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Production and in vivo evaluation of a new potential anti-cachexia drug based on Hsp60-containing nanovesicles

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INTRODUCTION

Cachexia is a multifactorial metabolic disorder it is associated with reduced physical function, reduced tolerance to anticancer therapy, and reduced survival (Fearon K. et al., 2011; Stubbins R. et al., 2020). Has been recognized that the onset and course of cachexia are always linked to the presence of serious underlying pathological conditions like; chronic diseases (Yoshida T et al., 2015), cancer (Wolf I et al., 2006; Fearon K. et al., 2011; Kasprzak A 2021), malnutrition, heart disease, AIDS (Chang HR et al., 1998), multiple sclerosis (Burfeind KG et al., 2016), other progressive and degenerative diseases (Wang YX. et al., 2014), including autoimmune (An HJ et al., 2020). The prevalence of cachexia is as high as 87% in patients with pancreatic and gastric cancer, and 61% in patients with colon, lung, and prostate cancer (Dewys W.D. et al., 1980; Ni J. et al., 2020). The adverse effects of cachexia cannot be completely mitigated through conventional nutritional support alone, resulting in gradual functional deterioration (Fearon K. et al., 2011; Maccio A. et al., 2021). Recent research indicates that exercise can partially preserve muscle mass in various cachectic conditions. This preservation occurs not only due to exercise's anti-inflammatory and antioxidant properties but also its capacity to modulate autophagy (Bowen TS et al., 2015; Pigna E. et al., 2016). Increased physical activity protects skeletal muscle from atrophy, and has been observed to stimulate Peroxisome-activated receptor gamma coactivator 1-alpha (PGC-1α) expression, which promotes fiber-type switching from glycolytic toward more oxidative fibers (Sandri M. et al., 2006). PGC-1α is also directly involved in the mitochondrial biosynthesis of muscle cells and this activity is regulated by heat shock protein 60 (Hsp60), which increases during physical activity and activates PGC1α (Barone R. et al., 2016). Furthermore, scientific evidence demonstrates that following physical training, the promotion of myogenesis is observed in adults (Ultimo S. et al.,2018; Sailani MR. et al., 2019; Chen J. et al., 2022). Myo-D activity contributes to the upregulation of the expression of several miRNAs during myogenesis (Ultimo S. et al., 2018), such as miR152-3p, an inhibitor of the proliferation of mesenchymal stem cells (Msc) which contributes to the differentiation of myoblasts (Gan M. et al., 2018; Dalle Carbonare L. et al., 2022;). Myo-D is one of the main proteins involved in the differentiation of satellite cells (SC) that participate in the formation of new muscle cells (Kong Y. et al., 1997; Macaluso F. et al., 2012; Dalle Carbonare L. et al., 2022).

In pathological conditions such as cachexia, an alteration of the pathways that regulate the expression of proteins involved in muscle homeostasis leads to an imbalance towards atrophy, mainly due to the activity of the forkhead transcription factor (FoxO) (Sandri M. et al., 2004; Reed SA, et al., 2012; Kang SH. et al., 2017), the latter is also involved in the inhibition of myogenesis (Kitamura T. et al., 2007). To date, several drugs have been used in preclinical models in the treatment of cachexia, but only anamorelin is used as a drug in phase 3 studies (Garcia JM. et al., 2013; Currow D et al., 2017). Despite these advances, there are still no effective treatments to counteract cachexia. Resistance training so far is the main strategy to promote muscle mass gain in cachexia conditions (Haredee JP. et al., 2017; Cortiula F. eta al., 2022). In accordance with these findings, the aim of this study is to produce, and evaluate the activity of Hsp60-containing nanovesicles obtained from a conditioned medium (Physiactisome (PHY)), to be used as a treatment in trained (TR) and sedentary mice (SED), inoculated with colon cancer fragments (C26) for develop cachexia. This strategy could be relevant to the biotechnology and pharmaceutical sectors, as it provides a process for the in vitro preparation of vesicles containing the heath shock protein Hsp60, which can be used to prepare pharmaceutical compositions.

1.1 Extracellular Vesicles (EVs)

In recent decades, there has been an increasing interest in the development of new and effective biopharmaceutical products for drug delivery in the field of rehabilitation and diagnosis (Herrmann I.K. et al. 2021; Bhatia S., 2016). One area of research aimed at improving these applications is the methodology based on extracellular vesicles (EVs). EVs are structures enclosed by a phospholipid bilayer that are released by almost all cell types under both physiological and pathological conditions, including immune responses, tissue regeneration, and cancer progression (Bhatia S., et al., 2016). Originally thought to be merely waste products excreted by cells, EVs carry a number of biological cargoes such as nucleic acids, lipids, and proteins that may have both metabolic significance and signaling potential, and are therefore now recognized as key components in cell-cell communication (Kalra H., 2016; Doyle L.M. et al., 2019; Zaborowski M.P. et al., 2015). EVs are released in body fluids and have been observed in saliva, plasma, tears, and cerebrospinal fluid (Neri T., et al., 2022; Cai J. et al., 2022; Ullah L. et al., 2022). Technological advancements have revolutionized the field of EV research. The development of novel isolation and purification methods, such as ultracentrifugation, density gradient centrifugation, and size-exclusion chromatography (SEC), has enabled researchers to obtain highly pure EV populations for downstream analysis (Akbar A. et al., 2022; Zhao Z. et al., 2021). Furthermore, the rise of advanced imaging methods such as electron microscopy and super-resolution microscopy has enabled the observation of EVs (Rikkert L.G. et al., 2019) and their interactions with recipient cells, offering valuable insights into the ways they function. Lately, there has been a growing emphasis on the potential medical uses of EVs. Scientists have investigated their utility as diagnostic indicators for different conditions, like cancer, neurodegenerative disorders, and cardiovascular diseases (Ciferri MC. Et al., 2021). Moreover, the potential of EVs for therapy has captured considerable interest, as research has shown their capacity to transport therapeutic cargoes to specific cells and influence the advancement of diseases (Sanz-Ros J. et al., 2023). The nomenclature and classification of extracellular vesicles (EVs) have been the subject of extensive discussion and refinement within the scientific community, intending to establish a standardized and comprehensive framework. Various classification systems and terminologies have been proposed based on different criteria, including biogenesis pathways, size, and cargo composition. The International Society for Extracellular Vesicles (ISEV) has played a pivotal role in promoting standardized

nomenclature and classification guidelines. EVs are released from cells in a variety of ways, and although their nomenclature is still controversial (Théry C. et al., 2018), are generally categorized into three main subtypes (**fig.1**): microvesicles (MVs, large EVs: 100 – 1000 nm diameter), exosomes (EXs, small EVs: 30 – 150 nm diameter), and apoptotic bodies (ApoBDs: 1000-5000 nm diameter), which are distinguished based on their biogenesis, release pathways and size (Yáñez-Mó M, et al., 2015; Doyle LM. Et al., 2019; Ullah L. et al., 2022). In this study, we will talk about nanovesicles to refer to the large and small extracellular vesicles.

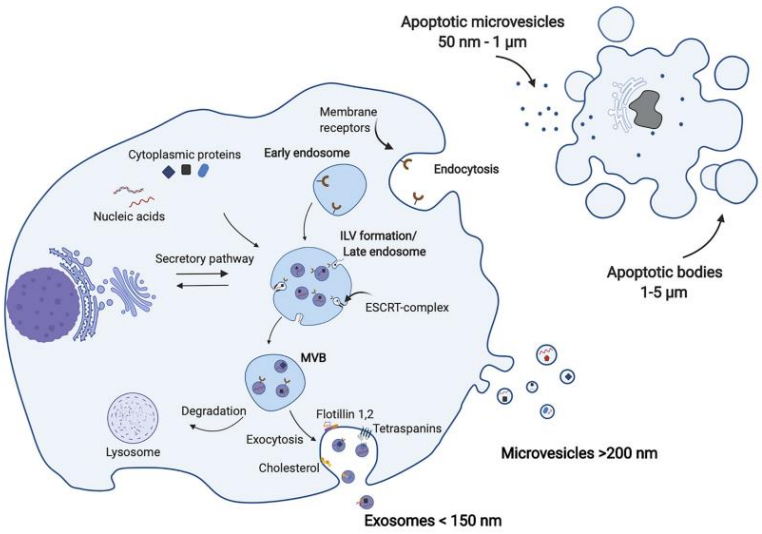

Figure 1 Extracellular vesicles are produced through several cellular processes, including the budding of the plasma membrane (microvesicles), the release of intraluminal vesicles (ILV) for exosomes production, and apoptotic cell disassembly (apoptotic bodies). (Brakhage A.A. et al., 2021)

1.1.1 Apoptotic bodies (ApoBDs) biogenesis

Based on the different biogenesis, and different release mechanisms, it has been observed that the ApoBDs are produced during the late-stage apoptosis process which is an active process that occurs in normal cell turnover to remove many cells every single day, an adult human loses about 50 billion cells per day on average (Raj D. et al., 2006). ApoBDs are the largest subclass of EVs, they typically have a diameter range of 1000-5000 nm (**fig.2**) (Shpacovitch V. et al., 2017). They typically contain cytosolic contents including proteins, RNA, and fragmented DNA, as well as cellular organelles (Kakarla R. et al., 2020). Apoptosis plays a crucial role during the process of development and aging and it represents a homeostatic mechanism for maintaining regular cell populations in tissues. Based on the different initial activation pathways, it can be classified into intrinsic and extrinsic apoptosis. In the intrinsic pathway, also called the mitochondrial pathway, apoptosis begins with internal signals and depends on the cytoplasmic release of cytochrome C (cyt C) protein through the mitochondrial membrane (Kakarla R. et al., 2020). *B-cell lymphoma 2* (BCL-2) family proteins are the major regulators of mitochondrial outer membrane (MOM) permeabilization, allowing the release of cytochrome C from the mitochondrial (Shamas-Din A. et al., 2013). BCL-2 family proteins can be pro-apoptotic (BAX, BAD, BAK, Bok) or anti-apoptotic (Bcl-2, Bcl-xL Bcl-w, Mcl-1, and A1) (Kakarla R. et al., 2020). After being released into the cytoplasm, cyt C binds Apaf-1 and forms a molecular complex that activates caspase-9. The latter activated further activates the caspase cascade, leading to apoptosis (Shamas-Din A. et al., 2013). The extrinsic pathway is activated by extracellular ligands binding to cell-surface death receptors, which leads to the formation of the death-inducing signaling complex (DISC). DISC is a multi-protein complex formed by members of the death receptor family, the activation of the DISC complex transduces a downstream signaling cascade, activating caspase-8 and caspase-3 resulting in apoptosis (Raj D. et al., 2006; Kakarla R. et al., 2020; Shamas-Din A. et al., 2013). In the last part of both apoptotic processes, ApoBDs are formed by the fragmentation of the nucleus and cytoplasm, they may contain a wide variety of cellular components: micronuclei, chromatin remnants, cytosol portions, degraded proteins, DNA fragments, or even intact organelles (Shpacovitch V. et al, 2017). After

releasing in extracellular space, ApoBDs are phagocytosed by macrophages and degraded within phagolysosomes (Raj D. et al., 2006; Kakarla R. et al., 2020).

	Exosomes	Microvesicles	Apoptotic bodies
Size of vesicles / Shape	30- 100 nm, regular	100- 1000 nm, irregular	50- 5000 nm, irregular
Markers	LAMP-1, tetraspanins, Alix, MHC -I, -II, HSP70, TSG100	Selectins, integrins, tissues factor and cell-specific markers	Histones, organelles
Origin	Endosomal compartments of cells	Cell surface plasma membrane	Cells which undergo apoptosis

Figure 2 General characterization of extracellular vesicle population based on size origin and contents (Suchorska WM. et al., 2016).

1.1.2 Microvescicles (MVs) biogenesis and release

Microvesicles unlike ApoBDs are not produced during apoptosis but are released physiologically by directly budding from the plasma membrane (**fig.1**) (Kramer Albers E.M. et al., 2016) and, over the years there has always been a strong interest in understanding their role in intercellular communication. Microvesicles are also smaller than apoptotic bodies as their diameter range is approximately 100 – 1000 nm (**fig.2**) (Zhao F. et al., 2020). Microvesicles also contain components such as proteins, lipids, ROS regulators, mitochondria, and nucleic acids such as messenger RNAs (mRNAs) and DNA (Clancy JM. et al., 2021; Waldenstrom A. et al., 2012). Microvesicles showing surface markers such as integrins and selectins (Ekstrom K. et al., 2022; Baci D. et al., 2020), and are documented to contain a broad array of molecular cargo, which reflects not only the cell type from which the MV was released, but also the intracellular trafficking pathways delivering that cargo to the cell surface (Clancy J.W. et al., 2021). Scientific evidence shows that the mechanism underlying the biogenesis of microvesicles is based on the redistribution of membrane phospholipids and the coordination of actomyosin contractile

machinery. Formation of MVs also requires cholesterol, its depletion results in the loss of microvesicle release (Muralidharan-Chari V. et al., 2009; Del Conde I. et al., 2005). Microvesicles are formed by direct outward budding of the plasma membrane and differents pathways regulate the capture and release of MVs. Microvesicle regulatory pathways are influenced by small GTPases, many of which have known roles in regulating the contractile machinery, such as Ras-related ADP-ribosylation factor GTPase 6 (ARF6) that collaborates with endosomal sorting complexes required for transport (ESCRT) (Muralidharan-Chari V. et al., 2009; Clancy JM. Et al., 2021). ARF6 signaling regulates and coordinates the contraction of actomyosin for MVs release, his activity is important for phospholipase D activation and recruitment of extracellular signal-regulated kinase (ERK) to the plasma membrane (Muralidharan-Chari V. et al., 2009). At the plasma membrane, ERK activity results in an activating phosphorylation on myosin light chain kinase (MLCK) which in turn phosphorylates the myosin light chain (MLC), which when activated allows fission, releasing microvesicles into the extracellular space (Muralidharan-Chari V. et al., 2009). It has also been observed that under certain stress conditions, the shedding of MVs can be modulated, a hypoxic tumor environment leads to an increase in the expression of hypoxia-inducible factors (HIFs) which can increase the release of MVs (Hockel M. et al., 2001). Under hypoxic conditions, RAB22A is a gene target of HIF-1, from experiments done with cultures of breast cancer cells grown in 1% of oxygen, it has been observed that HIF-1 binds to a 5'-untranslated sequence in the RAB22A gene to induce RAB22A expression. Rab22A colocalizes with budding vesicles and knockdown of RAB22A leads to less vesicle release suggesting a direct role for Rab22A in MVs release, this highlights that modulation of microvesicle secretion may have a potential therapeutic role (Lima LG. et al., 2011).

