# THE MOLECULAR MECHANISMS INVOLVED IN DOXORUBICIN-INDUCED SKELETAL MUSCLE WASTING

## MECANISMOS MOLECULARES SUBJACENTES AO CATABOLISMO MUSCULAR PROMOVIDO PELA DOXORRUBICINA

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**ABSTRACT**

Chemotherapeutic agents like doxorubicin (DOX) are the foundation for the treatment of a variety of malignancies; however, these therapies have several side-effects. DOX may trigger or potentiate the muscle wasting observed in cancer patients, which is particularly worrying in frail old patients. Therefore, it is important to comprehend the mechanisms responsible for DOX-induced toxicity in skeletal muscle, to identify therapeutic targets envisioning the improvement of survival rates and quality of life of these patients. Hence, this review discusses the molecular players that may be involved in DOX-induced muscle wasting. From the analysis performed herein, DOX seems to induce the activation of the proteolytic ubiquitin proteasome pathway (UPP), which in turn can also be enhanced by DOX-induced increase in myostatin and tumor necrosis factor (TNF)-α signaling pathways, as well as insulin resistance. Furthermore, DOX-induced oxidative stress and mitochondrial dysfunction may also be critical contributors for muscle wasting. All these mechanisms may contribute to the loss of skeletal muscle mass and function observed after DOX exposure, which may lead to or aggravate cachexia, responsible for more than 20% of all cancer-related deaths.

***Keywords:*** *Adriamycin, cachexia, oxidative stress, muscular toxicity, proteolytic pathways.*

**RESUMO**

Os fármacos utilizados na quimioterapia como a doxorrubicina (DOX) são essenciais para o tratamento de vários tipos de cancro. No entanto, esta terapia tem vários efeitos secundários associados. A DOX pode potenciar a perda de massa muscular observada em pacientes com cancro, o que é particularmente preocupante em pacientes idosos. Assim, é necessário compreender os mecanismos responsáveis pela toxidade da DOX no músculo esquelético, de forma a identificar alvos terapêuticos e a aumentar as taxas de sobrevivência e qualidade de vida destes pacientes. Esta revisão discute os mediadores moleculares que poderão estar envolvidos na perda de massa muscular induzida pela DOX. Da análise realizada, a DOX parece promover a ativação da via da ubiquitina-proteassoma, ativação essa que pode ser intensificada pela elevação, induzida pela DOX, da atividade das vias da

miostatina e do fator de necrose tumoral alfa, bem como pela presença de resistência à insulina. A DOX parece também induzir stress oxidativo e disfunção mitocondrial, o que poderá contribuir para a perda da massa muscular. Todos estes mecanismos parecem ser cruciais para impulsionar a perda de massa e de função muscular observadas após a exposição à DOX, o que poderá resultar ou agravar a caquexia, que é responsável por mais do que 20% de todas as mortes relacionadas com o cancro.

***Palavras-chave:*** *Adriamicina, caquexia, stress oxidativo, toxicidade muscular, vias proteolíticas.*

## 1. INTRODUCTION

The global burden of cancer is rapidly increasing with more than 18 million new cases and 9.5 million deaths in 2018 and these numbers are expected to increase[1]. In part this is because of the rising number of older adults worldwide with 13.5% being 60 years or older in 2020[2]. Chemotherapy is still one of the most successful therapeutic strategies against cancer. Doxorubicin (DOX, also known as Adriamycin) is a widely used agent and a member of the anthracycline anticancer drug group[3]. DOX acts on cancer cells by intercalating into DNA, inhibiting or poisoning topoisomerase-II, which results in DNA damage and cell death, and by generating reactive oxygen species (ROS), leading to lipid peroxidation, damage to cellular membranes and DNA, oxidative stress and activation of apoptotic pathways of cell death[4]. This highly potent and effective cytotoxic chemotherapeutic drug is used in the treatment of many cancers, such as breast, liver, lungs, ovaries, thyroid and colon cancer, lymphoma and leukemia[5,6]. Nonetheless, its use is limited by dose-dependent acute and chronic toxic side effects in many organs, such as in the heart[7], brain[8], liver[6], kidney[9] and skeletal muscle[10]. Regarding the latter, muscle weakness and fatigue are frequently reported in patients receiving DOX treatment[11,12], and muscle wasting may be the main contributor to the alterations in body mass experienced by cancer patients undergoing DOX treatment. The loss of muscle mass in cancer is usually associated to cancer cachexia that affects 25-80% of all cancer patients[13,14] and is responsible for more than 20% of cancer deaths[15]. Nonetheless, the muscle mass loss in cancer can also be associated to sarcopenia, a condition primarily diagnosed in ages above 65 years[16], since sarcopenia is diagnosed in 15-50% of all cancer patients[14]. Hence, chemotherapeutic agents like DOX may play a key role in the onset or aggravation of cachexia or sarcopenia. Hitherto, the magnitude of DOX contribution to the loss of skeletal muscle mass and function is not clear. Moreover, the mechanisms underlying DOX-induced toxic effects on skeletal muscle of older patients are even less studied. This constitutes a huge gap in knowledge, since individuals with more than 65 years of age comprise the fastest growing portion of the population[17], and indeed, 47.5% of all cancers were diagnosed in the eldest worldwide in 2012[18]. The management of older cancer patients is one of the most challenging tasks to the clinical oncologist[17], and therefore, uncovering the molecular basis of DOX-induced skeletal muscle wasting is needed and will certainly improve the clinical management of both younger and older cancer patients, their quality of life and survival rates.

