U.PORTO
FACULDADE DE DESPORTO
UNIVERSIDADE DO PORTO

Sarcopenia vs. Cachexia: Explaining Skeletal Muscle Wasting on Aging, Breast Cancer or Chemotherapy in Preclinical Models

Academic thesis submitted to the Faculty of Sport of the University of Porto to fulfill the necessary requirements to obtain the doctoral degree in Physical Activity and Health under the Decree-Law No. 74/2006 of 24 March, amended by Decree-Law No. 65/2018 of 16 August.

Supervisor

José Alberto Ramos Duarte, MD, PhD

Co-supervisors

Rita Maria Pinho Ferreira, PhD

Paula Alexandra Martins de Oliveira, DVM, PhD

Alexandra Moreira Pais

Porto, 2024

FICHA DE CATALOGAÇÃO

Moreira Pais, A. (2024). *Sarcopenia vs. Cachexia: Explaining Skeletal Muscle Wasting on Aging, Breast Cancer or Chemotherapy in Preclinical Models*. Porto: A. Moreira Pais. Doctoral Thesis in Physical Activity and Health submitted to the Faculty of Sport of the University of Porto.

Keywords: AGING, CANCER, MITOCHONDRIAL REMODELING, SEXUAL DIMORPHISM, SKELETAL MUSCLE WASTING.

This thesis was supported by Alexandra Moreira Pais's grant (SFRH/BD/144396/2019) attributed by the Portuguese Foundation for Science and Technology (FCT)/Ministry of Science, Technology and Higher Education (MCTES) and the European Social Fund (ESF) through PT2020, and by Research Center in Physical Activity, Health and Leisure (CIAFEL, UIDB/00617/2020), Laboratory for Integrative and Translational Research in Population Health (ITR, LA/P/0064/2020), Associated Laboratory for Green Chemistry (LAQV, UIDB/50006/2020), and Centre for Research and Technology of Agro Environmental and Biological Sciences (CITAB, UIDB/04033/2020, https://doi.org/10.54499/UIDB/04033/2020) research units projects through national founds by FCT/MCTES.

Para a minha mamã, Maria.

To my mommy, Maria.

Agradecimentos

Quero começar por agradecer às pessoas que tornaram o começo deste projeto possível ao dizerem “sim” e ao confiarem em mim para o seu desenvolvimento, Professora Doutora Rita Ferreira, Professora Doutora Paula Oliveira e Professor Doutor José Alberto Duarte. Professores, o vosso apoio, disponibilidade *24/7*, críticas construtivas, conhecimento extenso, incentivo, e motivação, foram vitais para o desenvolvimento deste trabalho. Tenho de salientar o vosso profissionalismo e capacidade de trabalho que me inspiraram todos os dias. À Professora Doutora Rita Ferreira, com quem trabalhei de mais de perto, agradeço a paciência, as incontáveis oportunidades que me proporcionou, todas as correções e sugestões dos primeiros *drafts*, todas as conversas e apoio; é das pessoas mais inspiradoras que conheço. Obrigada!

Quero agradecer ao Professor Doutor Rui Vitorino pela disponibilidade, pelas colaborações em trabalhos, pelo conhecimento partilhado e pelas oportunidades extraordinárias que me proporcionou. Obrigada!

Quero agradecer à Doutora Cristina Barros por estar sempre disponível a ajudar, pelo apoio incondicional, pelas palavras, pelo sorriso e pelo carinho. Obrigada!

Quero agradecer ao Professor Doutor Francisco Amado pelo conhecimento partilhado e pelas oportunidades proporcionadas. Obrigada!

Quero agradecer aos coautores dos trabalhos que constam desta tese e aos coautores dos restantes trabalhos realizados ao longo destes quatro anos pela colaboração, em particular à Doutora Rita Nogueira-Ferreira pela sua disponibilidade e envolvência incomparáveis. Obrigada!

Quero agradecer às unidades de investigação LAQV-REQUIMTE, CIAFEL e CITAB. Quero agradecer à FCT e MCTES pelo financiamento através da bolsa de doutoramento que possibilitou a realização desta tese. Obrigada!

Quero agradecer à Patrícia Tavares pelos cafés às oito da manhã, pelas conversas, pelo apoio e pelas aventuras. Obrigada!

Quero agradecer às minhas amigas, Sara Pinheiro e Inês Belchior pelo apoio incondicional, pelas mensagens de força e pelo amor. Obrigada!

Quero, por fim, agradecer à minha família, mãe, pai, tio e avó, pelo apoio incondicional e pelo amor. Mamã, sem ti nada seria possível. Obrigada!

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List of publications

This doctoral manuscript includes the following papers, which are here referred according to the subchapters they are included in:

Subchapter IIA Moreira-Pais, A., Ferreira, R., Oliveira, P. A., & Duarte, J. A. (2022). A neuromuscular perspective of sarcopenia pathogenesis: deciphering the signaling pathways involved. *GeroScience*, *44*(3), 1199–1213. https://doi.org/10.1007/s11357-021-00510-2

Subchapter IIB Moreira-Pais, A., Ferreira, R., Oliveira, P. A., & Duarte, J. A. (2021). Sarcopenia versus cancer cachexia: the muscle wasting continuum in healthy and diseased aging. *Biogerontology*, *22*(5), 459–477. https://doi.org/10.1007/s10522-021-09932-z

Subchapter IIC Moreira-Pais, A., Ferreira, R., Costa, V. M., Oliveira, P. A., & Duarte, J. A. (2021). The molecular mechanisms involved in doxorubicin-induced skeletal muscle wasting. *Revista Portuguesa de Cirurgia*, *51*, 13–22. https://doi.org/10.34635/rpc.930

Subchapter IIIA Moreira-Pais, A., Vitorino, R., Sousa-Mendes, C., Neuparth, M. J., Nuccio, A., Luparello, C., Attanzio, A., Novák, P., Loginov, D., Nogueira-Ferreira, R., Leite-Moreira, A., Oliveira, P. A., Ferreira, R., & Duarte, J. A. (2024). Mitochondrial remodeling underlying age-induced skeletal muscle wasting: let's talk about sex. (**submitted**)

Subchapter IIIB Moreira-Pais, A., Ferreira, R., Aires, I., Oliveira, P. A., & Duarte, J. A. (2024). Age and cancer in skeletal muscle wasting: which one to blame? (**in preparation**)

Subchapter IIIC Moreira-Pais, A., Ferreira, R., Baltazar, T., Neuparth, M. J., Vitorino, R., Reis-Mendes, A., Costa, V. M., Oliveira, P. A., & Duarte, J. A. (2024). Long-term impact of doxorubicin on skeletal muscle remodeling in aged mice. (**submitted**)

Resumo

Esta tese teve por objetivo estudar os mecanismos moleculares subjacentes à remodelação do músculo esquelético induzida pela idade, cancro e/ou quimioterapia e os mediadores envolvidos que possam suportar o diagnóstico diferencial. Com o intuito de melhor compreender a remodelação muscular associada à idade e o efeito do sexo analisou-se o proteoma de mitocôndrias isoladas do *gastrocnemius* de ratos fêmea e macho, novos e velhos. Os resultados mostraram um comprometimento da fosforilação oxidativa e um aumento da oxidação de ácidos gordos e da suscetibilidade das proteínas à oxidação associado à idade, nas mitocôndrias das fêmeas, devido, possivelmente, à diminuição dos níveis séricos de 17beta-estradiol e dos níveis do recetor de estrogénio alfa mitocondrial. Estas alterações parecem ter contribuído para a atrofia e fibrose observadas no *gastrocnemius* das fêmeas velhas. Para distinguir as bases moleculares subjacentes ao catabolismo muscular na sarcopenia e na caquexia associada ao cancro, analisou-se o *gastrocnemius* de ratos fêmea novos, adultos e velhos e de ratos fêmea adultos com cancro da mama induzido quimicamente. Os resultados evidenciaram que a inflamação (níveis séricos elevados de interleucina-6) e a diminuição dos níveis musculares do fator neurotrófico derivado do cérebro parecem contribuir exclusivamente para a perda de massa muscular induzida pela idade. As alterações moleculares a longo prazo da administração crónica de doxorrubicina foram estudadas no *soleus* de ratinhos velhos. Os resultados mostraram que a atrofia do *soleus* está associada a níveis séricos elevados de miostatina e ao aumento da carbonilação de proteínas musculares. Em conclusão, a atrofia do músculo esquelético associada ao envelhecimento envolve a disfunção da mitocôndria (de forma dependente do sexo) e da regeneração muscular associada, em parte, à inflamação sistémica, mecanismos estes que não parecem contribuir para a atrofia muscular associada ao cancro. A atrofia muscular a longo prazo promovida pela administração crónica de doxorrubicina parece decorrer da proteólise e do comprometimento da regeneração muscular.

Palavras-chave: CANCRO, DIMORFISMO SEXUAL, ENVELHECIMENTO, CATABOLISMO MUSCULAR, REMODELAÇÃO MITOCONDRIAL.

Abstract

The overarching aim of this thesis was to delve into the molecular mechanisms underlying age-, cancer- and/or chemotherapy-induced skeletal muscle remodeling and the mediators that might retain propitious clinical differential value. To better understand the age-induced skeletal muscle remodeling and the role of sex, the proteome of mitochondria-enriched fractions isolated from the *gastrocnemius* of young and old, female and male rats was analyzed. The results demonstrated an age-induced compromised oxidative phosphorylation, increased fatty acid oxidation and higher proneness of the mitochondrial proteins to oxidative modifications, possibly triggered by diminished levels of 17beta-estradiol in serum and of estrogen receptor alpha in mitochondria. These alterations appear to have contributed to the atrophy and fibrosis noticed in the *gastrocnemius* of old females. With the purpose of distinguish the molecular foundations underlying the skeletal muscle wasting that characterizes sarcopenia and cancer cachexia, the *gastrocnemius* of young, adult and old female rats, and of adult female rats with chemically-induced breast cancer was explored. The results revealed that inflammation (increased serum levels of interleukin-6) and decreased muscle levels of brain-derived neurotrophic factor seem to exclusively contribute to the loss of skeletal muscle induced by aging. The long-term molecular alterations of the chronic administration of doxorubicin were investigated in the *soleus* of old mice. The results evinced the atrophy of the *soleus* associated with elevated levels of myostatin and an increased carbonylation of muscle proteins. In conclusion, the atrophy of the skeletal muscle induced by aging involves the dysfunction of the mitochondrion (in a sex-specific manner) and of the skeletal muscle regeneration associated, in part, to the presence of systemic inflammation. These mechanisms seem not to participate in cancer-induced skeletal muscle atrophy. The long-term skeletal muscle atrophy prompted by the chronic administration of doxorubicin seems to be ensued from proteolysis and a compromised skeletal muscle regeneration.

Keywords: AGING, CANCER, MITOCHONDRIAL REMODELING, SEXUAL DIMORPHISM, SKELETAL MUSCLE WASTING.

List of abbreviations

3-NT: 3-nitrotyrosine

4-HNE: 4-hydroxynonenal

A: adult

AA: amino acid

ABCa: adult breast cancer

AC: accession number

ACh: acetylcholine

AChE: acetylcholinesterase

AChR: acetylcholine receptor

ActRIIB: activin receptor type-2B

AFG3L2: AFG3 like matrix AAA peptidase subunit 2

ALDH5A1: succinate-semialdehyde dehydrogenase

ALDH6A1: methylmalonate-semialdehyde dehydrogenase [acylating]

AMPK: 5'-adenosine monophosphate-activated protein kinase

ANOVA: analysis of variance

APOA1: apolipoprotein A1

AR: androgen receptor

ATF4: activating transcription factor 4

ATG: autophagy-related protein

ATP: adenosine triphosphate

ATP5A1: adenosine triphosphate synthase subunit alpha

ATP5B: adenosine triphosphate synthase subunit beta

ATP5E: adenosine triphosphate synthase subunit epsilon

ATP5MPL: adenosine triphosphate synthase subunit ATP5MPL

ATPB: adenosine triphosphate synthase subunit beta

BAX: Bcl-2-associated X protein

BCa: breast cancer

BDNF: brain-derived neurotrophic factor

BSA: bovine serum albumin

BW: body weight

CAF: C-terminal agrin fragment

cAMP: cyclic adenosine monophosphate

CASCO: CAchexia SCOre

CGRP: calcitonin gene-related peptide

CHOP: CCAAT-enhancer-binding protein homologous protein

CKK: cholecystokinin

CKMT2: creatine kinase S-type

CLR: calcitonin-like receptor

CoA: coenzyme A

CONT: control

COX5A: cytochrome c oxidase subunit 5A

COX5B: cytochrome c oxidase subunit 5B

COX6C2: cytochrome c oxidase subunit 6C-2

COX7A2: cytochrome c oxidase subunit 7A2

CRAT: carnitine O-acetyltransferase

CREB: cyclic adenosine monophosphate response element-binding protein

CRP: C-reactive protein

CS: citrate synthase

CSA: cross-sectional area

CSQ: calsequestrin

DEXA: dual-energy X-ray absorptiometry

DGAV: General Directorate of Food and Veterinary Medicine/Direção Geral de Alimentação e Veterinária

DHPRs: dihydropyridine receptors

DLAT: Dihydrolipoyllysine-residue acetyltransferase component of pyruvate dehydrogenase complex

DLST: Dihydrolipoyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex

DNA: deoxyribonucleic acid

DNP: dinitrophenol

DNPH: dinitrophenylhydrazine

DOK7: downstream of kinase 7

DOX: doxorubicin

DTNB: 5,5'-dithiobis-(2-nitrobenzoic acid)

ECI1: enoyl-CoA delta isomerase 1

ECL: enhanced chemiluminescence

eIF2α: eucaryotic translation initiation factor 2

ELISA: enzyme-linked immunosorbent assays

EMA: European Medicines Agency

EPA: eicosapentaenoic acid

EPP: endplate potential

ERα: estrogen receptor alpha

ERRα: estrogen-related receptor alfa

ESF: European Social Fund

ETC: electron transport chain

ETFB: electron transfer flavoprotein subunit beta

ETFDH: electron transfer flavoprotein-ubiquinone oxidoreductase

EWGSOP2: European Working Group on Sarcopenia in Older People 2

FAO: fatty acid oxidation

FCT: Foundation for Science and Technology

FDA: Food and Drug Administration

FDR: false discovery rate

FGFs: fibroblast growth factors

FKBPs: FK506-binding protein

FoxO: forkhead box, class O

FoxO1: forkhead box, class O 1

GAP-43: growth-associated protein

GDNF: glial cell line-derived neurotrophic factor

GFRα1: glial-cell-line-derived neurotrophic factor family receptor alpha 1

GLUD1: glutamate dehydrogenase 1

GLUT4: glucose transporter type 4

GO: Gene Ontology

GS: *gastrocnemius*

HADH: hydroxyacyl-coenzyme A dehydrogenase

HADHB: trifunctional enzyme subunit beta

H&E: hematoxylin and eosin

His: histidine

HSPA9: stress-70 protein

HSPE1: 10 kDa heat shock protein

ICBAS: School of Medicine and Biomedical Sciences Abel Salazar

IDH2: isocitrate dehydrogenase [nicotinamide adenine dinucleotide phosphate]

IDH3A: isocitrate dehydrogenase [nicotinamide adenine dinucleotide] subunit alpha

IGF-1: insulin-like growth factor 1

IL: interleukin

IL-6: interleukin-6

i.p.: intraperitoneal

IR: insulin receptor

IRS-1: insulin receptor substrate-1

IVD: isovaleryl-coenzyme A dehydrogenase

JAK/STAT: Janus kinase/signal transducer and activator of transcription

LC: liquid chromatography

LC3: microtubule-associated protein light chain 3

LDH: lactate dehydrogenase

LOC685596: cytochrome b-c1 complex subunit 7

LRP4: low-density lipoprotein receptor-related protein 4

Lys: lysine

MCCC1: methylcrotonoyl- coenzyme A carboxylase subunit alpha

MCTES: Ministry of Science, Technology and Higher Education

MDH2: malate dehydrogenase

MEF: mitochondria-enriched fractions

Met: methionine

MHC: myosin heavy chain

MHCe: myosin heavy chain embryonic isoform

MHCn: myosin heavy chain neonatal isoform

MnSOD: manganese superoxide dismutase

MNU: *N*-methyl-*N*-nitrosourea

MRGs: myogenic regulatory genes

MRPs: multidrug resistance proteins

MS: mass-spectrometry

MT-ATP6: mitochondrial encoded adenosine triphosphate synthase membrane subunit 6

MT-CYB: mitochondrial encoded cytochrome B

mtDNA: mitochondrial deoxyribonucleic acid

mTOR: mammalian target of rapamycin

mTORC1: mammalian target of rapamycin complex 1

mtUPR: mitochondrial unfolded protein response

MuRF1: muscle RING-finger protein-1

MuSK: muscle-specific kinase

MW: molecular weight(s)

MYF5: myogenic factor 5

MyoD: myoblast determination protein 1

nAChRα1: nicotinic acetylcholine receptor alpha 1

NADH: nicotinamide adenine dinucleotide

NCAM: (N-)neural cell adhesion molecule

NDUFA2: nicotinamide adenine dinucleotide dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 2

NDUFA7L: nicotinamide adenine dinucleotide dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 7

NDUFS7: nicotinamide adenine dinucleotide dehydrogenase [ubiquinone] iron-sulfur protein 7

NDUFV2: nicotinamide adenine dinucleotide dehydrogenase [ubiquinone] flavoprotein 2

NF-κB: nuclear factor kappa light-chain enhancer of activated B cells

NMJ: neuromuscular junction

$^{\bullet}$NO: nitric oxide

NOS: nitric oxide synthase

NOXs: nicotinamide adenine dinucleotide phosphate oxidases

NPY: neuropeptide Y

NRF: nuclear respiratory factor

NSAID: nonsteroidal anti-inflammatory drug

NTRK3: neurotrophic tyrosine kinase receptor type 3

NTs: neurotrophins

O: old

$O_2^{\bullet-}$: superoxide anion

OD: optical density

OF: old female

OM: old male

$ONOO^-$: peroxynitrite

ORBEA: Orgão Responsável pelo Bem-Estar e Ética Animal

OXPHOS: oxidative phosphorylation

p75NTR: p75 neurotrophin receptor

PCA: principal component analysis

PCCB: propionyl-coenzyme A carboxylase beta chain

PDHB: pyruvate dehydrogenase E1 component subunit beta

PFKM: 6-phosphofructokinase muscle type

PGC1α: peroxisome proliferator-activated receptor gamma coactivator 1-alpha

PGK1: phosphoglycerate kinase 1

Phe: phenylalanine

PI3K: phosphatidylinositol 3-kinase

PINK1: PTEN-induced kinase 1

PKA: protein kinase A

PLS-DA: partial least squares discriminant analysis

PM: position of the modification

p-MuSK: phosphorylated muscle-specific kinase

p-NA: p-nitroanilide

PPI: protein-protein interaction

pSMAD: phosphorylated mothers against decapentaplegic (SMAD) family member

pSMAD3: phosphorylated mothers against decapentaplegic (SMAD) homolog 3/family member 3

PTMs: posttranslational modifications

PUFA: polyunsaturated fatty acids

RNS: reactive nitrogen species

ROS: reactive oxygen species

RyR(s): ryanodine receptor(s)

TBS: Tris-buffered saline

TBST: Tris-buffered saline with Tween 20

TCA: tricarboxylic acid

TFA: trifluoroacetic acid

TFEB: transcription factor EB

TGF-β: transforming growth factor beta

TL: tibia length

TNF-α: tumor necrosis factor alpha

TNF-αR1: tumor necrosis factor alpha receptor 1

TopII: topoisomerase II

TrkB: tropomyosin-related kinase

Trp: tryptophane

TUFM: elongation factor Tu

Tyr: tyrosine

sACVR2B-Fc: soluble ligand binding domain of the activin receptor type 2B

SC(s): satellite cell(s)

SD: standard deviation

SDHB: succinate dehydrogenase [ubiquinone] iron-sulfur subunit

SERCA: sarcoplasmic/endoplasmic reticulum calcium ATPase regulator

SIRT1: sirtuin 1

SLC25A3: solute carrier family 25 member 3

SLC25A4: adenosine monophosphate/adenosine triphosphate translocase

SLC25A12: electrogenic aspartate/glutamate antiporter SLC25A12

SMAD3: mothers against decapentaplegic homolog 3

SMN: spinal motor neuron

SNAP-25: synaptosomal-associated protein of 25 kDa

SNARE: soluble N-ethylmaleimide sensitive factor attachment protein receptor

SOCS3: supressor of cytokine signaling 3

SR: sarcoplasmic reticulum

STAT3: signal transducer and activator of transcription 3

SUCLG1: succinate-CoA ligase [adenosine diphosphate/guanosine-5'-diphosphate-forming] subunit alpha, mitochondrial

UniprotKB: UniProt Knowledgebase

UPP: ubiquitin-proteasome pathway

UPS: ubiquitin proteasome system

UQCRC1: cytochrome b-c1 complex subunit 1

UQCRC2: cytochrome b-c1 complex subunit 2

UQCRQ: cytochrome b-c1 complex subunit 8

V: volume

VDAC: voltage-dependent anion-selective channel protein

VDAC2: voltage-dependent anion-selective channel protein 2

VDAC3: voltage-dependent anion-selective channel protein 3

VGCC(s): voltage-gated calcium channel(s)

XBP1: X-box binding protein 1

Y: young

YF: young female

YM: young male

Chapter I

General introduction

Population aging is the most pervasive and prevailing global demographic trend assignable to declining fertility levels and increasing life expectancy (United Nations Department of Economic and Social Affairs, 2020). Globally, adults aged 60 years or older constituted 13.5% of the population in 2021, a number that is expected to increase up to 16.0% by 2050 (Geneva: World Health Organization, 2021; United Nations Department of Economic and Social Affairs, 2020). Consequently, there is a growth of the incidence of age-related conditions, namely sarcopenia. Sarcopenia is a progressive skeletal muscle disease defined by low levels of muscle strength, muscle quantity or quality and physical performance that affects 10-27% of people aged 60 years or older (Cruz-Jentoft et al., 2019; Petermann-Rocha et al., 2022). Sarcopenia is related to an increased risk of disability and consequent hospitalization, a higher hospital stay length, an increased risk of postoperative complications, frailty, mobility limitations, and premature mortality (Coletta & Phillips, 2023). The etiology of sarcopenia is multifactorial and may involve neurological, systemic, and skeletal muscle insults (Tian et al., 2023). Regarding the neurological point of view, it is proposed an age-related remodeling of motor units where the number of motor units decreases with aging, jointly with insufficient denervation-reinnervation cycles at neuromuscular junction (NMJ) and the emergence of denervated fibers that undergo atrophy (Kwon & Yoon, 2017). Systemically, it is suggested that a chronic low-grade inflammation coupled with an impaired lipid metabolism and hormonal alterations are involved in sarcopenia pathogenesis, whereas locally in the skeletal muscle, mitochondrial dysfunction is one of the proposed causes (Tian et al., 2023). The specific role and contribution of each one of these processes and their mediators to sarcopenia development and progression are not yet completely comprehended.

