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UNIVERSITA' DEGLI STUDI DI SIENA

Department of Biotechnology, Chemistry and Pharmacy

PhD School in Biochemistry and Molecular Biology BIBIM 2.0

XXXIV Cycle

A metabolic change towards fermentation drives cancer cachexia in myotubes

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1. Introduction

1.1 Background

It has long been known that tumours not only trigger local tissue function alterations but also promote systemic effects on the whole-body metabolism. The role of inflammatory cytokines clearly emerged as crucial for many of systemic paraneoplastic effects observed in cancer patients. Particularly, the uncoordinated production of pro-inflammatory cytokines, secreted by both tumour cells and host cells, promotes the onset of a condition defined cancer cachexia, which is observed in a significant proportion of advanced cancer patients. One of hallmarks of this cancer-related syndrome is massive skeletal muscle wasting, leading to a progressive bodyweight loss in response to cancer growth. Besides, cancer cachectic patients show a drastic worsening of both prognosis and quality of life. Thus, cachexia managing represents an important and unmet need. At present, an effective therapeutic option is not available to counteract cancer cachexia, due to the limited understanding of the underlying molecular mechanisms of the disease process. This highlights the need to better define mediators and factors governing the molecular basis of cancer cachexia. However, cancer-associated cachexia describes a complex multifactorial disease which may involve multiple signalling pathways and candidate molecular approaches may therefore be limited due to the overall molecular interaction underlying cancer cachexia onset and progression.

Cancer cachexia is considered an energy-balance disorder caused by pronounced metabolic alterations. Despite the evidence suggesting that cancer-induced cachexia is associated with a broad range of metabolic rearrangements, the potential role of muscle metabolic abnormalities in the activation and development of cancer cachexia has been little studied so far.

1.2 Cancer cachexia: clinical relevance

Cachexia is a complex multi-organ syndrome characterized by unchecked and progressive loss of body weight associated with dysregulated control of energy and protein balance [1]. Although it occurs in a wide range of chronic or end-stage diseases, cachexia plays a prominent role in association with cancer [1,2]. Globally, approximately 8.2 million people die from cancer each year, and at least half of the deaths are attributed to the tumours most frequently associated with cachexia, such as pancreatic, esophageal, gastric, pulmonary, haepatic and colorectal cancers [3]. Particularly, cachexia affects 50–80% of patients with advanced cancer and accounts for about 20% of cancer-related deaths [4,5], mainly due to impaired respiratory or cardiac function [6,7]. Cachectic patients show a drastic worsening of both prognosis and quality of life, as well as reduced tolerance and response to antineoplastic treatments [8]. In skeletal muscle, cachexia induces wasting and atrophy due to several metabolic alterations [4,9,10]. Muscle loss is due to the great increase in protein degradation, not counterbalanced by protein synthesis, in which activation of the ubiquitin-dependent proteasome pathway plays a crucial role [11]. Weight loss due to muscle mass loss has been associated with poor prognosis and, notably, it has been shown that the inhibition of weight loss would significantly improve overall survival in patients with gastrointestinal cancer [12]. Therefore, cancer cachexia has a negative impact on cancer patient prognosis and quality of life. Thus, the most in-dept study and the discovery of new strategies able to prevent or reverse cancer cachexia would significantly be beneficial to the management of malignancy in general.

Heterogeneity of clinical manifestation and the multifactorial nature of cancer cachexia have greatly hindered the diagnosis of this syndrome, as a universal definition has only quite recently been established. Although our understanding of cachexia has progressed over the past decade [13], a lack of a definition, diagnostic criteria and classification has impeded advanced in both clinical trials and clinical practice [14,15,16]. Starting from 2006, cachexia was defined as the unintentional

weight loss of at least 5% in 12 months with underlying illness, with the concomitant presence of at least three of five overall criteria including i) decreased muscle strength, ii) anorexia, iii) chronic fatigue, iv) low fat-free mass index and v) abnormal biochemical analyses (e.g. increased inflammatory markers) [17]. In 2011 several experts participated in a formal consensus process to develop a framework for the better definition and classification of cancer cachexia. The consensus defined cachexia as a multifactorial syndrome characterised by an ongoing loss of skeletal muscle mass (with or without loss of fat mass) that cannot be fully reversed by conventional nutritional support and leads to progressive functional impairment. The pathophysiology is characterised by a negative protein and energy balance driven by a variable combination of reduced food intake and altered metabolism [2]. For what staging is concerned, cachexia was defined as a continuum with three stages of clinical relevance: precachexia, cachexia and refractory cachexia. In precachexia, early clinical signs and metabolic alterations occur before the involuntary weight loss (ie, ≤ 5%). Not all patients experience the entire spectrum as the risk of progression depends on a wide range of factors such as cancer type and stage, the presence or the severity of systemic inflammation and the lack of response to anticancer therapy. However, precachexia evolves in cachexia when patients exhibit i) more than 5% loss of stable body weight over the past six months, or ii) body mass index (BMI) less than 20 kg/m^2 and ongoing weight loss of more than 2%, or iii) sarcopenia and ongoing weight loss of more than 2%. Ultimately, refractory cachexia, characterized by active catabolism, low performance status and a life expectancy of less than three months, emerges as a result of a very advanced cancer or the presence of rapidly progressive cancer unresponsive to anticancer therapy. In this final stage, therapeutic interventions focus typically on alleviating the consequences and complications of cachexia [2].

Although cancer cachexia is associated with poor prognosis, notably, no simple correlation can be established regarding the tumour burden, anatomical position of the tumour, staging of tumour or metastasis [6]. Furthermore, not all types of cancer lead to cachexia with the same incidence. Indeed, cachexia has been found to be most

commonly associated with gastric or pancreatic cancer (60%), while in other cancer such as breast, sarcomas and non-Hodgkin's lymphomas cancer cachexia is less frequently seen (15%), in according with the previous mentioned ≥ 5% weight loss definition [18]. Although cancer cachexia occurs with high frequency in some types of cancer leading to a dramatic worsening of patient prognosis, there is no effective early detection technique or treatment that can prevent or reverse its debilitating effects to date.

1.3 General mechanisms involved in cancer cachexia

The complexity of cancer cachexia is revealed as it affects many tissues and organs, whose functional changes contribute to the resulting symptoms observed in patients with cancer cachexia. Altered molecular processes, that appear to be initially adaptive in response to the tumour, and chronic dysregulation of key metabolic pathways lead to a hypercatabolic state of body wasting as seen in cancer cachexia. The main systemic physiological changes observed in cancer cachexia include: i) anorexia and dysregulated neurological signalling; ii) hypermetabolism; iii) increased acute phase response (APR) in liver in addition to depletion of adipose tissue and skeletal muscle. The observed physiological modifications during cancer cachexia are mainly due to the release into the systemic circulation of pro-inflammatory cytokines and cachectic factors produced by both cancer cells and host immune cells in response to cancer growth [19]. In this paragraph, therefore, will be described the role played by cancer in the genesis of cachectic pathology, with particular attention to metabolic alterations and chronic systemic inflammation induced by tumour presence and growth, which ultimately promote cachexia.

1.3.1 Cancer as a metabolic entity

Cancer is able to reprogram cellular metabolism in order to promote its growth and progression, evading the signals that block growth and induce cell death. The transformation into "cancer cell" requires the onset of a major genomic instability that allows the overexpression of oncogenes (such as c-Myc), encoding receptors for growth factors, signal transduction proteins (such as Ras and phosphatidyl-inositol 3-kinase) and inhibitors of the apoptotic process (such as Bcl-2). This is accompanied by decreased expression of genes encoding proteins involved in the inhibition of cell division and proliferation (such as p53) [20]. Metabolism, composed of interconnected biochemical reactions that promote proliferation, survival, and controlled growth of cells in the body, can be divided into catabolic pathways, which generate energy in the form of adenosine triphosphate (ATP) from complex molecules, and anabolic pathways, which instead consume energy to synthesize useful substances for cells [21]. While under physiological conditions metabolism is tightly regulated according to cellular needs, in the cancerous condition cancer cells are genetically reprogrammed in order to promote key metabolic pathways, such as aerobic glycolysis, glutaminolysis and fatty acid synthesis [22]. These modifications are, in fact, functional and essential for tumour growth and progression [23]. Many tumour cells, having numerous altered signalling pathways [24], exhibit high avidity towards glucose and glutamine [25], a more glycolytic metabolism and an increased ability to produce and secrete lactate [26]. Aerobic glycolysis not only plays a key role in allowing cancer cells to grow rapidly but also facilitates cancer progression by increasing the invasive and metastatic capacity of cancer cells [27]. Furthermore, considering that aerobic glycolysis is a very energy inefficient process, it is not surprising that cancer cells make sure to uptake as much glucose as possible [28]. All these anabolic and catabolic alterations are involved in the state of systemic inflammation, imbalance of energy metabolism, and tissue wasting caused by the cancer-induced stress condition involved in the onset of cachexia [29].

1.3.2 Chronic systemic inflammation

One of the main features of cancer cachexia is the establishment of a critical inflammatory condition, due to the increased production and secretion of pro-inflammatory cytokines [30]. Cytokines are proteins that act as intracellular paracrine mediators, capable of inducing (pro-inflammatory cytokines) or inhibiting (anti-inflammatory cytokines) the immune response. Chronic inflammation, found in cancer and cachectic patients, is ultimately the consequence of a consistently high level of pro-inflammatory cytokines [31]. Another factor contributing to the systemic inflammatory state is, certainly, the ability of cancer to promote the hepatic Acute Phase Response (APR). Indeed, it has been observed in cancer patients that the liver is among the main organs in which protein metabolism is altered [32]. Indeed, under physiological conditions, the liver synthesizes structural and secretory proteins in equal proportion. In patients with cancer cachexia, however, this balance is strongly shifted towards the hepatic production of secretory proteins, in particular Acute Phase Proteins (APPs) [33]. Hepatic APPs can be functionally divided into i) proinflammatory APPs, which play a role in the innate immune response and therefore their synthesis is increased in the presence of an ongoing inflammatory state (including c-reactive protein and fibrinogen) and ii) anti-inflammatory APPs, whose levels instead decrease in the presence of inflammation (such as albumin). Therefore, in cancer cachexia patients, APR is promoted at the hepatic level, which induces an increased production of pro-inflammatory APPs and a concomitant decrease in the production of anti-inflammatory APPs [34]. As evidence of this, elevated plasma levels of c-reactive protein and hypoalbuminemia are often found in the presence of cancer cachexia [35]. APR is not only a highly energy-intensive process, but also requires a surplus of proteins and amino acids to reach the liver via the bloodstream [36]. Therefore, to support the increased rate of production of pro-inflammatory APPs, protein mobilization from peripheral tissues, particularly muscle tissue, is promoted, a phenomenon that certainly accelerates the skeletal muscle wasting typical of cancer cachexia patients [33]. Ultimately, it is the systemic

inflammation state established in cancer patients, due to the production of pro-inflammatory cytokines, to be attributed in part to the tumour cells, in part to the cells of the immune system, that promotes tissue wasting and thus the onset and maintenance of the cachectic state [37].

1.3.3 Anorexia and neurological regulation

Although anorexia may contribute to the weight loss observed in cancer cachexia, the anorexia-related reduced food intake appears to be not crucial as skeletal muscle is usually preserved with predominant adipose depletion in anorexia, whereas in cancer cachexia, similar loss was observed in skeletal muscle and adipose tissue [38,39]. Furthermore, nutritional supplementation has been shown to have minimal effectiveness in reversing wasting in cancer cachexia [40], even if counteracting malnutrition with nutritional supplementation seems to be beneficial in the restoration of body mass in patients affected by gastrointestinal cancer and receiving radiotherapy [41]. Therefore, anorexia alone cannot justify the skeletal muscle wasting observed in cancer cachexia patients, even if reduced nutritional intake could coherently result in weight loss.

Induction of anorexia in cancer patients experiencing cachexia could result from i) the dysregulated traffic of pro-inflammatory cytokines such as interleukin-6 (IL-6), interleukin-1 (IL-1) and tumour necrosis factor α (TNF-α) across the blood-brain barrier [42]; ii) the enhancement of anorexigenic (appetite-suppressing) signals or the inhibition of orexigenic (appetite-stimulating) signals [43]. In this context, the block of anorexigenic melanocortin signalling in the central nervous system has highlighted anorexia reversal in rats with prostate cancer [44] and tumour-bearing rats and cancer patients with anorexia show reduced levels of orexigenic signals such as neuropeptide Y (NPY) [45]. Therefore, complex molecular mechanisms attributed to dysregulation of cytokines and neuropeptides guide anorexia induction.

1.3.4 Hypermetabolism and futile metabolic cycles

Cancer patients suffering from cachexia usually manifest changes in metabolism; energy expenditure has been found to vary between 60-150% of the expected values of resting patients [46]. Cancer cachexia is considered an energy balance disorder due to pronounced metabolic alterations. Cancer patients have resting energy expenditure (REE) higher than healthy individuals [47], probably induced by inflammatory stimuli [48]. The increase in REE can be attributed to increased protein turnover, inefficient energy metabolism including unnecessary heat production and futile metabolic cycles such as the Cori cycle (lactate recycling) to satisfy the glucose demand of the tumour [36]. In addition, the elevated REE could also be attributed to increased thermogenesis in skeletal muscle or brown adipose tissue (BAT), which usually exists in minimal amounts in adult humans [49]. Thermogenesis in the BAT and skeletal muscle is regulated by uncoupling proteins (UCP), which dissipate the proton gradient along the inner mitochondrial membrane leading to decreased ATP generation [4,50,51]. The increase in UCP1 gene expression has been shown to be significantly elevated in the BAT of cachectic mice MAC-16 tumour-bearing mice [52]. Similar results were reported in a clinical study highlighting a significant increase in UCP3 mRNA in the skeletal muscle of cancer patients undergoing weight loss compared to stable weight controls [53]. Coherently, increased thermogenesis could contribute to the disrupted energy homeostasis observed during cancer cachexia.

Energy is also wasted through futile metabolic cycles such as the Cori cycle during cancer cachexia. The overall preference of cancers to generate energy through anaerobic glycolysis over oxidation regardless of oxygen concentration (known as the Warburg effect) is less energy efficient and generates lactate as an end product [54]. To meet the enormous demand for glucose, lactate produced by the tumour and amino acids, mainly from the protein reserves of skeletal muscle, are fed into gluconeogenesis for glucose production in the liver. This recycling of lactate accounts for up to 300kcal of energy loss per day in patients with cancer cachexia [55]. Therefore, during cancer cachexia, the body experiences a state of energy stress

whereby energy is wasted through inefficient metabolic cycles that support tumour growth.

All these phenomena, taken together, are responsible for the alteration of energy metabolism that we find in patients with cancer cachexia, in which the cancer determines an increase in energy expenditure that leads to a strongly negative energy balance. The homeostatic tissue balance is therefore shifted towards an overall catabolic state, which leads to the typical tissue degeneration mainly affecting the skeletal muscles of patients affected by the disease.

1.4 Skeletal and adipose tissue wasting

As mentioned above, cancer cachexia preferentially affects skeletal muscle and adipose tissue. In fact, tumour growth determines the release of substances that alter tissue homeostasis, promoting metabolic alterations that lead to the loss of muscle tissue and the depletion of the body fat reserves. While in skeletal muscle tissue this is primarily mediated by the promotion of proteolysis [37], at the adipose level the effect is primarily due to the promotion of lipolysis [56]. This paragraph will describe the role played by proteolysis and lipolysis in the onset of the cachectic state, with particular attention to the mechanisms that regulate these two processes, which are crucial for the maintenance of muscle and adipose homeostasis.

1.4.1 Lipolysis and Browning in cancer cachexia

Adipose tissue, other than its function as an energy reserve, can be considered as an organ with endocrine function, capable of secreting hormones and adipokines that modulate appetite and metabolism. This tissue is composed of lipids such as triglycerides, which constitute approximately 90% of the body energy reserves, released from adipose tissue in the absence of energy [57]. In addition, adipose tissue secretes adipokines such as leptin, adiponectin, TNFα, and IL-6, which can regulate

appetite, energy expenditure, insulin sensitivity, and the inflammatory response [57]. Although it does not always occur and in any case occurs secondary to muscle tissue loss [19], marked adipose tissue loss has recently been counted among the main peculiarities of neoplastic cachexia, which can contribute to the negative energy balance seen in cachectic patients [58]. The depletion of adipose reserves is due to increased lipid mobilization, mainly attributable to the promotion of lipolysis in adipocytes [59]. Lipolysis, characterized by the hydrolysis of triglycerides with release of fatty acids and glycerol into the bloodstream, is a degradative mechanism mediated by lipolytic hormones such as glucagon and adrenocorticotropic hormone (ACTH), via signalling pathways involving cyclic AMP [19]. In cancer cachexia, promotion of lipolysis in adipocytes due to increased levels of some mediators such as Lipid-Mobilizing-Factor (LMF), TNFα, IL-1, IL-6 and IFN-γ has been shown [60,61]. Interestingly, increased lipolysis in adipocytes is a phenomenon peculiar to cancer cachexia, considering that subjects who lose weight due to other factors do not manifest changes in the lipolytic pathway [56]. Furthermore, cancer cachexia is associated with increased Hormone-sensitive lipase (HSL) enzyme activity and blood levels of fatty acids [62], reflecting how the HSL enzyme is able to promote lipolysis in superficial adipose reserves [63]. An interesting feature of cancer cachexia is the occurrence of a progressive change in the type of fat tissue, from white adipose tissue (WAT) to brown adipose tissue (BAT), whose name comes from the darker colour, associated with enrichment regarding the number of mitochondria. These mitochondria exhibit high levels of UCP-1, which, as mentioned above, directly promote thermogenesis by decoupling the electrochemical gradient from ATP generation. The progressive switch from WAT to BAT contributes most strongly to increased energy expenditure, a common condition in cachectic patients [64]. The cytokine IL-6, whose levels are significantly increased in cancer cachexia [57], plays a crucial role in the phenomenon defined "browning", being able to overexpress UCP-1 [65]. Overall, adipose tissue loss and its degeneration can be interpreted as a critical point in the cachectic process, able to further contribute to the propagation of cachexia, through the promotion of muscle tissue loss [66].

1.4.2 Skeletal muscle protein breakdown in cancer cachexia

Skeletal muscle tissue is a very plastic tissue, capable of functional and metabolic changes in response to physiological stimuli. However, pathological conditions, such as tumour growth, can compromise the mechanisms that regulate muscle homeostasis, leading to loss of muscle mass, loss of function and compromised metabolism. This tumour-induced condition, either directly as a result of substances produced by the tumour cells or indirectly by substances produced by the host immune system, is characterized by the promotion of the proteolytic process. The skeletal muscle is therefore one of the major targets of cachexia. The promotion of the proteolytic pathway inevitably leads to an increase in the degradation of muscle proteins with a consequent increase in the release of amino acids into the circulation. If on the one hand these amino acids are used for the synthesis of tissue proteins, on the other hand, reaching the liver through the bloodstream, go to support gluconeogenesis. As a consequence, this could increase the hepatic production of glucose, which tumour cells need in high quantities [67]. Proteolysis, a fundamental process for the maintenance of muscle homeostasis, is mainly regulated by two important cellular degradative systems: i) the autophagy-lysosome degradative system and ii) the ubiquitin-proteasome degradative system. Given the importance of the process, it is not at all surprising that these degradative systems are finely modulated by different signalling pathways. As a result of the alteration of these intracellular signalling pathways there is, in fact, a modification of the homeostatic balance between protein synthesis and degradation at the muscle level. This induces a loss of efficiency and functionality of skeletal muscles [67].