This review discusses the current knowledge on key molecular players that may contribute to DOX--induced loss of skeletal muscle mass and function in cancer patients. Nonetheless, the reader should keep in mind that most of the available data on this topic come from preclinical experiments given the invasiveness of skeletal muscle collection and most of these studies harbor young or adult animals. Moreover, when not otherwise mentioned, the (cumulative or single) dose of DOX administered in the animals was between 15-20 $mg.kg^{-1}$ *per* body weight. 20 $mg.kg^{-1}$ in rodents corresponds to the usually used human clinical dose when is scaled

according to the generally applied methods[19,53]. Doses lower than 20 $mg.kg^{-1}$ also mimic the treatment of human patients receiving low DOX doses and were used to induce muscle wasting but without treatment-related deaths. The maximum cumulative dose recommended for DOX treatment in humans is of 450-550 $mg.m^{-2}$ [3].

## 2. DOXORUBICIN EFFECTS ON SKELETAL MUSCLE

Skeletal muscle may, in part, modulate DOX pharmacokinetics and therapeutic effect by sequestering the drug (observed in rats' *gastrocnemius*, *soleus*, *extensor digitorum longus* and *plantaris*)[20], which induces several modifications, such as vacuolation of sarcoplasmic reticular membranes, myofibrillar degeneration, interstitial edema, and mitochondrial swelling with breakdown of organelle membranes[21]. This accumulation seems to be dose-dependent[20] and may occur preferentially in the mitochondria through binding to cardiolipin, which accounts for about 15% of the phospholipid content of mitochondrial membranes[5,22]. In line with this, it is plausible to think that a greater accumulation of DOX may occur in oxidative skeletal muscles comparatively to glycolytic ones[22]; however, this idea has been refuted[20]. Skeletal muscle has a high mitochondria density and so it is probable that DOX-induced mitochondrial toxicity can culminate in skeletal muscle-specific symptomatology, namely muscle wasting, fatigue, impaired regenerative capacity and exercise intolerance[5]. The accumulation of DOX in skeletal muscle seems to markedly increase with aging (4 to 24 weeks rats' *extensor digitorum longus*)[23]. This may be due to an age-related decrease in multidrug resistance proteins (MRPs) muscle levels, which extrude anticancer drugs out of the cell[24]. DOX is in fact a substrate for, at least, MRP-1, MRP-2 and MRP-6[24], and with aging, the levels of MRP-2 were found to decrease in the *extensor digitorum longus* of rats[23]. This greater accumulation of DOX in older skeletal muscles indicate that older cancer patients may present a higher risk of developing muscle wasting and/or a higher rate of wasting progression.

