Universidade de Aveiro Departamento de Química
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Impacto do exercício físico na remodelação do tecido adiposo branco induzida pela caquexia associada ao cancro

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Bioquímica, ramos em Bioquímica Clínica, realizada sob a orientação científica da Doutora Rita Maria Pinho Ferreira, Professora Auxiliar do Departamento de Química da Universidade de Aveiro e da Doutora Rita Marisa Nogueira Ferreira, Investigadora Auxiliar do Departamento de Fisiologia e Cirurgia Cardiotorácica da Faculdade de Medicina da Universidade do Porto.

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For my mom
(the shiniest of them all)
my sister and my father

o júri

presidente

Professor Doutor Pedro Miguel Dimas Neves Domingues

Professor Auxiliar com Agregação do Departamento de Química da Universidade de Aveiro

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Palavras-chave

Cancro; Caquexia associada ao cancro; acastanhamento do tecido adiposo branco; remodelação do tecido adipos branco; exercício de endurance; UCP1; PGC-1α; análise morfológica de adipócitos.

Resumo

A prática de exercício tem vindo a ser aclamada pela sua capacidade em induzir múltiplas adaptações benéficas, não só em indivíduos saudáveis, como também em pacientes diagnosticados com um largo conjunto de patologias, incluindo cancro. No contexto da caquexia associada ao cancro (CC), contudo, o exercício pode representar uma "espada de dois gumes". Se por um lado os pacientes podem beneficiar dos muitos efeitos benéficos induzidos pela prática de exercício, por outro lado, evidencias apontam o exercício como um promotor do acastanhamento que ocorre no tecido adiposo branco (WAT). De facto, o exercício parece ser capaz de induzir a expressão da UCP1 e da PGC-1α, o que pode contribuir para aumentar o gasto energético experienciado pelos indivíduos com CC. Esta aparente dicotomia faz do exercício físico uma variável interessante e crucial a ser estudada ao nível do tecido adiposo no âmbito na caquexia associada ao cancro. O objetivo do presente trabalho foi, portanto, avaliar o impacto do exercício de endurance nas remodelações que ocorrem no tecido adiposo branco na caquexia relacionada com o cancro. Para tal, um protocolo de exercício em tapete rolante foi implementado em dois modelos pré-clínicos, de cancro da mama e da próstata, usando ratos *Sprague-Dawley* e ratos *Wistar Unilever*, respetivamente. Foram recolhidas amostras de tecido retroperitoneal e o seu conteúdo proteico, no que diz respeito a marcadores de acastanhamento e metabolismo, foi analisado. Foi ainda realizada uma avaliação histológica em ratos com caquexia associada ao cancro da próstata, com o objetivo de analisar a área e o número de adipócitos, bem como outros processos de remodelação a ocorrerem no tecido adiposo branco. Os resultados obtidos sugerem que o exercício de endurance não piora nem melhora o dispêndio energético que é exibido na caquexia associada ao cancro, já que não foram detetadas alterações nos níveis de expressão dos marcadores analisados nos animais com caquexia em resposta ao exercício. Não obstante, alterações ao nível da morfologia dos adipócitos, caracterizadas por uma diminuição das, foi notada nos animais sujeitos ao protocolo de exercício de endurance. Estas evidencias experimentais realçam a capacidade do exercício em induzir adaptações no tecido adiposo e evidenciam a prática de exercício como uma ferramenta potencialmente benéfica no controlo da caquexia associada ao cancro.

Keywords

Cancer; Cancer cachexia; white adipose tissue browning; white adipose tissue remodeling; endurance exercise; UCP1; PGC-1α; adipocytes morphological analysis.

Abstract

Exercise training has been claimed for its capability of inducing several beneficial adaptations, not only on healthy individuals, but also on a large range of pathologies, cancer included. In the cancer cachexia (CC) set, however, exercise may represent a “double-edged sword”. If by one hand patients may benefit from the many healthy effects induced by exercise training, by other hand, some evidences are pointing exercise as a promoter of the browning that occurs in white adipose tissue (WAT). Indeed, exercise seems to be capable of upregulate the expression of UCP1 and PGC-1α, which can contribute to further enhance the energy expenditure experienced by subjects with CC. This apparent dichotomy makes exercise an interesting and crucial variable to be studied on adipose tissue remodeling level in the cancer cachexia set. The aim of the present study was to evaluate the impact of endurance exercise on the WAT remodeling taking place on cancer-related cachexia. To do it so, a treadmill exercise protocol was implemented in two pre-clinical models, of mammary and prostate cancer using female Sprague-Dawley rats and male Wistar Unilever rats, respectively. Retroperitoneal tissue samples were collected and its protein content, regarding some selected markers of browning and metabolism, were analyzed. In addition, a histological evaluation, aiming to analyze the cross-sectional area and adipocytes number, as well as other remodeling process occurring on WAT, was performed on rats bearing prostate cancer. The obtained results suggested that endurance exercise does not worsen or ameliorates the energy expenditure and the metabolic impairment scenario taking over WAT in cancer cachexia, once no alterations were detected in the expression levels of the analyzed markers. Nevertheless, alterations regarding adipocytes morphology, characterized by a significant decrease of adipocytes areas, was notice for the animals subjected to the endurance exercise protocol. These findings highlight exercise capacity in inducing adaptations on the adipose tissue and evidence exercise practice as a potential beneficial tool on the managing of cancer cachexia.

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List of Abbreviations

APR	*acute-phase response*
AT	*adipose tissue*
BAT	*brown adipose tissue*
BMI	*body mass index*
cAMP	*cyclic-AMP*
Casco	*Cachexia Score*
CC	*cancer cachexia*
C/EBP	*cytosine-cytosine-adenosine-adenosine-thimidine/enhancer binding protein*
CHO	*chinese hamster ovary*
COPD	*chronic obstructive pulmonary disease*
CRP	*C-reactive protein*
DGC	*dystrophin glycoprotein complex*
E1	*ubiquitin-activating enzyme*
E2	*ubiquitin-conjugating enzyme*
E3	*ubiquitin-protein ligase*
ECM	*extracellular matrix*
FFA	*free fatty acids*
FoxO	*Forkhead box O*
GH	*growth hormone*
GHSR	*growth hormone secretagogue receptor*
GSK3β	*glycogen synthase kinase 3β*

HUVECs	*human umbilical vein endothelial cells*
IFN	*Interferon*
IGF	*insulin-like growth factors*
IL	*Interleukin*
LMF	*lipid mobilizing factor*
LPL	*lipoprotein lipase*
MAPK	*mitogen-activated protein kinase*
mTOR	*mammalian target of rapamycin*
NF-κB	*nuclear factor-κB*
NPY	*neuropeptide Y*
PPARγ	*peroxisome proliferator-activated receptor-γ*
PIF	*proteolysis-inducing factor*
PI3K	*phosphatidylinositol-3-kinase*
PKB	*protein kinase B*
PTHrP	*parathyroid hormone-related protein*
REE	*resting energy expenditure*
SREBP-1c	*sterol regulatory element-binding prontein-1c*
sWAT	*subcutaneous white adipose tissue*
Tfam	*mitochondrial transcription factor A*
TNF-α	*tumor necrosis factor-α*
UCP	*uncoupling proteins*
vWAT	*visceral white adipose tissue*
WAT	*white adipose tissue*
ZAG	*zinc α_2-glycoprotein*

1. Introduction

The formal definition of cachexia has been subject of alterations as new information's come to light. The current most accepted definition states that cachexia describes a complex multifactorial syndrome associated with an underlying disease and characterized by an ongoing loss of skeletal muscle associated with or without loss of adipose tissue and increased protein catabolism [1,2]. The main clinical features displayed in this condition include not only an involuntary and progressive weight loss in adults or growth failure in children, as well as a reduced physical function and decreased survival [3,4]. Anorexia, inflammation, insulin resistance, and impaired protein, carbohydrate and lipid metabolism are also frequently related to this syndrome [3,5]. Even though cachexia is recognized to be a serious consequence of many chronic diseases such as cancer, chronic obstructive pulmonary disease (COPD), sepsis, chronic heart failure, acquired immunodeficiency and multiple sclerosis, it continues to be frequently undiagnosed and rarely treated [6,7]. Accordingly to a recent consensus, cachexia should be diagnosed whenever a patient presents a body weight loss of at least 5% during the previous 12 months or less [3,8]. The time frame may be disease-specific, being reported to be shorter in cancer patients (3 to 6 months) and longer in COPD (12 months) [3]. A body mass index (BMI) of < 20.0 Kg/m^2 may also be used as a key factor to diagnose cachexia in cases where a history of weight loss cannot be accessed [1,3]. In addition to weight loss, at least 3 of the following 5 parameters must be identified: decreased muscle strength; fatigue; anorexia; low fat-free mass index; and an abnormal biochemistry (such as increased inflammatory markers anemia and low serum albumin) [3].

In the scope of cancer, cachexia has been pointed, for a long time, as a significant adverse outcome, often associated with poor responses to chemotherapy and poor prognosis [1,9]. It affects around 50 to 80% of cancer patients, depends on the tumor type, and may account for up 20% of cancer deaths [10]. In some cases, cachexia arises as one of the earliest manifestations of the tumor-host interaction [11], and may markedly affect patients quality of life. The highest frequency seems to appear in patients with pancreatic or gastric cancer, while patients with non-Hodgkin's lymphoma, breast cancer, acute nonlymphocytic leukemia, and sarcomas are reported to be the ones with the lowest frequency of weight loss [7,12]. Although cancer cachexia (CC) is more incident in some types of tumors, its extent may present variations from host to host bearing the same type of tumor [11]. In situations of advanced stages, when patients often present a severe muscle and adipose tissue wasting, signs of a reduced physical performance and metastatic refractory disease with reduced tolerance to anticancer therapy, even a

multimodal approach intended to regain function and lean tissue is unlikely to be successful [1]. In order to easily recognize and efficiently counteract the progression of this disease, a better understanding of the pathogenesis and the implementation of novel therapies becomes crucial.

1.1. The role of inflammation in cancer cachexia

Multiple processes consort with the progression of cancer cachexia to force important metabolic changes and often leading to anorexia, decreased physical activity and an abnormal metabolic response regarding protein, lipid, and carbohydrate metabolism [3]. A proposed path for the development of cancer cachexia states that there are alterations in substrate mobilization driven by inflammation, that is currently considered a cancer hallmark [13]. Indeed, numerous pro-inflammatory cytokines are released in response to cancer cachexia-related inflammation and due to a tumor-induced activation of the host immune system [14]. These cytokines are thought to have important roles in the pathological mechanisms of CC and include the tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, IL-6, IL-8, interferon (IFN)-γ, parathyroid hormone-related peptides (PTHrP) and the macrophage migratory factor (MIF) [10,14]. They share some of the same metabolic effects and their activities are closely correlated, often presenting synergistic effects [14]. These cytokines are transported across the blood-brain barrier and interact with brain endothelial cells causing the release of substances that affect appetite, contributing to the anorectic state that is often identified in cachectic patients [15,16]. They are likely to be the primary catabolic triggers of skeletal muscle loss, inducing signaling pathways that upregulate enzymes involved in skeletal muscle protein turnover [17,18]. Currently, the analysis of the C-reactive protein (CRP), an acute-phase response (APR) protein, is considered to be an accurate method to measure the impact of pro-inflammatory cytokines burst, serving as an index of these proteins activity and allowing to investigate the potential role that they may have in several aspects of the cachectic syndrome [19]. Due to their importance in the CC etiology, these mediators are important targets in the current research for therapeutics and to reach a better comprehension of adipose tissue remodeling and skeletal muscle loss.

1.1.1. Pro-inflammatory cytokines – an integrated view in CC

It is of general acceptance that the genesis of cancer cachexia is strongly associated with the inflammatory status promoted by the tumor, contributing to many of the observed features[14]. TNF-α, for instance, a powerful cytokine involved in the maintenance of the immune system and inflammation, has been subject of extensive studies in the scope of cancer cachexia. This pro-inflammatory cytokine can be released by activated macrophages or by other types of cells, such as neutrophils, mast cells, CD4+, eosinophils and neurons [20]. TNF-α is reported to promote apoptotic cell death, inflammatory response and to induce a direct catabolic effect on skeletal muscle, leading to the loss of this tissue through the induction of the ubiquitin-proteasome system (UPS) [20]. Indeed, increased levels of both free and conjugated ubiquitin and mRNA ubiquitin were detected in rats' limb muscles after an intravenous injection of TNF-α, supporting the idea of an induced muscle catabolism stimulated by this cytokine [21]. At a cellular level, a wide number of pathways can be mediated by TNF-α to promote complex post-receptor signaling events. Out of them, the nuclear factor kappa B (NF-κB)-induced catabolic signaling is thought to be a key path in protein degradation in cachexia [20]. In this pathway, TNF-α activates the NF-κB, a primary mediator in the control of transcription and an important signaling applicant during catabolism [22]. This activation occurs in skeletal muscle cells through the binding of TNF-α to the type 1 TNF-α receptor (TNFR1), that has been linked to protein loss by affecting the expression of genes involved in the regulation of the UPS [20,21]. Indeed, mice transplanted with Lewis carcinoma lacking TNFR1 showed decreased muscle wasting when compared with wild-type mice and suggested an involvement of this type of receptor in muscle protein degradation rather than type 2 TNF-α receptor [23]. Normally, TNF-α is not detected in plasma or serum of healthy individuals, but it can be detected in most cancer patients, especially in those with advanced disease and poor prognosis [24]. A study conducted by Pfitzenmaier *et al.* [25] in a group of patients with advanced prostate carcinoma and diagnosed with cachexia, revealed a significant elevation on the serum levels of TNF-α, as well as of IL-6 and IL-8 when compared with those without cachexia, supporting the idea that these cytokines involvement in cachexia development. Furthermore, a pairwise correlation between the levels of TNF-α and IL-8 was also found, establishing the idea of a coordinate expression regulation [25]. In addition to induce protein breakdown, other important roles have been associated with TNF-α activity in the scope of CC. This

cytokine seems to be capable of stimulating gluconeogenesis and lipolysis and to decrease the levels of protein synthesis, lipogenesis and glycogen synthesis [10,24,26]. It has also been reported that TNF-α can stimulate the expression of the uncoupling proteins (UCP)-2 and UCP3 in the skeletal muscle, and induce the expression of IL-1 [7,27–29]. In adipose tissue, particularly, TNF-α was reported to downregulate the expression of some transcription factors, such as peroxisome proliferator-activated receptor γ (PPARγ), retinoid X receptor α (RXRα) and CCAAT enhancer binding protein α (C/EBPα) [30]. These are essential for adipocyte function and differentiation, and therefore, these findings expose the role of TNF-α in adipose tissue loss by inhibiting lipogenesis.

