MECHANISMS OF MUSCLE WASTING IN CACHEXIA MODELS: THERAPEUTIC IMPLICATIONS

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Nothing in life is to be feared, it is only to be understood.
Now is the time to understand more, so that we may fear less.

Marie Curie

A la meva família (especialment als meus pares i a la Núria)
Al David, a la Cora i a la Sónia
Als amics d'aquí, del poble i de la uni
Als companys de laboratori
A l'Esther i al Francesc

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Abstract

Cachexia negatively affects patients with chronic diseases and especially in cancer. Therapeutic strategies are still limited. The $beta_2$ agonists (formoterol) and the nutritional support (L-carnitine) can attenuate the deleterious effects in the muscle. In this thesis, treatment with formoterol and L-carnitine induced beneficial effects (total body and muscle weights, structure, apoptosis, proteolysis and signaling pathways) in the diaphragm and limb muscles in an experimental model of cancer cachexia (AH-130 Yoshida hepatoma ascites cells, in rats). In mice with cancer cachexia (LP07 lung adenocarcinoma cells), treatment of the tumor with monoclonal antibodies (anti-PD-1, anti-CTLA-4, anti-CD-137, and anti-CD-19) induced beneficial effects of the same kind as a consequence of the decrease in size and tumor burden. This thesis has shown that various signaling pathways and mechanisms involved in protein and muscle degradation are attenuated, improving the phenotypic and functional characteristics of the diaphragm and peripheral muscles in response to various therapeutic strategies.

Keywords: cancer cachexia, respiratory and peripheral muscles, $beta_2$ agonists, L-carnitine, immunomodulators and cancer.

Resumen

La caquexia afecta negativamente a los pacientes con enfermedades crónicas y sobre todo en el cáncer. Las estrategias terapéuticas son aún limitadas. Los beta$_2$ agonistas (formoterol) y el soporte nutricional (L-carnitina) pueden atenuar los efectos deletéreos en el músculo. En la presente tesis, el tratamiento con formoterol y L-carnitina indujo efectos beneficiosos (peso corporal y muscular, estructura, apoptosis, proteólisis y vías de señalización) en el diafragma y músculos de las extremidades en un modelo experimental de caquexia cancerosa (hepatoma ascitico Yoshida AH-130, en ratas). En ratones con caquexia cancerosa (células de adenocarcinoma del pulmón LP07), el tratamiento del tumor con anticuerpos monoclonales (anti-PD-1, anti-CTLA-4, anti-CD-137, y anti-CD-19) indujo efectos beneficiosos de la misma índole como consecuencia de la disminución del tamaño y la carga tumoral. En esta tesis se ha demostrado que diversas vías de señalización y mecanismos implicados en la degradación proteica y muscular se ven atenuadas, mejorando las características fenotípicas y funcionales de los músculos diafragma y periféricos en respuesta a diversas estrategias terapéuticas.

Palabras clave: caquexia cancerosa, músculos respiratorios y periféricos, beta$_2$ agonistas, L-carnitina, inmunomoduladores y cáncer.

Preface

Scientific collaborations

The research described in the current thesis has been conducted in the Muscle Wasting and Cachexia in Chronic Respiratory Diseases and Lung Cancer Research Group, Institute of Medical Research of Hospital del Mar (IMIM)-Hospital del Mar, Barcelona, Spain. In addition, four of the five studies have been performed in collaboration with other research groups as described below.

Study #1 was conducted in collaboration with Biomarker and New Therapeutic Targets in Lung Cancer Research Group and Preclinical Models and Analysis Tools Research Group, Solid tumors program, Center for Applied Medical Research (CIMA), University of Navarra, Pamplona, Spain.
Studies #2, **#3** and **#4** were conducted in collaboration with Cancer Research Group, Biochemistry and Molecular Biomedicine department, University of Barcelona, Barcelona, Spain.

Publications

All the studies that are included in the current thesis have been published in international journals:

Study #1
Salazar-Degracia A, Blanco D, Vilà-Ubach M, de Biurrun G, de Solórzano CO, Montuenga LM, Barreiro E. Phenotypic and metabolic features of mouse diaphragm and gastrocnemius muscles in chronic lung carcinogenesis: influence of underlying emphysema.
J Transl Med. 2016 Aug 23;14(1):244

IF: 3.786, Q1 in medicine, research & experiments

Study #2

Salazar-Degracia A, Busquets S, Argilés JM, López-Soriano FJ, Barreiro E. Formoterol attenuates increased oxidative stress and myosin protein loss in respiratory and limb muscles of cancer cachectic rats.

PeerJ. 2017 Dec 13;5:e4109.

IF: 2.118, Q2 in multidisciplinary sciences

Study #3

Salazar-Degracia A, Busquets S, Argilés JM, Bargalló-Gispert N, López-Soriano FJ, Barreiro E. Effects of the $beta_2$ agonist formoterol on atrophy signaling, autophagy, and muscle phenotype in respiratory and limb muscles of rats with cancer-induced cachexia.

Biochimie. 2018 Jun;149:79-91

IF: 3.188, Q2 in biochemistry and molecular biology

Study #4

Salazar-Degracia A, Busquets S, Argilés JM, Serpe R, Pérez-Peiró M, Rojano-Toimil A, López-Soriano FJ, Barreiro E. Differential structural features in soleus and gastrocnemius of carnitine-treated cancer cachectic rats.

J Cell Physiol., (under review)

IF: 3.923, Q1 in physiology

Study #5

Salazar-Degracia A, Granado-Martínez P, Millán-Sánchez A, Tang J, Pons-Carreto A, Barreiro E. Reduced lung cancer burden by selective immunomodulators elicits improvements in muscle proteolysis and strength in cachectic mice.

J Cell Physiol., 2019 *(in press)*

IF: 3.923, Q1 in physiology

Communications

The data obtained as a result of the thesis have been previously shown in the form of an abstract (either poster or oral communication) in national and international conferences.

1. Pérez-Peiró M, **Salazar-Degracia A**, Busquets S, Serpe R, Argilés JM, López-Soriano JM, Barreiro E. Differential structural features in soleus and gastrocnemius of carnitine-treated cancer cachectic rats. III Research based on pulmonology conference, Terrassa, Spain, April 2019.

2. **Salazar-Degracia A**, Busquets S, Serpe R, Argilés JM, López-Soriano JM, Barreiro E. Differential structural features in soleus and gastrocnemius of carnitine-treated cancer cachectic rats. 11th International SCWD Conference on Cachexia, Sarcopenia and Muscle wasting**,** Maastricht, The Netherlands, December 2018.

3. **Salazar-Degracia A**, Granado-Martínez P, Millán-Sánchez A, Barreiro E. Effects on cachexia mechanisms of reduced tumor burden by selective immunomodulators of lung cancer in mice. 4th cancer cachexia conference, Philadelphia, USA, September 2018.

4. **Salazar-Degracia A**, Granado-Martínez P, Millán-Sánchez A, Barreiro E. Effects of reduced tumor burden by selective treatment of lung cancer on muscle mass loss in cachectic mice. The 43rd FEBS Congress, Prague, Czechoslovakia, July 2018.

5. **Salazar-Degracia A**, Busquets S, Puig-Vilanova E, Argilés JM, López-Soriano JM, Barreiro E. Proteolysis, autophagy and signaling of respiratory and limb muscles in rats with cancer-induced cachexia: Effects of the $beta_2$

agonist formoterol. I Research based on pulmonology conference, Barcelona, Spain, March 2017.

6. **Salazar-Degracia A**, Blanco D, Vilà Ubach M, de Biurrun G, de Solórzano CO, Montuenga LM, Barreiro E. Phenotypic and metabolic features of mouse diaphragm and gastrocnemius muscles in chronic lung carcinogenesis: influences of underlying emphysema. IX CIBERES and CIBER-BBN conference sessions, Madrid, Spain, September 2016.

7. **Salazar-Degracia A**, Blanco D, Vilà Ubach M, de Biurrun G, de Solórzano CO, Montuenga LM, Barreiro E. Phenotypic and metabolic features of mouse diaphragm and gastrocnemius muscles in chronic lung carcinogenesis: influences of underlying emphysema. 15th Symposium COPD, Barcelona, Spain, April 2016.

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Abbreviations

ActRII, activing type II receptors
ADP, adenosine diphosphate
AIDS, acquire immune deficiency syndrome
Akt, protein kinase B
ALK, activin type I receptors
AMPK, activated kinase
Ang, angiotensin
ANOVA, one-way analysis of variance
APC, adenomatous polyposis coli
Atg, autophagy-related gene
ATP, adenosine triphosphate
BAX, BCL-2 associated X protein
BCAA, branched-chain amino acid
BCL-2, B cell leukemia/lymphoma 2
BMI, body mass index
cAMP, cyclic adenosine monophosphate
CD, cluster differentiation
CK, creatine phosphate
CMA, chaperone-mediated autophagy
COPD, chronic obstructive pulmonary disease
CREB, cAMP response element binding
CRH, corticotrophin-releasing hormone
CTLA-4, T-lymphocyte protein-4
DNA, deoxyribonucleic acid
eiF3-f, eukaryotic translation initiation factor 3 subunit
EPA, eicosapentaenoic acid
ERK, extracellular signal-related kinase
FDA, food and drug administration
FoxO, forkhead box O class
GDF-8, growth and differentiation 8
GPx, gluthatione peroxidase
H_2O_2, hydrogen peroxide
HMB, β-hydroxy-β-methylbutyrate
HNE, 4-hydroxy-2-nonenal
IFN, interferon
IGF-1, insulin-like growth factor 1
IKK, IκB kinase
IL, interleukin
JAK, Janus kinase
JNK, jun amino-terminal kinase

LC, lung cancer
LC3B, microtubule-associated protein 1 light chain 3B
MA, megestrol acetate
mAbs, monoclonal antibodies
MAPK, mitogen-actived protein kinase
MDA, malondialdehyde
MRC, mitochondrial respiratory chain
MRF, myogenic regulatory factors
mTOR, mammalian target of rampamycin
MuRF-1, muscle RING-finger-1
MyOD, myogenic differentiation 1
NDEA, nitrosodiethylamine
NF-κB, nuclear factor-kappa B
NO, nitric oxide
NO_2, nitrogen dioxide
NT, nitrotyrosine
O, oxygen atom
O_2, singlet oxygen
O_2^-, superoxide anion
OH, hydroxyl radical
$ONOO^-$, peroxynitrite
P62, nucleoporin p62
PARP, polyadenosine diphosphate-ribose polymerase
PD-1, cell death protein-1
PI3K, phosphatidylinositol-3-kinase
PKA, protein kinase A
PUFA, polyunsaturated fatty acids
RNS, reactive nitrogen species
ROS, reactive oxygen species
Smad, similar to mothers against decopentaplegic
SOD, superoxide dismutase
STAT, signal tranducer and activators of transcription
TFG-β, transforming growth factor-β
TNF, tumor necrosis factor
TRIM32, tripartite motif containing 32
UPS, ubiquitin-proteasome system

Introduction

Introduction

1. SKELETAL MUSCLE

Muscle tissue is composed of three types: cardiac, smooth and skeletal muscle. Skeletal muscle is the most abundant type, it works mainly in a voluntary manner and it contributes to multiple body funcions [1]. Skeletal muscle, which comprises approximately 40% of total body weight, includes 50-75% of all body proteins and represents 30-50% of whole-body protein turnover. Muscle is principally composed of 75% water, 20% proteins and 5% of other elements such as carbohydrates, fats, minerals, and inorganic salts [2]**.** Muscle mass is based on the balance between protein degradation and synthesis in order to transform chemical energy into mechanical energy and carry out the various functions of the skeletal muscle [2,3]. The main function of skeletal muscle is to produce movement and generate power and force together with maintaining posture. In addition, it is a reserve of carbohydrates and amino acids for the muscles, contributing to the preservation of the levels of blood glucose during starvation periods [2,3]. The maintenance of skeletal muscle health status is crucial for the prognosis of various chronic diseases including cancer.

1.1. Muscle development, structure, and organization

Skeletal muscle has a particular and well-described organization of muscle fibers in association with connective tissue. Muscles fibers also are known as muscle cells or myofibers [1]. Muscle fibers are generated by the process described named myogenesis, which is divided into two distinct time-phases: embryogenic and adult myogenesis. Briefly, embryogenic myogenesis consists in the generation of the first muscle fibers of the body from mesoderm-derived structures [3,4]. In contrast, adult myogenesis depends on the activation of satellite cells to maintain the tissue

homeostasis in adult skeletal muscles. However, it has been described that the two time-phases of myogenesis share similarities in the transcription factors and signaling molecules that regulate both processes [4]**.** Besides, this process can also be divided into two stages, muscle development and muscle differentiation [3,4]. Firstly, muscle progenitor cells, widely named myoblasts, exclusively proliferate, then the absence of growth factors of these myoblasts, they start to differentiate and fuse into multi-nucleated fibers called myotubes. These two processes of myogenesis are strictly regulated by a family of transcription factors known as myogenic regulatory factors (MRFs), which are responsible for the differentiation of myoblast into functional myotubes [4,5].

The architecture of skeletal muscle comprises various integrated tissues such as skeletal muscle fibers, nerve fibers, blood vessels and connective tissue. Each skeletal muscle has three layers of connective tissue that provide structure to it and compartmentalize the muscle fibers within the muscle. The epimysium is the outermost layer and is responsible to connect the muscle to tendon and insertion to the bone segment, allowing the independent movement of the muscle [1,3]**.** Within each skeletal muscle, 10 or more muscle fibers are organized into individual bundles which form a fascicle. This is surrounded by a layer of connective tissue known as perimysium. Muscle fibers are also surrounded by a reticular connective tissue which constitutes the endomysium [1]**.** Muscle fibers are composed of a plasma membrane called sarcolemma (**Fig. 1**). Moreover, within the cytoplasm of fibers or sarcoplasm, there are myofibrils, mitochondria, myoglobin, potassium, magnesium, and multiple enzymes to supply energy during muscle contraction [1,3,6]**.**

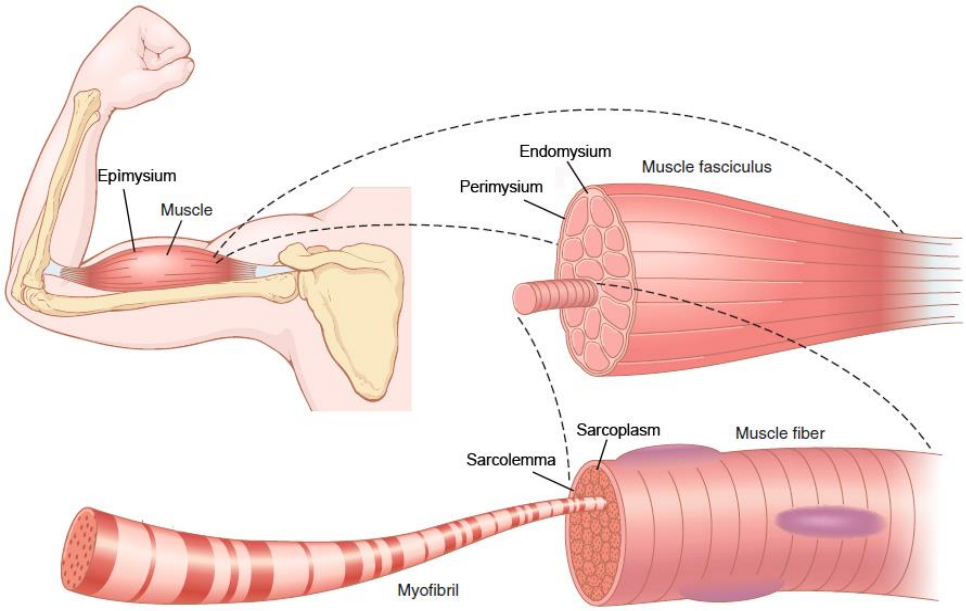

Figure 1. Skeletal muscle organization and its connective tissue coverings. A skeletal muscle comprises individual muscle fibers (cells) bundled into fascicles and surrounded by three connective tissue layers. Muscle fiber contains the contractile elements of myofibrils in their sarcoplasm *(Adapted from Hall JE 2015* [6]*).*

Myofibrils consist of two types of myofilaments, thick and thin, arranged in a well-defined order for the typical cross-banding being directly involved in the process of contraction. The filaments within a myofibril do not extend through the entire length of a muscle fiber (**Fig. 1**) [1,6].