1.1.3 Exosomes (EXs) biogenesis and release

The presence of exosomes in the extracellular space was first demonstrated in the late 1980s, exosomes were obtained by ultracentrifugation of sheep reticulocyte culture medium (Johnstone R.M. et al., 1987). Exs secreted from cells were initially proposed as cellular waste resulting from cell damage, but only recently it has been proven that these extracellular vesicles are functional vehicles that carry a complex cargo of proteins, lipids, and nucleic acids (Simpson R.J. et al., 2009; Valadi H. et al.,2007). Exosomes are the smallest subclass of EVs in terms of size with a diameter range of approximately 30 – 150 nm (**fig.2**) (Doyle LM. Et al., 2019). Although the exosome cargo is similar to microvesicles, they have specific distinct surface proteins such as tetraspanins (CD9, CD63, and CD81) and also integrins (α_2, α_6, β_1, and β_4) and other proteins (TSG101, and ALIX), due to the high content of these tetraspanins and proteins in EXs, they are most often used as specific exosome markers (Simpson RJ. Et al., 2012; Deng F. et al., 2019). The mechanism of biogenesis for exosomes (**fig. 3**) occurs starting from the invagination of late endosomes, which are formed by inward budding of the limited multivesicular body membrane (MVB). Invagination of late endosomal membranes results in the formation of intraluminal vesicles (ILVs) within large MVBs (Minciacchi VR. et al., 2015; Krylova SV. et al., 2023). During this process, some proteins are incorporated into the invaginating membrane, while the cytosolic components are internalized within the ILVs. MVBs, after fusion with the plasma membrane, release exosomes into the extracellular space (**fig.3**) (Sahu R. et al., 2011; Krylova SV. et al., 2023). ILVs formation utilizes the endosomal sorting complex required for transport function (ESCRT). It is an intricate protein machinery composed of four separate protein ESCRTs (0, I, II, III) that work to facilitate MVB formation, vesicle budding, and protein sorting (Henne WM. Et al., 2011; Krylova SV. et al., 2023). The ESCRT mechanism is initiated by the recognition and sequestration of ubiquitinated proteins to specific domains of the endosomal membrane via ubiquitin-binding subunits of ESCRT-0. After interaction with the ESCRT-I and -II complexes, this complex interacts with ESCRT-III, a protein complex that is involved in promoting the budding processes. Finally, after ILVs formation, the ESCRT-III complex separates from the MVB membrane due to the activity of the sorting protein Vps4 (Krylova SV. et al., 2023; Henne WM. Et al., 2011). From the analysis of exosomes released by various cell types, different ESCRT components and ubiquitinated proteins have already been

identified. The typical exosomal protein Alix, which is associated with ESCRT proteins, has been reported to participate in endosomal membrane budding, abscission, and exosomal cargo (Krylova SV. et al., 2023; Henne WM. Et al., 2011). Further recent studies also propose an alternative pathway for sorting exosomal cargo into MVBs in an ESCRT-independent manner, that depends on microdomains which should be highly enriched in sphingomyelinase, ceramides and cholesterol (Elsherbini A. et al., 2018; Krylova SV. et al., 2023). The ceramide might cause spontaneous negative curvature of the endosomal membrane, by collaborating with other proteins such as tetraspanins, promoting biogenesis, protein loading, and budding of exosomes (Castro BM. et al., 2014). Probably several specialized mechanisms exist to ensure the specific sorting of bioactive molecules into exosomes, either the ESCRT-dependent or ESCRT-independent mechanism may act variously depending on the origin of the cell type.

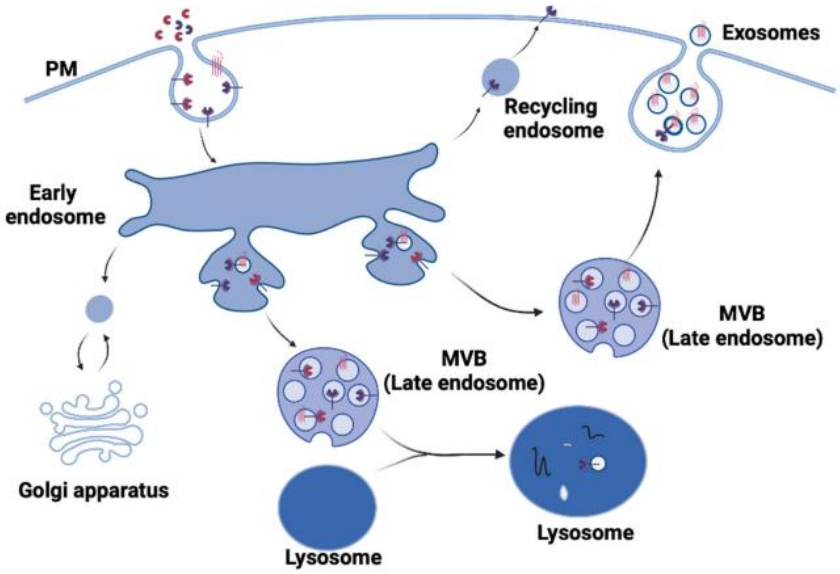

Figure 3 Schematic pathway for exosome biogenesis. Cargos are sorted by endocytosis to the early endosomes, which upon maturation form the multivesicular body (MVB). Finally, MVBs fuse with the plasma membrane (PM) to release exosomes. (Krylova SV. et al., 2023)

1.2 Skeletal muscle

Muscle tissue specializes in generating movement through the contraction of its cells. It performs different types of activities and can vary in its morphology and structure. Skeletal muscle is part of neuromuscular system and consists of striated muscle cells innervated by somatic motor nerves, it is considered a voluntary muscle. The skeletal muscle is able to contract and cause movement voluntary, and the contraction requires energy produced by ATP, and this step produces heat, during this activity (Dave H.D. et al., 2022). Each skeletal muscle is an organ that consists of various integrated tissues; the skeletal muscle bundles, blood vessels, nerve fibers, and three layers of connective tissue that enclose it, and also compartmentalize the muscle fibers within the muscle. The epimysium wraps the entire muscle and separates it from other tissues and organs, allowing the muscle to contract and move independently. Inside each skeletal muscle its cellular units, the muscle fibers, are organized in fascicles, separated by a layer of connective tissue called perimysium. Inside each fascicle, each muscle fiber is surrounded by a connective tissue layer of collagen called the endomysium. The muscle fibers contain the cytoplasm, called sarcoplasm, that is surrounded by the plasma membrane which in muscle cells is called the sarcolemma. Into the sarcoplasm, there are myofibrils long structures (1–2 μm in diameter) that represent the contractile machinery. Numerous nuclei are located below the sarcolemma and are always at the peripheral level of the muscle fiber. Myogenic satellite cells (SC) are positioned between the sarcolemma and the surrounding basal lamina. The functional unit of a skeletal muscle fiber is a modular structure repeated along the length of the fiber, the sarcomere, a highly organized structure of myofilaments, actin (thin filament), myosin (thick filament) and their regulatory proteins, troponin and tropomyosin, and other support proteins (Frontera W.R. et al., 2015). Each sarcomere is bordered by structures called Z-discs to which the actin myofilaments and its troponin-tropomyosin complex are anchored projecting toward the center of the sarcomere. The A-band consists of thick filaments, together with thin filaments that interdigitate by overlapping. The central region of the A-band is the H-zone, containing at the center the M-line, where the thick filaments are linked together. The I-band consists of the adjacent portions of two neighboring sarcomeres, in which only thin filaments are present and anchored in the Z-disc (Lange S. et al., 2020). The fibers contain other organelles essential for cellular function, such as ribosomes, Golgi apparatus, mitochondria, and a network of tubules and

cisterns. Tubular invaginations of the sarcolemma penetrate between the myofibrils, and the lumen of these transverse tubules (T) are in continuity with the extracellular space. The sarcoplasmic reticulum (SR) is a specialized form of smooth endoplasmic reticulum and forms a plexus of cisterns that expand into larger sacs, terminal cisterns, they come into close contact with T-tubules, forming the structures called triads (Jayasinghe I.D. et al., 2014). When a signal by a motor neuron arrives up to a skeletal muscle at the level of the neuromuscular junction, the signal propagates through the T tubules and opens the calcium channels of the sarcoplasmic reticulum allowing the release of Ca2+ ions, which enter the sarcoplasm bind to troponin showing binding sites on actin filaments (Bolaños P. et al., 2022). This allows the myosin heads to bind to these exposed binding sites and form cross-bridges, according to an ATP-dependent reaction, the thin filaments and thick filaments slide on each other, contracting the muscle fiber (Bolaños P. et al., 2022). Skeletal muscles are composed of different types of fibers based on their different type of speeds of contraction and fatigue, number of mitochondria, and myoglobin concentration. Molecular analyses have revealed that fibers may be further identified according to their content of myosin heavy-chain isoforms (Stuart C.A. et al., 2016). Myosin heavy-chain I (MHCI) are generally oxidative fibers and slow, myosin heavy-chain IIB (MHCIIB) are glycolytic fibers with a fast contraction, and myosin heavy-chain IIA (MHCIIA) are called intermediate fibers, they are moderately oxidative and glycolytic (**Fig.4**).

Characteristic	Type I	Type IIA	Type IIB
Nomenclature	Slow Red Fatigue resistant Oxidative	Fast White Fatigue resistant Oxidative/glycolytic	Fast White Fatiguable Glycolytic
Capillary density	High	Intermediate	Low
Mitochondrial density	High	High	Low
Oxidative capacity	High	High	Low
Glycolytic capacity	Low	High	High
Activity used for	Aerobic (long term)	Aerobic (short term)	Anaerobic (short term)
Force production	Low	High	Very high
Major storage fuel	Triglycerides	Glycogen	Glycogen

Figure 4 Metabolic characteristics of type I, IIA and type IIB fibers (Kruger S.D., 2007).

During embryonic development, the trunk and limb muscles derive from somites which are divided into two parts, the epaxial and hypaxial. After differentiation, the somites regionalize and form the dermomyotome (DM), which is the origin of the limbs, trunk, and head (Parker MH. Et al., 2003; Chal J. Et al., 2017). From epaxial dermomyotome derives the muscles of the back whereas the hypaxial dermomyotome forms the skeletal muscles and the limbs (Buckingham M. et al., 2003; Chal J. Et al., 2017). The dermomyotome is subdivided into dermatome and myotome from which the precursors of the myogenic lineage will originate, as well as satellite cells (SC), which are named after their histological localization around the muscle fibers, where they provide a stem cell population for muscle repair (Gros J. et al., 2005; Chal J. Et al., 2017). Prechordal mesoderm and paraxial mesoderm are the primary source of myogenic cells, the myoblasts. Myoblasts express the transcription factors Pax3 and Pax7, induced by Wnt signaling (Ben-Yair R. et al., 2005; Buckingham M. et al., 2003). These factors play an important role in activating the myogenic program. Pax3 and Pax7 are factors involved in the formation of muscles and the migration of myogenic progenitors (Buckingham M. 2006; Ben-Yair R. et al., 2005). Indeed, Pax7 induces the expression of genes such as Id3 (inhibition of differentiation 3), which inhibit the expression of MyoD in particular (Kumar D. et al., 2009). Progenitor cells can subsequently initiate the myogenic program by downregulating the expression of Pax3 and Pax7 while upregulating the expression of Myf5 and MyoD, thereby facilitating myogenesis (Hernandez-Torres F.H. et 2017). Thus, the myogenic progenitors are determined to express the myogenic factors MRFs: Myf5 (myogenic factor 5), MyoD (myogenic transcription factor), Mrf4 (myogenic regulatory transcription factor 4), and Myogenin (myogenic factor 4). In adults, a population of stem cells, the satellite cells, are still present in the muscle, SCs under different stimuli still have the ability to form myotubes and muscle fibers, and are responsible for the maintenance and repair of skeletal muscle (Zammit PS., 2017; Chal J. Et al., 2017) (**fig.5**).

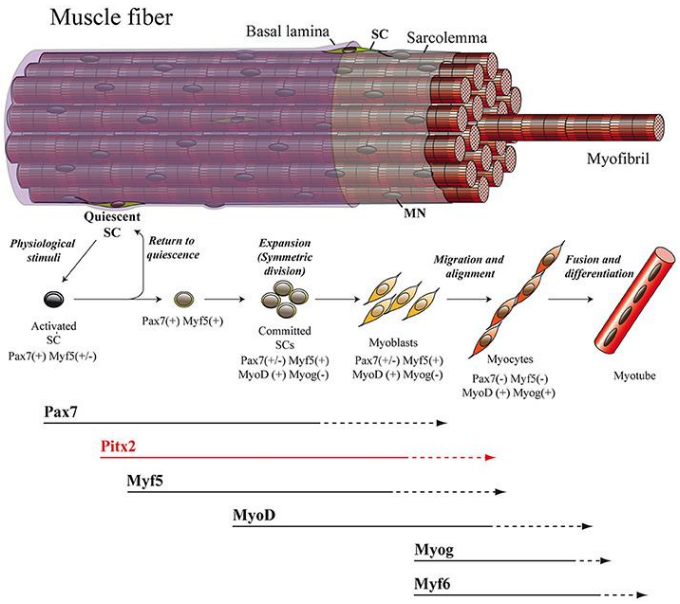

Figure 5 The myogenic differentiation pathway involves the activation of quiescent SCs, following inhibition of pax proteins and increased expression of MRF family proteins, particularly Myo-D. Stem cell differentiation produces myoblasts resulting in the formation of myocytes and myotubes. (Hernandez-Torres F.H. et 2017)

1.2.1 Muscle molecular homeostasis

The morpho-functional properties of skeletal muscle during homeostasis are governed by a dynamic turnover between protein synthesis and degradation (**fig.6**) and by cell turnover, in fact, skeletal muscle is a highly plastic organ that undergoes many changes throughout life to adapt to different functional demands (Poortmans JR. et al., 2012; Nishimura Y., et al 2021). Muscle cell turnover in adult skeletal muscle during the homeostatic process is guaranteed by the activity of satellite cells, and by other precursor cells, which deal with the differentiation and production of new cells (Chargé S.B. et al., 2004). The signaling pathway that regulates protein turnover in muscle fibers is mediated by the Insulin-like Growth Factor 1 (IGF1) and by a cascade of intracellular effectors, including Akt, which has the ability to control both protein synthesis, by activating the kinase mTOR, and protein degradation by repressing the forkhead box subfamily O (FoxO) transcription factor (O'Neill BT. et al., 2016; Schiaffino S. et al., 2013). Upon binding to IGF-1, IGF-1 receptor (IGF-1R) phosphorylates an intracellular adaptor protein insulin receptor substrate-1 (IRS-1), which recruits and phosphorylates phosphoinositide 3-kinase (PI3K). PI3K converts PIP2 to PIP3, which activates PDK1 and Akt, and the latter promotes protein synthesis.