Age-related neuromuscular perturbations are deemed to be a major focal point regarding sarcopenia development. As individuals age, it may occur structural alterations in the NMJ that compromise the seamless communication between the nervous and muscular systems (Arnold & Clark, 2023). It is suggested that the diminished reinnervation rate in older ages fails to adequately counterbalance the emergence of denervated fibers, possibly attributed to age-induced limitations

in enhancing reinnervation-related processes, including the neural cell adhesion molecule (NCAM) response (Gillon & Sheard, 2015). The integrity and functionality of the NMJ hinge on intricate signaling pathways involving key players that ensure the stability of both pre- and postsynaptic apparatus, as well as proper signal transmission through acetylcholine (ACh) and successful reinnervation post-denervation (Rodríguez Cruz et al., 2020). Signaling involving agrin, ACh, and neurotrophins (such as brain-derived neurotrophic factor (BDNF) and glial cell-derived neurotrophic factor (GDNF)) seems to become impaired with aging, leading to a compromised neuromuscular transmission (Dobrowolny et al., 2021; Gonzalez-Freire et al., 2014). Furthermore, investigations point out that the function of satellite cells (SCs) and of proteins crucial in the reinnervation process, notably calcitonin gene-related peptide (CGRP) and growth-associated protein-43 (GAP-43), faces challenges during the aging process, contributing to the accumulation of denervated muscle fibers that will potentially atrophy (Blasco et al., 2020; Huo et al., 2022). Still, the relative contribution of NMJ dysfunction to sarcopenia pathogenesis remains elusive, posing a compelling question that demands thorough exploration to advance our understanding and enrich strategies for the management of sarcopenia.

Along with a compromised NMJ function, mitochondrial dysfunction is advocated as one of the central factors driving skeletal muscle aging with a growing body of evidence provided by both animal and human studies, as reviewed elsewhere (Bellanti et al., 2021). In skeletal muscle, the mitochondrion plays a key role in the regulation of numerous cellular processes involved in energy supply, cellular proteostasis, reactive oxygen species (ROS) production, calcium homeostasis, and cell apoptosis (Tian et al., 2023). It is believed that during skeletal muscle aging, the mitochondrion might suffer an adverse remodeling that comprises an impaired uptake and utilization of glucose, fat and protein, a defective production of energy and a shift in the redox status towards oxidation, which is accompanied by overloaded or dysfunctional antioxidant defense mechanisms, augmenting the susceptible of cellular and subcellular environments to damage (Bellanti et al., 2021; Ferri et al., 2020; Hood et al., 2019). Coupled with mitochondrial remodeling, the age-related decrease in sex steroid hormones in both women

and men has been suggested as an important driver of sarcopenia (Geraci et al., 2021; Shigehara et al., 2022), despite the limited number of studies on this topic. Placing these facts together, it is not a surprise that steroid sex hormones seem to regulate mitochondrial biogenesis, dynamics, and autophagy to maintain mitochondrial function in skeletal muscle (Tian et al., 2023). Consequently, skeletal muscle mitochondrion might present sex specificities in its performance. For example, it was suggested that the function of the oxidative phosphorylation (OXPHOS) complexes is lower in old females than in old males (Kramer et al., 2023; Triolo et al., 2022). It was also proposed that skeletal muscle aging involves similar mitochondrial-related processes in both sexes but that the magnitude of the differential expression of these processes in old versus young subjects is sex specific (de Jong et al., 2023). Nonetheless, the study of sex differences in the mitochondrion is in its infancy with paradoxical data being frequently reported and with most of the studies evaluating young or adult individuals, leaving a gap in sarcopenia knowledge. Undoubtedly, further investigations focused on age-induced mitochondrial remodeling would shed light on sarcopenia pathogenesis, principally if sex dissimilarities are also approached, paving the way to the elaboration of more successful preventive and therapeutic interventions tailored to the sex of each patient, expanding their chances of survival and quality of life.

Sarcopenia is frequently misperceived with cancer cachexia, contributing to their misdiagnosis, which is aggravated by the co-presentation of sarcopenia in 15-50% of all cancer patients (Peterson & Mozer, 2017). Cachexia is a syndrome defined by the loss of body weight (more than 5% in six months or over 2% in individuals with a body mass index below 20 $kg.m^2$) and skeletal muscle mass with or without adipose tissue loss that cannot be entirely reversed by conventional nutritional support (Setiawan et al., 2023). It occurs in consequence of an underlying illness, such as cancer (termed “cancer cachexia”) (Setiawan et al., 2023). It affects 25%-80% of all cancer patients (Aoyagi et al., 2015; Peterson & Mozer, 2017) and is responsible for more than 20% of cancer-related deaths (Porporato, 2016). At older ages, cancer cachexia can affect up to 65% of cancer patients older than 65 years of age and is significantly related with poorer overall survival (vs. non-cachectic cancer patients) (Dunne et al., 2019). Skeletal muscle

wasting is a significant feature of cancer cachexia, and its severity and phenotypic presentation might diverge depending on tumor- and treatment-related factors and succeeding systemic inflammation. These factors instigate skeletal muscle remodeling by ensuing mitochondrial dysfunction, oxidative stress, and muscle protein turnover towards increased protein breakdown (Fairman et al., 2022). The intricate and complex nature of these multifaceted processes makes the management of this syndrome fiendishly difficult. Furthermore, the accelerated skeletal muscle loss substantially contributes to an increased risk of cancer-related and all-cause mortality and to a decreased quality of life and physical function (Fairman et al., 2022). Despite cancer cachexia and sarcopenia sharing some pathophysiological mechanisms, an amplified inflammation is believed to have a higher contribution to cancer cachexia and the neuromuscular impairment to sarcopenia. To date, however, these represent only theoretical hypothesis since the data is limited and often inconsistent. The dissimilarities between these two conditions commence on the apparent differences in the remodeling of the skeletal muscle phenotype but studies on this field in cancer cachexia are very limited, which hampers the draw of conclusions. Still, during aging type II muscle fibers seem to be more susceptible to denervation, atrophy and loss, while in cancer cachexia information dictates that type I muscle fibers are preferentially lost or no changes in skeletal muscle innervation occurs (Dowling et al., 2023; Martin & Freyssenet, 2021). Despite this being a possible factor for the differential diagnosis, the non-representative nature of skeletal muscle biopsies of the whole muscle fiber type distribution and the risk of complications especially in more vulnerable cohorts that can contraindicate its performance (Goryachev et al., 2022; Wilson et al., 2018), emphasizes the magnitude of unravel protein markers that can express more reliably the skeletal muscle adaptations, such as the ones involved in the neuromuscular system, which as discussed above, it is believed to be swayed by age. In cancer cachexia, the reduced information available raises more questions than answers since it dictates either a stable NMJ morphology as the presence of central myonuclei and upregulation of NCAM, which suggests NMJ impairment, motor neuron loss and/or the occurrence of reinnervation (Boehm et al., 2020; Daou et al., 2020). An altered ACh signaling

might also be involved in cancer cachexia (Brown et al., 2018). Still in this context, neurotrophins like BNDF seem not to be modulated in cancer cachexia (de Castro et al., 2020), hence variations in their levels are very promising for the differential diagnosis of sarcopenia. Nevertheless, there is, noticeably, a void in the knowledge regarding the involvement of the neuromuscular environment in the development of cancer cachexia that deserves to be fulfilled. Notwithstanding the presence of a chronic inflammatory state in both sarcopenia and cancer cachexia, the more prominent increase in the levels of circulating pro-inflammatory mediators appears to highly contribute to skeletal muscle wasting in cancer cachexia. This hypothesis, however, relies more on the data from independent studies than from studies that simultaneously investigated both conditions (either pre- or clinically), which might bias the conclusions obtained. Either the acute-phase protein C-reactive protein (CRP) or the pro-inflammatory cytokines interleukin (IL)-6 and tumor necrosis factor alpha (TNF-α) are reported to be elevated in both conditions and to correlate with skeletal muscle atrophy and function impairment (Pan et al., 2021; Siddiqui et al., 2020). The activation of the Janus kinase/signal transducer and activator of transcription (JAK/STAT) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling by IL-6 and TNF-α, respectively, triggers the ubiquitin-proteasome pathway (UPP) and inhibits the anabolic Akt/mammalian target of rapamycin (mTOR) signaling, promoting skeletal muscle wasting (Bowen et al., 2015). The dearth of studies delved into the simultaneously breakdown of the contribution of inflammation to age- and cancer-related skeletal muscle wasting obstructs the assessment of condition-specific cut-off values to succor the differential diagnosis or to recognize individuals at risk.

Besides cancer *per se*, it is also proposed that chemotherapeutic agents negatively affect the skeletal muscle (Campelj et al., 2021). However, this constitutes an overlooking adverse effect in existent studies. One of the most widely used anticancer drugs is doxorubicin (DOX), which has been associated with dose-dependent acute and chronic myotoxicity (Hiensch et al., 2020; van der Zanden et al., 2020). This association derives mostly from the clinical manifestations reported by patients and not from studies dedicated to the

mechanisms accountable for the effects of DOX on skeletal muscle. DOX acts on cancer cells by intercalating into deoxyribonucleic acid (DNA) and inhibiting topoisomerase-II, and by exacerbating ROS production, which leads to oxidative stress, lipid peroxidation, damage to cellular membranes and DNA, and activation of cell death pathways (Thorn et al., 2011). In skeletal muscle, the limited number of studies indicates that DOX-induced toxicity results in a hasty atrophy of both limb and respiratory muscles, exercise intolerance, muscle fatigue and muscle weakness (Powers et al., 2019; Smuder, 2019). The skeletal muscle weakness and fatigue frequently endure after termination of the treatment and can ensue acutely or within the years after the therapy ended (Gilliam & St. Clair, 2011; Huertas et al., 2020; Neidhart et al., 1986). The mechanisms encompassed in DOX-induced skeletal muscle toxicity remain to be fully uncovered, particularly the ones that promote the long-term effects of DOX in this organ. The knowledge is even scarcer pertaining to the effects of DOX in older skeletal muscle, which might already be impaired by aging, contributing to the complex management of the eldest cancer patients (Rao & Cohen, 2004). In addition, the animal protocols implemented in the preclinical studies focused on this subject usually do not mimic human therapy, given the acute administration of the drug to the animals, as well as their young ages that do not represent the human reality of cancer patients. The furtherance of the clinical management of both younger and older cancer patients towards improved quality of life and survival rates will be achievable if the mechanisms responsible for cancer- and chemotherapy-induced skeletal muscle wasting are uncovered.

Cogitating the review of the literature performed, one major question emerges: “How to explain muscle loss in the context of aging, cancer and/or chemotherapy?”. Hence, the overarching aim of this thesis was to delve into the molecular players involved in age-, cancer- or chemotherapy-induced skeletal muscle remodeling to add insights into the distinct skeletal muscle phenotypes behind each condition and to identify mediators that might hold promising clinical differential value. To attain this aim, specific purposes were delineated:

i) to elucidate the sex-specific molecular phenotype of age-induced skeletal muscle wasting with a specific focus on mitochondrial remodeling and the influence of sex hormone signaling on these alterations;

ii) to assess the role of NMJ remodeling and inflammation in the distinction between age- and cancer-induced skeletal muscle wasting;

iii) to evaluate the long-term effects of the chemotherapeutic agent DOX on skeletal muscle remodeling focusing on players of metabolism, proteolysis, apoptosis, oxidative stress and NMJ remodeling.

To accomplish these goals, preclinical models were implemented, and several experimental approaches were performed, ranging from histology to proteomics. With this research we intended to bring to light some cues about the players behind age-, cancer- and chemotherapy-related skeletal muscle wasting accompanied by conceivable biomarkers and therapeutic targets specific to each condition.

Thereunto, this doctoral manuscript was organized in five chapters (**Figure 1**).

In **Chapter I** (***General introduction***), a general and integrative introduction is presented along with the aims and organization of this manuscript.

In **Chapter II** (***State of the art***), the state of the art concerning the age-, cancer- and DOX-induced skeletal muscle remodeling is discussed. Specifically, **Subchapter IIA** (***A neuromuscular perspective of sarcopenia pathogenesis: deciphering the signaling pathways involved***) pretended to gather data pertained to the not properly comprehended age-related neuromuscular remodeling that might contribute to the development of sarcopenia. The focus was on elucidating the molecular players responsible for the regulation of NMJ function and examining how they are influenced by the aging process. The critical analysis conducted in this subchapter not only underscores the pivotal role of NMJ-related factors in sarcopenia pathogenesis but also highlights the potential diagnostic value of mediators like C-terminal agrin fragment (CAF), BDNF, and CGRP that merit consideration in future investigations involving older individuals.

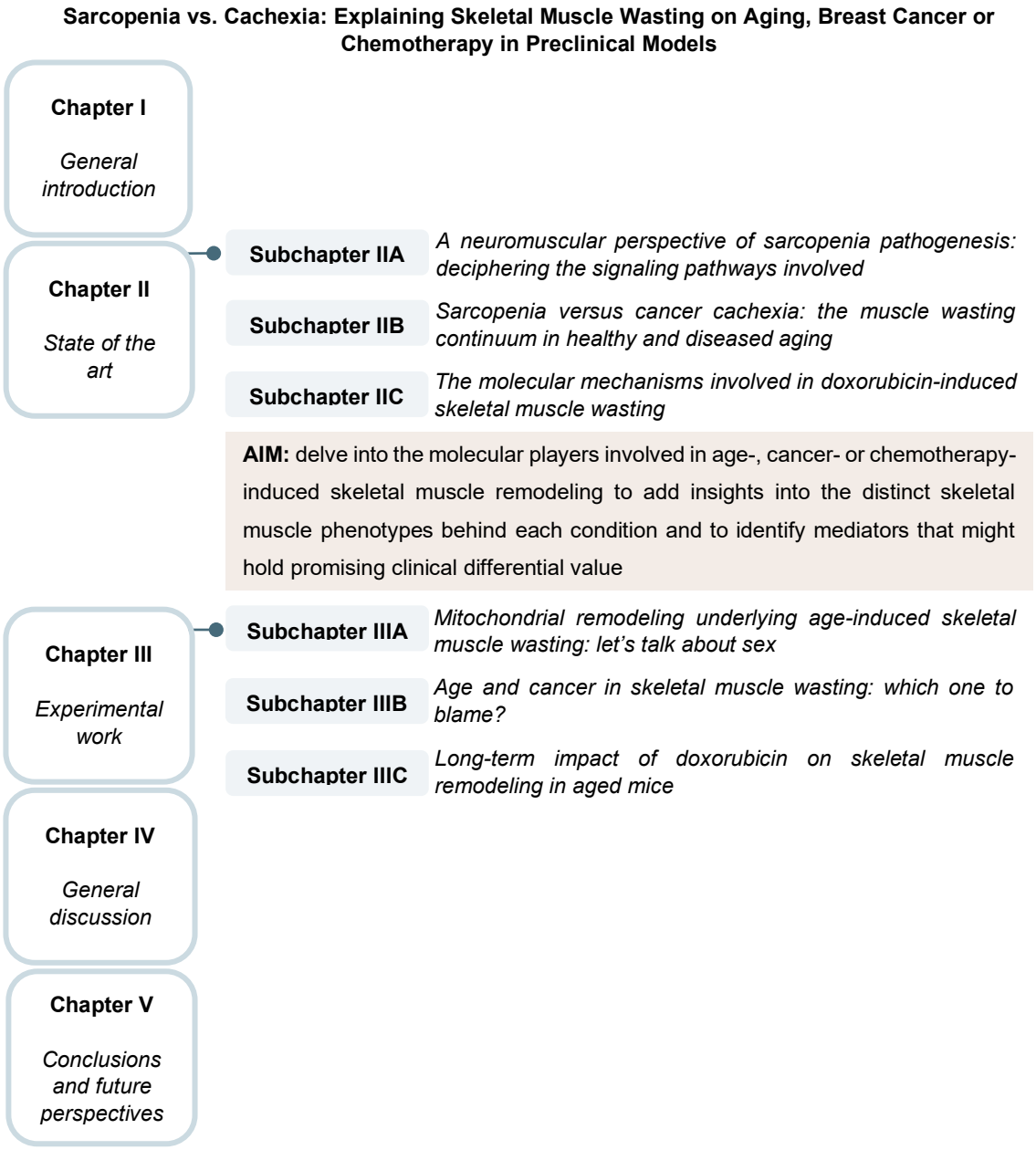

Figure 1 Schematic representation of the organization of this doctoral manuscript.

Subchapter IIB (***Sarcopenia versus cancer cachexia: the muscle wasting continuum in healthy and diseased aging***) critically compared the mediators involved in the shared processes that are believed to participate in the pathogenesis of both sarcopenia and cancer cachexia with the intention of recognize the dissimilar modulations. The literature review undertaken in this subchapter has brought to light several intriguing insights. These include the distinctive skeletal muscle phenotype remodeling that occurs in sarcopenia and

cancer cachexia, as well as the involvement of factors, such as NMJ remodeling, skeletal muscle disuse, systemic inflammation, and nutrition in their development. Despite these findings, the precise magnitude of the contribution of each factor to the development of each condition still lacks a comprehensive understanding. In **Subchapter IIC** (***The molecular mechanisms involved in doxorubicin-induced skeletal muscle wasting***), the mechanisms possibly involved in the overlooked DOX-induced myotoxicity were explored. This chapter underlines the significant role of the proteolytic UPP in the context of DOX-induced skeletal muscle wasting, a process that may be triggered by myostatin and TNF-α signaling pathways, along with insulin resistance. Furthermore, heightened oxidative stress and mitochondrial dysfunction emerge as potential key drivers for DOX-associated skeletal muscle wasting. It is important to note, however, that the majority of the evidence from the literature is derived from preclinical studies, often involving adult and healthy animals. Consequently, these findings may not fully capture the intricate interplay between cancer and/or aging and the impact of DOX on skeletal muscle wasting.