The autophagy system allows cells to degrade organelles and macromolecules in order to obtain recyclable molecules. There are three different types of autophagy: i) microautophagy, ii) chaperone-mediated autophagy, and iii) macroautophagy. Although all involved in the maintenance of muscle homeostasis, only macroautophagy appears to be clearly and distinctly implicated in cancer cachexia [68]. Given its involvement in cachexia, I will here analyse only the process of

macroautophagy, from now simply referred as autophagy. Autophagy is a catabolic process that occurs through sequential steps : i) induction; ii) formation of the phagophore by membrane folding from a wide variety of cellular compartments such as endoplasmic reticulum, mitochondria, golgi apparatus, and plasma membrane [69]; (iii) elongation of the membrane to form the autophagosome, which goes on to enclose the cellular components that need to be degraded; (iv) fusion of the autophagosome with a lysosome; and (v) degradation of the sequestered components as a result of the action of lysosomal enzymes; vi) reconstitution of the cellular lysosomal pool by regeneration and biogenesis [67]. All these steps are regulated by a pool of genes called autophagy-related genes (Atg), whose expression is controlled at the transcriptional level, mainly by the transcription factors TFEB and FOXO. In particular, Atg12, Atg5, Atg7, Atg10, Atg4 and Atg8, involved in membrane elongation and cargo to be degraded recognition, represent a pool of genes that have been very often found overexpressed in the cachectic state [70]. Although autophagy was initially considered a non-selective degradative system, there is increasingly strong evidence for the presence of forms of autophagy capable of mediating a controlled degradative phenomenon of organelles and protein aggregates within the cell [67]. Activation of autophagy in muscle cells has been demonstrated in animal models of cancer cachexia [71], cirrhosis [72], sepsis [73], as well as in a variety of dystrophic and myopathic conditions [68,74,75,76]. The central role played by the induction of the autophagy-lysosome degradative system in the reduction of muscle proteins that characterizes cachexia has been highlighted in vivo, both in animal models and in cancer patients. In particular, autophagy activation has been observed in skeletal muscle of mouse models affected by colon cancer, Yoshida AH-130 hepatoma, and Lewis lung cancer (LLC) [71,77]. On the other hand, regarding human studies, the autophagy-lysosome degradative system was found promoted in skeletal muscles of oesophageal cancer patients [78], and in particular in pancreatic and gastrointestinal cancer patients [79]. To date, research has not been able to fully understand whether and how autophagy contributes to the genesis of cachexia. Nevertheless, the importance of the autophagy process in the muscle homeostatic

balance has clearly emerged. Indeed, the reduction of basal levels of mitophagy, a process by which mitochondria are selectively degraded, results in an intracellular accumulation of damaged mitochondria, a condition that leads to oxidation of contractile muscle proteins and consequently muscle function alteration [80]. Moreover, it has been observed that physical exercise, capable of counteracting cachectic progression and prolonging life expectancy of cancer patients [81], is able to induce the autophagic process. Nevertheless, the role of autophagy in cancer cachexia still appears to be highly controversial. In fact, while taking into account that the autophagic process would be able to contribute directly to muscle degeneration in cachexia, it should be considered that autophagy may also have a protective function [67].

The ubiquitin-proteasome degradation system is a finely regulated ATP-dependent protein degradation system in which intracellular proteins that need to be degraded are tagged through the formation of a covalent bond with a series of ubiquitin molecules (polyubiquitination), through the sequential action of three enzymes: i) E1 or activation enzyme, ii) E2 or conjugation enzyme, and iii) E3 or ubiquitin-ligase. Binding to the polyubiquitin chain allows proteins to be selectively sent to the proteasome, an intracellular structure where proteins are cleaved into peptides that are spilled into the cytosol. The importance of the proteasomal degradation system in protein catabolism has long been known [82]. With particular reference to cancer cachexia, findings confirm that it is precisely the promotion of this degradative system that is mainly involved in the increased degree of protein degradation that characterizes the pathology [83,84]. The ubiquitin-proteasome degradation process is a highly selective process, both due to the presence of different functional E2-E3 enzyme pairs involved in the degradation of different proteins and due to the fact that the various E3 ubiquitin-ligase enzymes exhibit specificity for specific groups of proteins. Although the content of E2 and E3 enzymes is not constant but rather varies not only in a tissue-specific manner but also dependent on particular physiological conditions [67], in the past decade the first ubiquitin-ligases playing a key role in muscle mass loss in cancer cachexia have been highlighted: muscle atrophy F-box

(MAFbx/atrogin-1) and muscle RING finger 1 (MuRF1) [85,86]. The involvement of these two enzymes in the development of cancer cachexia clearly emerged as the transcriptional levels of MAFbx and MuRF1 were shown to be seven to ten times higher in animal models affected by skeletal muscle atrophy, when compared with healthy controls [83]. These two ubiquitin ligases have differential substrates, although still related to the maintenance of muscle function. Indeed, MAFbx appears to be involved in the ubiquitination of proteins related to growth and survival processes, as well as in the degradation of MyoD, a key transcriptional factor in muscle differentiation [87], and of an important activator of protein synthesis such as eIF3-f [88]. MuRF1 has instead been shown to mediate the ubiquitination of many proteins critical for the maintenance of muscle structure, such as troponin I [89], actin [90], and myosin heavy chain [91]. Furthermore, TRAF6 is another ubiquitin-ligase whose role in the genesis of cancer cachexia has been shown to be central [92]. Indeed, TRAF6 has been found to be highly overexpressed, along with ubiquitin, in the skeletal muscles of gastric cancer patients [93]. The list of ubiquitin-ligases involved in the onset of cachectic muscle degeneration has recently expanded with the entry of MUSA1 and Fbxo31, belonging to the F-box containing complex (SCF) family, found to be strongly overexpressed in the muscles of cachectic C26 colon cancer mouse models [94]. Nevertheless, the role of these two ligases in muscle loss associated with the cancerous condition remains to be elucidated.

1.5 Mediators of cancer cachexia

A range of mediators have been associated with the pathogenesis of cancer cachexia, including pro-inflammatory cytokines such as tumour necrosis factor-α (TNF-α), IL-6 and interferon-gamma (IFN-γ); hormones such as insulin-like growth factor 1 (IGF-1) and glucocorticoids; and tumour-derived factors such as proteolysis-inducing factor (PIF) [6]. Several mediators have been found to be associated with specific tumour types, largely due to the variety of animal models used in the field of cancer cachexia.

Patient studies have also shown controversial results regarding serum levels of cytokines. However, these discrepancies could be attributed to the different sensitivity and specificity of the assay method, the stability and half-life of the molecules, and also to the type of tumour and the time at which the measurements were made [95]. In addition, some cytokines and hormones may be produced locally and therefore serum levels may not truly reflect their levels and activity within the tissue.

The research has focused on the study and understanding of the mediators of cachexia, in order to discover new therapeutic targets and therefore new therapies against muscle degeneration induced by cancer. As mentioned, these mediators, capable of promoting the loss of muscle mass, are not only produced by tumour cells (which would secrete these substances in order to receive a greater supply of nutrients through the bloodstream and thus promote their growth and progression), but also by the mesenchymal tissues undergoing degeneration and by the immune system [37]. Chronic inflammation not only plays a major role in carcinogenesis, where cancer cells may become capable of producing pro-inflammatory mediators in order to evade the apoptotic process and promote the process of angiogenesis and metastasis, but it also turns out to be one of the phenomena most involved in the onset of cachexia [96]. Indeed, many pro-inflammatory cytokines promote the onset of the cachectic state, including: TNFα, IL-6, IL-1, and IFN-γ [60,36].

Pro-inflammatory cytokines, however, are not the only mediators of the onset of the cachectic state, being involved also tumour-specific catabolic factors (called "tumour-derived") such as lipid mobilization factors (LMF) and proteolysis-inducing factor (PIF) [36], as well as substances belonging to the transforming growth factor (TGF-β) family such as myostatin and activin [37], glucocorticoids, and angiotensin II. Despite their heterogeneity, these substances are involved in promoting degradative mechanisms in cancer cachexia models [82].

In the subsections I will illustrate how these substances, having often a synergistic action, manage to mediate the onset of the cachectic state leading to strong alterations in metabolism and energy expenditure of the host.

1.5.1 Tumour Necrosis Factor-Alpha (TNF-α)

TNF-α is a pro-inflammatory cytokine produced by macrophages, monocytes, B and T lymphocytes and other cell types in response to invasive stimuli which influences growth differentiation and immune function [97]. This cytokine is considered one of the most involved in the induction of the cachectic process, being able to promote skeletal muscle atrophy, mainly through the NF-kB signalling cascade [98]. *In vitro* experiments have shown that TNF-α is able to inhibit both skeletal myocytes and adipocytes differentiation processes, and also contributes to the establishment of strong insulin resistance [99,100]. Furthermore, treatment with TNFα is sufficient to cause atrophy of cultured myotubes, as a result of gene overexpression of E3 ligase enzymes involved in the ubiquitin-proteasome controlled protein degradation process. This inevitably leads to a considerable decrease in muscle proteins [101].

TNF-α has been demonstrated to be secreted from tumour cells and infiltrating macrophages in the tumour microenvironment [102]. Nevertheless, TNF-α is primarily secreted by activated macrophages, often found in the adipose reserves of neoplastic and cachectic patients. This suggests that immune system cells and adipocytes could be the main sources of TNFα, but also of all cytokines that are involved in chronic inflammation, regulation of lipid mobilisation and signalling pathways related to energy consumption [37]. However, some data contradict this thesis. In particular, microarrays experiments carried out on biopsies of adipose tissue from neoplastic patients suffering from cachexia, have shown no changes in gene expression of genes involved in the inflammatory phenomenon [103]. Furthermore, reduced gene expression of TNF-α has been demonstrated in the muscles of rats carrying Yoshida AH-130, thus making skeletal muscle an unlikely source of TNF-α during cancer cachexia [104]. In support of this, no differences were observed in the amount of TNFα mRNA in the skeletal muscles of patients with cancer cachexia compared to healthy subjects [105]. TNF-α is believed to be the core mediator of cachexia in the Yoshida AH-130 hepatoma, Yoshida sarcoma and Lewis Lung

Carcinoma (LLC) models of cachexia [106]. However, clinical data have shown a discrepancy in the correlation between serum TNF-α levels and weight loss in cancer patients [107]. Administration of an anti-TNF-α antibody in AH-130 rats appears to increase the protein content of the gastrocnemius muscle and heart compared to the untreated group; however, a partial but significant preservation of tissue mass was only demonstrated in gastrocnemius [108]. TNF-α blockade was associated with a delayed decline of insulin and an increase in corticosterone; reduced proteolytic activity in tumour-bearing mice, which suggested a role for TNF-α in muscle wasting [108,109]. Nevertheless, recent clinical trials using anti-TNFα antibodies have shown no clinical benefit [110], suggesting that TNFα probably acts only as a promoting factor, but not sufficient to induce the typical wasting observed in cachectic muscles. The onset of the cachectic condition would therefore be due to a synergistic action between several factors, both of immune and tumour origin, which would be able, cooperating, to lead to the cachectic state.

1.5.2 Interleukin-6 (IL-6)

Among the pro-inflammatory cytokines that appear to be able to cooperate with TNF-α in promoting the systemic inflammatory state underlying cachexia there is certainly IL-6. Considering that many tumour types have been shown to actively secrete IL-6 [111] and that, unlike TNFα, high circulating levels of IL-6 have been shown to strongly correlate not only with decreased body weight in cancer patients, but also with reduced survival of such patients [112], IL-6 appears to play a relevant role in cancer cachexia. This cytokine, produced and secreted also by activated macrophages, contributes significantly to the systemic inflammation observed in cachexia. Indeed, IL-6 has been shown to promote the acute phase response (APR) in liver and muscle tissue through STAT3, whose activation is correlated with the onset of skeletal muscle degeneration [113].

Having, therefore, an established role in the development of cachexia, research has focused mainly on studying the effects of inhibition of signal transduction triggered

by the binding of IL-6 to its receptor. In this regard, using anti IL-6 receptor antibodies, the arrest of the progression of the cachectic state could be observed [114]. Recent clinical trials using monoclonal antibodies directed specifically against the cytokine itself performed on cancer cachectic patients, have shown that blocking the action of IL-6, the health status of the patient improves, with a reduction of anorexia, fatigue and anaemia, although there were no significant effects on weight loss [115].

The involvement of IL-6 in the cachectic state would be mainly attributed to the triggering of the JAK/STAT signalling pathway and the subsequent activation of the transcriptional factor STAT3, by which IL-6 would be able to modulate the expression of acute phase proteins [116], promote the expression of ubiquitin-ligase E3α-II (and therefore proteasomal degradation of muscle proteins) and, finally, also promote the autophagic process [117]. At the level of adipose tissue, IL-6 was found to be able to directly promote the lipolytic process in adipocytes [57] and the progressive transition to brown adipose tissue [65].

1.5.3 Interleukin-1β (IL-1β)

IL-1β is a pro-inflammatory cytokine secreted by activated macrophages that can regulate and amplify the inflammatory response by promoting the expression of other cytokines such as IL-6 and IL-12. Although IL-1β seems to play a synergistic role with other cytokines (in particular TNFα) in promoting muscle protein degradation [82], the involvement of this cytokine in cachectic disease is, to date, still unclear and controversial. Indeed, there are works that have obtained results in clear antithesis. In Turrin et al., the expression of anorexigenic cytokines (such as IL-1β, IFN-γ and TNFα) in the central nervous system and tumour tissue in murine models (affected by Fisher-344 sarcoma) was examined, observing that the anorexia induced by the cancer condition is mainly due to the overexpression of IL-1β and its receptor [118]. In contrast, the role of IL-1β as a factor promoting cachexia does not seem to emerge in Argilés et al., in which in vivo administration of a receptor antagonist for IL-1β did not result in any improvement in cachectic status in the mouse models used for the

study. This would seem to indicate at least a minor role of IL-1β in the genesis of cachexia [119].

Ultimately, we can conclude that IL-1β cannot be considered a crucial mediator in cachectic pathogenesis, although it may cooperate with other cytokines (such as TNFα, IL-6, and IFN-γ) in promoting muscle wasting [83] and under-expression of MyoD, a gene that regulates myogenesis and muscle differentiation, in mouse models of cancer cachexia [120].

1.5.4 Interferon-γ (IFN-γ)

IFN-γ is a proinflammatory cytokine produced and secreted by cells involved in the cell-mediated immune response, particularly T lymphocytes and NK cells [121].

The role of IFN-γ in cachexia has been well illustrated in Smith et al. and Pijet et al., in which has been observed how IFN-γ manages to promote, in cooperation with TNFα, the expression of the ubiquitin ligases MAFbx and MuRF1 and thus the proteasomal degradation of muscle proteins [122,123].

Ultimately, while both IFN-γ and TNFα would seem in themselves incapable of leading to the onset of the cachectic state, these two cytokines would have a strongly complementary and synergistic action in promoting the loss of muscle tissue found in cachectic patients [124].

1.5.5 Myostatin and activin

Myostatin belongs to the Transforming-Growth-Factor β (TGF-β) family, and it represents another crucial factor on which research has been focused in order to find the main mediators of the cachectic state. Indeed, myostatin is well-known for negatively regulating satellite cell activation and differentiation as well as muscle growth [125]. In this regard, it has been observed how the lack of myostatin is able to determine a strong imbalance in muscle homeostasis, leading to severe muscle hypertrophy, both in animal models [126] and in humans [127]. The role of myostatin in maintaining proper muscle function has also been highlighted. Specifically, overexpression of myostatin has been

shown to lead to a major skeletal muscle atrophic state [128] and, consistently, decreasing its expression has been shown to promote a significant increase in both muscle mass and fiber size. [129,130]. Myostatin, preferentially secreted by skeletal muscle cells, has a signal pathway that begins with its binding to the receptor for activin type II (ACTRII). This results in the recruitment of an Alk protein kinase that leads to the activation of the transcriptional complex consisting of Smad2 and Smad3 factors [131]. Although the mechanism by which myostatin is able to promote muscle loss is still not fully known, the research carried out leads to the conclusion that several signalling pathways may be involved, having in common the inhibition of Akt [132]. Activin A is another factor, belonging to the TGF- β family, that would seem to play a role in the pathogenesis of the cachectic state. The synthesis and secretion of activins is stimulated by pro-inflammatory cytokines, via activation of the Toll-like receptor, and by oxidative stress [133]. In particular, activin A has been observed to strongly be induced by pro-inflammatory cytokines and therefore is found overexpressed, mainly as a result of activation of the TNFα/TAK-1 cascade, in the presence of cancer cachexia [134]. However, published data once again seem to highlight that the synergistic action of multiple factors is required for the development of the cachectic state.

1.5.6 Glucocorticoids

Glucocorticoids are a class of steroid hormones produced primarily in humans in the fasciculate zone of the adrenal cortical. These hormones present the important biological function of modulating glucose metabolism, specifically regulating the mobilization of gluconeogenic amino acids and consequently gluconeogenesis. By binding to intracellular receptors (GRs), glucocorticoids are able to modulate the expression of genes involved in a variety of cellular responses. In particular, among the most important genes transcriptionally regulated by glucocorticoids are those for pro-inflammatory cytokines and growth factors, and those for their respective receptors [135].

Glucocorticoids may contribute to the manifestation of the cachectic state [136]. In fact, glucocorticoids promote skeletal muscle atrophy through overexpression of the ubiquitin ligase MAFbx, which is accompanied by increased expression of the lysosomal enzyme cathepsin L [137]. These findings were confirmed more recently as glucocorticoids were shown to increase the expression of atrophy-related genes such as MAFbx, MuRF1, and MUSA1 in myoblasts and myotubes [138]. Ultimately, glucocorticoids promote the activity of the two most important cellular degradative systems involved in the development of cachexia, the ubiquitin-proteasome and autophagy-lysosome degradative systems through the activation of FOXO transcription factors [139]. This is consistent with the findings that activation of the Foxo3 factor was able to lead to increased protein degradation in murine myotubes through promotion of both the proteasomal and lysosomal degradation systems [140]. Specifically, Foxo3 stimulates autophagy-lysosomal activity by inhibiting the IGF-1/PI3K/Akt signalling pathway. Glucocorticoids, in this regard, induce the activation of FoxO factors by decreasing the activity of the PI3K/Akt pathway and thereby prevent phosphorylation of these factors that keep them inactive in the cytosol [19]. Calcium (Ca^{2+}) may also play a role in glucocorticoid-induced muscle proteolysis. As evidence, the increased protein degradation induced in myotubes by dexamethasone treatment is significantly reduced using both BAPTA, a chelator of Ca^{2+} ions, and KN-62, a calmodulin kinase II inhibitor [141].

1.5.7 Angiotensin II

Angiotensin II (ANGII) is a hormone responsible for blood pressure and electrolyte control in the kidney. In particular, ANGII plays a crucial role in the renin-angiotensin-aldosterone system, a hormonal mechanism by which blood pressure, circulating plasma volume and tone of arterial muscles are regulated. Renal juxtaglomerular cells release the hormone renin into the circulation in response to (i) reduction in circulating blood volume, (ii) lowering of blood pressure, and (iii) stimuli from the orthosympathetic nervous system. When renin enters the circulation, it is able to activate angiotensinogen,

a peptide present in the bloodstream in an inactive form and produced in the liver. Angiotensin I is thus formed in the circulation, which in order to be converted into the active form angiotensin II must become a substrate of Angiotensin Converting Enzyme (ACE), which is mainly present in the pulmonary capillaries. The ANG II formed acts at the systemic level, determining: (i) arteriolar vasoconstriction; (ii) increased systemic pressure and decreased glomerular blood flow; (iii) the release of aldosterone by adrenal cortical, a hormone with hyper pressor action that acts on the renal tubules (distal convoluted tubule and collector duct) favouring the reabsorption of sodium ions and water molecules, thus leading to an increase in circulating volume and consequently in arterial pressure; (iv) increased hypothalamic release of antidiuretic hormone or vasopressin. Ultimately, the hormonal action of ANG II, both directly and indirectly, is expressed in an increase in blood pressure. ANGII was firstly considered as a possible factor with catabolic action on muscle in 2001, when has been observed that in patients with congestive heart failure, treatment with an ACE inhibitor resulted in an increase in fat reserves and muscle mass in those patients who showed obvious signs of cachexia [142]. This effect was probably related to the lowering of ANGII levels. As evidence, *in vivo* experiments have shown that ANGII infusion in mouse models causes loss of body weight [143]. *In vitro* experiments in murine myotubes subsequently attributed to ANGII the ability to promote a strong decrease in muscle protein levels, an effect mediated by induction of the ubiquitin-proteasome degradation system [144]. Furthermore, it has been demonstrated that ANGII is able to inhibit protein synthesis [145]. ANG II, finally, promotes ROS formation by activating the enzyme NADPH oxidase [146], and this results in the activation of NF-kB [19], a transcriptional factor whose involvement in cachexia has already been widely demonstrated.

1.6 Signalling pathways involved in cancer cachexia

Tumour progression leads to a situation of high energy stress in the patients, due to increased catabolism, and a systemic inflammatory state. Moreover, it is well known that at the basis of cancer cachexia there are intracellular signalling mechanisms controlling phenomena such as cell growth, ATP production and cellular response to cytokine stimulation [147]. The most relevant signalling pathways in cachexia, having the function to regulate the expression of genes involved in the onset of muscle atrophy (called atrogens) are mainly four: i) the IKK-NF-kB pathway, ii) the JAK-Stat3 pathway, iii) the TGFβ/Myostatin-Smad2/3 pathway and iv) the Akt-FoxO pathway (particularly important for the control of protein synthesis and degradation within the cell). These pathways are activated by the main mediators of the cachectic state (such as TNFα, IL-1, IL-6, IFN-γ and myostatin), which can modulate the expression of genes involved in the maintenance of muscle tissue homeostasis [67].