The PI3K, Akt pathway plays a critical role in myotube hypertrophy (Peng XD. et al., 2003) it has been observed that activation of Akt in rat muscle prevents denervation-induced atrophy (Pallafacchina G. et al., 2002). The Insulin-IGF1-AKT-mTOR pathway promotes muscle growth. The mTOR kinase interacts with several proteins, recent studies have identified two different complexes for mTOR; mTORC1 complex that contains Raptor, and mTORC2 that contains Rictor (Sartori R. et al., 2021). These two complexes deal with different activities, The mTORC2 complex is related to glucose and lipid homeostasis and is involved in the regulation of reactions involved in cytoskeletal rearrangement and cell survival (Balasubramanian S. et al., 2009), mTORC1 integrates information coming from outside the cell such as stress factors, oxygen, growth factors (insulin or IGF-1), regulating protein synthesis, the biosynthesis of lipids and nucleotides, promoting muscle hypertrophy and inhibiting autophagy (Bodine S.C. 2022). FOXO transcription factors protein is activated by upstream signals under conditions of low nutrient availability (Brown AK. et al., 2018). FOXO in muscle promotes autophagy, and in particular, mitochondria are degraded by the lysosomal autophagy pathway (Liesa M. et al., 2018). Overexpression of FOXO1 or FOXO3 activates the expression of mitochondrial E3 ubiquitin ligase (Mul1), this induces the degradation of mitofusin 2 (MFN2) (Webb A.E. et al., 2014). Mitofusin 2 degradation results in the induction of autophagy and skeletal muscle atrophy (Webb A.E. et al., 2014). The reactions involved in the regulation of muscle homeostasis may undergo alterations. An imbalance in autophagy impairs myofiber homeostasis, causing excessive removal of cellular components that are needed for normal activities and leading to muscle atrophy (Scicchitano B.M. et al., 2018). There are many causes for the onset of atrophy and it can occur following the presence of various pathologies (Da Silva S.P. et al., 2020). A particular type of atrophy typical of cancer patients, in whom during the progression of the disease they present a state of muscular atrophy, is defined as cachexia (Baracos VE. Et al., 2018; Aoyagi T. et al., 2015).

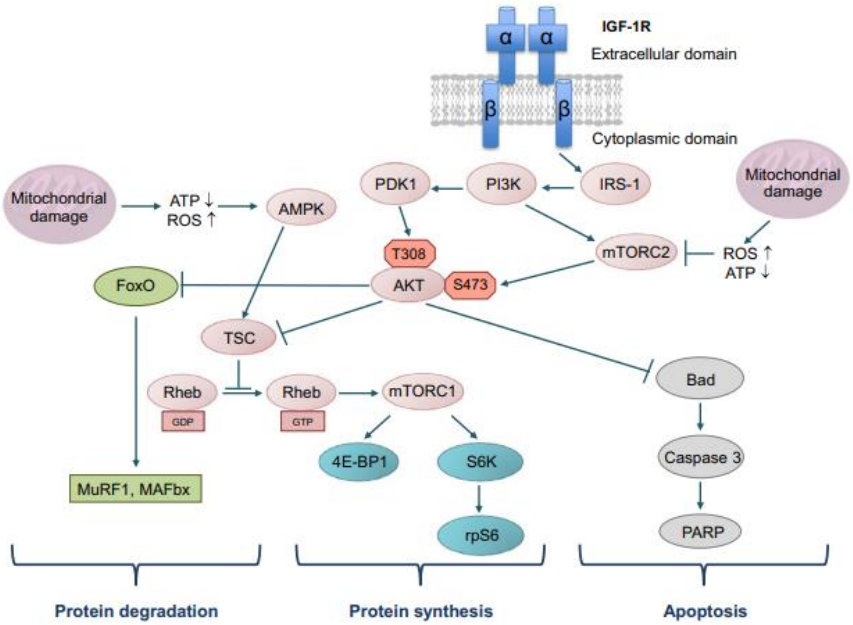

Figure 6 Simplified scheme of the molecular reactions involved in protein degradation and protein synthesis (muscle homeostasis), regulated by FoxO and mTOR. (Bonifacio A. et al., 2017)

1.3 Muscle and cachexia

Severe muscle atrophy typically is a direct effect of protein degradation induced by different pathophysiologic states such as denervation (Daou N. et al., 2020), immobilization (Appell HJ 1990; Ji LL . et al.,2019), aging (Volpi E. et al., 2004), cancer-associated cachexia (Baracos VE. Et al., 2018; Aoyagi T. et al., 2015), diabetes, renal failure, cardiac failure, burns and trauma. Atrophy is defined as a decrease in the size of a tissue or organ due to cellular decrease in cell size by the loss of organelles, cytoplasm, and proteins (Dumitru A. et al., 2018). The rapid loss of muscle mass and strength primarily results from excessive protein breakdown, which is often accompanied by reduced protein synthesis. This loss of muscle function can lead to reduced quality of life, increased morbidity, and mortality. Cancer-associated cachexia is a complex metabolic syndrome with profound changes in energy balance (Fredrix E.W. et al., 1990; Argilés J.M. et al., 2014), characterized by loss of muscle with or without loss of fat mass (Evans

W.J. et al., 2008). From a diagnostic point of view it is possible to talk about pre-cachexia, cachexia and refractory cachexia (Fearon K. et al., 2011):

- **Pre-cachexia**: when weight loss is more than 1kg but less than 5% with the presence of anorexia or metabolic abnormalities such as glucose intolerance.
- **Cachexia:** when weight loss is more than 5% or when weight loss is more than 2% in patients with body mass index (BMI) <20 kg/m2 or the presence of sarcopenia.
- **Refractory cachexia**: when weight loss is more than 15% with BMI less than 23kg/m2 or when weight loss is more than 20% with BMI less than 27 kg/m2. These patients have a reduced life expectancy (<3 months), which does not allow for effective anti-cachectic treatment to be implemented (Fearon K. et al., 2011; Law M.L., 2022).

The progression of cachexia is variable and can be influenced by tumor type, cancer progression, food intake, severity of inflammation, and response to anti-cancer therapies (Law M.L., 2022; Lim S. et al., 2020). Patients with cancers of the gastrointestinal tract, colon, lung, and liver have a risk of developing cachexia between 50 and 90% (Law M.L., 2022; Wan Q. et al., 2022). Cachexia has clear clinical relevance as 80% of cancer patients develop cachexia and 25% die from cardiac or respiratory failure associated with cachexia, rather than from the presence of the tumor itself (Lim S. et al., 2020). It has been known that the tumor releases pro-inflammatory cytokines such as interferon-gamma (INF-γ), tumor necrosis factor α (TNFα), interleukin 6/1 (IL-6, IL-1), and are responsible for the loss of muscle mass during cancer progression (Klampfer L. 2011; Acharyya S. et al., 2004; Borowczak J. et al., 2022). These cytokines originate from both the host and the tumor (de Matos-Neto EM. Et al., 2015), and activate the ubiquitin-proteasome pathway (UPP) leading to increased protein degradation altering the autophagic mechanism (Pigna E. et al., 2016; Zhang L. et al., 2013). There are many factors involved in the onset and maintenance of cachexia, which regulates autophagy and protein degradation pathways, mainly stimulating the ubiquitin-proteasome pathway (UPP) that involves three key enzymes- E1 activating, E2 conjugating, and E3 ligase. An ubiquitin molecule is first activated via the E1 enzyme in an ATP-dependent manner that is further conjugated to E2 enzymes and ubiquitin and finally transfers to the targeted substrate via E3 ligase leading the protein to be degraded (Tu Y. et al., 2012). An increase in UPP expression and activity

was observed in cancer patients with a 10% weight loss (Bossola M. et al., 2003). In vivo studies also demonstrated an increased level of proteasome subunits C2 and C5, as well as the levels of ubiquitin-conjugating enzyme E2 in gastrocnemius and pectoral muscles of mice bearing the MAC16 adenocarcinoma that showed cachexia (Khal J. Et al., 2005). Several transcription factors, particularly FOXO factors, have been identified to play important roles in muscle atrophy and cachexia, (Sandri M. et al., 2004; O'Neill BT. et al., 2019). In cultured myotubes undergoing atrophy, it has been observed that the activity of the PI3K/AKT pathway decreases, leading to activation of FoxO transcription factors and *atrogin-1* (Sandri M. et al., 2004)(**Fig.7**). Furthermore, active FOXO3 acts on the Muscle Atrophy F-box gene (MAFbx) promoter to cause transcription of MAFbx and increase atrophy of myotubes and muscle fibers (Sandri M. et al., 2004). In limb muscles and diaphragm of animal models of tumor, cachexia inoculated with colon-26 (C26) cells, FOXO1 and FOXO3 activity increased the levels of STAT3, Fos, C/EBPβ which are transcription factors linked to atrophy (Judge SM. Et al., 2014). Blocking FoxO prevented C26-induced muscle fiber atrophy of both locomotor muscles and the diaphragm and significantly spared force deficits (Reed SA. Et al.,2012; Judge SM. et al., 2014). FOXO activity appears to be involved not only in the control of protein degradation but also in the modulation of protein synthesis by inhibiting myogenic activity through Myo-D inhibition. Indeed, as was demonstrated when FOXO1 expression was downregulated both in cells and in mice, an increase in the level of MyoD and a decrease in the levels of the muscle regulator myostatin were observed, stimulating the myogenic pathway (Liu CM. Et al., 2007). Myostatin is a responsible factor for stopping the growth of muscle, and it's secreted by murine and human neoplasms. Myostatin probably is involved in the generation of cancer cachexia (Kawaguchi Y. et al., 2023). Different studies show that muscle wasting in advanced cancer patients with cachexia is characterized by impaired Akt activity and suppressed mTOR signaling, and analysis of gastrocnemius muscle from colon cancer mice that develop cachexia reveals a progressive decrease in mTORC1 activity (White JP. et al., 2011). Furthermore, in vitro studies with C2C12 cells demonstrated that the activation of mTORC1 following stretching was subsequently inhibited by cachectic factors contained in the culture media from Lewis cell carcinomas (Gao S. et al., 2016). These studies confirm that cachexia is a complex metabolic syndrome, characterized by drastic loss of body weight, decline in muscle mass and function, wasting and inflammation, higher metabolism increased fatigue, and weakness (Evans WJ. et al., 2008; Ali S. et al.,2014), which cannot be completely corrected with conventional nutritional

support and leads to progressive functional damage. Physical exercise is the only accepted approach to prevent or slow atrophy, but there may be some limitations and it is not always feasible on patients, for this reason in recent years much research has been conducted to study the effect of training both in cachectic and healthy subjects, in order to better understand the molecular mechanisms that can be used for the development of new therapies.

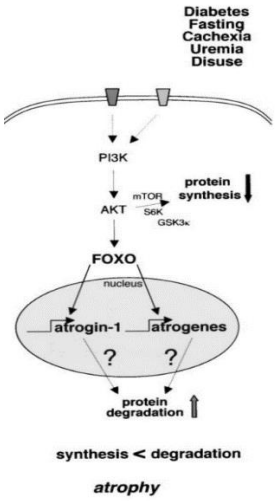

Figure 7 A Summary of the Roles of the IGF-1/AKT Pathway and Foxo in Muscle Atrophy (Sandri M. et al., 2004)

1.3.1 Cachexia treatments and training like treatment

To date, different types of therapies have been implemented and different drugs used in an attempt to counteract the loss of muscle mass induced by cachexia, despite various attempts, poor results have been achieved in the treatment. Tumors produce a lot of proinflammatory cytokines like IL-6, which is most likely involved in reducing muscle protein synthesis (Chonov DC. et al., 2019; Hardee JP. et al.,2018). Thus, inhibiting the effects of IL-6 would be important to reduce cachexia directed at skeletal muscle in cancer. The Food and Drug Administration-approved drug Sylvant (siltuximab) is an antibody to IL-6, which counteracts the effects of IL-6. However, it can lead to various side effects such as difficulty with breathing, nausea, vomiting, and body aches. Therefore, it is used with great caution (Lambert CP. et al., 2021). With the presence of cancer, a reduction in circulating testosterone concentrations is also observed, low testosterone

levels were seen in more than 70% of cancer-cachexia cases, this is the case for predominantly 'female cancers' because some of the testosterone is converted to estrogen (Burney BO. et al., 2012). It has been observed that the use of anabolic steroids such as nandrolone decanoate, and oxandrolone leads to an increase in muscle mass and low androgenic effect, but it can bring sexual and other side effects (Lambert CP. et al., 2021; Batterham MJ. et al., 2001). In cachexia conditions appetite stimulants, such as megestrol acetate (MA), were used, this is currently used to improve appetite and to increase weight in cancer-associated anorexia, but the mechanism by which MA increases appetite is unknown (Ruiz Garcia V. et al., 2013). In recent studies it has been observed that the use of megestrol acetate (treatment with 100 mg/kg/day in vivo model), leads to the downregulation of autophagy and attenuated body weight loss, however, this effect was mainly found in the cardiac level and its effectiveness for anorexia and cachexia in neoplastic patients is under investigation (Musolino V. et al.,2016). Another attempt was cyproheptadine, a histamine antagonist, that improved in appetite but unfortunately, cyproheptadine did not significantly abate progressive weight loss in these patients with advanced malignant diseases (Kardinal CG. et al.,1990). Corticosteroids also improve appetite and performance, but have had no positive effects on body weight and cause more serious adverse effects such as insulin resistance, fluid retention, and adrenal suppression, therefore, corticosteroids are not suitable, have many side effects and should be used in a limited manner. (Loprinzi CL et al., 1999; Tazi E. et al.,2010). Another type of experimental anti-cachectic therapy uses anamorelin which is used as a drug in phase 3 trials (Garcia JM et al., 2013; Currow D et al., 2017). Anamorelin is mainly used as an anti-anorexia drug, this drug is a high-affinity agonist of the ghrelin receptor, which when activated has an anabolic effect and stimulates the appetite (Naito T. et al.,2022; Hoon SN. et al., 2020).