In **Chapter III** (***Experimental work***), the elaborated experimental studies are presented, including the methods used, the results obtained and its discussion. Particularly, in ***Subchapter IIIA*** (***Mitochondrial remodeling underlying age-induced skeletal muscle wasting: let's talk about sex***), the contribution of mitochondrial remodeling to age-induced skeletal muscle atrophy along with the susceptibility of muscle mitochondrial proteins to posttranslational modifications were investigated with a sex viewpoint using proteomics approaches towards the uncover of the degree of mitochondrial dysfunction in skeletal muscle aging and to acknowledge the involvement sex specificities with the objective of an improved and individualized sarcopenia management. **Subchapter IIIB** (***Age and cancer in skeletal muscle wasting: which one to blame?***) was dedicated to the investigation of inflammatory and NMJ-related mediators with the intention of exposing the specific role of inflammation and neuromuscular environment to the pathogenesis of sarcopenia and cancer cachexia, and putative markers that might be attractive to the clinical differential diagnosis. In **Subchapter IIIC** (***Long-term impact of doxorubicin on skeletal muscle remodeling in aged mice***),

the long-term effects of the chronic administration of DOX were examined raging from systemic to skeletal muscle alterations with the purpose of disclose the mechanisms behind the ill-defined DOX-induced myotoxicity in aged skeletal muscle.

In **Chapter IV** (***General discussion***) a general and integrative discussion of the data obtained in the experimental studies was assembled, which was followed by **Chapter V** (***Conclusions and future perspectives***) where the main conclusions and future perspectives are reported.

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Chapter II

State of the art

Chapter II

Subchapter IIA

A neuromuscular perspective of sarcopenia pathogenesis: deciphering the signaling pathways involved

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Moreira-Pais, A., Ferreira, R., Oliveira, P. A., & Duarte, J. A. (2022). A neuromuscular perspective of sarcopenia pathogenesis: deciphering the signaling pathways involved. *GeroScience*, *44*(3), 1199–1213. https://doi.org/10.1007/s11357-021-00510-2

GeroScience (2022) 44:1199–1213
https://doi.org/10.1007/s11357-021-00510-2

ORIGINAL ARTICLE

A neuromuscular perspective of sarcopenia pathogenesis: deciphering the signaling pathways involved

Alexandra Moreira-Pais · Rita Ferreira · Paula A. Oliveira · José A. Duarte

Received: 30 September 2021 / Accepted: 26 December 2021 / Published online: 4 January 2022
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Abstract The escalation of life expectancy is accompanied by an increase in the prevalence of age-related conditions, such as sarcopenia. Sarcopenia, a muscle condition defined by low muscle strength, muscle quality or quantity, and physical performance, has a high prevalence among the elderly and is associated to increased mortality. The neuromuscular system has been emerging as a key contributor to sarcopenia pathogenesis. Indeed, the age-related degeneration of the neuromuscular junction (NMJ) function and structure may contribute to the loss of muscle strength and ultimately to the loss of muscle mass that characterize sarcopenia. The present mini-review discusses important signaling pathways involved in the function and maintenance of the NMJ, giving emphasis to the ones that might contribute to sarcopenia pathogenesis. Some conceivable biomarkers, such as C-terminal agrin fragment (CAF) and brain-derived neurotrophic factor (BDNF), and therapeutic targets, namely acetylcholine and calcitonin gene–related peptide (CGRP), can be retrieved, making way to future studies to validate their clinical use.

Keywords BDNF · CAF · Denervation · Muscle wasting · Neuromuscular junction · Neurotrophins

A. Moreira-Pais · J. Duarte
CIAFEL, Faculty of Sport, University of Porto, Dr. Plácido da Costa 91, 4200-450 Porto, Portugal

A. Moreira-Pais (✉) · R. Ferreira
LAQV-REQUIMTE, Department of Chemistry, University of Aveiro, 3810-193 Aveiro, Portugal
e-mail: alexandrapais@ua.pt

A. Moreira-Pais · P. Oliveira
Centre for Research and Technology of Agro Environmental and Biological Sciences (CITAB), Inov4Agro, University of Trás-Os-Montes and Alto Douro (UTAD), Quinta de Prados, 5000-801 Vila Real, Portugal

J. Duarte
TOXRUN – Toxicology Research Unit, University Institute of Health Sciences, CESPU, CRL, Gandra, Portugal

Introduction

The global population is aging, with about 13.5% being 60 years of age or older in 2021, a number that is expected to increase during the next decades [1]. This rise in longevity exposes age-related conditions, such as sarcopenia. Sarcopenia is a progressive and generalized skeletal muscle disease defined by low muscle strength, muscle quantity or quality, and physical performance [2]. It affects 5–13% of people between 60 and 70 years old, and 11–50% of the adults older than 80 years [3–5]. In addition, sarcopenic subjects have a higher mortality risk than non-sarcopenic ones, with this risk being greater in people older than 79 years [6]. Sarcopenia etiology is multifactorial, with many systemic factors being related to its development [7, 8]; however, the local neuromuscular environment is emerging as an important contributor to sarcopenia pathogenesis [9].

Voluntary movement and the maintenance of muscle mass and strength require an efficient communication between the nervous and muscular systems through a normal innervation [10]. Age-related changes in the neuromuscular system manifest functionally as loss of muscle strength and coordination, which precedes the loss of muscle mass [11]. Indeed, in older people, the loss of muscle strength is significantly more rapid than the loss of muscle mass, and maintaining or gaining muscle mass does not avert the aging-related decrease in muscle strength [12]. More specifically, after 75 years, strength is lost at a rate of 2.5–3% *per* year in women and 3–4% in men, while muscle mass is lost at a rate of 0.64–0.70% *per* year in women and 0.80–0.98% in men [13]. The early identification of the age-related impairment of the neuromuscular system will create the opportunity to clinically intervene and to avoid the irreversible loss of muscle mass and related negative health outcomes [2, 14], envisioning the improvement of the clinical management of sarcopenic subjects.

This mini-review discusses the current knowledge on key neuromuscular system–related mechanisms and players that may be responsible for the age-related impairment of the neuromuscular junction (NMJ), and so, that may contribute to the loss of muscle strength and mass with aging. The reader, however, should keep in mind that most of the available data on this topic come from experiments in animal models given the invasiveness underlying skeletal muscle collection, and that among the available clinical studies, most of them enrolled healthy older adults. Regarding the data provided from human muscle biopsies, it is important to be aware that it is difficult to obtain several samples *per* subject [15], and one tissue sample represents less than 0.01% of the whole muscle and contains only about hundreds of fibers of even less motor units. This means that the data obtained from biochemical, histochemical, and morphometric analyses may not be representative of the whole muscle [16]. This mini-review hopes to make way to future studies, aiming to identify biomarkers and therapeutic targets of sarcopenia.

Neuromuscular junction aging

The NMJ is a highly specialized synapse that ensures the efficient transmission of electric impulses from the presynaptic innervating motoneuron to the postsynaptic innervated muscle fibers to stimulate contraction. Briefly, when the motor nerve action potential arrives, calcium enters the presynaptic terminal. Consequently, acetylcholine (ACh) is released from their synaptic vesicles into the synaptic cleft through the presynaptic active zones [17, 18]. ACh binds to its receptors (AChRs), which are tightly clustered in the postsynaptic membrane of the muscle fiber (also known as motor endplate), depolarizing it, which initiates the muscle action potential, resulting in muscle contraction [19]. Besides ACh, other mediators, namely neurotrophins (NTs), agrin, and calcitonin gene–related peptide (CGRP), are also released by the motoneuron. On another hand, signals derived from the skeletal muscle influence neuron survival, axonal growth, and maintenance of synaptic connections [20]. This bidirectional communication is vital for the survival of both muscle and nerve and for their mechanism of action [20]. Since both presynaptic and postsynaptic mediators are fundamental to the function of the neuromuscular system, the functional impairment of the NMJ may be a causative factor to the age-related loss of muscle strength [21, 22]. Nonetheless, there is a slight chance that the opposite, i.e., the sarcopenia-associated alterations (e.g., inactivity), may promote maladaptive changes in the NMJ.

Age-related morphological/structural changes

Classic morphological features of NMJ impairment, like significant thinning of axons, increased number of sprouting axons and AChR clusters, lower density of postsynaptic AChRs, and synaptic detachment and fragmentation of the postsynaptic apparatus, were observed in aged *extensor digitorum longus* (30 months) [23] and *tibialis anterior* (24 months) of mice [24] (Fig. 1). Comparative to rodents (mice and rats), human NMJs are significantly smaller and more fragmented, with smaller axon diameter and average area of AChR clusters [25]. In some human studies [26, 27], the age-related features of NMJs observed were similar to those found in animals. For instance, in intercostal muscles (4–77 years), postsynapses were fragmented with increased length and degeneration of the junctional folds. Moreover, a higher number of smaller conglomerates of AChRs were found in the postsynaptic side, suggesting a possible degeneration of the NMJs and muscle fibers. Nonetheless, a recent study

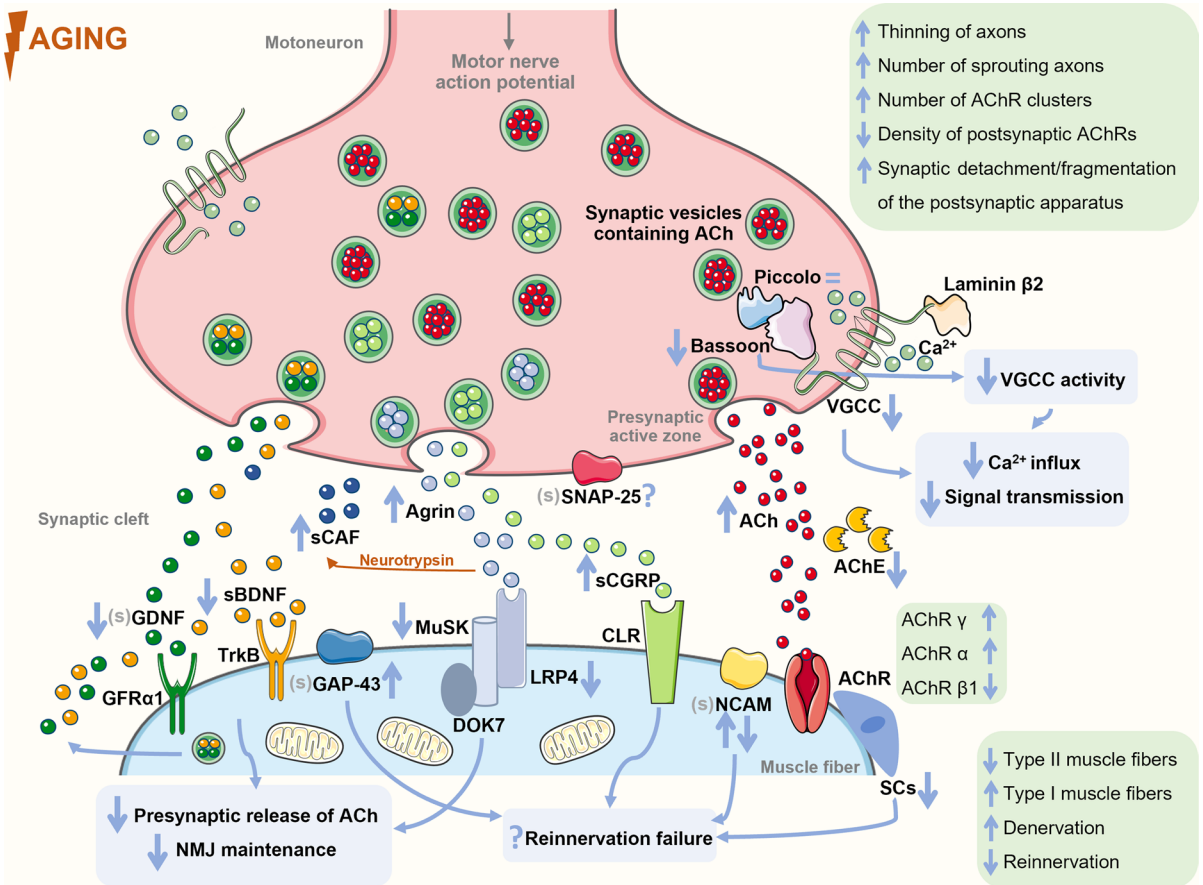

Fig. 1 The age-induced alterations that seem to preclude sarcopenia. Changes in the levels of key neuromuscular system–related mediators associated to the impairment of the neuromuscular junction function and morphology are highlighted as well as alterations in signal transmission between the motoneurons and muscle fibers, and of the reinnervation process that culminate in impaired muscle function. The mediators analyzed in the serum in aging or sarcopenia contexts are preceded by "s", while the ones not analyzed in these contexts (but in other conditions) are preceded by "(s)". Figure produced with *Servier Medical Art*. Abbreviations: ACh: acetylcholine; AChE: acetylcholinesterase; AChR: acetylcholine receptor; BDNF: brain-derived neurotrophic factor; CAF: C-terminal agrin fragment; CGRP: calcitonin gene–related protein; CLR: calcitonin-like receptor; DOK7: downstream of kinase 7; GAP-43: growth-associated protein; GDNF: glial-cell-line-derived neurotrophic factor; GFRα1: glial-cell-line-derived neurotrophic factor family receptor alpha 1; LRP4: low-density lipoprotein receptor–related protein 4; MuSK: muscle-specific kinase; NCAM: neural cell adhesion molecule; SCs: satellite cells; SNAP-25: synaptosomal-associated protein of 25 kDa; TrkB: tropomyosin-related kinase; VGCC: voltage-gated calcium channel

challenged these findings by declaring that human NMJs remain remarkably stable across the adult lifespan (34–92 years) [25]. The disparity between these studies may be explained by, for instance, different methodologies used and the analysis of fresh versus postmortem tissue. Clearly, further studies are needed to better understand human NMJ age-related plasticity.

With chronological aging is observed a process of remodeling of motor units, resulting mostly in denervation of type II muscle fibers (fast-twitch and glycolytic) that tend to be reinnervated by small neurons that innervate type I motor units [21, 28–31]. This remodeling results in the co-expression of multiple myosin heavy chain (MHC) isoforms and large fiber type clustering, i.e., an unusual number of adjacent fibers having the same fiber type [21, 32]. Since type II fibers are the ones capable of generating higher maximum force levels [33], changes in motor

unit composition may be the main cause responsible for the loss of muscle strength that is reported in adults over 55 years [29], as shown in [34].

When the denervation rate outpaces the reinnervation one, due, for instance, to the age-related impairment of the reinnervation process, the resulting denervated muscle fibers [11] will atrophy [28] and be lost [11]. Some examples of this impairment were reported. On one hand, aging muscle has a blunted neural cell adhesion molecule (NCAM) response to denervation. This molecule, which is nearly absent in adult muscles [35], reappears in the extrasynaptic region of the adult muscle fibers in response to denervation to help in the reinnervation process [35–37]. For instance, the partial denervation of the *extensor digitorum longus* of aged mice (22–30 months) did not produce a NCAM response, while in young mice (2 months), an enhanced NCAM response was observed [37]. On another hand, in older humans (65–94 years), the *vastus lateralis* had small muscle fibers co-stained with NCAM and MHC neonatal isoform (MHCn) [38]. These small fibers may be long-term denervated fibers that have atrophied over time and reverted to an immature MHC configuration. In fact, it has been suggested that the presence of either MHCn or embryonic (MHCe) isoforms indicates denervated muscle fibers [38, 39]. In aged rat *soleus* (30 months), the number of MHCe-positive fibers increased with the degree of sarcopenia, so being associated with a poor outcome [39].

The maintenance and function of the NMJs involve highly synchronized and fine-tuned actions of, for instance, (i) mediators involved in the organization of presynaptic active zones, fundamental for ACh release [40]; (ii) presynaptic mediators, such as agrin and ACh, and of their postsynaptic receptors and downstream proteins [22]; and (iii) neurotrophins [41]. Hence, the disruption of the action of any of the mediators involved in these processes may play a role in age-related neuromuscular dysfunction, and therefore, in sarcopenia.

Age-related changes in presynaptic signaling

Active zones

Upon arrival of an action potential, the synaptic transmission at the adult NMJs initiates by the opening of P/Q-type voltage-gated calcium channels (VGCCs) in the active zones, causing local calcium influx, fusion of the synaptic vesicles with the presynaptic membrane, and the release of the neurotransmitter ACh into the synaptic cleft [42, 43]. Presynaptic active zones are sites of accumulation and release of synaptic vesicles, and harbor specialized multidomain scaffold proteins, such as Bassoon, CAST/Erc2, Munc13, Piccolo, and Rim1 [19, 44]. These proteins seem to function as structural organizers of the active zone and as regulators of the exocytosis of ACh, being also involved in the recruitment of VGCCs [45, 46]. Specifically, Bassoon and Piccolo have been implicated in the local regulation of protein ubiquitination and proteasome-mediated proteolysis at presynapse, which may contribute to short-term presynapse plasticity [46]. In addition, these two scaffold proteins seem to connect presynaptic activity to neuronal gene expression reprogramming, potentially contributing to long-term alterations of the presynaptic function. This demonstrates the importance of the regulation of the levels of active zone proteins to ensure a normal NMJ function. The organization and maintenance of NMJ active zones rely on cues provided by the synaptic basal lamina, a layer of extracellular matrix placed at the synaptic cleft [43]. A key component of this lamina is laminin-β2 that binds to the presynaptic receptor P/Q-type VGCC, which is concentrated at the presynaptic terminus, initiating active zone assembly [47]. The VGCC β1b- or β4-subunit binds to the cytosolic active zone protein Bassoon or CAST/Erc2 [44].

Changes in the levels of these active zone proteins were observed during aging (Fig. 1). For instance, in old rodents (24 and 29 months old), the levels of P/Q-type VGCC and Bassoon were significantly lower than in adult ones [42, 48]. The low levels of Bassoon led to an attenuation of P/Q-type VGCC activity, and consequently, to an attenuation of calcium influx, signal transmission, and muscle contraction [42] (Table 1). The decrease of these levels seems to precede denervation, since the presence of the neuronal proteins Bassoon and Piccolo in aged NMJs indicates that nerve terminals are present [48]. Indeed, Bassoon levels were decreased at innervated NMJs (of *sternomastoid*) of old mice (27 months), while in denervated ones, a complete absence of Bassoon was observed [40]. Interestingly, the levels of the protein Piccolo were not decreased [48], suggesting a selective reduction of active zone proteins during aging. The correct function of both Bassoon and

Table 1 Effect of aging on key neuromuscular junction (NMJ) mediators and the possible outcomes in the NMJ that may contribute to sarcopenia

Mediator	Effect of aging	Possible outcomes in NMJ	References
		Presynaptic signaling	
P/Q-type VGCC	↓	• ↓ P/Q-type VGCC activity • ↓ Calcium influx	[48]
Bassoon	↓	• ↓ Signal transmission • ↓ Muscle contraction	[40, 42, 48]
ACh	↑	• NMJ fragmentation • Denervation • Axon sprouting	[58]
Agrin	↑	• NMJ fragmentation • Nerve terminal branching • Denervation • Smaller AChRs clusters	[77]
LRP4 p-MuSK	↓ ↓	• ↓ Agrin-LRP4-MuSK signaling	[68]
BDNF (serum)	↓	• Denervation (?) • ↓ ACh release (?) • ↓ Synapse maintenance (?)	[89–91]
BDNF (mRNA)	=	• ————————	[67]
GDNF (mRNA) GDNF	↓ =/↓	• ↓ ACh release (?) • ↓ Postsynaptic maintenance (?)	[95]
		Postsynaptic signaling	
CGRP	↑	• ↓ Reinnervation (?)	[77]
GAP-43	↑		[77, 113]

ACh, acetylcholine; *AChRs*, acetylcholine receptors; *BDNF*, brain-derived neurotrophic factor; *CGRP*, calcitonin gene–related protein; *GAP-43*, growth-associated protein; *GDNF*, glial-cell-line-derived neurotrophic factor; *LRP4*, low-density lipoprotein receptor–related protein 4; *p-MuSK*, phosphorylated muscle-specific kinase; *VGCC*, voltage-gated calcium channel

Piccolo seems to be supported by integrin-α3, a key adhesion receptor of the presynaptic active zone [43]. The NMJs of integrin-α3-knockout mice presented a reduction of the localization of Bassoon and Piccolo and typical structural changes that have been related with aging, namely fragmentation, less AChRs clusters, terminal nerve sprouting, and excessive axon branching, which can result in a deficient synaptic transmission [43]. Therefore, integrin-α3 may contribute to NMJ dysfunction and consequent denervation in aging, but the

effects of aging on presynaptic active zone proteins in humans are still unknown.