The sarcomere is the functional unit of the muscle fiber and it is situated between two dense regions of proteins called Z-disks. In the middle of the sarcomere, there is the M-line, where the thick filaments are attached. The thin and thick filaments elongate from the Z-disk and M-line respectively, overlapping on each side of the M-line and performing distinctive regions inside the sarcomere. The A-band region extends the entire length of the thick filaments and the zone that overlaps between thick and thin filaments.

In the center of the A-band, there is the H-zone which includes exclusively regions of thick filaments. The I-band contains the rest of the thin filaments that do not overlap with thick filaments, and a Z-disk gets through the center of each I-band (**Fig. 2A**)[1,7].

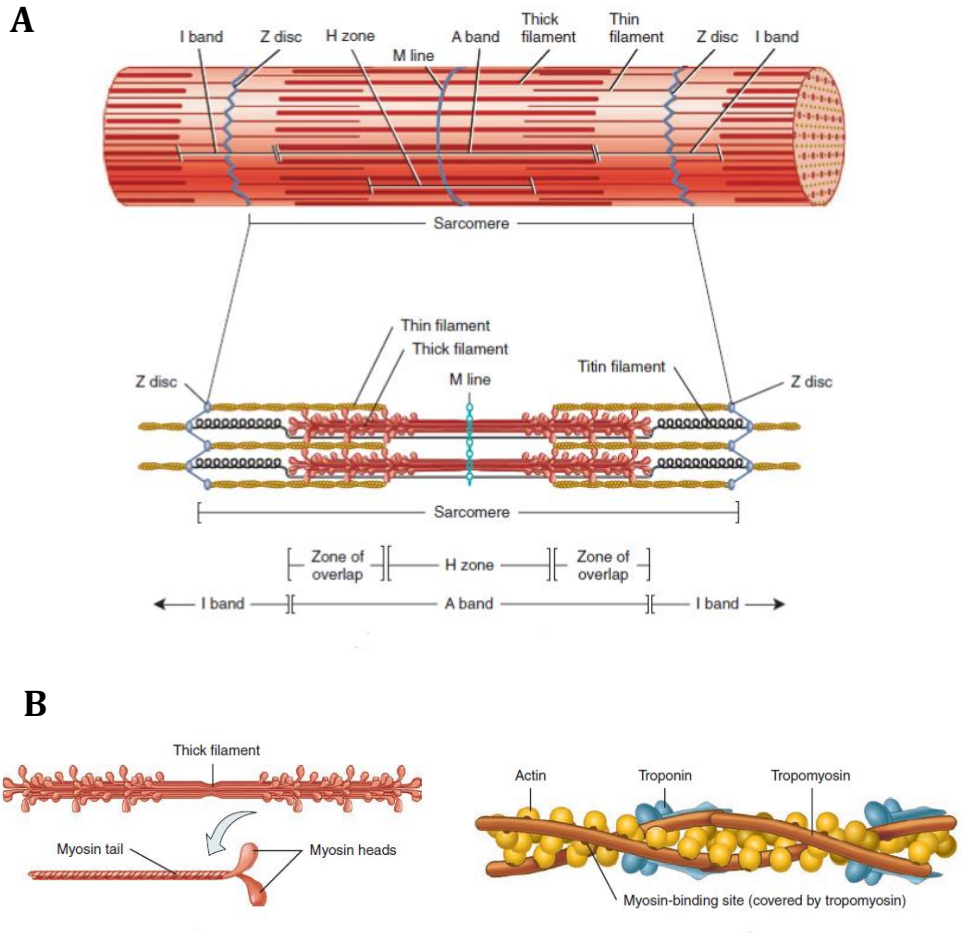

Figure 2. The arrangement of filaments within a sarcomere. A) Myofibrils contain thick and thin, which are two types of myofilaments that arrange to form the sarcomere. **B)** Structure of thick and thin filaments. Thick filaments contain myosin tails that form their axis, whiles, and the myosin heads project the union with thin filaments. Thin filaments comprise actin and regulatory proteins as troponin and tropomyosin *(Adapted from Tortora GJ et al. 2012* [1]*).*

As mentioned above, myofibrils are built of thick and thin filaments that are composed of contractile, regulatory and structural proteins. The components of thick filaments are myosin, myosin-binding protein C, titin and obscurin. Moreover, the major components of thin filaments are actin, troponin, tropomyosin, and nebulin [7,8]. The two contractile proteins in muscle are actin and myosin, acting the last one as a motor protein transforming the chemical energy of adenosine triphosphate (ATP) into mechanical energy of motion and force. In small amounts, troponin and tropomyosin are two regulatory proteins which function is to regulate the union between myosin-binding protein C and actin for muscle contraction (**Fig. 2B**). Finally, titin, obscurin, and nebulin are the major structural proteins which provide the stability, alignment, extensibility, and elasticity of myofibrils [1,7,8].

1.2. Muscle types

1.2.1. Respiratory muscles

The respiratory muscles are striated skeletal muscles that contribute to the physiological process of breathing by supporting the expansion and contraction of the thoracic cavity. Due to their function in the breathing process, the movements of these muscles are involuntary, and they are essential for life. Respiratory muscles are classified in inspiratory and expiratory depending on their mechanical functions. The inspiratory muscles lead to the air into the lungs, while expiratory muscles remove this air from the lungs. The primary inspiratory muscles are the diaphragm, the external intercostal and the parasternal. In contrast, the expiratory muscles group are composed of the internal intercostal, the external and internal oblique, rectus abdominis and transverse abdominis muscles [1,9,10].

1.2.1.1. Diaphragm

The diaphragm muscle is composed of two areas, the costal and crural diaphragm. The costal diaphragm is a thin layer of myofibers that extends from the ribs to the central tendon. Unlike the crural domain that is a thicker layer and is located further back, where myofibers attach to the vertebrae and surround the esophagus and aorta to central tendon. The central tendon is found at the apex of the diaphragm, keeping the different domains of diaphragm muscle together [11]. The diaphragm is composed of slow- and fast-twitch fibers *(see below, section 1.3)*. Although the diaphragm principal function is breathing, it has functional roles in emesis and swallowing [11,12]. Besides, diaphragm also has a passive function, serving as a barrier between the thoracic and abdominal cavities [11]. Due to its importance and its needed interrupted function, diaphragm muscle is more resistant to fatigue and recovers up to 10 times faster than other muscles [12]. Despite this resistance, some diseases interfere with correct diaphragmatic functions resulting in diaphragmatic dysfunctions [13–15].

1.2.2. Limb muscles

The limb muscles contribute to the physiological process of movements and support, so their movements are totally voluntary. They can be divided depending on the region where they are, being upper and lower limbs in humans, but for animals, the terminology is forelimbs and hindlimbs. In human studies, the fast twitch-muscles widely-used are quadriceps and tibialis anterior, whereas in animal models are the gastrocnemius, tibialis anterior, and extensor digitorum longus muscles. In contrast, soleus is the most widely-used slow-twitch muscle *(see below, section 1.3)*. In our group, gastrocnemius (fast-twitch) and soleus (slow-twitch) are the two widely-studied muscles in rodent animals and they are localized in the hindlimbs of these animals.

1.2.2.1. Gastrocnemius

The gastrocnemius is the most superficially located muscle from the hindlimb in the leg and is composed by two heads, one medial and one lateral. Both heads form a single elongated muscle, identified as the calf. The gastrocnemius is mostly composed of fast-twitch fibers for the functions that it performs [16,17]. Its principal functions are plantar flexing the foot at the ankle joint and flexing the leg at the knee joint. The fiber type composition and their functions allow running, jumping and any other "fast movements" of the leg, but also walking and posture in minor grade [18].

1.2.2.2. Soleus

The soleus is a small and broad flat muscle that is localized under the gastrocnemius. It is attached to the proximal end of the tibia and fibula, and to a tendinous ligament. As gastrocnemius, soleus participates in the function of plantarflexion of the foot. Soleus muscles are vital for walking, running and keeping body balance, playing an important role in posture maintenance. For this reason, this muscle is mainly composed of slow-twitch fiber [18].

1.3. Fiber type composition

Muscle fibers are characterized by the heterogeneity and the plasticity, which allow the same muscle to contribute a wide variety of functional capabilities [17,19,20]. Heterogeneity of muscle fibers is demonstrated by the notable variability in their metabolic phenotypes, the mechanical and biochemical characteristics [2,21]. In contrast, the plasticity of muscle fibers refers to its ability to modify the structural and functional properties or the proportion of distinct fiber types to achieve an adaptive response to a prolonged change in muscle functional requests. Besides fiber-type transition, changes in fiber size represent the basis of plastic adaptation in

muscles [19,22]. This plasticity takes place in response to age [23], chronic disease [24–26], exercise or training [21], and environmental factors.

Muscle fibers have been classified using different criteria, being the most common: 1) morphological color (red vs. white), 2) contractile properties, 3) fatigability, which correlates with the mitochondrial content (fatigue-resistant vs. fatigable), 4) predominance of certain metabolic or enzymatic pathways (glycolytic vs. oxidative), 5) enzyme-histochemical stain reaction (ATPase or succinate dehydrogenase), 6) calcium handling by the sarcoplasmic reticulum (slow vs. fast), and 7) expression of protein isoform, among others [27,28]. From them, the most widely used classification is based on protein isoform expression, being considered as molecular markers of fiber types [27]. Mammal species muscles express four major isoforms of myosin called type I, type IIa, type IIx and type IIB, however in specific conditions and muscles, other types of myosin are expressed such as embryonic, neonatal and extraocular [2,17,29]. Focusing on the principal isoforms, not all mammals present the same profile. Rodent muscles present the four isoforms, however, in human muscles the type IIb is not detected [17,21]. In addition to the differences related with the expression of myosin isoforms, it has also been described that there are differences in the proportions of isoforms depending on which mammal species are and the gender [17]. Each of the four isoforms shows different morphological, biochemical, physiological and metabolic characteristics that are illustrated in **table 1** [6,17,21,27,29].

Table 1. Main metabolic and structure characteristics of skeletal muscle fiber types [6,17,21,27,29].

	Type I	**Type IIa**	**Type IIx**	**Type IIb**
Expression	All mammals	All mammals	All mammals	Only rodents
METABOLIC PROPERTIES				
Speed contraction	Slow	Fast	Very fast	Fastest
Myofibrillar ATPase activity	Low	High	Very high	Very high
Metabolism	Oxidative aerobic	Oxidative-glycolytic aerobic-anaerobic	Glycolytic anaerobic	Glycolytic anaerobic
Endurance	Fatigue-resistant	Moderately fatigue-resistant	Moderately fatigable	Fatigable
STRUCTURAL CHARACTERISTICS				
Fiber diameter	Small	Medium-size	Large	Large
Muscle color	Red	Red	White	White
Myoglobin content	High	Medium	Low	Low
Mitochondria content	High	Medium	Low	Low
Capillary density	High	Medium	Low	Low

Type I muscle fibers are the slow-twitch fibers and are characterized by slow speed of contraction and oxidative metabolism. This type of fibers is supplied by a high content of blood capillaries, myoglobin, and mitochondria that generate ATP by aerobic cellular respiration. In addition, this type of fibers is characterized by being the smallest in diameter and by their red color due to large amounts of myoglobin content. All these characteristics mentioned above provide to these fibers resistance to fatigue [6,19,27,29]. The type IIa muscle fibers are the fast-twitch oxidative-glycolytic fibers and are characterized by fast speed of contraction and oxidative-glycolytic metabolism. This type also presents large amounts of

blood capillaries, myoglobin and mitochondria and they can generate ATP by aerobic cellular respiration or anaerobic glycolysis. Compared to type I fibers, the high-speed contraction of type IIa fibers cause faster ATP hydrolyzation and consequently a moderately fatigue-resistant [17,19,27,29]. Type IIx and IIb are the fast-twitch glycolytic fibers and have a high speed of contraction with a glycolytic metabolism. Therefore, generating ATP by glycolysis, ATP hydrolyze faster and the muscle fatigue easily [17,19,27,29]. Many muscle fibers only express one type of myosin isoform, but it is frequent that muscle fibers co-express multiple isoforms being classified as hybrid fibers. This type co-expresses type I and IIa, or IIa and IIx, or IIx and IIb myosin isoforms [17,19,26,29].

As mentioned above, the proportions of muscle fiber type depend on mammal species and the gender [12,17,23]. In diaphragm muscle, a continuously active respiratory muscle, the proportions of fast fibers are greater in rodents than in humans. The diaphragm of murine animals contains 10% of type I fibers and 90% of type II fibers [16,30], while in humans the proportions are around 55% of type I and 45% of type II fibers [12,27,31,32]. In contrast, in the limb muscles, type II fibers are predominant in forelimbs and hindlimbs, although it depends on each muscle. For example, vastus lateralis of humans, which is the most representative limb muscle, contains around 30% of type I fibers and 70% of type II fibers [26,33,34]. In rodents, the most representative limb muscle is gastrocnemius which contains 13% of type I fibers and 87% of type II fibers, in contrast with soleus where the proportion of type I is the predominant [16,17].

1.4. Energy production and consumption in muscles

Skeletal muscle fibers use ATP energy for muscle contraction. This ATP is required for metabolic reactions and pump calcium ions from the sarcoplasm into the sarcoplasmic reticulum. Due to this huge amount of

ATP muscle fibers have three basic energy pathways to produce and store it [1,6]. The first source of energy used to reconstitute the ATP is creatine phosphate, an energetic molecule that is found in muscle fibers. Briefly, the enzyme creatine kinase (CK) catalyzes the reaction of creatine phosphate and ADP to ATP [1,2,6]. The second source is the anaerobic glycolysis, which does not require oxygen. In this pathway, glycogen is catalyzed by pyruvic acid and lactic acid that liberates energy that is used to gain ATP. Then this ATP can be used directly for muscle contraction or to re-form the stores of creatine phosphate [6]. Finally, the third source is oxidative metabolism also known as aerobic respiration. In this case, ATP is produced from a series of reactions that require oxygen, which pyruvic acid get into the mitochondria where it is completely oxidized, and it generates ATP, carbon dioxide, water and heat. Even if this reaction is the slowest to get ATP, it yields much more ATP for each reaction and more than 95% of all energy used by the muscle is derived from this type of source [1,6].

2. SARCOPENIA AND CACHEXIA

One of the main features in sarcopenia and cachexia is muscle dysfunction. Muscle dysfunction is defined as the situation where skeletal muscle show reduced strength and/or endurance, which are the two main functional properties of muscle [25,35]. Briefly, strength is the ability of the muscle to generate force through muscle contraction, while endurance is the ability to maintain that force throughout time [35].

2.1. Sarcopenia

Sarcopenia has been recognized as a condition by itself [36]. It is defined as a syndrome characterized by the loss of muscle mass and muscle strength that occurs with advanced age or secondary to chronic diseases including cancer [37,38]. Moreover, sometimes a shift in body composition may be

observed, with an increase in fat mass and a decrease in lean body weight (**Table 2**) [39]. Therefore, anorexia is not a characteristic feature of sarcopenia (**Table 2**) [38,39]. In sarcopenia, several processes contribute to muscle mass loss (**Table 2**). For instance, one of the most important is a decline in the number of motor neurons together with a reduction of anabolic hormones and changes in mitochondrial function [38,40]. In addition, chronic levels in oxidative stress and inflammatory cytokines contribute to a decrease in the ability to synthesize proteins [38,40]. Although the rate of protein synthesis decreased in sarcopenia, a higher rate of protein degradation was shown but it seems to be less important than the process of synthesis [40]. Furthermore, a deficient intake of energy and proteins, as well as a decrease in physical activity, also contribute to sarcopenia development [38,40]. In fact, it has been described that sarcopenia may be attenuated with resistance training accompanied by nutritional intervention (**Table 2**) [38–40].

Once sarcopenia is diagnosed, it can be classified into three conceptual stages: pre-sarcopenia, sarcopenia and severe sarcopenia. Pre-sarcopenia is when low muscle mass is observed, while sarcopenia is when low muscle mass and physical performance or muscle strength is altered, and severe sarcopenia is when the three conditions mentioned above are poorly. This classification may help in selecting the appropriated therapeutic approach [41].

It is well-demonstrated that muscle mass loss initiates slowly around the age of 30 years and accelerates around 65 years. Therefore, around 30% of muscle mass is lost in 80 years [42]. The prevalence of sarcopenia is around 10% in adults after 60 years of age, and around 30% after 80 years old [43–46]. This prevalence may be vary depending on the age of the population, gender, chronic diseases or ethnicity [43]. It is reported that around 15-50%

of cancer patients present sarcopenia. This varying prevalence is expected attributed to the cancer population heterogeneity [42].