Even though TNF-α is widely recognized to be an important player in cachexia progression, as it contributes to induce many of the clinical features seen in this condition, it has been demonstrated that the inhibition of this mediator activity, by itself, cannot stop or reverse cancer cachexia. For this reason, there is a wide agreement that TNF-α may be involved but is not the only responsible for the effects observed [31]. In fact, levels of IL-1, another pro-inflammatory cytokine, are also generally found increased in cachectic patients [14]. This cytokine has been reported to cause similar effects to the ones described for TNF-α, such as the promotion of an anorexic state [14,15]. Indeed, IL-1 can contribute to increase the concentration of tryptophan in plasma, which in turn leads to an increase in serotonin levels. High levels of serotonin can cause an early satiety and suppression of hunger [32]. When it comes to TNF-α, the anorexic state is stimulated by the enhanced production of the corticotrophin-releasing hormone, and the consequent suppression of food intake [20]. IL-1 is also capable of stimulate the production of IL-6, whose levels are usually increased in cachectic patients and may have different functions in the organism defense by regulating the immune response [7,33]. Further evidences were given by Soda *et al.* [34], who described serum levels of IL-6 35% lower in non-cachectic mice relative to the cachectic ones. These findings led to the conclusion that IL-6 may be an enabler for the development CC, although, and similarly to TNF-α, cannot by itself induce all the observed symptoms [34]. Furthermore, circulating levels of IL-6 have been related to weight loss, reduced survival and an induced APR, stimulated by the liver in cancer patients [35–37]. Other studies also implicate IFN-γ, a cytokine produced by activated T-cells and macrophages, as an important player in cancer cachexia [38]. This protein has proven to stimulate the release of free fatty acids (FFA) from adipocytes by inhibiting the activity of the lipoprotein lipase (LPL) [39,40]. Additionally, in cultures of rats adipocytes, IFN-γ demonstrated the ability to inhibit the activity of the enzyme

glycerol phosphate dehydrogenase, that is involved in lipogenesis, thus contributing to the adipose tissue wasting [41]. A study conducted by Matthys *et al.* [42] in mice inoculated with chinese hamster ovary (CHO)/IFN-γ cells, has shown that this cytokine enhances body weight loss and reduce food intake. These features were not observed in the control group that was only inoculated with CHO cells, evidencing the role of IFN-γ as an inducer of cachexia [42]. Even though IFN-γ is widely recognized for having a prominent role in CC development, the idea that this cytokine does not work alone seems to be very clear [38,42]. In fact, it very well stablished that the effects caused by the systemic inflammation are an outcome from the interplay of various cytokines such TNF-α, IL-6, IL-1 and IFN-γ, leading not only to an increased proteolysis and lipolysis, but also influencing the rates of energy expenditure and food intake [14,25,31,38,42–47].

1.2. Energy balance disruption associated to CC

Cancer cachexia is a type of energy balance disorder in which energy intake is decreased and/or resting energy expenditure (REE) is increased [7]. The contributions of both intake and expenditure are related to the type and stage of the tumor [7]. Patients with pancreatic and lung cancer often indicate an elevated REE, while in opposition, patients with gastric and colorectal cancer are reported to have no elevation in the REE [46,48]. Even though alterations in energy intake are often associated with cachectic patients, the increased energy expenditure has been reported as the one that mostly contributes to the wasting syndrome [49]. These findings are supported by patients on total parental nutrition, and therefore with a controlled energy intake, that still lose weight and present symptoms of cachexia [49].

The molecular mechanisms underlying energy expenditure, and therefore contributing to the involuntary weight loss, can be of different types. About 70% of the total energy expenditure in sedentary people arises from the REE [7]. One example is the lactate recycling associated with *Cori Cycle* that occurs between the liver and the tumor mass [18]. Indeed, most cancer cells are glycolytic, using glycolysis to generate ATP over mitochondrial oxidative phosphorylation, even when the oxygen supply is not compromised [50,51]. This phenomenon is called Warburg effect [52], also known as aerobic glycolysis. In tumor cells, glucose is converted into lactic acid, an energy-inefficient process that ends up to requier about 40 times more glucose than if it was completely oxidized through the citric acid cycle [7]. In addition, the lactate passes from

the tumor to the liver, where it is resynthesized into glucose, in another inefficient process. It takes 6 mol of ATP to generate 1 mol of glucose from 2 mol of lactic acid. Higher rates of glucose synthesis and recycling have been observed in patients with metastatic colorectal cancer than in subjects without cancer that suffer from weight loss [53]. Moreover, the increased energy expenditure may also emerge as a consequence of mitochondrial DNA mutations, a scenario often observed in cancer patients that may prevent the use of *Krebs cycle* to fully oxidize the pyruvic acid, leading cancer cells to resynthesize lactate and rely almost exclusively upon glycolysis. [55, 56, 57]. The consequently use of *Cori Cycle* to regenerate glucose leads to an increased energy expenditure. Recent studies have also been pointing cachexia-related alterations in adipose tissue as significant contributors to the energy expenditure. In fact, an increased REE have been consistently reported and justified as a consequence of the enhanced thermogenesis that takes over adipose tissue in the progression of CC [28]. In this case, the upregulation of uncoupling proteins, which mediate proton leakage across the inner mitochondrial membrane, thus disrupting mitochondrial ATP synthesis, is thought to constitute the determinant factor [7].

1.3. Skeletal muscle loss underlying CC

Cancer cachexia is invariably associated with skeletal muscle loss and atrophy. It is estimated that almost one-third of the deaths in cancer patients are related to muscle catabolism and weakness, especially when the respiratory muscles are involved [55]. In healthy individuals, a balance in skeletal muscle metabolism, that accounts for nearly half of whole-body protein mass, is maintained through catabolic and anabolic processes that occur concomitantly [18]. The result is a continuous renewal of muscle protein without a net change in the global muscle mass [7]. Several studies in cachectic patients provide evidence for a decreased protein synthesis, an inhibited uptake of amino acids and an increased protein degradation, particularly, the myofibrillar proteins actin and myosin, as a reason for the muscle wasting [55–59]. However, the relative importance of both processes has not reached a consensus.

One of the most studied proteolytic pathways in the scope of CC, and considered to be the predominant player in the degradation of the myofibrillar proteins, is the ATP dependent ubiquitin/proteasome pathway [57]. This system has been consistently reported to be hyperactive in several animal models of cancer cachexia, revealing

increased mRNA levels of ubiquitin and proteasome subunits, as wells as increased proteasome activity being detected [60,61]. Supporting evidence for the role of UPS are given by muscle biopsies from cachectic gastric patients, that showed an elevated expression of ubiquitin mRNA and the 20S proteasome subunits, and also an increased activity in the muscle proteasome activity [62]. Moreover, skeletal muscle seems to be selective in the degradation of specific proteins rather than general. For instance, cachectic-induced mice by colon-26 tumors showed that myosin heavy chain was selectively reduced above all the others, what was correlated with wasting [63]. In addition, using the same model, was also reported degradation of the respiratory muscles, what may suggest cachexia as an increasing factor for the risk of respiratory failure [64]. This enhanced activity for the UPS is thought to be mediated by the activation of the FoxO and the NF-κB transcription factors through the induction of the key atrogenes MuRF-1 and MAFbx, which are responsible for the selective polyubiquitination of proteins targeted for degradation [55]. Furthermore, FoxO transcription factors may additionally suppress the PI3K/Akt pathway, and therefore inhibit protein synthesis [55]. Protein degradation through the autophagic-lysosomal system (ALS) is also getting a lot of attention in the context of CC. Indeed, autophagy is thought to play a central role in the regulation of muscle homeostasis, either constitutively or as a response to different stimuli such as fasting and exercise [65]. An inefficient autophagic process may lead to an impaired turnover of cellular components with an accumulation of misfolded and aggregated proteins and dysfunctional organelles [65]. Recent findings have suggested an impaired autophagosome clearance in the skeletal muscle of cachectic patients [65,66]. Abnormalities in the dystrophin glycoprotein complex (DGC), a membrane structure that connects the cytoskeleton of a muscle fiber to the extracellular matrix and is involved in muscular dystrophy, has been implied in the wasting noticed in cachectic patients [67]. In fact, studies performed in muscles from cachectic mice and gastrointestinal cancer patients revealed reduced levels of DGC with an increased glycosylation of DGC proteins, suggesting an important role of DGC abnormalities in the muscle atrophy [67]. Furthermore, the proteolysis-inducing factor (PIF), whose levels have been found elevated in cachectic patients [68,69], seems capable of inhibiting protein synthesis and to stimulate protein degradation by inducing the expression of components of the UPS through the activation of the transcription factor NF-κB [70]. This activation involves the phosphorylation of RNA-dependent protein kinases, leading to the inhibition of protein synthesis [71].

Several intracellular signals, that can be modulated by inflammatory cytokines secreted either by immune cells or tumor-derived, are also involved in the protein turnover that becomes compromised in the development of the wasting process [72,73]. The NF-κB and the JAK/STAT, when triggered by pro-inflammatory or pro-cachectic cytokines such TNF-α, IL-6, and IL-1, are two established pathways that can lead to muscle wasting [73]. Additionally, and beside all the factors inducing skeletal muscle atrophy, the study of the mechanisms underlying muscle hypertrophy has also been providing important insights into the processes leading to muscle loss. Multiple reports have implied the insulin-like growth factor 1 (IGF-1) pathway as an inducer of protein synthesis and satellite cells proliferation and differentiation [55,72]. From a molecular perspective, IGF-1 activates the insulin receptor substrate 1, which through the PI3K-Akt pathway can induce protein synthesis via the mammalian target of rapamycin (mTOR) and glycogen synthase kinase 3β (GSK3β) [72]. Furthermore, the activation of the PI3K/Akt/mTOR pathway may also result in the down-regulation of MuRF-1 and MAFbx by the inhibition of FoxO, resulting in a decreased protein degradation [73]. A study performed by Thomas L. Schmitt *et. al* in muscle and liver biopsies from 16 patients undergoing pancreatectomy for the suspect of carcinoma, revealed that the muscle samples from cachectic patients presented decreased levels of the Akt protein, as well as a reduced phosphorylation of the transcription factors FoxO1 and FoxO3a [73]. Decreased levels of phosphorylated protein kinases from mTOR were also found, what led to the idea of a cachexia-associated loss of Akt-dependent signaling in human skeletal muscle, with a decreased activity of regulators of protein synthesis and increased protein degradation [73].

Even though substantial amounts of studies have already been conducted in animals aiming to characterize and understand the molecular mechanism of muscle wasting in CC, there is still the need of more research on human tissue samples to clarify the signaling pathways and to confirm some pre-selected drug targets.

1.4. Principal features of White and brown adipose tissue

Adipose tissue (AT) functions as a central metabolic organ in the regulation of the whole-body energy homeostasis. In conditions of nutrient abundance, AT stores the excess in the form of neutral lipids [74]. By opposition, in conditions of nutrient depletion AT supplies nutrients to other tissue through lipolysis [74]. It is possible to identify two major types of adipose tissue in humans: white adipose tissue (WAT), that is mainly responsible

of providing insulation and energy storage; and brown adipose tissue (BAT), a specialized form that participates in non-shivering thermogenesis through lipid oxidation [75,76]. BAT is mainly present in neonates and is gradually replaced by WAT with aging [77]. However, and despite to what has been thought for quite some time, it is also present and functional in adults, exhibiting higher masses in females [78,79]. Compared with WAT, BAT presents a rich vascularization, abundant mitochondria and multilocular lipid droplet [80]. The characteristic brown color of BAT is due to a high mitochondrial density, which is necessary to support the high levels of lipid oxidation and heat generation [75]. BAT also expresses elevated levels of UCP1, a mitochondrial protein responsible for dissipating energy as heat instead of producing ATP [28]. When activated, UCP1 allows the FFA to flip-flop across the inner and outer mitochondrial leaflets, increasing its permeability and the bypassing of ATP-synthase, thereby allowing the electrochemical energy to dissipate as heat and resulting in thermogenesis [81].

The realization that brown functional adipose tissue does exist in adult humans, and that is possible to promote the switch from white into brown tissue under the action of specific conditions and mediators, has brought into light a new line of investigation aiming to develop new therapies based on adipose tissue itself.

1.4.1. Adipose tissue remodeling during cancer cachexia

In response to alterations in energy balance, adipose tissue can rapidly and dynamically undergo morphological and molecular changes. Some of these alterations have been reported to occur within adipose tissue depots during the progression of cancer cachexia, and even though there are some conflicting evidence regarding the relative contribution of lean *versus* fat loss, the current knowledge seems to indicate that AT loss precedes and occurs more rapidly than muscle wasting [82,83]. Additional studies, performed in cachectic patients with colorectal and lung cancer, documented accelerating rates of loss for all fat depots in terminal stages, within 3 to 7 months before death. However, clinical studies aiming to characterize changes in advanced cancer patients are limited due to ethical issues in obtaining AT biopsies from this group of patients. So, most studies are performed on patients with early staged operable tumors [47]. Alternatively, animal models can be used [84,85].

In addition to mature adipocytes, adipose tissue also contains endothelial cells, preadipocytes, mast cells and immune cells. Collectively, during the progressions of CC,

each of these elements contribute to the alterations that progressively occur in the tissue and in the extracellular matrix components [14,86]. One of the most relevant changes is undoubtedly the enhanced lipolysis, normally associated with an increased turnover of glycerol and FFA, and a decreased volume (but not the number) of adipocytes [85,87]. This process is mediated by the lipid mobilizing factor (LMF), a tumor-induced catabolic factor that directly affects adipocytes of WAT and stimulates lipolysis, leading to the release of FFA and glycerol in a cyclic-AMP (cAMP)- dependent process [88]. LMF binds with high affinity to β_3-adrenergic receptors [89], which have already been studied in the cancer cachexia set and suggested to be elevated and to play an important role in the regulation of lipolysis and energy expenditure [90]. A similar effect has been reported for the zinc α_2-glycoprotein (ZAG), whose production by WAT is enhanced in some cachectic patients. This protein is capable of stimulating glycerol release and increase UCP1 expression in BAT by activating β_3-adrenoreceptors [91,92]. Thus, LMF and ZAG not only increase lipid mobilization but also lead to substrate utilization through the stimulation of the mitochondrial oxidative pathway in BAT, further contributing to the energy expenditure observed in cachectic patients [88,93].

When it comes to morphological alterations, and complementarily to the marked loss of WAT due to lipolysis, shrunken and heterogeneous in size adipocytes were reported in cancer cachexia [85]. An increased fibrosis in the tissue matrix and other numerous alterations were also detected in the ultrastructural components of adipocytes. Cell membrane reveals irregular cytoplasmic projections, and the mitochondria are different from the typical WAT mitochondria, being electron dense and with an increased cristae [85]. The transcription of the key adipogenic factors PPARγ, C/EBP β e α and the SREBP-1c were also reported very decreased, what may inhibit lipogenesis and disrupt new adipocytes recruitment [85,94]. These findings highlight the existence of pronounced molecular and morphological alterations within WAT during the progression of cancer cachexia, revealing an impairment in the formation and lipid storage. Furthermore, inflammatory cell infiltration, primarily represented by macrophages and monocytes, have also been reported, which can further contribute to the inflammatory state noticed and lead to a set of negative effects on the tissue, including adipocytes death [95].