Another fundamental active zone protein is the synaptosomal-associated protein of 25 kDa (SNAP-25), a component of the soluble N-ethylmaleimide sensitive factor attachment protein receptor (SNARE) protein complex. SNAP-25 is presented in motor nerve endings, being responsible for the fusion of ACh-containing vesicles with the plasma membrane during synaptic transmission [49, 50]. Moreover, SNAP-25 also inhibits VGCC function, controlling calcium responsiveness to depolarization [51, 52]. However, the effects of aging on the levels of SNAP-25 in the NMJ are still unknown. Despite this, some studies suggest that SNAP-25 may be a target of reactive oxygen species (ROS), which leads to dysfunction in neurotransmitter release [53, 54]. Therefore, it is plausible to think that the age-related increase in oxidative stress [55, 56] may be involved, at some extent, in the deficient synaptic transmission observed in sarcopenia. It would be important to investigate this possible association.

Acetylcholine signaling

The principal mediator to NMJ function is ACh, which activates the AChRs located in the postsynaptic membrane, allowing sodium ions to enter the muscle and the generation of the endplate potential (EPP). If the EPP reaches the threshold, the sodium channels located in the borders of the motor endplate open, allowing a higher influx of positive ions, initiating the muscle action potential, which spreads in a wave-like effect throughout the sarcolemma [17, 18]. The action potential activates the voltage-gated dihydropyridine receptors (DHPRs) in the t-tubule membrane, and by induction, the ryanodine receptors (RyRs), releasing calcium from the sarcoplasmic reticulum and culminating in force production and muscle contraction [57]. It was observed an increase in the basal levels of ACh in the *gastrocnemius* of old mice (28 months vs. 8 weeks), which may indicate a compensation for decreased NMJ signals, i.e., denervation [58] (Table 1). Moreover, the mRNA levels of acetylcholinesterase, the enzyme that catalyzes the breakdown of ACh, was decreased in these animals, which might have contributed to the ACh levels observed [58]. In line with these results, it was demonstrated that increased levels of ACh at the synaptic cleft prematurely result in age-related structural alterations in the NMJs, including fragmentation, denervation, and axon sprouting, prior to muscle atrophy [59] (Table 1), which may indicate that ACh modulates the NMJ in a quantity-dependent manner. In fact, moderately reducing ACh levels promoted several positive effects on aged NMJs (17 months mice) and muscle fibers, such as an increase in NMJ area, muscle fiber cross-sectional area (CSA), and in the number of satellite cells (SCs) [60]. Therefore, moderately reducing ACh may constitute a therapeutic strategy to sarcopenia, as already suggested by [60].

The AChR in adult skeletal muscle forms a heteropentamer that harbors two α, one β, one δ, and one ε subunit (the ε subunit in adult AChR replaces the γ subunit in fetal AChR) [61]. These subunits also suffer changes in their expression during aging [62]. For instance, in the *vastus lateralis* of older women (71–78 years) was observed a robust increase of AChR γ mRNA expression and a decrease of AChR β1, compared to young women (20–28 years) [63]. These older women had a great amount of denervated fibers [63], which may explain the increase in the γ subunit, since its levels increase in response to denervation and neurotransmitter blockage [63, 64]. In another study, however, age (65–94 years) was negatively associated with AChR γ mRNA in the *vastus lateralis*, but existed a large interindividual variation in the mRNA levels of the participants of this study [38]. Human muscle biopsies may be accompanied by variability in the levels of the studied mediators due to, for instance, sampling site selection [65]. Thus, preclinical studies were considered. The mRNA levels of AChR α and γ were increased in the *gastrocnemius* (24 months vs. 4 months; 28 months vs. 8 weeks) [58, 66] and *vastus lateralis* (35 months vs. 8 months) of aged rodents [67], as well as the protein levels of AChR δ on the *diaphragm* of old mice (24 months vs. 8 months) [68]. However, no differences were found in protein levels of AChR α, β, and ε subunits in the muscles of aged animals compared to young ones [66, 68]. It was also suggested that the simple activation of AChRs by the ACh released from motoneurons is sufficient to prevent alterations associated to denervation, like the *de novo* synthesis and incorporation of connexins hemichannels into the sarcolemma, which initiates a sequence of deleterious changes, including sarcolemma permeabilization, increased cytosolic calcium and sodium levels,

and upregulation of atrogenes, which leads to protein catabolism and muscle atrophy [69]. Moreover, AChR subunits may play a critical role in NMJ stability after denervation with the main goal of maintaining NMJ function [70]. However, in old animals and contrarily to what occurs in young ones, the protein levels of AChR α did not increase after nerve injury [66], which may jeopardize NMJ functions.

Agrin-LRP4-MuSK signaling

Data suggest that agrin-low-density lipoprotein receptor–related protein 4 (LRP4)-muscle-specific kinase (MuSK) signaling pathway may be impaired during aging (Fig. 1). Neural agrin, which is released from motoneurons and accumulates at the synaptic basal lamina, binds to its co-receptor LRP4 on the cell surface of muscle fibers, activating the tyrosine kinase domain of the receptor MuSK to self-phosphorylate [71, 72]. Activated MuSK is transported along the axons and released into the synaptic basal lamina of the NMJs, transmitting the extracellular signal into the myotubes, where it triggers the assembly of the postsynaptic apparatus, including AChRs clustering and stabilization of presynaptic structures [73]. In muscle fibers, activated MuSK also recruits and phosphorylates downstream of kinase 7 (DOK7), promoting DOK7 dimerization that, in turn, enhances MuSK phosphorylation through a positive feedback loop that has to be finely controlled for normal NMJ structure and function [71]. Activated DOK7 leads to the recruitment of two adapter proteins Crk and Crk-L [74]. This signaling cascade stimulates the cytoplasmatic membrane structural protein rapsyn to self-aggregate and interact with agrin and MuSK, which also induces AChRs clustering and postsynaptic specialization [74–76].

With aging, the activity of this signaling pathway in the NMJs appears to become dysregulated. A significant increase of the expression of agrin in the NMJs of the *tibialis anterior*, *extensor digitorum longus*, and *soleus* of old mice (24–30 months) was observed [77]. These animals had a deficient locomotor activity and the NMJs presented typical age-related structural alterations, namely fragmentation, nerve terminal branching, denervation, and smaller AChRs clusters [77] (Table 1). However, LRP4 levels were found markedly reduced in the *diaphragm* of old mice (24 months vs. 3 months), which may be explained by the enhanced LRP4 ubiquitination observed [68]. Accordingly, MuSK phosphorylation was found reduced in aged *diaphragm* (24 months vs. 3 months) [68], despite its mRNA levels being increased in the *vastus lateralis* of old mice (35 months) comparatively to young animals (8 months) [67]. These results suggest a compromised agrin-LRP4-MuSK activation with aging (Table 1), which may contribute to the alterations in NMJ function and structure. On the other hand, the levels of the intracellular adaptor protein rapsyn were increased in the *vastus lateralis* of old rats (35 months vs. 8 months) [67], but mutations in human rapsyn gene were already associated with NMJ-related diseases [78].

Another cause to age-related NMJ impairment may be the augment of the proteolytic cleavage of agrin by the neuronal serine protease neurotrypsin (Fig. 1) [79]. This agrin inactivation occurs by its cleavage at two homologous, highly conserved sites and results in the release of the soluble 22-kDa C-terminal agrin fragment (CAF) into circulation making it detectable in serum [80], and therefore a great candidate as sarcopenia biomarker. The increase in the serum levels of CAF with aging [73, 81, 82] translates an enhanced agrin degradation that may symbolize reduced levels of agrin at the NMJs, compromising agrin role in the maintenance of a functional NMJ. Actually, overexpression of neurotrypsin in motoneurons of young adult mice established the sarcopenic phenotype (reduced number of muscle fibers, increased heterogeneity of fiber thickness, fiber type grouping, and increased proportion of type I fibers) and led to an excessive fragmentation of the NMJ [83]. A neurotrypsin inhibitor (NT-1474) showed efficacy *in vivo* (mice) in reducing CAF serum levels by 44% [81]. Unfortunately, NMJs were not investigated. Nevertheless, in another study, muscular and junctional sarcopenic alterations were less pronounced in neurotrypsin-overexpressing aged mice (24 months; *soleus*) by transgenic co-expression of cleavage-resistant agrin, which seems to protect NMJs from disassembly and also exerts a survival-promoting effect for denervated fibers by enhancing reinnervation [83]. This was further strengthened in a posterior study where injection of neurotrypsin-resistant agrin activated agrin signaling and almost fully reversed the sarcopenia-like phenotype in neurotrypsin-overexpressing mice and also accelerated muscle reinnervation after nerve crush [84].

Neurotrophins

The skeletal muscle and nerves interact through electrical activity and neurotrophic regulation [41]. The latter occurs by the release of neurotrophic factors, like NTs [41]. The family of NTs harbors nerve growth factor (NGF), NT-3, NT-4, brain-derived neurotrophic factor (BDNF), and glial-cell-line-derived neurotrophic factor (GDNF), which regulate motoneuron survival, enhance presynaptic release of ACh, and promote the maintenance of the postsynaptic region [85, 86]. NTs are synthesized and released from both neurons and muscles [87]. NT signaling is mediated by the tropomyosin-related kinase (Trk) receptor and by the p75 neurotrophin receptor (p75NTR) [85]. BDNF is one of the most studied NTs and potentiates ACh release through TrkB phosphorylation and PKC activation [41]. In addition, it is involved in the regulation of synapse function and maintenance in the neuromuscular system and also in muscle development and metabolism [41]. Indeed, inhibition of BDNF-TrkB signaling at early old age (18 months) induced NMJ denervation [88]. Studies suggest that an age-related decrease in BDNF serum levels occurs [89–91] (Table 1). This may be explained by the hypothesis that BDNF is a contractile-inducible protein [92, 93] and older people tend to be more sedentary [94]. In the *vastus lateralis* of old mice (35 months), no changes were observed in the mRNA levels of NT-3, NT-4, BDNF, or GDNF compared to young animals (8 months), despite the presence of marked denervation and increased mRNA levels of the neurotrophic tyrosine kinase receptor type 3 (NTRK3) [67]. This may suggest that a normal neurotrophic signaling is insufficient to a successful maintenance of the neuromuscular system.

A decrease of GDNF mRNA was also observed in both aged rats' *gastrocnemius* and *soleus*, but GDNF protein levels were maintained without alterations in the NMJs throughout adulthood [95] (Table 1). Nevertheless, the time analyzed was very low (birth to 3 months). In another study, 17-week rats showed lower GDNF levels on the *soleus* and *extensor digitorum longus* comparatively to 4-week animals [96].

Age-related changes in postsynaptic signaling

Calcitonin gene–related peptide

CGRP is a neuropeptide present in motoneurons where it is stored in dense-core vesicles, possibly coexisting with ACh, being released upon nerve stimulation [97–99]. In the skeletal muscle, CGRP binds to calcitonin-like receptor (CLR), a Gs protein–coupled receptor highly presented in the NMJs, and increases the levels of intracellular cyclic adenosine monophosphate (cAMP) that leads to the activation of cAMP-dependent protein kinase (PKA) and phosphorylation of cAMP response element-binding protein (CREB) [97], suggesting that CGRP regulates gene expression. It is known that CGRP increases the number of AChRs and the rate of AChR desensitization and decreases the expression of acetylcholinesterase, potentiating muscle contraction [97, 100]. This may be indicative that this neuropeptide acts on skeletal muscle to regulate the elements of the postsynaptic apparatus [100]. Despite CGRP levels being high during NMJ development, they markedly decrease in mature NMJs [101]; however, CGRP is upregulated following nerve injury, suggesting a function in NMJs during the process of reinnervation and nerve sprouting, with its levels declining with the morphological and functional recovery of the NMJ [77, 102]. Recent data also suggest that upon muscle denervation, CGRP is involved in NMJ stabilization through the transcriptional inhibition of forkhead box O (FoxO)/transcription factor EB (TFEB)–regulated autophagic genes, stimulation of mammalian target of rapamycin complex 1 (mTORC1), and inhibition of the calpain system. Hence, CGRP may be used as both a potential biomarker for age-induced denervation and a therapeutic target [103]. Indeed, mammalian target of rapamycin (mTOR) signaling must be fine-tuned to maintain NMJ function. A role for mTORC1 in NMJ morphology and function has been discussed. Typical morphological features of age-related NMJ instability like significant thinning of axons, increased number of sprouting axons, higher number of AChR clusters, and lower density of postsynaptic AChRs were observed in mice with sustained activation of mTORC1 (TSCmKO, 9 months, *extensor digitorum longus*) [23]. In contrast, mTORC1 inhibition (10 months mice, *tibialis anterior*) also resulted in denervation, NMJ fragmentation, and higher number of AChRs clusters [104]. Furthermore, partial mTORC1 inhibition (via RAD001, a rapalog, administrated at a low dose) reduced the age-induced transcriptional upregulation of denervation-associated gene markers in aged *tibialis anterior* (24 months); however, no differences were verified in the *gastrocnemius* or *plantaris* muscles [105]. Still, more studies are needed to clarify the role of mTOR signaling in NMJ function.

A significant increase in CGRP immunoreactivity was observed in the NMJs of old *tibialis anterior*, *soleus*, *extensor digitorum longus*, and *gracilis* muscles (24–30 months vs. 4 months) [77]. This continuous increase with aging without a subsequent decline may symbolize the occurrence of denervation without reinnervation, which may indicate failure in the functional recovery of the NMJs (Fig. 1 and Table 1). In fact, those animals exhibited frequent endplate denervation and classic age-related structural changes on their NMJs [77]. More studies on the effect of aging in the function of this signaling pathway are necessary, given that CGRP levels can be measured in human plasma [106], which makes it an attractive biomarker. Additionally, *in vivo* application of CGRP on the *soleus* of adult rats induced a local accumulation of AChRs on the extrajunctional surface where the AChRs are usually absent [98], indicating CGRP as a possible therapeutic strategy to counteract the impaired neuromuscular transmission observed with aging.

Growth-associated protein

Another mediator that is upregulated upon denervation is growth-associated protein (GAP-43) that is present in virtually all neurons during axonal growth, being particularly abundant in axonal growth cones [77]. GAP-34 is widely expressed in the central nervous system during the perinatal period, with its levels decreasing as maturation progresses [107, 108]. Mature motoneurons and their corresponding nerve terminals display low levels of GAP-43 [77]. Nonetheless, GAP-43 seems to be involved in the muscle regeneration process [109] with an upregulation of its levels following denervation [110], and also in the modulation of calcium dynamics and its intracellular roles [111, 112]. Indeed, GAP-43$^{-/-}$ mice showed, among others, decreased body weight, reduced muscle strength, altered myofiber ultrastructure, and dysregulated calcium homeostasis in the muscle [111], possible by the lack of the interaction of GAP-43 with DHPR and RyR [111, 112]. In old animals, a significant increase of GAP-43 levels was observed in the NMJs of the *tibialis anterior*, *soleus*, *extensor digitorum longus*, and *gracilis* muscles (24–30 months vs. 4 months) [77]. Moreover, aged (30 months vs. 2–3 months; rat) motoneurons revealed increased GAP-43 immunoreactivity and GAP-43 levels seem to be associated to the severity of the neuromuscular dysfunction [113] (Fig. 1). Thus, the increased levels of GAP-43 observed in old age may be indicative of denervated muscle fibers (Table 1). Indeed, increased muscle levels of this protein are present in denervation [114] and myopathies [115], and no immunoreactivity was found in the absence of those conditions; thus, GAP-43 may be a good candidate as sarcopenia biomarker. Hence, its study in age and sarcopenia contexts would be important.

Satellite cells

Muscle regeneration also relies on muscle stem cells designated SCs [116] that under resting conditions are quiescently located underneath the basal lamina [117]. Upon tissue injury, SCs exit quiescence and proliferate to myoblasts that later differentiate to myocytes (muscle cells) to regenerate the damaged tissue [117]. This regenerative capacity of skeletal muscle is greatly affected by aging, since SCs abundance declines with age in both human (22–76 years; *vastus lateralis*) [118] and animal muscles (3–33 months; *soleus*, *extensor digitorum longus*, *tibialis anterior*) [119, 120] (Fig. 1), which may suggest that SC population is not adequately replenished throughout life. Despite this decline in SC pool, some studies demonstrated that the myogenic potential of SCs does not decline with aging, despite a slower rate of SCs activation after an injury in both old (22–30 months) and geriatric (29–33 months) muscles [119, 121]; however, it was also suggested that in geriatric mice (28–32 months), resting SCs lost their reversible quiescence state and switch to an irreversible presenescence state, being unable to activate and repair the muscle upon injury [122]. Specifically, it was also demonstrated that SCs frequency declines in both synaptic and extra-synaptic regions in old age (18 and 24 months) [123]. In line with this, depletion of SCs resulted in an impaired NMJ reinnervation, reduction in postsynaptic morphology, and loss of post-synaptic myonuclei in response to denervation [124]. Moreover, SCs depletion at 12 months accelerated the onset of age-related impairment of NMJ integrity, and a decline in postsynaptic myonuclei and myofiber size (vs. 18 and 24 months) [123]. Therefore, the important role that SCs have in maintaining NMJ function is noteworthy, a role that may be impaired as a result of aging and that is important to explore in future

studies. Immunostaining of muscle sections with SCs markers has been the methodological approach used to evaluate the age effect on SCs remodeling. In human skeletal muscles, SCs have been identified using the NCAM/CD56 antigen [125]. Despite being considered a reliable molecular marker to identify SCs, NCAM is also expressed in myoblasts, myotubes, and muscle fibers during regeneration, as described before [125]. Thus, other markers are used, namely M-cadherin (M-Cad), c-Met, and Pax7, which are thought to be exclusively expressed in SCs of mature muscles [125].

Conclusions

The study of the effect of aging on the NMJ morphology and function is challenging, particularly in the clinical set. Muscle collection from aged subjects may raise ethical questions and the obtained samples may not be representative of the whole muscle. Besides, the NMJs may not be collected in the biopsy, and if so, may have a negative impact on the older subjects' muscle function, potentially aggravating an already impaired muscle. Hence, preclinical studies allow the generation of data from whole muscle that cannot be collected from humans. Despite all these constrains in the study of NMJs, the analysis of the literature highlights several interesting findings. With aging, the NMJ structure alters, jeopardizing the communication between the nervous and muscle systems. The reinnervation rate in older ages is not sufficient to counterweight the rate of the appearance of denervated fibers, possible due to an age-induced incapacity to enhance reinnervation-related processes, such as the NCAM response. The maintenance and function of the NMJ rely on different signaling pathways involving players that ensure the stability of the pre- and postsynaptic apparatus and a proper signal transmission through ACh and reinnervation following denervation. Agrin, ACh, and NTs (such as BDNF and GDNF) signaling is impaired at old ages, resulting in a deficient neuromuscular transmission. Moreover, the role of SCs and proteins involved in the reinnervation process, namely CGRP and GAP-43, is compromised during aging, contributing to the accumulation of denervated muscle fibers. The effect of aging on these key mediators and the consequent outcomes on the NMJ are overviewed in Table 1. The evidence provided here suggests a key role of NMJ-related players to sarcopenia pathogenesis and highlights potential biomarkers and therapeutic targets, paving the way to future studies. Nonetheless, it remains elusive the relative contribution of NMJ dysfunction to sarcopenia pathogenesis, a question that deserves to be explored to improve sarcopenia management. Future investigations should consider analyzing older individuals (animals, at least) and evaluate the mediators that have the potential to be analyzed in serum, such as CAF, BDNF, and CGRP, envisioning the translation to the clinical set.