2.2. Cachexia

Cachexia is defined as a multi-organ syndrome defined by body weight loss, inflammation, muscle and adipose tissue wasting, and regularly anorexia, which is associated with severe illness (**Table 2**). Cachexia is a complication in many chronic diseases like cancer, sepsis, acquired immune deficiency syndrome (AIDS), renal or cardiac failures and chronic obstructive pulmonary disease (COPD) [40,47–49].

Table 2. Differences between parameters involved in sarcopenia and cachexia [38,40,50].

	Sarcopenia	**Cachexia**
Body weight loss	Yes/no	Yes
Muscle mass loss	Yes	Yes
Fat mass loss	No	Yes/no
Anorexia	No	Yes/no
Resting energy expenditure	No	Yes
Comorbid condition	Yes/no	Yes
Neuromuscular degeneration	Yes	No
Systemic inflammation	Yes/no	Yes
Chronic oxidative stress	Yes	Yes
Increased muscle protein degradation	Yes/no	Yes
Impaired muscle protein synthesis	Yes	Yes/no
Increased muscle apoptosis	Yes	Yes
Reverts with nutritional support	Yes	No
Improved with physical activity	Yes	Yes

In cachexia, the loss of skeletal muscle mass accompanied or not accompanied by the loss of fat mass cannot be completely counteracted by nutritional support, therefore leading to a progressive muscle function impairment (**Table 2**) [51]. Moreover, it seems that appropriate physical training can counteract muscle wasting in cachexia [52,53] (**Table 2**). In addition, the patient's quality of life is reduced, and the hospitalization risk is increased. The progression of body wasting has poor prognostic implications and may reduce the tolerance to treatment [54,55].

In order to diagnose cachexia in humans, an international consensus statement defined that cachexia is present when at least one of the following clinical conditions are found in patients; 1) Weight loss >5% over the past 6 months (in absence of simple starvation); or 2) Body mass index (BMI) <20 and any degree of weight loss >2%; or 3) appendicular skeletal muscle index consistent with sarcopenia and any degree of weight loss >2% [51]. Once cachexia is diagnosed, it can be classified into three clinical stages: pre-cachexia, cachexia and refractory cachexia (**Fig. 3**). It is significant to highlight that not all patients traverse the whole spectrum [51,54].

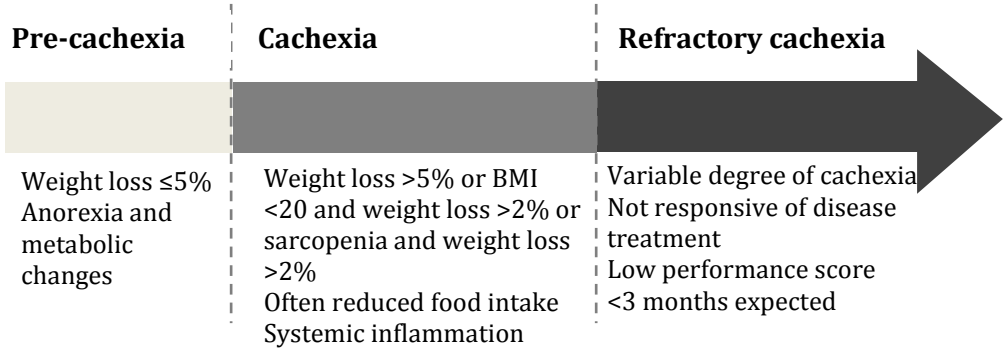

Figure 3. Stages of cachexia. Cachexia is classified into three clinical stages: pre-cachexia, cachexia, and refractory cachexia. Refractory cachexia is the most critical stage and usually, the expectancy survival is not more than three months *(Adapted from Fearon K. et al. 2011 [51]).*

Cachexia is usually present in the late stages of almost chronic diseases mentioned above, affecting 16-42% of cardiac failure patients, 30% of those with COPD, 60% of renal diseases and 50-80% of cancer patients determined by the tumor type [56,57]. For example, in pancreatic and gastric cancer patients, the incidence is more than 80%, while it is present in 60% of patients with lung cancer, and 40% in patients with leukemia or breast cancer [58]. These important percentages of prevalence directly correlate with mortality rates of cachectic patients. This range varies from 15-25% per year in COPD patients, 20-40% per year in chronic renal or cardiac failures patients, and up to 80% per year in some cancers with advanced-stages [56].

2.3. Conditions associated with muscle wasting and cachexia

2.3.1. Chronic obstructive pulmonary disease (COPD)

COPD is defined as a common, preventable, and treatable disease characterized by persistent airflow obstruction that is not fully reversible. This airflow obstruction is associated with airway and/or alveolar abnormalities response after the exposition to noxious particles or gases [59,60]. The main clinical manifestations of COPD are chronic bronchitis (inflammation and remodeling of the large-airway) and emphysema (parenchymal destruction), whereas the main symptoms are chronic dyspnea, cough, sputum, and weight loss [60,61]. Cigarette smoke is the main risk factor of COPD pathogenesis, but other less important factors such as environmental exposure (biomass fuel exposure and air pollution) and genetic factors may contribute to it [60]. Nowadays, COPD is one the leading causes of morbidity and mortality worldwide, and it is prognosticated to get the third leading cause of death by 2030 [59].

As abovementioned, COPD is one of the diseases associated with muscle dysfunction and cachexia. The prevalence of muscle wasting in COPD

patients is between 15-40%, and although these values are relatively high, it is the only determinant in the mortality, but independent of airflow obstruction [62]. The most common and best-studied manifestation of COPD is muscle dysfunction, and this affects differenty in respiratory and limb muscles. These phenotypic differences are due to the different activity pattern that these two muscles perform [61,63,64].

It is well described that one of the major features is the fiber-type switch from slow-twitch to fast-twitch fibers and the last ones reduced their size [34,61]. It is also shown a sarcomere disruption in respiratory and limb muscles [65,66]. Increased levels of oxidative stress but non-local inflammatory events have been consistently demonstrated in the respiratory and limb muscles *(see below, sections 5.1 and 5.2)* [67,68]. In addition, an increase in myofibrillar protein breakdown on account of an increase of catabolic signaling in skeletal muscle through nuclear factor-kappa B (NF-κB) and forkhead box O class (FoxO) was observed in both muscles. Thus, NF-κB and FoxO can induce gene expression of both ubiquitin-proteasome system (UPS) and the autophagy pathway *(see below, section 5.3)* [34,65,69,70]. As well as, Muscle RING-finger-1 (MuRF-1) and atrogin-1 expression, two E3 ubiquitin ligases of UPS, are altered in the skeletal muscle of COPD patients and animals compared to controls *(see below, section 5.4.1)* [34,65,71]. Moreover, the number of autophagosomes, the ratio of microtubule-associated protein 1 light chain 3 beta (LC3B) and nucleoporin p62 (p62) are increased in COPD *(see below, section 5.4.2)* [65,72]. In the case of COPD patients, these catabolic alterations were only observed in the limb muscles, while these differences were not found in the respiratory muscles [64,73]. In contrast, anabolic alterations even less increase in the limb muscles than in diaphragm, in spite of its expression is also higher in COPD patients compared to healthy controls [74].

2.3.2. Cancer cachexia

Cancer is one of the main conditions associated with muscle dysfunction and cachexia. Although cachexia is a complication present in several diseases, the muscle mass loss has been observed to occur most briefly in patients with cancer [75]. Whereas skeletal muscle mass loss is the main characteristic of cancer cachexia, the depletion of cardiac muscle it is also observed together with the affection of the liver, heart and brain [76,77]. Moreover, cancer cachexia presents anorexia, a reduction in protein content, albumins, and hemoglobin [75,78,79]. Patients with cancer cachexia experience numerous complications that provoke a decrease in their quality of life and limit their survival due to a poor response to chemotherapy [75,78]. As mentioned above, depends on the tumor type, the prevalence and the gravity of cachexia can vary though these two parameters are not being correlated between them [80].

2.3.2.1. Evidence from studies in patients

In previous studies of our group, it has been demonstrated that cancer cachexia affects differently in respiratory and limb muscles [32,65]. In lung cancer cachectic patients exhibited a reduction in type II fiber size in vastus lateralis but no differences were observed in the diaphragm [32,65]. This fiber size reduction accompanied by an increase in muscle structural abnormalities, and detrimental the exercise capacity and quadriceps muscle force [65]. Although it is demonstrated high levels of oxidative stress and inflammation in respiratory and vastus lateralis in cancer-cachectic humans, there is a controversial role in the ubiquitin-proteasome pathway *(see below, section 5)* [65,81]. In various studies in patients with lung and gastrointestinal cancers, the activity of the ubiquitin-proteasome system has shown similar level to healthy controls [82,83]. In contrast, other studies with lung and gastric cancer patients demonstrated higher levels of

ubiquitin compared with the controls [65,84]. Therefore, autophagic-lysosomal pathways would be the main proteolytic system in the muscles of cancer-cachectic patients [85,86].

2.3.2.2. Evidence from studies in animals

In animals studies, it has been demonstrated that diaphragm and gastrocnemius muscles behave in a more similar way than in humans [16,87]. In contrast from humans, rodents models of cancer cachexia exhibited a reduction in type I and type II fiber size accompanied with an increase in muscle structural abnormalities [15,16]. Moreover, it is well-described that oxidative stress and inflammation levels are higher in tumor-bearing animals *(see below, sections 5.1 and 5.2)*[88–90]. In cancer cachexia rodents with a weight loss between 15-30% showed a significant reduction (60%) in protein synthesis following by a relevant increase in protein degradation [91]. Three major pathways as the ubiquitin-proteasome, autophagy and calpains contribute to the increase of protein degradation in respiratory and limb muscles *(see below, section 5.4*)[16,92]. In cachectic animals, these pathways are activated mainly by NF-κB and FoxO signaling families *(see below, section 5.3)* [16,93].

3. EXPERIMENTAL MODELS OF CANCER CACHEXIA

Experimental models are useful to elucidate information about which are the underlying mechanisms that provoke cancer cachexia or muscle regeneration. They are also valuable to explore different therapeutic strategies and treatments to revert cancer cachexia. These approximations can be performed *in vitro* or *in vivo*. In this thesis, we focused on *in vivo* models.

3.1. *In vivo* murine models of cancer cachexia

Several *in vivo* models are widely used to mimic the clinical scenario of cancer cachexia and to discern which mechanisms underly muscle wasting. The used species go from mammal models like rat and mice until invertebrates such as *Drosophila melanogaster*, having each one their anatomical and molecular differences. The model of *Drosophila melanogaster* has been recently described as a cancer cachexia model and it is characterized by a relatively short-time regeneration, the facility to obtain wild-type or mutant strains, and it is a easily model to develop drug screenings [94]. However, mammal species are still the most commonly used in cancer cachexia studies, taking advantage of its capacity to reproduce the pathology and the clinical features of the humans. Several murine models are used to study the mechanisms involved in cancer cachexia and the effectiveness of the treatments, although there is not an ideal murine model that mimics the heterogeneity and complexity of this pathology.

Although the animal models mimic the characteristics of cachexia in humans, it is important to perform studies with human samples on account of their heterogeneity. In humans, the reason of such heterogeneity is not only related to in the clinical stage of the patients and the tumor-specific effects, but it also resides in age-related sarcopenia, morbidities, and the individual-genetic predisposition to develop cachexia. The main difficulty of human studies is the recollection of samples event though the high amount of patients are focused on cancer treatment, and their participation in cancer cachexia studies is limited [95]. The limitation of human samples makes it fundamental to adopt standard procedures and provide guidelines to help investigators to choose an appropriate animal model and compare their results between models.

The murine models that develop cancer cachexia can be classified into four groups depending on the way that the tumor growth is induced.

3.1.1. Syngeneic models

Syngeneic models are the most widely used models and involves injectin cells from rodent cancer cell lines subcutaneously, intramuscularly, or intraperitoneally in recipient mice or rats. This is characterized by being reproducible, synchronized, and rapid tumor growth [96]. Main cell lines that are used are MAC16 adenocarcinoma (MAC16), Lewis lung carcinoma (LLC), Yoshida ascites hepatoma AH-130, Walker 256 carcinosarcoma and colon 26 adenocarcinoma (C26). Although other types of cell lines less known are also used like lung P07 adenocarcinoma (LP07) that used in our group. In these cases, it is important to know the characteristics of each model to choose carefully the best option to test the scientific hypothesis. Briefly, these types of model present in common a systemic inflammation and body weight loss but they differ in the tumor development and the day that trigger the loss of body weight. Moreover, most models develop anorexia except C26 and MAC16 adenocarcinomas. By contrast, metastasis in only detectable in LLC and LP07 mice models [16,97,98]. In our group, we normally use Yoshida ascites hepatoma AH-130 and LP07 cachectic models because they are two models that are well-validated with different carcinogenic potential.

3.1.2. Xenograft models

Xenograft models are similar to syngeneic models described above, but the source of cell cultures or tissues implanted into immunosuppresed animals are from human origin. The main advantage of these models, compared to syngeneic models, is that they better-mimic the human tumor. In contrast,

these animals cannot reproduce the important interactions of the immune system with the tumor-host [96].

3.1.3. Genetically engineered models

Genetically engineered animal models are genetically manipulated to develop spontaneous tumors. This model overcomes at least two weaknesses of the models mentioned above, one is the ectopic localization and the second is the growth rate that allows longer prospective studies [94,97]. The adenomatous polyposis coli (Apc) Min/+ mouse, which develops colon cancer because it has a mutation in the APC gene, it is the most widely used genetically engineered model used in cancer cachexia studies [99].

3.1.4. Carcinogen-induced models

Carcinogen-induced models are animals exposed to known carcinogens that will develop tumors after a certain time. This model has similar advantages to genetic engineered models although the way to induce carcinogenesis is simpler [96]. Several chemical carcinogeneses are described and they used to study cancer cachexia like Ethyl carbamate (urethane) [100] and N-Nitrosodiethylamine (NDEA) [101,102].

4. MOLECULAR MECHANISMS INVOLVED IN CANCER CACHEXIA

4.1. Oxidative and nitrosative stress

The oxidative and nitrosative stress are widely accepted distinguished players that regulate protein synthesis and degradation in skeletal muscle by modulating the signaling pathways, and therefore they contribute to atrophy. They are characterized by an increment in the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) in the mitochondria [103]. Reactive species are defined as free radicals which, in this case, are associated with the oxygen atom (O) or their equivalent, and have

stronger reactivity with other molecules [104]. The ROS species include singlet oxygen (O_2), superoxide anion (O_2^-), hydroxyl radical (OH), and hydrogen peroxide (H_2O_2). Instead, peroxynitrite (ONOO-), nitrogen dioxide (NO_2) and nitric oxide (NO) are RNS [104,105].

In physiological conditions, basal levels of oxidative and nitrosative stress are necessary because they regulate various physiological processes like the maintenance of vascular tone or a defense against infectious agents, so there is a balance between the formation of ROS and RNS and the antioxidants systems. On the other hand, an imbalance due to an increase of oxidative and nitrosative stress usually describes a condition in which antioxidants levels are enough to reduce ROS or RNS produced. This imbalance can be caused by excessive ROS and RNS products, loss of antioxidants defenses, or both [103,106].

Oxidative and nitrosative stress usually damage cell structures such as membranes proteins, lipids and deoxyribonucleic acid (DNA). Lipid peroxidation may contribute to increased cellular damage from the formation of oxidized products like 4-hydroxy-2-nonenal (HNE) and malondialdehyde (MDA), which can be measured as oxidative stress markers [105]. Protein carbonylation and the production of nitrotyrosine (3-NT) are physiological manifestations of oxidative and nitrosative stress [105,106]. The assessment of these markers has been widely studied in our group to demonstrate that redox imbalance is involved in muscle wasting in both respiratory and limb muscles of COPD and lung cancer patients [34,65,107] and in different animal models [88,89].