Administration of DOX significantly decreases body weight in preclinical models[25–29]. This deleterious effect on body weight can be caused by only one single administration of DOX and it can be observed as soon as 48-72 hours after the exposure[25,27,28]. Animals treated with DOX presented a significant decrease in food consumption comparatively to healthy counterparts[26,30], which may contribute, to some extent, to body weight loss. However, the key factor to the outcome of body weight loss may be the DOX-induced loss of skeletal muscle mass. Animals treated with DOX showed a marked decrease in muscle mass (*extensor digitorum longus*)[25,26,31], which was noticeable just 72 hours after DOX administration[25]. These data are strengthened by the significant reduction of the cross-sectional area (CSA) of the *extensor digitorum longus*[25,26] and of type I, IIa and IIb muscle fibers of the *diaphragm*, *plantaris* and *soleus* of animals treated with DOX[32,33]. This reduction in the CSA of muscle fibers may be a consequence of denervation[34]; however, studies focused on DOX-related denervation are scarce. For instance, an elevation of ROS levels, which is associated with DOX administration[33] (explored in the next section), seems to be sufficient to induce neuromuscular junction (NMJ) abnormalities, including increased NMJ fragmentation[35], a typical signal of denervation[34]. The NMJ is responsible for the transmission of electric impulses from innervating motoneuron to the innervated muscle fibers. This transmission relies on acetylcholine (ACh) release and binding to its receptor (AChR), culminating in muscle contraction[36]. DOX administration reduced mRNA expression of AChR α and δ subunits in the *soleus* of animals that had lower CSA of type I and IIb fibers comparatively to healthy ones[37], which may indicate neuromuscular transmission impairment. Future studies should investigate the putative effect of DOX-induced denervation to the loss of

muscle mass and ffunction, particularly in older classes, to not aggravate an underlying age-induced denervation, which is associated to sarcopenia. Furthermore, DOX exposure seems to decrease satellite cells content[38], which may jeopardize skeletal muscle regeneration **(Figure 1)**.

In line with these results, DOX seems to impair muscle function, by worsening physical performance[39] and inducing muscle fatigue[26]. For instance, animals treated with DOX had decreased maximal twitch force, rate of force development and rate of force decline of the *extensor digitorum longus* and *soleus*[22,29], and decreased specific force of *diaphragm*[27,40] and *extensor digitorum longus*[26]. This impairment in muscle function may be dependent of the time after DOX exposure[22] and DOX may affect different muscles in distinct ways, since the onset of muscle function impairment was observed earlier in the *extensor digitorum longus* than in the *soleus* of the animals[22].