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

Acknowledgments This work was supported by CIAFEL (UIDB/00617/2020), LAQV (UIDB/50006/2020), and CITAB (UIDB/04033/2020) research units and by A.M.P.'s fellowship (SFRH/BD/144396/2019) through national funds by the Portuguese Foundation for Science and Technology (FCT) and co-financed by the European Regional Development Fund (FEDER), within the PT2020 Partnership Agreement.

Declarations

Conflict of interest The authors declare no competing interests.

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Chapter II

Subchapter IIB

Sarcopenia versus cancer cachexia: the muscle wasting continuum in healthy and diseased aging

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Moreira-Pais, A., Ferreira, R., Oliveira, P. A., & Duarte, J. A. (2021). Sarcopenia versus cancer cachexia: the muscle wasting continuum in healthy and diseased aging. *Biogerontology*, *22*(5), 459–477. https://doi.org/10.1007/s10522-021-09932-z

Biogerontology (2021) 22:459–477
https://doi.org/10.1007/s10522-021-09932-z

REVIEW ARTICLE

Sarcopenia versus cancer cachexia: the muscle wasting continuum in healthy and diseased aging

Alexandra Moreira-Pais · Rita Ferreira · Paula A. Oliveira · José A. Duarte

Received: 26 April 2021 / Accepted: 21 July 2021 / Published online: 29 July 2021
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Abstract Muscle wasting is one of the major health problems in older adults and is traditionally associated to sarcopenia. Nonetheless, muscle loss may also occur in older adults in the presence of cancer, and in this case, it is associated to cancer cachexia. The clinical management of these conditions is a challenge due to, at least in part, the difficulties in their differential diagnosis. Thus, efforts have been made to better comprehend the pathogenesis of sarcopenia and cancer cachexia, envisioning the improvement of their clinical discrimination and treatment. To add insights on this topic, this review discusses the current knowledge on key molecular players underlying sarcopenia and cancer cachexia in a comparative perspective. Data retrieved from this analysis highlight that while sarcopenia is characterized by the atrophy of fast-twitch muscle fibers, in cancer cachexia an increase in the proportion of fast-twitch fibers appears to happen. The molecular drivers for these specificmuscle remodeling patterns are still unknown; however, among the predominant contributors to sarcopenia is the age-induced neuromuscular denervation, and in cancer cachexia, the muscle disuse experienced by cancer patients seems to play an important role. Moreover, inflammation appears to be more severe in cancer cachexia. Impairment of nutrition-related mediators may also contribute to

A. Moreira-Pais · J. A. Duarte
CIAFEL, Faculty of Sport, University of Porto, Dr. Plácido da Costa 91, 4200-450 Porto, Portugal

A. Moreira-Pais · R. Ferreira
LAQV-REQUIMTE, Department of Chemistry, University of Aveiro, 3810-193 Aveiro, Portugal

A. Moreira-Pais · P. A. Oliveira
Centre for Research and Technology of Agro Environmental and Biological Sciences (CITAB), Inov4Agro, University of Trás-os-Montes and Alto Douro (UTAD), Quinta de Prados, 5000-801 Vila Real, Portugal

A. Moreira-Pais (✉)
Departamento de Química, Universidade de Aveiro, Campus Universitário de Santiago, 3810-193 Aveiro, Portugal
e-mail: alexandrapais@ua.pt

J. A. Duarte (✉)
Faculdade de Desporto, Universidade do Porto, Rua Dr. Plácido da Costa 91, 4200-450 Porto, Portugal
e-mail: jarduarte@fade.up.pt

J. A. Duarte
TOXRUN – Toxicology Research Unit, University Institute of Health Sciences, CESPU, CRL, Gandra, Portugal

sarcopenia and cancer cachexia, being distinctly modulated in each condition.

Keywords Aging · Circulating players · Disuse · Inflammation · Neuromuscular · Nutrition

Introduction

The world's population is aging, leading to a global burden of late-life diseases (Jaul and Barron 2017; Borras et al. 2020). One of the most disturbing conditions to older people's health is the decline of skeletal muscle mass and function, which is traditionally associated to sarcopenia. This condition is primarily diagnosed in ages above 65 years, but can be observed in younger ages secondary to, for example, chronic diseases (Yazar and Olgun Yazar 2019). According to the European Working Group on Sarcopenia in Older People 2 (EWGSOP2) (Cruz-Jentoft et al. 2019), age-related sarcopenia (hereafter referred as sarcopenia) is clinically defined by decreased levels of muscle strength, muscle quantity or quality and physical performance (as an indicator of severity) (Cruz-Jentoft et al. 2019). Nonetheless, in the presence of cancer the loss of skeletal muscle mass may also occur (independently of age) (Baracos et al. 2018; Dunne et al. 2019), and in this case it is associated to cancer cachexia. This condition is clinically defined as an involuntary loss of skeletal muscle mass, with or without loss of fat mass, which cannot be completely inverted by conventional nutritional support and results in a progressive functional impairment (Fearon et al. 2011). However, sarcopenia is also diagnosed in 15–50% of all cancer patients (Peterson and Mozer 2017), which raises the question if the muscle wasting observed in older cancer patients is age- or tumor-induced, or a combined effect of both.

There are some tools that have been proposed for the diagnosis of sarcopenia and cancer cachexia. Briefly, the EWGSOP2 proposed the following criteria to sarcopenia diagnosis: (i) the assessment of muscle strength (grip strength or chair stand test); (ii) if low, sarcopenia is probable and muscle quantity or quality should be assessed (dual-energy X-ray absorptiometry (DEXA)); (iii) if low, sarcopenia is confirmed and physical performance should be evaluated (gait speed, short physical performance battery, timed-up-and-go test, or 400 m walk test) to asses severity; (iv) if low, sarcopenia is considered severe (Santilli et al. 2014; Cruz-Jentoft et al. 2019). For cachexia diagnosis the CAchexia SCOre (CASCO) was proposed and harbors: (i) body weight loss and composition; (ii) inflammation/metabolic disturbances/immunosuppression; (iii) physical performance; (iv) anorexia; and (v) quality of life (Argilés et al. 2017). Despite these tools, the development of a panel of biomarkers specific for each condition, which may include potential circulating (pro-inflammatory cytokines, nutrition-related mediators) and tissue (neuromuscular system-related mediators) markers, has been investigated to complement and strength the diagnosis of the condition underlying muscle wasting in (not only) older subjects with cancer, and to monitor the effectiveness of treatments against these wasting conditions.

The present review discusses the current literature on key molecular players responsible for muscle wasting that may have a clinical differential value between sarcopenia and cancer cachexia. Thus, only mediators that have been analyzed in both conditions were considered. Clinical studies were the main focus of this review, since animal models do not always mimic what happens in humans; however, most of the available studies are done in preclinical models due to the ethical constrains in obtaining muscle samples in humans, and therefore they were also considered whenever necessary. Focus was given to the biological processes and the mediators involved in each condition and not to the molecular pathways taken place in skeletal muscle.

Skeletal muscle phenotype remodeling in sarcopenia versus cancer cachexia

Between the second and tenth decades of humans' life, 25% of motoneuron cells are lost and this number can increase up to 50% in adults older than 60 years (Tomlinson and Irving 1977), which is accompanied by a decline in the motor unit pool (Wilkinson et al. 2018). Similar results were observed in a rat model of sarcopenia (Rowan et al. 2012). This leads to denervation of muscle fibers that in the majority of the cases are reinnervated by nearby surviving axons, constituting the denervation-reinnervation cycles (Wilkinson et al. 2018). In these cycles, axonal sprouts are

originated from non-myelinated areas of the axon to save the denervated fibers and in this way try to preserve muscle mass and function (Wilkinson et al. 2018). In older muscle, type II fibers are more susceptible to denervation, being reinnervated by type I motor units (Fig. 1) (Kwon and Yoon 2017). This process results in the co-expression of multiple myosin heavy chain (MHC) isoforms and large fiber type I grouping, which characterizes aged skeletal muscle (Rudolf et al. 2014; Joanisse et al. 2016). However, when the denervation rate outpaces the reinnervation process, a portion of denervated fibers may not be successfully reinnervated (Hepple and Rice 2016a), which in older adults, results in a specific atrophy of type II fibers and their consequent loss (Rudolf et al. 2014; St-Jean-Pelletier et al. 2017; Roberts et al. 2018). This may be responsible for the lower muscle strength that is reported in adults over 55 years comparatively to younger individuals (Roberts et al. 2018), since type II fibers are the ones capable of generate higher maximum force levels (Li and Larsson 2010). Thus, failure to reinnervate denervated fibers (or expand motor unit size) may be determinant to distinguish sarcopenic older individuals from pre-sarcopenic ones (Piasecki et al. 2018).

Regarding cancer cachexia very few studies have been conducted to evaluate the process of skeletal muscle phenotype remodeling, but contrarily to what happens in sarcopenia, in cancer cachexia both clinical and preclinical studies suggest a specific decline in the expression of slow-twitch fibers accompanied by an increase in fast-twitch ones (Fig. 1). For instance, it was observed a progressive decline in type I expression over type II (*rectus abdominis*) from control to

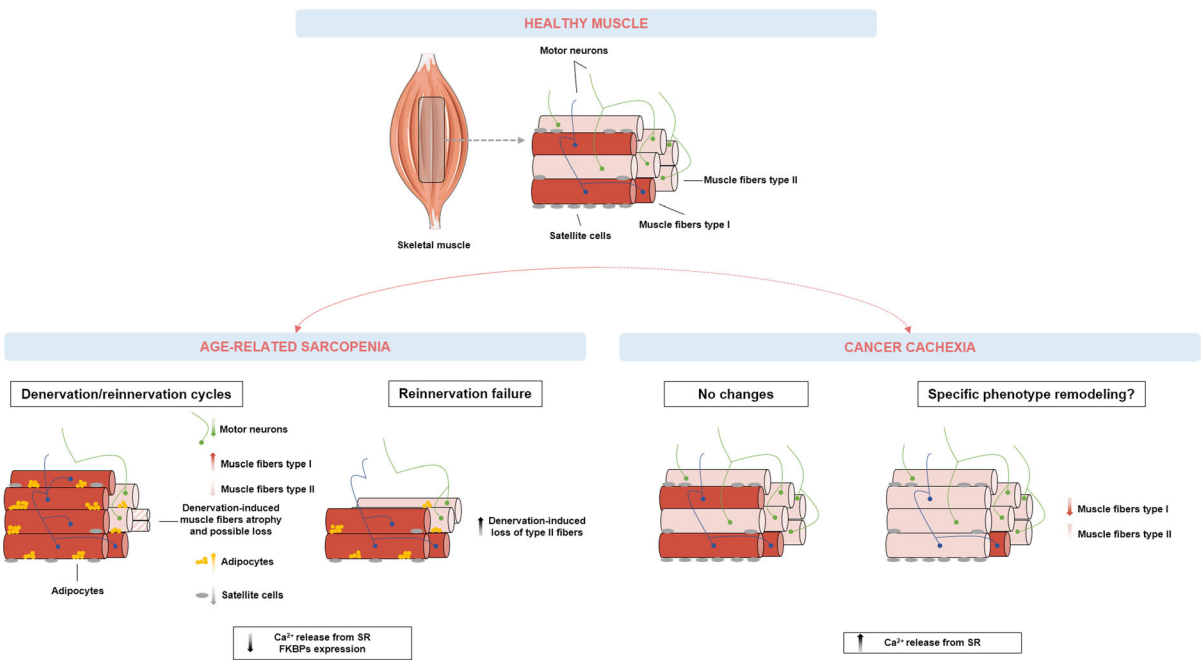

Fig. 1 Overview of the skeletal muscle phenotype remodeling that occurs in age-related sarcopenia and in cancer cachexia. In age-related sarcopenia, it seems to occur a specific denervation of type II fibers followed by reinnervation through sprouting of nearby motor neurons, usually, type I ones, increasing the number of type I fibers. When reinnervation fails, atrophy of type II fibers happens with subsequent loss. In cancer cachexia, the poor existing evidence suggests that the muscle innervation does not change or that an increase in type II fibers and a decrease of type I ones occurs; however, due to the lack of studies, these data are inconclusive (dashed line). The figure was produced using the *Servier Medical Art*. Abbreviations: *FKBPs* FK506-binding protein, *SR* sarcoplasmic reticulum

non-cachectic and cachectic patients (Taskin et al. 2014), suggesting a progressive slow-to-fast transition of muscle fibers from cancer to cachexia development. This progressive transition may be useful to precociously identify cancer patients at risk of developing cachexia and muscle dysfunction. These results are supported by data obtained from animal models of cancer cachexia, where in the *soleus* was observed a decrease in the number of type I fibers and an increase in type IIa and IIb (Diffee et al. 2002; Roberts et al. 2013) and in the *extensor digitorum longus* a decrease in type IIa and an increase in IIb fibers (Roberts et al. 2013) comparatively to healthy animals. No differences were found in the *plantaris* or *gastrocnemius* muscles (Diffee et al. 2002). This specific decline in type I fibers expression may be driven by the muscle disuse experienced by cancer patients (Hardee et al. 2019) and not by the disease itself (overviewed in the "muscle disuse" section). Still, no changes in the expressions of the MHC isoforms of the *rectus abdominis* were reported in cancer cachectic patients, when compared to patients with a stable weight, despite decreased cross-sectional area (CSA) of all fiber types (Johns et al. 2014). Clearly more information is needed to uncover the muscle phenotype in cancer cachexia, namely the basis of how oxidative and glycolytic muscle fibers/skeletal muscles are affected, since it can influence the outcome of therapies, like exercise programs.

Histological analyses of muscle sections would be important to differentiate between sarcopenic and cachectic older adults. Nevertheless, and despite muscle biopsies been sometimes ordered during the diagnostic procedure of muscle diseases (Joyce et al. 2015), it is difficult to obtain several samples *per* subject due to the invasive nature of the procedure (Hayot et al. 2005). One tissue sample represents less than 0.01% of the whole muscle and contains only about hundreds of fibers of even less motor units, and therefore, it is not representative of the whole muscle fiber type distribution (Baguet et al. 2011), especially when exists a MHC co-expressing phenotype. Hence, minimally- (microbiopsy) or non-invasive (proton magnetic resonance spectroscopy) approaches have been proposed (Hayot et al. 2005; Baguet et al. 2011); however, in the former the inconsistencies with sample weight and morphological integrity can comprise histological analyses (Townsend et al. 2016), and the latter technique is an indirect estimation of the muscle phenotype and the results can be biased by extrinsic factors such as diet (Baguet et al. 2011).

The contribution of neuromuscular system-related players to muscle phenotype remodeling in sarcopenia and cancer cachexia

The impairment of the interaction between motoneurons and myofibers, i.e., the impairment of the neuromuscular junction (NMJ) is emerging as an important contributor to sarcopenia, since it leads to the loss of muscle strength, which precedes skeletal muscle wasting (Hepple and Rice 2016b). In cancer cachexia, data point to stable morphology and function of the NMJ in patients with cachexia (Boehm et al. 2020). Hence, neuromuscular system-related players will certainly be useful for distinguish sarcopenia from cachexia in older cancer patients. Nevertheless, there are few experimental studies focused on the interplay between neuromuscular control and cancer cachexia (Diffee et al. 2002; Johns et al. 2014), and most of the experimental evidence on this topic was obtained from preclinical studies.

Classic morphological features of NMJ impairment, such as thinning of axons, increased number of sprouting axons and of acetylcholine receptor (AChR) clusters, low density of postsynaptic AChRs and synaptic detachment and fragmentation of the postsynaptic apparatus were observed in aged *extensor digitorum longus* (Ham et al. 2020) and *tibialis anterior* of mice (Valdez et al. 2010). This NMJ dysfunction is further strengthened by the presence of N-Cell adhesion molecule (NCAM) positive fibers in aged muscle (Mosole et al. 2014). The levels of this molecule increase in adult muscle in response to denervation to induce reinnervation (Covault and Sanes 1985). However, in aged muscle the reinnervation process is impaired (Carlson et al. 2001), hence NCAM presence may not reflect the occurrence of reinnervation, since its levels remain elevated in denervated fibers (for up 300 days, at least) (Covault and Sanes 1985). Indeed, aged muscle seems to have a diminished capacity to enhance NCAM response following denervation (Gillon and Sheard 2015). Regarding cancer cachexia, no changes were observed in the morphology of the NMJ of the *rectus abdominis* of cachectic cancer patients comparatively to cancer patients without cachexia and to age-matched subjects without cancer (Boehm et al. 2020). Nonetheless, the

presence of central myonuclei was reported in the *rectus abdominis* of cancer patients with cachexia as well as the upregulation of NCAM, which may indicate NMJ impairment, motor neuron loss (Daou et al. 2020) or the occurrence of reinnervation (Covault and Sanes 1985). More studies in this field are needed, principally because a soluble form of NCAM has been suggested to be involved in the reinnervation process (Olsen et al. 1993) and it was already identified in serum of patients with, for instance, neurodegenerative disorders and cancer (Secher 2010), which makes it an attractive biomarker.

Alterations in the flow of information between the nerve and the muscle in wasted conditions may involve acetylcholine (ACh) (Bassel-Duby and Olson 2006; Kuo and Ehrlich 2015). In aged *gastrocnemius* (Apel et al. 2009; Uchitomi et al. 2019) and *vastus lateralis* (Aare et al. 2016) of rodents, the mRNA levels of AChR α, δ and ε were increased, as well as the *diaphragm* protein levels of AChR δ (Zhao et al. 2018). This increase in the levels of AChRs is probably a compensatory mechanism in response to NMJ impairment and denervation (Tsay and Schmidt 1989). However, no age-related differences were observed in the protein levels of AChR α and ε subunits (Zhao et al. 2018). In rat's *gastrocnemius* muscle, the release of Ca^{2+} from the sarcoplasmic reticulum (SR) was reported to diminish with aging, possible as a result of a reduction of FK506-binding protein (FKBPs) expression and FKBPs-ryanodine receptor (RyR) binding, culminating in RyR impairment (Russ et al. 2011). The FKBPs family controls the activity of RyR channels, by promoting the dissociation of FKBP12 from RyR channels, activating them (Ozawa 2010). However, to the best of our knowledge, no age-related changes were found in muscle sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) and calsequestrin (CSQ) levels (Russ et al. 2011). In contrast, in tumor-bearing mice with cachexia, it was observed an increased expression of *RyR1*, *SERCA1* and *SERCA2* genes in the *extensor digitorum longus*, which may reflect an increased leak of Ca^{2+} from the SR that can result in the activation of proteolytic and apoptotic processes (Fontes-Oliveira et al. 2013)' molecular pathways traditionally associated to muscle atrophy (Bowen et al. 2015). In addition, mRNA levels of AChR α seem not to be altered in (the *gastrocnemius*) experimental cancer cachexia, while AChR δ and AChR ε were downregulated. However, AChR density is usually higher than needed for an efficient capture of ACh, making difficult to conclude about the contribution of ACh signaling to cachexia with only these results (Brown et al. 2018).

The regulation of AChR clustering and NMJ formation and function is controlled by spinal motor neuron (SMN)-derived molecules besides ACh, such as fibroblast growth factors (FGFs), brain-derived neurotrophic factor (BDNF) and glial cell line-derived neurotrophic factor (GDNF) (Li et al. 2018). However, few studies have been dedicated to the analysis of these factors in any of the wasting conditions (Ming et al. 1999; Baspinar et al. 2017). For instance, BDNF serum levels were significantly decreased in older adults with sarcopenia and these levels were positively correlated with muscle strength, physical performance and body weight (Miyazaki et al. 2020). Moreover, neuromuscular transmission was reported to decrease in 18 and 24months mice (versus 6 months mice) with the consequent reduction in muscle strength, and BDNF treatment prevented this age-related diminishing of neuromuscular transmission (Greising et al. 2015). In contrast, in patients with cancer cachexia, no differences were found in the levels of circulating or *rectus abdominis* muscle BDNF (de Castro et al. 2020). There are studies regarding the effect of age or sarcopenia in the levels of other mediators, such as GDNF and FGFs, that link low levels to age and impaired NMJ morphology and function (McCullough et al. 2011; Taetzsch et al. 2017); however, to the best of our knowledge, in the set of cancer cachexia no studies were found.

