of cancer disease were studied [10]. In this study, 13 weeks of moderate endurance training (see above) were sufficient to reduce fibrosis and cardiomyocyte atrophy, while significantly increasing Mn-SOD and modulating NF-κB. Additionally, elevated protein expression of c-kit was detected in the heart of trained tumor-bearing rats compared with sedentary tumor-bearing rats, strengthening the idea that the heart is a self-regenerative organ and exercise training is a good modulator of the regenerative pathways [10]. However, cardiac functionality was not evaluated in these studies. Overall, exercise training appears to modulate some of the main features of cancer-induced cardiac cachexia, such as cardiomyocyte disorganization and fibrosis, possibly through the induction of antioxidant defenses and stimulation of stem cell proliferation.

Concluding Remarks

Although the presence of cardiac cachexia in therapy-naïve patients with cancer is now recognized, it is an underappreciated syndrome with a tremendous burden on the patient's chances of survival (Box 4). There is a need for therapies to counteract the effects of cancer on the heart, and exercise training has emerged as a high-quality adjuvant therapy, given the tremendous potential already demonstrated in several diseases, such as CVDs [77]. The little experimental evidence reported so far points towards the potential of using exercise training as a therapy for cancer-induced cardiac cachexia through the modulation of tumoral activity,

Outstanding Questions

What type of exercise training is more beneficial to target cancer-induced cardiac cachexia: endurance, resistance, or a combination of both? What is the best dosage? Both endurance and resistance exercise are known to induce hypertrophy of the heart; however, endurance exercise training is known to best improve cardiovascular performance. Endurance training might be difficult to implement initially, given the poor conditioning of these patients; resistance training may be better tolerated and could be used to increase the initial physical fitness. Future research should determine what combination of training is best: potentially both, given that endurance training has anti-inflammatory properties and resistance training has greater anabolic potential. The optimal amount should also be further scrutinized.

Are the benefits a result of positive effects of exercise on the heart or in the tumor? Preclinical evidence suggests that exercise training modulates tumoral size and activity, and consequently modulates inflammatory cytokines released to the blood stream. This will have an impact on the heart through modulation of upstream triggers of cardiac catabolism (e.g., IL-6, TNF-α, and TWEAK) but the cardioprotective effects of exercise might also be mediated through indirect mechanisms, such as skeletal muscle-derived myokines, and should be explored because it might lead to the identification of molecular targets.

Is it better to use exercise training as a preventive or therapeutic measure? While there is insufficient clinical evidence to assure the safety and effectiveness of exercise in patients with established cancer cachexia, there is satisfactory data suggesting that performing exercise after cancer diagnosis can prevent or delay the progression to cachexia by decreasing skeletal muscle wasting and improving strength. Preclinical data provide preliminary insights by showing that exercise, performed before or after cancer diagnosis, can counteract some of the structural and molecular changes of cancer-induced cardiac cachexia.

Box 4. Clinician's Corner

Cancer-induced cardiac cachexia is an underappreciated syndrome present in some therapy-naïve patients with cancer. Cardiotoxic therapies should be administered taking into account this information.

New therapies, with a lower cardiac toxicity should be investigated and designed to prevent an aggravation of the patient's health status.

Exercise training is a positive modulator of several molecular pathways in the heart in both physiological (e.g., athlete) and pathological conditions (e.g., the patient with CVDs). Preclinical evidence highlights the potential role of exercise training as an adjuvant and/or therapy for cancer-induced cardiac cachexia.

As with all therapies, efforts should be made to establish the safety and tolerability of an exercise intervention in these patients, and to tailor the load of exercise training to each patient's condition, which will not only provide better results, but also encourage the patient to comply with the exercise plan.

inflammation, cardiac fibrosis and cardiomyocyte organization, and metabolism and oxidative defenses. Despite all the positive developments, a glaring lack of information remains regarding the direct effect of exercise training in this particular setting, and several potentially difficult crossroads exist along the way (see Outstanding Questions). Future studies should focus on the crosstalk between cancer-induced cardiac cachexia and distinct exercise training programs, considering cancer type and stage, age, and gender, for devising personalized treatment strategies to improve the cardiac health of patients with cancer.

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CHAPTER III

Experimental Work. **Crosstalk Between Serum Testosterone Concentration and Exercise in Prostate Cancer-Induced Cardiac Remodelling**

1. Introduction

Cardiomyopathy and heart failure in surviving cancer patients is a well-established complication secondary to chemotherapy or radiotherapy-induced cardiotoxicity, adversely impacting the overall morbidity and mortality [1]. A relatively underexplored topic is related with the influence of cancer itself on cardiac function and remodelling of chemotherapy- and radiotherapy-naive patients, as recently reviewed by our group [2]. Briefly, clinical studies showed increased circulating levels of biomarkers of cardiac damage [3–5], myocardial fibrosis [6] and cardiac dysfunction [7,8] in therapy-naive cancer patients, which is further supported by several pre-clinical models [9–13]. While the mechanisms underlying such changes remain to be disclosed, it has been hypothesized that cytokines released by the tumour cells could play a major role [14]. Cancer-induced changes may directly worsen the patient's prognosis by limiting or delaying the use of certain therapies and, in addition, it may also contribute for some of the cardiovascular complications occurring later in life in cancer survivors [15]. Thus, it is mandatory to characterize the changes taking place in the heart of therapy-naïve cancer patients, to understand if they are cancer-type and/or stage specific, to clarify the molecular mediators for the cross-talk between the heart and cancer cells and, ultimately, to develop preventive or therapeutic strategies for such changes.

The cardioprotective effects of exercise training have been extensively acknowledged both in physiological and pathological scenarios [16], from which cardiovascular diseases are of particular interest given that cancer seems to cause cardiac dysfunction [2]. While there are specific medical conditions that may limit or even contraindicate exercise [17], the truth is that, for stable and well medicated cardiac patients, exercise training is safe, beneficial and highly recommended [16]. For instance, in patients with heart failure, exercise training was shown to reduce hospitalization rate, to increase survival, and to improve cardiac function and remodelling [17]. In accordance, it has been hypothesised that exercise training could potentially be used as an adjuvant therapy to prevent cancer-induced cardiac cachexia [2]. Early pre-clinical studies in the setting of bladder and breast cancer support this hypothesis, showing that exercise training positively modulated cardiac structural and metabolic remodelling and improved antioxidant defences in the myocardium [18,19]. Because the remodelling of the heart may be cancer-specific, it is imperative to design studies in different types of cancer [2]. Considering that prostate cancer is the fifth most incident and the second most prevalent worldwide, with a 41%

increase in years living with disability during the last decade [20], we explored the preventive potential of life-long exercise training in cancer-induced cardiac remodelling in an animal model of chemically- and hormonally-induced prostate cancer submitted to 53 weeks of exercise training.

2. Material and Methods

2.1. Animals

Fifty-four male WU rats (age= 4 weeks) were obtained from Charles River Laboratories (France). During the experimental protocol, the animals were housed in the University of Trás-os-Montes e Alto Douro's (UTAD) bioterium. After arrival, the animals remained in quarantine for two weeks, after which they were randomly divided according to the experimental groups and allocated in cages (5 rats per cage) and maintained under controlled conditions: 18±2ºC temperature, 55±5% relative humidity, inverted 12-hour day/night (20:00-8:00) cycles, with free access to food (standard laboratory diet 4RF21®, Mucedola, Italy) and water. The experimental protocol was approved by UTAD's Committee for Animal Well-Being (*Órgão Responsável pelo Bem-Estar Animal*), and by the Portuguese Ethics Committee for Animal Experimentation (*Direção Geral de Alimentação e Veterinária*) (license nº 021326).

2.2. Experimental Protocol

After the quarantine period, animals were assigned to one of four experimental groups: sedentary control (CONT+SED, n=10), exercised control (CONT+EX, n=10), sedentary with prostate cancer (PCa+SED, n=15) and exercised with prostate cancer (PCa+EX, n=19). Animals from the exercise training groups started the training programme at the 8th week of age. First, in order to determine the training speed (70% of maximal running speed), a maximal running speed test was performed. After that, the animals started the training program that consisted of running on an electric treadmill (Treadmill Control LE 8710, Harvard Apparatus, USA) for 60 min/day, at 70% of maximal running speed, 5 days per week, for 53 weeks. In the first two weeks of training, the load (intensity and duration) of exercise was progressively increased until the target duration and running speed was reached. During the course of the exercise programme, running speed was adjusted based on a maximal running speed test that was performed every 6 weeks. In order for the

sedentary groups (CONT+SED and PCa+SED) to be subjected to the same handling stress as those in the exercise training groups, sedentary rats were regularly placed on a non-moving treadmill for a couple minutes.

At the 12th week of age, prostate cancer was induced in the animals from PCa groups (PCa+SED and PCa+EX) (**Figure 1**). Initially, flutamide (20 mg/Kg, prepared in 10% propylene glycol and 5% ethanol) was administered subcutaneously for 21 consecutive days. Two days after the end of flutamide administration, testosterone propionate (100 mg/Kg, dissolved in corn oil) was subcutaneously administered, and two days later, methylnitrosurea (MNU, 30 mg/Kg, prepared in 0.1 M pH 4.8 citrate buffer) was administered intraperitoneally. Fifteen days after MNU administration, crystalline testosterone capsules [3.5 cm length, silicone tubes (*Silastic® Tubbing*) filled with crystalline testosterone and sealed with medical glue (*Silastic® Medical Adhesive Silicone Type*] were implanted subcutaneously through a small incision in the interscapular region, followed by closure with a surgical suture. The procedure was done under anaesthesia (75 mg/Kg of ketamine and 10 mg/Kg of xylazine, i.p.).