The systemic inflammation, characterized by an increase of pro-inflammatory cytokines, is also closely related to many of the alterations occurring in adipose tissue [14]. The serum levels of TNF-α have been demonstrated to be positively correlated with adipocyte size and volume, as well as with a stimulated lipolysis and a suppressed activity of LPL,

needed for the hydrolysis of fatty acids from plasma lipoproteins [96]. Moreover, IL-1 and IFN-γ can directly stimulate lipolysis, and along with IL-6, further contribute to inhibiting the expression of LPL mRNA [7]. TNF-α has also been reported to prevent preadipocytes differentiation and inhibit the expression of lipogenic transcription factors [96,97]. Additionally, the chronic inflammation that characterizes CC, particularly the increased levels of IL-6, can enhance the expression of UCP1 in WAT, leading to the tissue browning, increased lipid mobilization and energy expenditure [98].

1.4.2. How adipokines interplay with CC

Besides its primary role in storing excess lipids, AT defines a major endocrine organ, secreting hormones and adipokines capable of modulating appetite and nutrient metabolism [99]. Therefore, alterations in AT mass may lead to significant effects on organism energy homeostasis. Among the CC set of players, alterations on adipokines levels have been fundamentally implied in the regulation of appetite and inflammatory status [99]. Leptin, the first discovered adipokine and a key mediator in the regulation of body mass, has indeed been proven to have its own role in the development of CC [100]. Its main functions have been reported to be the suppression of food intake and the stimulation of energy expenditure [101]. This hormone is produced by the obese gene in differentiated adipocytes in proportion to fat mass and acts in the central nervous system by inhibiting the orexigenic pathways, with a decreased expression of the neuropeptide Y (NPY), and by stimulating anorexigenic pathways [100]. Moreover, leptin may affect several metabolic pathways, such as GH signaling and lipogenesis [102,103]. Normally, leptin presents higher levels among females and its secretion is regulated not only by food intake but also by insulin, glucocorticoids and catecholamines [104–106]. In fact, the transport of leptin to the brain seems to increase by pretreatment with glucose and insulin and to be reduced by fasting [107,108]. Moreover, it also seems to be influenced by the presence of leptin binding proteins in the blood such as CRP, that limits leptin receptor binding and the transport across the blood-brain barrier [109]. In fact, a correlation between the levels of CRP and leptin have already been established. Some *in vitro* studies reported that leptin is able to promote CRP production from hepatocytes and endothelial cells and suggested that *in vivo* leptin administration may modulate plasma CRP levels [110,111]. Additionally, it has also been shown that CRP directly binds leptin in extracellular settings, thus impairing its biological properties [110]. Indeed, some patients

with CC exhibit significantly decreased levels of leptin when compared to cancer patients without cachexia and healthy controls [112]. However, this decrease is thought not to trigger a compensatory mechanism in order to promote food intake or diminish energy expenditure [100]. This lack of response has been attributed to a hypothalamic inflammation, that causes a reduced response of leptin targets to the diminished levels of this adipokine [100]. In addition, an intracerebroventricular administration of leptin in rats seems to induce the expression of UCP1, UCP2, and UCP3, what may contribute to the increased energy inefficiency [113]. The relation between leptin and cancer cachexia is still, however, somewhat hypothetical, with several studies showing contradictory results [112,114,115].

Another member of the adipocyte-secreted proteins, and also an important body weight regulator is adiponectin, which is exclusively secreted by adipose tissue [116]. Such as leptin, adiponectin serum levels are gender dependent, being normally higher in females [117]. Its circulating levels have been reported to be inversely correlated with body weight and to be reduced in situations of insulin resistance [118]. In fact, the administration of adiponectin has been shown to increase glucose uptake by muscles and increase insulin sensitivity [119]. In several tissues, such as liver and muscles, an increased fatty-acid oxidation, as well as a stimulated expression of uncoupling proteins and thermogenesis have also been attributed to adiponectin action [119,120]. Even though the mechanisms that coordinate the regulation of this adipokine still poorly understood, some evidences suggest a down-regulation of this molecule by TNF-α [121]. An inverse correlation between the circulating levels of adiponectin and both free and total leptin concentration in cancer patients have also been reported, with a suggested antagonizing action of adiponectin on leptin effects after weight loss [95]. In cancer patients exhibiting signs of cachexia, some contradictory data regarding adiponectin serum levels have been presented. In fact, a study conducted by Jamieson *et al.* [122] reported low levels of adiponectin in individuals with advanced lung cancer who experienced weight loss, whilst a different study, performed by Kim *at al.* [101] in lung and colorectal cancer patients, revealed no significant differences between the cachectic and the non-cachectic subjects. Opposite results were obtained by Batista Jr. *et al* [99], that reported significantly higher plasma adiponectin levels in cachectic cancer patients when compared to stable weight cancer patients. An explanation to these controversial data may pass through the realization that the adiponectin levels may differ with the location and stage of the tumor, as well as with other variables such as age, gender, BMI and the fat depot in analysis [99].

Indeed, higher levels of adiponectin were detected especially in the subcutaneous rather than in the visceral adipose tissue and thought to be a result of a specific fat depot alteration, such as increased production of TNF-α and IL-6 [99].

In addition to leptin and adiponectin, some other hormones such as resistin and ghrelin have also been arousing much interest in the scope of CC. Resistin, for instance, is an adipose tissue-derived hormone that has been suggested to be capable of inducing endothelial cell proliferation and to promote angiogenesis by upregulating VEGF expression [123]. Furthermore, processes like the regulation of glucose homeostasis, adipogenesis and the modulation of the inflammatory response have also been reported to be under the action of resistin activity [124]. Even though increased levels of resistin were associated with an increased risk of cancer and several inflammatory processes, there are a limited number of studies regarding the interplay between resistin and the pathophysiology of CC, as well as a clear perception of the link between body weight loss and appetite [95,124]. Ghrelin, by another side, has been a target of extensive studies in the CC context. This peptide is mostly produced by the stomach and its main properties have been cited to be the regulation of food intake [125]. Ghrelin is able to stimulate the secretion of the growth hormone (GH) through the growth hormone secretagogue receptor (GHSR), and to inhibit the production of anorectic pro-inflammatory cytokines, such as IL-1, IL-6 and TNF-α, which may help to overcome some of the symptoms observed in CC, in addition to anorexia [126]. Furthermore, the GH, whose release is induced by ghrelin, regulates the levels of insulin-like growth factors (IGF)-1, therefore promoting the preservation of protein stores at the expense of fat utilization during periods of caloric restriction [125]. Indeed, the GH and IGF-1 are recognized to be the major metabolic mediators in the regulation of energy balance [125]. Moreover, ghrelin seems to be a powerful orexigenic factor, promoting food intake and inducing adiposity. Li *et al.* [127] observed in human umbilical vein endothelial cells treated with and without TNF-α, that ghrelin was capable of inhibiting both basal and TNF-α-induced activation of the NF-κB, a transcription factor involved in the production of some pro-inflammatory cytokines and in the regulation of skeletal muscle protein degradation [128]. In some CC patients, the circulating levels of ghrelin (both acylated and deacylated forms) have been reported elevated, what may represent a compensatory response to the negative energy balance state underlying the disease [129,130]. Data obtained by others also support the favorable effects of ghrelin in minimizing the inflammatory status and the muscle loss that occurs in patients with cachexia [131,132]. Additionally, a chronic repeated

treatment with ghrelin in mice demonstrated its ability to promote adiposity and to decrease the expression of UCP-1 in BAT [84].

Ghrelin seems, indeed, a beneficial factor to counteract some of the cachexia symptoms and a promisor therapeutic target, enclosing the potential to increase appetite, muscle mass, and adiposity, and to decrease thermogenesis in BAT by lowering the UCP-1 levels. Although, further studies still vital to understand the influence of this mediator on tumor growth, since ghrelin may increase growth factors such the GH and IGF-1, and therefore, promote cancer to further expand [125]. A better understanding of how leptin and adiponectin levels may vary according to the type and location of the tumor and the molecular pathways underlying its action in CC are still required and of great interest, since these cytokines are exclusively secreted from adipose tissue and may serve as important biomarkers in staging CC and in following fat alterations.

1.4.3. Browning of adipose tissue – a novel feature in energy expenditure on CC

Increased energy expenditure is one of the most relevant features in CC, and has been closely associated with AT activity [48,98]. Attending to the origin and the anatomic features, it is possible to identify two types of thermogenic adipocytes: brown and beige cells [133]. In both, sugars and lipids are burned to generate heat instead of ATP, and to maintain body temperature through adaptive thermogenesis [98,134]. Brown and beige cells express high levels of UCP1 to allow the uncoupling of mitochondrial respiration and present a high mitochondrial density [133,134]. While brown adipose cells are the major content of BAT, beige cells are present within white fat depots and have a molecular signature of their own that includes the expression of Tbx1, Tmem26, and Cd137 gene markers, that are not expressed in brown adipocytes [135]. When stimulated either by cold exposure, sympathetic stimulation mediated by the β_3-adrenoreceptor, exercise, or by a long treatment with PPARγ agonists, an increased expression of thermogenic genes and in the number of beige cells can be triggered [134,136]. This process is often called “browning” of WAT, and the beige adipocytes formed express levels of UCP1 in similar amounts to the ones of brown adipocytes and present thermogenic capacity [135,137]. The overexpression of UCP-1 is probably the major event triggering the conversion of white into brown adipocytes [138]. This protein is located in the inner mitochondrial membrane and is thought to be activated via the PKA-

p38 mitogen-activated protein kinase (MAPK) signaling pathway, that induces the phosphorylation and activation of transactivators of UCP-1 [138,139]. Once active, this protein acts as a proton channel and uncouple the ATP production by the ATP synthase. Moreover, FFA are thought to be potent activators of UCP-1, what further supports the high levels detected for this protein in cancer cachexia, since an increased lipolysis is one of the main features of the syndrome (Figure 1) [138,140]. There are two proposed hypotheses that have been trying to explain the beige adipocytes differentiation: one of them, states that beige adipocytes are formed by transdifferentiation from mature white adipocytes; the second hypothesis postulate that the differentiation of beige adipocytes is mediated by specific precursors [141–144]. There are also some reports indicating that beige adipocytes derived from exposure to cold stimulation can be reverted to typical adipocytes once the stimulus is finished [143]. If once again stimulated by cold exposure, they can turn again into beige adipocytes, suggesting a potential for repeated transdifferentiation of white adipocytes into beige adipocytes [143]. Even though the brown adipocyte is the thermogenic unit of BAT, its activity is dependent on a proper stimulation and an adequate supply of oxygen and substrates through the capillaries surrounding each cell [145]. Furthermore, and despite that brown adipocytes constitute the major content in volume of BAT, the largest number of cells within adipose tissue are represented by endothelial and interstitial cells, as well as preadipocytes [145]. Under conditions of increased thermogenic stimulation, these cells can proliferate and differentiate to form, not only new brown adipocytes but also new capillaries and terminal nerves in order to properly support the new demands. In fact, VEGF-A, a strong angiogenic factor, is found overexpressed in BAT [81]. This marker of vascularization has been correlated with the induction of adipose tissue browning, an increased vascularization and an up-regulated expression of both UCP1 and the peroxisome proliferators-activated receptor gamma coactivator-1α (PGC-1α) in BAT, thus increasing thermogenesis and energy expenditure [81,146]. These alterations are intended to support the high energy consumption of BAT, control adipose tissue expansion and the overall metabolic health [81]. Supporting this idea, a study conducted in mice with adipose VEGF deletion, revealed a reduced adipose vascular density, increased hypoxia, apoptosis, and inflammation. In contrast, induction of VEGF expression led to an increased adipose vasculature and reduced hypoxia [81]. These findings clearly highlight the essential role of VEGF signaling for a proper adipose function, although, its importance in the CC set still yet to be completely understood. It is important to acknowledge that even though

VEGF seems to be essential for a healthy adipose function, it is also an important factor involved in tumorigenesis by stimulating proliferative signaling pathways [146]. In fact, increased levels of VEGF have been found in obese patients, and its possible involvement in the obesity-cancer link has been put into discussion [147]. Therefore, further studies aiming to understand and characterize the role of this marker in the CC scope are still needed.

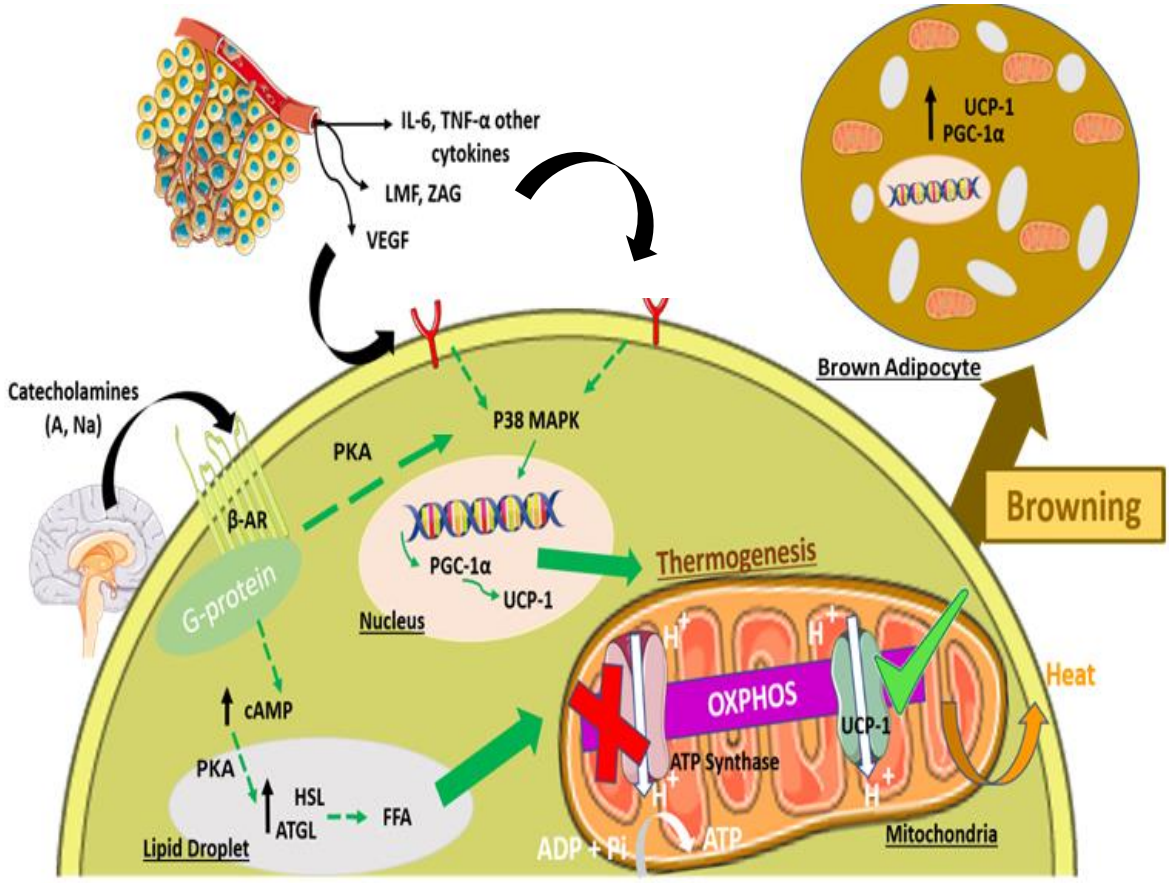

Figure 1- Schematic overview of the molecular mechanisms and signaling pathways leading to the browning of the adipose tissue. Signaling molecules tumor-derived, produced by the SNS and muscles are able to trigger signaling pathways leading to the WAT browning. The catecholamines Adrenalin (A) and Noradrenalin (NA) through the activation of β-adrenoreceptors (β-AR) are also able to enhance thermogenesis either by stimulating the production of FFA or by promoting the expression of PGC-1α via the p38 MAPK pathway. Similarly, cytokines such IL-6 and TNF-α, and other factors LMF, ZAG and VEGF can activate PKA and may also be able to induce thermogenesis and WAT browning. PTHrP has also been proven to cause WAT browning, however the mechanisms of its action are still unknown.