The sources that cause cytosolic and mitochondrial ROS production in skeletal muscle are via increased pro-inflammatory tumor necrosis factor (TNF)-alpha and plasmatic angiotensin II (Ang II) levels [108,109]. The

mechanisms by which TNF-α and Ang II have produced ROS it is well described. Briefly, on the sarcolemma TNF activates TNF-1 receptors, and thus triggers a cascade of signaling events that lead to an increase in the production of superoxide [108,109]. Furthermore, Ang II binds to angiotensin I receptors of the sarcolemma and activates NAD(P)H oxidase, which also produces superoxide molecules [108]. Finally, it is observed that myostatin also participates in the ROS production by TNF mechanisms. Instead, the increase of RNS production is given by high levels of circulating cytokines like TNF [110]. As mentioned above, high levels of ROS and RNS participate in the inhibition of protein synthesis and the promotion of proteolysis system in skeletal muscle. One of the main signaling pathways that regulate protein synthesis in muscle is the insulin-like growth factor-1 (IGF-1)- protein kinase B (Akt) signaling pathway. Although there are controversial studies, it seems that ROS and RNS molecules block the phosphorylation of AKT and consequently promotes the inactivation of mammalian target of rapamycin complex 1 (mTORC1) causing inhibition of protein synthesis [111]. Furthermore, oxidative stress can accelerate proteolysis by different proteolytic systems like autophagy, proteasome system, calpains, and caspases. This acceleration can be given by the stimulation of gene expression and the increase on the abundance of key proteins involved in this system, increasing the 20S proteasome activity and the activation of calpains and caspase-3 markers, and finally oxidizing proteins to be more susceptible to degradation [108].

Antioxidants are present at low concentrations in the cell and their main function is to reduce or prevent oxidation. As mentioned above, antioxidants help to maintain the balance between ROS or RNS production. Skeletal muscle fibers have strong oxidant systems to preserve myocytes from ROS production [105]. Antioxidants can be classified in enzymatic oxidants, non-enzymatic oxidant, and thiol antioxidants. The most efficient

enzymatic antioxidants include catalase, glutathione peroxidase, and superoxide dismutase (SOD), being this last one divided into CuZn-SOD and Mn-SOD that are present in sarcoplasm and mitochondria, respectively. Moreover, non-enzymatic antioxidants components such as vitamin C and E, and thiol oxidants such as thioredoxins and peroxiredoxins, also contribute to antioxidants defenses in skeletal muscle [103]. Moreover, antioxidants can interact with each other to regenerate their original characteristics [104–106].

4.2. Inflammation

Inflammation is a tightly regulated mechanism aimed to protect the organism against alterations in homeostasis due to different stimuli such as biological, physical, or chemical agents on one side, and damaged, infected or neoplastic cell on the other. Therefore, the inflammatory response auto-regulate the equilibrium between pro- and anti-inflammatory stimuli, but when this balance is persistent, lost chronic inflammation occurs [112,113]. In this regard, this chronic inflammation is associated with a rise in the levels of pro-inflammatory circulating cytokines that are capable to influence in different metabolic pathways related to cachexia causing damage in muscle structure and altering the muscle contraction [25,113].

4.2.1. Inflammatory cytokines

Cytokines are involved in the initiation and the progression of cancer. It is well accepted that are the main determinants of the progression of cachexia, response to treatment, quality of life, and patient survival [114]. They regulate the interactions between cells by connecting with specific receptors in the membrane of the target cells, inducing proliferation, activation, differentiation or death. Cytokines are not only regulators of the immune response but it also has been described that they have a wider function in cellular communication forming an interactive network [115].

In cancer cachexia, it can be distinguished two types of cytokines; the pro-inflammatory and anti-inflammatory. The pro-inflammatory or pro-cachectic cytokines include interleukin (IL)-6, IL-1, TNF-α and interferon (IFN)-γ [115]. These cytokines are shown to be involved in muscle wasting through activation of protein catabolism inducing protein degradation and impaired myogenesis [116]. As mentioned above, oxidative and nitrosative stress and inflammation are related to act in the triggering of muscle wasting since inflammation can modulate the production of ROS and oxidative stress can activate the expression of inflammatory mediators [108–110]. On the other hand, the anti-inflammatory cytokines (IL-10, IL-4, IL-13, and IL-15) modulate the results of the pro-inflammatory cytokines [115,117,118].

TNF-α is pro-inflammatory cytokine involved in the progression of cancer cachexia by the increase of the corticotrophin-releasing hormone (CRH) levels and a reduction in food intake, leading to anorexia [119]. It is reported that TNF-α induces metabolic energy production, uncoupling of mitochondria respiration, and the degradation of myofibrillar proteins [114,119]. TNF-α also induces the activation of catabolic pathways by the ubiquitin-proteasome system. Briefly, binding TNF-α to its receptors initiates a signaling cascade that activates NF-κB and causes its translocations into the nucleus. Inside it activates the ubiquitin-proteasome system through the increase of MuRF-1, and it decreases the expression of the myogenic differentiation 1 (MyoD) [119–122]. The pro-inflammatory cytokine TNF-α not only activates catabolic pathways, it also downregulates the anabolic pathways by the inhibition of the Akt/mTOR signal transduction pathway [123].

IL-6 is an important inflammatory cytokine that has been associated with increased mortality and correlated with survival rates in cancer patients [58]. This cytokine has a property of exercise both pro- and anti-inflammatory

effects. Although IL-6 pro-inflammatory mechanisms are well reported, the mechanisms of anti-inflammatory effects remain unknown [124]. The IL-6 pro-inflammatory induces the activation of Janus kinase (JAK) that activates signal transducer and activators of transcription (STAT) proteins, triggering protein degradation [81,120]. Moreover, IL-6 may indirectly inhibits Akt/mTOR pathway and induces NF-κB and AMP-activated kinase (AMPK) [125].

Finally, IL-1 and IFN-γ are two cytokines that play a secondary role in cancer cachexia. Both induce the activation of the ubiquitin-proteasome system through NF-κB signaling pathway and the inhibition of the Akt/mTOR pathway, in similar-way mechanisms that TNF-α [120,123]. In addition, IFN-γ also induces the activation of JAK/STAT triggering anti-myogenic properties and protein degradation [120].

4.3. Signaling pathways

Cell signaling pathways are responsible for the transmission of information within the cell in order to regulate cellular activities. Signal transduction occurs when an external or internal stimulus activates a specific receptor that triggers a number of biochemical events inside the cell to originate one or more cellular responses. This signal transduction process usually involves multiple steps amplifying the signal response. In skeletal muscle, the signaling pathways involved in the homeostatic balance between protein synthesis (anabolism) and protein degradation (catabolism) are mitogen-activated protein kinase (MAPK), nuclear factor-kappa B (NF-κB), forkhead box O class (FoxO), myostatin/similar to mothers against decapentaplegic (Smad), and insulin-like growth factor-I (IGF-1)/phosphatidylinositol-3-kinase (PI3K)/Akt/mTOR pathways.

4.3.1. Catabolic signaling pathways

4.3.1.1. Mitogen-activated protein kinase (MAPK) pathway

The mitogen-activated protein kinase (MAPK) signaling pathway is composed by four different cascades; p38 MAPK, Jun amino-terminal kinase (JNK1/2/3), the extracellular signal-related kinase (ERK1/2), and ERK5 [126,127]. In general, MAPK pathway is described to be associated with cell proliferation, migration, differentiation, senescence, and apoptosis, while in cancer cachexia also controls cellular processes of protein synthesis and degradation [126,128]. These cellular processes are given by the activation through the phosphorylation mechanisms of distinct MAPK pathways stimulated by growth factors, cytokines like TNF-α and ROS species [127,129,130].

Focus on p38 MAPK pathway, it is well described that their activation through the cytokine TNF-α and oxidative stress stimuli increase the expression of atrogin-1, which participates in the ubiquitin-proteasome and autophagy-lysosome proteolytic mechanisms [122,129]. In addition, the activation of p38 leads to increased caspase activity, and thus, apoptosis [131]. Moreover the activation of different catabolic pathways, p38 MAPK dephosphorylates Akt and thus, inhibits Akt/mTOR pathway [130]. In the case of the ERK MAPK pathway, their activation increases the ubiquitin-proteasome mechanism and inhibits the protein synthesis by the Akt/mTOR pathway [128].

4.3.1.2. Nuclear Factor-kappa B (NF-κB) pathway

Nuclear factor κB (NF-κB) transcription factors are a conserved signaling family that plays a crucial role in various biological processes and it is composed of five members, RelA/p65, RelB, c-Rel, p50 and p52 [132]. Each member of NF-κB family can form homodimers or heterodimers between them, and they act either as repressor or as inducer of gene expression,

respectively. The transcriptional repression forms of NF-κB are the homodimers p50/50 or p52/52, while heterodimers such as p65/p50 or p65/p52 are transcriptionally active [132,133]. As mentioned in previous sections, inflammatory cytokines (TNF-α, IL-1), oxidative stress, and growth factors are the upstream signals responsible for the activation of NF-κB [133,134]. Depending on the upstream signals and the involved subunits of IκB kinase (IKK) complex, NF-κB activation can occur in either the classical or the alternative signaling pathway (**Fig. 4**) [134]. NF-κB remains in an inactive form by binding IκB family of inhibitory proteins. In the classical signaling pathway, the increasing levels of upstream signals like TNF-α induce the activation of the IKK complex, specifically IκB. The phosphorylation of IκB promotes its polyubiquitination and subsequent immediate the degradation of the proteasome. This lead to NF-κB nuclear translocation and following activation mediated gene transcription (**Fig. 4**) [106,133–135]. Finally, NF-κB interferes in the myogenic differentiation process that it is necessary for regeneration [136,137]. Evidence shows that the downregulation of classical pathway coincides with the alternative signaling activation [134].

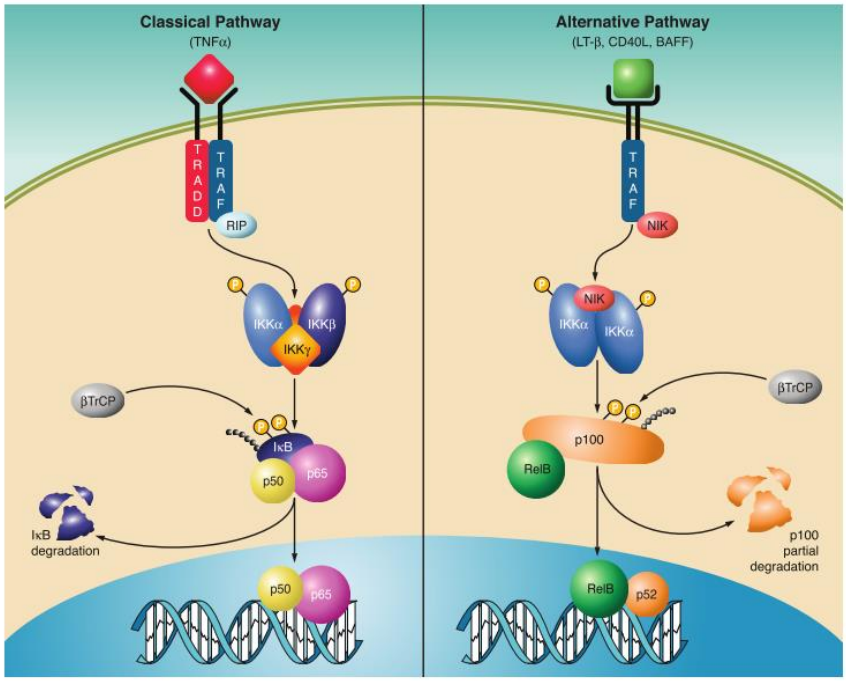

Figure 4. Classical and alternative NF-κB signaling pathway. The classical signaling pathway is activated by the binding of TNF-α to its receptor and the consequent assembly of IKK complex and the phosphorylation of IκB. This promotes its phosphorylation and classical p65/p50 heterodimer can translocate to the nucleus and mediate transcription of NF-κB genes. Alternative NF-κB signaling is activated by different ligands and activates NIK. NIK phosphorylates IKKα and this phosphorylates p100 that generate the p52 subunit. The resulting RelB/p52 complex translocates to the nucleus and transcribes NF-κB target genes *(Extracted from Bakkar N et al. 2010* [134]*).*

4.3.1.3. Forkhead box O class (FoxO) pathway

The forkhead box class O (FoxO) proteins form a subclass of the large family of forkhead proteins characterized by the presence of a "winged-helix" DNA-binding domain named Forkhead box, which gave the name to these proteins [138]. They participate in various cellular processes like cell growth, differentiation, apoptosis, oxidative stress resistance, and energy

metabolism as a regulator [139,140]. Four FoxO species (FoxO1, FoxO3, FoxO4 and FoxO6) are identified in mammals and all of them are expressed in muscles [138,140]. Especially, FoxO6 is predominantly expressed in the central nervous system and in less quantity in oxidizing muscles so when it is referred in skeletal muscle, only there are three FoxO members [140].

Several post-translational modifications like acetylation, phosphorylation and ubiquitination are responsible for the regulation of FoxO activity, and the changes in its transcriptional activity are specific for each FoxO member [135]. A critical negative regulator of FoxO activity is the IGF-1/PI3K/Akt signaling pathway by phosphorylation. Low levels of Akt activity cause a decrease in phosphorylated FoxO levels and promote their translocation from the cytoplasm to the nucleus. This translocation is sufficient to promote an increase in atrogin-1 and MuRF-1 expression [135,141]. Moreover, AMPK is another regulator that is associated with the regulation of FoxO3 and the atrogin-1 activity [140]. It is well-reported that FoxO1 activation is associated with an upregulation of atrogin-1 and MuRF-1, in contrast to FoxO3 that only is associated with an increase of atrogin-1 expression [142,143]. Therefore, FoxO1 and FoxO3 seem to be involved in the ubiquitin-proteasome pathways, only FoxO3 has a role in the activation of the autophagy-lysosomal system and the suppression of protein synthesis [135,140,141].

The activation of FoxO1, 3 and 4 inhibits cell proliferation, and especially FoxO1 repress myoblast differentiation reducing MyoD gene expression [140,144]. Furthermore, FoxO1 was correlated to the distribution of muscle fiber and it was observed that FoxO1 may negative regulates type I fiber formation but positively regulates type II. This could be because type II fibers express more MyoD than type I fibers [144]. In energy metabolism, it is reported that FoxO1 is the main regulator of the metabolism of muscle

energy through the regulation of lipolytic and glycolytic flux across FoxO3 [140].

4.3.1.4. Myostatin/Similar to mothers against decapentaplegic (Smad) pathway

Myostatin, or growth and differentiation factor 8 (GDF-8), is a member of the transforming growth factor-β (TFG-β) that is expressed and secreted predominantly by skeletal muscle, and it has emerged as a key regulator of skeletal muscle mass [145].

The activated form of myostatin interacts to one of the two activin type II receptors (ActRIIB or ActRIIA), which phosphorylates and activates the activating type I receptors (ALK4 and ALK5) which leads to the phosphorylation and thereby the activation of Smad2 and Smad3. Phosphorylated Smad2 and Smad3 constitute a complex with Smad4. The key role is an intracellular mediator of myostatin signaling, which translocates into the nucleus, and activates the transcription of several target genes [145–147]. Even though Smad2 and Smad3 are the transcription factors interceding myostatin functions, the downstream targets and the mechanisms of Smad-dependent atrophy remains unknown [141,147]. Furthermore, Smad7 is a member of the Smad family and its acts as a negative inhibitor for the myostatin signaling pathway [148].

It is now widely accepted as an inhibitor of skeletal muscle growth and the upregulation of myostatin increases protein degradation, decreases myogenesis and reduces protein synthesis exacerbating skeletal muscle atrophy [145,146]. Specifically, myostatin negatively regulates the protein synthesis through Akt signaling [147]. Moreover, the blockage of Akt/mTOR signaling by myostatin, activates FoxO1 allowing increased expression of ubiquitin E3 ligase atrogin-1 and MuRF-1 in an independent way of the NF-

kB pathway. This fact supports the concept that the myostatin pathway synergies with Akt-FoxO signaling [141,149]. In addition, myostatin can promote increased ROS production in skeletal muscle fibers, and as mentioned in previous sections, this inhibits Akt signaling and induces the ubiquitin E3 ligases atrogin-1 and MuRF-1 [108,110]. Interestingly, it has been reported that myostatin is also a potent negative regulator of myoblast proliferation and differentiation through down-regulating expression and activity of myogenin and MyoD [144,147,150]. Finally, it was demonstrated that the complete loss of myostatin improves muscle functions and cause muscle hypertrophy [151].

4.3.2. Anabolic signaling pathways

4.3.2.1. Insulin-like growth factor-I (IGF-1)/ phosphatidylinositol 3-kinase (PI3K)/ Akt/ mammalian target of rapamycin (mTOR) pathway

As mentioned above, for the muscle homeostasis is required a balance between protein synthesis and degradation, and an imbalance in favor of protein degradation causes muscle wasting. So far, several signaling pathways have been described that let to protein degradation and catabolism, but IGF-1/PI3K/Akt/mTOR is the main signaling pathway leading to protein synthesis and anabolism in skeletal muscle [152].