At the 61st week of age (53 weeks after the initiation of the treadmill exercise programme and 45 weeks after MNU administration), animals were euthanised with an overdose of ketamine/xylazine (i.p), followed by exsanguination by cardiac puncture. Blood was collected immediately to obtain serum, and thereafter a complete necropsy was performed. Heart, prostate, lung, liver, mesenteric adipose tissue and *gastrocnemius* were removed and weighted (**Figure 1**). The heart was divided in two sections, with one being stored at -80ºC for biochemical analysis and the other (apex) immediately processed for histological analysis.

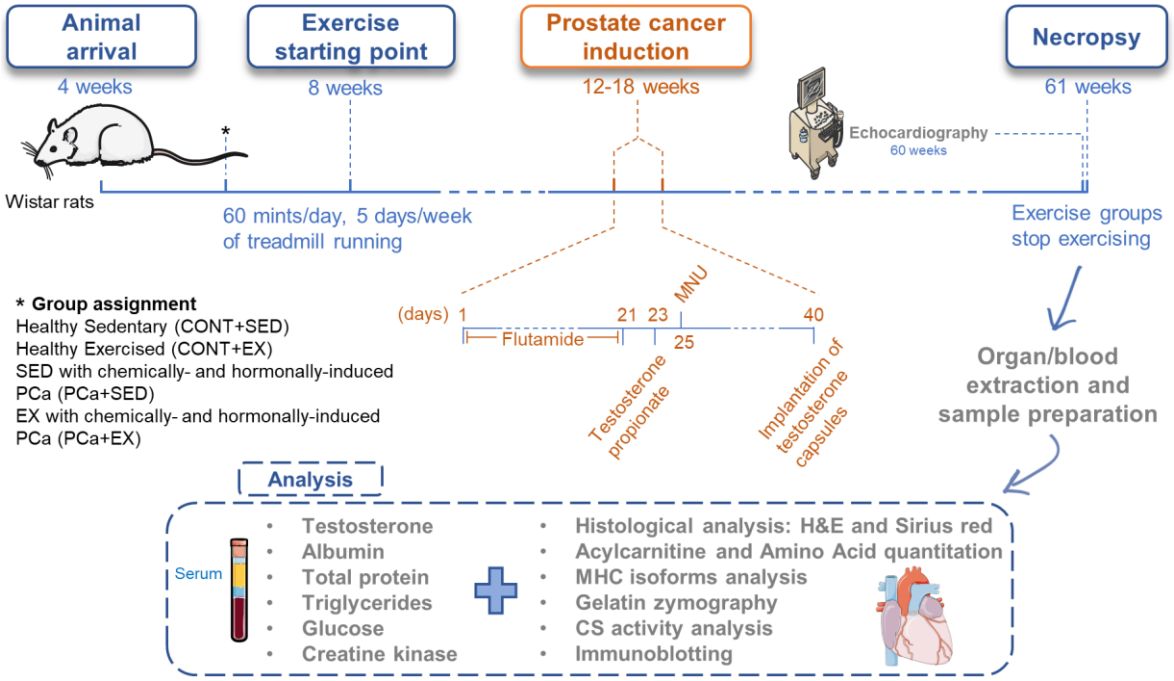

Figure 1. Overview of the experimental protocol followed in this study. After arrival, animals spent two weeks in quarantine, after which they were divided into four experimental groups: sedentary control (CONT+SED, n=10), exercised control (CONT+EX, n=10), sedentary with prostate cancer (PCa+SED, n=15) and exercised with prostate cancer (PCa+EX, n=19). Animals from EX groups started the exercise-training programme with 8 weeks of age. It consisted of treadmill running 60 mints/day, 5 day/week at 70% their maximal capacity, during 53 weeks. Four weeks after exercise initiation, cancer was induced in PCa groups through the sequential administration of flutamide, testosterone propionate, MNU and implantation of testosterone capsules. One week prior to the end of the protocol, echocardiographic evaluation was performed. Twenty-four hours after the last training session, rats were euthanised, and the organs and blood were extracted, followed by evaluation of several parameters. Figure was made using Servier Medical Art by Servier (https://smart.servier.com/), which was modified by the authors under the Creative Commons Attribution 3.0 Unported License (CC BY 3.0).

2.3. Analysis of biochemical parameters in serum samples

Serum testosterone concentration was determined using an ELISA Kit (582701; Caymann Chemical, MI, USA), following the manufacturer's instructions. Briefly, this is a competitive assay based on the competition between testosterone and a testosterone-acetylcholinesterase conjugate, with a concentration range from 3.9 to 500 pg/mL and a sensitivity of approximately 6 pg/mL. Eight testosterone standards with decreasing testosterone concentrations were prepared and the corresponding absorbance was measured at 412 nm using a microplate reader (Tecan® Infinite M200), generating a standard curve.

The serum samples were diluted according to the manufacturer's instructions, the absorbance was measured and the circulating levels of testosterone were determined by using the standard curve. Values are presented in pg/mL.

Regarding serum albumin, total protein, triglycerides, glucose and creatine kinase, these parameters were measured in duplicate on a Prestige 24i automated analyser (PZ Cormay, Poland).

2.4. Echocardiographic assessment

Echocardiographic evaluation was conducted with animals at rest one week prior to the end of the protocol. Transthoracic echocardiography was performed by an Acuson Sequoia C512 (Siemens, Germany) ultrasound device with a 15 MHz linear cardiac transducer. Measurements were obtained from standard views according to accepted standards for dogs and humans, using transthoracic two-dimensional, M-mode, tissue and colour Doppler imaging [21,22]. Rats were anaesthetised with inhalation of 8% sevoflurane (Sigma Delta Anaesthetic Vaporizer; Penlon, United Kingdom) through an in-house manufactured co-axial breathing system, and endotracheally intubated (14–16G iv catheter). Anaesthesia was maintained with 2.5–3 % sevoflurane and adjusted according to toe-pinch reflex. All data were collected using a track ball-driven cursor and the help of the ultrasound system software. The measured beats were selected on the basis of quality of the recording and presence of a regular cardiac rhythm. Three representative cardiac cycles were analysed, and a mean value was calculated for each measurement.

2.5. Histological analysis of cardiac muscle and prostate

Cardiac muscle samples (collected from the apex) and prostates were fixed by diffusion in 4% (v/v) buffered paraformaldehyde for 24 hours, subsequently dehydrated through graded ethanol and then included in paraffin blocks (xylene was used in the transition from ethanol to paraffin). Serial sections (5 μm of thickness) of paraffin blocks were cut utilizing a microtome and mounted on silane-coated slides. The slides were dewaxed in xylene, hydrated through graded ethanol and washed in water. Thereafter, deparaffinised sections of cardiac tissue were stained with Haematoxylin-Eosin or Picrosirius red for cardiomyocyte cross-sectional area or cardiac fibrosis analysis, respectively, as previously described by our group [23]. Prostate sections were stained with

Haematoxylin-Eosin and slides were observed blindly, under a light microscope, by two independent researchers from UTAD.

2.6. Biochemical analysis of cardiac muscle

2.6.1. Cardiac muscle preparation

Portions of cardiac muscle of approximately 50 mg were homogenised in 1 mL of 100 mM phosphate buffer (50 mM KH_2PO_4, 50 mM $Na_2HPO_4.H_20$ pH 7.4 with 200 mM PMSF and protease inhibitor cocktail (P0044 and P5726, Sigma 1:1000)). The protein content of the cardiac muscle homogenates was assayed with RC-DC™ Protein Assay (Bio-Rad, CA, USA) following the manufacturer's instructions. In brief, to 5 µL of cardiac muscle sample or protein standard (bovine serum albumin; concentrations ranging from 0.3 to 10.0 mg/mL), 25 µL of Reagent A' (mixture of reagent S with reagent A in a 20 µL:1 mL ratio) was added, which was followed by mixture. Subsequently, 200 µL of Reagent B was added, followed by mixture and incubation at room temperature for 15 minutes. Thereafter, absorbance was measured at 750 nm in a Multiskan GO microplate spectrophotometer (Thermo Scientific, MA, USA).

2.6.2. Myosin Heavy Chain isoforms analysis

Myosin Heavy Chain (MHC) isoforms were separated according to Talmadge and Roy [24]. Briefly, 20 µg of cardiac muscle protein of each sample were diluted in loading buffer (125 mM Tris, pH 6.8; 4% SDS (w/v); 15% glycerol (v/v); 20% β-mercaptoethanol (v/v); 0.1% bromophenol blue) in a 1:2 ratio and incubated at 97ºC for 5 minutes. Subsequently, loaded samples were electrophoresed in modified polyacrylamide gels – the stacking gel consisted of 30% (v/v) glycerol, 4% acrylamide, acrylamide:bis-acrylamide (50:1), 0.07 mM Tris (pH 6.8), 4 mM EDTA and 0.4% (w/v) SDS. The separating gel was composed of 30% (v/v) glycerol, 8% acrylamide, acrylamide:bis-acrylamide (50:1), 0.2 mM Tris (pH 8.8), 0.1 M glycine and 0.4% (w/v) SDS – for 20h at 80V. After electrophoresis, gels were stained in Coomassie Brilliant Blue G250 and scanned in a Gel Doc™ XR+ System (Bio-Rad).