Recent studies have identified the parathyroid hormone-related protein (PTHrP), a tumor-derived polypeptide involved in calcium homeostasis, as an inducer of thermogenic gene expression, and therefore, a promoter in the browning of adipose tissue [147,148]. In fact, PTHrP seems to potently induce UCP1 mRNA in similar amounts to noradrenaline, the

classic thermogenic catecholamine produced by the sympathetic nervous system [147]. Furthermore, PTHR knockout mice revealed not only resistance to cachexia driven by tumors, but also an improvement in muscle strength and a preserved muscle mass, highlighting its role in other features observed in CC, such as muscle atrophy, and thus suggesting a possible crosstalk mechanism between the loss of AT and skeletal muscle [149,150]. In fact, elevated levels of PTHrP were found in patients with metastatic colorectal and lung cancer exhibiting signs of cachexia [151]. Supporting evidences were given in studies performed with mice bearing tumors in which the neutralization of PTHrP or loss of its receptor in fat cells resulted in a blockage of the browning process and the tumor-induced hypermetabolism as well as in the promotion of adipose tissue wasting [150,151]. Therefore, PTHrP plays an important role in cancer cachexia, promoting energy expenditure by stimulating the expression of thermogenic genes in AT [151].

An important relation between IL-6 and WAT browning has also been established, as represented in Figure 1. When an IL-6-deficient colon cancer tumor was implanted in mice, WAT browning became significantly impaired [98]. It was reported that IL-6 is required for maximal induction of UCP1 in subcutaneous WAT [98]. Indeed IL-6 plays an important role in the browning of AT, although, it has been suggested to be less important than PTHrP [98].

Attending to the thermogenic properties and the high levels of UCP1 expressed, brown and beige cells are capable to dissipate energy to produce heat, resulting in an increased energy inefficiency [152]. There are some evidences reporting the existence of activated brown fat in some patients diagnosed with cachexia, including large peri-adrenal brown fat depots and increased UCP1 expression in white fat tissue, what may contribute to the increased energy expenditure that characterizes the syndrome [151,152].

1.4.4. Regulation of the lipolytic pathway

Lipolysis in adipocytes is achieved by a sequential action of some lipases, such hormone sensitive lipase (HSL) and adipose triglyceride lipase (ATGL), that have been found increased in the cachexia syndrome [153]. ATGL, since its discovery in 2004, has been well accepted to mediate the initial step of triglyceride breakdown, leading to the formation of diacylglyceride (DAG) and FFA [153,154]. This process is continued by the intervention of HSL and monoglyceride lipase (MGL), that further completes the hydrolysis to produce additional FFAs and glycerol, being released to the vasculature to

be used by other tissues during energy shortage [153]. These lipases are key regulators of the lipolytic pathway, precisely determining the rates in which each of the breakdowns occurs [153,155]. Therefore, alterations in the expression of these molecules are often detected in cachectic patients, supporting the altered lipolysis rates that characterize this syndrome. Several signals, either hormonal or biochemical, are able to modulate the activity of lipolytic enzymes and other accessory proteins, therefore allowing an adequate response of AT to changes in energy requirements. Under conditions of a negative energy balance, such as fasting and exercise, lipolysis is primarily stimulated upon the release of, especially, adrenaline and noradrenaline, to activate the β-adrenergic receptors on the surface of adipocytes [155,156]. By turn, the activation of the receptors causes the G_S subunit of G-protein to interact with adenylyl cyclase, whose activation leads to the conversion of ATP into cAMP, resulting in an increase of the intracellular cAMP levels (Figure 1) [155]. This messenger is able to promote the phosphorylation of both HSL and perilipin-1 via protein kinase A [155]. The phosphorylated HSL is then translocated from the cytosol to the surface of lipid droplets to induce lipolysis, while the phosphorylated perilipin-1 enables the docking of the phosphorylated HSL and promotes the activation of ATGL, thus initiating the lipolytic cascade and providing DAG to the action of HSL [155]. Indeed, it is currently well accepted that a coordinate regulation of ATGL and HSL is fundamental to control AT lipolysis in situations of high energy demand. The expression of HSL mRNA and protein, as well as the ratio of plasma glycerol/body fat (an index of *in vivo* lipolysis), have been found elevated in cancer cachexia [140]. A similar result regarding the mRNA expression, however, has not been obtained for ATGL, that revealed no significant differences between cachectic cancer patients and controls [140]. This result is thought to be due to a lack of translation between ATGL mRNA expression and enzyme activity since its function is regulated by post-translation modifications [157,158]. In fact, elevated activities have been reported for both HSL and ATGL in the AT of cachectic patients compared to non-cancer and cancer patients without cachexia [159]. Furthermore, the inhibition of lipolysis through genetic ablation of ATGL and HSL has proven to ameliorate some features displayed in cancer-associated cachexia [159]. In fact, both HSL and ATGL deficient mice with tumors were reported to be protected against increased WAT lipolysis, although, in different extents [153,159]. Indeed, ATGL seems to play a more prominent role in AT lipolysis than HSL [159,160]. Supporting evidences are given by Das *et al.* [159], in a study conducted on ATGL-deficient mice with cachexia-inducing Lewis Lung carcinomas or B16 melanomas, that

in addition to the increased protection against WAT lipolysis, also reported an enhanced resistance to myocyte apoptosis and proteasomal muscle degradation for both HSL and ATGL-deficient mice, although, in an extensive degree to the last ones. These findings support the critical role that ATGL seems to play in cancer cachexia and suggest the idea of a cross-talk between adipose tissue and muscle during the progression of cachexia.

In addition to catecholamines, there are other factors that can modulate HSL and ATGL activities to regulate lipolysis in adipocytes. These factors include natriuretic peptides, that signals through guanylyl cyclase and cGMP, and insulin, that is able to down-regulate the expression of both HSL and ATGL via the anabolic signaling of Akt/PKB [153,155,156]. It is the balance kept by the anabolic and catabolic factors that determine the net lipid flux in adipocytes, as well as the aberrant lipid turnover that occurs in pathological diseases, such cachexia, when this balance is disrupted [155]. Even though much has recently been revealed in spite of how these lipases contribute to the adipose tissue remodeling, the signaling mechanisms involved in fat loss still poorly understood. Furthermore, little is known about the interplay between the systemic inflammation and the enhanced lipolysis in cancer cachexia, and therefore, more studies are essential.

Moreover, AMP-activated protein kinase (AMPK) has also been reported as being capable to phosphorylate both ATGL and HSL, however with different outcomes [153,161,162]. AMPK is reported to inhibit HSL by a Ser565 phosphorylation, preventing protein kinase A phosphorylation at Ser563 and Ser660 under stimulatory conditions [162]. ATGL, in turn, is activated when phosphorylated by AMPK at Ser406 [160,163]. The overall outcome of AMPK activity is a suppressive effect on FA oxidation and energy usage within adipose tissue, by lowering the lipolysis rates [161,162]. Even though during states of low cellular energy, AMPK is normally activated in the peripheral tissues, the cachectic condition seems to be accompanied by an inactivation of AMPK, thus preventing its beneficial role in counteracting the chronic lipolysis taking place in cachexia [162]. Interestingly, Rohm *et al.* [162] developed an AMPK-stabilizing peptide, the ACIP, which was able to ameliorate WAT wasting *in vitro* and *in vivo* by shielding the Cidea targeted interaction surface on AMPK, proposing ACIP as a preserver of AMPK activity in WAT and a promisor therapeutic agent for cachexia.

1.5. Exercise training in the modulation of CC

Indeed, several studies have already associated exercise training with anti-inflammatory properties [164–166]. In this context, and since systemic inflammation is considered to be one hallmark of cancer cachexia, playing a significant role in many of the symptoms that characterize this condition, exercise might be seen as a potential strategy in counteracting the progression of cachexia [51,167,168]. In fact, it is well established that acute exercise induces an immune response, leading to an increased production of cytokines that are involved in the acute-phase response and of those that limit the inflammatory status, as represented in Figure 2 [164–166,169]. Increased levels of IL-6 in circulation have consistently been reported following exercise training [166,169]. This is the first cytokine detected in circulation during exercise, and increases with the duration and intensity of the training and with the muscle recruitment, declining in the post-exercise period [169–171]. Even though often associated to inflammatory responses, some data report that IL-6 does not induce inflammation directly and even present anti-inflammatory properties by exerting inhibitory effects on pro-inflammatory cytokines such as TNF-α and IL-1 [164,172]. In fact, the infusion of elevated levels of IL-6 and the practice of exercise were demonstrated by Starkie *et al.* [172] to attenuate endotoxin-induced increases in TNF-α. Furthermore, the stimulation of IL-1 receptor antagonist and IL-10, a very important anti-inflammatory cytokine, has also been shown for IL-6, further supporting the anti-inflammatory effect of exercise [173]. The IL-10/ TNF-α ratio, an indicator of individual's inflammatory status and disease-related morbidity [174], has been reported increased in WAT from rats during exercise, promoting an anti-inflammatory environment in adipose tissue and further emphasizing a beneficial role for exercise [175].

Moreover, resistance exercise can be a powerful stimulant of protein synthesis, leading to an increase of muscle fiber area and stimulating both myofibrillar and mitochondrial protein synthesis [176,177]. In fact, the concentration of IGF-1 mRNA, an important regulator of protein synthesis in skeletal muscle and an inducer of hypertrophy, was found increased after resistance exercise (Figure 2) [178]. IGF-1 is thought to act through a highly conserved signaling pathway involving a cascade of intracellular components. The binding of IGF-1 to its receptor leads to the activation of the protein kinase B (PKB), also known as AKT, having as intermediate step the activation of the phosphatidylinositol-3-kinase (PI3K) [179]. In turn, the activated AKT is responsible for mediating cell growth

and survival in a different set of tissues [180]. Indeed, AKT has been reported to be capable of inhibiting protein degradation by repressing the transcription factors of the FoxO family and to stimulate protein synthesis via the mTOR and GSK3β [181]. In this context, resistance exercise leads to an increase of IGF-1 levels, that have been reported to be downregulated in cancer cachexia [181], which in turn stimulates protein synthesis and induce hypertrophy. Supporting this idea, the use of rapamycin, an inhibitor of mTOR, has shown to be capable of completely block muscle hypertrophy [182].

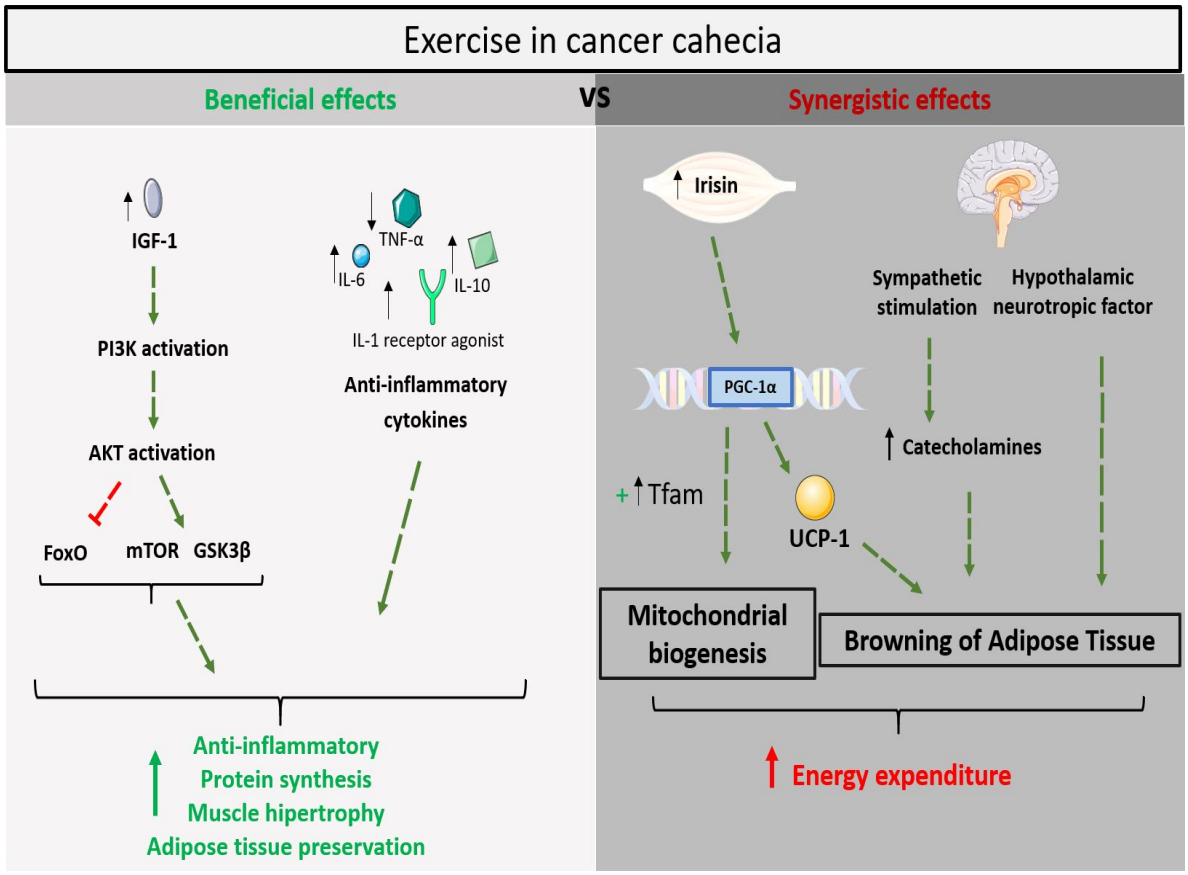

Figure 2- Schematic overview of how exercise training impact may modulate cancer cachexia. Exercise may counteract the systemic inflammation that characterizes CC by inducing the release of IL-6, IL-10 and IL-1 receptor agonist and by reducing the levels of TNF-α. Since pro-inflammatory cytokines are associated with adipose tissue wasting, it may lead to the preservation of the tissue. Furthermore, through the increase of IGF-1 levels, exercise training promotes an increased protein synthesis and a decreased protein degradation by stimulating the UPS. Exercise training also enhance the browning of WAT either by increasing the mitochondrial biogenesis, through the expression of PGC-1α and Tfam, or by enhancing the expression of the UCP-1. Exercise can also promote the browning of WAT either by sympathetic stimulation, through the action of catecholamines, or by stimulating the release of hypothalamic neurotropic factors. Taken together this alteration may lead to an increased thermogenesis and consequently, an enhanced energy expenditure.