In skeletal muscle, it is well-known that IGF-1 activates PI3K pathway which triggers a series of steps that finally activates Akt [108,152]. Although IGF-1 is the main activator of PI3K, it also can be activated by extracellular signals like cytokines, growth factors or hormones [153]. Activated Akt promotes the mTOR activation and the inactivation of FoxO transcription factors, playing a wide range of biological effects, such as anti-apoptosis, promoting cell survival and other functions [153–155]. Particularly, the

activation of Akt on mTOR is indirect and it forms two functionally distinct complexes, known as mTORC1 and mTORC2. The mTORC1 complex require an adaptor protein known as Raptor, which is essential for muscle maintenance [129,155]. The main function of mTORC1 is protein synthesis stimulation and it is also involved in the translation process [108,129]. In contrast, mTORC2 is involved in positive feedback which phosphorylates Akt leading its maximum activation [129]. The inactivation of FoxO by Akt causes the inhibition of mTORC1 and the ubiquitin-proteasome system [152].
In rodent cachexia models it is not clear how dysregulation of protein synthesis occurs, it is described that IL-6 may be negatively regulated mTOR by the activation of AMPK [156]. On the contrary, there are studies that despite seeing a mTORC1 suppression, do not observe changes in the phosphorylation of Akt, mTOR, or AMPK suggesting that mTORC1 signaling is controlled by another upstream regulator [157].

4.4. Proteolytic systems

Muscular atrophy is defined as a decrease in the fiber size caused by the loss of proteins, organelles and cytoplasm. This atrophy is due to the hyper-activation of the process of protein degradation or proteolysis and a reduction in protein synthesis [135,158]. In physiological conditions, its main function is to maintain the homeostasis through the continual hydrolyzation of proteins to amino acids and to be finally reused for new protein synthesis. Furthermore, protein degradation acts as a quality control to eliminate damaged proteins or remove of regulatory proteins essential for the regulation of cell growth and metabolism [159].

The two main mechanisms of cell degradation in cancer cachexia are the ubiquitin-proteasome system (UPS) and the autophagy-lysosome pathways [135,141,158]. However, calpain-calpastatin system and apoptosis through caspases are other two mechanisms involved in cell degradation [160,161]. It

has been reported that UPS is responsible for short-lived proteins degradation, whereas autophagy-lysosomal and calpains system seem to be involved in the long-lived proteins and damaged organelles [159,162]. The signaling pathways described previously are responsible for the regulation of proteolytic systems.

4.4.1. Ubiquitin-proteasome system

In the ubiquitin-proteasome system, targeted proteins are degraded by the 26S proteasome. The proteins are targeted through covalent attachment of a ubiquitin molecules chain [158]. Protein ubiquitination is a finely regulated process mediated by the consecutive action of three classes of enzymes named ubiquitin-activating enzymes (E1), ubiquitin-conjugating enzymes (E2), and ubiquitin-protein ligases (E3). The process begins with the E1, which activates the ubiquitin COOH-terminal end by producing a highly reactive thiol ester between it and the enzyme cysteine active site. The activated ubiquitin is translocated onto the E2 cysteine residue active site. Then E2 interacts with E3 that binds to the substrate, and E3 promotes the binding of the ubiquitin onto the substrate. Once ubiquitinated-substrate is recognized by the 26S proteasome for degradation, the deubiquitinating enzymes remove the ubiquitin chains to allow using the ubiquitin for other reactions [142,163] (**Fig. 5**). Exist different E2-E3 pairs which degrade a specific group of proteins. The selectivity in the degradation is provided by the specificity of E3s. The variety of E2 and E3 proteins depends on tissue type and the physiological conditions of this one, but only some of them it has been described to regulate and to be transcriptionally induced under atrophy process [141,158].

Although many types of E3 are known, only some of them are muscle-specific ubiquitin ligases and are upregulated in muscle wasting of cancer cachexia. The two first ubiquitin ligases identified were MuRF-1 and

atrogin-1 [158,164]. These two E3 are considered as master genes because have an exclusive role in muscle wasting. It was described that MuRF-1 and atrogin-1 knockout mice are resistant to muscle atrophy induced by denervation [164], while MuRF-1 or atrogin-1 knockout mice individually are not resistant to muscle atrophy [165,166]. MuRF-1 is responsible for ubiquitinate various muscle structural proteins like troponin-I, actin, myosin heavy chains, myosin binding protein C, and myosin light chains 1 and 2 (**Fig. 5**) [158,167].

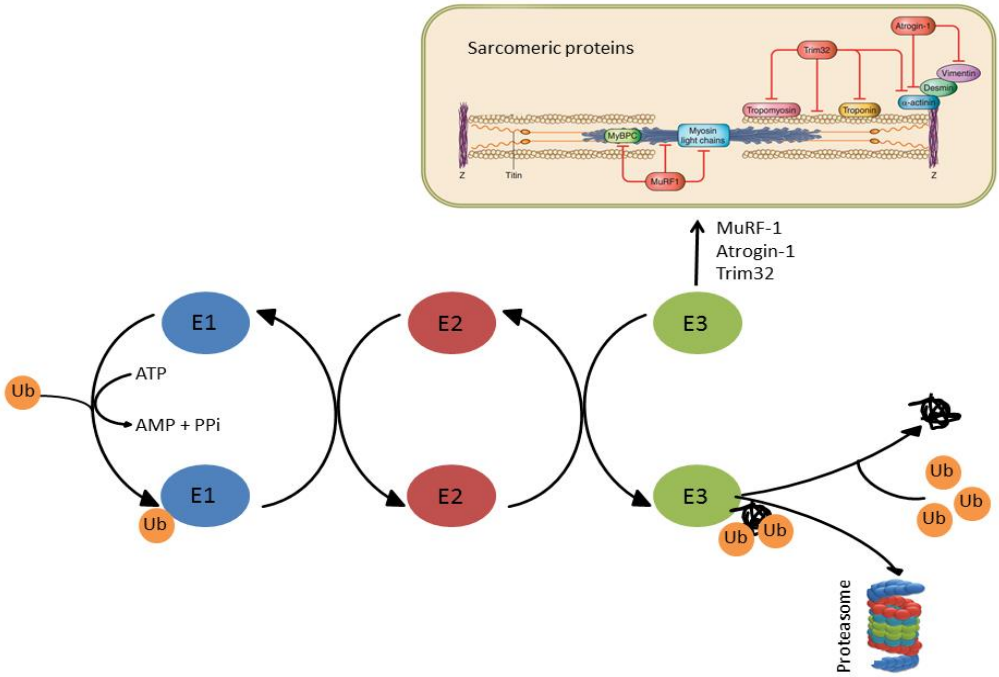

Figure 5. Ubiquitin-proteasome system in cancer cachexia. E1 enzymes activate ubiquitin proteins and consequently activates E2. Then E2 interacts with E3 that binds the substrate and transfer the ubiquitin onto it. Once the 26S proteasome recognizes the ubiquitinated substrate, the ubiquitin chains are removed, and substrates are degraded. E3 ubiquitin ligases (MuRF-1, atrogin-1, and TRIM32) are the responsible for the ubiquitination of sarcomeric proteins (*Adapted from Boldano P et al. 2013* [141] *and Bodine SC et al. 2014*[142]*).*

In contrast, atrogin-1 substrate seems to be involved in growth-related processes or survival pathways. Additionally, atrogin-1 stimulates the degradation of MyoD and eukaryotic translation initiation factor 3 subunit f (eiF3-f), a key muscle transcription factor and an activator of protein synthesis, respectively [142,168]. Atrogin-1 is also responsible for the ubiquitination of sarcomeric proteins including myosin, desmin and vimentin (**Fig.5**) [169]. MuRF-1 and atrogin-1 are transcriptionally upregulated together so it suggests that the two E3 ligases are under the regulation of similar transcription factors. As mentioned in previous sections, the FoxO transcription factor regulates both E3 ligases opposed to NF-κB that only regulates MuRF-1 or p38 MAPK that only regulates atrogin-1[122,141,142,146,170].

Since these two ubiquitin ligases cannot degrade all muscle structural proteins, further E3s are involved in muscle wasting. In support of potential involvement of tripartite motif containing 32 (TRIM32) in promoting muscle atrophy, it has been shown that several structural muscular proteins of thin filaments (actin, tropomyosin, and troponins), α-actinin and desmin were identified as substrates of TRIM32 ubiquitination activity (**Fig. 5**)[171]. Furthermore, TRIM32 can negatively regulate the PI3K/Akt/FoxO signaling pathway and it has a role in muscle growth through the regulation of satellite cell proliferation and differentiation [172,173]. Although TRIM32 is another E3 ubiquitin ligase that it is not upregulated in all forms of atrophy and its role as a mediator of muscle wasting remains unclear [142].

4.4.2. Autophagy-lysosomal

The autophagy-lysosomal is a highly preserved homeostatic mechanism forming part of the proteolytic system, which plays an essential role in the upset of cell components in basal conditions and in response to different stimuli like cellular stress or cytokines, and acts as essential mechanisms

for cell survival [174,175]. Initially, autophagy was considered as a non-selective degradation pathway compared to the ubiquitin-proteasome system, but it is now widely-acknowledged that autophagy can promote protein degradation in a selective manner [176,177]. Three main types of autophagy are described, micro- and macroautophagy and chaperone-mediated autophagy (CMA), and they differ with the components that they use for the degradation and their mechanisms. In microautophagy, lysosome has the capacity to directly cover the cytosolic compounds, and macroautophagy involves in the formation of double-membrane vesicle called autophagosome to engulf organelles and larges structures. In contrast, CMA degrades only soluble proteins [175,178]. In this thesis, macroautophagy is referred to as autophagy such as more research studies.

In skeletal muscle is required a basal level of autophagy to preserve the homeostasis of muscle mass. Nevertheless, an alteration or inhibition of autophagy activity can induce muscle protein breakdown, and therefore muscle wasting [179]. As mentioned above, the process of autophagy in skeletal muscles engulf portions of the cytoplasm, organelles and protein aggregates by autophagosomes, which are then fused with lysosomes for the degradation. This process is often divided into five steps: 1) induction; 2) expansion; 3) elongation and completion of autophagosome; 4) fusion with lysosome; and 5) degradation of proteins and organelles [108]. Firstly begins with the generation of the preautophagosome structure which it is negative regulate by mTORC1 [180–182]. The expansion consists of the assembly of fractional autophagosome membranes called phagophore, and the recruitment of several autophagy-related gene (atg) proteins like beclin-1 [108,183]. During the elongation and completion of autophagosomes, the inactive form of LC3 (LC3B-I) is posttranslationally modified to its active form (LC3B-II), which is a component of autophagosomes [184]. Next, the autophagosome is fused with a lysosome, and the autophagosome's

content is transferred to lysosomal proteases. The final step of autophagy involves the degradation of proteins and organelles [108,183]. The autophagy p62 adaptor interacts with ubiquitin or polyubiquitin chains to autophagy and then delivered to autophagosomes and binds LC3B-II to facilitate degradation by autophagy [185,186].

Among mTOR signaling pathways, transcription factors like FoxO and p38 MAPK also participate in the regulation of autophagy [187,188]. It well described that FoxO3 is required for the upregulation of autophagy-related genes such a LC3B [189]. Interestingly, oxidative stress can regulate the induction of autophagy by regulating the activation of mTORC1 and p38 MAPK transcription factors in cachexia models [188,190].

4.4.3. Calpain-calpastatin system

Calpains family is calcium-dependent cysteine proteases that are responsible for cleavage of target proteins in response to calcium signaling. Although it has been described 15 different calpains in mammals, only three distinct calpains are expressed in muscle tissue; calpain 1 (μ-calpain), calpain 2 (m-calpain) and calpain 3 (p94) [191]. In addition to calpains, calpastatin is also found in the muscle tissue and it is an inhibitor of calpains [192]. Therefore, the calpain activity is regulated by the concentration of cytosolic calcium and the activity of calpastatin [191,193]. It is reported that oxidative and nitrosative stress links with the levels of cytosolic calcium and as a consequence promote calpain activation, promoting its translocation from the cytosol to the membrane [108].

In skeletal muscle, the main function of calpains is breaking the cytoskeletal proteins and consequently degrade them by ubiquitin-proteasome system, whereas it also plays role in cell cycle, cell motility and apoptosis [160,194]. Calpains are localized within the Z-disc of muscle fibers and they can cleave

the cytoskeletal proteins near Z-disc like titin and nebulin. Furthermore, calpains are also cleft sarcomeric proteins like troponin, tropomyosin and protein C, and contractile proteins like myosin and actin and subsequently be ubiquitinated and degraded by the proteasome [195,196]. Hence, there is an interaction between calpains and the ubiquitin-proteasome system, and it would seem that calpains could act as the upstream of the ubiquitin-proteasome pathway in muscle wasting [160,196]. This interaction between these two proteolytic pathways could be given because it has been described that calpains reduce the activation of Akt and, as a result, indirectly activate FoxO transcription factors to regulate the expression of MuRF-1 and atrogin-1 [197].

4.4.4. Caspase-mediated apoptosis

Apoptosis or programmed cell death is a crucial regulated process for the maintenance of tissue homeostasis [198]. In skeletal muscle, a distinguishing feature of muscle fibers that form skeletal muscles is that they are multinucleated, so when apoptosis is activated and cause muscle wasting, the number of nuclei and fiber size are decreased but the muscle fiber is not eliminated [198–200].

The family of proteolytic enzymes responsible for the apoptosis initiation in muscle wasting is caspases. The caspases are typically divided as initiator caspases such as caspase-2, 8, 9 and 10, or effector caspases such as caspase-3, 6 and 7 [161]. Interestingly, caspase-3 participates in muscle wasting by protein degradation, and it is also required for muscle differentiation [201]. In muscle wasting, the apoptosis can be classified into two main pathways: extrinsic and intrinsic. The extrinsic pathway is activated by oxidative stress and cytokines that activate death receptors and induce the activation of initiator caspases [108,202]. In contrast, the intrinsic pathway is dependent of mitochondria and is regulated by B cell

leukemia/lymphoma 2 (BCL-2, anti-apoptotic factor) and BCL-2-associated X protein (BAX, apoptotic factor) [203]. However, both pathways converge into the activation of the execution caspases like caspase-3. Finally, caspase-3 activates endonuclease G, which triggers DNA fragmentation into apoptotic bodies and are eliminated by phagocytic cell [190,204].

5. THERAPEUTIC APPROACHES IN CANCER CACHEXIA

5.1. Anti-cachectic treatments

Several therapeutic strategies to ameliorate cancer cachexia have been described during the last years. The development of new therapeutic approaches should focus on the following targets: 1) increase body mass, 2) reduce the energy expenditure, 3) diminish anorexia, 4) improve the quality of life, 5) ameliorate performance status, and 6) reduce the pro-inflammatory cytokines levels [205,206]. Currently, several drugs are being in clinical trials to test the efficacy in cachectic patients (**Fig. 6**) [80].

Studies conducted so far can be classified into two major groups: as those using pharmacological compounds or those based on nutritional supports. Pharmacological compounds are divided into four groups depending on their biological function: appetite-modifying, anti-inflammatory, anabolic or another type of drugs (**Fig. 6**). In lower proportions, there are studies that use herbal or alternative compounds, exercise or multimodal approaches (**Fig. 6**) [80]. The last one refers to combine two types of anti-cachectic strategies like pharmacological treatment with nutritional support or including physical activity [207]. Recent studies using multimodal approaches have realized promising results and it would be an effective strategy to reduce muscle mass loss [208,209].