2.6.3. Gelatin zymography

Zymography assays were performed according to Vitorino *et al.* [25]. Briefly, 40 µg of cardiac muscle protein of each sample were diluted in charging buffer (100 mM Tris pH 6.8, 5% SDS (w/v), 20% glycerol (v/v), 0.1% bromophenol blue, and completed with deionised water) in a 1:2 ratio and incubated at room temperature for 10 minutes. Subsequently, samples were electrophoresed in 10% gelatin-containing polyacrylamide gels (0.1% gelatin) for 90 minutes at 125V. After the run, gels were incubated 2x30' in renaturation buffer (2.5% Triton X-100) at room temperature with soft agitation. Thereafter, gels were incubated in development buffer (50 mM Tris, 5 mM NaCl, 10 mM $CaCl_2$, 1 µM $ZnCl_2$, pH 7.4, 0.02% (v/v) Triton X-100) for 30 minutes at room temperature with soft agitation, which was followed by overnight incubation (approximately 16 hours) at 37ºC in new development buffer. For specific inhibition of metalloproteinases, zymograms were incubated in a development buffer containing 10 mM EDTA. After incubation, gels were stained in 0.4% (w/v) Coomassie Brilliant Blue G250 prepared in 50% (v/v) ethanol and 10% (v/v) acetic acid. Gels were distained through graded ethanol solutions starting at 25% (v/v) ethanol with 5% (v/v) acetic acid and scanned in a GS-800™ Calibrated Densitometer (Bio-Rad).

2.6.4. Acylcarnitine and Amino Acid Quantitation

Acylcarnitine (AC) and Amino Acid (AA) quantitation was performed according to Petucci *et al.* [26]. In brief, 100 µL of cardiac muscle homogenate of each sample were added to 360 µL of methanol, followed by vortexing and centrifugation at 14000 rpm (10ºC, 5 minutes). Afterwards, 140 µL of supernatant were collected to a 96-well plate and 100 µL of methanol containing deuterated acylcarnitine internal standard solutions (Cambridge Isotope labs, MA, USA) was added to each well. The mixture was subsequently dried using nitrogen at 45ºC and shortly thereafter derivatised to the corresponding methyl esters by incubation with 95 µL of 3N methanolic HCl at 50ºC for 15 minutes. The samples were then dried once again using nitrogen at 45ºC and reconstituted with 200 µL of 80% methanol for flow injection MS/MS in an API 4000 QTRAP (Sciex, Washington, D.C., USA).

2.6.5. Citrate synthase activity

Citrate synthase (CS) activity was measured in cardiac muscle homogenates according to the method described by Coore *et al.* [27]. In brief, a determined amount of cardiac muscle homogenate (4 μL) was diluted in reaction buffer (0.1% of Triton X-100, 200 μM DTNB, 200 μM acetyl-CoA and 100 mM of buffer [Tris pH 8.0]). The solution was completed to a final volume of 200 μL with deionised water, stirred and the absorbance was measured at 412 nm for 2 minutes. Thereafter, oxaloacetate (OAA) was added to achieve a 100 μM concentration, the solution was stirred, and absorbance was immediately measured at 412 nm for 2 minutes. The first measurement is a measure of mitochondrial integrity, while the second is the measurement of CS activity, measured by the appearance of DTNB-CoA (product of DTNB reaction with released SH-CoA from OAA reaction with acetyl-CoA, catalysed by CS; molar extinction coefficient of 13.6 $mM^{-1}.cm^{-1}$). All absorbance measurements were done in a Multiskan GO microplate spectrophotometer (Thermo Scientific).

2.6.6. Immunoblotting analysis

Forty μg of cardiac muscle protein of each sample were diluted in loading buffer (125 mM Tris, pH 6.8; 4% SDS (w/v); 15% glycerol (v/v); 20% β-mercaptoethanol (v/v); 0.1% bromophenol blue) in a 1:2 ratio and incubated at 97ºC for 5 minutes. Subsequently, loaded samples were electrophoresed in 12.5% (or 15% for CITED4) polyacrylamide gels as described by Laemmli [28]. After adequate separation, gels were blotted onto a nitrocellulose membrane (Amersham™ Protan™, GE Healthcare Lifesciences) in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol) for 2 hours at 200 mA. After membrane preparation, non-specific binding was blocked for 1h in 5% (w/v) non-fat dry milk in TBS-T (100mM Tris, 1.5 mM NaCl, 0.5% Tween 20). Immediately, membranes were incubated with primary antibody [diluted 1:1000 in 5% (w/v) non-fat dry milk in TBS-T] for 1h at room temperature, followed by overnight incubation at 4ºC (mouse monoclonal anti-ATP synthase subunit β, ab14730, Abcam; rabbit anti-GAPDH, ab9485, Abcam; rabbit anti-PGC-1α, ab191838, Abcam; rabbit anti-ETFDH, ab91508, Abcam; rabbit anti-PPARα, ab24509, Abcam; rabbit anti-AR, 06-680, Merck Millipore; rabbit anti-ErRα, 07-662, Merck Millipore; rabbit anti-CITED4, MBS833529, MyBioSource; all antibodies are polyclonal, unless otherwise stated). Thereafter, membranes were washed 3 x 10 minutes in TBS-T,

which was followed by incubation with secondary HRP-conjugated anti-mouse or anti-rabbit (GE Healthcare Life Sciences; diluted 1:1000 in 5% (w/v) non-fat dry milk in TBS-T) for 2 hours at room temperature. Finally, membranes were again washed 3 x 10 minutes in TBS-T and immunoreactive bands were detected by enhanced chemiluminescence (WesternBright™ ECL, Advansta) according to the manufacturer's procedure. Images were recorded using a ChemiDoc™ Imaging System (Bio-Rad) and analysed with Image Lab (version 5.0, Bio-Rad). The optical densities obtained were expressed in arbitrary units. Equal protein loading was confirmed by Ponceau S staining.

2.7. Statistical analysis

Values are presented as mean ± standard deviation for all variables. The Kolmogorov-Smirnov test was performed to check the normality of the data. Statistical analysis was performed using a two-way analysis of variance (ANOVA) followed by the Tukey multiple comparisons post hoc test. Results were considered significantly different when $p < 0.05$. GraphPad Prism (version 7.0) was the software used.

3. Results

3.1. Characterisation of morphometric and serum parameters

Morphometric parameters are displayed in **Table 1**. Body weight was significantly reduced in PCa+SED, CONT+EX and PCa+EX in comparison to CONT+SED ($p < 0.001$), while no differences where noted between the exercised groups. Exercise resulted in greater body weight loss in the presence of PCa, as PCa+EX presented a lower body mass than their sedentary counterpart ($p < 0.001$ *vs.* PCa+SED). No differences were detected in *gastrocnemius* mass, suggesting that body weight loss was not related to muscle mass. Mesenteric fat mass was used in representation of adipose tissue depots and was significantly reduced only in CONT+EX ($p < 0.01$ *vs.* CONT+SED) and PCa+EX ($p < 0.01$ *vs.* PCa+SED). These differences were confirmed when mesenteric fat mass was adjusted to tibia length - in morphometric characterisation, adjustment to tibia length has been suggested as an ideal alternative to body mass normalisation in situations in which body mass might be affected by the experimental conditions [29]. Regarding heart mass, we found an intriguing reduction in the heart mass of CONT+EX group ($p < 0.05$ *vs.* CONT+SED), which was prevented in PCa+EX ($p < 0.01$ *vs.* CONT+EX). These differences were confirmed

when heart mass was adjusted to tibia length. The liver was also weighted and its mass was significantly reduced by exercise in both CONT+EX ($p < 0.001$ *vs.* CONT+SED) and PCa+EX ($p < 0.01$ *vs.* CONT+EX). Cancer per se had no effect. These differences were confirmed when liver mass was adjusted to tibia length. Lung mass was unaffected by PCa or exercise in isolation. However, when combined, it resulted in lung mass reduction as observed in PCa+EX group ($p < 0.01$ *vs.* PCa+SED and $p < 0.01$ *vs.* CONT+EX), which was also confirmed when lung mass was adjusted to tibia length. Finally, prostate mass was significantly increased in both PCa+SED and PCa+EX groups ($p < 0.001$ *vs.* CONT+SED and $p < 0.001$ *vs.* CONT+EX, respectively), but also in CONT+EX group ($p < 0.05$ *vs.* CONT+SED), suggesting that exercise per se had an impact on prostate mass. Similar findings were observed after adjustments to tibia length.

Consistent with prostate mass changes, all animals from PCa groups developed pre-neoplastic and neoplastic prostate lesions. Invasive carcinomas alone or associated with PIN and/or dysplasia were observed in both sedentary and exercised animals. Curiously, exercised animals developed more frequently multiple neoplastic and pre-neoplastic lesions (data not shown). Intriguingly, a small number of control animals also developed pre-neoplastic and neoplastic prostate lesions. From these, only exercised animals developed invasive carcinomas (data not shown).

Table 1. General morphometric parameters.