Until now, in recovering lost mass, the treatment that brings promising results is physical training. Specifically, what would stimulate muscle protein synthesis and increase the positive effects on muscle protein growth is progressive resistance exercise also known as resistance training (RT). The muscular system has attracted attention thanks to the discovery of the muscle secretome and its high plasticity and adaptation, and the physiological effects of physical exercise are ubiquitously reported as beneficial to the cardiovascular and musculoskeletal systems. (Zunner B.E.M. et al.,2022). Exercise induces considerable physiological change in the immune system, and has a strong impact on the production and release of cytokines, the relationship between exercise and the

immune system provides an opportunity to explore the complex interaction between basic physiological and immunological mechanisms in musculoskeletal health and disease (Pedersen BK et al., 2000; Rhind SG et al., 2002). Exercise induced neuromuscular adaptations lead to increased muscle strength and power, regulate the release of cytokines and hormones (testosterone, cortisol, growth hormone (GH), insulin-like growth factor-1 (IGF-1), promotion of muscle growth stimulating hypertrophy (Vingren JL. et al., 2010; Bermejo JL et al., 2022; Manini TM et al.,2012). Exercise training is able to decrease myostatine and cytokines expression in skeletal muscle, it may reduce pro-inflammatory cytokine expression, mainly of TNF and IL-6. (Jaworska J et al., 2020; Calle MC et al., 2010). Resistance training, moreover, induces significant phosphorylation on mTOR favoring protein synthesis (Shirai T et al., 2020). Exercise is known to be associated with the production of reactive oxygen species (ROS), the contraction of skeletal muscles produces oxygen radicals and other reactive species capable of oxidizing cellular molecules. Importantly, the failure to eliminate these oxidant molecules during exercise results in the oxidation of cellular proteins and lipids but, under physiological conditions, muscle fibers and other cells contain endogenous antioxidant enzymes capable of eliminating oxidants (Kawamura T et al., 2018; Thirupathi A et al., 2021). The anabolic component of exercise training increases the activation of antioxidant enzymes (Powers SK et al., 2022). Oxidative stress can increase strongly following the presence of various pathologies and is the key factor that triggers skeletal muscle atrophy (Powers SK et al., 2007; Powers SK. et al., 2022). At the cellular level, the main organelle involved in the regulation of reactive oxidant species is the mitochondrion. Chronic excess oxidative stress leads to an alteration of mitochondrial activity and is observed suppression of mitochondrial fusion and fission, inhibition of the electron transport chain and a reduction in ATP production, furthermore, there is an increase in damage to mitochondrial DNA and reduced mitochondrial biogenesis (Alizadeh Pahlavani H. et al., 2022; VanderVeen BN et al., 2017). Cachexia also leads to a decrease in mitochondrial dynamics, and the efficiency of the mitochondrial network by increasing the level of ROS and apoptosis (VanderVeen BN et al., 2017). The role of ROS in promoting skeletal muscle dysfunction and atrophy is well known and examined (Ferreira LF et al., 1985; Li YP et al., 2003). Pgc1-α (peroxisome proliferator gamma coactivator 1) is a transcriptional coactivator, that regulates the expression of numerous genes, including those responsible for regulating mitochondrial biogenesis and fat oxidation. Several studies show how exercise rapidly activates PGC-1α and increases mitochondrial biogenesis improving muscle functions

(Jung S et al., 2014, Lira VA et al., 2010). In conditions of cachexia, alterations and decreases in the regular activity of Pgc1-α have been observed, with related alterations in mitochondrial function and increased muscle atrophy. (Kim Y.M. et al. 2021; Huot JR et al., 2022). Several studies done on the overexpression of Pgc1-α show an improvement in muscle tissue in cachectic conditions but are not sufficient to re-establish the initial condition (Huot JR et al., 2022; Morena da Silva F. et al., 2022). It has also been observed that skeletal muscle training is also responsible for a strong increase in a particular family of proteins, the heat shock proteins (HSPs). HSPs are molecular chaperones involved in folding proteins, in general, are prosurvival molecules, and their overexpression can lead to protection against and improvements after a variety of stressor factors (Noble EG et al., 2008; Hu C. et al., 2020; Barone R. et al., 2016). Under conditions of stress, HSPs are required for the stabilization of denatured or misfolded proteins (Kiang J.G. et al.,1998; Liu Y. et al., 2001). Several studies demonstrate that the activity of Hsp proteins is directly related to improved mitochondrial function and biogenesis in fact, some chaperones are localized in subcellular compartments such as mitochondria, which contain several different chaperones (including HSP60, HSP10, HSP70) (Böttinger L. et al., 2015; Voos W., 2013). HSP72 overexpression in mice has been shown to increase endurance, and an increase in mitochondrial content by 50% was also observed (Archer AE et al., 2018). Furthermore resistance exercise increases Hsp60 levels in the soleus muscle in trained mice. In conjunction with this, an increase in the number of mitochondria and PGC1α isoforms expression was also found (Barone R. et al., 2016). A better and greater understanding of the molecular pathways involved in physical activity that regulate mitochondrial biogenesis and myogenesis is still necessary, as in certain pathological conditions it is not possible to physically implement training sessions to exploit their beneficial effects. Therefore, more information could help us in the design and preparation of new drugs and therapies to combat cachexia.

1.4 Peroxisome proliferator-activated receptor-gamma coactivator (PGC-1α)

Peroxisome proliferator-activated receptor-gamma coactivator 1 is a member of the family of transcription coactivators and regulates critical processes in muscle physiology and promotes the remodeling of muscle tissue, including mitochondrial biogenesis, lipid metabolism, and angiogenesis (Liang H et al., 2006). PGC-1α has been identified in tissues rich in mitochondria, such as skeletal, muscular, and cardiac, but also in organs such as kidney, liver and brain (Finck BN et al., 2006). Multiple stimuli including calcium ions, ROS, insulin, thyroid hormones and hypoxia, ATP demand and cytokines lead to the activation of PGC-1α which interacts in the nucleus with various transcription factors (Puigserver P et al., 2003). Mitochondrial biogenesis is regulated by complex signaling pathways, this requires replication of mitochondrial DNA and the synthesis, import and incorporation of proteins and lipids into mitochondria (Mootha VK et al., 2003; Knutti D et al., 2001; Russell AP. et al., 2005; Olmos Y et al., 2009; Housley MP et al., 2009). The collaboration between PGC-1α and nuclear respiratory factors 1 and 2 (NRF-1, 2) promotes the expression of numerous mitochondrial proteins, such as mitochondrial transcription factor A (Tfam), which directly stimulates mitochondrial DNA (mtDNA) replication and transcription (Kelly DP et al., 2004; Lin J et al., 2005). Different signaling pathways may be involved in causing muscle atrophy, and this depends on the type of upstream signal that triggers the negative stimulus leading to reduced nutritional and energy intake (Hunt LC et al., 2021). The activity of PGC-1α could be very important to counteract the course of muscle atrophy and cachexia, in this regard it has been demonstrated that in muscle atrophy a downregulation of PGC-1α is observed, this leads to a lower inhibition of FoxO activity resulting in an increase in phosphorylated FoxO which promotes protein degradation. Instead, transgenic mice with overexpression of PGC-1α showed reduced production of inflammatory cytokines and protein degradation after undergoing limb denervation (Sandri M. et al., 2006).

1.5 Satellite cells and role of MyoD

Myoblast determination protein 1, plays a major role in regulating muscle differentiation (**fig.5**). MyoD belongs to a family of proteins known as myogenic regulatory factors (MRFs), MRF family members include MyoD, Myf5, myogenin, and MRF4 (Myf6) (Rudnicki M.A. et al.,1995). MyoD is one of the earliest markers of myogenic commitment (Cooper R.N. et al., 1999; Cornelison D.D. et al., 2000). and is expressed at extremely low and essentially undetectable levels in quiescent satellite cells, but expression of MyoD is activated in response to exercise or muscle tissue damage (Silver J.S. et al., 2021). As it is known skeletal muscle is capable of phenotypic adaptation (Hoppeler H. et al., 2002), and shows enormous plasticity to adapt to extrinsic and intrinsic stimuli, such as contractile activity, endurance exercise, electrical stimulation, denervation, nutritional interventions or hypoxia, by modifying the balance between muscle catabolism and anabolism (Holloszy JO 1984; Hoppeler H et al., 2001). Skeletal muscle satellite cells are considered to play a crucial role in muscle fiber maintenance, repair and remodeling. Their discovery dates back to the 1960s when they were identified as the main source of new myonuclei in postnatal skeletal muscle tissue (Mauro A. 1961; Reznik M. 1969; Moss FP., Leblond C.P.,1970). In adult muscle, satellite cells typically reside in a quiescent state; however, after stimulation they are activated and undergo proliferation and/or differentiation (Zhou S et al., 2022), playing an important role in the re-adaptation of muscle tissue. The myogenic program of satellite cells is highly regulated by a fine mechanism of up or down-regulation of the paired box (Pax3, Pax7) transcription factor and myogenic regulatory factors (MRFs, such as MyoD, Myf5). In satellite cells, Pax7 induces the expression of genes such as Id3 (inhibition of differentiation 3), which inhibit the expression of MyoD and other myogenetic factors (Kumar D. et al., 2009). So these quiescent cells, following appropriate environmental stimuli, can enter the myogenic program, it is therefore observed a decreased expression of Pax3 and Pax7 and a progressive increase in expression of Myf5 and MyoD promoting myogenesis (Zammit PS., 2017). Several studies done both in vitro and in vivo have demonstrated that upregulation of Myf5 marks the first phase of myogenic commitment followed by concomitant expression of MyoD, which marks the majority of newly activated satellite cells (Grounds MD et al., 1992; Smith C.K. 2nd et al., 1994; Cooper RN et al., 1999; Cornelison D.D. et al., 2000). The activated satellite cell undergoes a symmetrical division allowing the formation of myoblasts, which migrate inside the muscle fiber, aligning themselves with each other, and forming myocytes.

Ultimately these undergo fusion and complete cellular maturation forming myotubes. Myostatin is a member of the transforming growth factor-β (TGF-β) superfamily and acts as a potent negative regulator of skeletal muscle growth (McPherron AC et al., 2002). In vivo studies with myostatin knockout mice demonstrate that the gene mutation produces mice with 2-3 times greater muscle mass than wild-type mice (McPherron et al., 1997). Myostatin, in addition to having a role in protein degradation, also inhibits myogenesis by inhibiting the proliferation of satellite cells (McCroskery S et al2003; Taylor WE et al., 2001). This activity has been observed in several in vitro experiments demonstrating how myostatin blocks proliferation and differentiation by the down regulation of MyoD (Langley B et al., 2002). Furthermore, using the single exercise model it was demonstrated that the number of myostatin-positive satellite cells in both type I and type II muscle fibers decreases substantially, and this is consistent with a concomitant increase in the number of MyoD-positive satellite cells and with a greater number of satellite cells in the S phase of the cell cycle (McKay B.R. et al., 2012; Snijders T et al., 2014). Myostatin has been shown to induce muscle wasting, there is evidence that its activity induces up-regulation of genes like atrogin-1, FoxO1, and MuRF-1, which have a role in the ubiquitin proteolytic system (McFarlane C. et al., 2006). Particularly, FoxO is the main player in promoting muscle atrophy and can directly regulate and inhibit the activity of Myo-D by interfering with the myogenic pathway (Xu M et al., 2017) (**fig. 8**). These in vitro and in vivo studies suggest that under various stimuli MyoD is an important regulator involved in the plasticity and adaptation of muscle tissue, allowing the differentiation of satellite cells.

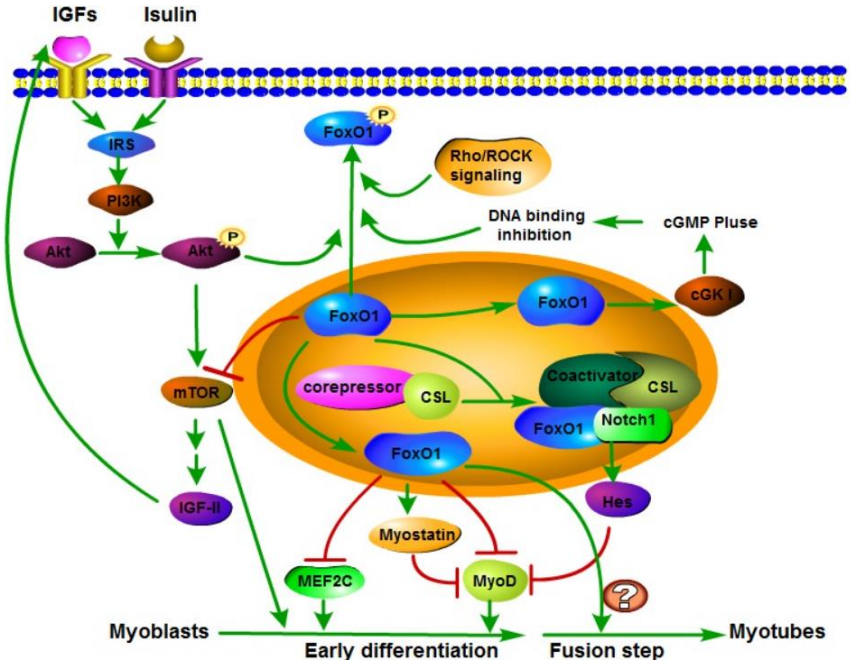

Figure 8: FoxO1 signaling pathway involved in skeletal muscle differentiation. FoxO1 negatively regulates early myoblast differentiation by promoting the activity of myostatin and instead inhibiting that of MEF2C, MyoD and mTOR. This leads to a block or delay in myogenesis. (Xu M et al., 2017)

1.6 Heat shock proteins (HSPs)

Several heat shock proteins (Hsps) function as molecular chaperones (Whitley D. et al., 1999). Furthermore, there are other molecular chaperones that do not belong to the Hsp family but play crucial roles in helping cellular proteins reach their native state, achieving correct folding or conformational, and reaching their intended cellular destinations such as the endoplasmic reticulum or the mitochondria. The HSPs are generally considered protective molecules against different types of stress and have numerous intracellular functions (Macario AJ., 1995; Rappa F. et al., 2012). In living organisms, cells are under constantly changing conditions and maintenance of cellular protein homeostasis, all cells respond to various stress conditions, and the HSPs expression is induced by numerous and different stress stimuli (Pockley AG et al., 2018), but they are recognized as heat shock protein as their discovery occurred following the application of heat shock which

demonstrated an increase in their transcription (Ritossa s.d., 1962; Mirault ME et al., 1978). The temperature at which heat shock proteins are induced varies depending on the normal growth temperature of the species. These proteins are primarily molecular chaperones involved in protein transport by assembling multimolecular complexes and in folding and triggering protein degradation by the proteasome. HSPs play crucial roles in the regulation of gene expression, signal transduction, cell differentiation, intercellular communications, apoptosis and cellular senescence (Czarnecka A.M. et al., 2006; Ikwegbue P.C. et al., 2017). Several of the HSPs are members of gene families that include proteins physiologically present and in organisms, in most cases, essential for cell function (Hu C. et al., 2020). and are classified based on their molecular weight (Schlesinger MJ.,1990). The heat shock proteins proved to be incredibly highly conserved among organisms, for example, the heat shock protein, hsp70, has about 50% of its sequence conserved between E. coli and humans, and some domains are 96% similar (Schlesinger MJ.,1990) . Although they are the most highly conserved, ubiquitous, and abundant proteins in all organisms, their cellular stress response can depend on the class and stimulus.

1.6.1 Heat shock protein 60 (Hsp60)

The HSPD1 gene (17 kb) with 12 exons and it is localized at chromosome locus 2q33.1. This gene encodes a protein of 573aa corresponding to a molecular weight of 61.05 kDa known as HSP60 or Hsp60 (Mukherjee K. et al., 2010). Hsp60 belongs to group I of chaperonins (Caruso Bavisotto C. et al., 2020), its ATP-dependent chaperon mechanism was thoroughly investigated for the prokaryotic homolog GroEL. Three domains were identified for GroEL: apical, intermediate, and equatorial. To carry out its chaperoning function, GroEL needs to generate a tetradecamer complex with its co-chaperon GroES (the homolog of Hsp10) (Ishii N., 2017). Thus, the chaperon complex is made up of GroEL, structured in two rings with seven identical subunits, and GroES, which binds to the apical domains of GroEL to close the cage (Vilasi S et al., 2017). The chaperon mechanism is a

multistep process that involves the unfolded protein binding to GroEL apical domains, concomitantly, ATP binds to GroEL's equatorial domain and its hydrolysis allows the conformational change (from trans to cis) of the GroEL apical and intermediate domains for the substrate encapsulation in the central cavity of the chaperon (Dekker C et al., 2011; Horwich A.L., 2011). Consecutively, the dissociation of the cis-complex and the release of the folded protein, ADP, and GroES occurs (Yan X. et al., 2018). Although the chaperon mechanism of the mammalian mitochondrial Hsp60-Hsp10 complex is similar, the structure appears as a symmetrical football-shaped complex by X-ray (Nisemblat S. et al., 2014), structural and biochemical analyzes of mitochondrial Hsp60 establish a unique toroidal structure of seven subunits, in contrast to the bacterial homolog (Viitanen P.V. et al.,1992).