The above-mentioned signalling pathways involved in the onset of the cachectic state will be then described, with regard to the interconnections by which these pathways cooperate in promoting muscle tissue loss.

1.6.1 The IKK-NF-kB pathway

NF-kB is a dimeric transcription factor mainly involved in the regulation of the expression of genes that mediate the cellular response to stress conditions, as well as, more generally, of genes involved in the inflammatory and immune response. In many human cancers it is possible to observe how the NF-kB signalling pathway is strongly promoted, demonstrating how its fine regulation plays a certainly central role in carcinogenesis [148]. There are many receptors whose activation has been shown to activate the transcriptional factor NF-kB. These include receptors for TNFα and IL-1, which, although belonging to different receptor families, would appear to act similarly in activating the NF-kB factor. Under resting conditions, the dimer of NF-kB, whose expression level is

kept constant, is sequestered in the cytosol by binding to an inhibitory protein, IKB, which prevents its translocation into the nucleus. In fact, the binding of the three possible inhibitory proteins (IKB-α/β/ε) functions to mask the region of import into the nucleus (NLS) present on NF-kB, which therefore remains in the cytosol. When receptors involved in NF-kB activation are activated by binding to their ligand, a signalling pathway that leads to the activation of a multiprotein complex called IKK (IKB kinase) is triggered. IKK is composed of two serine/threonine kinases (IKKα and IKKβ) and a regulatory subunit (IKKɣ), which can phosphorylate the inhibitory NF-kB-binding protein. The conformational change induced by phosphorylation results in the release of the inhibitor, which is polyubiquitinated and degraded by the proteasome. Then, the NF-kB dimer, with its import region made accessible, can migrate into the nucleus, where it promotes transcription of genes under its control (Figure 1).

The importance of NF-kB factors in muscle degeneration has been widely highlighted. The muscle-specific overexpression of the IKKβ subunit, part of the IKK kinase complex able to promote the activation of NF-kB, has been shown to determine a strong state of muscle degeneration in mouse models mediated, at least in part, by the ubiquitin-ligase MuRF1. Furthermore, the inhibition of the classic NF-kB signalling pathway, achieved by overexpression of the IkBα-SR super repressor, was found able to significantly arrest muscle tissue loss in mouse models of Lewis lung carcinoma [149]. Although its involvement in cancer cachexia is not completely clear, since, for example, the C-26 colon cancer-induced cachectic state does not appear to be due to the promotion of the NF-kB pathway [150], findings have been shown that there is a strong involvement of the IKKβ kinase and the IKB inhibitory protein in the onset of the cachectic state. In this regard, knockout mice for IKKβ have been shown to be resistant to muscle atrophy and show hyperphosphorylation of Akt [151]. This study highlights the existence of a cross-talk between the signalling pathways capable of activating IKK and those capable of inducing Akt phosphorylation, a phenomenon that could explain the effect on muscle mass obtained through IKKβ inhibition [67].

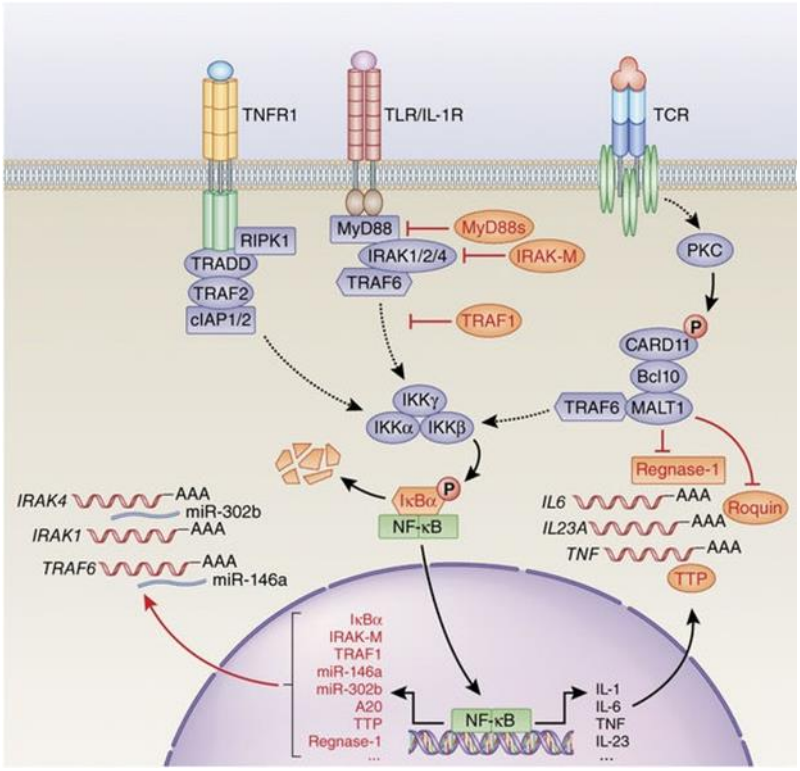

Figure 1. NF-kB signalling pathway. The dimeric transcription factor NF-kB, following phosphorylation of its inhibitor IkBα, mediated by the IKK complex, migrates into the nucleus and promotes the expression of genes that mediate the cellular response to stress conditions and are involved in the inflammatory response, including IL-1, IL-6, TNFα, and IL-23.

1.6.2 The JAK-STAT3 pathway

Cytokine receptors are associated with cytosolic tyrosine kinase enzymes called JAK (Janus kinase) that can phosphorylate and activate proteins called STAT (Signal Transducer and Activators of Transcription) proteins. STATs are transcription factors and therefore, once migrated into the nucleus they regulate the expression of target genes. Cytokine receptors are associated with one or two of the known JAKs (JAK1, JAK2, JAK3, and Tyk2). When a cytokine binds to its receptor, the binding induces the dimerization of two adjacent receptors. In this conformation, two JAK proteins are so spatially close that they activate each other by autophosphorylation with their respective tyrosine kinase activities. The activated JAK proteins phosphorylate the receptor in its intracellular

domain, thus allowing the subsequent recruitment of two STAT proteins. The recruited STATs are in turn phosphorylated by the JAKs themselves, and the phosphorylation allows the STAT proteins to leave the receptor domain and thus migrate in dimeric form into the nucleus, where they bind to DNA activating the transcription of target genes (Figure 2). The JAK-STAT pathway is subject to regulation by negative feedback. STAT dimers, in fact, in addition to promoting the expression of genes in response to cytokine stimulation, can also activate genes encoding inhibitory proteins, which perform their action by binding and inactivating phosphorylated JAKs, or by binding to STAT dimers preventing their binding to DNA. Nevertheless, negative feedback is not able to permanently block the response, as this requires the JAKs/STATs inactivation by dephosphorylation. The JAK-STAT signalling pathway, activated by the main cachectic mediators (TNFα IL-1 and IL-6) [67], seems to play a relevant role in neoplastic cachexia, being involved in the regulation of both main cellular degradative mechanisms (the ubiquitin-proteasome system and autophagy). The role of the JAK-STAT signalling pathway in controlling proteasomal degradation was well highlighted in 2015, when increased STAT3 expression sufficient to induce muscle atrophy by overexpressing the ubiquitin-ligase MAFbx has been reported. Moreover, knockout mouse model for STAT3 is strongly resistant to muscle tissue loss induced by Lewis lung carcinoma or C26 colon cancer [152]. Regarding the regulation of the autophagic-lysosomal process, STAT3 has been shown to block the expression of VPS34, thus leading to a significant alteration in the formation of the Vps34-Beclin1-Vps15-Atg14 complex, crucial for the induction of the autophagic process. Indeed, STAT3 overexpression leads to autophagy inhibition by a STAT3-dependent mechanism [153]. In conclusion, findings suggests that STAT3-mediated regulation constitutes a nodal point in the genesis of cancer cachexia, considering that its promotion is able to: i) increase degradative activity at the proteasomal level and ii) inhibit degradative activity at the lysosomal level, leading to the alteration of muscle homeostasis.

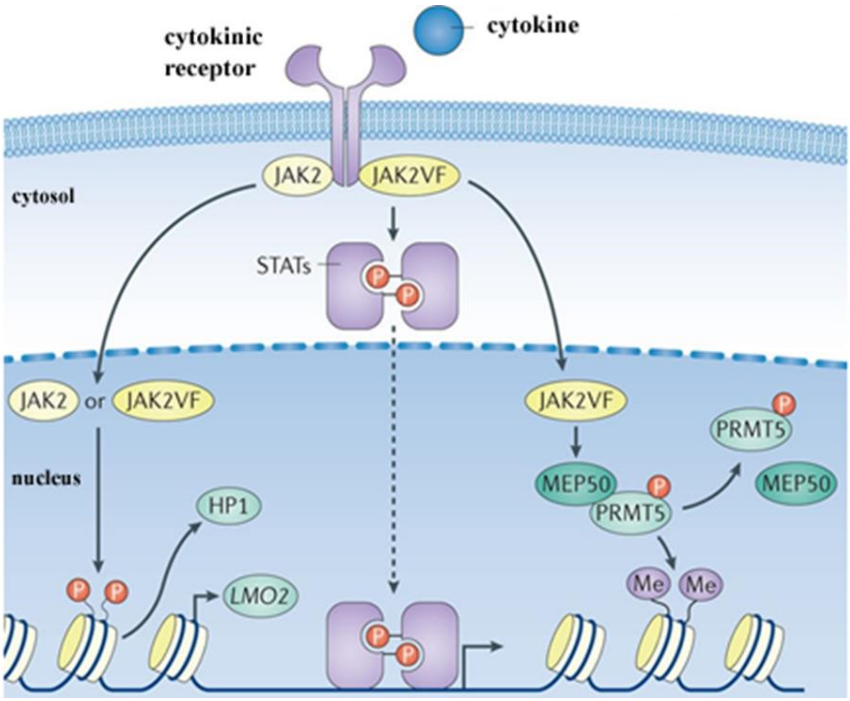

Figure 2. JAK-STAT signalling pathway. Binding of the cytokine (TNFα, IL-1, or IL-6) to its receptors results in their dimerization, which leads to activation of their associated JAK proteins. JAK proteins phosphorylate the cytosolic domain of the receptors, allowing the recruitment of two STAT proteins. The STATs are in turn phosphorylated by the JAKs and associate to form a dimer that in this conformation is able to enter the nucleus, where it modulates the expression of target genes.

1.6.3 The TGFβ/myostatin-Smad2/3 pathway

The TGFβ (Transforming Growth Factor β) family is composed of a whole series of structurally related and dimeric proteins that, once secreted, can act as hormones or as local mediators, capable of modulating innumerable biological functions such as development, proliferation processes, differentiation, extracellular matrix synthesis and programmed cell death (apoptosis), as well as tissue repair and immune response. The family includes BMPs (bone morphogenic proteins) and TGFβ/activins, such as TGFβ, myostatin, and activin, which perform their function by binding to transmembrane receptors having a cytosolic domain with serine/threonine kinase activity, which can be subdivided into two receptor types that are structurally homologous: type I receptor and type II receptor. Each member of the TGFβ family is able to bind to a characteristic combination of type I and type II receptors. The binding results in receptor dimerization

that makes the cytosolic domains of the two receptors spatially close, allowing the type II receptors to phosphorylate and consequently activate the type I receptors, forming an active tetrameric receptor complex. The activated receptor complex recruits and phosphorylates a Smad protein. TGFβ-activated receptors phosphorylate Smad2 or Smad3, whereas BMP-activated receptors phosphorylate Smad1, Smad5, or Smad8. Once the Smad protein has been activated by the receptor through phosphorylation (called R-Smad), it dissociates from the receptor and binds to Smad4 (called co-Smad), which can form a complex with any of the five so-called R-Smads. The Smad complex translocates into the nucleus where it associates with cofactor proteins and regulates the transcription of specific target genes. As previously observed for the JAK-STAT pathway, also the Smad pathway is often regulated by negative feedback. Among the genes controlled by Smad complexes there are in fact genes encoding Smad Inhibitors (Smad6 and Smad7), proteins that can bind to activated receptors and inhibit their signalling capacity (Figure 3).

The role of the TGFβ family in muscle and adipose tissue loss in model animals with cancer is well known. In particular, inhibition of myostatin signalling via blockade of ActRIIB was shown to prevent and reverse skeletal muscle tissue loss and cardiac atrophy in cancer animal models. The effect also occurs in those models in which tumour growth and pro-inflammatory cytokine production are not inhibited [154]. The catabolic effects of the myostatin-dependent pathway depend on the recruitment and activation of Smad2/3 transcription factors. Indeed, the inhibition of Smad2 or Smad3 has been found to be sufficient to promote muscle growth [131]. The degenerative effects on muscle mediated by Smad2/3 involve the activation of MAFbx [132] and the inhibition of the IGF/Akt/mTOR pathway [155], resulting on the one hand in the promotion of ubiquitin-proteasomal degradative activity and on the other hand in the inhibition of protein synthesis.

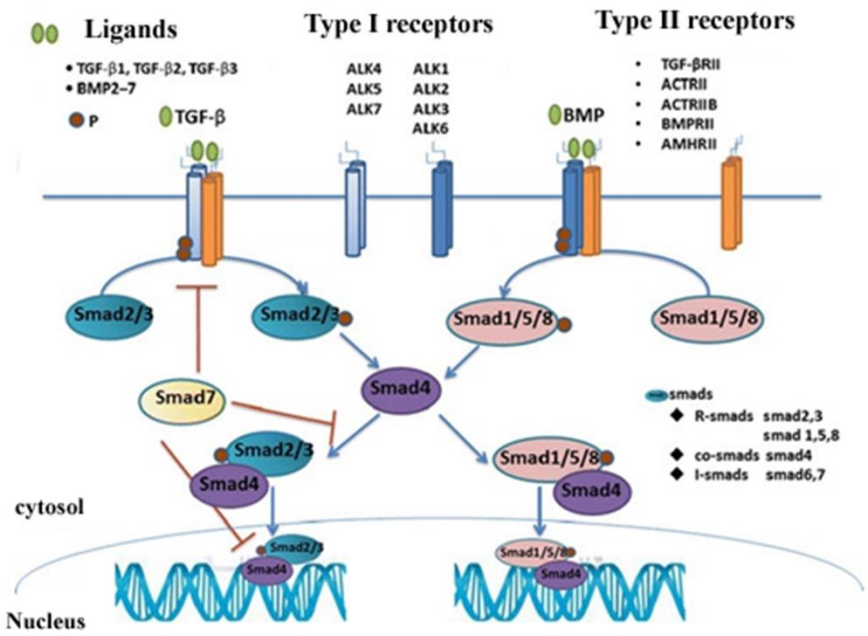

Figure 3. TGFβ-Smad2/3 signalling pathway. TGFβ results in the formation of an active tetrameric receptor complex, which is the result of the interaction of a type I receptor and a type II receptor. The latter phosphorylates the type I receptor via its cytosolic kinase domain, and thereby allows recruitment of a Smad2/3 protein, which is in turn phosphorylated (R-Smad). R-Smad binds to Smad4 (Co-Smad) and the Smad complex can then migrate into the nucleus and promote the expression of target genes.

1.6.4 The Akt-FoxO pathway

FoxO transcription factors belong to the Forkhead box (FOX) family. The genes encoding these factors have been rightfully included in the list of atrophy-related genes, as FoxO factors have been shown to coordinate the activities of the most important cellular degradative systems, the ubiquitin-proteasome system and autophagy-lysosome [140,156]. Therefore, an alteration at the level of these factors is capable of heavily altering muscle homeostasis (Figure 4). In this regard, FoxO factors have been found to be strongly overexpressed in the muscles of mouse models and patients with cancer cachexia [86], and their inhibition was found to be able not only to arrest the atrophic process, but to induce a state of muscle hypertrophy [157]. Of particular relevance would

be the factor FoxO3, whose overexpression has been shown to be sufficient to induce muscle tissue loss through the induction of E3 ubiquitin-ligase enzymes (such as MAFbx and MuRF1), thus promoting muscle protein degradation [139]. FoxO3 factor is, in addition, required for the activation of the autophagic degradation process under catabolic conditions [156]. The expression of a dominant negative FoxO3 factor in the muscles of cachectic C-26 colon cancer mouse models, on the one hand, strongly inhibited the expression of MAFbx and MuRF1 and autophagy-related genes (dramatically reducing muscle tissue loss), on the other hand, the inhibition of FoxO3 factors would even appear to promote protein synthesis and muscle regeneration [157]. Therefore, such inhibition would be able to reverse the cachectic condition. The gene expression profile of cachectic muscle cells, transfected ad hoc with a dominant negative FoxO3 factor has been obtained [94]. Findings showed that there are many genes under the transcriptional control of FoxO factors, able to modulate the expression of genes encoding proteasome proteins and E3 ubiquitin-ligase enzymes including Fbxo031, MUSA1, MAFbx and MuRF1, as well as genes involved in the autophagic process (Catepsin-L, Bnip3 and Gabarapl1).

Considering their involvement in regulating the expression of many genes in response to various types of stress, the action of FoxO transcriptional factors is finely regulated. In this regard, the expression of FoxO genes has been shown to be under the control of p38 MAPK and ERK [158], JNK [159], SGK1 [160], AMPK [161], MST1 [162] and IKK factors [163], as well as under the negative control of Akt, a key protein in the process of cell growth and survival. Indeed, Akt, in addition, determines i) the activation of mTOR and ii) the inhibition of the apoptotic process, thus promoting protein synthesis and cell survival (Figure 4). Transcriptional control is not the only one to be present, but rather FoxO factors can undergo post-translational modifications that can alter their functionality [164].

In conclusion, as emerges from what has been stated so far, FoxO factors play a crucial role in cachexia by promoting increased muscle protein degradation and consequently muscle tissue loss.

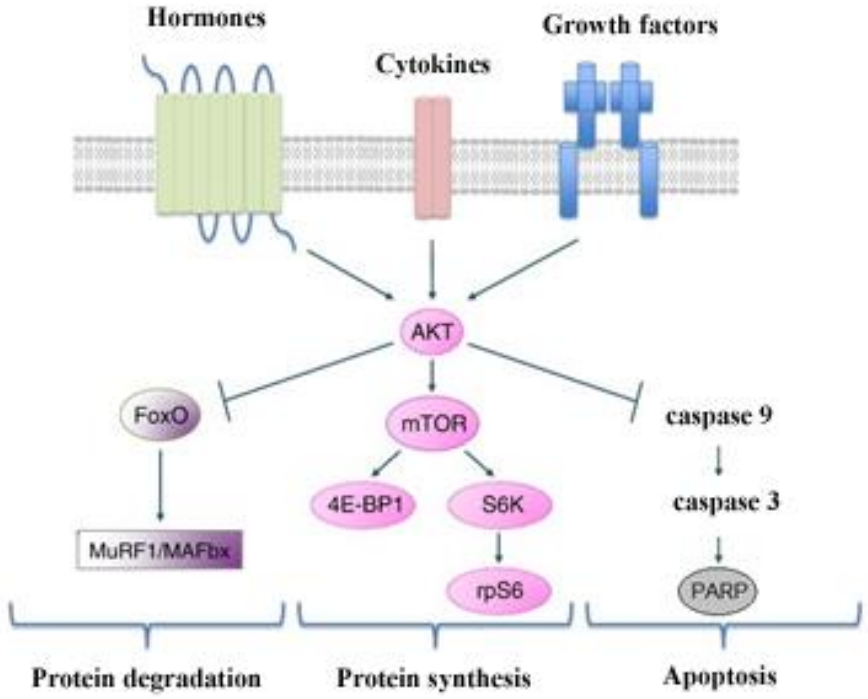

Figure 4. The Akt-FoxO signalling pathway. The signalling pathway is strongly implicated in maintaining muscle homeostasis by maintaining the proper balance between protein synthesis and degradation.