	Experimental groups			
	CONT+SED	PCa+SED	CONT+EX	PCa+EX
Body mass (g)	554.69±25.70	494.59±34.12***	442.02±19.03***	416.81±24.36***§§§
Gastrocnemius mass (g)	4.408±0.279	4.438±0.266	4.386±0.308	4.325±0.364
Gastrocnemius-to-tibia length (g/mm)	0.956±0.042	0.956±0.039	0.974±0.056	0.946±0.067
Mesenteric fat mass (g)	0.943±0.363	0.756±0.171	0.485±0.168**	0.430±0.265***§§
Mesenteric fat-to-tibia length (g/mm)	0.208±0.078	0.157±0.051	0.120±0.053*	0.096±0.060***§
Heart mass (g)	1.506±0.113	1.423±0.118	1.316±0.118*	1.505±0.176$^{\#\#}$
Heart-to-tibia length (g/mm)	0.334±0.028	0.314±0.019	0.287±0.025**	0.329±0.037$^{\#\#}$
Liver mass (g)	12.917±2.083	12.610±0.847	10.000±0.239***	11.918±1.756$^{\#\#}$
Liver-to-tibia length (g/mm)	2.619±0.545	2.740±0.172	2.123±0.077*	2.552±0.475$^{\#}$
Lung mass (g)	1.589±0.201	1.589±0.196	1.591±0.197	1.364±0.081$^{**\#\#§§}$
Lung-to-tibia length (g/mm)	0.355±0.040	0.345±0.047	0.348±0.049	0.300±0.018$^{**\#§}$
Prostate mass (g)	2.889±0.283	4.685±0.534***	3.450±0.257*	4.799±0.415$^{***\#\#\#}$
Prostate-to-tibia length (g/mm)	0.637±0.058	1.041±0.139***	0.759±0.073*	1.054±0.097$^{***\#\#\#}$

[a]Values are expressed as mean ± standard deviation (*p < 0.05 *vs.* CONT+SED; #p < 0.05 *vs.* CONT+EX; §p < 0.05 *vs.* PCa+SED; **p < 0.01 *vs.* CONT+SED; ##p < 0.01 *vs.* CONT+EX; §§p < 0.01 *vs.* PCa+SED; ***p < 0.001 *vs.* CONT+SED; ###p < 0.001 *vs.* CONT+EX; §§§p < 0.001 *vs.* PCa+SED).

Serum parameters analysis results are displayed in **Table 2**. Testosterone levels were greatly increased in both PCa groups when compared with the respective controls ($p < 0.001$ *vs.* CONT+SED and $p < 0.001$ *vs.* CONT+EX, respectively), as expected considering the pre-clinical model used in this study [30]. Moreover, exercise exacerbated the increase in the levels of serum testosterone in tumour bearing animals ($p < 0.001$ *vs.* PCa+SED). No significant difference was detected between control groups regarding this parameter. When we assessed the interaction between morphometric and serum testosterone data, we detected a significant positive correlation between serum testosterone concentration and prostate ($p < 0.001$, $r = 0.8351$; **Figure 2*A***) and heart mass ($p < 0.05$, $r = 0.3625$; **Figure 2*C***); and a significant, but negative correlation between serum testosterone and body ($p < 0.05$, $r = -0.3785$; **Figure 2*B***) and lung mass ($p < 0.05$, $r = -0.3458$; **Figure 2*D***). No correlation was

detected between serum testosterone concentration and liver or mesenteric fat mass (data not shown).

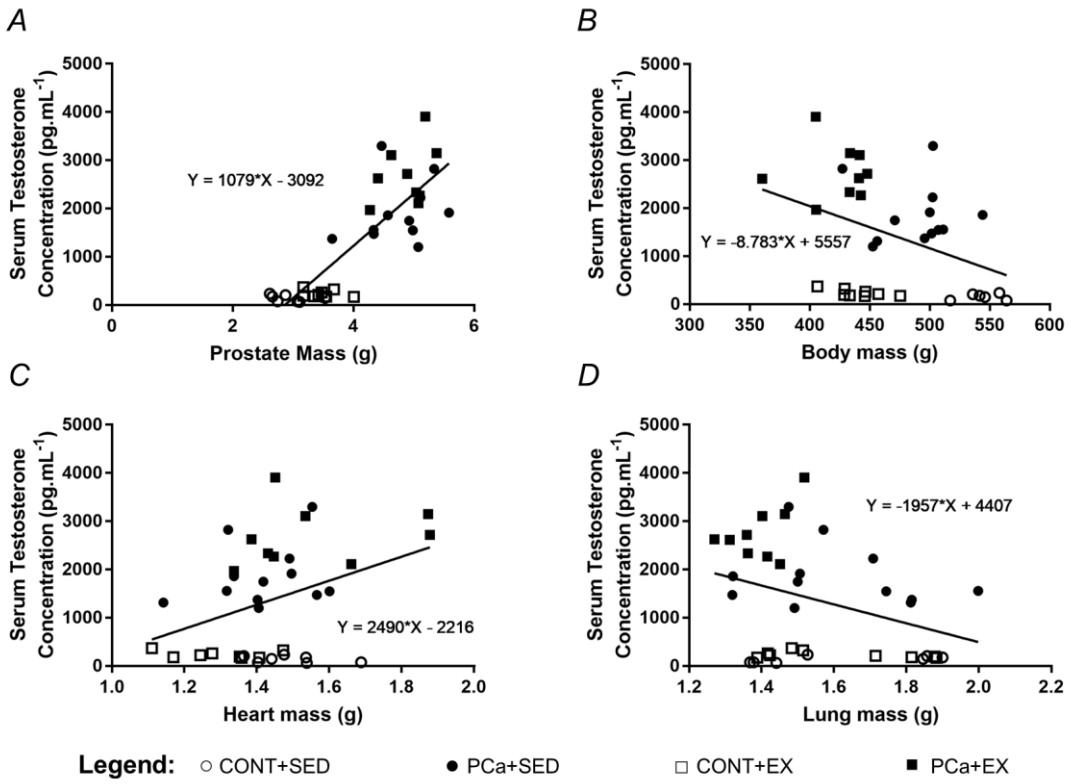

Figure 2. Correlation analysis between morphometric parameters and serum testosterone concentration. *A*, prostate mass; *B*, body mass; *C*, heart mass; *D*, lung mass.

Albumin was decreased in all groups but significance was obtained only for PCa+EX ($p < 0.01$ *vs.* CONT+SED and $p < 0.05$ PCa+SED), while total protein was significantly reduced in all groups ($p < 0.01$ *vs.* CONT+SED). Triglycerides were also decreased in all groups, but significance was found only for the PCa groups ($p < 0.01$ *vs.* CONT+SED). Glucose was significantly reduced in both EX groups ($p < 0.05$ *vs.* CONT+SED), as previously described in human patients [31]. Finally, CK levels were increased in both exercised groups, though significance was found only for PCa+EX in comparison to its sedentary counterparts ($p < 0.01$ *vs.* PCa+SED), possibly an acute manifestation of the last training session that was performed 24 hours before blood collection.

Table 2. General serum parameters.

	Experimental groups			
	CONT+SED	PCa+SED	CONT+EX	PCa+EX
Testosterone (pg/mL)	140.08±64.28	1862.17±608.66***	233.74±66.98	2680.54±550.25*** $^{\#\#\#\S\S\S}$
Albumin (g/L)	42.45±6.11	40.57±4.02	39.77±1.36	37.04±2.60**§
Total protein (g/L)	63.14±7.33	56.33±2.03**	56.04±2.25**	54.03±3.23***
Triglycerides (mg/dL)	52.59±18.81	28.60±8.81**	41.74±17.49	32.88±11.07**
Glucose (mg/dL)	285.39±43.67	254.16±49.00	184.24±41.59**	201.14±81.42*
Creatine kinase (U/L)	22.33±7.55	19.55±5.54	29.44±6.71	30.98±9.75§§

[a]Values are expressed as mean ± standard deviation (§p < 0.05 *vs.* PCa+SED; **p < 0.01 *vs.* CONT+SED; §§p < 0.01 *vs.* PCa+SED; ***p < 0.001 *vs.* CONT+SED; ###p < 0.001 *vs.* CONT+EX; §§§p < 0.001 *vs.* PCa+SED).

3.2. Characterisation of cardiac function

Cardiac function parameters of the four groups determined by echocardiographic evaluation are shown in **Table 3**. Heart rate was significantly increased in trained groups, though significantly only in the CONT+EX group (p < 0.01 *vs.* CONT+SED). Overall, systolic function was similar between the different groups, except for CO that was greater in PCa+EX in comparison to its sedentary counterparts (p < 0.05 *vs.* PCa+SED) and ET that was smaller in PCa+EX in comparison to sedentary control rats (p < 0.05 *vs.* CONT+SED). Diastolic dysfunction was unchanged in PCa groups except for peak mitral inflow velocity during early diastole in PCa+SED (p < 0.05 *vs.* CONT+SED). Exercise training improved diastolic function in both exercised groups, as supported by their lower E/A ratio and DT (p < 0.05 *vs.* CONT+SED), though the decrease in DT was not significant in PCa+EX. Regarding cardiac dimension, exercise training induced a significant increase in aortic diameter of both exercised groups (p < 0.05 *vs.* CONT+SED).

Table 3. Cardiac function parameters determined by echocardiography.