For quite some time exercise has been postulated to cause adaptations in WAT, including a decrease in adipocyte size and lipid content, and an increase in mitochondrial biogenesis and activity [183,184]. Recently, exercise has been reported to promote the expression of beige adipocytes in WAT, especially in subcutaneous WAT, with a markedly increased expression of UCP1 [185,186], as represented in Figure 2. Although the mechanisms underlying the beiging of WAT cells as a result of physical exercise are still not fully understood, some hypotheses have already been suggested. Among them, one states that exercise can increase sympathetic innervation in subcutaneous WAT, what can be responsible for the beiging that takes place in the cells of this type of tissue; another one suggests that exercise training induces the beiging as a response to the increased secretion of hypothalamic neurotropic factor [80,187]. Indeed, moderate to high-intensity endurance training has been proven to enhance SNS activity, causing the release of catecholamines and, as a result, stimulating the browning of white adipose tissue and promoting whole-body energy expenditure [95]. Furthermore, markers of vascularization such as VEGFA and PGDF, as well as the number of blood vessels, are also found increased in the subcutaneous WAT from exercised animals and thought to be mediated by the increased sympathetic innervation [95,188]. Additionally, irisin, a cleaved form of FNDC5 and a major inducer of the beneficial properties of exercise, has also been found increased following exercise training [189]. Exercise is thought to promote increases in irisin levels by up-regulating PGC-1α expression in skeletal muscle, causing the release of irisin from this tissue by myocytes into the circulation [95,189]. In turn, irisin can act on adipose tissue, preferentially on the subcutaneous WAT, and cause the browning of the tissue by increasing the expression of UCP-1 and other thermogenic genes [189]. In fact, some clinical studies have been confirming the positive correlation between the increased levels of FNDC5 and the circulating irisin with exercise training, although, the role of irisin is still creating some controversy among the research community, since a consistently increase of FNDC5 and irisin has not been detected after endurance exercise in humans [95,190,191].

Moreover, exercise training is reported to increase PGC-1α and the mitochondrial transcription factor A (Tfam) mRNA expression, as well as COX IV and citrate synthase activity in both epididymal and retroperitoneal fat pads, promoting mitochondrial biogenesis and improving its function (Figure 2) [184,192]. However, even though the up-regulation of PGC-1α after exercise is thought to be necessary to increase the mitochondrial biogenesis, an enhanced expression of Tfam is not always detected. After

a 2 hours bout of acute exercise, Sutherland *et al.* [193] reported an elevated expression of PGC-1α, but not of Tfam. This finding is thought to be related with a delayed induction of Tfam expression, that is expected to reach its peak after a 12 hours exposure, and therefore evidencing a lack of effect for this transcription factor immediately following acute exercise [192]. Alterations in the mitochondrial gene expression in subcutaneous WAT also seem to arise as a response to several training program durations and modalities [95]. Furthermore, the mitochondrial activity, that was accessed by measuring the activity of the enzyme cytochrome c oxidase from the respiratory chain, and the enzyme malate dehydrogenase from the tricarboxylic acid cycle, were also significantly increased in the visceral WAT of rats in response to 10 weeks of swim training [95,194].

2. Aims

Our aim was to study the influence of exercise training on the white adipose tissue remodeling associated to cancer-related cachexia. To fulfill our goal, we evaluated the impact of 35 and 55 weeks of treadmill endurance exercise on retroperitoneal adipose tissue collected from two pre-clinical models of cancer, breast and prostate cancer, focusing on thermogenic alterations, metabolism regulation, mitochondrial biogenesis and morphological adaptations.

3. Materials and Methods

3.1. Experimental design

In order to evaluate the effect of exercise training on cancer-induced WAT remodeling, histological and biochemical analysis were performed through the assessment of the expression of target proteins, following the sequence of steps summarized in the figure 3.

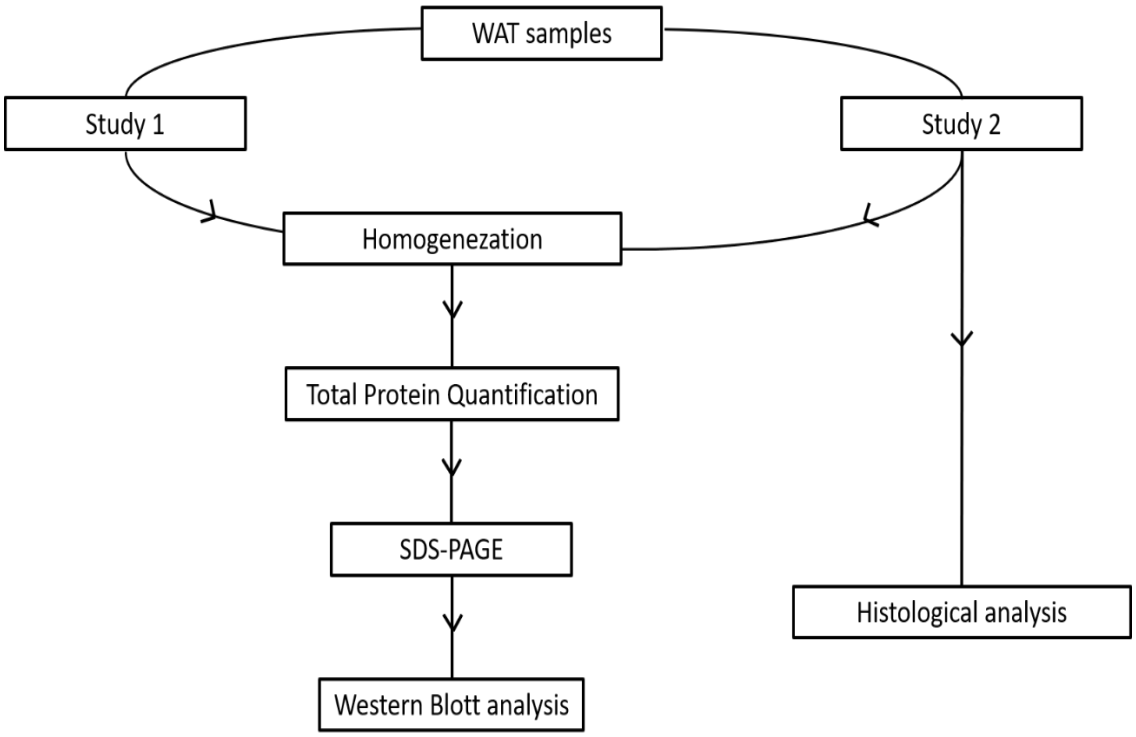

Figure 3- Schematic overview of the selected experimental design followed in the present work.

In the present work two different studies using distinct pre-clinical models of cancer were conducted. The *Study 1* was performed using WAT samples from retroperitoneal tissue of rats with mammary tumors. In *Study 2*, WAT samples from a pre-clinical model of prostate cancer were used. In this study, a morphometric analysis of retroperitoneal adipose tissue was conducted and some molecular players putatively modulated by cancer and/or exercise training were assessed by western blotting. In both studies, four experimental groups were considered (n=4 *per* group): control and sedentary, control and exercised, with cancer and sedentary, with cancer and exercised. The exercise program was distinct in both studies (described below).

3.1.1. Study 1 – Induction of mammary tumorigenesis and implementation of exercise training

Fifty female Sprague-Dawley rats were obtained at the age of 38 days from Harlan (Barcelona, Spain). During the experimental protocol, animals were housed in groups of 4 rats *per* cage under controlled conditions at 22 ± 2 ºC and 60 ± 5% of relative humidity with 12/12 h dark-light cycle, with free access to food (standard laboratory diet 4RF21® (Mucedola, Italy)) and water. After a week of acclimatization, the animals were randomly divided into four experimental groups: Control Sedentary (CONT + SED, n=10), N-Methyl-N-nitrosourea (MNU) Sedentary (MNU + SED, n=15), Control Exercised (CONT + EX, n=10) and MNU Exercised (MNU + EX, n=15). The animal protocol was approved by the Portuguese Ethics Committee for Animal Experimentation, *Direção Geral de Alimentação e Veterinária* (license number 008961) and was performed in accordance to European Parliament Directive 2010/63/EU. Mammary tumorigenesis was chemically induced by the administration of MNU (N-Methyl-N-nitrosourea, ISOPAC®, Sigma chemical Co., Spain). At the age of 50 days, rats from MNU groups were intraperitoneally (i.p.) injected with a single dose of 50 mg MNU/Kg body weight. Rats from CONT groups were i.p. injected with a single dose of vehicle.

Animals from EX groups started a treadmill training protocol (Treadmill Control LE 8710, Harvard Apparatus, USA) at 52 days of age. In the first two weeks, exercise duration and treadmill speed were gradually increased until reaching 60 min *per* day at 20 m *per* min, 5 days *per* week, which was maintained for 35 weeks.

At the end of the experimental protocol, animals were sacrificed with ketamine/xylazine (Imalgen® and Rompun®, respectively) and blood was collected for serum preparation, mammary tumors were counted and collected for histological analysis and retroperitoneal adipose tissue was removed and prepared for biochemical analysis.

3.1.2. Study 2- Induction of prostate cancer and implementation of exercise training

Forty Wistar Unilever (WU) male rats were purchased from the *Charles River Laboratories* company (France) ate the age of 4 weeks. After arriving, rats were placed in quarantine for two weeks and randomly housed in 5 cages. The animals were randomly divided in four experimental groups: Control Sedentary (CONT+SED), PCa Sedentary (PCa+SED), Control Exercised (CONT+EX) and PCa Exercised (PCa+EX) and kepted

in *Trás-Os-Montes e Alto Douro University (UTAD)* bioterium under controlled conditions at 18±2ºC and a relative humidity of 55±5% with 12/12 h dark-light cycle. The animal protocol was authorized by the Responsible Organ of animal well-being from UTAD and by the General Direction of Veterinary Alimentation (license nº 021326). In order to chemically induce prostate lesions in 20 rats (PCa groups), a subcutaneous injection of flutamide was administered (20 mg/kg, prepared in 10% of propylene glycol and 5% of ethanol) during 21 consecutive days. Two days following the flutamide administration, a subcutaneous injection of testosterone propionate was performed (100 mg/Kg, dissolved in starch oil (Sigma)). After two days, an i.p. administration of MNU (30 mg/Kg prepared in citrate buffer 0,1 M, pH 4,8) was given. Fifteen days after MNU administration, subcutaneous implants with crystalline testosterone (Sigma) were placed in the interscapular region through a small incision followed by suture. This procedure was performed under anesthesia (75mg/Kg of ketamin and xilazin) and the implants were prepared with medium silicone tubes, 4 cm filled with testosterone, and the extremities sealed with medical glue (G.E. RTV-108).

The animals from EX groups were exercised since six weeks old in a leveled treadmill (Treadmill Control LE 8710, Harvard Apparatus, USA) for 50 weeks. The exercise program included five days per week, 30 minutes per day during the first week (habituation period) and then 60 minutes per day until the end. The speed of treadmill was set for 70% of the maximal speed capacity of the animals with PCa and every fifteen days the speed capacity was re-evaluated to correct the exercise intensity.

At the end of the protocol, animals were sacrificed using an overdosage of ketamin and xilazin, followed by a cardiac puncture exsanguination. The blood was collected and centrifugated to obtain serum, and the prostates collected for anatomopathological analysis and retroperitoneal adipose tissue was weighed and stored for histological and biochemical analysis.

~~3.3.~~3.2. Adipose tissue preparation for biochemical analysis

A portion (~40-50 mg – Study 1; ~20 mg- Study 2) of retroperitoneal adipose tissue was homogenized in homogenization buffer (8 M urea, 2 M thiourea, 2 % CHAPS, 50 mM DTT, 2 % ampholytes pH 3-10, 1 % NP-40 supplemented with the protease inhibitor PMSF (200 mM)), in the proportion of 20 mg of tissue/mL of buffer, using a Teflon pestle on a motor-driven Potter-Elvehjem glass homogenizer at 0-4ºC. The protein content of

the adipose tissue homogenate was assayed with the Bio-Rad RC-DC method, following the instructions of the manufacturer, using bovine serum albumin (BSA) as a standard and then the samples were preserved at -80ºC.