1.7 Skeletal muscle in cancer cachexia

1.7.1 Skeletal muscle structure, physiological characteristics, and function

Skeletal muscle is one of the most dynamic and plastic tissues of the human body. In humans, skeletal muscle comprises approximately 40% of total body weight, contains 50-75% of all body proteins and accounts for 30-50% of whole-body protein turnover [165]. The architecture of skeletal muscle is characterized by a well-described arrangement group of fibers, called muscle fascicles, which are formed by muscle cells or muscle fibers organized with their longitudinal axes in parallel, and associated connective tissue. An individual muscle is surrounded by a layer of connective tissue

known as the epimysium. Group of fibers within that muscle are arranged in bundles and surrounded by another layer of connective tissue known as the perimysium. Each skeletal muscle fiber is surrounded by a cell membrane known as sarcolemma, and has a cytoplasm named sarcoplasm. Multiple nuclei for each muscle fiber, mitochondria, and the main intracellular structures in striated muscles, the myofibrils, are located within the sarcoplasm of muscle cells. Every myofibril is formed by different types of proteins: the muscle contractile proteins actin and myosin, the regulatory proteins tropomyosin and troponin, and the accessory proteins titin and nebulin. While actin is grouped forming clusters in order to constitute the thin filaments, myosin molecules are joined together forming the thick filaments.

At the whole muscle level, muscle size is determined mostly by the number and size of individual muscle fibers [165].

Satellite cells are the adult stem cells of skeletal muscle. These cells are located between the sarcolemma and the basal lamina and contribute to muscle growth, repair, and regeneration [166,167]. When activated by myogenic factors, satellite cells proliferate and differentiate into new muscle fibers [168].

Myosin is the most abundant protein in the skeletal muscle and has the ability to create movement and generate force through actin-myosin cross-bridge mechanism. Several isoforms of myosin take place in different types of muscle and contribute to determine the muscle speed and contraction. Each fiber type confers to the muscle different structural and functional properties. In this regard, each muscle can perform different activities depending on the fiber type composition. Proportions and sizes of each muscle fiber type can change in response to different conditions such as exercise, training, and environmental factors, which regulate muscle phenotype with the purpose of adapt to the different functional requirements [166,167,168]. Muscle fibers can be classified into three main types according to the morphological, biochemical, physiological, and metabolic characteristics: types I, IIa and IIb. The type I muscle fibers are the slow-twitch fibers, characterized by high myoglobin content. This type of fibers has an oxidative metabolism owing to the elevated mitochondrial

content that makes it fatigue resistant [168,169]. Both type IIa and type IIb muscle fibers are the fast-twitch fibers, which are found in the white muscles (low myoglobin content) and are characterized for having a glycolytic metabolism. Type IIa fibers possess high mitochondrial content that confers them an oxidative glycolytic metabolism, which make them fatigue resistant, but less than type I fibers. However, type IIb fibers have low mitochondrial content and they do not have oxidative metabolism. This type of fibers has exclusively glycolytic metabolism that make them easily fatigable [166,169]. Any particular skeletal muscle is comprised of a variable proportion of several different fibre types. The predominance of these fibre types in various skeletal muscles contributes to the metabolic and functional capability of that muscle [170]. Fibre types are ultimately determined by the expression of a specific myosin heavy chain (MHC) protein isoform and a distinct set of genes [171]. Skeletal muscle fibre types directly influence lipid and glucose metabolism and determine the physical properties of a muscle. Skeletal muscle is able to remodel itself (e.g. hypertrophy, myofibre type switching) to adapt to different environmental stimuli including physical activity. Different myofibre types may also display differential susceptibility to a pathological condition thanks to the unique metabolic profile.

Skeletal muscle is also metabolically active. Indeed, skeletal muscle plays a crucial role in managing whole body metabolism being the largest insulin-sensitive tissue in the body and coherently the primary site for insulin-stimulated glucose uptake [172]. Furthermore, skeletal muscle, producing cytokines (myokines), exerts metabolic roles on whole-body homeostasis and it is now considered as an endocrine organ which communicates with other organs via myokines [173]. Particularly, data shown that contracting skeletal muscle fibers release IL-6, thus exerting both local and systemic effects in regulating glucose homeostasis [174,175].

1.7.2 Cancer cachexia impact on skeletal muscle

Muscle wasting represents the main cancer cachexia hallmark, leading to asthenia that is one of the most debilitating features of patients experiencing this syndrome [176]. The maintenance of skeletal muscle is important to the long-term prognosis of cancer cachexia, as it has been shown that the preservation of skeletal mass improved survival in mice bearing the C26 tumour [154]. Skeletal muscle wasting results from imbalance between protein synthesis and degradation, thus altering muscle protein homeostasis. Cachectic cancer patients exhibit increased whole-body protein turnover and breakdown coupled with decreased protein synthesis [177]. The increased breakdown of skeletal muscle protein can partly be explained by the increased demand of amino acid substrate(s) for tumour development as well as liver acute phase protein synthesis [36]. However, visceral proteins are generally conserved in cancer cachexia, thus highlighting that other mechanisms are involved, which directly lead to the breakdown of skeletal muscle [178].

Myofiber type switching has also been observed in skeletal muscle during cancer cachexia. A shift from slow (type I) to fast (type II) fiber has been reported in C26 and Apc $^{min/+}$ tumour models [179,180]. Considering the higher susceptibility of type II glycolytic myofiber to atrophy [181], a shift in myofibre type towards the more glycolytic isoforms may indeed be associated with the progression of muscle wasting. Given the clinical relevance in muscle wasting, it is important to improve our understanding of the molecular mechanisms driving these phenotypical and metabolic changes in skeletal muscle, and to more in dept study the potential role of muscle metabolic rearrangements in the activation and development of cancer cachexia.

2. Aim of the thesis

Despite the evidence suggesting that cancer-induced cachexia is associated with broad metabolic alterations, the potential role of muscle metabolic abnormalities in the activation and development of cancer cachexia has been little studied so far. For this reason, the aim of this thesis was to in depth study the cachectic muscle metabolism and to evaluate the possible role of muscle metabolic changes in the induction and development of cancer cachexia. Considering the strong negative impact of cancer cachexia on both prognosis and life expectancy of cancer patients, the identification of specific metabolic rearrangements underlying the induction of cancer cachexia, could open the way for new and innovative therapeutic strategies able to counteract cancer cachexia acting precisely at the metabolic level in order to restore healthy muscle metabolism. This could significantly improve both quality of life and prognosis of patients suffering from this insidious disease against which there is no approved drug treatment to date.

3. Materials and Method

3.1 Materials

C2C12 murine myoblasts were a gift of Dr. P. Porporato, University of Turin, Italy. The 4T1 and CT26 cell lines were from ATCC. Unless differently specified, all reagents were obtained from Sigma-Aldrich, Inc. (St. Louis, MO, USA); SDS-PAGE materials and ECL detection reagents were from Bio-Rad Laboratories, (Hercules, USA); anti-OXPHOS, anti-Atrogin-1, anti-MuRF and anti-STAT3 primary antibodies were from Abcam; anti-ubiquitin (sc-8017), anti-Pyruvate Dehydrogenase (PDH)-E1 (sc-377092) and anti-citrate synthase primary antibodies were from Santa Cruz; anti-pSTAT3 primary antibody was from Cell Signalling; the inhibitor of mitochondrial pyruvate carrier (MPC) UK5099 and the inhibitor of STAT3 WP1066 were from Santa Cruz; IL-6 primary antibodies (#500-p56) were from Peprotech (London, UK); IL-6 was from Biovision (Milpitas, CA, USA); Tetra-methyl-rhodamine methyl ester (TMRM) probes was from Molecular Probe (Eugene, OR, USA); K-LATE kit for lactate assay was from Megazyme (Bray, Ireland); [3H] 2-deoxy-glucose was from Perkin Elmer (Waltham, MA, USA).

3.2 Cell Culture

Murine C2C12 myoblasts were cultured in a medium consisting of Dulbecco's Eagle's modified (DMEM, #ECB7501 Euroclone, Milan, Italy) supplemented with with 10% fetal bovine serum in a 5% CO_2 humidified atmosphere. For differentiation, subconfluents C2C12 were shifted from growth medium to differentiating medium consisting of DMEM containing 2% horse serum (HOS). C2C12 cells were maintained for 4 days in differentiating medium until well-differentiated myotubes were obtained from myoblast fusion. All cell lines used (C2C12, CT26 and 4T1) were cultured in DMEM supplemented with 10% fetal bovine serum in a 5% CO_2 humidified atmosphere.

3.3 Conditioned Media preparation

Carcinoma cells were cultured in DMEM containing 10% fetal bovine serum until 80% confluence. Then, the medium was replaced with DMEM without serum for 48 hours. Medium, which became conditioned medium (CM) from the tumor cell secretome, was centrifuged at 1,000 rpm, for 10 minutes, to remove cell debris. The CM was then diluted in differentiation medium to 20% final and used for myotube treatment. CM were used at 20% final since lower CM dilution did not induce any cachectic effect, whereas higher CM progressively led to cell death.

3.4 Immunoblot analysis

Cells were lysed for 20 minutes on ice in 500 µL of complete radioimmunoprecipitation assay (RIPA) buffer (150 mM NaCl, 100 mM NaF, 2 mM EGTA, 50 mM Tris HCl pH 7.5, 1 mM orthovanadate, 1% triton, 0.1% SDS, and 0.1% protease inhibitor cocktail). Lysates were clarified by centrifugation, and total protein content was obtained using the Bradford assay (Bio-Rad Laboratories, Hercules, USA). For detection of IL-6 by immunoblot, 6 mL of CM from CT26 and 4T1 cell lines were concentrated using Amicon Ultra-4 centrifugal filtration unit (Millipore Sigma, St. Louis, USA) up to 100 µL. Then, 20 µg of total protein for each sample were separated by SDS-PAGE and transferred to PVDF membranes. PVDF membranes were incubated in 2% milk or 2% bovine serum albumin (BSA), probed with primary antibodies, and incubated with horseradish peroxidase-conjugated secondary antibodies. Bands were quantified using ImageJ software.

3.5 Glucose uptake

Glucose uptake was performed using [^{3}H]2-deoxy-glucose (0.5 mCi/mL, final concentration) diluted in a buffer solution (140 mM NaCl, 20 mM HEPES/Na, 2.5 mM $MgSO_4$, 1 mM $CaCl_2$, and 5 mM KCl, pH 7.4) for 15 min at 37 ◦C. The cells were subsequently washed with cold PBS (ECB4004, Euroclone, Milan, Italy) and lysed with 0.1 M NaOH. The incorporated

radioactive glucose was assessed with a scintillation counter, and the obtained value was then normalized on total protein content.

3.6 Oxygen Consumption Assay

Myotubes were treated with CM CT26 for 24 h. Regarding 2-DG, oxamate and pyruvate treatments, 2-DG (1 mg/mL final), oxamate (75 mM final) and pyruvate (20 mM final) were added to the cells together with CM CT26 and maintained for 24 h. Regarding the MPC inhibitor UK5099 treatment, healthy myotubes were treated with UK5099 (10 µM final) for 24 h. Myotubes were detached, washed with PBS, and suspended in 1 mL of culture medium. The cell suspension was transferred to an airtight chamber maintained at 37 ◦C. Oxygen consumption was measured using a Clark-type O_2 electrode (Oxygraph Hansatech). The rate of oxygen consumption (nmol/min/mL) was monitored for 10 minutes and taken as an index of respiratory capacity. This value was then normalized on total protein content.

3.7 PDH Activity

Pyruvate dehydrogenase (PDH) activity was assessed in cell lysates using the PDH Activity Assay Kit (#MAK183, Sigma Aldrich, St. Louis, USA) according to the manufacturer's instructions. The 2-DG (1 mg/mL final), oxamate (75 mM final) and pyruvate (20 mM final) were added to myotubes in combination with CM CT26 and maintained throughout the experiment. PDH activity (nmol/min/mL) was normalized on the total protein content in each sample.

3.8 Lactate Assay

The amount of lactate was assessed in the culture medium using the K-LATE kit (Megazyme, (Bray, Ireland)) according to the manufacturer's instructions. Before treatment of myotubes, the amount of lactate was measured in the CMs. To obtain the amount of lactate produced exclusively from the myotubes, the amount of lactate in the CMs was subtracted from the lactate in the medium obtained from the treated myotubes. The value obtained was normalized to the total intracellular protein content and reported in the bar graph.

3.9 Confocal Analysis

C2C12 myoblasts were grown to subconfluence on glass coverslips and then differentiated for four days. The obtained myotubes were treated with CM CT26 for 24 hours, washed in PBS, and immediately fixed in 4% paraformaledehyde (PFA) for 20 minutes at 4°C. Subsequently, three 5-minute washes were performed using a solution consisting of 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.1% Triton X-100 (PBST) in order to permeabilize the cells. A blocking solution (5.5% horse serum in PBST) was incubated for 60 minutes and then coverslips with permeabilized cells were incubated with anti-MHC primary antibody (Abcam) diluted 1:100 in 3% BSA for 24 hours at 4°C. At the end of incubation with the primary antibody, cells were first washed for 15 min with PBST solution and then for 15 min with a solution of PBST with 0.1% BSA. The biotinylated secondary antibody was then incubated (1:400) in a solution of PBST with 3% BSA for 1 hour at room temperature. Cells were finally labelled with DAPI (250 nM final) for 12 minutes at room temperature for nuclei staining. Mitochondrial membrane potential analysis was performed by treating viable myotubes with the TMRM probe (40 nM final) and with DAPI (10 µM final) for nuclei labelling for 15 minutes at 37 ◦C and immediately observed. In all experiments, the emitted fluorescence was analyzed using a confocal fluorescence microscope Leica TCS SP8.

3.10 Gas chromatography-Mass Spectrometry (GC-MS)

Gas chromatography–mass spectrometry (GC–MS) analysis of myotube intracellular metabolomic profile was performed using selected ion monitoring (SIM) mode MS. Myotubes were scraped in 80% methanol and phase separation was achieved by centrifugation at 4 ◦C. The methanol-water phase containing polar metabolites was separated and dried using a vacuum concentrator. Dried polar metabolites were dissolved in 10 µL of 2% methoxyamine hydrochloride in pyridine (Pierce, Thermo Fisher Scientific) and kept at 37 ◦C for 2 hours. After dissolution and reaction, 50 µL of N-tert-Butyldimethylsilyl-N-methyltrifluoroacetamide with 1% tert-Butyldimethylchlorosilane MBTSTFA + 1% TBDMCS (Thermo Fisher Scientific) was added, and the samples were incubated at 60 ◦C for 60 min. Gas chromatography runs were performed with helium as

carrier gas at 0.6 mL/min. The split inlet temperature was set to 250 ◦C and the injection volume of 1 µL. The temperature of the GC oven was from 70 to 280 ◦C. The first temperature ramp was from 70 to 140 ◦C at 3 ◦C/min. The second temperature ramp was from 140 to 180 ◦C at 1 ◦C/min. Finally, the last temperature ramp was from 180 to 280 ◦C at 3 ◦C/min. The data acquisition rate was 10 Hz. For the quadrupole, a source EI (70 eV). The ion source and transfer line temperatures were set to 250 and 290 ◦C. For determination of relative abundances of metabolites, the integrated signal of all ions for each metabolite fragment was normalized on total protein content of each sample.

3.11 Statistical Analysis

Data are presented as mean ± SD from at least three independent experiments. Statistical analysis of the data was performed by Student's t test or by one-way ANOVA, using Graph Pad Prism (Graphpad Holdings, LLC, USA), version 6.0. A p-value < 0.05 was considered statistically significant.

4. Results

4.1 The treatment with CM CT26 triggers cachexia in myotubes

Firstly, the validity of the *in vitro* cachexia model was tested. For this task, myotubes from the differentiation of C2C12 murine myoblasts were 24 h-treated with conditioned medium (CM) obtained by growing CT26 murine colon carcinoma. CT26 cell line has been selected as carcinoma cell model able to trigger cachexia in murine models, as already reported [182].

To investigate the induction of cancer cachexia in myotubes by CM CT26, the phenotypic effects induced by CM CT26 on myotubes have been examined. Results reveal that CM-CT26 clearly impairs the myotube structure, as myotubes appear thinner than control myotubes (Figure 5A). Indeed, CM CT26-treated myotubes display muscle wasting phenotype characterized by significant decrease in myotube size compared to control myotubes (Figure 5B). Consistently, confocal microscopy analysis showed that treatment with CM CT26 results in a significant decrease in myosin heavy chain (MHC)-related fluorescence, a well-known muscle differentiation marker (Figure 5C).

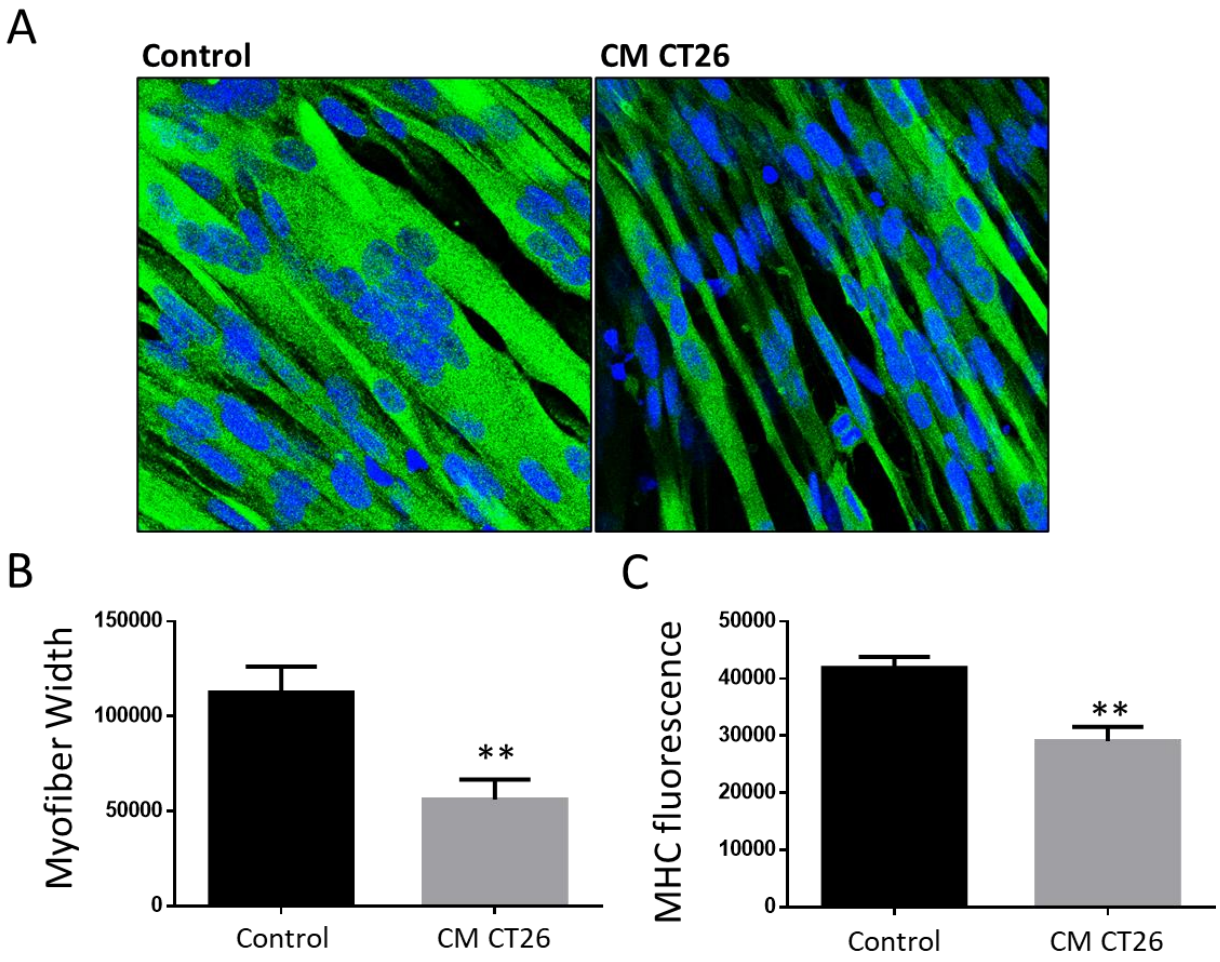

Figure 5. CM CT26 treatment triggers cachexia activation in myotubes. Four-day-differentiated myotubes were treated with CM CT26 for 24 h. Untreated myotubes (indicated as Control) are maintained in differentiating medium for the same period. (**A**) Representative confocal images of control and CM CT26-treated myotubes. MHC (green) has been labelled using a secondary antibody conjugated with Alexa Fluor 488, while nuclei (blue) have been labelled with DAPI. (**B**) Myotube width measurement using ImageJ software. (**C**) MHC fluorescence reported as the mean fluorescence in at least ten randomly chosen fields. n = 4; ** $p < 0.01$.