	Experimental groups			
	CONT+SED	PCa+SED	CONT+EX	PCa+EX
Systolic Function				
HR (bpm)	215.5±21.9	215.4±29.3	264.3±48.1**	236.9±37.8#
CO (mL/min)	39.2±6.7	35.6±10.3	48.9±8.6	45.1±8.0§
SV (mL)	0.178±0.027	0.173±0.056	0.191±0.010	0.176±0.037
IVCT (ms)	17.2±4.4	16.3±4.3	15.3±3.6	14.3±3.6
ET (ms)	85.0±9.9	80.6±6.4	82.3±3.6	76.2±8.8*
AoVTI (cm)	6.6±1.0	6.2±1.0	6.2±0.4	6.4±1.1
EF (%)	78±11	83±7	78±9	80±5
FS (%)	41±10	45±7	41±7	42±5
Diastolic Function				
Tei index	0.54±0.14	0.51±0.09	0.47±0.09	0.52±0.13
E (cm/sec)	0.62±0.08	0.71±0.10*	0.63±0.05	0.67±0.09
A (cm/sec)	0.35±0.10	0.40±0.06	0.42±0.09	0.43±0.08
E/A	2.06±0.60	1.81±0.29	1.55±0.31*	1.60±0.30*
IVRT (ms)	26±6	25±4	23±4	24±5
DT (ms)	68.1±13.4	65.8±22.6	48.5±13.1*	55.9±12.8
Wall dimensions				
LV mass (mg)	0.91±0.13	0.88±0.21	0.92±0.21	0.93±0.18
LVd (mm)	7.12±0.56	7.28±0.63	6.68±0.88	7.56±0.80#
IVSd (mm)	1.82±0.16	1.71±0.24	1.79±0.20	1.72±0.28
LVPWd (mm)	1.75±0.28	1.80±0.40	2.07±0.62	1.75±0.28
Aod (cm)	0.37±0.03	0.39±0.02	0.40±0.02*	0.40±0.02**
LVs (mm)	4.21±0.80	4.13±0.85	3.96±0.74	4.40±0.70
IVSs (mm)	2.86±0.56	2.88±0.27	2.80±0.47	2.77±0.36
LVPWs (mm)	2.68±0.56	2.86±0.26	2.86±0.32	2.74±0.40

[a]Values are expressed as mean ± standard deviation (*p < 0.05 *vs.* CONT+SED; #p < 0.05 *vs.* CONT+EX; §p < 0.05 *vs.* PCa+SED; **p < 0.01 *vs.* CONT+SED; ##p < 0.01 *vs.* CONT+EX; §§p < 0.01 *vs.* PCa+SED; ***p < 0.001 *vs.* CONT+SED; ###p < 0.001 *vs.* CONT+EX; §§§p < 0.001 *vs.* PCa+SED).

[b]Abbreviations: A, peak mitral inflow velocity at atrial contraction; Aod, aortic diameter; AoVTI, aortic velocity/time integral; CO, Cardiac Output; DT, mitral deceleration time; E, Peak mitral inflow velocity during early diastole; EF, Ejection Fraction; ET, Ejection Time; FS, Fractional Shortening; HR, Heart Rate; IVCT, isovolumic contraction time; IVRT, isovolumic relaxation time; IVSd, interventricular septum at the end of diastole; IVSs, interventricular septum at the end of systole; LV, Left Ventricle; LVd, LV dimension at the end

of diastole; LVPWd, LV posterior Wall thickness at the end of diastole; LVPWs, LV posterior Wall thickness at the end of systole; LVs, LV dimension at the end of systole; SV, Stroke Volume.

3.3. Evaluation of the impact of prostate cancer and/or exercise training on molecular markers of cardiac remodelling

As shown in **Figure 3,** no switch in myosin heavy chain (MHC) isoforms was detected. The more beneficial MHC isoform (MHCα) was the most abundant isoform, contributing with 92% of overall MHC content [32]. Cardiac expression of each of the isoforms of MHC did not significantly changed between groups.

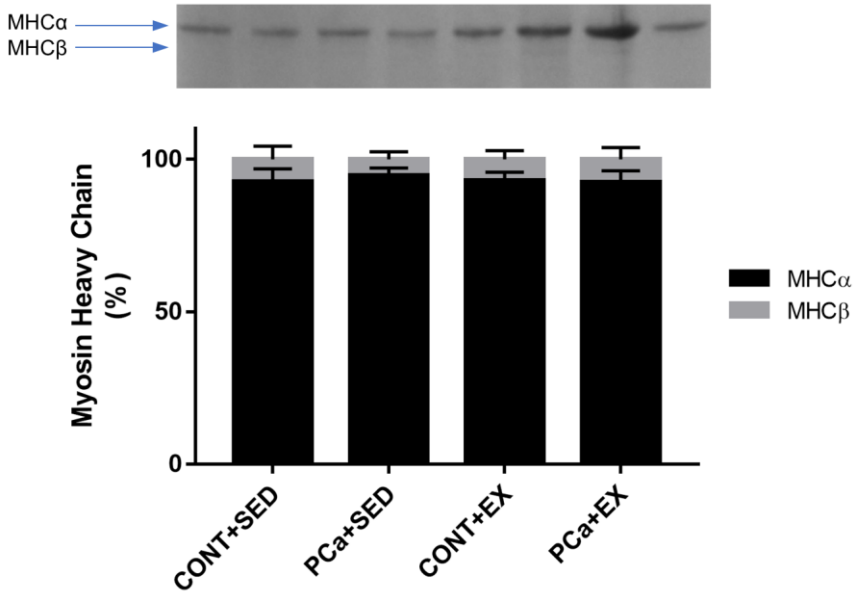

Figure 3. Effect of PCa and/or exercise on the expression of MHC isoforms. A representative gel is shown above the graphic – samples were loaded in the gel two per group side-by-side. Values are expressed as mean ± standard deviation.

Activity of cardiac metalloproteinases (more specifically MMP2 and MMP9) was assessed by gelatin zymography. The results shown in **Figure 4*A*** indicate an increase in extracellular matrix turnover by MMP2 in PCa+SED group ($p < 0.05$ *vs.* CONT+SED). Exercise training was not able to reverse this state. MMP9 activity had no differences between groups (**Figure 4*B***). Unfortunately, due to technical problems in the histological preparation of the samples, we have, to this point, been unable to relate with collagen accumulation and its consequences at the structural level.

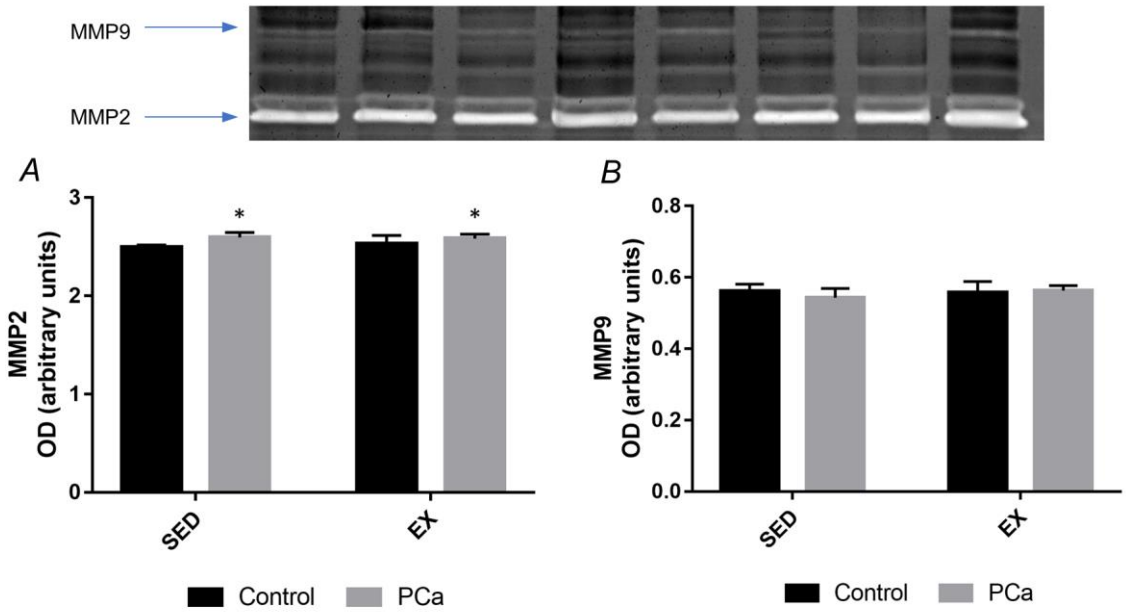

Figure 4. Effects of PCa and/or exercise on MMPs activities. *A*, MMP2; *B*, MMP9. A representative gel is shown above the graphics – samples were loaded in the gel two per group side-by-side. Values are expressed as mean ± standard deviation (*p < 0.05 *vs.* CONT+SED).

CBP/p300-interacting transactivator with ED-rich carboxy-terminal domain-4 (CITED4) was shown to be greatly overexpressed in the PCa+EX rats ($p < 0.001$ *vs.* CONT+SED, $p < 0.01$ *vs.* CONT+EX and $p < 0.01$ *vs.* PCa+SED; **Figure 5**). Despite not significant, a tendency was also found in PCa+SED rats and CONT+EX rats. Currently, analysis of c-kit and CCAAT-enhancer binding protein β (C/EBPβ) expression is underway to better evaluate the contribution of regeneration to PCa and/or exercise-induced cardiac remodelling.

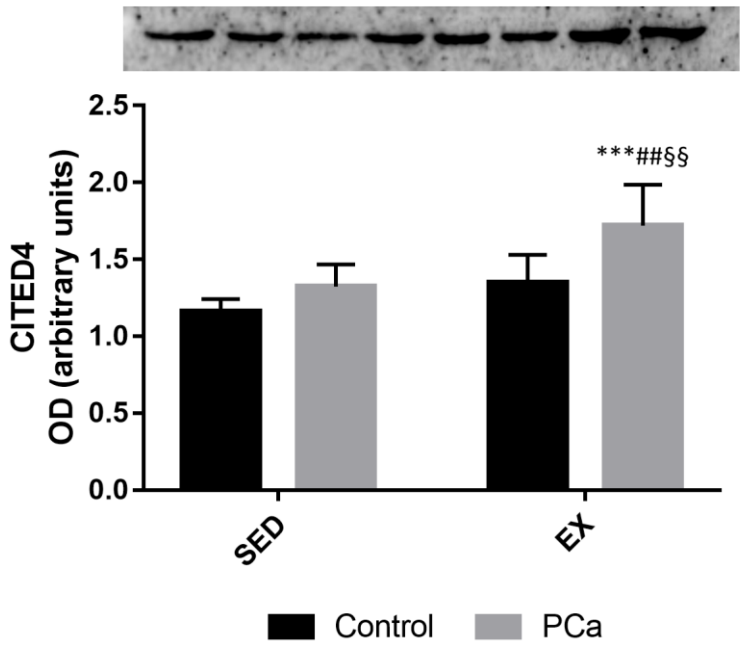

Figure 5. Effects of PCa and/or exercise on CITED4. A representative membrane is shown above the graphic – samples were loaded in the gel two per group side-by-side. Values are expressed as mean ± standard deviation (##p < 0.01 *vs.* CONT+EX; §§p < 0.01 *vs.* PCa+SED).