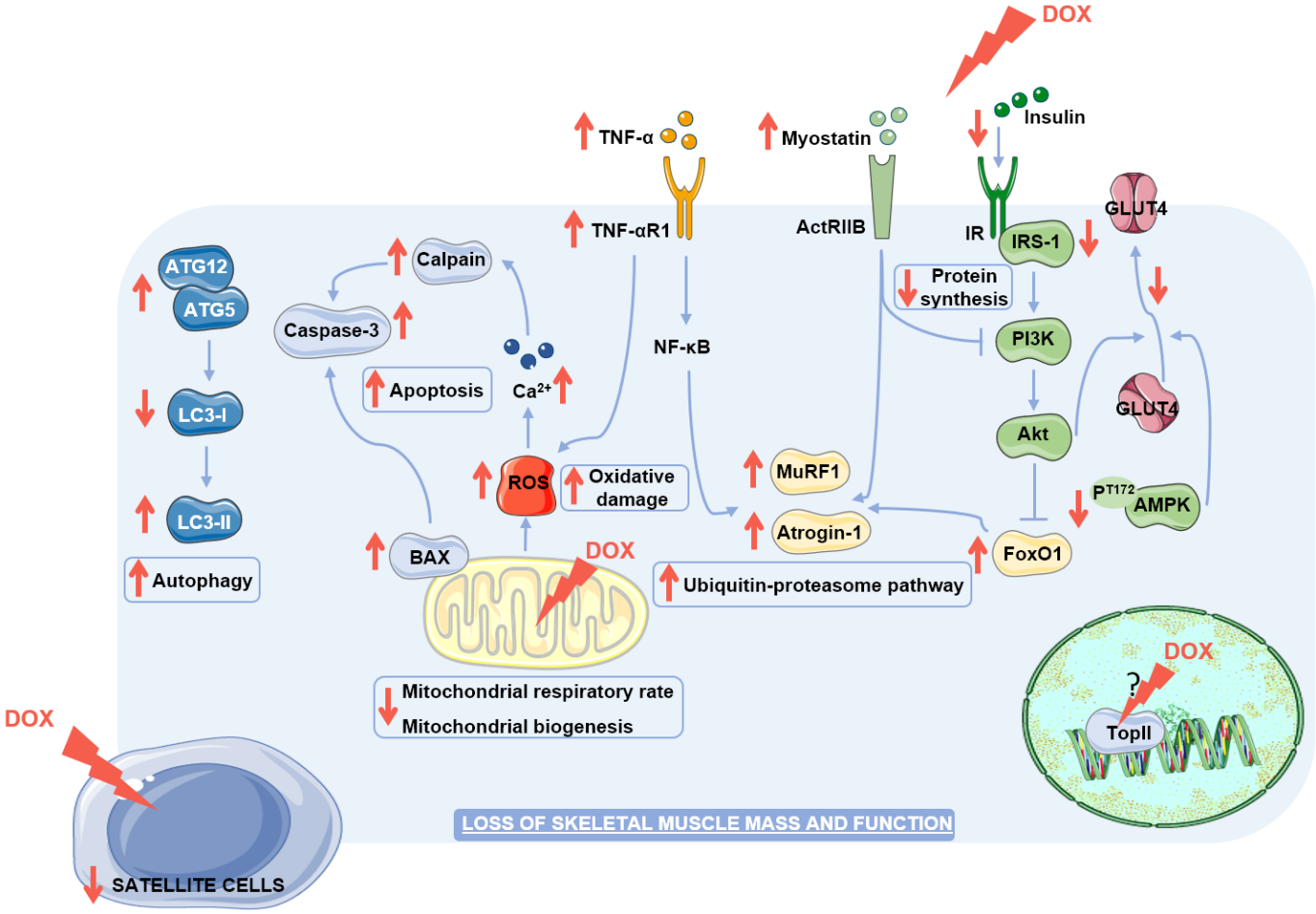


FIGURE 1 – **Key molecular mediators involved in doxorubicin (DOX)-induced skeletal muscle wasting. Administration of DOX seems to increase the activity of the ubiquitin-proteasome pathway, increasing muscle proteolysis, and decrease the activity of protein synthesis, in part by inducing insulin resistance. Moreover, DOX also appears to increase oxidative damage, through the increase of ROS levels and mitochondrial dysfunction. Autophagy and apoptosis are also upregulated by DOX. A decline in the satellite cells content is also associated with DOX exposure. The figure was produced using *Servier Medical Art*.**

**Abbreviations: ActRIIB: activin receptor type 2B; AMPK: 5'-adenosine monophosphate-activated protein kinase; ATG: autophagy-related protein; BAX: Bcl-2-associated X protein; FoxO1: forkhead box O 1; GLUT4: glucose transporter type 4; IR: insulin receptor; IRS-1: insulin receptor substrate-1; LC3: microtubule-associated protein light chain 3; MuRF1: muscle-specific RING-finger 1; NF-κB: nuclear factor kappa light-chain enhancer of activated B cells; PI3K: phosphatidylinositol 3-kinase; ROS: reactive oxygen species; TNF-α: tumor necrosis factor alpha; TNF-αR1: tumor necrosis factor alpha receptor 1; TopII: topoisomerase II**

## 3. POTENTIAL MOLECULAR MECHANISMS INVOLVED IN DOXORUBICIN-INDUCED SKELETAL MUSCLE WASTING

Currently, the exact molecular mechanisms underlying DOX-induced muscle loss are not completely clarified. The loss of muscle mass occurs due to increased protein degradation possibly driven by enhanced ubiquitin-proteasome pathway (UPP) activity, increased autophagy and oxidative stress, as well as decreased protein synthesis possibly driven by an impaired response to growth-promoting mediators[10].

### 3.1. Activation of proteolytic signaling pathways in skeletal muscle

The activity of the UPP increased following DOX administration **(Figure 1)**[30,41]. Indeed, the content of ubiquitinated proteins was increased in the *gastrocnemius* of aged mice exposed to DOX[41]. In line with this, an increase in the muscle levels of UPP-related mediators, namely forkhead box O (FoxO)1[30], muscle-specific RING-finger 1 (MuRF1)[41] and atrogin-1 (both muscle and C2C12 myotubes)[42,43] was also observed in mice treated with DOX. This augment in the activity of the UPP may be associated to the DOX-related increase of myostatin signaling **(Figure 1)**[44]. In fact, inhibition of the myostatin signaling by a soluble ligand binding domain of the activin receptor type 2B (sACVR2B-Fc) administered in mice treated with DOX prevented the DOX-induced increase in atrogin-1[42]. Moreover, myostatin or other mediators that belong to the transforming growth factor beta (TGF-β) superfamily of proteins, may be important factors for DOX-induced muscle wasting, since inhibition of its signaling through sACVR2B-Fc in mice exposed to DOX prevented the DOX-induced muscle wasting and was even able to increase muscle mass, effect that was not due to a decrease on the muscle concentration of DOX[42].