Skeletal muscle wasting is mainly driven by a drastic promotion of catabolic pathways, leading to an unbalanced ratio between protein synthesis and degradation [68]. Specifically, the ubiquitin-dependent proteasome pathway has clearly emerged as a key degradative system involved in muscle protein degradation leading to cachexia [37]. In agreement, our results show that CM-CT26-mediated phenotypic effect on myotubes is associated with the enhancement of the ubiquitin-proteasome system, as evidenced by the increased expression of E3 ubiquitin-ligase Atrogin-1 and MuRF (Figure 6A) and, coherently, by the

increase in total protein ubiquitination in comparison to control myotubes (Figure 6B). Obtained results show that treatment with CM CT26 induces the typical cachectic features in myotubes.

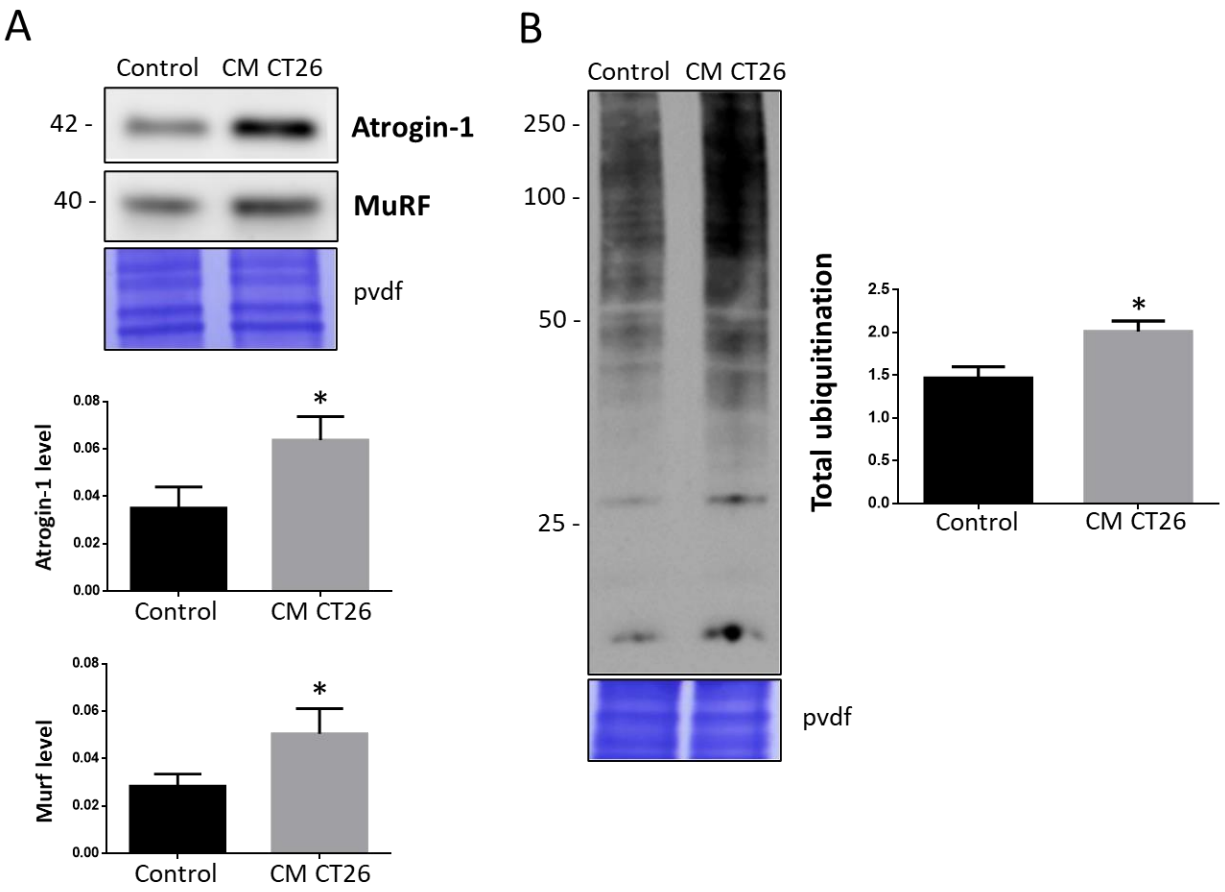

Figure 6. CM CT26 treatment promotes the ubiquitin-proteasome system in myotubes. Four-day-differentiated myotubes were treated with CM CT26 for 24 h. Untreated myotubes (indicated as Control) are maintained in differentiating medium for the same period. (**A**) Immunoblot analysis of Atrogin-1 and MuRf levels. Atrogin-1 and MuRf levels reported in bar graphs were obtained by using Coomassie-stained PVDF membranes for normalization. (**B**) Myotube ubiquitination level. Total ubiquitination level reported in the bar graph was obtained by using Coomassie-stained PVDF membranes for normalization. n = 4; * $p < 0.05$.

4.2 CM CT26-related cachexia is associated to a metabolic modification in myotubes

Cancer cachexia is considered an energy-balance disorder caused by pronounced metabolic alterations. Despite the evidence suggesting that cancer-induced cachexia is associated with a broad range of metabolic rearrangements, the potential role of muscle metabolic abnormalities in the activation and development of cancer cachexia has been little studied so far.

Therefore, we planned to study carbohydrate metabolism in myotubes treated with CM CT26. In this context, glucose uptake and extracellular lactate measurement represent useful tools to detect change in glucose catabolism, whereas oxygen consumption of myotubes provides information on mitochondrial oxidative phosphorylation. Glucose Uptake analysis shows that CM CT26 treatment significantly improves myotube glucose uptake compared to control myotubes (Figure 7A). To evaluate the effects of CM CT26 on mitochondrial respiration, myotube oxygen consumption rate (OCR) has been investigated. The results show that CM-CT26-treated myotubes have a lower OCR than control myotubes (Figure 7B). The lactate production assay in myotube culture medium highlights that CM-CT26 strongly increases myotube lactate production compared to control myotubes (Figure 7C).

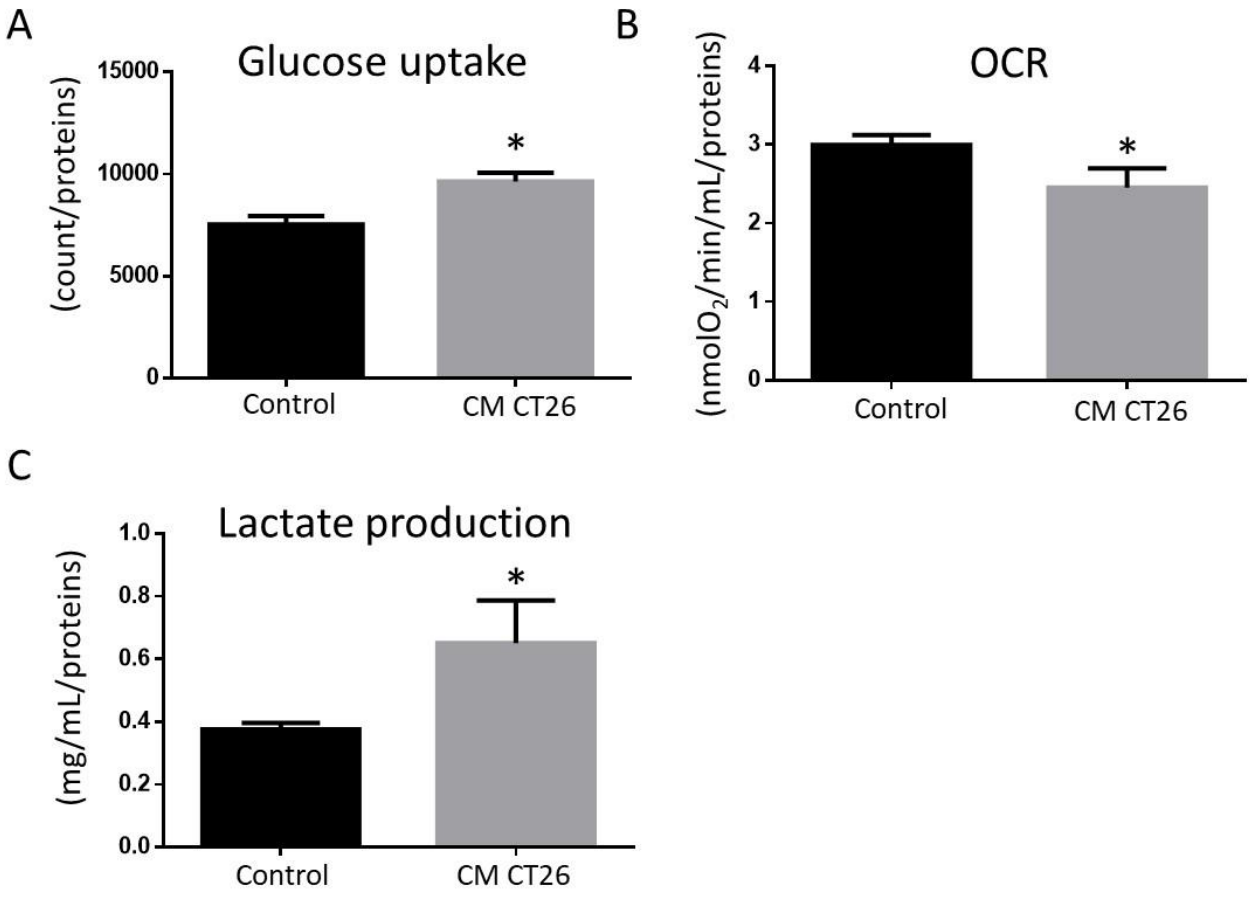

Figure 7. CM CT26 treatment modifies glucose metabolism in myotubes. Twenty-four hours after the treatment of myotubes with CM CT26 or with differentiating medium (reported as Control) (**A**) glucose uptake, (**B**) Oxygen Consumption Rate (OCR), and (**C**) extracellular lactate amount was assayed. Data were normalized on total protein content of each sample. n = 4; * $p < 0.05$.

The finding that CM CT26 treatment significantly impacts oxygen consumption in myotubes suggests that CM CT26 could trigger relevant alterations in mitochondria. To investigate this, mitochondrial membrane potential has been evaluated using TMRM, a cell-permeable dye that accumulates in functionally active mitochondria with intact membrane potential and decreases upon loss of potential. Confocal images reveal that CM CT26-treated myotubes have impaired mitochondrial membrane potential compared with control myotubes (Figure 8A). Furthermore, CM-CT26-treated myotubes show a decreased level of OXPHOS complexes compared with control myotubes. Finally, immunoblot analysis of the citrate synthase level, normally used as a marker of mitochondria quantity [183] shows that CM CT26 treatment does not affect mitochondria quantity (Figure 8B).

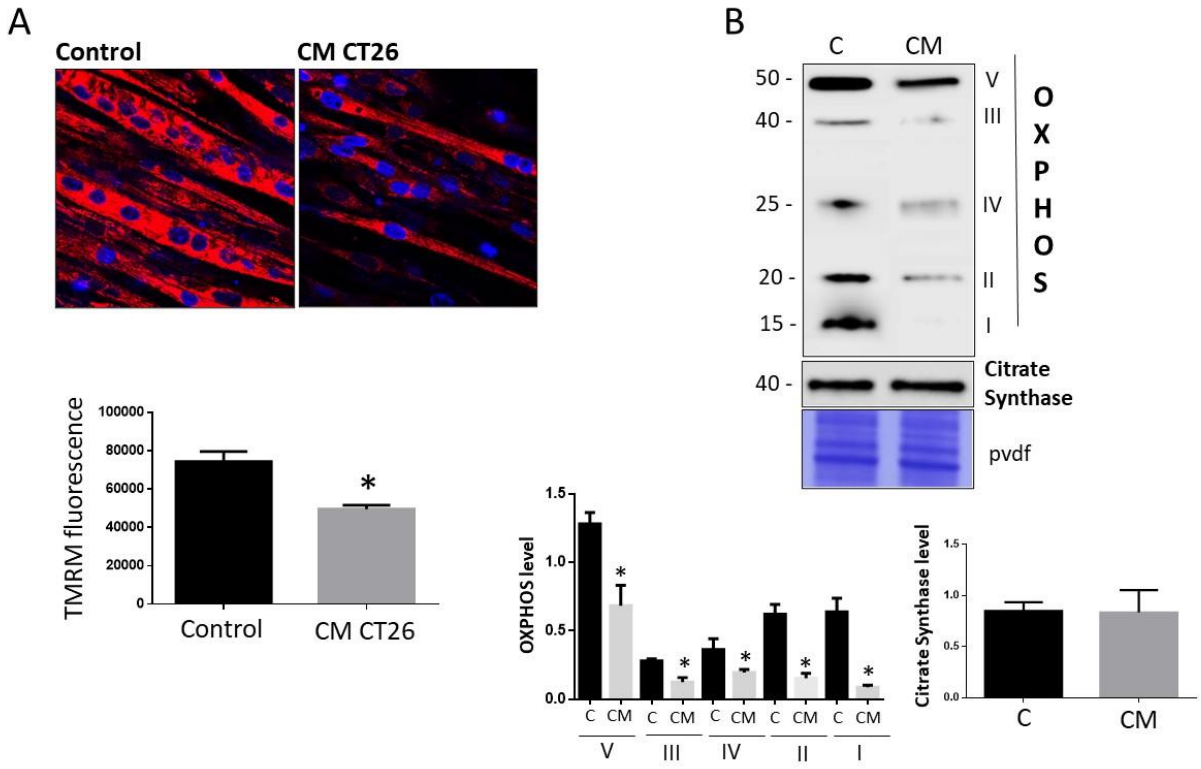

Figure 8. Analysis of mitochondria in CM CT26-treated myotubes. Four-day-differentiated myotubes were treated with CM CT26 for 24 h. Untreated myotubes (indicated as Control) are maintained in differentiating medium for the same period. (**A**) Mitochondria have been labelled with TMRM (40 nm final) and immediately analyzed under confocal microscope. Representative images of myotubes labelled with TMRM. Mitochondria appear red, while nuclei are blue due to DAPI staining. (**B**) Immunoblot analysis of mitochondrial OXPHOS complexes (I: NADH dehydrogenase; II: succinate ubiquinone oxidoreductase; III: ubiquinol cytochrome C oxidoreductase; IV: cytochrome C oxidoreductase; V: ATP synthase) and citrate synthase. Expression levels has been obtained using Coomassie-stained PVDF membranes for normalization (C: control myotubes; CM: CMCT26-treated myotubes). n = 4; * $p < 0.05$.

These results highlight that CM CT26 promotes a metabolic shift toward fermentation in myotubes, enhancing glucose uptake and conversion of glucose to lactate under aerobic conditions. Furthermore, CM CT26 mediates significant alterations in myotube mitochondria, ranging from modification of mitochondrial membrane potential to decreased level of OXPHOX complexes. This suggests that these alterations could be implicated in the impaired oxygen consumption observed in CM CT26-treated myotubes.

4.3 Inhibition of glycolysis or LDH prevents the CM CT26-induced cachexia in myotubes

Although the molecular mechanisms underlying cancer cachexia are very well studied [9], the possible role of metabolic changes in the onset of cachexia has never been thoroughly investigated so far. Therefore, we decided to investigate the possible involvement of the metabolic shift toward fermentation in the induction of cachexia in CM CT26-treated myotubes. First, block of glycolysis in order to decrease the amount of pyruvate converted to lactate by the LDH enzyme has been performed. Inhibition of glycolysis was achieved using 2-deoxy-D-glucose (2-DG) which consists of a modified glucose molecule that cannot undergo further enzymatic modification. Thus, myotubes were treated with CM CT26 (with or without 2-DG) for 24 hours. Results show that inhibition of glycolysis was effective in preventing the cachectic phenotype induction in myotubes. Indeed, myotubes treated with CM CT26 and 2-DG were morphologically similar to control myotubes, as shown by optical microscope images (Figure 9A) and myotube width measurement (Figure 9B). Consistently, immunoblot analysis demonstrated that inhibition of glycolysis is able to considerably reduce the high levels of total protein ubiquitination observed in CM CT26-treated myotubes. Specifically, the level of ubiquitinated proteins in CM CT26-2DG cotreated myotubes becomes comparable to that of control myotubes (Figure 9C). Furthermore, as it clearly emerges from the Figure 9D and Figure 9E graphs, respectively, inhibition of glycolysis was effective in preventing the decreased oxygen consumption and increased lactate production observed in CM CT26-treated myotubes.

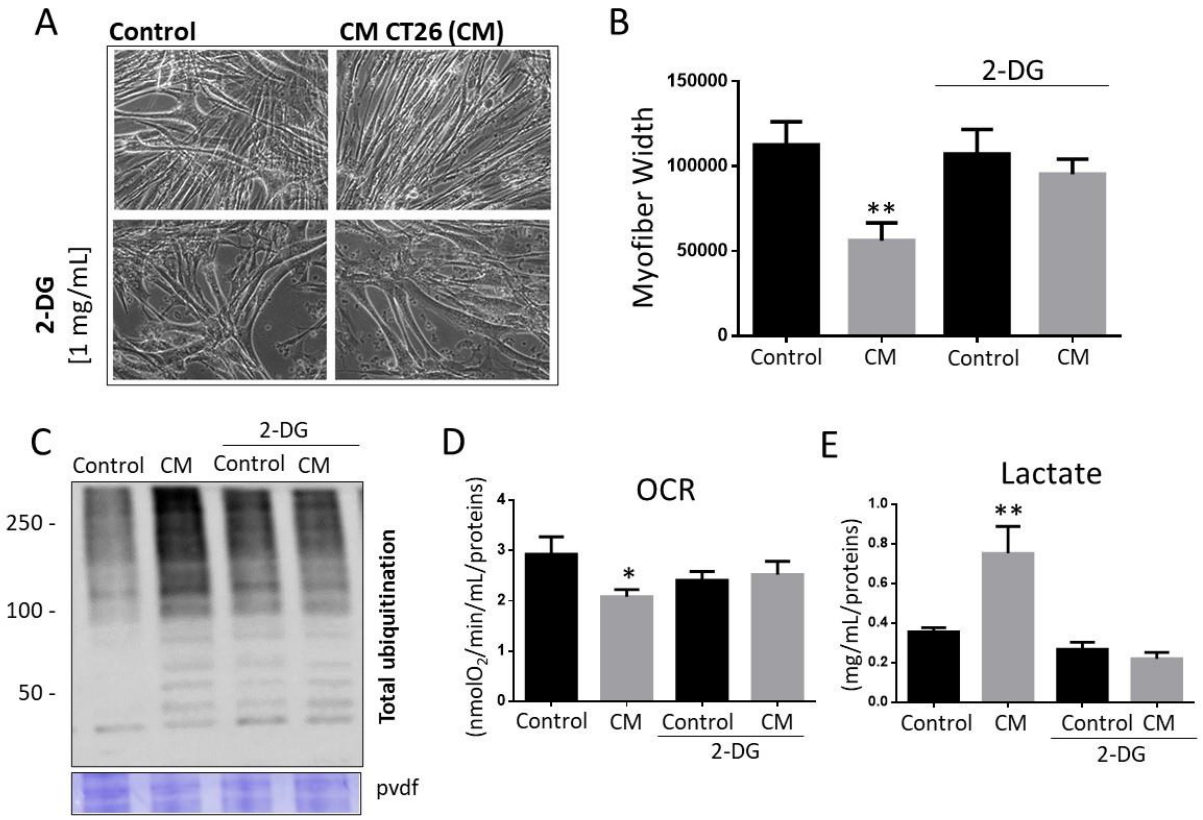

Figure 9. Glycolysis inhibition prevents cancer cachexia onset in CM CT26-treated myotubes. Four-day-differentiated myotubes were treated with CM CT26 for 24 h. Where indicated, 2-DG (1mg/mL final) was added to the media. (**A**) Representative optical microscope images of treated myotubes with or without 2-DG. (**B**) Myotube width measurement after 24 h of treatment. (**C**) Ubiquitination level of myotubes. (**D**) Analysis of myotube oxygen consumption rate (OCR). Data were normalized on total protein content of each sample. (**E**) Myotube lactate production assay. Data were normalized on total protein content of each sample. (CM: myotubes treated with CM CT26; A.U: arbitrary units). n = 4; * $p < 0.05$; ** $p < 0.01$.

In order to analyse the involvement of increased lactate production in the induction of cachexia in CM CT26-treated myotubes, the specific LDH inhibitor oxamate capable therefore of blocking the conversion of pyruvate to lactate was used. Myotubes were treated with CM CT26 (with or without oxamate) for 24 h. In particular, for myotube treatment we used a concentration of oxamate (75 mM final) capable of inhibiting the production of lactate without having an effect on cell viability (data not shown).