In order to unravel the metabolic adaptations of cardiac muscle to prostate cancer, glycolytic and oxidative pathways were assessed (**Figure 6**). No changes were found between groups for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), electron-transferring-flavoprotein-dehydrogenase (ETFDH) or peroxisome proliferator-activated receptor alpha (PPARα) (**Figure 6*A*-*C***). On the other hand, PCa led to a reduction of the expression of ATP synthase in the PCa+SED group (p < 0.05 *vs.* CONT+SED), which was prevented by exercise in PCa+EX (p < 0.01 *vs.* PCa+SED; **Figure 6*D***). No changes were found in CONT+EX for this parameter. Regarding the ratio GAPDH to ATP synthase, which informs about the shift in the metabolic status, no significant changes were detected between groups (**Figure 6*G***).

Since fatty acid oxidation mainly occurs in mitochondria, the contribution of mitochondrial function and biogenesis was evaluated by assessing the protein expression of peroxisome proliferator-activated receptor γ coactivator-1 α (PGC-1α), a transcription regulator highly involved with the PPAR family of transcription factors and with significant functions in OXPHOS [33]. While no changes were detected in both control groups (CONT+SED and CONT+EX), PGC-1α protein expression was significantly increased in

PCa+SED ($p < 0.05$ *vs.* CONT+SED; **Figure 6*E***), and this was prevented by exercise in PCa+EX group ($p < 0.001$ *vs.* PCa+SED). The activity of the Krebs cycle enzyme citrate synthase, a rough marker of mitochondrial density [34], was also measured, which was increased in all groups in comparison to CONT+SED ($p < 0.05$; **Figure 6*F***). When PGC-1a was normalized to CS activity, an indicator of mitochondrial efficiency, it was noticed that this ratio was significantly decreased in both exercised groups ($p < 0.001$ *vs.* CONT+SED and $p < 0.001$ *vs.* PCa+SED; **Figure 6*H***).

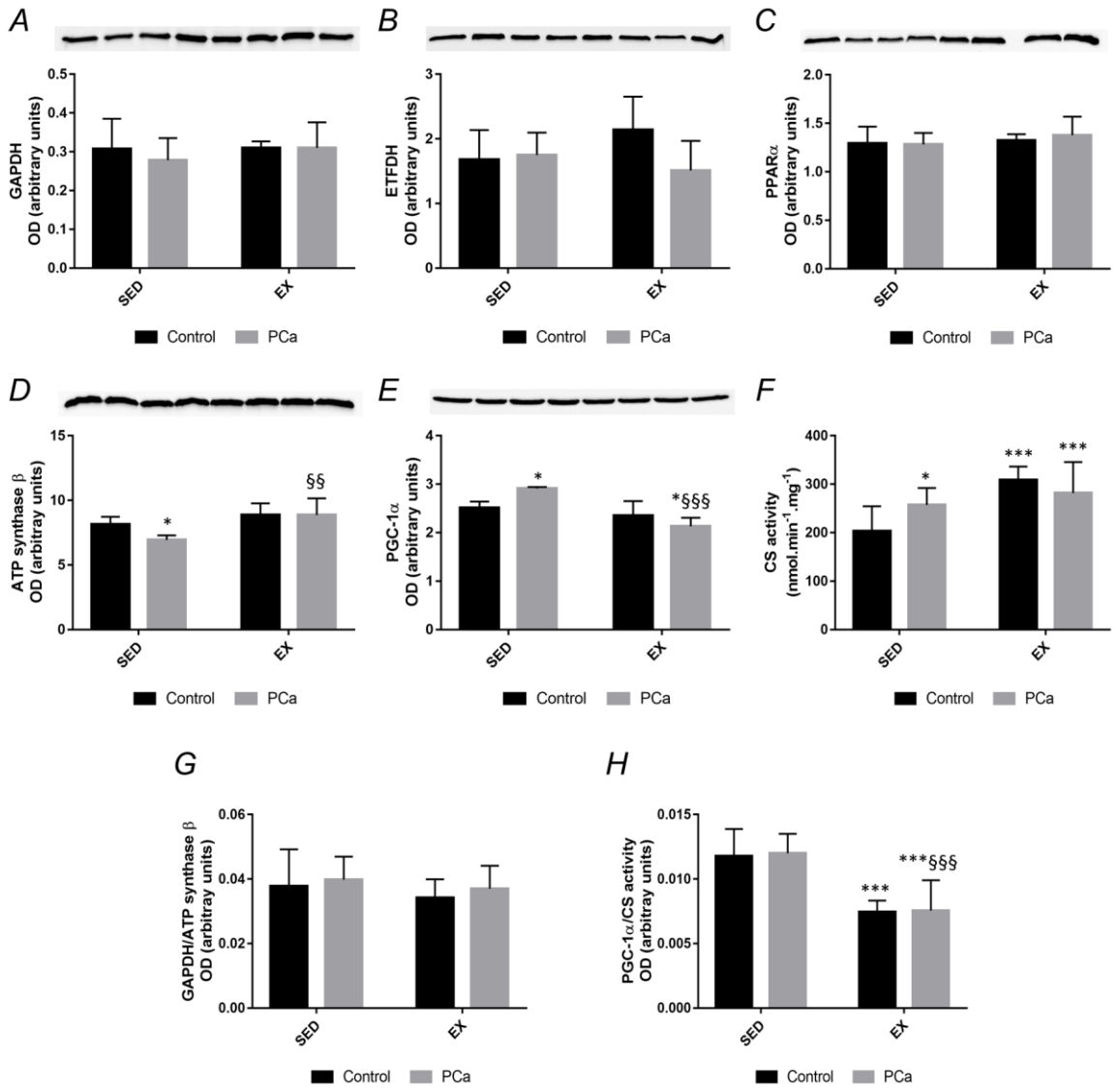

Figure 6. Effects of PCa and/or exercise on different molecular markers of metabolism. *A*, GAPDH protein expression; *B*, ETFDH protein expression; *C*, PPARα protein expression; *D*, ATP synthase subunit β protein expression; *E*, PGC-1α protein expression; *F*, CS activity; *G*, ratio GAPDH to ATP synthase subunit β; *H*, ratio PGC-1α to citrate synthase activity. Representative blots are shown above the corresponding graphic

– samples were loaded in the gel two per group side-by-side. Values are expressed as mean ± standard deviation (*p < 0.05 *vs.* CONT+SED; §§p < 0.01 *vs.* PCa+SED; ***p < 0.001 *vs.* CONT+SED; §§§p < 0.001 *vs.* PCa+SED).

To better comprehend the effect of PCa and/or exercise on cardiac metabolism, metabolite profiling was performed by MRM MS/MS. Regarding acylcarnitine profile, the lipidic profile of the heart was altered in both PCa+SED and CONT+EX, leading to greater concentrations of β-oxidation intermediates (**Figure 7**). This was particularly evident in the hydroxylated intermediates, resulting in a greater accumulation of C6 and C4 fatty acids. Similar results were found for PCa+EX. However, on several intermediates, this was not verified, which can be explained, at least partially, by the lower fat storages in these animals, given by the visceral adipose tissue mass (**Table 1**). Of note, no change was detected between groups in 3-hydroxybutyrilcarnitne (C4-OH) concentration (data not shown), the carnitine bound form of the ketone body β-hydroxybutyrate, suggesting neither PCa nor EX promoted alterations in ketone bodies metabolism. Again, a tendency was detected for lower levels of this metabolite when both stimuli were combined.