3.4.3.3. SDS-PAGE and Western blot analysis

Equal amounts of protein (20 μg) from each sample were dissolved in loading buffer (0.5 M Tris-HCl pH 6.8, 4 % (w/v) SDS, 15 % (v/v) glycerol, 1 mg/mL bromophenol blue and 20 % (v/v) β-mercaptoethanol) and heated 5 minutes at 100 ºC and then electrophoresed on a 12.5% SDS-PAGE prepared as described by Laemmli. Gels were run for 45 minutes at 180 V in running buffer (250 mM glycine, 25 mM Tris, pH 8.6 and 0.1 % (w/v) SDS) and the resolved proteins were blotted onto a nitrocellulose membrane (Whatman®, Protan®) in transfer buffer (25 mM Tris, 192 mM glycine, pH 8.3 and 20% methanol) during 2 h at 200 mA. Then, nonspecific binding was blocked with 5% (w/v) nonfat dry milk in TBS-T (100 mM Tris, 1.5 mM NaCl, pH 8.0 and 0.5% Tween 20) for 1 h at room temperature with mild shaking. The membrane was incubated with primary antibody diluted 1:1000 in 5% (w/v) nonfat dry milk in TBS-T (rabbit anti-HSL, ab45422, abcam; rabbit anti-VEGFA, ab46154, abcam; rabbit anti-UCP1, ab10983, abcam; rabbit anti-FNDC5 (irisin), ab174833, abcam; rabbit anti-ETFDH, ab91508; rabbit anti-PGC-1alpha, ab54481; anti-mtTFA, ab47548; rabbit anti-GAPDH, ab9485, abcam; rabbit anti-Adiponectin, ab22554, abcam; rabbit anti-ATGL, ab99532, abcam). After a 2 h incubation at room temperature with agitation, the membrane was washed 3 times with TBS-T during 10 min each, to remove the unbonded antibody, and incubated with anti-mouse or anti-rabbit IgG peroxidase secondary antibody (NA931 or NA934, respectively from GE Healthcare, UK) diluted 1:1000 in 5% (w/v) nonfat dry milk in TBST for 1 h at room temperature. After, once again, washed 3 times, 10 min. each, with TBST, immunoreactive bands were detected with enhanced chemiluminescence reagents (ECL, WesternBright™ ECL, advansta, CA, USA) according to the manufacturer's procedure and images were recorded using X-ray films (Kodak Biomax Light Film, Sigma®, St. Louis, USA). Films were scanned in Molecular Imager Gel Doc XR + System (Bio-Rad®, Hercules, CA,) and semi-quantitative analysis of optical density (OD) was performed with and QuantityOne® 1-D Analysis Software version 4.6.3 (Bio-Rad®, Hercules, CA, USA). For mtTFA, Irisin, ETFDH, GAPDH, HSL and VEGF (Study 2) the detection method used was the fluorescent one, once this technique is more sensitive

then chemiluminescence. In this protocol after the first 2h incubation using a primary antibody, a second wash was performed during 1h using a fluorescent secondary antibody. The membranes were than washed 3 times with TBS-T and the fluorescence of membranes was automatically measured using the Odyssey Infrared Imaging System (LI-COR® Biosciences, US). Protein loading was controlled by Ponceau S staining once the content of cytoskeletal proteins was found to be modulated by the conditions in study.

~~3.5.~~3.4. Histological analysis

Rats retroperitoneal pieces of adipose tissue collected within Study 2 were included in paraffin. The paraffin blocks were sectioned onto 5 µm sections using a manual microtome. For each sample two glass slides were prepared with three cuts *per* slide. One of the two glass slides of each sample was deparaffinized in xylol, dehydrated with alcohol in decreasing concentrations (100%, 95% and 75%) and stained with haematoxylin and eosin (H&E). The same procedure was applied to the other glass slide, although, instead of being stained with H&E, a Sirius Red staining was performed in order to access the collagen fiber content. Both glass slides of each sample were then examined in a bright-field optical microscopy and digital images were captured using ZEN Microscopy software. For the samples stained with H&E, a quantification of the number, using the automated cell counting system software AdipoCount by CSBIO, and adipocytes size, using ImageJ basic software for digital image processing, was performed, and the data analyzed. The cell perimeter and sectional area were measured in 100 adipocytes *per* field (three *random* fields for one rat and five animals per group). For the samples stained with Sirius Red, due to the friability of the tissue, it was only possible to perform a qualitative analysis.

~~3.6.~~3.5. Data analysis

Values are given as mean ± standard deviation (SD) for all variables. Significant differences between the groups were evaluated using Kruskal-Wallis test followed by Dunn's multiple comparisons post hoc test. The statistical significance between the four analyzed groups were measured based on P-value and the results were considered significantly different when P-value<0.05. Statistical analysis was performed with Graph Pad Prism software (version 6.01).

4. Results

4.1. Study 1 – Influence of exercise and/or mammary tumorigenesis on adipose tissue remodeling

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

The administration of MNU induced mammary lesions in 100% of the animals and there were no alterations in the food intake. No histopathological changes in the mammary tissue were observed in control animals (CONT+SED and CONT+EX groups). Endurance training modulated tumor development with lower incidence of mammary lesions observed in the MNU+EX than in MNU+SED group (54 vs. 75 lesions, respectively), and less malignant lesions, as already reported [195].

MNU administration induced a significant loss of body weight ($p<0.05$) on sedentary animals compared with the ones from the control sedentary group (CONT+SED). A significant decreased body weight was also found on exercised MNU-injected animals ($p<0.05$) compared to the control exercised (CONT+EX) group (Table 1). No significant alterations on body weight were induced by exercise in the control group (CONT+EX).

Table 1- Characterization of animal's response to MNU-induced muscle wasting and /or endurance training regarding body weight, gastrocnemius mass and gastrocnemius-to-body weight ratio. The results are presented as mean ± standard deviation.

Experimental Group	CONT+SED	MNU+SED	CONT+EX	MNU+EX
Body weight (g)	298.29±13.59	273.20±16.62*(a)	313.74±24.51	267.45±27.77*(b)
Gastrocnemius (g)	3.94±0.28	3.50±0.31*(a)	4.15±0.25	3.36±0.62*(b)
Gastrocnemius-to-body weight (mg g^{-1})	13.38±0.73	13.08±0.2	13.39±0.49	12.43±1.89

*p<0.05; **(a)** vs CONT+SED; **(b)** vs CONT+EX

Regarding gastrocnemius mass, MNU induced a significant decrease ($p<0.05$) in the muscle mass on sedentary animals (MNU+SED) compared to the control sedentary group (Table 1). It was also detected a significant ($p<0.05$) lower gastrocnemius mass on MNU-injected animals following endurance exercise (MNU+EX) compared to the exercised ones from the control group (Table 1). No significant differences on gastrocnemius-to-body weigh were detected between the analyzed groups as response to both exercise and

mammary tumors (Table 1), which suggest that body weight loss was related to muscle mass loss.

Data concerning adipose tissue mass was not collected in this study, although, attending that the loss of adipose tissue is reported to precede the loss of muscle in cachexia [158], one can speculate that animals also presented alterations towards a reduced adipose tissue mass.

4.1.2. Effect of exercise and/or cancer in the browning of WAT

The browning of adipose tissue is a key feature in the adaptations to cancer and to exercise training [98]. Therefore, to access the contribution of mammary tumorigenesis and/or exercise to the WAT browning, the expression of some major makers of this process, such as UCP1 and PGC-1α, were assessed by a Western blot analysis and results are presented in Figure 4.

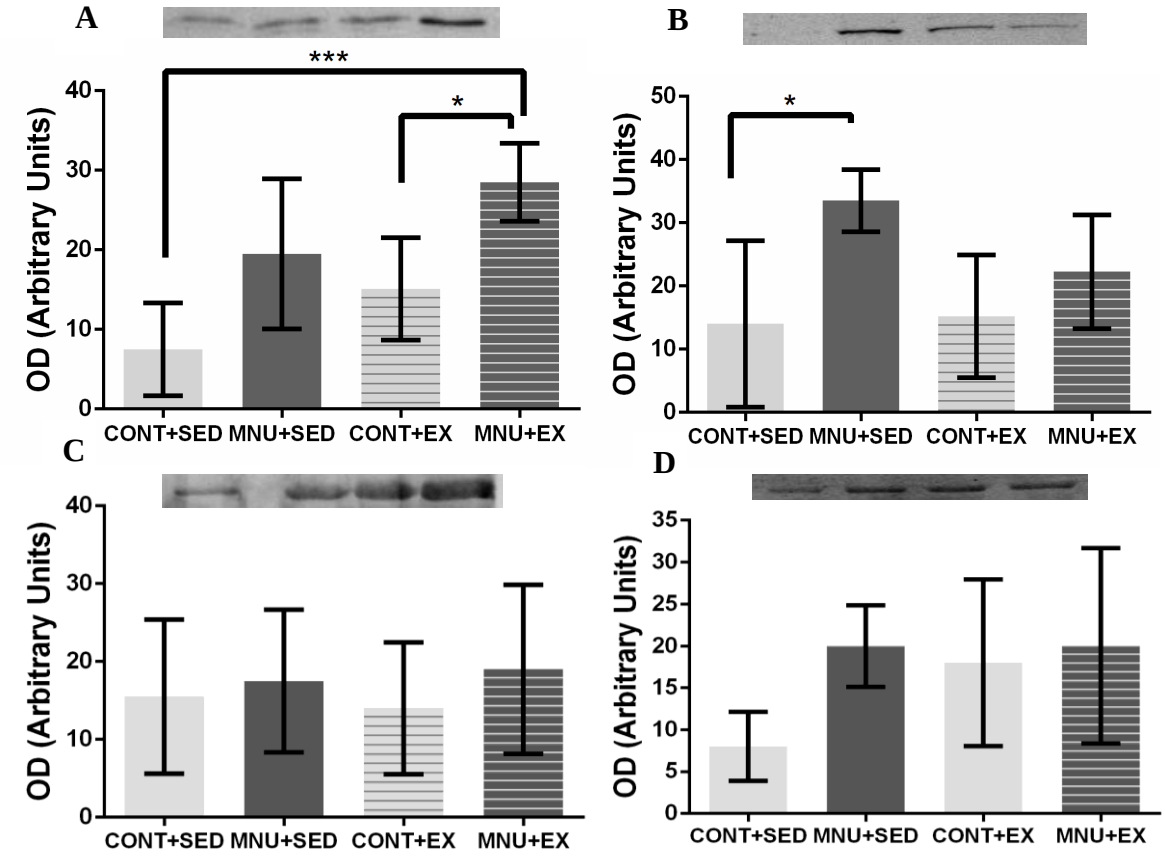

Figure 4- Effect of MNU-induced mammary tumorigenesis and/or exercise training on the expression levels of UCP-1 (A), PGC1-alpha (B); TFAM (C) and Irisin (D) in retroperitoneal tissue. A representative image of the immunoblot obtained is presented above the graphic. The values are presented as mean ± standard deviation and expressed in arbitrary units of OD (Arbitrary Units). *p<0.05; ***p<0.001.

UCP1 is probably the major marker of the browning displayed on adipose tissue, once this protein is responsible for the uncoupling of the respiratory chain that ultimately leads to energy expenditure [137]. The combination of both exercise and mammary tumorigenesis led to significant increases on UCP1 expression levels ($p<0.001$ vs. CONT+SED). The role of exercise as an inducer of UCP1 expression was also highlighted, once exercised animals from the control group reveal significant lower UCP1 levels compared to the exercised ones undergoing mammary tumorigenesis ($p<0.05$). Curiously, no significant differences were found for exercised MNU-injected rats compared to sedentary ones (vs MNU+SED).

A different scenario was found for PGC1-α, a marker of mitochondrial biogenesis [196]. Increased levels on PGC1-α expression levels were only found for sedentary tumor-bearing rats ($p<0.05$ vs. CONT+SED; Figure 4-B). No expression differences were observed among the remaining groups for PGC1-α. Moreover, no significant differences were detected for TFAM expression levels ($p>0.05$; Figure 4-C) neither for irisin levels ($p>0.05$; Figure 4-D), despite a tendency towards increased irisin levels for trained tumor-bearing rats.

4.1.3. Influence of exercise training and/or cancer on the adipose tissue metabolic status

In order to evaluate the effect of mammary tumorigenesis and/or exercise training on adipose tissue metabolic status, western blotting analysis of OXPHOS complexes subunits was performed. The expression levels of GAPDH were also measured using the same approach and the ratio GAPDH:ATP synthase was calculated as a rough marker of tissue's glycolytic profile. The results are presented in the figure 5.

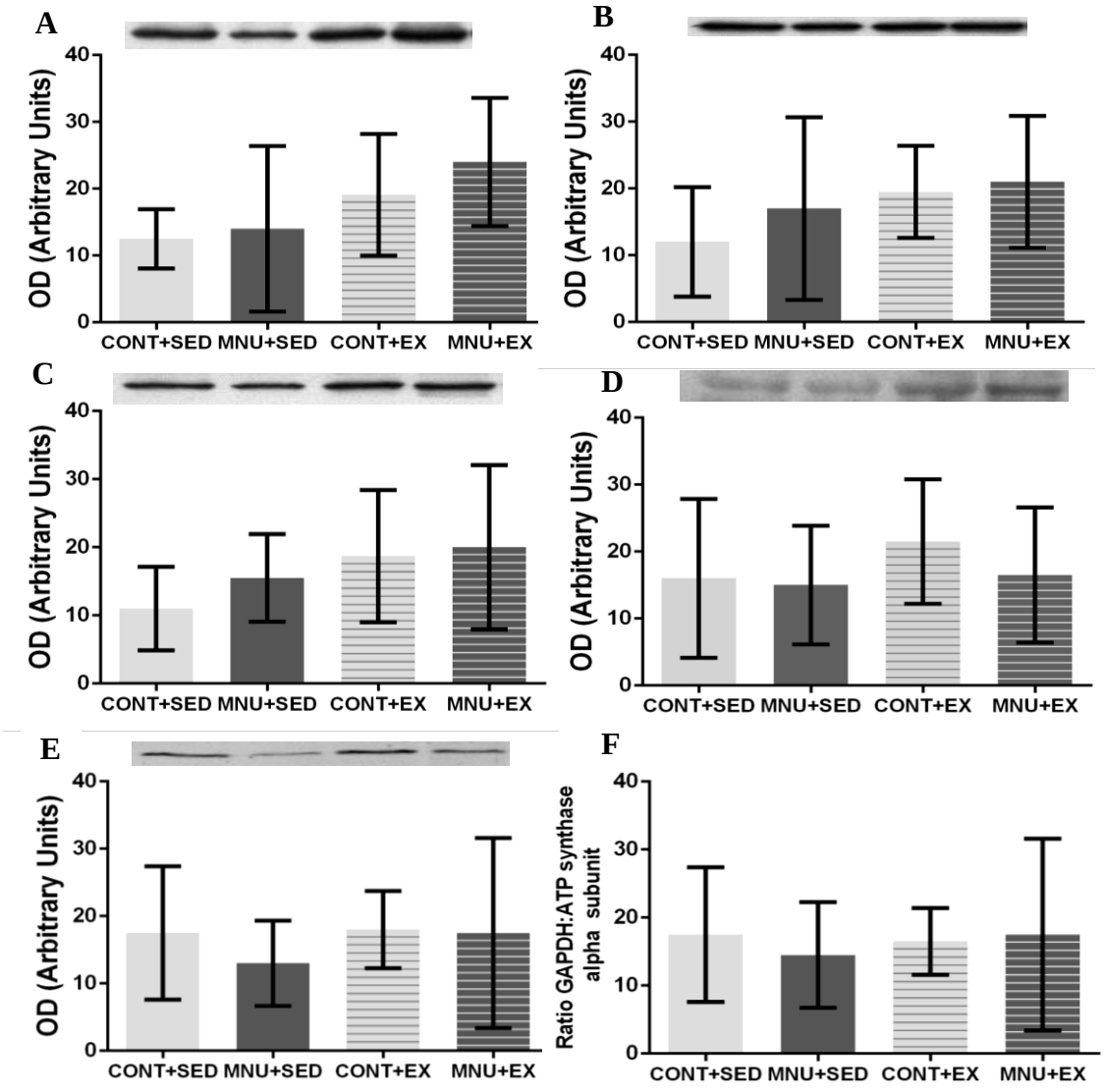
A
B
C
D
E
F
OD (Arbitrary Units)
Ratio GAPDH:ATP synthase alpha subunit
0
10
20
30
40
CONT+SED
MNU+SED
CONT+EX
MNU+EX

Figure 5- Effect of endurance exercise training and/or mammary tumors on the adipose tissue OXPHOS subunits and GAPDH levels. The Expression levels of Complex II iron sulfur subunit B **(A)**; complex III subunit 2 **(B)**; complex IV subunit 1 **(C)**; complex V subunit 5 **(D);** GAPDH **(E)** were measured by Western Blot. a representative image of the immunoblot obtained is presented above the graphic for each protein assayed. The ratio between GAPDH and ATP synthase alpha subunit is also presented **(F)**. Values are presented as mean ± standard deviation and expressed in arbitrary units of OD (Arbitrary Units) for A, B, C, D and E.