The results confirm the previous observations obtained by inhibiting glycolysis by using 2-DG. Indeed, LDH inhibition prevents the induction of the cachectic phenotype in CM CT26-treated myotubes, as evidenced by the images in Figure 10A and the corresponding

quantification of myotube width (Figure 10B). Consistently, the high levels of protein ubiquitination observed in CM CT26-treated myotubes become comparable to those in control myotubes in the presence of oxamate (Figure 10C). Interestingly, the absence in myotubes of the cachexia-hallmark phenotypic and molecular features induced by CM CT26 are also associated with the absence of the metabolic alterations. Indeed, inhibition of lactate production by oxamate (Figure 10E) is associated with normal oxygen consumption in CM-CT26-treated myotubes in the presence of LDH inhibitor (Figure 10D).

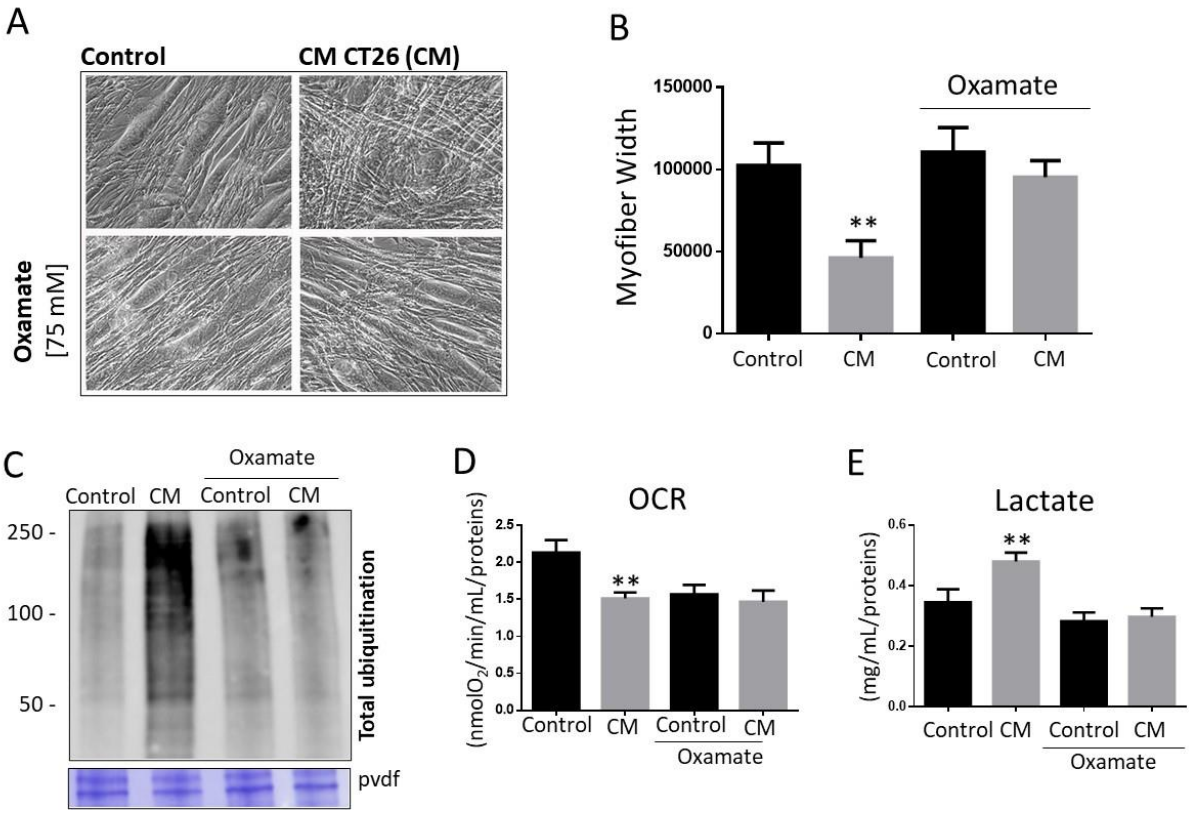

Figure 10. LDH inhibition impedes CM-CT26-induced cachexia. Four-day-differentiated myotubes were treated with CM CT26 for 24 h. Where indicated, oxamate (75 mM final) was added to the media. (**A**) Representative optical microscope images of treated myotubes with or without oxamate. (**B**) Myotube width measurement after 24 h of treatment. (**C**) Ubiquitination level of myotubes. (**D**) Analysis of myotube oxygen consumption rate (OCR). Data were normalized on total protein content of each sample. (**E**) Myotube lactate production assay. Data were normalized on total protein content of each sample. (CM: myotubes treated with CM CT26; A.U: arbitrary units). n = 4; ** $p < 0.01$.

Taken together, these results suggest that the metabolic shift toward fermentation induced in myotubes by CM CT26 could be the crucial point in the activation of cachexia, considering

that its abolition by inhibition of glycolysis or LDH turns prevents the onset of the cachectic condition in CM C T26-treated myotubes.

4.4 Inhibition of glycolysis or LDH impedes mitochondrial membrane potential and PDH activity alterations in CM CT26-treated myotubes

To further investigate the beneficial effects of inhibition of glycolysis and lactate production in CM CT26-treated myotubes, we next investigated whether abolition of the metabolic alteration could result in restoration of proper mitochondrial membrane potential in CM CT26-treated myotubes. As shown in Figure 11A,B, the block of the metabolic shift toward fermentation (either by inhibition of glycolysis by 2-DG or inhibition of LDH by oxamate) associated with induction of cachexia in CM CT26-treated myotubes results in maintenance of a functional and correct mitochondrial membrane potential in myotubes.

In addition, we analysed whether treatment of myotubes with CM CT26 in the presence or absence of 2-DG or oxamate could alter the activity of the enzyme pyruvate dehydrogenase (PDH). PDH constitutes the E1 subunit of the three subunits that are part of the pyruvate dehydrogenase complex (PDC) that catalyses the conversion of pyruvate to Acetyl-CoA. Specifically, PDH catalyses the first reaction of the PDC complex leading to the formation of Acetyl-CoA, which consists of the decarboxylation of pyruvate [184]. As already reported in published data [185], obtained results confirm that PDH activity is significantly decreased in CM CT26-treated myotubes. Interestingly, both 2-DG and oxamate treatment maintain PDH activity in CM CT26-treated myotubes at levels comparable with that in control myotubes (Figure 11C). Additionally, in no case a change in the expression levels of PDH has been highlighted (Figure 11D). These results demonstrate that both inhibition of glycolysis by 2-DG and lactate production by oxamate positively impact on mitochondria function in CM CT26-treated myotubes, where both mitochondrial membrane potential and PDH activity

were reported at the level of control myotubes due to the inhibition of the fermentative metabolic shift.

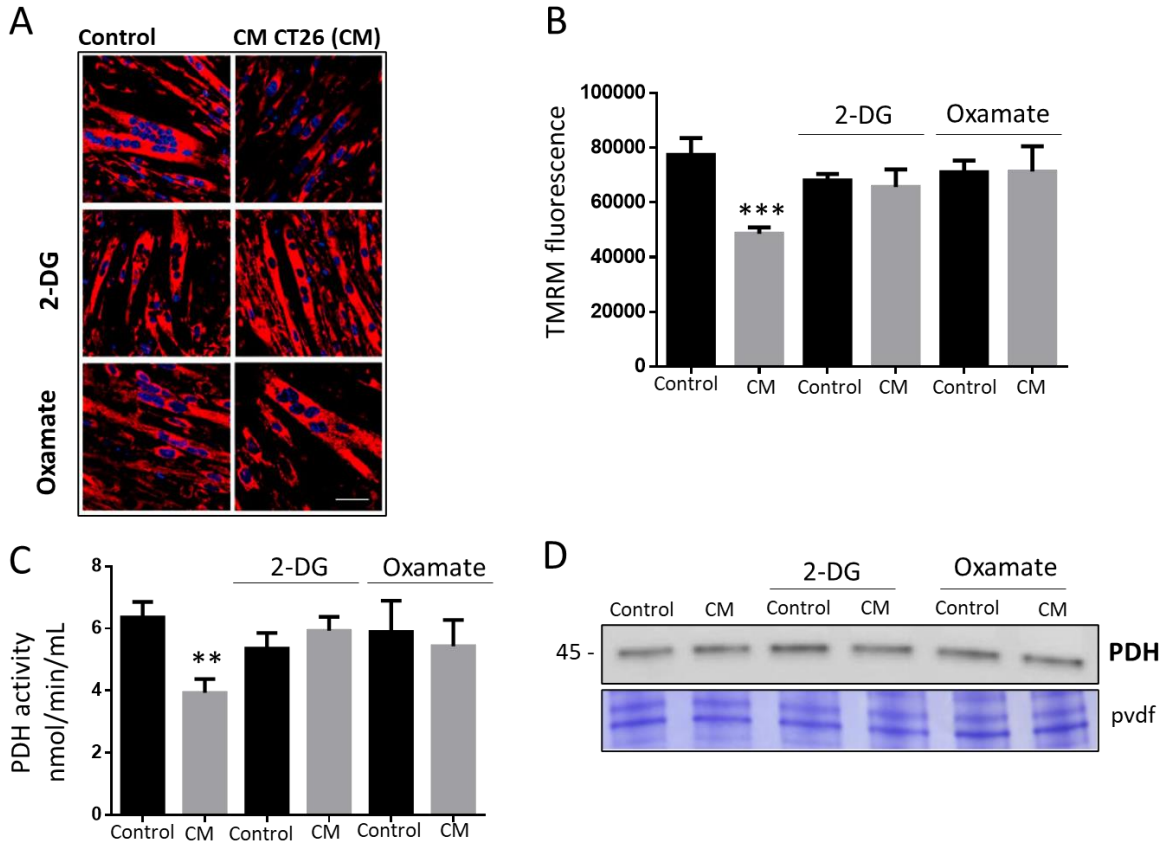

Figure 11. Analysis of mitochondrial membrane potential and PDH in CM CT26-treated myotubes with or without 2-DG or oxamate. Four-day-differentiated myotubes were treated with CM CT26 for 24 h. Where indicated, 2-deoxy-glucose (2-DG) (1 mg/mL final) or oxamate (75 mM final) was added to the media. (**A**) Representative images of myotubes labelled with TMRM (40 nM final). Mitochondria appear red, while nuclei are blue due to DAPI staining. (**B**) TMRM fluorescence reported as the mean fluorescence in at least ten randomly chosen fields. (**C**) PDH activity. PDH activity measured as nmol/min/mL is normalized on total protein amount of each sample. (**D**) PDH immunoblot. n = 4; ** $p < 0.01$; *** $p < 0.005$.

4.5 Interleukin-6 plays a role in the metabolic remodelling leading to cachectic features

As well known, the condition of cachexia is characterized by a markedly increased production and release of proinflammatory cytokines including interleukin-6 (IL-6) [186]. In order to evaluate the ability of CT26 tumour cells to secrete IL-6 in the conditioned medium, the murine breast carcinoma 4T1 cell line was used as a control cell line. The 4T1 cell line has been chose as counterpart carcinoma cell model that is unable to induce cachexia in the mouse model, as already reported [182]. As shown in Figure 12A, immunoblot analysis performed on conditioned media showed that CM CT26 exhibited a significantly higher level of IL-6 than CM 4T1. The result could suggest an involvement of IL-6 in the onset of the metabolic shift toward fermentation underlying the induction of cachexia in CM CT26-treated myotubes. To test this hypothesis, myotubes were treated for 24 hours with IL-6 (120 ng/mL) and were analysed both at phenotypical and metabolic levels. Results highlight that the addition of IL-6 to the culture medium determines the onset of the cachectic condition in myotubes, as evidenced by microscopic images (Figure 12B) where treated myotubes appear significantly thinner than control myotubes, and by the quantification of myotube width (Figure 12C). Furthermore, IL-6-treated myotubes showed the same metabolic alterations observed due to CM CT26 treatment, manifesting decreased oxygen consumption (Figure 12D) and increased lactate production (Figure 12E). Moreover, as previously observed with CM CT26, induction of the cachectic phenotype is linked with the metabolic shift toward fermentation. Indeed, co-treatment of myotubes with IL-6 and LDH inhibitor oxamate prevents the induction of the cachectic phenotype (Figures 12B,C) by blocking metabolic remodelling in myotubes (Figures 12D,E). These findings suggest that IL-6 is involved in the metabolic changes that leads to cachexia activation in myotubes.

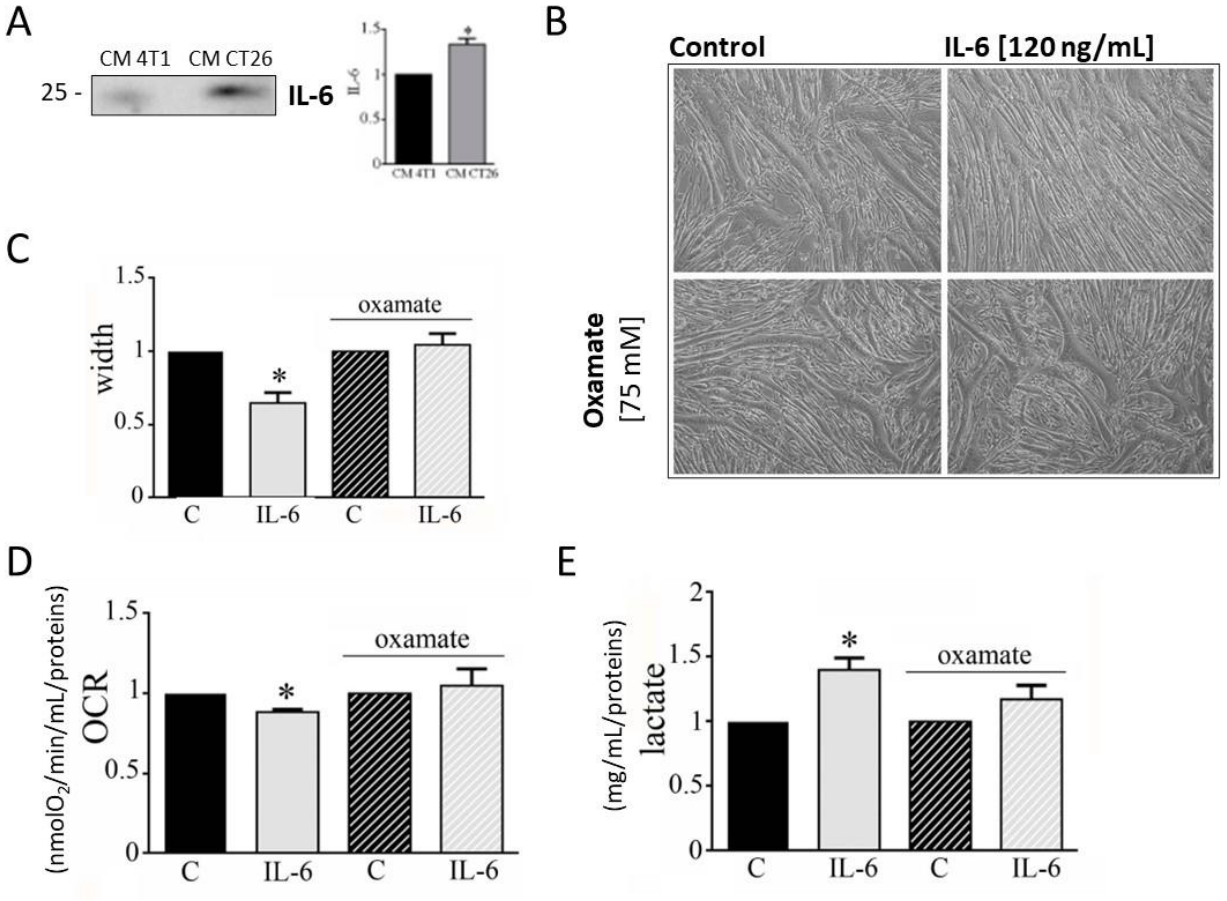

Figure 12. IL-6 secreted by CT26 colon carcinoma cells participates in metabolic shift leading to cachexia. (**A**) Anti-IL-6 immunoblot analysis performed on CM 4T1 and CM CT26. IL-6 level was normalized on CM protein content. Four-day-differentiated myotubes were treated with IL-6 (120 ng/mL) for 24 h, while control myotubes, indicated as C, were maintained in differentiating medium for the same period. Where indicated, the LDH inhibitor oxamate (75 mM final) was added to the medium. (**B**) Representative microscopic images of myotubes treated with IL-6 with or without oxamate. (**C**) Myotube width measurement after IL-6 24 h treatment with or without oxamate. (**D**) Oxygen consumption rate (OCR) analysis and (**E**) lactate production assay. n = 4; * $p < 0.05$.

4.6 CM CT26 removal restores myotube healthy phenotype and metabolism

In order to assess whether the phenotypic and metabolic effects triggered in myotubes by CM CT26 could be reverted, after the treatment of myotubes with CM CT26 for 24 h, CM CT26 was removed and replaced with differentiating medium for additional 48 h. Removal of conditioned medium has been shown to be able to revert the cachectic phenotype of

myotubes as shown by images (Figure 13A), myotube width measurement (Figure 13B) and decreased level of total protein ubiquitination (Figure 13C). Interestingly, reversion of the cachectic phenotype is associated with a restoration of metabolism which returns comparable to that of control myotubes. Indeed, OCR and lactate production, that appeared decreased and increased, respectively, in CM-CT26-treated myotubes return to control values after CM CT26 removal (Figure 13D,E). These findings suggest that induction of cachexia in myotubes due to CM CT26 treatment could be reverted, at least in the early phase of the process.

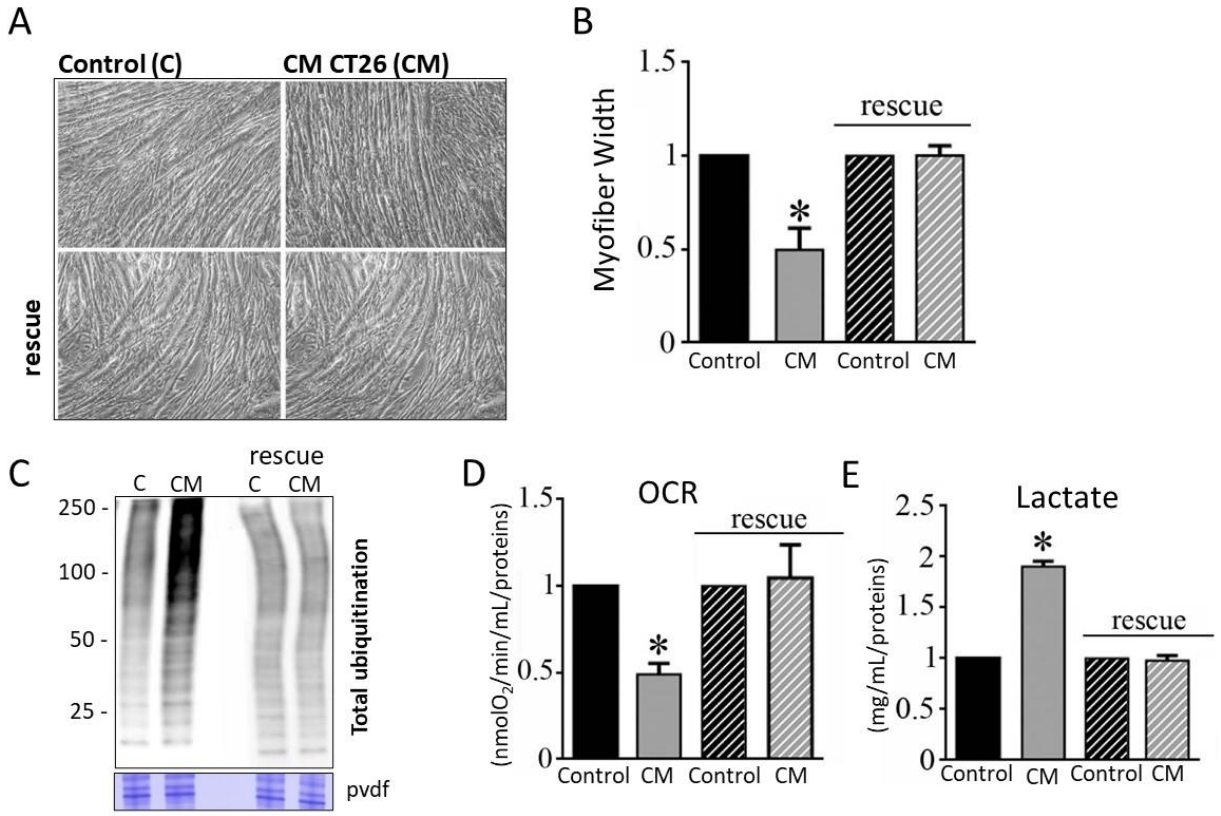

Figure 13. CM CT26 removal restores normal phenotype and metabolism in myotubes**.** Four-day-differentiated myotubes were treated with CM CT26 for 24 h and then CM CT26 were removed and replaced with differentiating medium for additional 48 h. Control myotubes were maintained in differentiation medium throughout the experiment. (**A**) Microscopic images of myotubes before CM removal (images above) and after the replacing with differentiating medium (images below). (**B**) Myotube width measurement. (**C**) Immunoblot analysis of total ubiquitinated proteins normalized by using Coomassie-stained PVDF membrane. (**D**) Oxygen consumption rate (OCR) analysis and (**E**) lactate production assay normalized on total protein content of each sample. n = 4; * $p < 0.05$.