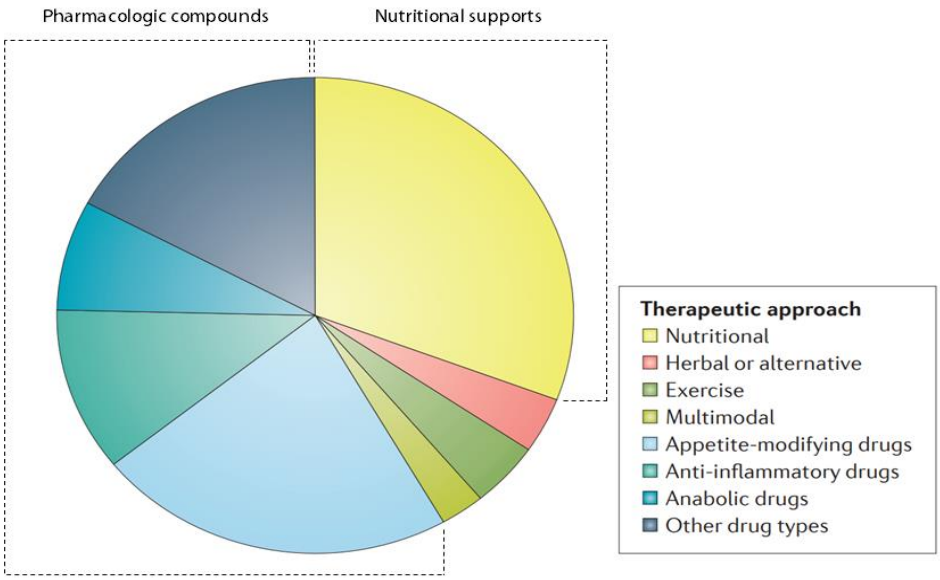

Figure 6. Distribution of anti-cachectic therapeutic approaches in clinical trials. At present, a total of 107 clinical trials in phase II-IV are reported in *www.clinicaltrial.gov.* All these clinical trials can be divided into two broad groups; pharmacological compounds and nutritional supports. Furthermore, in a lower proportion, there are clinical trials that use herbal or alternative approaches, exercise and multimodal treatments *(Extracted and adapted from Baraccos VE et al. 2018* [80]*).*

5.1.1. Pharmacologic compounds

The development of pharmacological compounds aims to increase appetite, reduce inflammation and modulate the catabolic and anabolic pathways involved in cancer cachexia [206].

Anorexia is an often condition in cancer patients with muscle wasting. This can also be associated with a reduction of food intake [51]. To counteract cancer anorexia and increase the ingestion, appetite stimulants have been tested in the last years. One appetite stimulant that is currently used in cancer cachectic patients is progesterone derivatives such as megestrol

acetate (MA), which was approved by Food and Drug Administration (FDA) in 1993. In particular, it has been observed that MA improves nutritional status, appetite and caloric intake in many clinical studies, although it has adverse effects [210]. Another potential appetite stimulant is Ghrelin, a growth hormone secretagogue receptor that secreted by the stomach and pancreas [211]. The administration of ghrelin has demonstrated consistent positive results in improving appetite, body and muscle weights [212]. Finally, several appetite stimulants such as cannabinoids and corticosteroids are being evaluated for their beneficial effects in muscle wasting but the adverse effects are important, and thus, they should be carefully tested in more studies or trials [213–215].

A key source to muscle wasting in cachectic patients is the overexpression of several pro-inflammatory cytokines, which makes anti-inflammatory drugs an interesting therapeutic strategy. Although several IL-6, IL-1α and TNF-α inhibitors have demonstrated beneficial effects in muscle mass loss in experimental models, unfortunately, most clinical trials that evaluate anti-cytokines drugs did not obtain positive results. Therefore, more studies are needed to clarify which mechanisms are involved and if they have beneficial effects on muscle mass [216,217].

During the last few years, specific anabolic agents have been developed to block proteolytic pathways, such as targeting myostatin and the ActRIIB pathway, a negative regulator of muscle mass. As mentioned above, myostatin inhibits muscle growth and promote muscle protein loss in cancer cachexia [145]. Therefore, blocking this pathway by the administration of actRIIB has been observed to prevent muscle wasting, ameliorate muscle strength and prolong survival without increase tumor growth in cachectic animals [218–220].

The inhibition of proteolytic pathways is another possible therapeutic strategy. One example is bortezomib, which is a potent proteasome and NF-κB inhibitor, that firstly has been shown beneficial effects in muscle mass in cancer patients [221]. Moreover, previous studies in our group [16] and Penna et al. [222] have demonstrated that bortezomib administration is not able to prevent muscle wasting. In contrast, Sulfasalazine and U0126, an NF-κB and MAPK inhibitors respectively, induced considerable prevention of muscle mass loss and improve muscle strength through a reduction of ubiquitin-proteasome system and autophagy with greater expression in myogenin and contractile and functional muscle proteins [16].

Finally, $beta_2$ agonists have also been studied as a potential anabolic agent in cancer cachexia [223]. Previous investigations reported that $beta_2$ agonist stimulation with formoterol activates guanine nucleotide-binding regulatory protein (Gα), and thus activates adenylate cyclase which converts ATP to cyclic adenosine monophosphate (cAMP) [223]. Therefore, formoterol would be increasing the production of cAMP in the muscles. Moreover, several studies have clearly shown the relevance of cAMP-dependent signaling in the skeletal muscle mass regulation. Binding of cAMP on protein kinase A (PKA) induce cAMP response element binding protein (CREB) phosphorylation, and thus CREB stimulates various transcription factors, which indirectly inhibits myostatin expression [223]. Numerous studies with animal models have shown that the $beta_2$ agonist formoterol improves muscle mass together with preserves muscle function and physical activity [224–227]. These improvements are caused by a reduction in muscle protein degradation and an increase in protein synthesis [225]. Moreover, it is also observed a reduction in myostatin and apoptosis levels that are present in higher levels in cancer cachexia [225,228]. Although there are several studies in animal models of cancer cachexia, only one study with cancer patients has been published. In this specific clinical trial, a

combination of formoterol with megestrol acetate has been administrated in cancer patients and showed improved muscle mass and function [229]. Further investigations to understand the molecular mechanisms and clinical trials with cancer patients are necessary to confirm that formoterol is an effective treatment in cancer cachexia.

5.1.2. Nutritional support

In cancer cachexia, a characteristic feature is that it cannot be fully counteracted by conventional nutritional support [51]. Therefore, nutritional interventions are an important part for the treatment of cancer cachexia and they also are essential within the multimodal strategies [207].

Besides, nutritional approaches providing energy and proteins supplementation could be also representing a potential approach to reduce inflammation and interfere with the molecular mechanisms related in the cancer cachexia pathogenesis. Several compounds like β-hydroxy-β-methylbutyrate (HMB), eicosapentaenoic acid (EPA) and L-carnitine are the most important nutritional supports that have been demonstrated to ameliorate the effects of cancer cachexia [222].

HMB is a metabolite of the branched-chain amino acids (BCAAs) leucine. BCAAs are an integral component of skeletal muscle proteins and their main function is provide energy and is able to initiate signal transduction pathways [230]. In animal models, BCAA leucine supplementation like HMB attenuates proteolysis through down-regulating the activation of FoxO and MuRF-1 levels which controls autophagy and ubiquitin-proteasome system [231,232]. Furthermore, it also enhances protein synthesis through the activation of the mTOR in muscle wasting conditions [233]. Also, it has been demonstrated, that HMB increases lean body mass and muscle strength in cachectic patients [234].

EPA is an omega-3 (ω3)-polyunsaturated fatty acids (PUFA), present in a huge amount in fish oil. It is well reported that PUFA has the ability to reduce either tumor growth as well as muscle wasting [75,207]. In numerous studies, the administration of EPA as a treatment of cancer cachexia has been shown anti-inflammatory properties [233]. Animals and patients studies have demonstrated that EPA ameliorates the levels of pro-inflammatory cytokines and ROS accompanied by the inactivation of NF-κB, and thus the expression of MuRF-1 is reduced [232,235]. Even though clinical trials in the past reported that EPA did not improve the muscle mass of the patients, recent studies have been obtained promising results in cancer cachexia patients especially if it is combined with another agent [236–238].

Finally, promising nutritional support for the treatment of cancer cachexia is L-carnitine. L-carnitine is mostly stored in the muscle and it is necessary for the correct function of the muscle [239]. One of the main functions of L-carnitine is the translocation of long-chain fatty acids from the cytosol into the mitochondria for the following β-oxidation. Furthermore, it is an essential cofactor for the production of acetyl coenzyme A and it also regulates the activity of pyruvate dehydrogenase complex, which is required to trigger the tricarboxylic acid cycle [233,239]. Although in different studies with animals models or patients demonstrated that L-carnitine reduces cachexia improving the body weight and body composition [240,241], just a few studies demonstrated in which molecular mechanisms are involved in L-carnitine supplementation. Liu et al. [242] and Laviano et al. [243] demonstrated that L-carnitine administration reduces the levels of inflammatory cytokines TNF-α and IL-6 and thus, ameliorates cancer cachexia. Moreover, it has also been reported that L-carnitine has the antioxidant capacity and therefore decreases ROS formation and increases glutathione peroxidase levels, leading to a reduction of apoptosis ratio [244]. In addition, L-carnitine is able to modulate proteolytic activity since it has

been shown a down-regulation of proteolytic mRNA expression in a rat model of cancer cachexia [245]. Based on these findings, more studies are needed to confirm which signaling and proteolytic pathways are ameliorated by L-carnitine supplementation.

5.2. Anti-tumoral agents

Over the last years, the diagnostic and treatments of cancer patients have progressed quickly. Besides the surgery, radiotherapy and chemotherapy, nowadays cancer patients also receive adjuvant treatments which are more specific and focused on molecular mechanisms that drive cancer growth and propagation [246]. Currently, immunotherapy is a promising approach to treat several cancers although the effects in the skeletal muscle remain elusive.

5.2.1. Chemotherapy agents

It is well known, that chemotherapy is a systemic treatment and thus, it has more secondary effects than surgery or radiotherapy. One of the secondary effects of chemotherapy is cachexia. Cisplatin-based chemotherapy modulates multiple molecular pathways involved in the regulation of muscle mass. Cisplatin induces the expression of proteolytic pathways and the pro-inflammatory cytokines accompanied by a reduction in the activation of protein synthesis mechanisms [247]. Another chemotherapy agent used in diverse cancers is doxorubicin which also induces adverse effects on skeletal muscle tissue including muscle wasting [248]. To counteract the adverse effects of chemotherapy, it should be necessary to administrate an anti-cachectic treatment, but not effective treatment is nowadays available.

5.2.2. Immunomodulator agents

For many decades, the role of the immune system in cancer was underestimated because tumors suppress immune response by activating negative regulatory pathways or escape of the immune system [249]. Nowadays a strong interest in cancer immunotherapy is emerging as a result of positive results in tumor microenvironment suppression [250]. The therapeutic approaches are the use of agonistic and antagonist immunomodulatory monoclonal antibodies (mAbs) to lymphocyte receptors and/or their ligands to induce a Th1 response and thus, reduce tumor burden [249]. Currently, the two checkpoints which are most present in clinical trials and demonstrate their efficacy are T-lymphocyte protein-4 (CTLA-4) and programmed cell death protein-1 (PD-1)[249,250]. Recent studies have demonstrated that a combination of immunomodulatory mAbs improved the therapeutic efficacy by different mechanisms of action compared to a single mAb administration [251–253]. Dai et al. [251] demonstrated that a combination of four mAbs (PD-1, CTLA-4, CD-137, and CD-19) cause tumor rejection owing to an induce of stronger Th1 response in melanoma and lung cancer mice model. In the current thesis, we assess the effects of the administration of four mAbs combination (PD-1, CTLA-4, CD-137, and CD-19) in body and muscle weight in cancer cachexia mice model.

Hypothesis

Hypothesis

We hypothesized whether the biological events and molecular mechanisms involved in the process of wasting in both diaphragm and gastrocnemius muscles between chronic carcinogenic and cellular syngeneic murine models are similar.

Moreover, we hypothesized whether the $beta_2$ agonist formoterol and the nutritional supplement L-carnitine, two known anti-cachectic therapeutic agents, with no known effects on tumor burden, may improve muscle wasting and cachexia in respiratory and limb muscles through attenuation of oxidative stress and atrophy signaling pathways while improving protein synthesis signaling and muscle phenotype.

Finally, we also hypothesized whether treatment of the tumor with immunomodulators (no known effects on muscles) may favor cachexia and muscle wasting through attenuation of atrophy signaling pathways in both diaphragm and gastrocnemius in lung cancer tumor-bearing mice.

Objectives

Objectives

According to the study hypothesis, the thesis has been divided into five different studies with the following objectives:

Chronic carcinogenic model

Study #1: Phenotypic and metabolic features of mouse diaphragm and gastrocnemius muscles in chronic lung carcinogenesis: influence of underlying emphysema.

In the diaphragm and gastrocnemius muscles of mice exposed to urethane-induced lung cancer (LC) with and without elastase-induced emphysema at two different time-points (20 and 35 weeks):

1.1 To assess total body and muscle weights in both 20- and 35-week cohorts.

1.2 To confirm lung tumor and emphysema by micro computed tomography and lung histology.

1.3 To analyze muscle fiber type composition and morphometry, muscle structural abnormalities and fiber apoptotic nuclei in both 20- and 35-week cohorts.

1.4 To identify protein levels of muscle structure, muscle mass maintenance and metabolic markers in the 35-week cohort.

1.5 To determine molecular mechanisms potentially involved in muscle wasting in the 35-week cohort: redox balance, inflammation, proteasome-system, and autophagy protein markers.

Anti-cachectic therapies

Study #2: Formoterol attenuates increased oxidative stress and myosin protein loss in respiratory and limb muscles of cancer cachectic rats.

In the respiratory and limb muscles of rats bearing the AH-130 Yoshida ascites hepatoma with and without treatment with formoterol beta$_2$ agonist for seven days:

2.1 To assess improvement of total body and muscle weight with formoterol beta$_2$ agonist administration.

2.2 To determine whether formoterol regulates redox balance (prooxidants and antioxidants).

2.3 To evaluate the effects of formoterol on inflammatory markers in muscles.

2.4 To elucidate whether formoterol administration ameliorates the susceptibility of degradation in muscle structural proteins (myosin, actin, CK and carbonic anhydrase-III).

2.5 To analyze the role of the mitochondrial respiratory chain (MRC) in response to formoterol treatment in the diaphragm and gastrocnemius muscles.

Study #3: Effects of the beta$_2$ agonist formoterol on atrophy signaling, autophagy and muscle phenotype in respiratory and limb muscles of rats with cancer-induced cachexia.

In the respiratory and limb muscles of rats bearing the AH-130 Yoshida ascites hepatoma with and without treatment with formoterol beta$_2$ agonist for seven days:

3.1 To assess improvement of total body and muscle weight with formoterol beta$_2$ agonist administration.

3.2 To analyze muscle fiber type composition and morphometry, and structural abnormalities in response to treatment with formoterol.

3.3 To study the molecular signaling pathways (NF-κB, FoxO, and MAPK) that are regulated by the effects of beta$_2$ agonist formoterol.

3.4 To explore the proteolytic systems (proteolysis, autophagy or apoptosis) that are involved in the reduction of muscle mass loss with formoterol treatment.

3.5 To evaluate proteins levels of muscle mass maintenance and metabolic markers in response to formoterol treatment.

Study #4: Differential structural features in soleus and gastrocnemius of carnitine-treated cancer cachectic rats.

In the gastrocnemius (fast-twitch) and soleus (slow-twitch) muscles of rats bearing AH-130 Yoshida ascites hepatoma with and without treatment with the nutritional supplement L-carnitine for seven days:

4.1 To explore whether L-carnitine has any effects on total body and muscle weights.

4.2 To assess whether L-carnitine treatment has any effects on muscle fiber type composition and morphometry, and muscle structural abnormalities between fast- and slow-twitch muscles.

4.3 To determine whether L-carnitine has any effects on redox balance (protein oxidation and antioxidants).

4.4 To explore whether the molecular signaling pathways and proteolytic markers are modulated in response to L-carnitine treatment in fast- and slow-twitch muscles.

Anti-tumor agents

Study #5: Reduced lung cancer burden by selective immunomodulators elicits improvements in muscle proteolysis and strength in cachectic mice.

In the diaphragm and gastrocnemius muscles of mice bearing lung adenocarcinoma (LP07) with and without treatment with a combination of immunomodulators (PD-1, CTLA-4, CD-137, and CD-19):

5.1 To analyze the effects of monoclonal antibodies in the immune microenvironment of the lung adenocarcinoma tumors in mice.

5.2 To assess total body and muscle weights and tumor growth in response to treatment with immunomodulators against the tumor.

5.3 To evaluate metabolic parameters, physical activity and muscle strength in the cachectic mice with and without treatment with the immunomodulators.

5.4 To determine levels of inflammatory, proteolytic and apoptotic markers in blood and in both diaphragm and gastrocnemius

muscles in response to treatment with anti-tumor monoclonal antibodies.

5.5 To study muscle injury and structural alterations in response to treatments with the monoclonal antibodies.

Methods

Methods

The different methodologies that have been used in the five studies of the current thesis are described in the corresponding articles (main text and online suplement). However, a brief summary of those methodologies is also described below.