Taken together, the disruption of the communication between the nervous and muscular systems in aged muscle seems to contribute for the muscle wasting observed in sarcopenia, but not for cancer cachexia. Hence, neuromuscular system-related players, such as BDNF, may help to distinguish between these two conditions. There are some attractive mediators that can, or have the potential to, be measured in circulation, including NCAM, BDNF and GDNF. In this sense, future studies should investigate the putative diagnostic value of these molecular markers, envisioning to improve the differential diagnosis of sarcopenia and cancer cachexia.

The contribution of age- and cancer-induced muscle disuse to muscle phenotype remodeling

Physical inactivity may be experienced by older adults and cancer patients, which contributes to and aggravates sarcopenia- and cancer cachexia-related muscle wasting (Callahan et al. 2015). The reduced physical activity performed by older adults may be due to several factors, such as physical, cognitive and psychological impairment (Gomes et al. 2017), as well as due to the downregulation of the dopamine signaling (Kwon and Yoon 2017). With aging, dopamine levels suffer a decline, as well as, the presynaptic dopamine transporters (Troiano et al. 2010) and the postsynaptic dopamine receptors, resulting in decreased reward signals and motivation for movement, which culminates in reduced physical activity (Kwon and Yoon 2017). Concerning cancer patients, in overall, they usually have loss of motor control, reduced mobility, limitations in range of motion and a long length of hospital stay, which normally implies bedrest, and consequently, they can experience a generalized reduction in physical activity for long periods (Fialka-Moser et al. 2003; Peterson and Mozer 2017).

The contribution of muscle disuse to sarcopenia or cancer cachexia has been poorly investigated with most of the available studies being focused on muscle disuse in healthy individuals. For instance, recent evidence suggests that in healthy older adults, a 5-day bed rest can downregulate cell adhesion and mechanosensing pathways that may be involved in disuse-induced muscle loss (Mahmassani et al. 2019). Moreover, in healthy older adults, 10 days of bed rest resulted in the loss of lower extremity strength, power and aerobic capacity, but without affecting physical performance (Kortebein et al. 2008). No similar studies involving cancer patients are known (Wesley Peixoto et al. 2020).

Still, data from both preclinical and human studies have consistently showed that in muscle disuse type I fibers are more susceptible to atrophy than type II fibers (Gibson et al. 1987; Stevens et al. 2000; Degens and Alway 2006; Brocca et al. 2012). Moreover, muscle disuse has been associated to a decreased expression of type I fibers and an increased expression of type II fibers (Talmadge 2000; Brocca et al. 2012), suggesting a shift in MHC profile from slow to fast isoforms. This may function as a compensatory process by which the decreased capacity of the disused muscle to generate power is balanced by an increased shortening velocity, since in daily life most of the performed contractions are shortening contractions (Degens and Alway 2006). This specific pattern of alteration in the expression of MHC isoforms meets the muscle phenotype remodeling observed in clinical and preclinical studies of cancer cachexia, and so, muscle disuse may be a key contributor to the development of muscle wasting in cancer cachexia. In sarcopenia, muscle disuse may also contribute to the outcome of loss of muscle mass and strength but does not seem to be the main responsible for it.

The contribution of circulating inflammatory mediators to sarcopenia and cancer cachexia

Both sarcopenia and cancer cachexia are associated with a chronic inflammatory state (Cho et al. 2018; Ebner et al. 2019). Nonetheless, the low grade inflammation associated with sarcopenia is characterized by an insidious increase in the levels of circulating pro-inflammatory mediators, while in cancer cachexia this increase seems to be more prominent (Bye et al. 2016; Dalle et al. 2017). Thus, inflammation may have a higher contribution to muscle loss in cancer cachexia (Ali and Garcia 2014). The most widely measured biomarker of systemic inflammation is the C reactive protein (CRP). The levels of this acute-phase protein have been reported to be consistently increased in sarcopenic old subjects (Wåhlin-Larsson et al. 2017; Hida et al. 2018) and in cachectic cancer patients (Op den Kamp et al. 2013; Lima et al. 2019) (Fig. 2); however, it is not known if CRP contributes to the pathogenesis of these conditions or is upregulated by them. Still, CRP levels have been inversely associated with muscle mass (Wåhlin-Larsson et al. 2017) and physical function, such as grip strength, in sarcopenia (Hida et al. 2018), and with body weight and muscle fiber CSA in cancer cachexia (Loumaye et al. 2015; Kitagawa et al. 2017). In overall, CRP does not seem to be a distinguishing factor between cachexia and sarcopenia in older cancer patients. Despite the diagnostic value of CRP in the set of wasting conditions being questionable due to its non-specificity, in the absence of other clinical condition CRP may help to identify older subjects with or without cancer in risk of developing muscle wasting

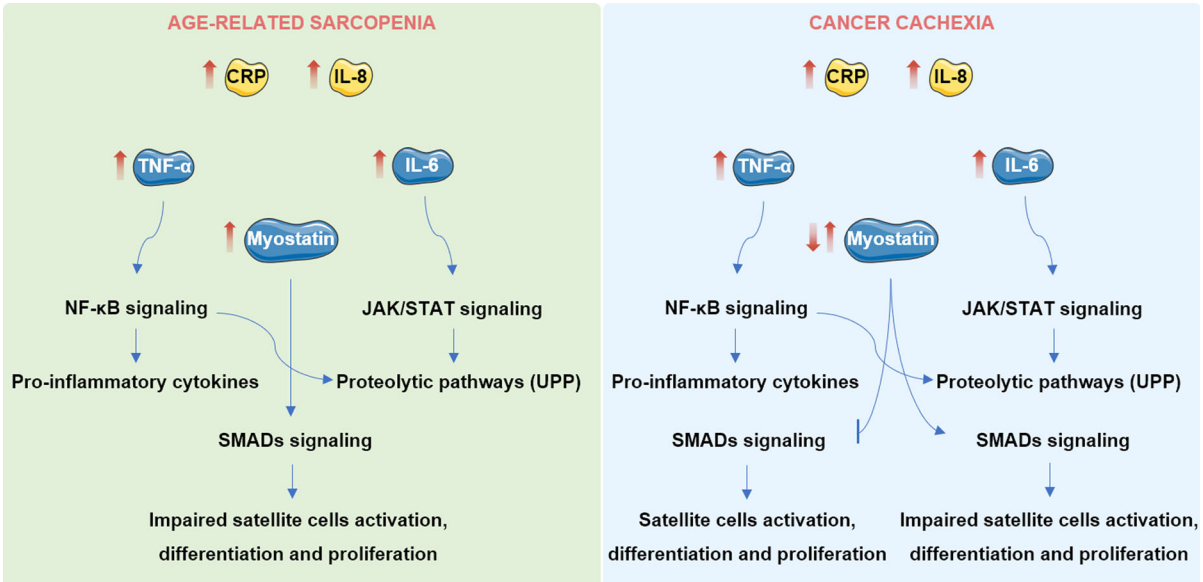

Fig. 2 The contribution of circulating inflammatory mediators to age-related sarcopenia or cancer-induced cachexia. The figure was produced using the *Servier Medical Art*. *FKBPs* FK506-binding protein, *SR* sarcoplasmic reticulum. *CRP* C reactive protein; *IL* interleukin; *JAK/STAT* Janus kinase/signal transducer and activator of transcription; *NF-κB* nuclear factor kappa-light-chain-enhancer of activated B cells; *TNF-α* tumor necrosis factor alpha; *UPP* ubiquitin-proteasome pathway

and to follow patient's response to anti-wasting treatments. For that, the definition of CRP threshold values is required.

Tumor necrosis factor (TNF)-α and interleukin (IL)-6 are the most studied pro-inflammatory cytokines in the set of sarcopenia and cancer cachexia (Argilés et al. 2006). IL-6 acts on Janus kinase/signal transducer and activator of transcription (JAK/STAT) receptors located on muscle fibers, activating proteolytic pathways, particularly the ubiquitin-proteasome pathway (UPP) (Muñoz-Cánoves et al. 2013). TNF-α, possible by acting through nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling, changes the MHC expression profile, and also enhances proteolytic pathways and the expression of pro-inflammatory cytokines (Guttridge et al. 2000; Karin and Ben-Neriah 2000; Oeckinghaus and Ghosh 2009). These cytokines not only impact muscle fiber remodeling but also the stem cell pool. For instance, chronic high levels of IL-6 and TNF-α decrease or even inhibit the proliferation of satellite cells, possibly through the activation of IL-6/STAT3/suppressor of cytokine signaling 3 (SOCS3) and TNF-α/NF-κB signaling cascades, and the inhibition of anabolic mediators (like Akt), abolishing the regenerative capacity of the skeletal muscle (Wåhlin-Larsson et al. 2014, 2017; Snijders et al. 2015; Dalle et al. 2017; Baar et al. 2018). High IL-6 circulating levels have been associated with the physical decline that occurs in older adults (Schaap et al. 2009) and evidence points to a gradual increase of IL-6 levels during aging, reaching higher values when age is superior to 60 years (Wyczalkowska-Tomasik et al. 2016). In addition to this, IL-6 levels have been inversely correlated with muscle area (Schaap et al. 2009) and strength in sarcopenic subjects (Visser et al. 2002) and with body weight in cancer patients (Garcia et al. 2005), but no cause-effect relation can be inferred from this. Moreover, the absence of differences in IL-6 levels was also observed between old individuals with severe and mild sarcopenia and young subjects (Ratkevicius et al. 2011), as well as, between cancer cachectic and non-cachectic patients (Hou et al. 2018). Despite being greatly analyzed in most of the studies, some issues must be considered when analyzing IL-6 results. For instance, circulating IL-6 levels are modulated by physical activity, with its basal levels decreasing by exercise training in some individuals (Fischer 2006). Therefore, different cut-off values should be considered in the analyzed

population based on their fitness status. Some authors also argue that IL-6 can act as both a pro- and anti-inflammatory mediator. The IL-6 produced by contracting muscle fibers (increasing up to 100 times the IL-6 levels) seems to inhibit pro-inflammatory TNF-α and to enhance anti-inflammatory IL-10 (Gleeson et al. 2011; Cole et al. 2018). This increase in IL-6 levels might occur without any muscle damage (Muñoz-Cánoves et al. 2013). However, a transient production with a short-term action as in exercise, cannot be compared with a chronic age- or cancer-induced IL-6 stimulation.

Old sarcopenic individuals present higher TNF-α levels than old non-sarcopenic ones (Li et al. 2019) and no differences were found between healthy young and healthy old individuals (Wyczalkowska-Tomasik et al. 2016). These data suggest that high circulating TNF-α may contribute to sarcopenia development/aggravation. As a matter of fact, higher TNF-α levels were associated with a greater decline in muscle mass and strength (Schaap et al. 2009). Regarding cancer cachexia, higher circulating levels of TNF-α were observed in cachectic patients when compared to healthy subjects (Bilir et al. 2015; Hou et al. 2018) and no differences were found between cancer cachectic and non-cachectic patients (Wu et al. 2013; Hou et al. 2018). The similar levels between cachectic and non-cachectic cancer patients may suggest that TNF-α is not involved in muscle wasting but it may be implicated in disease pathogenesis.

Circulating IL-8 levels have been associated to an increased risk for sarcopenia in older adults and have been inversely correlated with muscle mass (Westbury et al. 2018). However, no changes were observed in IL-8 levels between young and older subjects (Wyczalkowska-Tomasik et al. 2016). In contrast, circulating IL-8 was elevated in cachectic cancer patients in relation to non-cachectic counterparts (Lima et al. 2019), and it was associated with the loss of body weight and of *psoas* muscle area in cachectic patients (Hou et al. 2018; Lima et al. 2019). Serum IL-1α was also elevated in cachectic cancer patients, when compared to healthy subjects (Garcia et al. 2005; Bilir et al. 2015), but no differences were found in IL-1β levels between old and young individuals (Rivas et al. 2012).

Despite being widely studied, from the data above, TNF-α and IL-6 appear not to be the most useful biomarkers to distinguish sarcopenia from cancer cachexia in older individuals (Sebastián et al. 2016; Cho et al. 2018), because their levels are elevated in both conditions (Fig. 2). In addition, the levels of these cytokines (and others) are also increased in other conditions that may be simultaneously present in older adults with muscle wasting such as hypertension and diabetes (Kim et al. 2011), biasing diagnosis. Nonetheless, since the inflammatory process seems to be more severe in cancer cachexia, the definition of specific cut-off values for each condition may be helpful in the differential diagnosis. Besides the pro-inflammatory cytokines referred above, others have been investigated, but unfortunately most of the studies only explore their association to sarcopenia or to cancer cachexia, not allowing the comparation of their levels between conditions. Therefore, future studies should compare the levels of the same pro-inflammatory cytokines in subjects with sarcopenia or cancer cachexia to make way to a panel of inflammatory biomarkers with defined cut-off values to help in the differential diagnosis of these wasting conditions.

Circulating myokines related with sarcopenia and cancer cachexia

In response to aging and/or tumor burden, myostatin and activin A are secreted by muscle cells (Cole et al. 2018; Schmidt et al. 2018), and have been implicated in sarcopenia (McKay et al. 2012; Rooks and Roubenoff 2019) and cancer cachexia (Han et al. 2018; Schmidt et al. 2018) (Fig. 2). These catabolic cytokines can suppress the cell cycle, impairing the differentiation and proliferation of satellite cells (Carnac et al. 2007; McKay et al. 2012), thus jeopardizing skeletal muscle repair (Elliott et al. 2012; Desgeorges et al. 2017).

The role of myostatin or activin A in sarcopenia or cancer cachexia has been poorly investigated in humans and the results are often conflicted. Myostatin levels seem to gradually increase during aging and are inversely correlated with skeletal muscle mass (Yarasheski et al. 2002). The regulation of myostatin levels appears to be sex-dependent, since older sarcopenic women have higher myostatin levels than the corresponding group of men (Bergen III et al. 2015). However, no changes on myostatin serum levels were observed in old sarcopenic men comparatively to younger ones (Ratkevicius et al. 2011), and in another study younger men presented higher levels

than older sarcopenic men (Bergen III et al. 2015). In cancer cachexia, it has been suggested that myostatin can be produced by cancer cells and that once on bloodstream can induce muscle wasting (Han et al. 2018). However, recent studies with cancer patients indicate a different and opposite role of myostatin and activin A in cachexia. For instance, in cancer cachectic patients, activin A plasma levels were elevated, while myostatin levels were decreased, comparatively to cancer non-cachectic counterparts (Loumaye et al. 2015). Moreover, activin A levels were inversely correlated with body weight (Loumaye et al. 2015) and higher activin A levels were associated with lower overall survival (Loumaye et al. 2017). Contrarily, myostatin levels were positively correlated with body weight, muscle mass and strength (Loumaye et al. 2015). Nonetheless, circulating myostatin might not reflect its effective expression at muscle level, since it acts predominantly in an autocrine and paracrine manner. It may also indicate a protective downregulation of myostatin in response to muscle wasting.

Insulin-like growth factor 1 (IGF-1) can be secreted by skeletal muscles and it is involved in muscle regeneration and hypertrophy (Carosio et al. 2011; Ascenzi et al. 2019). Therefore, low levels are expected to occur in muscle loss-related conditions. Indeed, plasma IGF-1 levels gradually decrease from early adulthood into advanced old age (O'Connor et al. 1998), and old sarcopenic men had significantly lower IGF-1 levels than young healthy ones, but no correlation was found between these levels and muscle mass or strength (Ratkevicius et al. 2011). Likewise, cancer cachectic men had low levels of IGF-1 comparatively to cancer non-cachectic and even healthy patients (Garcia et al. 2005). These low levels of IGF-1 observed in both sarcopenia and cancer cachexia may contribute to the impairment of skeletal muscle regeneration that characterizes these conditions, exacerbating muscle proteolysis and wasting.

The contribution of age- or cancer-related malnutrition to muscle wasting

Sarcopenia and cancer cachexia normally coexist with malnutrition (Argilés 2005; Cerri et al. 2015), which is commonly unrecognized and undertreated (Deutz et al. 2019). One of the causes for the malnutrition observed in older adults may be the age-related loss of appetite, termed as "anorexia of aging" (Cox et al. 2019). Many age-related factors have been associated with this "anorexia", such as reduced sense of taste and smell (and so enjoyment of food); impaired sight (Hope et al. 2017); difficulties in chewing and swallowing; alterations in the gastrointestinal system, increased fullness and satiety; reduced activity of appetite-stimulating hormones and increased activity of satiety hormones (Robinson et al. 2018). Moreover, loneliness, depression (Chatindiara et al. 2020), limited finances, absence of personal transport, lack of nutrition knowledge and cooking skills (Wham and Bowden 2011) have been associated with the anorexia of aging. Cachexia is also characterized by the presence of anorexia (Ezeoke and Morley 2015). Cancer patients may experience changes in taste and difficulties in swallowing and chewing (Argilés 2005), along with competition for nutrients between the tumor and the patient's whole body, food aversion, malabsorption, nausea, diarrhea, vomiting, oral ulceration, reduced intestinal motility, side effects of drugs and other feeding-related changes induced by the tumor (Arends et al. 2017).

One of the most studied nutrition-related mediators is the peptide ghrelin. Ghrelin is produced by the stomach during periods of fasting, increasing appetite by stimulating the production of orexigenic factors, such as the neuropeptide Y (NPY). Sarcopenic individuals presented lower ghrelin levels than non-sarcopenic ones (Serra-Prat et al. 2015); however, when the patients were stratified by sexes no differences were observed (Serra-Prat et al. 2015). In line with this, NPY levels were reported to decrease in older adults (Chiodera et al. 2000). Preclinical studies showed an age-induced decrease in the expression of the NPY receptors Y1R and Y5R in several brain areas (Coppola et al. 2004; Veyrat-Durebex et al. 2013; Botelho and Cavadas 2015). In contrast to sarcopenia, cachectic cancer patients seem to have an overexpression of circulating ghrelin, despite no increase in appetite (Garcia et al. 2005). This may reflect a compensatory process to body weight loss and/or the presence of ghrelin resistance in cancer patients. The same seems to happen with NPY, since hypothalamic NPY mRNA was overexpressed in an animal model of cancer cachexia (Bing et al. 2001), but its feeding-stimulatory effect seems to be blunted in cancer (Chance et al. 1996). Hence, an impairment on the

NPY downstream targets or on the NPY transport or release from the hypothalamus may be hypothesized (Chance et al. 1994; Bing et al. 2001).

Changes in leptin levels have also been associated with these wasting conditions. On one hand, circulating leptin levels were reported to be elevated in old sarcopenic subjects compared to non-sarcopenic ones, and were negatively correlated with muscle mass (MacIntosh et al. 1999; Li et al. 2019). One another hand, leptin content seems to gradually decrease with aging with a more pronounced decline in women than in men (Isidori et al. 2000), suggesting a sex-dependent regulation. In fact, estradiol has a stimulatory effect on leptin secretion, while testosterone has an inhibitory action (Isidori et al. 2000). In cachectic cancer patients, circulating leptin levels seem to decrease comparatively to non-cachectic ones (Weryńska et al. 2009; Smiechowska et al. 2010). These low levels of leptin were not associated with increased appetite, despite increased expression of the hypothalamic leptin receptor (Bing et al. 2001), which may suggest a resistance to the orexigenic effects of hypoleptinemia (Smiechowska et al. 2010). With aging there is also an increased release of the appetite-suppressing hormone cholecystokinin (CKK) from the endocrine cells in the intestine (MacIntosh et al. 1999). Moreover, older adults seem to be more sensitive to the action of CKK than younger subjects (MacIntosh et al. 2001; Di Francesco et al. 2005). This may contribute to the rapid satiation and reduced food intake seen in older subjects (Chance et al. 1984; Kweder and Eidi 2018). In contrast, in tumor-bearing animals with body weight loss, hypothalamic CKK mRNA levels were found decreased (Chance et al. 1984), indicating a possible downregulation of CKK in response to weight loss.