1.6.2 Hsp60 in Skeletal Muscle

In the muscular system HSPs chaperone play an important role in cellular homeostasis, and heat shock proteins such as Hsp60 have an important role in muscular adaptation. The correlation between Hsp60 and exercise seems plausible, but the available literature is limited and occasionally controversial. Hsp60 does not appear to have fibro-specific expression, there is currently an ongoing debate regarding the localization of fibro-specific Hsp60 following resistance training. It was observed that men who did aerobic training had significantly higher levels of Hsp60 in the vastus lateralis muscle, also showing a high percentage of I and IIa fibers, compared to untrained subjects. However, no increase in Hsp60 levels was observed in the same muscle following a single bout of acute resistance training (Morton J.P. et al., 2008). In a study conducted with male rats after resistance training they were not observed significant differences of Hsp60 in muscles rich in type IIB and IIx fibers such as plantaris and gastrocnemius (Ogata T. et al., 2009, Moura C.S. et al., 2014). In contrast, it was observed that female rats, following an 8-week resistance training protocol, showed significantly elevated levels of Hsp60 in the plantaris muscle (which is rich in type IIB fibers), with no detectable difference in the soleus muscle compared to the untrained group (Mattson J.P. et al., 2000). However, resistance-trained

rats show increased Hsp60 levels in type I fibers of the soleus, and this does not occur in the gastrocnemius (rich in fibers type IIB) (Samelman T.R. et al., 2000). In agreement with the latter study, male mice trained in endurance for 6 weeks show an increase in Hsp60 expression levels in the soleus, but the same effect is not observed in the gastrocnemius muscle (Barone R. et al., 2016). Furthermore, this physiological adaptation with increased Hsp60 expression appears to be related to increased expression of peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PGC-1α), which can trigger mitochondrial biogenesis and thus avoids cytotoxic effects (Barone R. et al., 2016). Hsp60 is mainly localized at the mitochondrial level, the mitochondrial import signal (MIS) at the N-terminus drives Hsp60 from the cytoplasm to the mitochondria (Rodriguez A. et al.,2020). Hsp60 is also at the cytoplasmic level, plasma-cell membrane, inside exosomes, extracellular space, and circulation (Gupta S. et al., 2002; Cappello F. et al., 2008). Within mitochondria, Hsp60 activity allows for the correct folding of other mitochondrial proteins (Cheng M.Y. et al., 1989) and stabilizes misfolded proteins, providing for mitochondrial biogenesis and protein homeostasis (Lin Z. et al., 2008). Hsp60 is also involved in mitochondrial DNA (mtDNA) replication (Kaufman BA ET AL., 2003).

Research Aims

The aim of the study was to produce a new nanovesicle-based drug containing Hsp60 and to compare the effectiveness of this product with exercise training in cachexia management. Furthermore, it has been studied the expression levels of Hsp60, PGC-1α, and Myo-d in response to the treatments.

MATERIALS AND METHODS

2.1 Cell culture

C2C12 mouse myoblasts (from ATCC CRL-1772™) were maintained in DMEM high glucose, GlutaMAX™ Supplement (Gibco, Thtermo fischer)containing, 4.5 g/l glucose supplemented with 10% heat-inactivated Fetal Bovine Serum (FBS), 1000 UI/ml penicillin, 1000 UI/ml streptomycin and 2 mM L-Glutamine at 37 °C in humidified air containing 5% CO2.

2.2 Physiactisome production (C2C12 transfection with electroporation)

Electroporation (EP) was used to develop Physiactisome (PHY), an Hsp60-rich nanovesicle-based product obtained from the culture medium of electroporated C2C12 cells that were transfected with the plasmid Pcmv-6-Entry HSPD1-DDK-flag to overexpress Hsp60 in C2C12 cells. The electroporation was done by the use of Neon® Transfection System_Invitrogen. Two days before EP, C2C12 was grown in T-75 flasks to obtain a confluence of about 80-90%. On the day of the experiment, a 6-well was prepared containing 2 mL of fresh medium without antibiotic, DMEM Glutamax with L-

Glut 1%, 10% FBS. In six nuclease-free tubes 5 µg of Pcmv-6-Entry HSPD1-DDK-flag was inserted. Cells were detached from T-75 flasks with 1x trypsin, washed with PBS 1x (W/O Ca^{++}, Mg^{++}) and counted, $2X10^5$ cells for any condition; C2C12 (not EP), electroporated without plasmid (C2C12 CN), C2C12 electroporated with Pcdna 3.1 plasmid as a negative control (C2C12 pcdna 3.1), C2C12 electroporated with Pcmv-6-Entry plasmid without insert (C2C12 MOCK) as a second negative control and C2C12 electroporated with Pcmv-6-Entry HSPD1-DDK-flag plasmid (C2C12 HSPD1). The Neon® Tube in the Neon® Pipette Station was set up with 3 mL of E2 Electrolyte Buffer. C2C12 cells were resuspended for each condition mentioned in 100 µL of Buffer R. Finally, 100 µL of cell suspension containing the DNA was taken with the Neon® Tip 100ul, this was inserted inside the Neon® Tube in Buffer E2. After setting the working conditions for electroporation, which in our case were; Pulse voltage (V) 1200, Pulse Width 20ms, Pulse N°2, the samples were ready for electroporation. Once the electroporation was completed, the 100 µL were placed in the corresponding well. This was repeated for all samples. Finally, the plate was incubated at 37°C for 48h. The secretion obtained after electroporation from C2C12 HSPD1 cells (Physiactisome) was used for the in vivo study, and another portion was used to prepare the samples to be observed under the scanning and transmission electron microscope (STEM) to confirm the presence of extracellular vesicles, and for western blot analysis to investigate on nanovesicles contents. Instead the secretion obtained from C2C12 MOCK cells it was used as a placebo (PLA) in vivo study. Instead C2C12 cells for each condition were used for subsequent western blot and double-immunofluorescence studies to confirm cell transfection.

2.3 Immunofluorescence for electroporated C2C12

The plasmid used didn't have a fluorescent protein that could have been easily detected by microscope. For this reason, immunofluorescence was necessary to follow the evolution of the transfected cells. Briefly, after electroporation, the C2C12 cells were detached from the 6 weel, count and plate, $1x10^3$ cells per well with 500uL of culture medium in an 8-well slide chamber. After 24 h the medium was removed, made 3 washes with PBS 1X

(W/O Ca^{++}, Mg^{++}), subsequently, we proceeded with the fixation with Cold Methanol 100%: 20 min on ice and Parafolmaldeide 4%: 20 Min on ice. Permeabilization of the cells was done with Triton-x 100, 0,5%: at RT, for the blocking instead we used BSA 3% in PBS: 1H at RT, and finally, the primary antibody, Anti-DDK-flag (mouse monoclonal antibody, Origene TA50011-100, dilution 1:50) in PBS was incubated, 4 C°, O.N. The following day, the first primary antibody has been removed, three washes were carried out with PBS 1X (W/O Ca^{++}, Mg^{++}), then the second incubation was carried out with the primary anti-Hsp60 (rabbit monoclonal, abcam ab46798, dil 1:50). The next day, the antibody was removed and 3 washes with PBS were done, after incubation was performed with the secondary antibodies, anti-mouse (IgG-TRIC LOT: SLBJ3612V, SIGMA), and anti-rabbit (IgG-FITC, LOT: SLBJ6087V, SIGMA) diluted 1:100 in BSA 3%, 1h, in dark, after three washes with PBS, and the nuclei were stained with DAPI 1ug/ml in PBS for 1 Min. Finally, the coverslip was mounted and the result was subsequently visualized with confocal microscopy.

2.4 C2C12 Protein extraction

Cells were detached from culture plates using 1X trypsin. After 5 minutes the action of the trypsin was interrupted by adding in ratio 1:1 DMEM Gutamax + 10% FBS. The cells were centrifuged at 500 x g for 5 minutes, 4 ° C. The supernatant was discarded and a wash was performed with PBS 1x (W/O Ca^{++}, Mg^{++}), spin again at 500 x g, 5 minutes, 4°C. Finally the pellet was resuspended in 100uL of RIPA buffer (25mM Tris-HCl pH 7.6, 150mM NaCl - 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) with Protease Inhibitor Cocktail (cOmplete ULTRA Tablets, Mini, EDTA-free, EASYpack by Roche) for 1h on ice for cell lysis, then centrifuged at 12,000 x g, 20 minutes, 4 °C. Once the proteins were extracted, they were quantified through Qubit™ fluorometer (Invitrogen) to use the samples for western blot analysis.

2.5 Nanovesicles Isolation

The isolation of extracellular vesicles from the culture medium was done with, using a Total Exosomes Isolation kit ((TEI) invitrogen). Briefly, the cell medium was first Centrifuged at 300 x g, 10 min, + 4°C, to remove cells; a second centrifugation step was done at 2,500 x g, 30 min, +4 °C to remove dead cells. The culture medium was concentrated using concentrator tubes VIVASPIN® 20, 10k centrifugal concentrator filters (Sartorius, 10,000 MWCO PES, REF_VS2001) by centrifuging the samples at 5.000 g, 16 minutes, at 4 °C. The starting volume was 24ml for each experimental condition (C2C12-CN , C2C12-MOCK , C2C12-HSPDI), and was concentrated to obtain a final volume of 1mL. Subsequently 500uL of Total Exosome Isolation was added to these concentrated media for each sample. Once the mix was prepared, the samples were incubated overnight at 4 ° C. The following day the samples were centrifuged at 10,000 g, 1h at 4°C. The supernatant was discarded and the pellet was lysed in 100uL of RIPA buffer with Protease Inhibitor Cocktail, 1h on ice for lysis, then centrifuged at 12,000 x g, 20 minutes, 4°C. Finally, the protein quantization was done with Qubit™ fluorometer (Invitrogen) for western blot analyses.

2.6 Western Blot

Cells and extracellular vesicles were lysed in RIPA buffer for protein extraction, are previously described. Protein samples were mixed with 10X sample buffer and denatured for 5 min at 95 ° C and loaded onto a 12% SDS-PAGE gels. After the Electrophoresis, proteins were transferred onto a nitrocellulose membrane (Nitrocellulose Membrane Filter Paper Sandwich 0.45 µm) checked with Red Ponceau and blocked for 1h at room temperature with 5% BSA in Tris-buffered saline/0.05% Tween20 (T-TBS). The membrane was then incubated overnight at 4 °C in gentle shaking with anti-Alix (3A9 sc-53538, mouse monoclonal, Santa Cruz, diluted 1:1000), Anti-Hsp60 (K-2, SC-1722, Santa cruz, mouse, diluted 1:1000), Anti-GAPDH (ABS15, LOT: 3557958, Millipore Corp, rabbit, diluted 1:10000). All diluted in 0,5% BSA in T-TBS. The secondary antibody used

are, anti-mouse (Gt X Ms IgG (H+L), LOT: 0605031259 AP124P, Chemicon International, Fisher Scientific) and anti-Rabbit IgG antibody, GTX26795, LOT: 821502963) diluted 1:10000 in 0,5% BSA in T-TBS 1X. The signal was detected by manual development in the dark room.

2.7 Scanning and transmission electron microscopy (STEM)

The culture medium obtained from C2C12 cells (10ml) was centrifuged at 2500 x g for 30 minutes to eliminate cell debris and cells left in suspension. Subsequently using polyethersulfurone concentrator tubes with 10000 MWCO Vivaspin® filter, the sample was concentrated to a final volume of 1ml. The obtained sample was resuspended with 0.5 ml of total exosomes isolation kit (Invitrogen) and left incubating at 4°C overnight. The following day the sample was centrifuged for 1 h at 10,000 X g, the supernatant was discarded and the pellet containing the extracellular vesicles was resuspended in 30ul of 1x PBS (W/O Ca^{++}, Mg^{++}). With a micropipette the sample was poured dropwise onto the copper grids (3mm diameter) and dried, then 1% uranyl acetate was used for 5 minutes of incubation in the dark, two washes with methanol were carried out for 5 minutes and allowed to dry. Using Reynolds' solution, the sample was incubated in the dark for 3 minutes and then washed with distilled water twice for 5 minutes. After being dried, the sample was ready for transmission and scanning electron microscope (STEM) observation.

2.8 In vivo model

The study was carried out using 64 mice, Seven-week-old male balb/c mice, that were divided into two groups, trained (TR) and sedentary (SED). During the first two weeks

habituation was performed by first placing the mice on the rotarod for a few minutes. The speed was then gradually increased to ensure that the mice became familiar with running. Two mice were initially inoculated with pieces of thawed colon tumor, and the grown tumor was used to inoculate the other groups. The two groups, trained and sedentary, were further divided into mice that received colon tumor fragments (C26) inoculation (T+), via dorsal subcutaneous injection, and mice that did not receive tumor inoculum (T-). After tumor inoculation the training and treatment period began. During the treatment period we administered Physiactisome once a week before training 10ul of PHY (0.01 mg/mL of proteins) and 10ul of PLA by intramuscular injection into the hind legs of the mouse.
In this way we organized a total of 8 groups; SED T- PLA, SED T- PHY, TR T- PLA, TR T- PHY, SED T+ PLA, SED T+ PHY, TR T+ PHY, TR T+ PLA (**fig. 9**). After tumor inoculation, mice were subjected to daily progressive resistance training (TRP) on the rotarod (5 days/week), described as follows; (third week, 30 minutes, speed 4 m/min), (fourth week, 45 minutes, speed 4.8m/min), (fifth week, 60 minutes, speed 4.8m/min) (**fig.10**). The animals were checked and weighed daily, when the animal exceeded a weight loss of 9%, confirming the cachectic spectrum of mice with the tumor, it was weighed and sacrificed by cervical dislocation. Organs and tissues; heart, liver, spleen, lungs, kidney, brain, tumor, gastrocnemius, soleus, tibialis were removed and weighed, some samples were frozen in liquid nitrogen and others were embedded in paraffin for further study.