1. Experimental models

1.1 Experimental design

	Laboratory-bred strain	Cancer cachexia induction	Nº animals /group	Experimental groups
Study #1	A/J mice	Carcinogenic-urethane carcinogenesis induction and emphysema induced by elastase	N=8	(1) Non-exposed control (2) Lung carcinogenesis mice (3) Lung emphysema-carcinogenesis mice
Studies #2 and #3	Wistar rats	Syngeneic-AH-130 Yoshida ascites hepatoma cancer cells injection	N=10	(1) Non-cachexia controls (2) Non-cachexia controls+formoterol (3) Cancer-cachexia (4) Cancer-cachexia +formoterol

Study #4	Wistar rats	Syngeneic-AH-130 Yoshida ascites hepatoma cancer cells injection	N=8	(1) Cancer-cachexia (2) Cancer-cachexia +carnitine
Study #5	Balb/c mice	Syngeneic-LP07 adenocarcinoma cancer cells injection	N=10	(1) Cancer-cachexia (2) Cancer-cachexia +mAbs (anti-PD-1, anti-CTLA-4, anti-CD-137, and anti-CD-19)

1.2 *In vivo* measurements and interventions

	In vivo measurements	Treatments
Study #1	Body weight Muscle weight measurements	
Studies #2 and #3	Body weight Tumor cell content Muscle and tumor weights measurements	0.3 mg/kg/24h of formoterol for 7 days (from day 1 to day 7)
Study #4	Body weight Muscle and tumor weights measurements	1 g/kg/24h of L-carnitine for 7 days (from day 1 to day 7)
Study #5	Body weight Tumor area Muscle and tumor weights measurements Limb strength Metabolic and physical parameters	5×10^{-3} mg/kg/72h of each antibody (anti-PD-1, anti-CTLA-4, anti-CD-137, and anti-CD-19 antibodies) during 15 days (from day 15 to day 30)

1.3 Study period and type of samples

	Study period	Sample collection		
		Muscle	Tumor	Blood
Study #1	20 and 35 weeks	Diaphragm and gastrocnemius	Yes	No
Studies #2 and #3	7 days	Diaphragm and gastrocnemius	Yes	No
Study #4	7 days	Gastrocnemius and soleus	Yes	No
Study #5	30 days	Diaphragm and gastrocnemius	Yes	Yes

2. Molecular biology analyses

2.1 Molecular biology techniques

	Technique	Analyses
Study #1	Immunohistochemistry/ Hematoxylin and eosin staining and optical microscopy	Lung histology Muscle morphometry and composition Muscle damage Muscle apoptotic nuclei
	Immunoblotting of 1D electrophoresis	Muscle contractile and functional proteins Metabolism and regeneration proteins Oxidants and antioxidants Proteolysis Autophagy
	Enzyme-linked immunosorbent assay (ELISA)	Muscle levels of TNF-α and IL-6 cytokines
Study #2	Immunoblotting of 1D	Protein carbonylation

	electrophoresis	Oxidants and antioxidants Muscle contractile and functional proteins
	2D electrophoresis and silver staining	Identification of carbonylated proteins in muscle specimens
	Mass spectrometry	Identification of carbonylated proteins in muscle specimens
	Immunohistochemistry and optical microscopy	Counts of leukocytes (CD-45) and macrophages (CD-68)
	Enzyme activity assay	Mitochondrial citrate synthase and complex I, II, IV activity
Study #3	Immunohistochemistry/ Hematoxylin and eosin staining and optical microscopy	Muscle morphometry and composition Muscle damage Muscle apoptotic nuclei
	Immunoblotting of 1D electrophoresis	Signaling pathways Proteolysis Autophagy Apoptosis Muscle anabolism and metabolism
	TaqMan based qPCR reactions (real-time PCR)	RNA expression of proteolytic genes
Study #4	Immunohistochemistry/ Hematoxylin and eosin staining and optical microscopy	Muscle morphometry and composition Muscle damage
	Immunoblotting of 1D electrophoresis	Carnitine palmitoyltransferase-1 Oxidants and antioxidants Signaling pathways Proteolysis

		Autophagy
Study #5	Enzyme-linked immunosorbent assay (ELISA)	Plasma levels of troponin-I Muscle levels of IL-6
	Tyrosine release assay	Protein catabolism
	Immunohistochemistry/ Hematoxylin and eosin staining and optical microscopy	Tumor immune system Muscle morphometry Muscle apoptotic nuclei
	Immunoblotting of 1D electrophoresis	Proteolysis Apoptosis

3. Statistical analyses

3.1 Statistical test

Study #1	One-way analysis of variance (ANOVA) and *Tukey* post-hoc analysis
Study #2	One-way ANOVA and *Tukey* post-hoc analysis
Study #3	Two-way ANOVA and *Tukey* post-hoc analysis
Study #4	Student's *T-test*
Study #5	Student's *T-test*

Results

Results

Summary of the main findings reported in this thesis:

In cancer cachectic rodents (chronic carcinogenic and cellular syngeneic models) compared to non-cachectic control animals:

- Total body and muscle weights (diaphragm and gastrocnemius) were decreased.
- Fiber cross-sectional area and myosin protein content were reduced in both respiratory and limb muscles.
- The proportion of structural abnormalities was increased in both respiratory and limb muscles.
- Protein levels of oxidative stress, inflammation, atrophy signaling, autophagy and apoptosis were greater in diaphragm and gastrocnemius muscles.
- Underlying emphysema induced a larger decrease in total body and muscle weights together with a greater reduction in diaphragm cross-sectional areas.

Effects of treatment with $beta_2$ agonist formoterol on muscles:

- Formoterol treatment induced an increase in total body and muscle weights.
- Formoterol treatment increased fiber cross-sectional area and reduced the proportion of muscle structural abnormalities in diaphragm and gastrocnemius muscles.
- Oxidative stress, atrophy signaling markers, autophagy and apoptosis were attenuated in diaphragm and gastrocnemius muscles in response to formoterol treatment.

Effects of treatment with L-carnitine on muscles:

- Total body and gastrocnemius and soleus muscle weights were increased in response to L-carnitine administration.
- L-carnitine treatment induced an increase in fiber cross-sectional area and reduced the proportions of muscle structural abnormalities in both limb muscles.
- Oxidative stress, atrophy signaling pathways, ubiquitin-proteasome system, and apoptosis were attenuated in gastrocnemius muscles in response to L-carnitine treatment.

Effects of treatment with anti-tumor monoclonal antibodies on muscle function and structure:

- Monoclonal antibodies induced a reduction in tumor size, while inducing a rise in total body weight and in function (grip strength).
- Muscle fiber area improved in the diaphragm and gastrocnemius muscles as a result of treatment with the anti-tumor monoclonal antibodies.
- Systemic troponin-I, tyrosine release, proteolytic, and apoptosis markers were attenuated in blood and in both diaphragm and gastrocnemius muscles in response to treatment with the anti-tumor monoclonal antibodies.

Study #1

Phenotypic and metabolic features of mouse diaphragm and gastrocnemius muscles in chronic lung carcinogenesis: influence of underlying emphysema.

Published article: Salazar-Degracia A, Blanco D, Vilà-Ubach M, de Biurrun G, de Solórzano CO, Montuenga LM, Barreiro E. Phenotypic and metabolic features of mouse diaphragm and gastrocnemius muscles in chronic lung carcinogenesis: influence of underlying emphysema. *J Transl Med*. 2016 Aug 23;14(1):244. doi: 10.1186/s12967-016-1003-9.

Study #2

Formoterol attenuates increased oxidative stress and myosin protein loss in respiratory and limb muscles of cancer cachectic rats.

Published article: Salazar-Degracia A, Busquets S, Argilés JM, López-Soriano FJ, Barreiro E. Formoterol attenuates increased oxidative stress and myosin protein loss in respiratory and limb muscles of cancer cachectic rats. *PeerJ*. 2017 Dec 13;5:e4109. doi: 10.7717/peerj.4109. eCollection 2017.

Study #3

Effects of beta$_2$ agonist formoterol on atrophy signaling, autophagy and muscle phenotype in respiratory and limb muscles of rats with cancer-induced cachexia.

Published article: Salazar-Degracia A, Busquets S, Argilés JM, Bargalló-Gispert N, López-Soriano FJ, Barreiro E. Effects of the beta$_2$ agonist formoterol on atrophy signaling, autophagy, and muscle phenotype in respiratory and limb muscles of rats with cancer-induced cachexia. *Biochimie*. 2018 Jun;149:79-91. doi: 10.1016/j.biochi.2018.04.009. Epub 2018 Apr 12

Study #4

Differential structural features in soleus and gastrocnemius of carnitine-treated cancer cachectic rats.

Under review article: Salazar-Degracia A, Busquets S, Argilés JM, Serpe R, Pérez-Peiró M, Rojano-Toimil A, López-Soriano FJ, Barreiro E. Differential structural features in soleus and gastrocnemius of carnitine-treated cancer cachectic rats. *J Cell Physiol 2020 Jan 26;235(1):526–37. DOI: 10.1002/ jcp.28992*

Study #5

Reduced lung cancer burden by selective immunomodulators elicits improvements in muscle proteolysis and strength in cachectic mice.

Published article: Salazar-Degracia A, Granado-Martínez P, Millán-Sánchez A, Tang J, Pons-Carreto A, Barreiro E. Reduced lung cancer burden by selective immunomodulators elicits improvements in muscle proteolysis and strength in cachectic mice. *J Cell Physiol.*

2019 Aug 9;234(10):18041–52. DOI: 10.1002/jcp.28437

Discussion

Discussion

In the present thesis, the hypothesis has been confirmed to a great extent. The two experimental models of chronic carcinogenic (urethane) and cellular-induced syngeneic (AH-130) cancer cachectic models have been well-validated. Although in both models have similarities in the phenotype, some differences are observed in the molecular mechanisms that trigger the protein breakdown. The administration of $beta_2$ agonist formoterol or the nutritional supplement L-carnitine attenuated muscle mass loss, while had no significant effects on tumor burden in AH-130 cancer cachectic models. These two antic-cachectic treatments reduced muscle wasting through the attenuation of oxidative stress and atrophy signaling pathways. On the other hand, the reduction of tumor burden with selective monoclonal antibodies attenuated muscles mass loss through a decreased in the proteolytic and apoptosis mechanisms in LP07 cancer cachectic animals. The most relevant findings observed in the thesis are discussed in more detail below.

Biological events involved in muscle mass during cancer cachexia

Muscle wasting and its dysfunction are two common features of cancer cachexia, which lead to muscle mass loss and to a progressive functional impairment of them [40,51]. In urethane-chronic carcinogenic and Yoshida AH-130 ascites hepatoma cancer cachexia models, muscle wasting was confirmed by the reduction of total body and muscle weights. These findings are in agreement with those reported in previous investigations, in which total body and muscle weights were reduced in these cancer cachexia models [100,208,254,255]. Interestingly, similar phenotypes were observed in the respiratory and limb muscles in the two cancer cachectic models. We observed a reduction in the cross-sectional area of slow- and fast-twitch

fibers in the studied muscles, but no differences in the fiber type proportions were found. Together with a reduction of fiber size, diminished levels of myosin protein were observed in all muscles of cancer cachexia models. Nonetheless, previous studies with advanced cachectic patients (LC and COPD) demonstrated a reduction in the size of fast-twitch fibers and a shift in fiber type composition toward a lower resistant phenotype in the limb muscles. In contrast, no differences were observed in the respiratory muscle of cachectic patients [32,34,256]. Furthermore, a novelty of this thesis is that in chronic carcinogenic model underlying emphysema induced a larger reduction in muscle fiber size in the diaphragm muscle than in gastrocnemius. Other remarkable findings in this thesis are the rise in proportions of muscle structural abnormalities and apoptotic nuclei in the chronic carcinogenic and AH-130 cellular syngeneic cancer cachectic models, as demonstrated in previous studies with experimental models [16,87,257] and LC and COPD patients [65,202]. In view of these results, we suggest that cancer cachexia experimental models present a similar phenotype between muscles, but are less fatigue-resistant than humans, especially in the diaphragm, due to their fiber composition.

Oxidative stress has been linked to muscle wasting, modulating the different signaling pathways that contribute to muscle atrophy [103,190]. Several investigations have consistently reported high levels of protein oxidation in cancer-induced cachectic animals [87–89] and patients [65]. The results obtained in the current thesis are in line with these studies, as levels of oxidative stress and protein carbonylation were increased only in the limb muscles of both study models. Besides, we observed alterations in the antioxidants levels, so these results may suggest that antioxidants activation may be inefficient and insufficient to reduce oxidative stress. Otherwise, no differences in oxidative stress and antioxidant markers were detected in the diaphragm muscles. The lack of oxidative stress present in

the diaphragm muscle compared to gastrocnemius could be explained by a better antioxidant system. Although no differences were observed in the studied antioxidants, other antioxidants such as glutathione peroxidase (GPx) could be regulating oxidative stress in the diaphragm, which exhibits high GPx levels than hindlimbs muscles [258,259]. Additionally, it has been described that rat diaphragm is an oxidative skeletal muscle that has greater levels of oxidative enzymes, and thereby it better reduces ROS production compared to glycolytic muscles [260,261].

Oxidative stress and inflammation trigger muscle wasting by the activation of signaling pathways [110]. A novel finding in this thesis is that NF-κB and FoxO pathways together with the myostatin system may be the main mechanisms involved in muscle mass loss in AH-130 cellular syngeneic cancer-induced cachexia model. These results are in accordance with previous studies conducted on cachectic patients [65,262], and in experimental models of cachexia [16,87,263]. Furthermore, the present thesis showed greater levels of total protein ubiquitin and 20S proteasome in both muscles of the syngeneic cachectic model, while no differences were observed in the chronic carcinogenic model. Nonetheless, MuRF-1, atrogin-1, and TRIM32 protein levels did not differ between study groups. In line with our results, previous investigations showed that E3 ubiquitin ligases levels did not differ in the LP07 cancer cachexia model [16,87] and lung cancer cachectic patients [262]. On the other hand, previous data reported a link between the activation of NF-κB and high levels of E3 ligases MuRF-1 and atrogin-1 in cancer cachexia [254,257,264]. It seems that these discrepancies can be given by the rapid development of tumor and muscle mass loss together with the differences in the experimental models [96].

Related to atrophy signaling, it was reported that the activation of FoxO3 induces autophagy in skeletal muscle [189]. This was observed in earlier

studies in which autophagy was induced in LP07 cancer cachexia experimental models [16] and cachectic patients [65,265]. In line with previous findings, we observed that levels of autophagy were increased in both muscles, especially in the diaphragm of urethane-chronic carcinogenic and AH-130 cancer cachectic models. Moreover, we observed that the levels of BAX were increased especially in the diaphragm of syngeneic cancer-cachectic rodents. Taken together, it seems that together with autophagy, apoptosis would also be one of the main mechanisms of muscle wasting in the study muscles.

In the syngeneic cancer cachexia model, the downregulation of muscle anabolism also contributes to the process of muscle wasting. These results are in line with those reported in previous studies in cancer cachexia models, where they observed a decrease in anabolic response [87,266]. Several factors such as inflammatory cytokines (TNF-α and IL-6) and myostatin may appear to contribute to the reduction of muscle anabolism during cancer cachexia. Previous investigations showed that TNF-α and IL-6 were enhanced [267,268] and they have been implicated to impair the anabolic response in the cancer cachexia experimental model [269]. On the other hand, the upregulation of myostatin during cancer cachexia can be impairing the anabolic response. These effects were attributed to the decrease of Akt phosphorylation, which could inhibit the rate of protein synthesis [147,270,271].

Therapeutic approaches in cancer-induced cachexia

Based on the mechanisms involved in muscle wasting in cancer cachexia, in the present thesis formoterol and L-carnitine were individually studied in cancer cachexia animal models. The $beta_2$ agonist formoterol or nutritional supplement L-carnitine did not elicit any significant effects on tumor burden, indicating that the muscular improvement would be given by direct effects of treatment towards muscle.

The administration of beta$_2$ agonist formoterol reverts the muscle mass loss, increasing the total body and muscle weights in cancer cachexia condition. Our results are in accordance with previous investigations that have shown an improvement of muscle mass in control animals [272], cancer cachexia [224,225,264] and other pathological conditions like sepsis [273] and rheumatoid arthritis [274] when they are treated with formoterol. In fact, apart from recovery muscle mass, previous studies observed that formoterol ameliorates the muscle function improving the physical activity and the grip force, and it also increased food intake in cancer cachexia animal models [224,264,273]. Related to the recovery of muscle mass, a relevant novel finding was the improvement of muscle fiber size and the increase of myosin protein in both respiratory and limb muscles of AH-130 cancer-induced cachexia treated with formoterol. Moreover, structural abnormalities and apoptotic nuclei were diminished in both treated muscles. These results are in agreement with a previous investigation that showed an increase in muscle fiber size with formoterol treatment in age-related rats [275].