Altogether, these humoral changes justify the decrease of appetite observed in both sarcopenic and cancer cachectic individuals. These changes can culminate in a decline of dietary protein intake, exacerbating muscle wasting (Robinson et al. 2018), since evidence suggests that both older adults (Campbell et al. 2001; Katsanos et al. 2006; Bauer et al. 2013; Moore et al. 2015) and patients with cancer (Winter et al. 2012; Arends et al. 2017) need higher values of dietary protein intake than younger or healthy individuals, respectively. Despite the association between malnutrition and sarcopenia or cancer cachexia, there are no mechanistic explanation integrating all the putative molecular players involved. Moreover, most of the attention is focused on the therapeutic side of nutrition, with few studies evaluating the preventive side of it (Chan et al. 2016; McClure and Villani 2017; Bloom et al. 2018).

Therapeutic strategies proposed to counteract muscle wasting in sarcopenia and in cancer cachexia

The management of muscle wasting in sarcopenia or in cancer cachexia is still a big challenge and no standard treatment or Food and Drug Administration (FDA) approved drug exists (Hardee and Lynch 2019). In the set of sarcopenia, testosterone is one of the available pharmacological strategies, but it seems to be effective only in men with symptoms and signs of androgen deficiency (Morley 2008; Rolland et al. 2011; Storer et al. 2017). Regarding cachexia, the existing therapeutic approaches are focused on the palliation of symptoms rather than on life prolongation (Aoyagi et al. 2015). An example of these are the appetite stimulants, such as megestrol acetate, corticosteroids and ghrelin analogs, but their use has potential adverse effects, including deep venous thrombosis, vomiting and worsening of pre-existing muscle wasting (Advani et al. 2018; Khatib et al. 2018; Moreira-Pais et al. 2018). Nutritional approaches relying on formulated nutrient mixtures by providing energy and protein may also be important for muscle wasting counteraction (Moreira-Pais et al. 2018). For instance, long-term n-3 polyunsaturated fatty acids (PUFA) supplementation seems to increase muscle mass and strength in older adults (Smith et al. 2015) and vitamin D supplementation seems to improve muscle strength, but only in vitamin D-deficient old individuals (Janssen et al. 2002). Supplementation with proteins/amino acids, particularly branched-chain amino acids, has also been studied for counteract muscle wasting in both conditions (Xia et al. 2017; Antoun and Raynard 2018). They decrease protein degradation in the skeletal muscle, possible by modulating inflammation, and by enhancing protein synthesis (Xia et al. 2017; Antoun and Raynard 2018). Recent evidence supports a multidisciplinary management of sarcopenia or cancer cachexia that combines diet modification plus drugs plus a personalized exercise program (Argilés et al. 2019; Hardee and

Lynch 2019). Nevertheless, exercise protocols and even nutritional advice can be challenging for both old sarcopenic or cachectic cancer patients, due to, for instance, their limited physical and/or mental capacity to perform exercise and prepare their meals (Rolland et al. 2011; Argilés et al. 2012).

In line with this, human studies focused on the effect of exercise interventions, alone or in combination with other strategies, highlighted an improvement of muscle mass and physical function in both sarcopenia (Beckwée et al. 2019) and cancer (Heywood et al. 2018) contexts. For instance, old sarcopenic women subjected to a resistance exercise protocol plus an intensive nutritional intervention (total energy of 30 kcal/kg/day, total protein of 1.5 g/kg/day and protein supplement containing, among others, whey protein and leucine) showed an improvement of muscle mass and a diminution of circulating pro-inflammatory mediators (e.g. TNF-α, IL-18) (Li et al. 2019). In experimental sarcopenia, the combination of a short-term treadmill exercise protocol with a myostatin inhibitor (PF-354) improved the physical function and decreased the circulating levels of catabolic mediators (e.g. muscle RING-finger protein 1 (MuRF1)) (LeBrasseur et al. 2009). In patients with advanced cancer (several types), 10 weeks of resistance exercise increased the muscle mass (versus baseline values) without adverse events being reported (Litterini et al. 2013). In experimental cancer cachexia, both low-intensity endurance exercise and resistance exercise training attenuated muscle mass loss by suppressing the activity of proteolytic pathways (e.g. UPP) and by activating the anabolic ones (Akt pathway) (Al-Majid and McCarthy 2001; Tanaka et al. 2019). Additionally, endurance exercise training combined with eicosapentaenoic acid (EPA) supplementation partially rescued the loss of muscle mass and strength in experimental cachexia; EPA alone did not had any effect on muscle loss, which highlights the importance of a multiapproach management (Penna et al. 2011).

Many studies have been investigating the therapeutic effect of anti-inflammatory drugs in the management of sarcopenia and cancer cachexia. In a cross-sectional study, usual nonsteroidal anti-inflammatory drug (NSAID) users evidenced a lower risk, of about 80%, of developing sarcopenia, when compared with age-matched non-NSAID users (Landi et al. 2013). This may suggest that long-term NSAID use may have a protective effect on muscle mass and even, may delay sarcopenia onset through the reduction of the inflammatory process. Several studies have been exploring the anti-inflammatory effect of herbal mixtures on muscle wasting in both sarcopenia (Rondanelli et al. 2016) and cancer cachexia (Park et al. 2019). For instance, the administration of a herbal combination of *Boswellia serrata*, *Cissus quadrangularis* and *Withania somnifera* (HIM-CHX) to sarcopenic rats decreased the levels of pro-inflammatory mediators, resulting in increased *gastrocnemius* muscle mass, locomotor activity and endurance (Azeemuddin et al. 2019). Similarly, in experimental cancer cachexia, the administration of *Paeonia lactiflora* root ethanol extract reduced the levels of the pro-inflammatory cytokines TNF-α, IL-6 and IL-1β and abolished the NF-κB signaling, resulting in improved muscle mass and strength (Bae et al. 2020). Creatine supplementation also inhibited skeletal muscle wasting in experimental cachexia, possibly by decreasing TNF-α and IL-6 levels (Cella et al. 2019). The inhibition of IL-6 by an IL-6 receptor antibody in a mice model of cancer cachexia attenuated the progression of cancer cachexia by reducing protein degradation and increasing IGF-1 mRNA expression, culminating in a positive impact on muscle mass (White et al. 2011).

Myostatin inhibitors have also been investigated for the management of muscle wasting. For instance, the administration of an anti-myostatin antibody (LY2495655) to old individuals (26% of which presented sarcopenia) increased lean mass and improved functional measures of muscle power, which is consistent with the LY2495655 mechanism of action that induces hypertrophy of fast-twitch fibers (Becker et al. 2015). Moreover, the administration of the anti-myostatin antibody PF-354 to aging rats prevented age-related sarcopenia (Morissette et al. 2009; Murphy et al. 2010). In experimental cachexia, the myostatin inhibitor named IMB0901 prevented the loss of body weight and muscle mass, possible by reducing the activity of proteolytic pathways and improving the activity of the anabolic ones (Liu et al. 2019). Moreover, IMB0901 had no influence on the anti-tumor effect of the chemotherapeutic agents cisplatin, doxorubicin or gemcitabine and even lowered the doxorubicin-induced upregulation of myostatin in myotubes (Liu et al. 2019).

Conclusions

The clinical management of sarcopenia and cancer cachexia constitutes a huge challenge due to, at least in part, the difficulties in their differential diagnosis. Hence, it is critical to better understand the pathogenesis of these wasting conditions in a comparative perspective. From the analysis of the literature herein performed some facts stood out (overviewed in Fig. 3): (i) muscle phenotype remodeling seems to discriminate both conditions, since in sarcopenia occurs a specific denervation of type II (fast-twitch) muscle fibers, followed by reinnervation by type I motor units, or atrophy, and in cancer cachexia occurs a specific decline in the expression of type I (slow-twitch) fibers and an increase in type II fibers; however, the molecular triggers for this phenotype remodeling are still unknown; (ii) age-induced neuromuscular denervation seems to be the principal driver for the loss of muscle mass and strength in sarcopenia; (iii) the muscle disuse experienced by cancer patients seems to have a key contribution to muscle loss in cancer cachexia; (iv) chronic inflammation is present in both conditions, but may contribute more to cancer cachexia pathogenesis; (v) nutrition-related mediators are distinctly regulated in each wasting condition. The integration of all these factors will certainly help in the definition of a panel of biomarkers to complement the current diagnostic tools, and therefore, to improve the clinical management of sarcopenia and cancer cachexia. Future investigations should consider: (i) the putative interplay between sarcopenia and cancer cachexia, since most of the clinical studies investigate aging/sarcopenia or cancer cachexia and preclinical ones also focus on cancer-induced muscle wasting in young animals (tumor induction between the 3rd and 4th weeks of age); and (ii) the impact of multidisciplinary approaches for the clinical management of

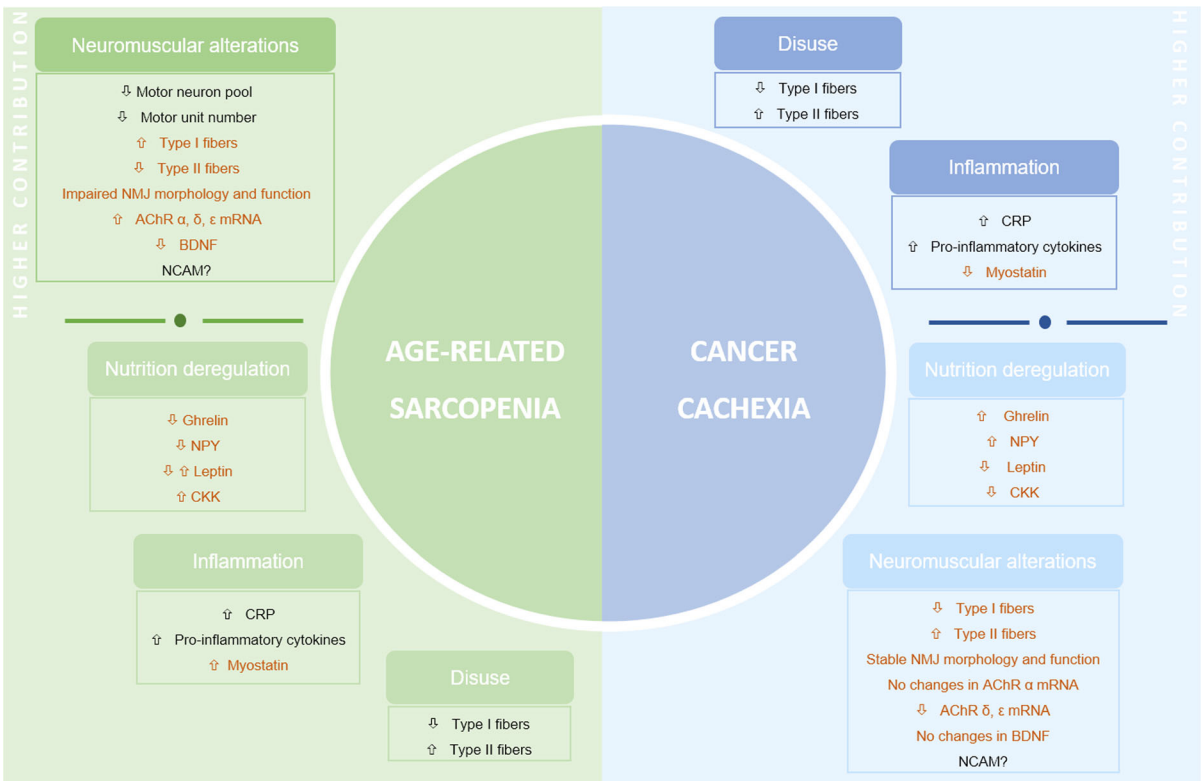

Fig. 3 An overview of the main mechanisms responsible for age-related sarcopenia or cancer cachexia. Age-induced neuromuscular alterations seem to have a higher contribution to the skeletal muscle phenotype remodeling that occurs in sarcopenia, whereas cancer-induced muscle disuse and inflammation appear to be key drivers for muscle remodeling in cancer cachexia. In orange are highlighted the differences between each condition. *AChR* acetylcholine receptor, *BDNF* brain-derived neurotrophic factor, *CKK* cholecystokinin, *CRP* C reactive protein, *NCAM* N-Cell adhesion molecule, *NPY* neuropeptide Y

these wasting conditions taking into consideration the physical and nutritional status, and sex.

Acknowledgements This work was supported by CIAFEL (UIDB/00617/2020), LAQV (UIDB/50006/2020) and CITAB (UIDB/04033/2020) research units and by A.M.P.'s fellowship (SFRH/BD/144396/2019) through national founds by the Portuguese Foundation for Science and Technology (FCT) and co-financed by the European Regional Development Fund (FEDER), within the PT2020 Partnership Agreement.

Authors' contributions AMP conducted the literature search and drafted the manuscript and PAO, RF and JAD critically revised the work.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval Not applicable.

Consent for publication Not applicable.

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Chapter II

Subchapter IIC

The molecular mechanisms involved in doxorubicin-induced skeletal muscle wasting

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Moreira-Pais, A., Ferreira, R., Costa, V. M., Oliveira, P. A., & Duarte, J. A. (2021). The molecular mechanisms involved in doxorubicin-induced skeletal muscle wasting. *Revista Portuguesa de Cirurgia*, *51*, 13–22. https://doi.org/10.34635/rpc.930

THE MOLECULAR MECHANISMS INVOLVED IN DOXORUBICIN-INDUCED SKELETAL MUSCLE WASTING

MECANISMOS MOLECULARES SUBJACENTES AO CATABOLISMO MUSCULAR PROMOVIDO PELA DOXORRUBICINA

ALEXANDRA MOREIRA-PAIS[1,2,3], RITA FERREIRA[2], VERA MARISA COSTA[4], PAULA A. OLIVEIRA[3], JOSÉ A. DUARTE[1,5]

1 CIAFEL, Faculty of Sport, University of Porto, Dr. Plácido da Costa 91, 4200-450 Porto, Portugal
2 LAQV-REQUIMTE, Department of Chemistry, University of Aveiro, 3810-193 Aveiro, Portugal
3 Centre for Research and Technology of Agro Environmental and Biological Sciences (CITAB), Inov4Agro, University of Trás-os--Montes and Alto Douro (UTAD), Quinta de Prados, 5000-801, Vila Real, Portugal
4 UCIBIO, REQUIMTE, Laboratory of Toxicology, Department of Biological Sciences, Faculty of Pharmacy, University of Porto, 4050-313 Porto, Portugal
5 TOXRUN – Toxicology Research Unit, University Institute of Health Sciences, CESPU, CRL, Gandra, Portugal

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].

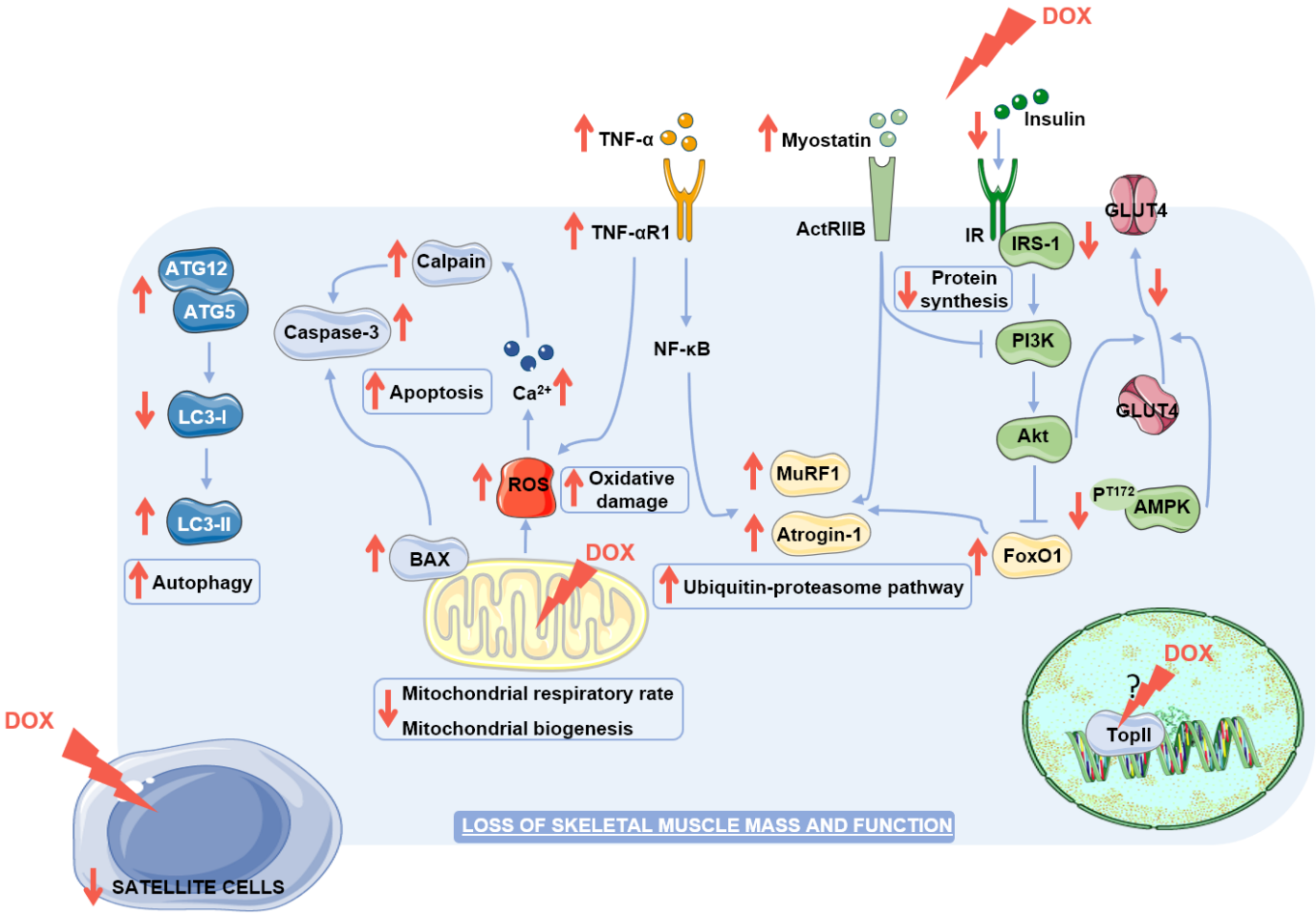

FIGURE 1 – **Key molecular mediators involved in doxorubicin (DOX)-induced skeletal muscle wasting. Administration of DOX seems to increase the activity of the ubiquitin-proteasome pathway, increasing muscle proteolysis, and decrease the activity of protein synthesis, in part by inducing insulin resistance. 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.

Acknowledgments

This work was supported by CIAFEL (UIDB/00617/2020), LAQV (UIDB/50006/2020) and CITAB (UIDB/04033/2020) research units and by A.M.P.'s fellowship (SFRH/BD/144396/2019) and V.M.C.'s grant (SFRH/BPD/110001/2015, under the transitional rule DL57/2016/CP1334/CT0006) through national founds by the Portuguese Foundation for Science and Technology (FCT) and co-financed by the European Regional Development Fund (FEDER), within the PT2020 Partnership Agreement.