4.7 Metabolomic analysis of CM CT26-treated myotubes

Given the previous results, we decided to focus our study on the metabolism of cachectic myotubes. To study in depth what happened within cachectic myotubes, we performed intracellular metabolomics analysis, focusing on glycolysis and Krebs cycle metabolites. As previously described, cachectic myotubes are obtained by the treatment for 24 hours with the conditioned medium from murine colon carcinoma CT26 cells (CM CT26). Metabolomic analysis highlights decreased glucose-6-phosphate level and increased pyruvate and lactate levels in CM CT26-treated myotubes with respect to control myotubes (Figure 14). These results are in agreement with the previous observations, showing increased glucose uptake and increased lactate production in cachectic myotubes. Concerning Krebs cycle intermediates, CM CT26-treated myotubes exhibit decreased amounts of citrate, succinate, and malate and increased amount of α-ketoglutarate, whereas the level of fumarate is unchanged when compared with control myotubes (Figure 14). In agreement with previous findings, these results demonstrate that glycolysis and lactic fermentation are up-regulated in cachectic myotubes.

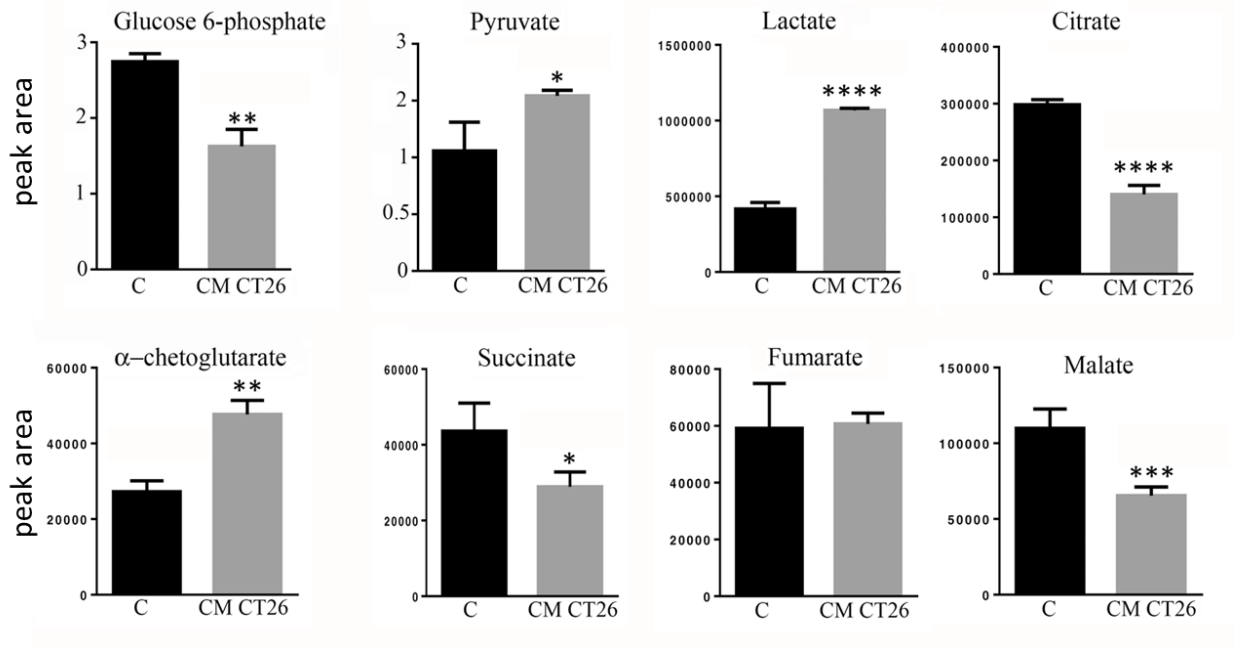

Figure 14. Intracellular metabolomic profiling of cachectic myotubes. Four-day-differentiated myotubes were treated with CM CT26 for 24h. Samples for metabolomic analysis were processed

as previously described in the “materials and methods” section. Metabolomic analysis of cachectic myotubes has been performed using gas chromatography-mass spectrometry (GC-MS) analysis and comparing the intracellular relative abundance of showed metabolites to that of control myotubes (indicated as C). Metabolite relative abundance has been normalized on total protein content of each sample. n = 3; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.005$; **** $p < 0.0001$.

4.8 Sodium pyruvate prevents the onset of cachectic hallmarks in myotubes

We hypothesized that the majority of pyruvate in cachectic myotubes is driven toward lactic fermentation, with a small proportion of the metabolite driven in mitochondria. In this context, the limiting step for the metabolic shift could be the use of pyruvate, produced by glycolysis, by LDH or pyruvate dehydrogenase complex (PDH). This is further supported by the previously showed observation that pyruvate dehydrogenase (PDH) activity is decreased in CM CT26-treated myotubes. Hence, the effect of pyruvate supplementation on the onset of cachexia in myotubes has been analysed. To this aim, sodium pyruvate (20 mM final) has been added to CM CT26 and used for treating myotubes for 24 hours. Images of myotubes from confocal microscope show that myotubes treated with CM CT26 containing sodium pyruvate display a phenotype similar to control myotubes (Figure 15A). The addition of pyruvate to CM CT26 greatly prevents the onset of cachectic myotubes. As shown in Figure 15B,C, CM CT26 induces the decrease of myotube width and the decrease of MHC level (as shown by the measure of green fluorescence) that are prevented by the addition of pyruvate. Moreover, myotubes treated with CM CT26 containing pyruvate show decreased Atrogin-1 level, which appears up-regulated in CM CT26-treated myotubes in comparison to control myotubes (Figure 15D). Collectively, these findings demonstrate that increasing sodium pyruvate concentration impedes the acquisition of cachectic features in myotubes treated with CM CT26.

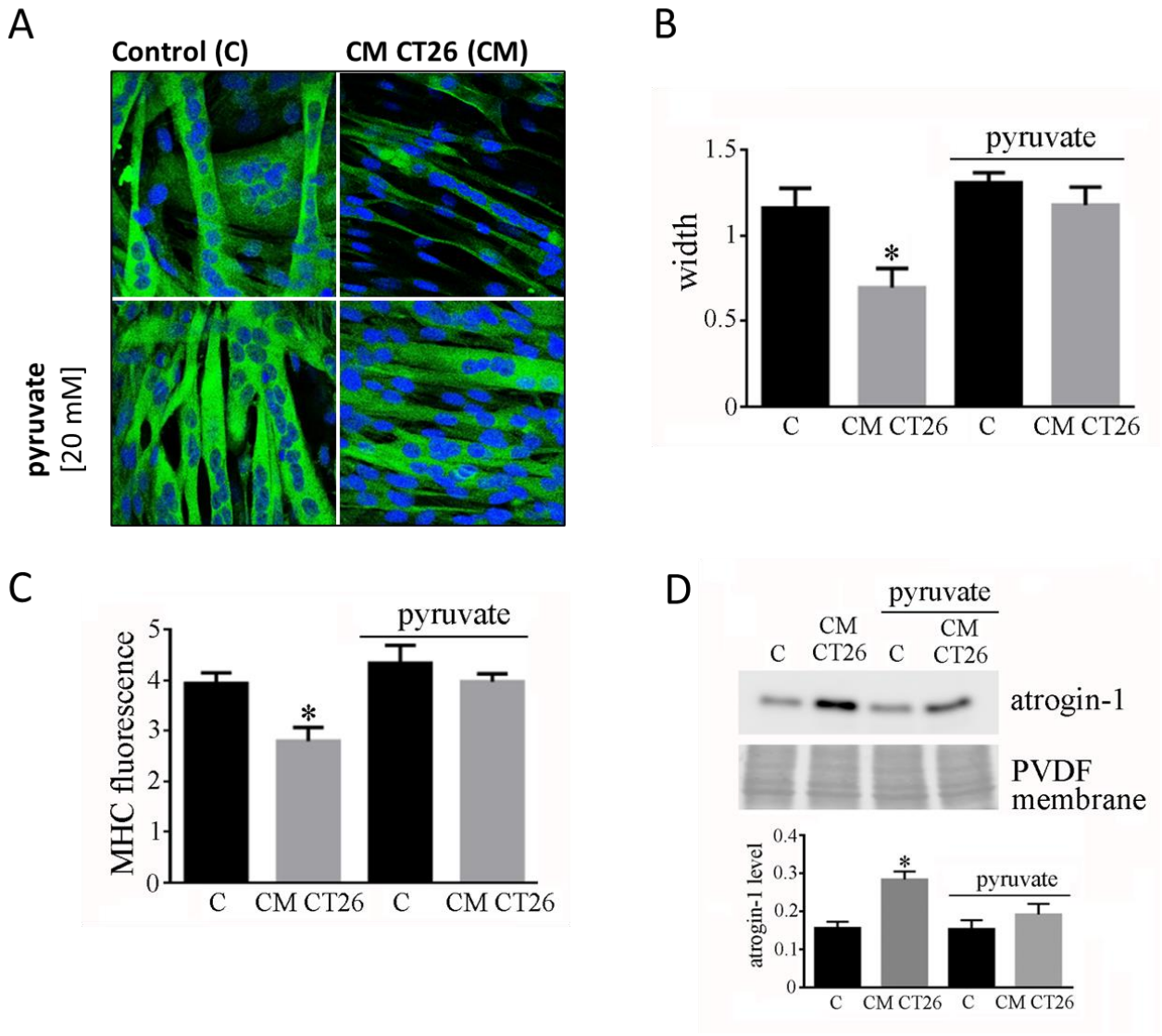

Figure 15. Pyruvate impedes cancer cachexia onset in CM CT26-treated myotubes. Four-day-differentiated myotubes were treated with CM CT26 for 24 h. Where indicated, pyruvate (20 mM final) was added to the media. (**A**) Representative confocal images of control and CM CT26-treated myotubes with or without pyruvate supplementation. MHC (green) has been labelled using a secondary antibody conjugated with Alexa Fluor 488, while nuclei (blue) have been labelled with DAPI. (**B**) Myotubes width measurement. (**C**) MHC fluorescence reported as the mean fluorescence in at least ten randomly chosen fields. (**D**) Immunoblot analysis of Atrogin-1 levels. Atrogin-1 levels reported in bar graphs were obtained by using Coomassie-stained PVDF membranes for normalization. n = 3; * $p < 0.05$.

4.9 Sodium pyruvate impedes metabolic alterations in CM CT26-myotubes

The impact of sodium pyruvate supplementation on the metabolic change that occurred in cachectic myotubes was evaluated. Results highlighted that myotubes treated for 24 hours with CM CT26 containing sodium pyruvate show lactate production similar to control myotubes whereas, as expected, CM CT26-treated myotubes without sodium pyruvate supplementation produce a higher amount of lactate than control myotubes, as previously reported (Figure 16A). For what mitochondria is concerned, myotubes treatment with CM CT26 induced the decrease of membrane potential and oxygen consumption [187]. Results show that sodium pyruvate supplementation prevents the CM CT26-mediated decrease in the oxygen consumption rate (Figure 16B) and depolarization of the mitochondrial membrane (Figure 16C). These findings demonstrate that the increased sodium pyruvate concentration impedes the metabolic shift induced by CM CT26, thus blocking the formation of the cachectic features in myotubes.

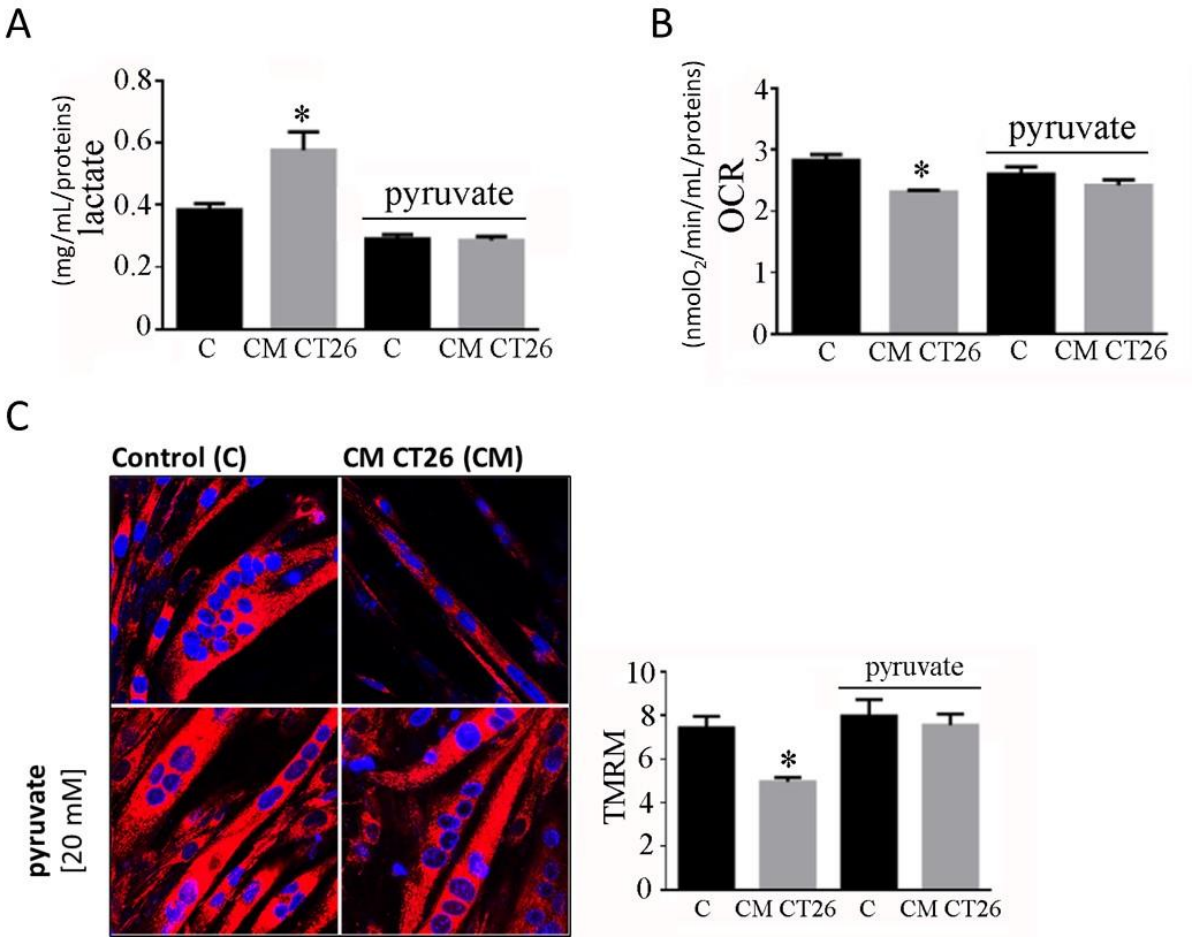

Figure 16. Pyruvate blocks metabolic change in CM CT26-treated myotubes. Four-day-differentiated myotubes were treated with CM CT26 for 24 h. Where indicated, pyruvate (20 mM final) was added to the media. (**A**) Lactate production assay and (**B**) oxygen consumption rate (OCR) analysis in CM CT26-treated myotubes with or without pyruvate supplementation. Data were normalized on total protein content of each sample. (**C**) Representative confocal images of control and CM CT26-treated myotubes with or without pyruvate supplementation. Mitochondria were labelled with TMRM (40 nM final) and appear red, while nuclei are blue due to DAPI staining. TMRM fluorescence is reported as the mean fluorescence in at least ten randomly chosen fields. n = 3; * $p < 0.05$.

4.10 PDH activity is not affected in myotubes treated with CM CT26 supplemented with sodium pyruvate

A decrease in PDH activity has been reported in cachectic myotubes [187]. However, PDH activity can be restored when the lactate dehydrogenase inhibitor oxamate is supplemented to CM CT26. In this condition, myotubes fail to increase lactate production and reduce oxygen consumption [187]. Thus, reactivation of PDH activity could be a key step to block the metabolic shift observed in myotubes after CM CT26 treatment, and to prevent the induction of cachectic features. Although metabolomic analysis shows increased pyruvate amount in cachectic myotubes, the majority of this metabolite could be driven towards lactate formation, thus subtracting the substrate for PDH. Hence, PDH activity could be recovered by adding sodium pyruvate to CM CT26. As expected, PDH activity is decreased in myotubes treated with CM CT26, while the addition of sodium pyruvate impedes the decrease of PDH activity (Figure 17A). At the same time, PDH expression level is not affect by any treatment (Figure 17B).

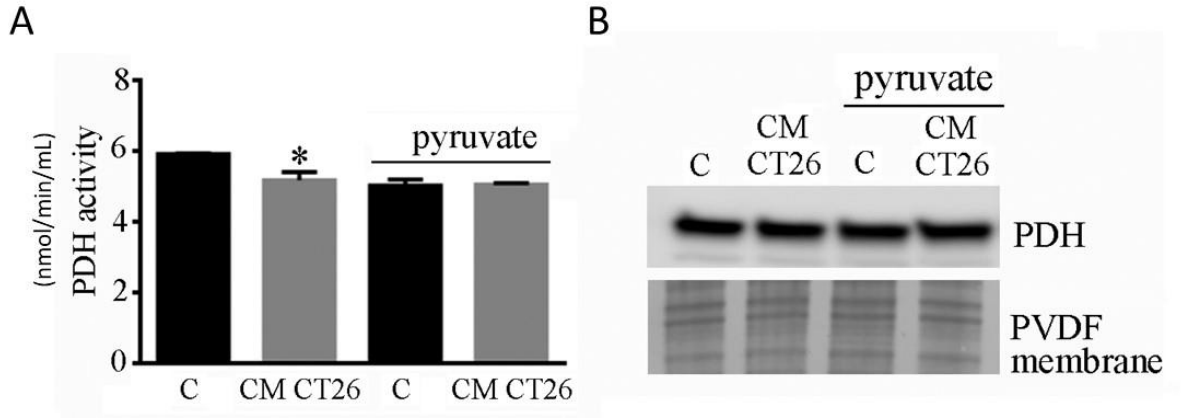

Figure 17. Pyruvate impedes the decreased PDH activity in CM CT26-treated myotubes. Four-day-differentiated myotubes have been treated with CM CT26 with or without pyruvate supplementation for 24h. (**A**) PDH activity. PDH activity measured as nmol/min/mL is normalized on total protein amount of each sample. (**B**) PDH immunoblot. n = 3; * $p < 0.05$.

4.11 Healthy myotubes treated with the pyruvate transporter inhibitor UK5099 manifest cachectic features

To investigate the role of the mitochondrial pyruvate flux in the onset of cachexia, myotubes have been treated with the mitochondrial pyruvate carrier (MPC) inhibitor UK5099. Then, the acquisition of the cachectic features, as well as the metabolic modifications, have been tested in UK5099-treated myotubes. We observed that treatment of myotubes with UK5099 (10 µM final) for 24 hours promotes the onset of the cachectic phenotype in myotubes, as evidenced by thinning of myotube width (Figure 18A and 18B) and promotion of ubiquitin (Figure 18C) and autophagic pathways (Figure 18D). Regarding metabolism, UK5099 treatment induces metabolic changes similar to CM CT26, with increased lactate production (Figure 18E) and decreased oxygen consumption (Figure 18F). Furthermore, UK5099-treated myotubes showed decrease in mitochondrial membrane potential and reduction in PDH activity, as shown in Figure 18G,H. These results highlight that mitochondrial pyruvate deprivation is involved in the formation of the cachectic feature, focusing the importance of the amount of suitable pyruvate into mitochondria to counteract the induction of cachexia in myotubes.