The activity of UPP-related mediators can also be enhanced by tumor necrosis factor alpha (TNF-α) **(Figure 1)**[45]. At systemic level, circulating TNF-α levels were found increase in both mice[26], and cancer patients who received DOX treatment comparatively to cancer patients without DOX treatment and healthy individuals[46]. This occurs because of DOX-induced apolipoprotein A1 (APOA1) oxidation, which compromises APOA1 role of negatively regulate TNF-α synthesis[54]. At muscle At muscle level, increased TNF-α mRNA levels were observed in rats' *soleus* following DOX exposure[31]. In addition, DOX administration in mice, increased *diaphragm* TNF-α receptor 1 (TNF-αR1) mRNA levels and stimulated TNF-αR1 translocation to the plasma membrane[40]. This suggests that DOX may improve the availability of TNF-αR1 at the cell surface favoring ligand binding, increasing in this way muscle sensitivity to TNF-α, which may promote muscle wasting through, for instance, nuclear factor kappa light-chain enhancer of activated B cells (NF-κB) signaling[47]. The deficiency of TNF-αR1 in mice abolished the decrease of specific force and lowered the decline in maximal absolute force observed in the animals treated with DOX[26,40], suggesting that TNF-α signaling is a great contributor to DOX-induced muscle dysfunction. In skeletal muscle, TNF-α can also stimulate the generation of ROS *via* a TNF-αR1-dependent mechanism, which can cause muscle impairment by affecting myofibrillar proteins or calcium homeostasis[48].

Increased muscle proteolysis through enhanced UPP activity may also be due to a decrease in phosphatidylinositol 3-kinase (PI3K)/Akt signaling induced by insulin resistance[49], which in turn seems to be a consequence of DOX exposure **(Figure 1)**. Indeed, DOX administration led to increased systemic insulin resistance and hyperglycemia, with a decrease of insulin receptor substrate-1 (IRS-1) protein levels in *extensor digitorum longus* of rats[25]; however, no differences were observed in protein levels of the insulin receptor or Akt[25]. Moreover, DOX administration resulted in a decrease of glucose

transporter type 4 (GLUT4) and 5'-adenosine monophosphate-activated protein kinase (AMPK) α (phospho-Thr172) levels in rats' *extensor digitorum longus*[25]. The DOX-induced disruption of the insulin signaling pathway can activate UPP-related mediators, contributing to muscle proteolysis and can also decrease protein synthesis[49]. As a matter of fact, muscle protein synthesis was blunted after DOX administration in mice[30,39].

### 3.2. Oxidative stress

Mitochondria function impairment due to DOX treatment may be involved in skeletal muscle damage, possible by contributing to the production of ROS **(Figure 1)**. This is given by the diminished mitochondrial respiratory control ratio [33] and state 3 of the respiratory chain[32] observed in animals treated with DOX that was accompanied by an increase of $H_2O_2$ formation[33]. Indeed, DOX treatment increased mitochondrial ROS production in the *diaphragm*, *soleus* and *plantaris* of rats, and decreased the mitochondrial respiratory control ratio in the *diaphragm*[32]. Moreover, the expression of peroxisome proliferator-activated receptor gamma coactivator 1 (PGC-1α) was decreased in the skeletal muscle of mice treated with DOX[42], suggesting decreased mitochondrial biogenesis. Administration of DOX also increased nitrotyrosine residues on *soleus*[31] and *diaphragm*'s desmin and tropomyosin, which may culminate in muscle weakness, increased susceptibility to damage and impaired actin and troponin complex stabilization[27]. Administration of DOX also increased the *soleus* muscle protein carbonyls[50]. Moreover, the elevated 4-hydroxynonenal (4-HNE) levels in the muscle of rats treated with DOX[32,50] suggest lipid peroxidation. In line with this, the administration of a mitochondrial-targeted antioxidant (SS31) prior to DOX administration prevented this oxidative damage of muscle proteins, as well as the DOX-induced atrophy of type I and IIa in *diaphragm* and *soleus* and type IIb in *plantaris* of rats[32], indicating that DOX-induced mitochondrial ROS production is an important mechanism to muscle damage. This DOX-induced increase in mitochondrial ROS generation can elevate cytosolic free calcium through, for instance, an increased ROS-mediated calcium release from the sarcoplasmic reticulum and a decreased calcium removal from the cell, which is a consequence of the oxidative damage to calcium channels located in the plasma membrane[51]. This rise in intracellular calcium concentration can activate apoptosis and autophagy[51]. Indeed, inhibition of autophagy in muscle attenuated the DOX-induced mitochondrial dysfunction and formation of $H_2O_2$[33], while treatment with SS31 prevented the DOX-induced increase in calpain activation[32].