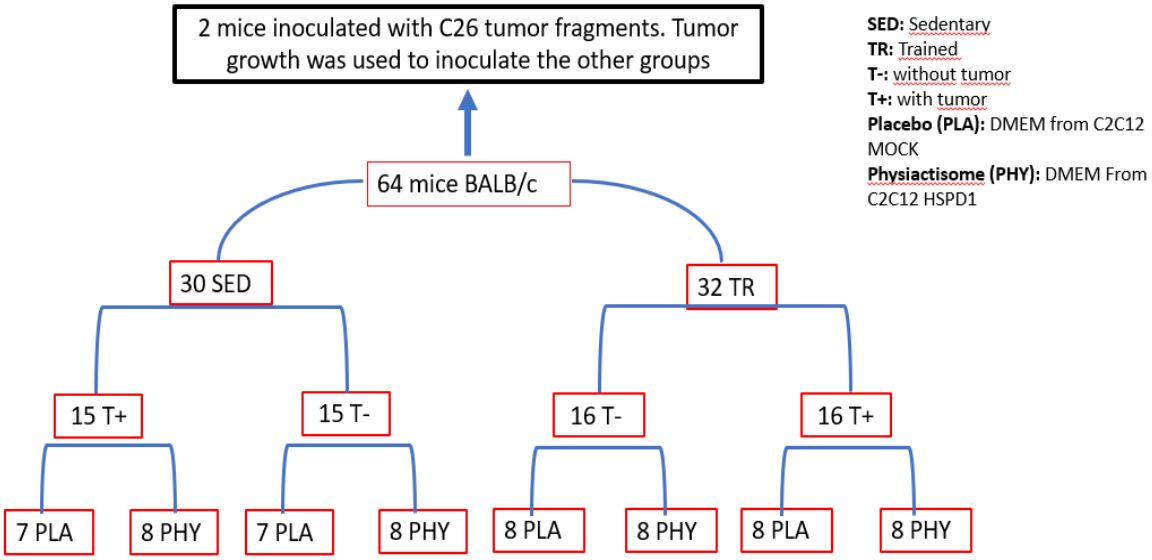

Figure 9 Schematic organization of the group of mice for the in vivo experiment

Week	Time (min)	Rotarod Speed (m/min)
1	Handling	
2	Handling	
3	30	4
4	45	4,8
5	60	4,8

Figure 10 The table shows the progressive resistance training program (TRP) on the rotarod to which the mice were subjected after inoculation and tumor growth.

2.9 Gastrocnemius (GA) hematoxylin-eosin staining

The gastrocnemius muscle was harvested from each mouse during surgery. After being weighed, the organs were preserved in formalin. We performed the protocol for embedding the samples in paraffin. The organs individually were placed in plastic boxes, washed in water and subsequently placed in 70% ethanol. After 24 hours they were placed in 96% ethanol for 12 hours, then in 100% ethanol for 2 hours, 3 hours in xylene at RT, and then they were incubated in a paraffin oven at 65°C for 3 hours. After that the samples were placed in metal boxes filled with paraffin at 65°C and allowed to cool at room temperature overnight. From the samples fixed in paraffin blocks we obtained the slides with sections of the gastrocnemius muscle (thickness 5um), the sections were obtained by microtome cutting and placed on polylysine slides (Thermo Scientific™) and dried at RT overnight. Slides obtained with gastrocnemius sections were stained with hematoxylin-eosin. The slides were placed in the oven at 55°C for 15 minutes and then in xylene for 10 minutes, then passed on a decreasing alcohol scale (100%, 95%, 80%) for 5 minutes for each. After being washed in water for 5 minutes they were incubated in hematoxylin (for nuclei staining) for 5 minutes. After being washed in water for 15 minutes they were incubated in eosin (for cytoplasmic staining) for 3 minutes and washed in water for 10 minutes. After making rapid passages in alcohol following an increasing scale (80%, 95%, 100%) the slides were placed in xylene for 5 minutes, and finally, after placing the coverslip, the slides were observed under the optical microscope to investigate the structure and size of muscle fibers.

2.10 Gastrocnemius western blot analysis

During surgery on mice, a portion of the tissue, gastrocnemius, after being weighed was frozen in liquid nitrogen for further investigation. These frozen gastrocnemius samples were subsequently processed for protein extraction. The tissues placed in 1.5 ml tubes were

homogenized and lysed using pestles and a lysis solution, 100uL of RIPA buffer (25mM Tris-HCl pH 7.6, 150mM NaCl - 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) + Protease Inhibitor Cocktail (complete ULTRA Tablets, Mini, EDTA-free, EASYpack by Roche in a ratio of 2:100) for each sample. The samples were processed for 30 minutes on ice and resuspended every 10 minutes, and then 30 minutes at -20°C. Subsequently, the samples were centrifuged at 13,500 x g, 20 minutes, 4 °C, the pellet containing the cell debris was discarded and the supernatant of each sample containing the protein part was taken and stored at -20°C. Protein samples were subsequently analyzed using the Qubit® Fluorometer spectrophotometer (Invitrogen) for protein quantization. From each sample, 45ug of protein was used for western blot analysis to investigate Hsp60, PGC-1α and Myo-D protein expression. The intensity signal was analyzed with ImageJ software.

2.11 Statistical analyses

Data were analyzed using Unpaired T for the analysis of western blots, in the in-vivo study the data were analyzed with ANOVA test ,and Tukey's multiple comparison test was performed, all data were analyzed in triplicate for each group. All data are presented as the means ± SD, and the level of statistical significance was set at $p<0.05$*, $p<0.001$**, $p<0.0001$***. GraphPad Prism 8.0.1 software (GraphPad Software Inc., San Diego, California, USA).

Results

3.1 Double-Immunofluorescence (IF) C2C12 cells post transfection

To evaluate whether the cell transfection occurred correctly, double immunofluorescence for DDK-flag and Hsp60 was performed on C2C12 cells. IF was done using anti-DDK-flag antibody. In figure11a, the IF carried out on control cells (C2C12 CN) shows negativity for the DDK-flag antibody. Cells transfected with a pcDNA3.1 (**fig.11b**) and cells transfected with the empty PCMV-6-Entry plasmid (**fig.13 c**) are negative for DDK-flag. Figure 11d shows positivity for DDK-flag for C2C12 HSPD1 cells transfected with the plasmid, Pcmv-6-Entry HSPD1-DDK.

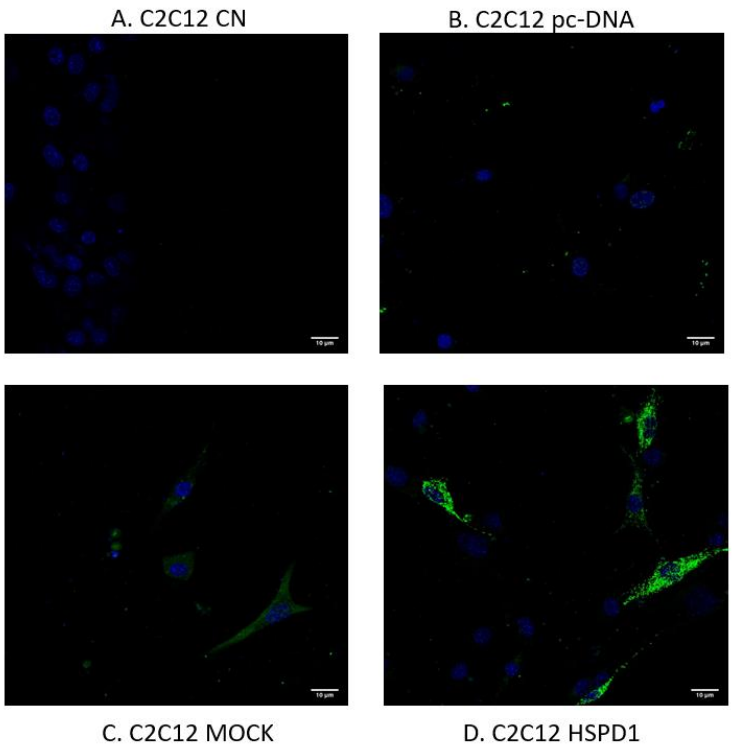

Figure 11: IF for anti-DDK flag performed on C2C12 cells transfected by electroporation. Control cells are negative (a), it is possible to observe the positivity of C2C12 HSPD1 that have been transfected with the plasmid Pcmv-6-Entry HSPD1-DDK-flag (d).

The IF for Hsp60 performed on C2C12 cells transfected with EP, shows that C2C12 HSPD1 (**fig.14 d**) cells have a greater presence of the Hsp60 protein than CN and MOCK and pcDNA3.1 cells.

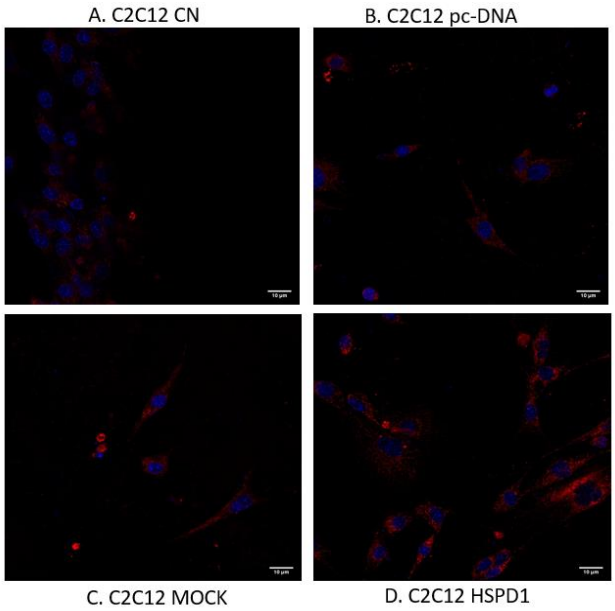

Figure 12: IF for anti-Hsp60 performed on C2C12 cells transfected by electroporation. Control cells are weakly positive for Hsp60 (a), it is possible to observe that C2C12 HSPD1 transfected with the Pcmv-6-Entry HSPD1-DDK-flag plasmid (a) show greater expression of Hsp60 protein.

The merged images of the immunofluorescences obtained for DDK and Hsp60 are shown in Figure 13.

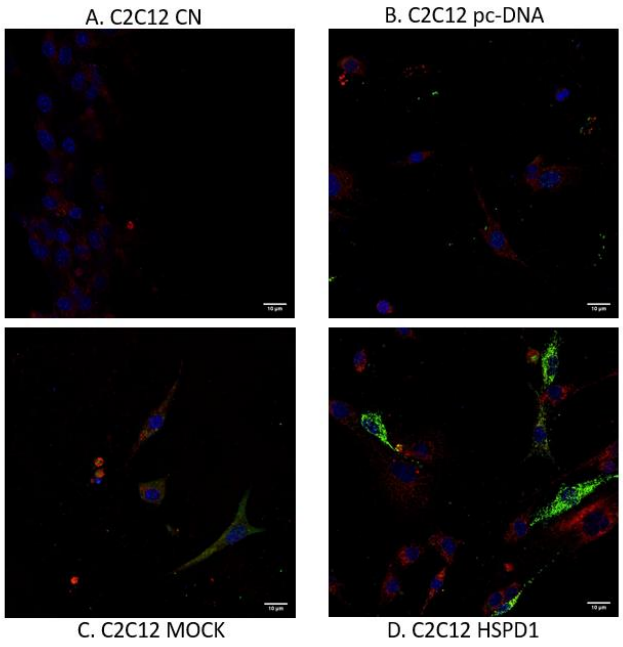

Figure 13: In the image it is possible to observe the merge of DDK + Hsp60. This can be appreciated in particular in the image (d) C2C12 HSPD1, where both proteins are more expressed.

3.2 Westernblot for C2C12 cells post transfection

Cell lysates from C2C12 cells were analyzed by western blot to investigate expression levels of Hsp60. In figure 14a it is possible to observe the developments of the WB carried out for the cell lysates. Densitometric analysis (fig.14b) was performed using ImageJ to evaluate the expression of the samples. It is possible to observe that Hsp60 is significantly expressed in the sample obtained from the C2C12 HSPD1 treated with the plasmid compared to the other samples control.

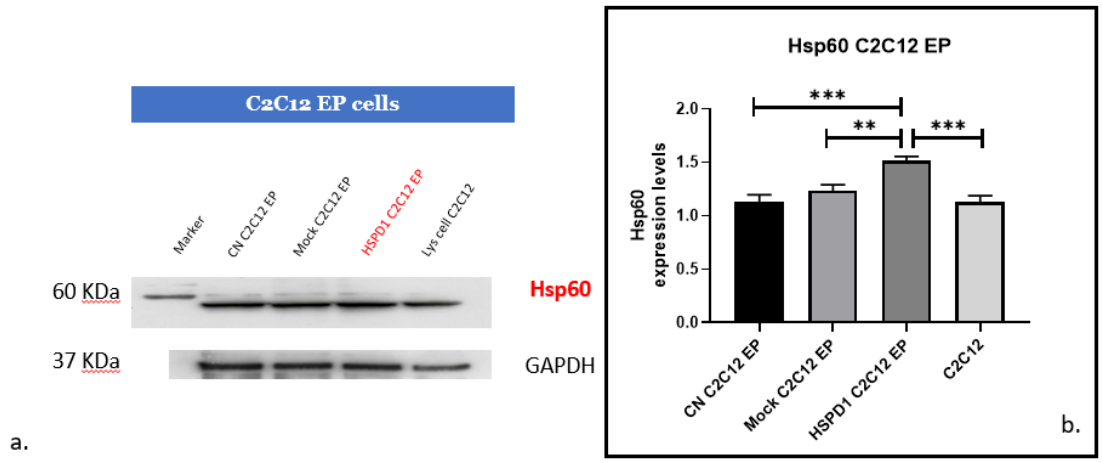

Figure 14: (a)The figure shows the expression of Hsp60 of the cell lysates from the western blot. 50ug of protein was loaded for each sample. (b) The graph shows the densitometric analysis of Hsp60 which is significantly expressed in the electroporated C2C12 transfected with the plasmid to over-express the heat shock protein 60.

3.3 Scanning and transmission electron microscopy (STEM), nanovesicles (NVs) from physiactisome

According to the previously described protocol, the EVs were isolated from the culture medium from electroporated cells (physiactisome), and the sample obtained was used to prepare the copper grids (3 mm diameter) to be observed at the STEM. As shown in the figure15 obtained from scanning and transmission electron microscopy, the presence of extracellular vesicles with a diameter of about 50-80nm obtained from the culture medium can be observed.