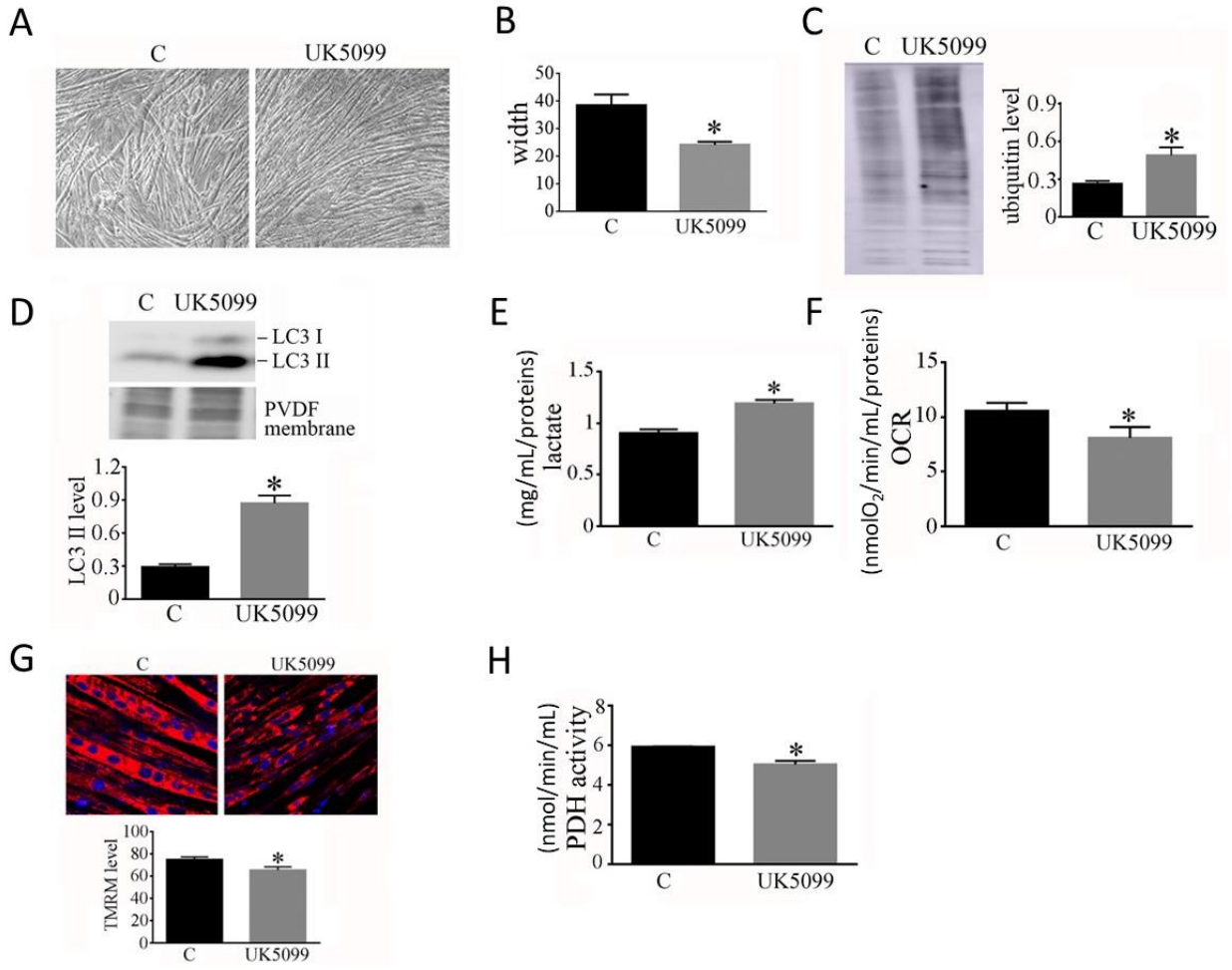

Figure 18. Pyruvate transporter inhibitor UK5099 triggers cachexia in healthy myotubes. Four-day-differentiated myotubes have been treated with UK5099 (10 µM final) for 24h. (**A**) Representative microscopic images of control and UK5099-treated myotubes. (**B**) Myotube width measurement after UK5099 24 h treatment. (**C**) Immunoblot analysis of total protein ubiquitination. The level of ubiquitinated proteins has been obtained using Coomassie-stained PVDF membrane for normalization. (**D**) Immunoblot analysis of the autophagy marker LC3-II. LC3-II levels has been obtained using Coomassie-stained PVDF membrane for normalization. (**E**) Lactate production assay and (**F**) Oxygen consumption rate (OCR) analysis performed on control and UK5099-treated myotubes. Data were normalized on total protein content of each sample. (**G**) Representative confocal images of control and UK5099-treated myotubes. Mitochondria were labelled with TMRM (40 nM final) and appear red, while nuclei are blue due to DAPI staining. TMRM fluorescence is reported as the mean fluorescence in at least ten randomly chosen fields. (**H**) PDH activity. n = 4; * $p < 0.05$.

4.12 CM CT26 supplemented with sodium pyruvate fails in the activation of STAT3 pathway

The activation of STAT3 signalling pathways plays a key role in the induction of cancer cachexia [152]. Hence, we analysed STAT3 phosphorylation in CM CT26-treated myotubes and how the addition of sodium pyruvate can affect the activation of this pathway. In agreement, as shown in Figure 19A we observed that CM CT26 induces high STAT3 phosphorylation after a short time treatment (15 minutes). To study the effective involvement of STAT3 signalling in the induction of cachexia, the STAT3 inhibitor WP1066 (10 μM final) has been added to CM CT26. The inhibition of STAT3 pathway impedes the onset of cachectic features, since CM CT26-WP1066 co-treated myotubes appear morphologically similar to control myotubes, as also evidenced by myotube width measurement (Figure 19B). Results showed that induction of cachexia by CM CT26 involves the activation of STAT3 pathway. Then how the addition of sodium pyruvate can affect the activation of this pathway has been evaluated. Addition of sodium pyruvate to CM CT26 decreases STAT3 phosphorylation (Figure 19C), thus blocking the cachexia onset induced CM CT26. In conclusion, these observations demonstrate that STAT3 signalling is strongly implicated in the promotion of cachexia by CM CT26, and sodium pyruvate attenuates the activation of this signalling cascade.

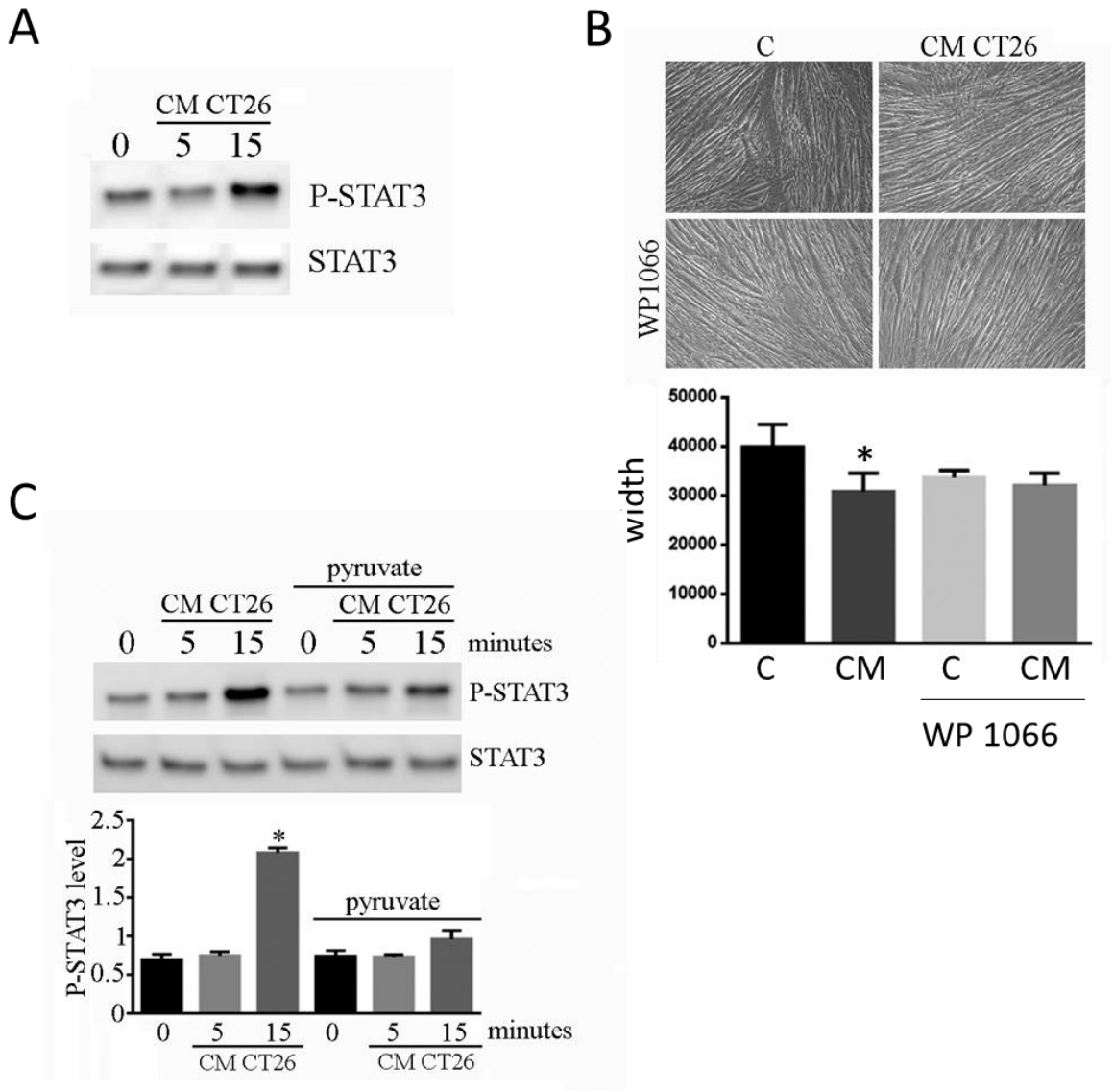

Figure 19. Pyruvate supplementation blocks STAT3 signalling pathways that is involved in CM CT26-related cachexia activation in myotubes. (**A**) Immunoblot analysis of p-STAT3 and total STAT3 in CM CT26-treated myotubes. Myotubes were treated with CM CT26 for the indicated times (5 and 15 minutes). (**B**) Representative microscopic images of CM CT26-treated myotubes with or without STAT3 inhibitor WP1066 (10 µM final). Myotube width measurement has been reported in the below graph. (**C**) Immunoblot analysis of p-STAT3 and total STAT3 in CM CT26-treated myotubes with or without the addition of pyruvate (20 mM final). Myotubes were treated with CM CT26 or co-treated with pyruvate for the indicated times (5 and 15 minutes). n = 3; * $p < 0.05$.

5. Discussion

Cancer cachexia is considered an energy-balance disorder caused by pronounced metabolic alterations. Despite the evidence suggesting that cancer-induced cachexia is associated with a broad range of metabolic rearrangements, the potential role of muscle metabolic abnormalities in the activation and development of cancer cachexia has been little studied so far.

The obtained results highlight that cachexia is induced in myotubes due to the manifestation of a metabolic shift from oxidative to fermentative metabolism. Indeed, our findings show that cachectic myotubes acquire the ability to secrete more lactate into the culture medium than control myotubes. This metabolic remodelling, also supported by an increased glucose uptake by cachectic myotubes, is very similar to that of cancer cells (Warburg effect) characterized by a forced glycolytic metabolism resulting in increased levels of lactate production in aerobic conditions [188]. These results highlight that conditioned medium derived from the culture of the murine carcinoma CT26 cell line capable of inducing cachexia *in vivo*, triggers in myotubes a sort of Warburg effect since treated myotubes manifest increased glucose uptake and activation of lactic fermentation in aerobic conditions. This suggests that CM obtained from CT26 tumour cells metabolically transforms healthy myotubes into "cancer myotubes." These findings are in agreement with published data showing an upregulated glycolytic pathway in muscles of cachectic mice [189].

In the *in vitro* model of cachexia used, cachectic myotubes showed significant mitochondrial alterations, in particular strong depolarization of the mitochondrial membrane associated with decreased oxygen consumption, reflecting a defect in oxidative phosphorylation. These results agree with previous findings showing disrupted mitochondrial structure in skeletal muscle of animal models of cancer cachexia [190,191,192]. Furthermore, cachectic patients show significant mitochondrial alterations in the skeletal muscles, such as a decreased oxidative

capacity [193] that is reflected in an increased oxidation of mitochondrial proteins [194], as well as alterations of the mitochondrial membrane [193].

Cancer-induced cachexia is associated with a broad range of metabolic rearrangements [195]. Particularly, animal models of cachexia are characterized by a decreased level of circulating glucose due to increased systemic glucose demand, depletion of hepatic glucose stores, and significant alterations in the tricarboxylic acid cycle pathway [196]. Nevertheless, there is no evidence to demonstrate a direct and causal involvement of metabolic alterations in the onset of cancer cachexia so far.

Cancer cachexia is associated to a systemic inflammatory manifestation [30]. Proinflammatory cytokines and in particular IL-6 plays a key role in muscle damage, such that high circulating levels of IL-6 have been correlated with body weight loss and survival in patients with cancer cachexia [124,37]. The role played by IL-6 in muscle wasting primarily includes the promotion of protein degradation mediated by the activation of the STAT3 pathway [197,120]. Moreover, high levels of IL-6 lead to severe mitochondrial alterations that result in functional defects characterized by increased levels of mitochondrial reactive oxygen species and decreased ATP production [198]. Obtained results show the presence of higher levels of IL-6 in CM CT26 and that treatment of healthy myotubes with IL-6 is able to induce the cachectic phenotype in myotubes in association with the metabolic shift toward fermentation. Furthermore, activation of the STAT3 pathway has been observed in CM CT26-treated myotubes, probably due to IL-6 action. These data demonstrate that IL-6 play a role in the metabolic change that leads to activation of cancer cachexia.

A crucial aspect is that inhibition of lactate production (performed either by blocking glycolysis or LDH activity) succeeds in preventing the induction of the cachectic phenotype in CM CT26-myotubes, and that this is associated with a restoration of an healthy myotube metabolism. Indeed, blockade of lactate production is also effective in preventing mitochondrial alterations, restoring metabolic parameters (PDH activity and oxygen consumption) to values comparable to those in control myotubes. However, understanding the mechanisms involved in blocking the induction of the cachectic phenotype by inhibition of glycolysis or LDH activity are not easy to argue.

IL-6 plays a role in muscle metabolism, resulting in increased muscle glucose uptake [199]. Thus, IL-6 is likely involved in the increased glucose uptake seen in cachectic myotubes treated with CM CT26. In addition, IL-6 has been shown to downregulate the activity of the pyruvate dehydrogenase (PDH) complex in muscle [200]. Specifically, PDH catalyses the pyruvate decarboxylation reaction that constitutes the first reaction of the PDC leading to the irreversible conversion of pyruvate to Acetyl-CoA, which can fuel the Krebs cycle [201,202]. The results demonstrate that CM CT26 promotes decreased PDH activity in myotubes, thereby blocking mitochondrial metabolism and forcing myotubes to use glycolysis-derived pyruvate for lactic fermentation. Inhibition of glycolysis by 2-DG or LDH by oxamate prevents the decrease in PDH activity. This is probably due to increased mitochondrial pyruvate availability in myotubes treated with the two inhibitors. The question to be addressed is the source of the pyruvate used by PDH. For what oxamate is concerned, pyruvate formed by glycolysis could be completely driven towards PDH. It is more complicated to argue what happens in the presence of the glycolysis inhibitor 2-DG. The use of the glucose analogue 2-DG results in a reduction in pyruvate formation. Hence, in 2-DG-treated myotubes, pyruvate could be derived from glucogenic amino acids, glycerol derived from triglyceride degradation, and glyceraldehyde-3-phosphate obtained from the pentose phosphate pathway, which in fact is upregulated in 2-DG-treated cells [203]. Whatever the source of pyruvate, in myotubes treated with 2-DG or oxamate, PDH could be restablished, thus allowing conversion of pyruvate to Acetyl-CoA and restoration of oxygen consumption similar to control myotubes. However, a role of the pyruvate dehydrogenase kinase 4 (PDK4) in the PDH reactivation cannot be excluded. Recently, a direct role for PDK4 in promoting cancer-associated muscle metabolic alterations and skeletal muscle atrophy has been reported [185]. Importantly, the increased lactate production of cachectic myotubes occurs in aerobic conditions, whereas healthy skeletal muscle activates lactic fermentation in oxygen-deficient conditions. Being unable to obtain energy from cellular respiration, in this case myotubes shift towards fermentation to establish with the liver the Cori cycle, which allows to recycle lactate to form new glucose molecules able to supply

the muscle. Subjected to significant inflammatory stress (i.e. IL-6), cachectic myotubes are forced to modify their metabolism by shifting toward lactic fermentation due to mitochondrial damage that does not allow them to utilize mitochondrial oxidative phosphorylation efficiently. Increased glucose uptake could allow energy-stressed myotubes to obtain ATP from glycolysis necessary for survival. From this perspective, the metabolic shift toward fermentation could be a mechanism to counteract cell death. In agreement with this hypothesis, we observe that myotubes after 24 h of CM CT26 treatment are viable and may reverse the cachectic phenotype after CM CT26 removal.

More interestingly, the obtained results focus the attention on the centrality of pyruvate utilization by myotubes in the induction of the cachectic phenotype. The majority of pyruvate in cachectic myotubes is driven toward lactic fermentation, with a small proportion of the metabolite driven in mitochondria due to blockage of mitochondrial metabolism. In this context, the limiting step for the metabolic shift could be the use of pyruvate, produced by glycolysis, by LDH or pyruvate dehydrogenase complex (PDH). Hence, LDH and PDH could compete for pyruvate in the early phase of cancer cachexia. In this period, pyruvate could be forced towards LDH, thus increasing lactate formation. Obtained results show that pyruvate supplementation blocks the induction of the cachectic phenotype in myotubes treated with CM CT26. In particular, the higher amount of pyruvate available could restore its flux in the mitochondria and thus the healthy mitochondrial metabolism of myotubes. Indeed, in myotubes treated with CM CT26 in the presence of a high concentration of pyruvate, the functional alterations at mitochondrial level do not occur as myotubes manifest PDH activity, mitochondrial membrane potential and oxygen consumption comparable to control myotubes. Furthermore, the use of a specific inhibitor of the mitochondrial pyruvate transporter (MPC) has been shown to mimic the establishment of cachectic features in myotube, thus reinforcing the concept that impaired pyruvate flux within the mitochondria could play a crucial role in the onset of CM CT26-related cachexia.

Cachectic patients show a drastic worsening of both prognosis and quality of life, as well as reduced tolerance and response to antineoplastic treatments [8]. Numerous efforts have therefore been made in order to counteract the onset of cancer cachexia. Particularly, these include a combination of specific nutritional supports associated with pharmacological interventions and muscle exercise [204]. Considering the obtained results highlighting the crucial role of a metabolic shift towards fermentation in the induction of cancer cachexia in myotubes, the use of therapeutic strategies aimed to restore healthy myotube metabolism could be an important step in the fight against cancer cachexia. Particularly, preventing higher lactate production by inhibiting LDH could represent an useful tool to counteract cancer cachexia. Given the key role of muscle pyruvate utilization in the induction of cachexia, inhibition of LDH would likely result in the restoration of healthy muscle metabolism, given that all available pyruvate would be forced into the mitochondria, thus reactivating the healthy mitochondrial metabolism in skeletal muscle. The end product of LDH, lactate, has long been considered to be just a "waste product" of aerobic glycolysis. Quite recently, the involvement of lactate in tumour progression, such as cell migration and metastasis formation, and the use of lactate as an energy source has been demonstrated [205]. Considering the role of the metabolic shift toward lactic fermentation underlying the induction of CM CT26-related cachexia, further in-depth studies will be required to evaluate the possible involvement of increased muscle lactate production in cancer cachexia development. Inhibition of LDH would lead, on the one hand, to a decrease in the amount of lactate produced by myotubes and, on the other hand, to an increased mitochondrial availability of pyruvate raised as a crucial point in the induction of CM CT26-mediated cachexia in this study. Hence, LDH inhibition could constitute a powerful tool to counteract cancer progression and those pathologies correlated to cancer, as cachexia. Conclusively, the obtained results show that the metabolic alterations underlying the induction of cachexia *in vitro* are prevented in the presence of increased muscle pyruvate availability (e.g. by inhibition of LDH with oxamate or by pyruvate supplementation), thus opening the way toward possible new approaches to counteract cancer cachexia-related muscle damage

based on increasing muscle-level availability of pyruvate, such as targeting specific pyruvate-rich diets.

6. References

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