No significant expression differences between groups were found for any of the analyzed protein targets ($p>0.05$), evidencing no alteration on the mitochondrial respiratory chain profile (Figure 5- A, B, C and D). Curiously, the expression levels of GAPDH obtained also revealed no significant alterations ($p<0.05$) in response to both exercise and the presence of mammary tumors (Figure 5-E). When the ratio between GAPDH:ATP synthase alpha subunit expression levels was calculated (Figure 5-F), no significant differences were evidenced for both exercised and cancer-induced animals ($p<0.05$).

The expression levels of ETFDH, the enzyme that links β-oxidation with the oxidative phosphorylation, were also evaluated by Western Blot; however, no significant differences were noticed among groups (Figure 6A). In addition, the expression levels of HSL and ATGL, some major markers key regulatory protein of lipolysis, were also accessed using the same approach.

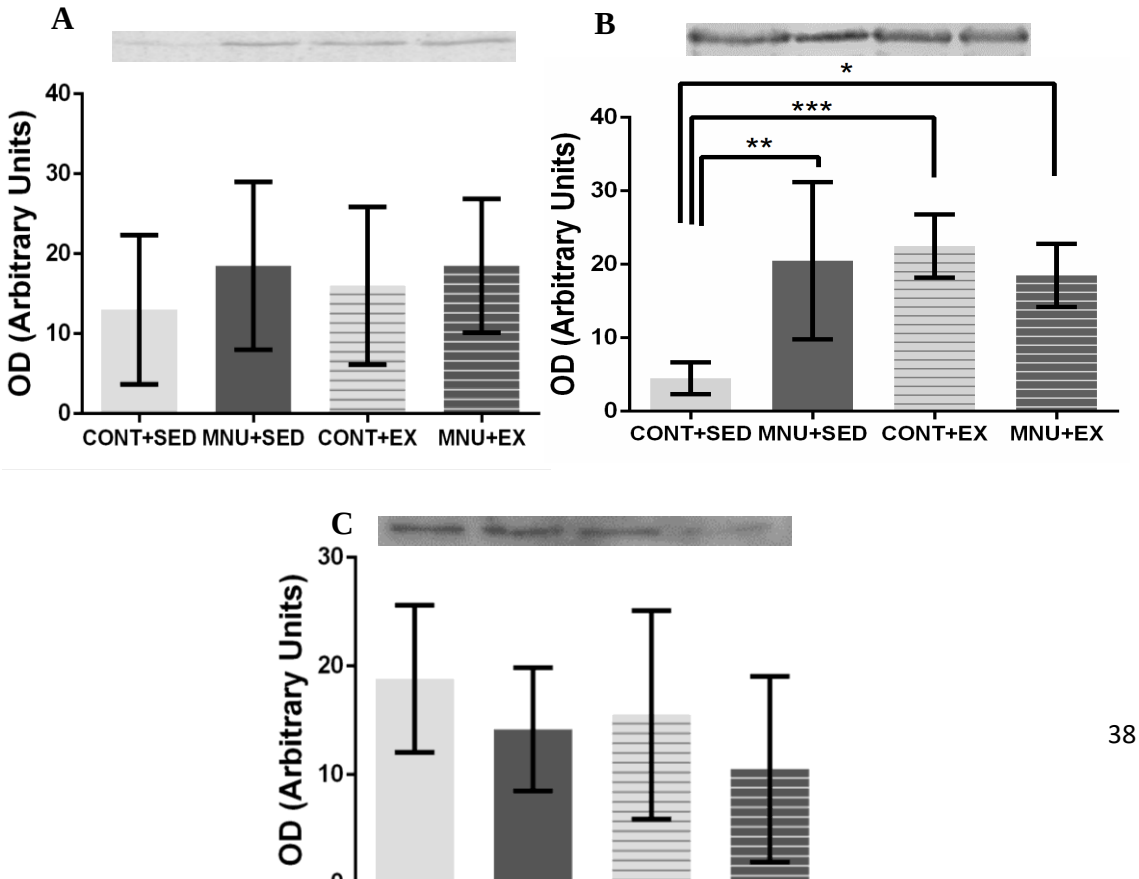

Figure 6- Effect of exercise training and/or mammary tumors on adipose tissue ETFDH **(A)**; HSL **(B)** and ATGL **(C) levels.** A representative image of the immunoblot obtained is presented above the graphic. The values are presented as mean ± standard deviation and expressed in arbitrary units of OI ιrbitrary Units). *p<0.05; **p<0.01; ***p<0.001

Regarding the regulation of the lipolytic path, a clear increase of HSL expression was detected following 35 weeks of exercise training either in health as in tumor-bearing animals (p<0.05 for MNU+EX vs CONT+SED; p<0.001 for CONT+EX vs CONT+SED). The presence of mammary tumors also induced a significant increase in the expression of HSL (p<0.01 for MNU+SED vs. CONT+SED; Figure 6-B). In addition to HSL, the levels of ATGL, also a key regulator of the lipolytic pathway, were also accessed. The results obtained, however, were not significant (p>0.05; Figure 6-C).

4.1.4. Influence of exercise training and/or mammary tumors on adipose tissue vascularization

In order to have some insights of the impact of cancer and/or exercise training in the angiogenesis taking place in the adipose tissue, the expression of VEGF was assessed by Western blot. Results are presented in the figure 7.

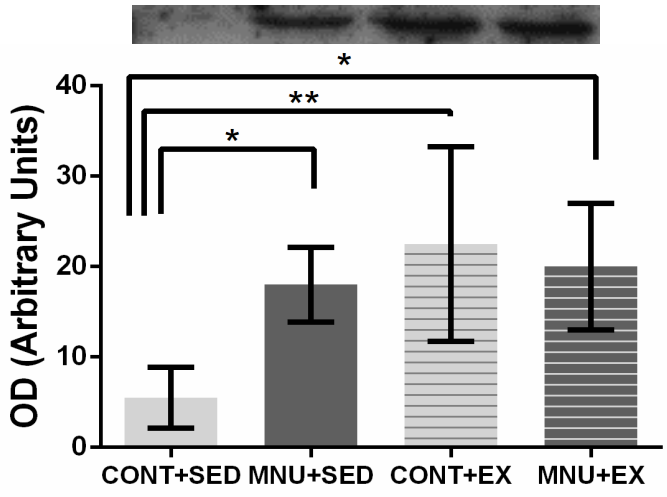

Figure 7- Effect of MNU and/or exercise training on the expression levels of VEGF in retroperitoneal adipose tissue A representative image of the immunoblot obtained is presented above the graphic. The values are presented as mean ± standard deviation and expressed in arbitrary units of OD (Arbitrary Units). *p<0.05; **p<0.01.

All the groups showed significant differences when compared to the control sedentary one (p<0.05- Figure 7). Curiously, it seems that even though exercise training and cancer induced an overexpression of VEGF, their combined action did not modify the expression levels of the protein, since the MNU+EX group presented similar levels to MNU+SED and CONT+EX.

4.2. Study 2 – Influence of exercise and/or prostate cancer on adipose tissue remodeling

4.2.1. Characterization of rat's response to prostate cancer and/or endurance training

Fifty-five weeks after PCa induction pre-neoplastic and neoplastic prostate lesions were observed in all animals. Invasive carcinomas alone or associated to prostatic intraepithelial neoplasia (PIN) and/or dysplasia were observed in both sedentary and exercised animals, even though, the exercised ones developed more multiple neoplastic and pre-neoplastic lesions. Regarding control groups, a small number of animals developed pre-neoplastic or neoplastic lesions; however, some exercised control animals developed invasive carcinomas (data not shown). Animals from PCa groups exhibited testosterone serum levels significantly higher than CONT groups (13% and 19% higher in the case of PCa+SED and PCa+EX, respectively; data not shown), which in agreement with the PCa induction protocol [197].

The induction of PCa led to significant lower body weights on both sedentary (p<0.01) and exercised (p<0.0001) animals compared to the control sedentary ones (CONT+SED). Animals from the control group also exhibit significant decreases on body weight (p<0.0001) in response to endurance exercise (Table 2). The major differences on body weight were presented by exercised tumor-bearing animals. Indeed, these animals revealed significant lower body weights when compared to the control sedentary group (p<0.0001) and to the control exercised group (p<0.0001). Regarding adipose tissue,

animals exhibit significant differences for both retroperitoneal and mesenteric tissue (Table 2). Retroperitoneal tissue mass presented significant lower values only for exercised animals with PCa ($p<0.05$ for PCa+EX vs PCa+SED) and no significant differences were notice for the retroperitoneal-to-body weight ratio. Mesenteric tissue mass presented significant lower values as response to both exercise and PCa induction ($p<0.01$ for PCa+SED vs CONT+SED; $p<0.0001$ for CONT+EX vs CONT+SED). Moreover, the ratio mesenteric-to-body weight also revealed significant differences as response to both exercise and PCa induction (Table 2).

Regarding muscle mass, neither endurance exercise nor PCa led to significant differences concerning gastrocnemius muscle mass. However, a significant decrease on the ratio gastrocnemius-to-body weight was presented by exercised animals with PCa compared to the ones from the control sedentary groups ($p<0.05$). A significant increased ratio gastrocnemius-to-body weight ($p<0.01$) was exhibited for the animals from the control group as response to exercise compared to the control sedentary ones.

Table 2- Characterization of animal's response to PCa induction and /or endurance training regarding body weight, retroperitoneal mass, mesenteric mass, gastrocnemius mass, retroperitoneal-to-body weight ratio, mesenteric-to-body weight ratio and gastrocnemius-to-body weight ratio. The results are presented as mean ± standard deviation.

Experimental Group	CONT+SED	PCa+SED	CONT+EX	PCa+EX
Body weight (g)	541.80±44.94	494.60±35.44 *(a)	434.80±29.70 ****(a)	427.20±39.30 ****(a); ****(b)
Retroperitoneal tissue (g)	0.94±0.38	0.76±0.35	0.55±0.26	0.43±0.27 **(a); *(b)
Mesenteric tissue (g)	11.84±3.27	9.07±1.80 **(a)	5.79±1.71 ****(a)	3.56±1.09 ****(a); ****(b); *(c)
Gastrocnemius (g)	4.41±0.30	4.44±0.28	4.39±0.33	4.11±0.82
Retroperitoneal-to-body weight (mg g^{-1})	1.73±0.69	1.56±0.76	1.31±0.72	1.04±0.69
Mesenteric-to-body weight (mg g^{-1})	21.63±5.04	18.38±3.47	13.25±3.55 ****(a)	8.44±2.45 ****(a); ****(b); **(c)
Gastrocnemius-to-body weight (mg g^{-1})	8.76±0.61	9.01±0.73	10.09±0.42 **(a)	9.62±1.78 *(a)

*p<0.05; **p<0.01; ****p<0.0001 **(a)** vs CONT+SED; **(b)** vs PCa+SED; **(c)** vs CONT+EX

4.2.2. Effect of exercise and/or prostate cancer in adipocytes morphology

In order to address the morphological alterations induced by exercise training and prostate cancer in the retroperitoneal adipose tissue, histological images were captured (40x ampliation) and analyzed. Representative images from each group and the measured cross-sectional area are presented in Figure 8.

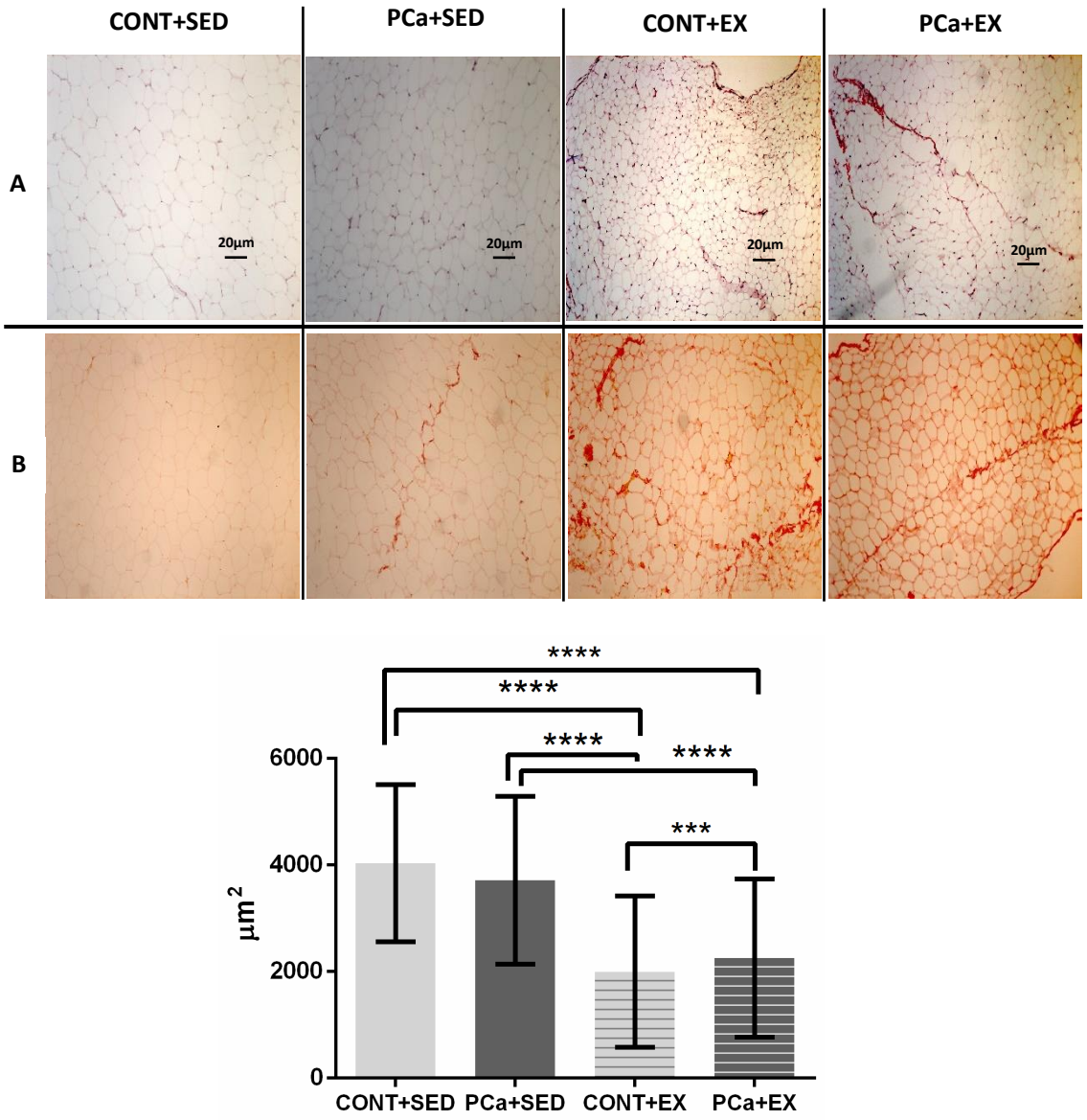

Figure 8- Effect of endurance exercise and /or PCa on adipose tissue morphology. A and **B** are representative images of the retroperitoneal adipose tissue morphology. Images from row **A** and **B** were photographed (40x amplification) in a light microscope after a H&E and Sirius Red staining, respectively. The presented images are divided into columns accordingly to the respective group from which they were obtained. Adipocytes cross-sectional area were measured **(C)** and the values presented as mean ± standard deviation and expressed in μm^2. ***p<0.001; ****p<0.0001.