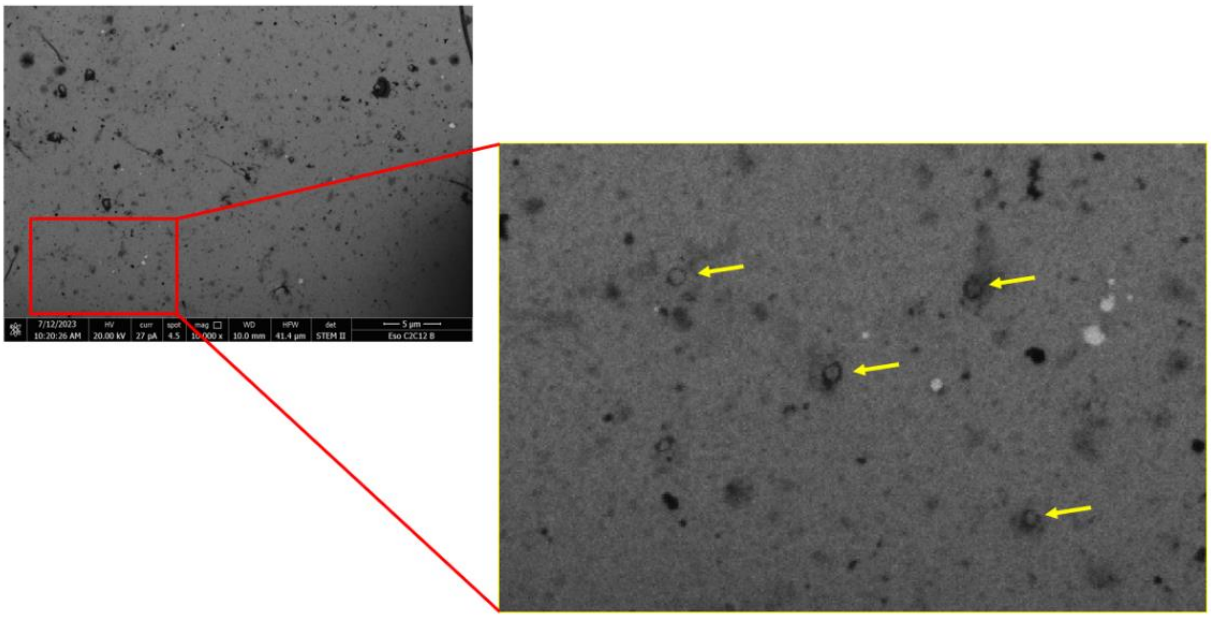

Figure 15: Nanovesicles from physiactisome observed by scanning and transmission electron microscopy (STEM)

3.4 Western blot nanovesicles characterization

According to the previously described lysis protocol, nanovesicles were lysed to isolate their protein content. Using an immunoblotting technique, the samples obtained were used to characterize the isolated extracellular vesicles. The development of immunoblotting obtained from western blot for ALIX (nanovesicle marker), Hsp60, and GAPDH is shown in figure 16 a. Figure 16c shows the densitometric analysis of the expression of all samples of both nanovesicles and cells, which are positive for ALIX. In figure 16b, the densitometric analysis of the Hsp60 expression levels is shown; in the sample of nanovesicles obtained from physiactisome, a significant increase in Hsp60 expression is observed compared to controls.

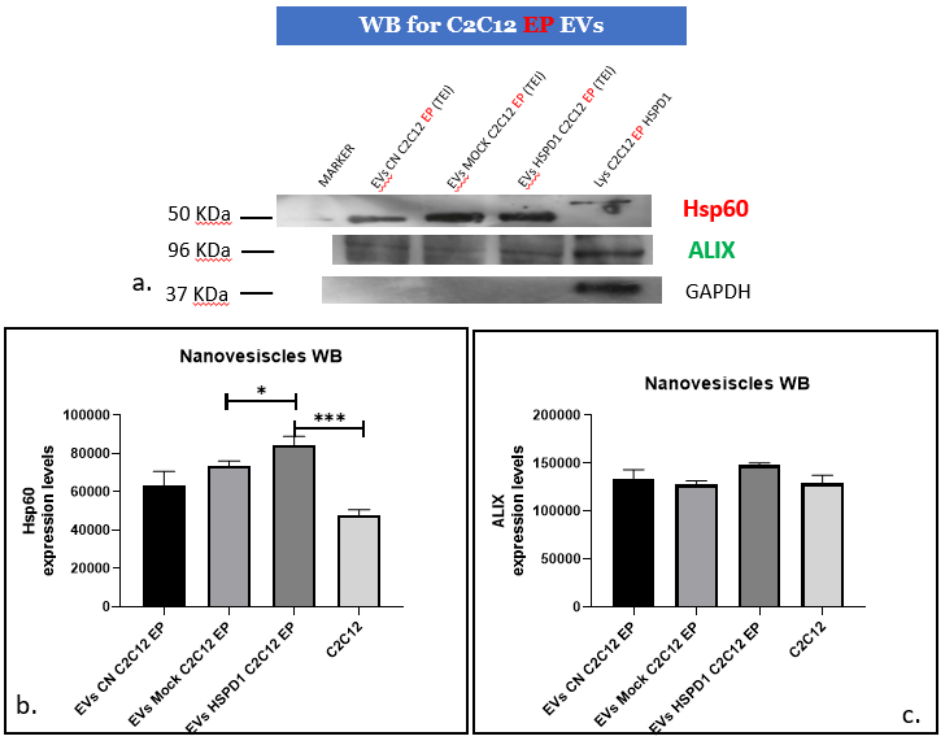

Figure 16: The images show the developments of the membranes obtained from the western blot for Hsp60, ALIX and GAPDH (a). 50ug of protein was loaded for each sample. The graphs show the results of densitometric analysis of Hsp60 (b) and ALIX (c). P<0.05*, P<0.0001***.

3.5 Hystologial comparison response to treatment in vivo

The analysis of data obtained from hematoxylin-eosin staining (fig.17, fig.18) of the gastrocnemius muscles (number of replicates n=3 for each group) documented a significant increase in the cross-sectional area of the muscles, this was observed for the trained group and in trained-physiactisome treated group compared to the sedentary. This result was obtained in both the tumor-free and cachectic mice (fig.19). It is also interesting to note that the CSA values in the groups of tumor-bearing mice, TR T+ PLA, TR T+ PHY and SED T+ PHY, do not present significant differences (ns), compared to the groups without cancer (fig. 20).

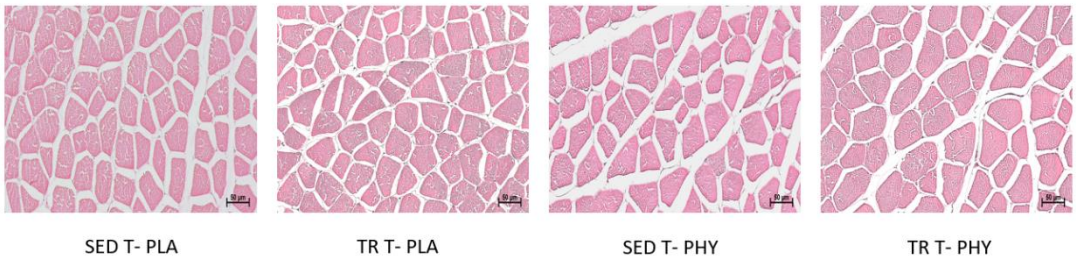

Figure 17 The image shows the hematoxylin-eosin staining performed on gastrocnemius tissue samples from the group of mice without tumor (sedentary without tumor placebo (SED TLA),sedentary without tumor physiactisome (SED T- PHY), trained without tumor placebo (TR T- PLA), trained without tumor physiactisome (TR T- PHY)).

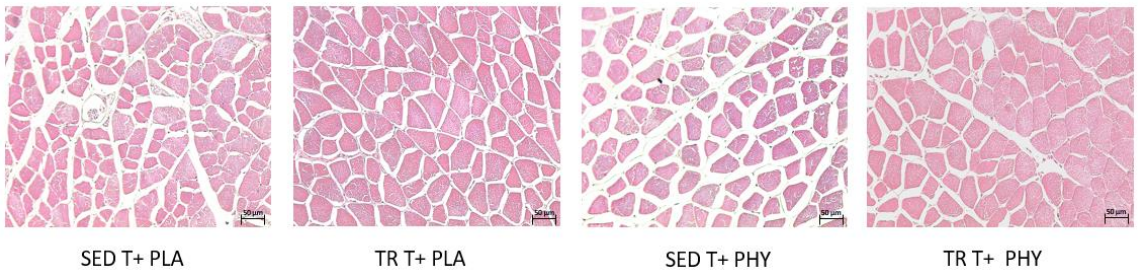

Figure 18 The image shows the hematoxylin-eosin staining performed on gastrocnemius tissue samples from the group of mice inoculated with the tumor (sedentary with tumor placebo (SED T+ PLA), sedentary with tumor physiactisome (SED T+ PHY), trained with tumor placebo (TR T+ PLA), trained with tumor physiactisome (TR T- PHY).

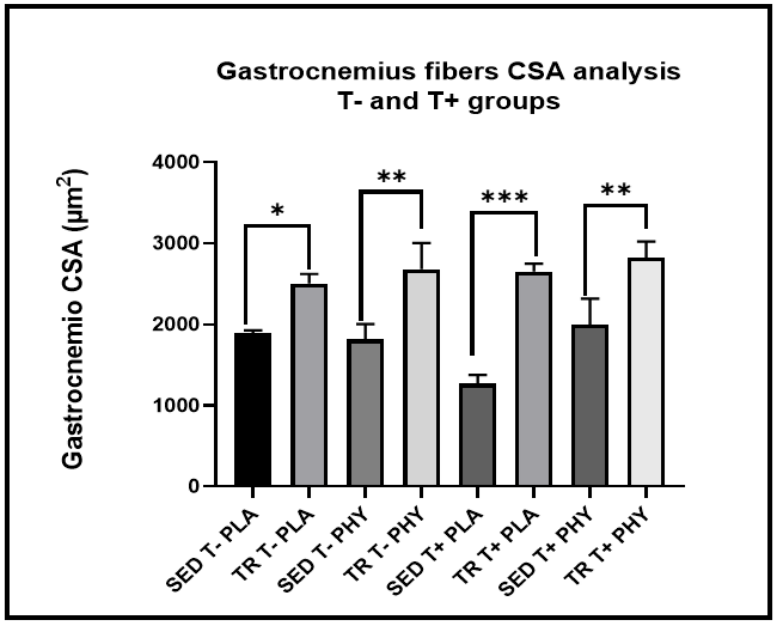

Figure 19 The graph shows the muscle fibers CSA values of all groups of mice, with and without tumor. An analysis of the values of the trained groups compared to the sedentary groups was carried out which shows an increase in CSA in the TR groups. Significance P<0,05*, P<0.001**, P<0.0001*** .

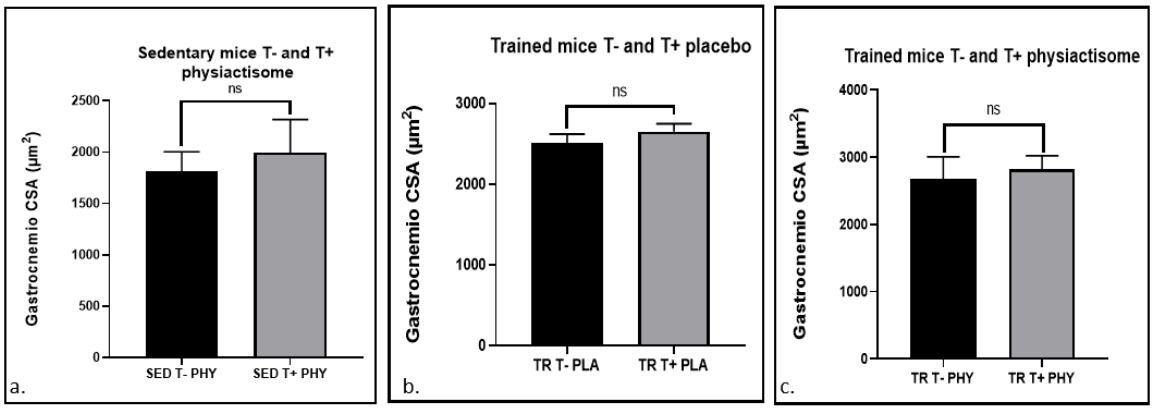

Figure 20: Comparison of cross-sectional area analysis between, (a) sedentary group tumor-free mice treated with PHY (SED T- PHY) and mice group with tumor, PHY treated (SED T+ PHY). (b) trained tumor-free mice (TR T- PLA) and mice with tumor trained (TR T+ PLA). (c) tumor-free mice trained and treated with PHY (TR T- PHY) and mice with tumor trained and treated with PHY (TR T+ PHY).

Furthermore, it was documented that the CSA of muscles from sedentary cachectic mice treated with physiactisome (SED T+ PHY) was significantly greater than muscles from untreated cachectic mice SED T+ PLA (fig. 21). In figure 21a it is possible to observe that the SED T+ PLA group presents a clear reduction of the muscle fiber bundles (black arrow) due to the cachectic syndrome.

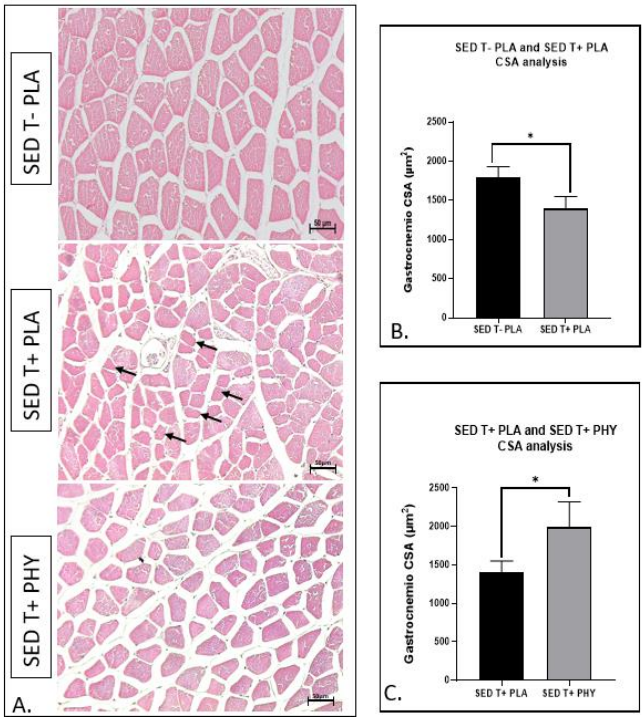

Figure 21 Comparison of CSA values obtained between groups of sedentary tumor-free placebo mice (SED T-PLA) and sedentary with tumor (SED T+ PLA)(b). Comparison between SED T+ PLA mice and sedentary with tumors treated with physiactisome (SED T+ PHY)(c). In figure 21a it is possible to observe that the SED T+ PLA group presents a clear reduction of the muscle fiber bundles (black arrow).

3.6 Molecular response to the treatment: Hsp60 expression

Quantification of Hsp60 proteins through the westerblot technique showed that in the group of tumor-free mice, Physiactisome administration (SED T- PHY) significantly increases Hsp60 levels compared to untreated and untrained mice (SED T- PLA). The same result was found in mice trained and treated with physiactisome (TR T+ PHY) compared to the control (SED T- PLA) (fig.22b). Training alone does not show a significant increase in Hsp60 compared to the sedentary group without tumor and without treatment (SED T- PLA) (fig.22b). The level of Hsp60 protein expression in cachectic mice was significantly reduced following physiactsome administration combined with exercise (TR T+ PHY). Single treatments (TR T+ PLA, SED T+ PHY) did not show a significant increase in Hsp60 (fig. 23c).