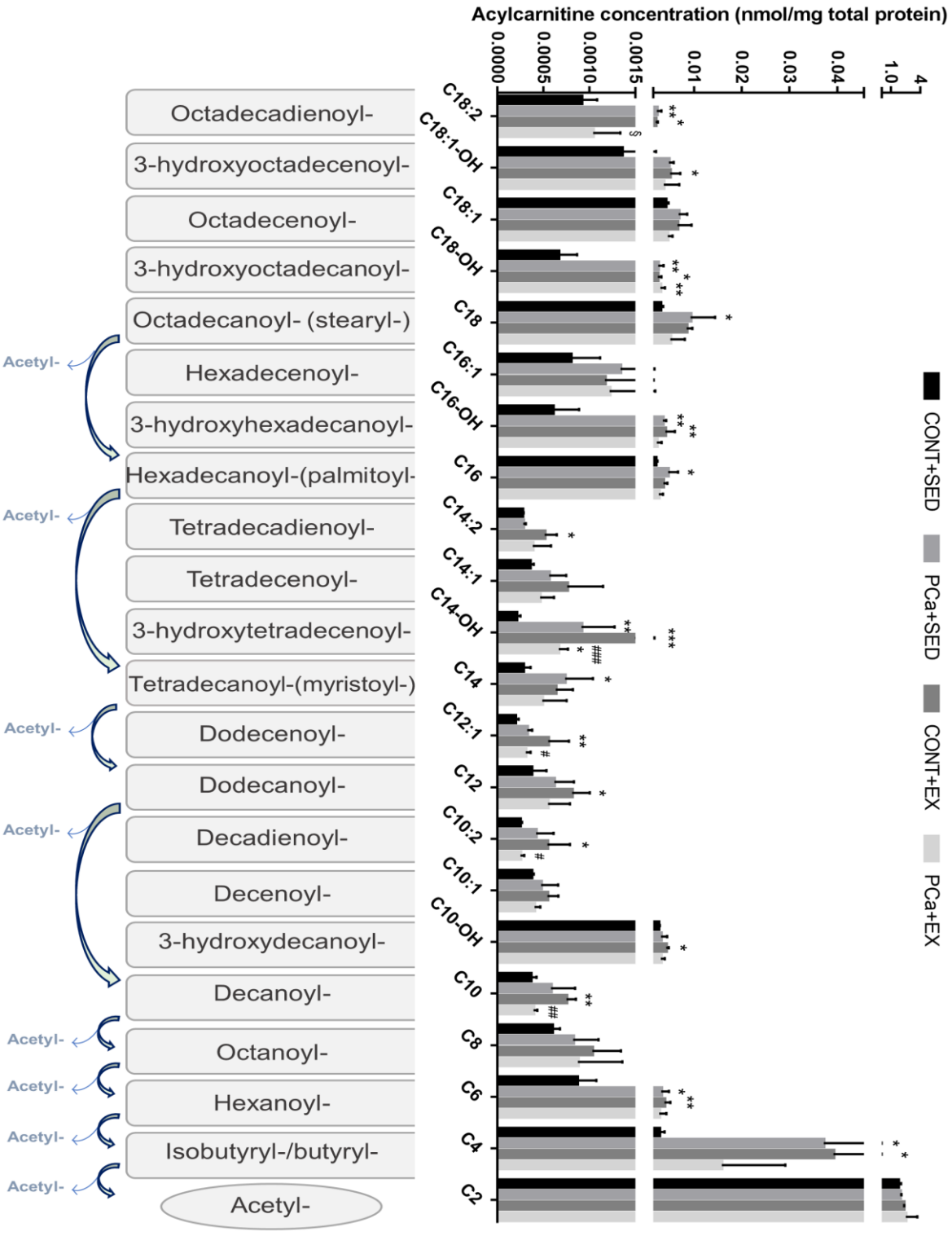

Figure 7. Effects of PCa and/or exercise on the heart's acylcarnitine profile. On the left, the complete sequence of oxidation of an octadecadienoyl fatty acid (C18:2) is shown; in total, nine acetyl molecules are yielded. On the right, the data resultant from the analysis of the heart's acylcarnitine profile. Values are expressed as mean ± standard deviation (*p < 0.05 *vs.* CONT+SED; #p < 0.05 *vs.* CONT+EX; §p < 0.05 *vs.* PCa+SED; **p < 0.01 *vs.* CONT+SED; ##p < 0.01 *vs.* CONT+EX; ***p < 0.001 *vs.* CONT+SED; ###p < 0.001 *vs.* CONT+EX).

Despite not being the main energetic substrates of the heart [35], the putative contribution of amino acids metabolism to cardiac remodelling was also assessed by MRM MS/MS. The changes observed (**Figure 8**) were subtler than the ones observed for the acylcarnitine profile. In general, PCa was able to affect amino acid content in sedentary animals, as shown by the significant increase in concentration of valine, leucine and isoleucine (analysed together with leucine due to their equal molecular weight) in PCa+SED group ($p < 0.01$ *vs.* CONT+SED). These branched amino acids are a preferential source of acetyl-CoA and succinyl-CoA, supporting the TCA cycle after transamination [36]. Isoleucine and leucine were also significantly increased in CONT+EX ($p < 0.05$ *vs.* CONT+SED), while valine, leucine and isoleucine, and aspartate where decreased in PCa+EX when compared to their sedentary PCa counterparts ($p < 0.01$ *vs.* PCa+SED). In this regard, aspartate is of particular importance since it is a regulator of TCA intermediates levels [36]. In PCa+EX, valine, leucine and isoleucine were also decreased in comparison to the respective control ($p < 0.05$ *vs.* CONT+EX).

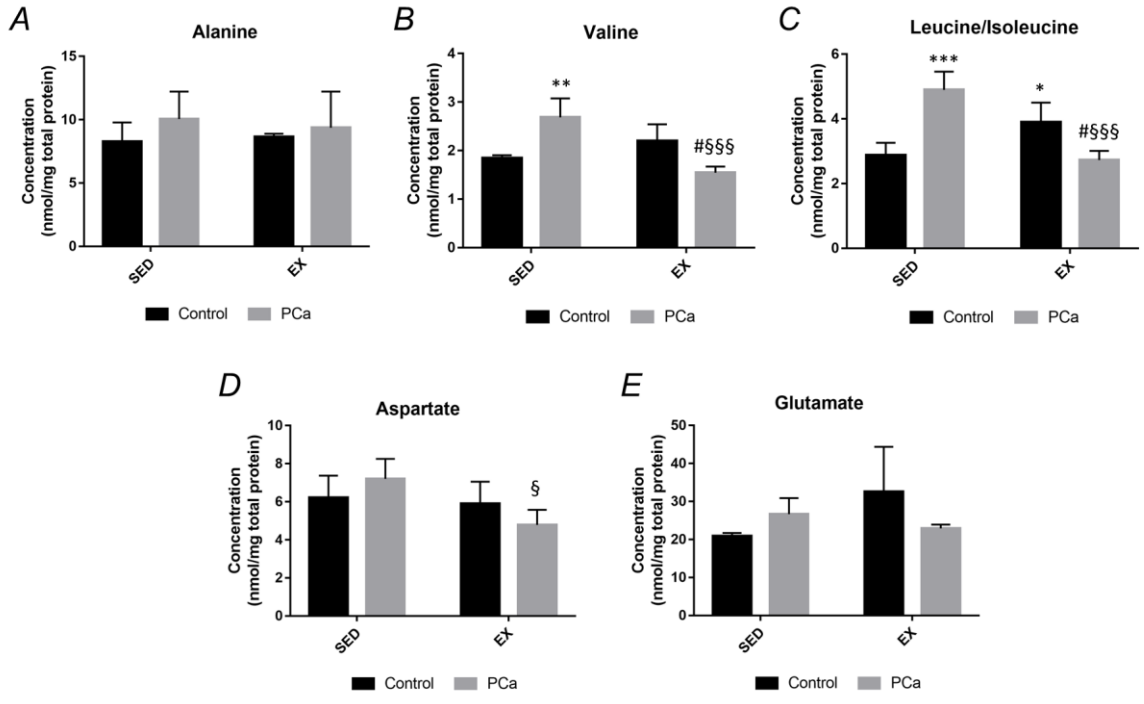

Figure 8. Effects of PCa and/or exercise on the heart's amino acid profile. *A*, Alanine; *B*, Valine; *C*, Leucine/Isoleucine; *D*, Aspartic Acid; *E*, Glutamic Acid. Values are expressed as mean ± standard deviation (*$p < 0.05$ *vs.* CONT+SED; #$p < 0.05$ *vs.* CONT+EX; §$p < 0.05$ *vs.* PCa+SED; **$p < 0.01$ *vs.* CONT+SED; ***$p < 0.001$ *vs.* CONT+SED; §§§$p < 0.001$ *vs.* PCa+SED).

3.4. The role of testosterone in cardiac remodelling

To evaluate the potential role of testosterone in the prostate cancer-induced cardiac remodelling, we evaluated androgen (AR) and oestrogen-related receptor α (ErRα) expression in the heart. We found increased expression of both receptors only for PCa+EX in comparison to PCa+SED or CONT+SED ($p < 0.05$ *vs.* PCa+SED or CONT+SED in AR – **Figure 9*A*** – and $p < 0.05$ *vs.* CONT+SED or $p < 0.01$ *vs.* PCa+SED in ErRα – **Figure 9*B***).

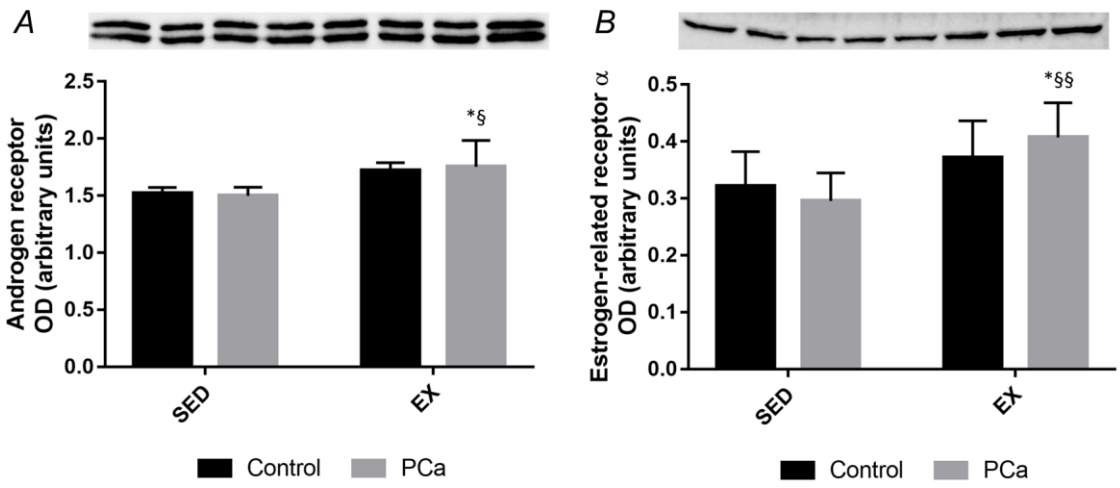

Figure 9. Effects of PCa and/or exercise on androgen and oestrogen receptors. *A*, AR protein expression; *B*, ErRα protein expression. Representative blots are shown above the corresponding graphic – samples were loaded in the gel two per group side-by-side. In A, only the upper bands were used to quantify protein. Values are expressed as mean ± standard deviation (§$p < 0.05$ *vs.* PCa+SED; §§$p < 0.01$ *vs.* PCa+SED).