Regarding adipocytes areas (Figure 8-C), endurance exercise induced significant decreases (p<0.0001- CONT+SED vs CONT+EX), and even overcome the effect promoted by prostate cancer (p<0.0001- CONT+EX vs PCa+EX), that also evidenced to induce decreases in adipocytes area (p<0.0001- CONT+SED vs PCa+SED). In fact, the mean adipocytes area presented by the sedentary PCa animals was significantly higher (p<0.0001) than the one exhibit by the exercised PCa group. In addition, a significant decrease in adipocytes area was found for the control exercised group compared to the

exercised PCa one. In overall, exercise has showed to induce decreases in adipocytes areas and increases in adipocytes number.

A qualitative analysis of the images stained with H&E (Figure 8-A) also confirmed the scenario displayed by the quantitative analysis. Indeed, adipocytes from exercised animals were smaller and numerous compared to the sedentary groups. In addition, adipocytes from the PCa groups seemed to be more heterogeneous in size and number, effect that seemed to be counteracted by endurance exercise (Standard Deviation of adipocytes area 28% lower for PCa+EX than for PCa+SED – Figure 8 C; Standard Deviation of adipocytes number 25% lower for PCa+EX than for PCa+SED – Figure 8 D). Exercised and PCa animals also evidenced a richer vascularization and, curiously, an increased amount of collagen deposition was also evidenced (Figure 8-B).

In order to have a molecular insight of the results from the histological analysis and attending to the significant differences regarding VEGF expression displayed by rats with mammary tumors (Figure 7), the levels of this protein were also analyzed in this study. Results are presented in the figure 9.

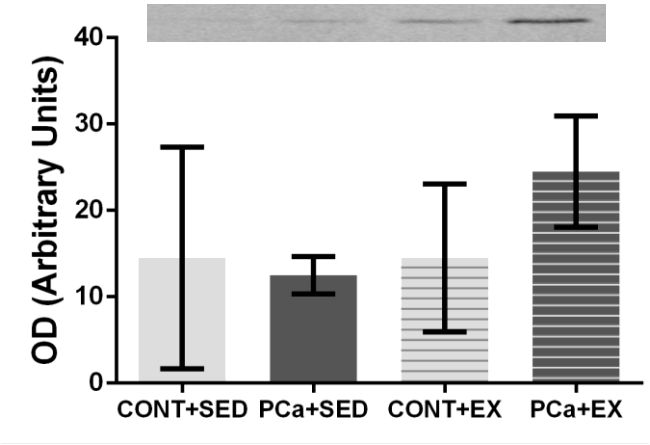

Figure 9- Expression levels of VEGF obtained by Western Blotting analysis of retroperitoneal adipose tissue. A representative image of the immunoblot obtained is presented above the graphic. The values are presented as mean ± standard deviation and expressed in arbitrary units of OD (Arbitrary Units).

The results reveal no significant expression differences for VEGF between groups ($p>0.05$; Figure 9).

4.2.3. Effect of exercise and/or cancer in the metabolic status of cachectic subjects

Attending to the significant differences found in study 1, HSL expression levels were also accessed on adipose tissue from rats with PCa. The results are presented in Figure 10.

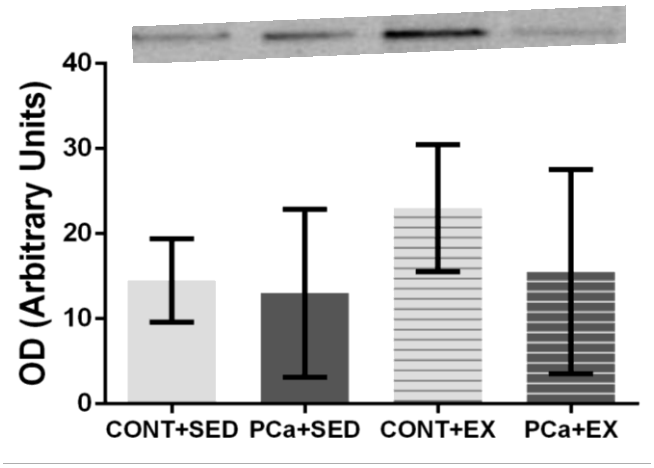

Figure 10- Effect of exercise training and/or PCa on expression levels of HSL obtained by Western Blot analysis of retroperitoneal adipose tissue. A representative image of the immunoblot obtained is presented above the graphic. The values are presented as mean ± standard deviation and expressed in arbitrary units of OD (Arbitrary Units).

The results obtained revealed no significant differences for HSL expression levels ($p>0.05$) following exercise training or as response to PCa (Figure 10).

5. Discussion

Exercise training has been evidencing to be a promising beneficial tool in counteracting low-grade inflammatory conditions, such as cancer cachexia. However, a limited amount of information, that is essentially hypothetical and somehow controversial, is currently known about how exercise training induces adaptations on WAT in this syndrome. In order to further comprehend the role of exercise and/or cancer in the remodeling processes occurring on WAT in cancer associated cachexia, a pre-clinical study of mammary tumorigenesis was performed using the MNU animal model. MNU carcinogen has often been used to induce mammary tumor and is recognized for providing a useful model to study mammary carcinogenesis [198]. Indeed, this model has proven to mimic several aspects displayed in human breast cancer, such as tumor's histopathology, origin and chronic inflammation, evidencing this model to be appropriate to study the influence of exercise training on the cancer-related cachexia set [198]. Mammary tumorigenesis induced by MNU injection led to an 8.4% lower body weight on female Sprague-Dawley rats (Table1), a value that is currently consider on humans as a sign of mild to moderate cachexia [4]. This decrease was related to a reduced gastrocnemius muscle mass [195], a finding that has already been reported on the cachexia set and that has been correlated with reduced physical performance on cancer patients [199]. This loss of muscle was particularly notorious on exercised animals undergoing mammary tumorigenesis, since the gastrocnemius-to-body weight ratio found for this group exhibit the lowest value. Findings from previous studies of our research group using the same animal model, reported less malignant mammary lesion on rats, as well as decreased tumor weights [195]. These effects were associated to the positive anti-inflammatory effects of exercise training, as it was capable to prevent increases in the pro-inflammatory cytokine TWEAK [195]. In addition, in the present work, a moderate intensity treadmill training was selected to induce adaptations on adipose tissue, including the promotion of WAT browning, which has been consistently reported [189,193,200]. In this regard, our results didn't evidence endurance exercise to induce significant alterations on cachectic rats undergoing mammary tumorigenesis (Figure 4). The cancer-induced increased thermogenic capacity of the tissue didn't seem to be promoted by exercise training, once no differences were found for UCP1, PGC-1α and irisin expression levels (Figure 4-A and D). In addition, a lack of effect was also exhibit in the mitochondrial biogenesis, which was evidenced by the unaltered PGC-1α and TFAM levels (Figure 4-B and C). PGC-1α is reported to be required for the exercise training-induced UCP1 expression on adipose tissue [201], which may justify, at some extension, the lack of response showed

by UCP1 following endurance exercise (Figure 4-A), once no alterations on PGC-1α were detected (Figure 4-B). A similar reason may be in the basis of the lack of response demonstrated by irisin levels to endurance exercise (Figure 4-D), once increased irisin levels are thought to be dependent of an up-regulation of PGC-1α [189]. Even though no effect was detected as response to exercise, increased expression levels of PGC-1α were found in the MNU sedentary group. This trend was not followed by the mitochondrial complexes, which expression levels weren't affected (Figure 5-A to D). In fact, no alterations on adipose tissue metabolism were evidenced. In addition to the mitochondrial complexes, no differences were found in the expression levels of GAPDH and the GAPDH:ATP synthase ratio (Figure 5- E and F, respectively). When ETFDH expressions levels were analyzed, no evidences pointing for an altered β-oxidation were revealed (Figure 6-A). Taken together, these results may suggest that glycolysis and β-oxidation do not contribute to WAT remodeling promoted by cancer and/or exercise. Even though, the analysis of the mitochondrial complex's activity are still necessary, once may not exist a correlation between the expression and the activity levels. Our results, given HSL levels, support the contribution of lipolysis in the CC-related remodeling, as previously reported [140]. Indeed, even though ATGL expression has been consistently reported as being modulated by cancer and exercise [154,159,202], no alterations induced by exercise or MNU were detected in this protein expression levels (Figure 6-C). HSL, by other hand, showed higher expression levels in response to both exercise and cancer in animals with mammary tumors, although, endurance exercise did not seem to affect significantly HSL levels (Figure 6-B). For the sedentary MNU rats, the obtained results may be explained by the inflammatory status induced by cancer. Indeed, TNF-α, a proinflammatory molecule often found overexpressed in the CC set, has been reported to induce lipolysis and even downregulate the expression of both ATGL and HSL [4]. The mechanism behind this process, however, is still unknown. In addition, TNF-α may also induce the phosphorylation of perilipin-1 in the sequence of an increase in cAMP levels that activates PKA, what may induce HSL phosphorylation and its translocation to the lipid droplet, further increasing lipolysis [203]. Indeed, increased levels were found for HSL in animals undergoing mammary tumorigenesis (Figure 6-B). Once both exercise and cancer have been consistently reported to promote an extensive vascular network in a differenced set of tissue, including adipose tissue [204,205], VEGF expression levels were measured, since this protein is reported to be a potent angiogenic factor. In the mammary tumorigenesis increased VEGF expression levels were found in exercised and cancer-

induced animals (Figure 7). No effect was, however, evidenced for endurance exercise on animals induced with mammary tumors. A possible explanation may arise from other factors, rather than VEGF, to promote adipose tissue vascularization. Indeed, along with VEGF, PGDF, a growth factor that plays an important role in blood vessel formation, have also been reported to increase in subcutaneous WAT from exercised animals [188].

Regarding the pre-clinical study conducted on rats with PCa, body weight results showed lower values following endurance exercise and PCa development. The presence of prostate cancer influenced rats body weight by inducing a decrease of 8.7%, a value that in humans indicates signs of mild to moderate cachexia [4]. Adipose tissue depots exhibit significant lower mass values as consequence of both endurance exercise and PCa. These results not only evidence the capability of the selected training program to induce adaptations on adipose tissue, as it also supports the current idea that adipose tissue loss underlies CC [57]. Moreover, the mass of gastrocnemius muscle didn't reveal significant differences between the analyzed groups, which supports the idea that the loss of adipose tissue precedes the loss of muscle in cancer cachexia [158]. To assess possible morphological alterations on adipose tissue morphology, a histological analysis was performed on the retroperitoneal tissue pad. This analysis revealed reduced adipocytes areas for the exercised and PCa animals. This effect has already been reported in the CC set [85], although, our study gives evidences of an intensified effect as consequence of endurance exercise. Indeed, several data have been suggested that smaller adipocytes are a frequent outcome of cachexia development and thought to result from an increased lipolysis and a decreased lipid accretion [83,85]. In this context, and to have some insights about how exercise influences the lipolysis occurring in prostate cancer-associated cachexia, HSL levels were measured in this study. Even though a decreased adipocytes area was possible to detect after endurance exercise, and thought to be correlated with higher lipolysis rates, HSL expression levels do not corroborated this idea, since no significant alteration were induced neither by exercise or prostate cancer. This effect, similarly to the one describer in the previous study, may be related with altered lipolysis regulation. Furthermore, the histological analysis also suggested increased amounts of connective tissue surrounding the adipocytes of exercised animals, including the ones with PCa (Figure 8-B), as well as evidences of a richer vascularization. In this context and aiming to evaluate if a possible correlation between the obtained images and the

molecular expression could be stablished, the levels of VEGF were assessed. Even though images suggest an increased vascularization for exercised and PCa induced rats, VEGF levels were not found. These findings may support other players, such as PGDF, as promoters of adipose tissue vascularization [188], what should be further explored.

It should be noticed that the pre-clinical study using rats induced with prostate cancer was performed intending to gain additional molecular e morphological insights of endurance exercise effect on PCa-related cachexia. Due to issues of time, some major marker of browning, such as UCP1 and PGC-1α, were not assessed, although preliminary results are pointing for increased expression levels of UCP-1 in a similar patter to the one encountered in the mammary tumorigenesis model.

6. Conclusion and further perspectives

In order to evaluate the effect of endurance exercise in the WAT remodeling e on cancer, two pre-clinical studies were performed on rats with prostate and mammary tumorigenesis. The obtained results led to following conclusions:

i) Endurance exercise did not promote adipose tissue thermogenic capacity in cachectic rats undergoing mammary tumorigenesis, once no difference was detected for the expression levels of UCP1 and PGC-1α; and did not influenced retroperitoneal adipose tissue metabolic status.

ii) Endurance exercise induced morphological alterations on adipose tissue by reducing adipocytes areas. This feature was correlated with a stimulated lipolysis in the breast cancer study, given the induced HSL expression levels.

Taken together, data suggests that endurance exercise does not worsen or ameliorates cancer-induced energy expenditure. These results may highlight exercise as a promising approach in counteracting cancer cachexia attending to its known anti-inflammatory properties and its capability of improving muscle strength. However, further studies aiming to uncover the signaling pathways mediating CC effects must be performed.

7. References

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