### 3.3. Activation of apoptosis and autophagy in the skeletal muscle

Treatment with DOX seems to increase both apoptosis and autophagy in the skeletal muscle, which are also associated with muscle wasting **(Figure 1)**[52]. Increased activity of caspase-3 was observed in the *gastrocnemius*[41], *diaphragm*, *plantaris*[32] and *soleus*[50] of animals treated with DOX. Moreover, an elevation of Bcl-2-associated X protein (BAX) levels, apoptotic DNA fragmentation[41], and of the number of TUNEL-positive nuclei[32] was observed in skeletal muscles of animals exposed to DOX. Furthermore, an increased calpain activation in animals' *diaphragm*, *plantaris*[32] and *soleus*[50] was also observed following DOX treatment. A specific calpain inhibitor (SJA 6017) administered prior to DOX, attenuated DOX-induced decline in *diaphragm* contractile function and protected against DOX-induced atrophy in adult rats' *diaphragm* (type I), *plantaris* (type IIa) and *soleus* (type I and type IIa)[32]. By activating sirtuin 1 (SIRT1) and consequently repressing apoptotic/catabolic pathways, resveratrol counteracted the

muscle loss associated with aging and the repeated use of DOX in cancer subjects[41], which reinforces the importance of these upregulated pathways to muscle wasting.

Administration of DOX increased autophagy-related protein (ATG)12-ATG5 conjugation, microtubule-associated protein 1A/1B-light chain 3 (LC3)-II/I ratio and autophagic vacuole formation in rats' *soleus*[33]. However, DOX administration did not altered the expression of activating transcription factor 4 (ATF4), CCAAT-enhancer-binding protein homologous protein (CHOP), X-box binding protein 1 (XBP1) nor the phosphorylation of eucaryotic translation initiation factor 2 (eIF2α) in the *soleus*[33]. Inhibition of autophagy prior to DOX administration increased *soleus* muscle transcription of PGC-1α and several downstream transcriptional mediators of mitochondrial biogenesis, such as nuclear respiratory factor (NRF)1/2, and also ROS detoxification in rats[33], suggesting a DOX-related interplay between autophagy and mitochondrial dysfunction.

## CONCLUSION

The chemotherapeutic drug DOX is widely used and presents a high efficacy against several types of cancer; however, its use is also associated with acute and chronic toxic side effects to certain organs, such as the skeletal muscle. In this way, it is important to unravel the mechanisms responsible for DOX-induced toxicity in muscle, which seems to result in loss of muscle mass and function, worsening the effects of cancer itself in skeletal muscle. DOX-induced muscle wasting appears to involve the activation of the proteolytic UPP, which in turn is also enhanced by DOX-induced increase in myostatin and TNF-α signaling pathways, as well as insulin resistance. Moreover, DOX-induced oxidative stress and mitochondrial dysfunction may also be crucial drivers for muscle wasting. It is noteworthy that the contribution of these pathways to the muscle response to DOX may be exacerbated or even distinct in aged muscle. However, the hypotheses advanced in this review were based on the available preclinical studies that in most cases involved adult and healthy animals, and therefore, do not comprehend the interplay between cancer and/or aging and DOX on muscle wasting. Future studies should investigate the effect of DOX on older skeletal muscle from cancer subjects, being aware that a great proportion of cancer patients experiencing cancer cachexia or sarcopenia belong to the older strata of the society. Unraveling key molecular players involved in DOX-induced muscle wasting may help to prevent the loss of muscle mass and strength in cancer patients, which will improve patients' quality of life and their prognosis and survival rates, by, for instance, increasing patients' tolerance to cancer treatments. Such knowledge will boost precision medicine in the set of cancer.

### Conflict of interest

The authors declare no conflict of interest.

### Authors' contributions

A.M.P. conducted the literature search and drafted the manuscript and R.F., V.M.C., P.A.O. and J.A.D. critically revised the work.


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