4. Discussion

The purpose of the present study was to explore the preventive potential of life-long exercise training in cancer-induced cardiac remodelling in an animal model of chemically- and hormonally-induced prostate cancer submitted to 53 weeks of exercise training. Our main findings are: a) prostate cancer does not seem to impair cardiac function or structure, b) myocardial metabolism seems to be modulated by prostate cancer and c) exercise training prevents cardiac metabolic remodelling.

While cardiac dysfunction and remodelling are relatively well studied in the setting of cancer as a side-effect of anti-cancer therapy, a relatively underexplored topic is related with the influence of cancer itself on cardiac function and remodelling of chemotherapy- and

radiotherapy-naive patients, as recently reviewed by us [2]. Cancer-induced changes may directly worsen the patient's prognosis by limiting or delaying the use of certain therapies and, in addition, it may also contribute for some of the cardiovascular complications occurring later in life in cancer survivors [15]. Because the remodelling of the heart may be cancer-specific, it is imperative to design studies in different types of cancer [2]. In the current study, we assessed for the first time the impact of prostate cancer on the heart and the preventive effects of exercise by using a chemically- and hormonally-induced model in Wistar rats. This model constitutes a good correlator to human prostate cancer not only due to the tumour slow growth (approximately 50 weeks), which allows for the study of the preventive effects of exercise training, but also due to the morphological similarities between human and rat prostates [30]. Data from our study suggest that PCa did not induce cardiac functional or structural alterations (**Tables 1 and 3**). Previous studies reported altered LVFS and LVEF induced by other types of cancer, both at the preclinical [11,37–40] and clinical [5] level. Even clinical studies in which LVEF was preserved, ventricle mechanics (strain analysis) were disrupted, suggesting that standard echocardiographic parameters, as we used in our study, might fail to detect early changes [7,8]. Thus, cancer-induced changes may be cancer-stage dependent. Previous studies have associated cardiac functional impairment with structural alterations, from which cardiac atrophy is of particular incidence [6,11,13,37–42], with concomitant wall thinning [11,38], chamber dilation [37,40] and cardiomyocyte atrophy [13,19]. The serum levels of testosterone could be a possible explanation for this lack of structural alteration. In our study, the levels of serum testosterone in the PCa groups were extremely elevated (13- (PCa+SED) and 12-fold (PCa+EX) compared with the respective CONT groups; as shown in **Table 2**). Testosterone has anabolic properties and previous studies showed that it induces cardiac hypertrophy and modulates cardiac function by regulating cardiac calcium homeostasis [43]. Another important factor that might be contributing to preserved heart mass is fibrosis [18]. We were not able to determine fibrosis histologically, but MMP2 activity was increased in the sedentary PCa rats (**Figure 4*A***), consistent with previous findings [12], suggesting that fibrosis could also contribute for the maintenance of heart mass. MHC isoform shift from the alpha to the beta isoform is also a traditional marker of maladaptation found to be elevated in cancer-induced cardiac remodelling [44,45]. In our study, this parameter was similar among the different groups (**Figure 3**), suggesting that the hypertrophic phenotype, at least until this time point, was

adaptive. Indeed, it has been shown that cardiac hypertrophy toward pathological state after treatment with every supra-physiological dose of testosterone is time-dependent [46]. Moreover, a great increase in CITED4 content was found in PCa+EX animals. This molecule has been reported to be associated with cardiomyocyte proliferation [47] and to work as a regulator of mTOR signaling that is sufficient to induce physiologic hypertrophy and mitigate adverse ventricular remodelling after ischemic injury [48]. Thus, while we do not have enough data to support that PCa induced a maladaptive phenotype, it seems that the combination of PCa with exercise training modulates cardiac hypertrophy in the direction of an adaptive phenotype.

At the metabolic level, exercised PCa animals retained the profile of the exercised control ones. Not only that, but compared to the sedentary PCa animals, exercise appeared to prevent the PCa-induced decrease of ATP synthase β and increase of PGC-1α expression, while increasing CS activity to the levels of the control exercised animals (**Figure 6*D*, 6*E* and 6*F*, respectively**), suggestive of increased mitochondrial efficiency, as evidenced by the ratio PGC-1α to CS activity (**Figure 6*H***), and thus increased metabolic efficiency, with higher yields of ATP. In the sedentary PCa animals, despite a similar pattern, the increase in PGC-1α correlates with increased mitochondrial biogenesis, which together with increases in CS activity and decreases in ATP synthase β expression indicates lower efficiency, despite increased mitochondrial activity. One previous paper reported increased maximal mitochondrial respiratory capacity in neonatal rat cardiomyocytes cultured in a cancer-conditioned medium, which led to lipid storages depletion [13]. In our study, both the sedentary PCa and exercised control animals presented themselves with increased levels of acylcarnitines (**Figures 7**), evidencing increased activity of fatty acid oxidation, the main source of ATP in the heart [49]. The reliance on fatty acids as energetic substrate was lower in exercised PCa animals compared both to the sedentary and control exercised counterparts (**Figure 7**), which could be explained by a higher reliance on glucose. Indeed, PCa animals presented lower body mass, which seemed to be the result of fat mass storages depletion (**Table 1**). Moreover, lower serum levels of triglycerides were observed in these animals (**Table 2**), suggestive of lower sources of available lipids to support heart metabolism. These results suggest a switch of the energetic substrate from fatty acids to glucose, a metabolic adaptation often reported in cardiac dysfunction [49]. However, the levels of the glycolytic enzyme GAPDH were not impacted by exercise or PCa, suggesting no changes of glucose

metabolism. In the setting of PCa, this could also be the result of increased metabolic needs by the tumour. On the other hand, the analysis of the amino acid profile highlighted significant changes (**Figure 8**), particularly of branched amino acids like leucine, isoleucine and valine in the heart of trained PCa rats compared with the sedentary PCa and control exercised animals, denoting their usage as acetyl-CoA and succinyl-CoA donors for the TCA [36]. To further understand the molecular mechanisms involved in the regulation of cardiac metabolism, the expression levels of AR and ErRα, two transcription factors with regulatory functions over various cardiac processes, were analysed (**Figure 9*A* and *B*)**, and interestingly, their levels were increased compared to both the sedentary PCa animals and the exercised controls. The genomic testosterone pathway has been previously reported to induce cardiomyocyte hypertrophy and control calcium cardiac metabolism [43], while estrogen-related receptors have been described in the regulation of fatty acid uptake and oxidation [49], supporting our hypothesis. PPAR levels were also probed, but no changes were found (**Figure 6*C***), despite previous reports on the impact of exercise training on the upregulation of this transcriptional factor [35].

Limitations

Contrary to what has been reported, we found a lower heart mass in the CONT+EX in comparison to their sedentary counterparts. Analysis of cross-sectional area of cardiomyocytes and quantification of fibrosis will be determinant to clarify the reasons for this observation. It is possible that sedentary aging could induce an increase in cardiac mass due to hypertrophy and fibrosis in sedentary animals, while training would prevent fibrosis (and thus its contribution to heart weight). It should also be noted that while we found no evidence of cardiac dysfunction, we evaluated traditional parameters, and as evidenced in recent clinical studies, sub-clinical dysfunction might occur even before traditional parameters are altered. Moreover, in this study we used a carcinogenic model of cancer, and while our study developed over the course of a year to maximize the effect, a longer prostate tumorigenesis could eventually impact cardiac function. One might wonder if increased exposure to PCa would eventually produce more pronounced cardiac effects. Several other caveats might be considered; however, we do think these are the ones deserving of some reflection.

Conclusion

In conclusion, prostate cancer did not lead to significant functional or structural alteration, but promoted metabolic remodelling of the heart in PCa animals. Exercise training in PCa animals prevented metabolic remodelling. Future studies should continue assessing the effects of different types of cancers on the heart and focus on the intensity and duration of the exercise programme.

5. References

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CHAPTER IV

Final Remarks

The presence of cardiac cachexia in therapy-naïve cancer patients is already established. It is an underappreciated syndrome with a tremendous burden on patient mortality and quality of life. Given the benefits already demonstrated by exercise training in several diseases, such as CVDs, it has emerged as a high-quality adjuvant therapy. Exercise training appears to exert its effects through the modulation of tumoral activity, inflammation, cardiac fibrosis and cardiomyocyte organization, metabolism and oxidative defences. In our experimental work, we further explored the effects of exercise on cancer-induced cardiac remodelling.

Data showed that while prostate cancer did not produce pronounced cardiac alterations, it did lead to metabolic remodelling through the increase of acylcarnitine and branched amino acid levels, PCG-1α expression and CS activity, and decreased ATP synthase content in the heart, which were modulated by exercise training. Moreover, by inducing CITED4 expression, it also appeared that exercise promoted an adaptive phenotype in cancer-bearing animals. However, further studies are necessary to support this claim. Importantly, in this study we studied the preventive effect of exercise, for the first time, supporting the benefit of life-long exercise training.

Despite the aforementioned positive developments, several questions still remain unanswered, namely which type of exercise, endurance or resistance, would prove more beneficial, or would it be a combination of both? Are the benefits a result of direct interaction between exercise and the heart or tumour? Future studies should help shed some light on these and other questions. Specifically, they should focus on the cross-talk between cancer-induced cardiac remodelling and distinct exercise training programs, considering cancer type and stage, age, and gender, envisioning an ever-increasing personalisation of treatment strategies not only to improve the cardiac health of cancer patients, but also to promote compliance with training programmes.