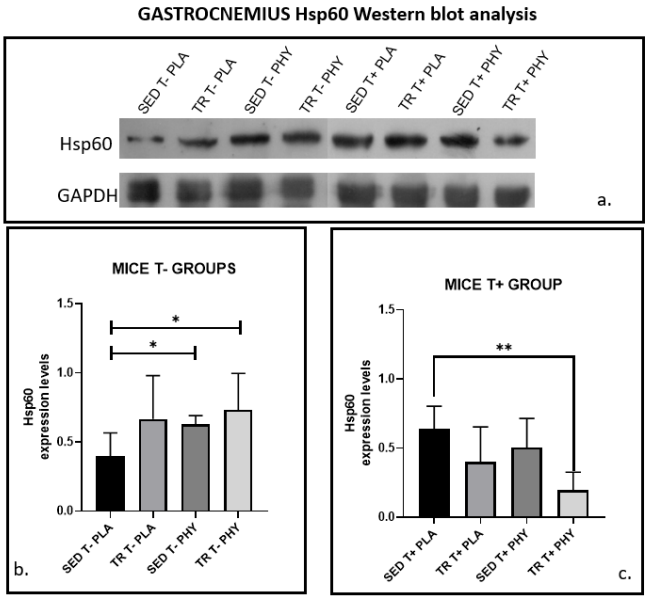

Figure 22 Western blot analysis for Hsp60 expression in gastrocnemius muscles(a). 40ug of protein was loaded for each sample. The values obtained from the densitometric analysis for the groups of trained and sedentary tumor-free (T-) mice are shown (b). The values obtained from the densitometric analysis can be observed for the groups of trained and sedentary mice with tumor (T+) (c).

3.7 Molecular response to the treatment: PGC-1 and Myo-d expression

The response to physiactisome and/or training treatments appears not to have involved the expression of PGC-1 in the gastrocnemius (fig. 23)

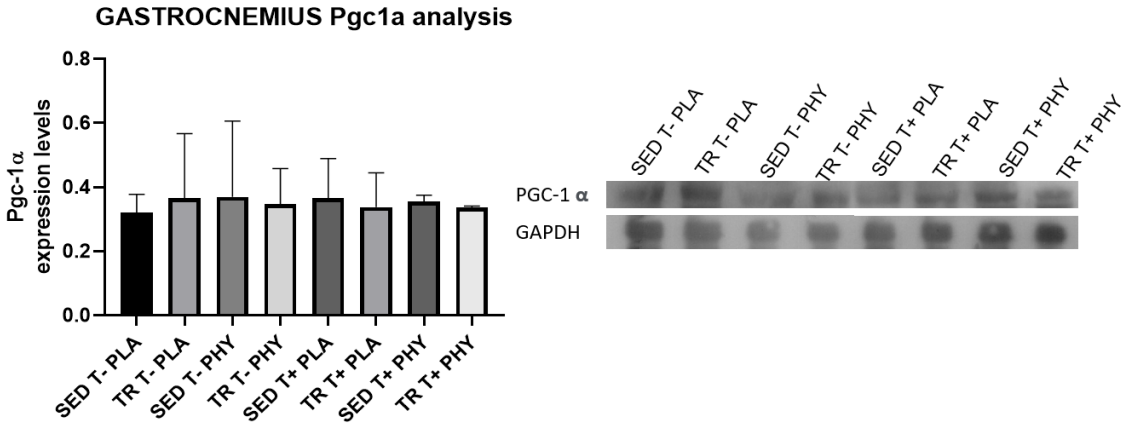

Figure 23 Western blot analysis for PGC-1α expression in gastrocnemius muscles. 40ug of protein was loaded for each sample

Furthermore, the expression of the Myo-D protein level of the SED T+ PHY, TR T+ PLA, TR T+ PHY groups does not undergo variations and maintains a value comparable to the groups of mice without tumors (fig. 24). Only a significant reduction in Myo-D expression is observed in the SED T+ PLA group compared to the TR T- PLA (fig. 25) .

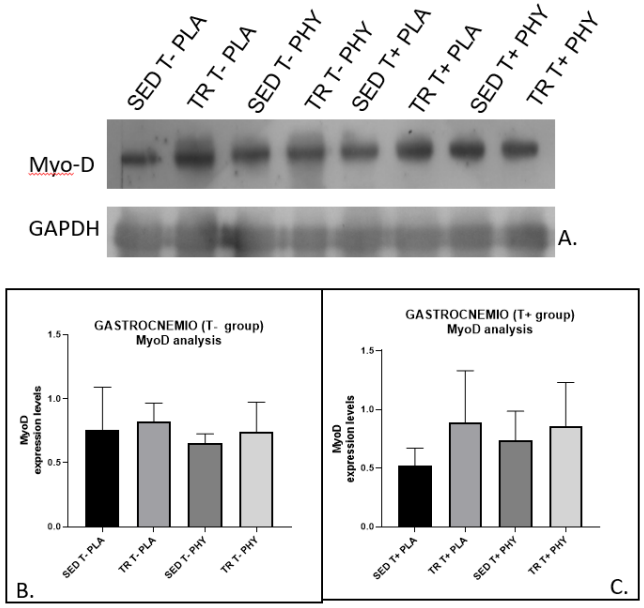

Figure 24 Western blot analysis for Myo-D expression in gastrocnemius muscles. 40ug of protein was loaded for each sample**.**

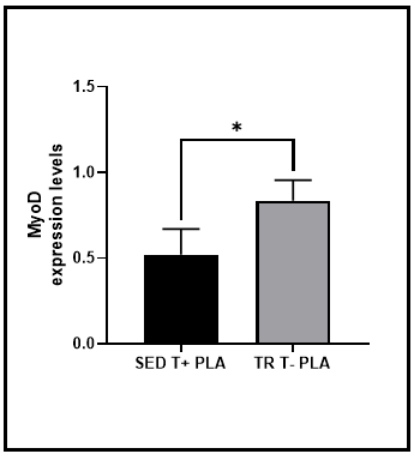

Figure 25 Comparison of Myo-D expression which appears to be significantly reduced in the SED T+ PLA group compared to the TR T- PLA group.

Discussion

In this study, we aimed to investigate the condition known as cachectic syndrome following two types of treatments: physical exercise and the use of treatment involving the use of nanovesicles containing Hsp60 (physiactisome; PHY). The PHY vesicles were obtained from the culture medium derived from C2C12 cells that had been transfected via electroporation with the pCMV6-Entry-HSPD1 plasmid. I showed the production of physiactisome and it was confirmed by western blot analysis and immunofluorescence staining of transfected C2C12 cells. The nanovesicles from PHY were isolated and observed by scanning transmission electron microscopy (STEM). Furthermore, using western blot techniques, the presence of ALIX (nanovesicle marker) was observed in nanovesicles, it was also demonstrated that there was a significant content of Hsp60 inside the nanovesicles compared to the control that did not undergo transfection. It is known that physical training has a positive effect on the recovery of muscle mass in wasting conditions (Bowen TS et al., 2015; Graham ZA et al., 2021). Exercise also has effects on the immune system and on the release of cytokines, playing a role in the control of pro- and anti-inflammatory proteins (Pedersen BK et al., 2000; Rhind SG et al., 2002), is able to reduce pro-inflammatory cytokine expression, mainly of TNF and IL-6. (Jaworska J et al., 2020; Calle MC et al., 2010). There is some evidence to suggest that exercise training may be beneficial during the treatment of cachexia and for survival (Hardee JP et al., 2017; Halle JL et al., 2020). On the other hand, it is important to develop new therapeutic strategies, as in certain clinical conditions it is not always possible to use physical exercise. In this study, I documented through the results obtained from the analysis of the cross-section area (CSA) of the gastrocnemius muscles stained with hematoxylin-eosin, that physical training and treatment with physiactisome help to preserve the muscle fibers from cachexia. In fact, in all trained groups both treated with physiactysome or placebo (TR T- PLA, TR T- PHY, TR T+ PLA, TR T+ PHY) a significant increase in the cross-sectional area of the muscle fibers was observed. It should be underlined that the CSA of the muscle fibers of the groups of cachectic mice, subjected to training and/or treatment with physiactisome; SED T+ PHY, TR T+ PHY, TR T+ PLA, show values comparable to the cross-section areas of the groups of mice without tumors. Furthermore, it was observed that the CSA of the group of cachectic mice treated with physiactisome (SED T+ PHY) had a significant increase compared to the sedentary cachectic group without treatment (SED T+ PLA), although a

notably greater and statistically significant increase in cross-sectional area was observed in the groups of tumor-bearing mice subjected to training alone (TR T+ PLA) and those that received physactisome treatment in conjunction with training (TR T+ PHY) when compared to the untreated cachectic mice.

Skeletal muscle training is responsible for a strong increase of heat shock proteins (HSPs) (like Hsp60), and their overexpression can lead to protection against and improvements after a variety of stressor factors (Venojärvi M et al., 2007; Nussbaum EL et al.,2007, Geiger PC et al., 2011). Furthermore, following resistance exercise, with mice for 6 weeks trained, increased Hsp60 levels were obtained, mainly observed in the soleus muscle (rich in MHC-I slow fibers) and in the blood. Type MHC-IIb fibers, on the other hand, showed lower levels of Hsp60 than any other fiber type (Barone R. et al., 2016). Based on the data available in the literature, in this study, I used western blot analysis to investigate the expression of Hsp60 in the gastrocnemius muscle of animals subjected to physical endurance training and/or physactysome treatment. Among the groups of mice without tumors, a significant increase in Hsp60 expression is observed in the groups of sedentary mice treated with physiactisome (SED T-PHY) and in mice treated with physiactisome and trained (TR T-PHY) compared to the control. So probably in healthy subjects physiactisome could be involved or support pathways stimulated by physical exercise. However, a significant increase in Hsp60 was not observed in the group of healthy trained mice (TR T-PLA) compared to the control. This could probably be due to the type of aerobic resistance training supported by the gastrocnemius muscle, which is a muscle made up mainly of fast fibers that carry out a predominantly anaerobic activity, in fact, the gastrocnemius muscle is composed primarily of MHCIIB-type fibers and has been shown to have lower levels of Hsp60 after resistance training than MHCI type fibers (Barone R. et al., 2016). Instead, in the group of mice with the tumor, contrary to what was expected, a notable reduction in the expression of Hsp60 was observed in the trained groups and treated with physiactisome compared to the sedentary ones. On the basis of the data collected, we do not have the possibility to understand why the contribution of the physiactysome treatment, which we have seen have a positive effect in the groups of healthy mice, did not have the same effect in the mice with tumors; one of the possible causes is that probably the concentration of the physiactisome was not sufficient in a pathological condition such as cancer cachexia to guarantee a stable increase in Hsp60 in the gastrocnemius muscle. As mentioned previously it is possible to hypothesize a close link between Hsp60 and PGC1α. In myoblast cell cultures following the overexpression of

Hsp60, an increase in the expression of the PGC1α isoform was observed (Barone et al., 2016).

In this study, the expression levels of PGC1α in the gastrocnemius muscle were studied by western blot analysis, but from the results obtained in none of the groups were changes in the levels of PGC-1α observed. It is known that the number of mitochondria is greater in tissues with MHCI fibers and less present in tissues with MHCIIB fibers. So probably due to the type of endurance training done by the mice, no variations are observed for PGC-1α in the different groups, and the muscle maintains a basal activity. PGC1α expression appears to be altered neither by the increase in Hsp60 observed in groups of tumor-free trained mice, nor by the reduction in Hsp60 observed in groups of tumor-trained mice (TR T+ PHY). Further investigation for Hsp60 and PGC1α expression should be conducted in other muscles, such as the soleus, which is characterized by its fibrous composition rich in MHCI-type fibers and mitochondria, and it is likely to have been more stimulated by the resistance training protocol employed in this study.

It is well established that physical exercise can stimulate the activity of muscle-resident stem cells, specifically the satellite cells (SCs) (Kadi F. et al.,2004; (Kvorning T. et al., 2015). Following the stimulus, the SCs emerge from their quiescent state and begin to proliferate and differentiate, one of the key proteins involved in the differentiation of muscle cells is Myoblast Determination Protein (Myo-D). (Kong Y. Et al., 1997; Legerlotz K et al., 2008; Bazgir B et al., 2017). Consequently, in response to appropriate environmental stimuli, the myogenic program can be initiated, leading to a reduction in the expression of Pax3 and Pax7, accompanied by a gradual upregulation of Myf5 and MyoD expression, thereby facilitating myogenesis (Hernandez-Torres F.H. et 2017). It is established that within the muscle tissue, a balance exists between catabolism and anabolism, primarily governed by the activity of the FoxO protein, which oversees the protein degradation pathway (atrophy) (Sandri M. et al., 2004), and the mTOR protein complex, which governs the protein synthesis pathway (hypertrophy) (Ogasawara R et al., 2019). Physiologically, the protein kinase AKT inhibits the activity of FOXO, thereby promoting protein synthesis. Furthermore, FOXO, in addition to its role in regulating pathways associated with protein degradation, is also involved in modulating myogenesis by interacting with the activity of MyoD (Xu M et al., 2017). In cancer cachexia, FOXO activity is upregulated leading to the inhibition of MyoD activity and activating the ubiquitin-proteasome system UPS, thereby promoting protein degradation (Yang W 2020).

Therefore, in this study, additional investigation was conducted to assess the expression of the MyoD protein in the gastrocnemius muscle through the utilization of the western blot technique. It was observed that the Myo-D protein expression levels in SED T+ PHY, TR T+ PHY, TR T+ PLA cachectic groups have a similar expression trend compared to the groups of mice without tumors. In fact, no significant differences are observed between these groups. On the other hand, it was observed that the group of untreated sedentary cachettitic mice (SED T+ PLA) showed a significant decrease in Myo-D expression compared to the TR T- PHY group. Probably both physical activity and treatment with physactisomes will have had a possible positive effect by preserving the satellite cells of the gastrocnemius muscle fibers in the groups of cachectic mice. To learn more and better understand this data, further investigations are needed both on other factors regulating myogenesis and on satellite cells.

Conclusion

This study showed the preparation and characterization of the product nanovesicle-based anticachectic drug containing Hsp60 (physiactisome). Furthermore, it has been demonstrated that following treatment with training and/or administration of physactysomes in cachectic mice, the gastrocnemius muscle fibers are preserved, confirmed by the significant increase in the cross-section area of the muscle fibers. The vesicles obtained from C2C12 following cell transfection may contain other cell-derived components such RNA or proteins other than Hsp60. Therefore, future studies regarding the action of heat shock protein 60 are necessary to better understand whether the effect of the vesicles in vivo is mediated by Hsp60 or by other possible factors contained in the vesicles. From a molecular point of view, Myo-D expression levels in the gastrocnemius muscle of mice with cachexia subjected to training and/or physactisome treatment appear preserved and show expression comparable to Myo-D levels of healthy mice. A significant increase in Hsp60 was observed following treatment with physiactisom and/or training in groups of tumor-free mice, however, this result was not repeated in the cachectic trained groups, and this leads to the hypothesis that probably the concentration of the drug must probably be modulated to better adapt it to the pathological condition of cachexia.

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