As far as we are concerned, the present thesis is the first to report the effects of formoterol treatment towards oxidative and nitrosative stress in skeletal muscle. We revealed an attenuation of oxidative and nitrosative stress accompanied by a reduction in protein carbonylation in the limb muscles treated with formoterol. Previous studies have described that myostatin is capable of induce oxidative stress and the formation of ROS through TNF-α and NF-κB signaling [110]. Moreover, other studies have shown a reduction in myostatin expression in response to formoterol treatment in control animals [272] or cachectic conditions [228,274]. In line with this, the present thesis corroborates the reduction of myostatin with formoterol treatment. Besides, our results showed a reduction in the activation of NF-κB in the limb muscles in response to formoterol treatment. These results are in accordance with previous reports that also showed a reduction in NF-

κB activity with formoterol administration in different cachectic conditions [273,274,276], although Busquets et al. [225] did not detect differences in the DNA-binding activity of NF-κB in cancer-cachectic rats.

Several studies described that formoterol administration reduces protein degradation by the inactivation of the ubiquitin-proteasome system in cancer cachexia [225,264], sepsis [273], and rheumatoid arthritis [274]. However, this thesis shows that formoterol treatment did not reduce the ubiquitin-proteasome pathway in cancer cachexia. Indeed, a novelty in this thesis was that formoterol blocks the autophagy and apoptosis. Interestingly, our results showed that formoterol administration inhibits LC3B protein content in both respiratory and limb muscles in cancer cachexia. In line with this, previous studies also described a reduction in autophagy levels with formoterol treatment in cachexia conditions [273,274] and control animals [272]. Moreover, it has been shown that myostatin could negative regulate protein synthesis by Akt signaling and activate FoxO, which is responsible for the induction of autophagy [147]. Our results suggest that autophagy could be inactivated by another mechanism, as no differences were observed in Akt and FoxO signaling in cancer cachectic animals treated with formoterol. It is interesting to point out that formoterol reduces apoptosis in cancer cachexia as demonstrated in this thesis and in a previous investigation [225].

The other therapeutic approach, object of study in this thesis, to counteract muscle wasting was the nutritional suplement with L-carnitine. Their potential beneficial effects on muscle mass loss and function were shown in several studies on animals [245,277], but also in humans [278–281]. Nonetheless, few of them demonstrate which molecular mechanisms are underlying the amelioration of muscle mass loss.

In this thesis, we have corroborated that L-carnitine reduces total body and muscle weights lost. Moreover, the administration of L-carnitine

ameliorated muscle fiber size and diminished the muscle structural abnormalities and apoptotic nuclei in limb muscles. These results are in accordance with previous studies that have shown a significant improvement in muscle mass with L-carnitine supplementation, and this was correlated with a significant increase in physical activity, grip force and food intake [242,245,282]. Furthermore, we observed a decrease in lipid peroxidation in the gastrocnemius muscles with L-carnitine treatment. This result is in accordance with previous investigations that reported an attenuation of lipid peroxidation in the muscles of amyotrophic lateral sclerosis [283] and in rats subjected to hypoxia [284] in response to L-carnitine treatment.

A Relevant finding observed in this thesis was a reduction in FoxO3 levels together with levels of ubiquitinated proteins and in E3 ubiquitin ligases MuRF-1 and atrogin-1 in the gastrocnemius muscles with the administration of L-carnitine in cachectic animals. These findings are supported by the previous investigation, in which it was found that ubiquitin and MuRF-1 gene expressions were reduced in gastrocnemius muscles of cachectic animals treated with L-carnitine [245]. In contrast, no differences in proteolytic systems were observed in soleus muscles of cancer cachexia animals in response to L-carnitine administration, although in other cachectic conditions, L-carnitine plays a key role in proteolytic systems [282,285]. In line with this, a previous study revealed a reduction in gene expression of E3 ubiquitin ligases in the soleus muscles in the hind limb suspension model [282]. These differences in the results could be given by the fact that the cachexia condition is different and by the duration of the cachectic state. On the other hand, we did not attempt to analyze apoptotic markers in the cachectic animals treated with L-carnitine, but Busquets et al. [245] reported that L-carnitine attenuates apoptosis by a reduction in the mRNA expression of caspase-3 in the gastrocnemius muscles in the same

cancer cachexia rat model. All these findings lead to the conclusion that L-carnitine can modulate the proteolytic activity and apoptosis in gastrocnemius muscle in cancer cachexia.

Nowadays, the first purpose in cancer cachexia patients must be to treat primary tumors and secondly, it must be to complement them with anti-cachectic strategy. It was described that the prevalence of cachectic patients is directly correlated with mortality rates [56]. Therefore, it is important to evaluate the effects of cancer therapies on skeletal muscle. During the last years, several investigations have evidenced the effects of chemotherapy [286–288] or the tumor resection by surgery [289,290] in the skeletal muscle, but the impact of adjuvant treatments such immunotherapy on the muscle has not been studied.

As far as we are concerned, the present thesis is the first to report the effects of a combination of immunotherapy to reduce tumor burden on muscle wasting. We corroborated a reduction in tumor burden after the administration of monoclonal antibodies recognizing PD-1, CTLA-4, CD-137 and CD-19 in LP07 lung adenocarcinoma Balb/c mice, as it was demonstrated in previous investigation with various carcinogenic models [251]. A novelty in this thesis was that a reduction in tumor burden by the immunomodulators attenuates muscle wasting. It seems that the administration of these monoclonal antibodies does not exert any significant direct effects on skeletal muscles, and the improvement of muscle may be a result raised from the decline in tumor burden.

As shown in this thesis, total body weight and grip force were significantly increased, while muscle weights did not differ in tumor-bearing mice treated with monoclonal antibodies compared to non-treated mice. Moreover, food intake and physical activity did not differ between animals.

Therefore, these results suggest that the amelioration of total body weight and grip force is not attributed to nutritional support or physical activity. In addition, the reduction of tumor burden by immunomodulators increased muscle fiber size and decreased apoptotic nuclei. Levels of systemic troponin-I, tyrosine release, ubiquitin-proteasome system, and apoptosis were increased in cancer cachexia animals [16,87], which were reduced by the treatment with monoclonal antibodies. Altogether, the data suggest that despite having an improvement of fiber size through a reduction in the levels of proteolysis and apoptosis, this is not enough to observe significant results in the improvement of muscle weight. It would probably take more time to see significant changes in muscle weight, so further studies are needed.

Taken together, the results obtained in this thesis have been shown that $beta_2$ agonist formoterol and nutritional supplement L-carnitine reverse muscle wasting by reducing the protein degradation. Moreover, immunotherapy did not accentuate muscle wasting in cancer cachexia rodents as chemotherapy or tumor resection. Therefore, it is important to observe the effects of anti-tumoral agents on the muscles, in order to select the correct anti-cachectic strategy. The immunotherapy accompanied by an anti-cachectic treatment could present beneficial effects on tumor and muscles in cancer patients.

Conclusions

Conclusions

1. Cancer cachexia is present in a similar fashion in both respiratory and peripheral muscles of that share phenotypic characteristics in the two studied models.

2. Cellular syngeneic and chronic carcinogenesis models haven been well-validated to induce experimental cancer cachexia.

3. Pharmacological strategies such as $beta_2$ agonist formoterol and nutritional supplement L-carnitine induced beneficial effects in the total body and muscle weights, muscle performance and structure, and in muscle biology as a result of the attenuation of mechanisms involved in increased muscle protein degradation and signaling.

4. Reduction of tumor burden in response to immunotherapy of lung cancer tumors in mice exerted beneficial effects on both respiratory and limb muscles as a result of attenuation of enhanced muscle proteolysis, and improvement of muscle structure and performance.

5. Cancer cachexia should be therapeutically targeted with anti-cachectic agents and by treating the primary tumors.

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Future perspectives

Future perspectives

Future research should aim to explore whether formoterol has any beneficial effects on slow-twitch muscles such as soleus and which biological mechanisms are involved. Otherwise, it would be interesting to study the diaphragm muscle of cancer cachexia rats with the treatment of L-carnitine, because despite of being fast-twitch muscle such as gastrocnemius, previous studies have shown differences in molecular pattern. Besides, future experiments should investigate whether a multimodal therapeutic approach with a combination of formoterol and L-carnitine could have a synergistic effect on muscle wasting in cancer cachexia animal models.

Moreover, future studies should also focus on investigating which signaling pathways are responsible for attenuation of ubiquitin-proteasome system and apoptosis in muscles of cancer cachexia mice treated with monoclonal antibodies, which could help to understand how the reduction of tumor burden may impact on the attenuation of muscle wasting. As only a trend of improvement in muscle weights has been found with the immunomodulators treatment, the time period of the next study would have to be longer than 30 days to observe if there are significant differences in muscle weights.

In addition, future investigations should be based on exploring the beneficial effects on muscles with polyadenosine diphosphate-ribose polymerase (PARP) inhibitors tumor treatment, due to it was observed that the PARP1 and PARP2 knockout mice has an attenuated muscle wasting in cancer cachexia models.

Finally, further research aim should be to explore the potential contribution of muscle regeneration in experimental models of cancer cachexia, as it was

observed that the regenerative ability of skeletal muscle is disturbed. Therefore, exploring the number of satellite cells and their state of activation or quiescence could help to determine the regenerative capacity of muscles, and to investigate new therapeutic approaches against cancer cachexia such as resveratrol and curcumin.

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resection on skeletal muscle mass and protein turnover in colorectal cancer patients. *Am. J. Clin. Nutr.* **96,** 1064–1070 (2012).

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Addendum

Addendum

Publications

During my formative stage in the Muscle Wasting and Cachexia in Chronic Respiratory Diseases and Lung Cancer Research group, under the supervision of Dr. Esther Barreiro, I have had the opportunity to collaborate in other investigations that have also been published in international journals:

Barreiro E, **Salazar-Degracia A,** Sancho-Muñoz T, Aguilo R, Rodriguez-Fuster A, Gea J. Endoplasmic reticulum stress and unfolded protein response in the diaphragm of Chronic Obstructive Pulmonary Disease patients.
J Appl Physiol (1985). *(under review)*
IF: 3.256, Q2 in physiology

Barreiro E, Sancho-Muñoz T, Puig-vilanova E, **Salazar-Degracia A**, Pascual-Guardia S, Casadevall C, Gea J. Differences in microRNA expression profile between vastus lateralis samples and myotubes in COPD cachexia.
J Appl Physiol (1985). 2019 Feb 1;126(2):403-412.
IF: 3.256, Q2 in physiology

Barreiro E, **Salazar-Degracia A**, Sancho-Muñoz T, Gea J. Endoplasmic reticulum stress and unfolded protein response profile in quadriceps of sarcopenic patients with respiratory diseases.
J Cell Physiol. 2018 Nov 22.
IF: 3.923, Q1 in physiology

Barreiro E, Puig-vilanova E, **Salazar-Degracia A**, Pascual-Guardia S, Casadevall C, Gea J. The phosphodiesterase-4 inhibitor Roflumilast reverts proteolysis in skeletal muscle cells of patients with COPD cachexia.

J Appl Physiol (1985). 2018 Aug 1;125(2):287-303.
IF: 3.256, Q2 in physiology

Paul T, **Salazar-Degracia A**, Peinado VI, Tura-Ceide O, Blanco I, Barreiro E, Barberà JA. Soluble guanylate cyclase stimulation reduces oxidative stress in experimental Chronic Obstructive Pulmonary Disease.
PLoS One. 2018 Jan 5;13(1):e0190628.
IF: 2.766, Q1 in multidisciplinary sciences

Barreiro E, Puig-Vilanova E, Marin-Corral J, Chacón-Cabrera A, **Salazar-Degracia A**, Mateu X, Puente-Maestu L, García-Arumí E, Andreu AL, Molina L. Therapeutic approaches in mitochondrial dysfunction, proteolysis, and structural alterations of diaphragm and gastrocnemius in rats with chronic heart failure.
J Cell Physiol. 2016 Jul;231(7):1495-513.
IF: 4.08, Q1 in physiology

Communications

Some of the results, which I have collaborated, have been presented in the form of an abstract (either poster or oral communication) in national and international conferences.

1. Tang J, Yélamos J, Ampurdanès C, **Salazar-Degracia A**, Wang X, Pijuan L, Curull V, Barreiro E. Expression of PARP in lung cancer of patients with and without COPD and in mice with experimental lung tumors. III Research based on pulmonology conference, Terrassa, Spain, April 2019.

2. Tang J, **Salazar-Degracia A**, Ramis-Cabrer D, Barreiro E. Oxidative stress as a mediator of the immunomodulation exerted by monoclonal antibodies

in the treatment of lung cancer in mice. III Research based on pulmonology conference, Terrassa, Spain, April 2019.

3. Tang J, Yélamos J, Ampurdanès C, **Salazar-Degracia A**, Wang X, Pijuan L, Gea J, Curull V, Barreiro E. Differential expression of PARP in lung cancer of patients with COPD and in mice with experimental lung tumors. Barcelona lung conference Boston, Barcelona, Spain, January 2019.

4. Tang J, Yélamos J, Ampurdanès C, **Salazar-Degracia A**, Wang X, Pijuan L, Gea J, Curull V, Barreiro E. Differential expression of PARP in lung cancer of patients with COPD and in mice with experimental lung tumors. XI CIBERES and CIBER-ONC conference sessions, Madrid, Spain, November 2018.

5. **Salazar-Degracia A**, Iczi-M, Barreiro E. Differential profile of muscle regeneration and regulatory mechanisms in respiratory and limb muscles of lung cancer cachectic mice. 4th cancer cachexia conference, Philadelphia, USA, September 2018.

6. Barreiro E, **Salazar-Degracia A**, Sancho-Muñoz A, Gea J. Genomic profile of endoplasmic reticulum stress and unfolded protein response in quadriceps of cachectic patients with respiratory diseases. 4th cancer cachexia conference**,** Philadelphia, USA, September 2018.

7. Barreiro E, **Salazar-Degracia A**, Puig-Vilanova E, Gea J. Expression profile of the unfolding protein response in the vastus lateralis of patients with respiratory cachexia: advanced COPD versus lung cancer. International Union of Physiological Societies (IUPS) 38th World Congress, Rio de Janeiro, Brasil, August 2017.

8. Barreiro E, **Salazar-Degracia A**, Puig-Vilanova E, Gea J. Activation of the unfolding protein response in the vastus lateralis of patients with

respiratory cachexia: advanced COPD versus lung cancer. American Thoracic Society 2017, International Conference, Washington, USA, May 2017.

9. Barreiro E, Pascual-Guardia S, Puig-Vilanova E, **Salazar-Degracia A**, Casadevall C, Gea J. Muscle-specific microRNA expression differs in vastus lateralis specimens from that of myotubes in COPD patients with cachexia. American Thoracic Society 2017, International Conference, Washington, USA, May 2017.

10. Balaña A, Martínez-Llorens J, Agustin-Rodríguez D, Admetlló M, **Salazar-Degracia A**, Ausin P, Barreiro E, Gea J. Skeletal muscle function and structure in patients with non-cystic fibrosis bronchiectasis. ERS International Congress 2016, London, United Kingdom, September 2016.

11. Paul T, **Salazar-Degracia A**, Peinado V, Cortes M, Ferrer E, Puig-Vilanova E, Tura O, Blanco I, Barreiro E, Barberà JA. Effects of Soluble Guanylate Cyclase (sGC) stimulation on extrapulmonary oxidative stress in guinea pigs chronically exposed to cigarette smoke. American Thoracic Society 2016, International conference, San Francisco, USA, May 2016.

12. Barreiro E, Pascual-Guardia S, Puig-Vilanova E, **Salazar-Degracia A**, Casadevall C, Gea J. Do differences exist in microRNA expression profile between vastus lateralis and myotues in COPD cachexia? 15th Symposium COPD, Barcelona, Spain, April 2016.

13. Paul T, **Salazar-Degracia A**, Peinado V, Barreiro E, Barberà JA. Stimulation of soluble guanylate cyclase (sGC) in guinea pigs chronicaly exposed to cigarette smoke attenuates oxidative stress in plasma. VIII CIBERES conference sessions, Valladolid , Spain, October 2015.