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Correspondência:
ALEXANDRA MOREIRA-PAIS
e-mail: alexandrapais@ua.pt

Data de recepção do artigo:
03/03/2021

Data de aceitação do artigo:
11/05/2021

Chapter III

Experimental work

Chapter III

Subchapter IIIA

Mitochondrial remodeling underlying age-induced skeletal muscle wasting: let's talk about sex

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Moreira-Pais, A., Vitorino, R., Sousa-Mendes, C., Neuparth, M. J., Nuccio, A., Luparello, C., Attanzio, A., Novák, P., Loginov, D., Nogueira-Ferreira, R., Leite-Moreira, A., Oliveira, P. A., Ferreira, R., & Duarte, J. A. (2024). Mitochondrial remodeling underlying age-induced skeletal muscle wasting: let's talk about sex. (**submitted**)

Mitochondrial remodeling underlying age-induced skeletal muscle wasting: let's talk about sex

Alexandra Moreira-Pais[a,b,c], Rui Vitorino[d], Cláudia Sousa-Mendes[e], Maria João Neuparth[a,f], Alessandro Nuccio[b,g], Claudio Luparello[g], Alessandro Attanzio[g], Petr Novák[h], Dmitry Loginov[h], Rita Nogueira-Ferreira[e], Adelino Leite-Moreira[e,i], Paula A. Oliveira[c], Rita Ferreira[b], José A. Duarte[a,f]

[a] Research Center in Physical Activity, Health and Leisure (CIAFEL), Faculty of Sports, University of Porto (FADEUP) and Laboratory for Integrative and Translational Research in Population Health (ITR), 4200-450 Porto, Portugal

[b] LAQV-REQUIMTE, Department of Chemistry, University of Aveiro, 3810-193 Aveiro, Portugal

[c] Centre for Research and Technology of Agro Environmental and Biological Sciences (CITAB), Inov4Agro, University of Trás-os-Montes and Alto Douro (UTAD), Quinta de Prados, 5000-801 Vila Real, Portugal

[d] iBiMED - Institute of Biomedicine, Department of Medical Sciences, University of Aveiro, 3810-193 Aveiro, Portugal

[e] Cardiovascular R&D Center - UnIC@RISE, Department of Surgery and Physiology, Faculty of Medicine of the University of Porto, 4200-319 Porto, Portugal

[f] TOXRUN – Toxicology Research Unit, University Institute of Health Sciences, CESPU, CRL, 4585-116 Gandra, Portugal

[g] Department of Biological, Chemical and Pharmaceutical Sciences and Technologies (STEBICEF), University of Palermo, 90128 Palermo, Italy

[h] Laboratory of Molecular Structure Characterization, Institute of Microbiology, Academy of Sciences of the Czech Republic, Videnska 1083, CZ-142 20 Prague, Czech Republic

[i] Department of Cardiothoracic Surgery, Centro Hospitalar Universitário São João, 4200-319 Porto, Portugal

ORCIDs and emails:

Alexandra Moreira-Pais: 0000-0003-2582-1481; alexandrapais@ua.pt

Rui Vitorino: 0000-0003-3636-5805; rvitorino@ua.pt

Cláudia Sousa-Mendes: 0000-0002-4452-6149; csmendes@med.up.pt

Maria João Neuparth: 0000-0002-1464-8700; mneuparth@hotmail.com

Alessandro Nuccio: 0009-0009-5067-5667;
alessandro.nuccio@community.unipa.it

Claudio Luparello: 0000-0001-9821-5891; claudio.luparello@unipa.it

Alessandro Attanzio: alessandro.attanzio@unipa.it

Petr Novák: 0000-0001-8688-529X; pnovak@biomed.cas.cz

Dmitry Loginov: 0000-0002-4164-6145; dmitry.loginov@biomed.cas.cz

Rita Nogueira-Ferreira: 0000-0001-7059-8237; rmferreira@med.up.pt

Adelino Leite-Moreira: 0000-0001-7808-3596; amoreira@med.up.pt

Paula A. Oliveira: 0000-0001-9519-4044; pamo@utad.pt

Rita Ferreira: 0000-0002-6872-4051; ritaferreira@ua.pt

José A. Duarte: 0000-0003-4756-5917; jose.duarte@iucs.cespu.pt

Corresponding author:

Alexandra Moreira-Pais

Departamento de Química

Universidade de Aveiro

Campus Universitário de Santiago

3810-193 Aveiro

Portugal

e-mail: alexandrapais@ua.pt

Abstract

Sarcopenia is associated with reduced quality of life and increased premature mortality. The sex disparities in the processes underlying sarcopenia pathogenesis, which include mitochondrial dysfunction, are ill-understood and can be decisive for the optimization of sarcopenia-related interventions. To improve the knowledge regarding the sex differences in skeletal muscle aging, the *gastrocnemius* muscle of young and old female and male rats was analyzed with a focus on mitochondrial remodeling through the proteome profiling of mitochondria-enriched fractions. Data demonstrated that age induced skeletal muscle atrophy and fibrosis in both sexes. In females, however, this adverse skeletal muscle remodeling was more accentuated and might be attributed to an age-related reduction of 17beta-estradiol signaling through mitochondrial estrogen receptor alpha, which was accompanied by a females-specific mitochondrial remodeling that encompassed increased fatty acid oxidation, impaired oxidative phosphorylation, and enhanced proneness to oxidative posttranslational modifications. This conceivable accretion of damaged mitochondria in old females might be ascribed to a defective Parkin-mediated mitophagy. Despite skeletal muscle atrophy and fibrosis, males maintained their testosterone levels throughout aging, as well as their androgen receptor content, and the age-induced mitochondrial remodeling was limited to increased abundance of pyruvate dehydrogenase E1 component subunit beta and electron transfer flavoprotein subunit beta. Altogether, age affects more severely the skeletal muscle mitochondrial proteome of females, reinforcing the necessity of sex-personalized approaches towards sarcopenia management, and the inevitability of the assessment of mitochondrion-related therapeutics.

Keywords: fatty acid oxidation, mitophagy, oxidative phosphorylation, sarcopenia, sexual dimorphism

1. Introduction

The world undergoes a sustained transformation in the age organization of the population driven by increasing life expectancy and decreasing fertility levels, with the global population aged 65 years or older being expected to increase from 9.3% in 2020 to 16.0% by 2050 (United Nations Department of Economic and Social Affairs, 2020). This event is accompanied by diverse diseases namely sarcopenia, which is a muscle disease defined by low levels of muscle strength, muscle quantity or quality and physical performance that develops gradually throughout lifetime (Cruz-Jentoft et al., 2019). Sarcopenia is related to an increased risk of disability and consequent hospitalization, a higher hospital stay length, an increased risk of postoperative complications, frailty, mobility limitations, and premature mortality (Coletta & Phillips, 2023). The pathogenesis of sarcopenia is multifactorial and may involve systemic changes like hormonal alterations and chronic inflammation, and local alterations such as neuromuscular junction (NMJ) degeneration, muscle fat infiltration, mitochondrial dysfunction, and oxidative stress (Tian et al., 2023). Nonetheless, the precise role and contribution of each one of these processes to sarcopenia development and progression are not yet entirely recognized (Petermann-Rocha et al., 2022), making sarcopenia management challenging.

Mitochondrial dysfunction is advocated as a central factor associated with skeletal muscle aging with a growing body of evidence provided by both animal and human studies as elegantly reviewed by Bellanti and collaborators (Bellanti et al., 2021). Besides energy production, the mitochondrion performs essential functions in skeletal muscle fibers, namely the regulation of intracellular calcium homeostasis and apoptotic signaling (Tian et al., 2023). It is believed that the age-related decline of skeletal muscle quality is associated with a reprogramming of the skeletal muscle metabolism that results in an impaired uptake and utilization of glucose, fat and proteins, and a defective production of energy (Bellanti et al., 2021). Aging also appears to induce a shift in the redox status towards oxidation, which is accompanied by overwhelmed or dysfunctional antioxidant defense mechanisms, ensuing an amplified susceptibility of cellular and subcellular environments to damage (Ferri et al., 2020). Along with mitochondrial remodeling,

an age-related decrease in the levels of sex steroid hormones in both women and men has been proposed as an important driver of sarcopenia (Geraci et al., 2021; Shigehara et al., 2022). In fact, these hormones can modulate *via* their receptors distinct signaling pathways in skeletal muscle, but the precise outcomes and sex disparities are not completely acknowledged, principally in skeletal muscle aging (Kim et al., 2016). In mitochondrion, this sex specificity seems to also be present, with the suggestion that old females have lower function of the oxidative phosphorylation (OXPHOS) complexes than old males (Kramer et al., 2023; Triolo et al., 2022). To date, the mechanisms behind the effect of sex on sarcopenia development and on age-related mitochondrial remodeling in the skeletal muscle are yet to be fully uncovered. Coupled with this gap are the equivocal data retrieved from clinical studies on the effect of sex on skeletal muscle remodeling, probably due to different selection criteria that can bias the results, such as physical status and comorbidities. In this regard, the use of preclinical models allows the control of not only physical activity but also nutritional status, both of which can influence the outcomes of the studies. The recognition of sex-specific players involved in age-related skeletal muscle impairment will enable the development of preventive and therapeutic interventions tailored to the sex of each patient towards improved chances of survival and quality of life.

Hence, the overarching goal of this study was to investigate the effect of sex on age-related skeletal muscle wasting with a focus on mitochondrial remodeling. Specifically, we examined the *per se* effect of sex and aging and their interplay on the *gastrocnemius* muscle mass, fiber cross-sectional area (CSA) and fibrosis, and its association with the circulating levels of sex hormones and the muscle content of their receptors. Mass-spectrometry (MS) profiling of mitochondria-enriched fractions was performed to uncover the age-induced skeletal muscle mitochondrial remodeling, comprising the identification of mitochondrial proteins more prone to posttranslational modifications (PTMs) that may contribute to the aging phenotype. The use of rats in this study was a well thought-through choice based on the chronic nature of sarcopenia that takes years to develop in humans and the consequent extended time course and ethical concerns surrounding the

invasive acquisition of skeletal muscle biopsies from potentially frail individuals and also from healthy ones. Rats were selected based on evidence reporting that the pattern of skeletal muscle impairment in the rat (vs. mice) seems to be more representative of the human (Börsch et al., 2021). The *gastrocnemius* muscle was elected because it is believed to be affected by aging, it is fundamental for lower limb activities (Xie et al., 2021), for comparative proposes with another studies (Börsch et al., 2021; Xie et al., 2021) and its thickness was proposed as a muscle mass index for sarcopenia diagnosis (Yuguchi et al., 2022).

2. Materials and methods

2.1 Animal protocol

Female and male Wistar rats were obtained from the Charles River Laboratories (FR) at four (young groups) or twelve (old groups) weeks. Before the initiation of the experiments all rats were acclimatized to the handler and the experimental protocol for one week and then randomly distributed (three to four rats *per* cage; 1500U Eurostandard Type IV S cages, Tecniplast, Varese, IT) into the experimental groups: young female (n=7), old female (n=13), young male (n=8), old male (n=11). The rats were housed at controlled temperature (22 ± 2°C) and relative humidity (50 ± 5%) with a 12:12h light-dark cycle with *ad libitum* food (standard diet 4RF21, Mucedola, Milan, IT) and water access. To mimic young adult and middle-age humans, the rats from the young groups were sacrificed at 6 months old (corresponds to approximately 18 years in humans (Sengupta, 2013)) and the rats from the old groups at 19 months old (corresponds to approximately 45-50 years in humans (Sengupta, 2013)) by an intraperitoneal overdose of ketamine (75 $mg.kg^{-1}$, Imalgene 1000, Merial SAS, Lyon, FR) and xylazine (10 $mg.kg^{-1}$, Rompun 2 %, Bayer, Kiel, DE) followed by exsanguination as indicated by the Federation of European Laboratory Animal Science Associations (Forbes et al., 2007). Blood was collected from the heart, and the two *gastrocnemius* muscles of each rat were collected and weighed (*vide infra* for sample-specific processing). The right and left tibiae were dissected to determine tibia length, which is an indicator of animal body size that is independent of alterations in muscle and fat masses (Yin et al., 1982). All the

procedures were approved by the University of Trás-os-Montes and Alto Douro Ethics Review Body ORBEA (Orgão Responsável pelo Bem-Estar e Ética Animal, reference 424-e-DCV-2016) and by the Portuguese Competent Authority DGAV (Direção Geral de Alimentação e Veterinária, license numbers 021326 and 004583), according to the European Guidelines, and following the Portuguese law (decree-law number 113/2013) on animal protection for scientific purposes.

2.2 Serum samples preparation and analyses

The blood collected from the heart during the necropsy was allowed to clot and then centrifuged at 4°C for 15 minutes at 3000 *g*. The obtained serum was stored at -80°C for the subsequent biochemical analyses that follow.

The serum levels of cholesterol, triglycerides, fasting glucose, and lactate were measured on an AutoAnalyzer (PRESTIGE 24i, Cormay PZ, Diamond Diagnostics, MA, US). Enzyme-linked immunosorbent assays (ELISA) were performed to determine the serum levels of 17beta(β)-estradiol (ab108667, abcam, Cambridge, UK) and testosterone (E-39, InterMedical Diagnostics, Naples, IT).

2.3 Histological analyses of the gastrocnemius muscle

Half of one *gastrocnemius* muscle (n=5 *per* group) was fixed in 4% paraformaldehyde and embedded in paraffin to prepare paraffin blocks, which were sectioned (3 µm of thickness) using a microtome and mounted on silane-coated slides. After deparaffinization through incubation at 60°C for 30 minutes and with xylol for 10 minutes, the slides were hydrated in 5-minute incubations with decreasing concentrations of ethanol aqueous solutions (100%, 90% and 70%, v/v) and water. The slides were then stained with hematoxylin and eosin (H&E; Hematoxylin H and Eosin Y 1%, BioGnost, Zagreb, HR) or Sirius Red (Direct Red 80, Sigma Aldrich, Missouri, US) to analyze the CSA and fibrosis area, respectively. Skeletal muscle images were acquired with a microscope (Olympus XC30 Digital Color Camera, Olympus, Tokyo, JP) and analyzed at 400x (CSA) or 200x (fibrosis) magnification with Cell^B (version 5.1.0.2640, Olympus Soft Imaging Solutions, Tokyo, JP) and Image Pro Plus (version 6.0) software.

2.4 Preparation of gastrocnemius muscle homogenates and mitochondria-enriched fractions

Approximately 50 mg of the other half of the *gastrocnemius* muscle was homogenized with a Teflon® pestle in a motor-driven Potter-Elvehjem glass homogenizer at 0-4°C in a lysis buffer (0.5 mM EGTA, 10 mM HEPES, NaOH pH 7.4, 0.1% Triton X-100, 1:1000 PMSF and 1:400 protease inhibitor cocktail (P8340, Sigma-Aldrich, Missouri, US)) in the proportion of 50 mg of muscle to 1 mL of lysis buffer. The protein content of the muscle homogenates (henceforward named "total homogenate") was assessed using the commercial kit DC™ Protein Assay (Bio-Rad, CA, US), according to the manufacturer's instructions and using bovine serum albumin (BSA) as standard. Then, the total homogenates were preserved at -80°C for the subsequent biochemical analyses described in the subsections 2.5 and 2.6.

The other *gastrocnemius* muscle was used for mitochondria isolation based on Padrão and collaborators (Padrão et al., 2012) with modifications. For MS analyses pooled tissue samples were considered (n=6 *per* group). Briefly, muscles were finely minced in isolation buffer (20 mM MOPS, 1 mM EGTA, KOH pH 7.5, 110 mM KCl, and 2 mM PMSF) and incubated in 0.25 $mg.mL^{-1}$ of trypsin (Sigma-Aldrich, Missouri, US). After removing the supernatant, the muscles were suspended in an isolation buffer with 0.1% fatty acid-free BSA and homogenized with a motor-driven Teflon® Potter-Elvehjem homogenizer, followed by a centrifugation at 1000 *g* for 5 minutes. The collected supernatant was centrifuged at 12500 *g* for 10 minutes, followed by pellet resuspension in a washing medium (10 mM HEPES, KOH pH 7.4, 250 mM sucrose, and 1:1000 PMSF) and centrifuged at 12500 *g* for 10 minutes, followed by new pellet resuspension and centrifugation. Lastly, the obtained pellet, which contained the mitochondrial fraction, was resuspended in washing medium supplemented with 1:400 of protease inhibitor cocktail (P8340, Sigma-Aldrich, Missouri, US) and stored at -80°C for the posterior biochemical analyses described in the subsections 2.5 and 2.6. Regarding the pooled samples, the described procedure was equally implemented, with the exception that the final pellet containing the mitochondrial fraction was resuspended in 10 mM HEPES, KOH pH 7.4 (without protease

inhibitors) and stored at -80°C for the posterior biochemical analyses described in the subsection 2.7. The procedures were all performed at 0-4°C. The protein content of the mitochondria-enriched fractions was assessed using the commercial kit RC-DC™ Protein Assay (Bio-Rad, CA, US), according to the manufacturer's instructions and using BSA as standard.

2.5 Enzymatic assays in total homogenates and mitochondria-enriched fractions

Citrate synthase (CS) activity was assessed in total homogenates based on the method described by Coore and collaborators (Coore et al., 1971). Briefly, 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) reacted with the thiol groups (-SH) of coenzyme A (CoA), previously released by the reaction of acetyl-CoA with oxaloacetate, which was spectrophotometrically measured at 412 nm (molar extinction coefficient of 13.6 $mM^{-1}.cm^{-1}$). The activity of CS was expressed in µmol *per* minute *per* milligram of *gastrocnemius* total protein.

Lactate dehydrogenase (LDH) activity was evaluated in total homogenates according to Šimaga and collaborators (Šimaga et al., 2008). Concisely, the reduction of pyruvate to lactate was followed spectrophotometrically by the measurement of the decrease in the absorbance of nicotinamide adenine dinucleotide (NADH), due to its oxidation, at 340 nm (molar extinction coefficient of NADH 6.21 $mM^{-1}.cm^{-1}$). The specific activity of LDH was expressed in units *per* milligram of *gastrocnemius* total protein. One unit of enzyme activity was defined as the amount of enzyme that transforms 1 µmol of substrate in 1 minute at room temperature and pH 7.0.

Adenosine triphosphate (ATP) synthase activity was evaluated in mitochondria-enriched fractions according to Morin and collaborators (Morin et al., 2000). Succinctly, the reaction of phosphate, previously produced by the hydrolysis of ATP, with ammonium molybdate resulted in the formation of a complex that was reduced in the presence of the reducing agent ferrous sulfate, originating a colored complex (molybdenum blue), which was spectrophotometrically measured at 610 nm. Potassium dihydrogen phosphate was used as standard. The activity of ATP was expressed in µmol *per* minute *per* milligram of *gastrocnemius* total protein.

2.6 Immunoblot analyses in total homogenates and mitochondria-enriched fractions

To determine the content of protein carbonyls in mitochondria-enriched fractions, the samples were derivatized before the immunoblot analyses, as previously described (Padrão et al., 2012). Briefly, a certain volume (V) of the mitochondria-enriched fraction correspondent to 20 μg of protein was mixed with 1V of SDS 12% and 2V of 10 mM dinitrophenylhydrazine (DNPH) in 10% trifluoroacetic acid (TFA), followed by 30 minutes of dark incubation. After this, 1.5V of a solution constituted by 30% of glycerol and 18% of β-mercaptoethanol in Tris 2M was added. The derivatized samples were electrophoresed on a 12.5% SDS-PAGE gel according to Laemmli (Laemmli, 1970) and then blotted onto a nitrocellulose membrane (Amersham™, Protan®, GE Healthcare, Illinois, US) during 2 hours at 200 mA. The immunodetection was made using an anti-dinitrophenol (DNP) antibody (*vide infra*).

Regarding the remaining western blot analyses, equivalent volumes to 50 μg of protein of total homogenate or mitochondria-enriched fraction were electrophoresed on a 12.5% SDS-PAGE gel according to Laemmli (Laemmli, 1970) and then blotted onto a nitrocellulose membrane (Amersham™, Protan®, GE Healthcare, Illinois, US) during 2 hours at 200 mA. Protein loading was controlled by Ponceau S staining, since most of the housekeeping markers used can be affected by stressful stimuli in the skeletal muscle (Vigelsø et al., 2015).

Nonspecific binding was blocked by the incubation of the membranes in a 5% (w/v) solution of non-fat dry milk in Tris-buffered saline (TBS) with Tween 20 (TBST) for 1 hour at room temperature and with agitation. Then, each membrane was incubated with the corresponding primary antibody: anti-6-phosphofructokinase muscle type (PFKM, ab154804), anti-estrogen receptor alpha (ERα, ab32063), anti-peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1α, ab191838), anti-mitochondrial encoded ATP synthase membrane subunit 6 (MT-ATP6, ab192423), and anti-mitochondrial encoded cytochrome B (MT-CYB, ab81215) from Abcam (Cambridge, UK); anti-estrogen-related receptor alpha (ERRα, 07-662), anti-androgen receptor (AR, 06-

680), and anti-DNP (MAB2223) from Sigma-Aldrich (Missouri, US); anti-PTEN-induced kinase 1 (PINK1, sc517353), and anti-Parkin (sc-32282) from Santa Cruz Biotechnology (Texas, US); and anti-ubiquitin (AUB01) from Cytoskeleton Inc. (Colorado, US). This incubation was followed by three 10-minute washes with TBST, incubation with the corresponding secondary antibody (anti-rabbit (NA934V) or anti-mouse (NA931V) from GE Healthcare, Illinois, US) and new washes. Both primary and secondary antibodies were diluted 1:1000 in 5% (w/v) non-fat dry milk in TBST. The primary antibody was incubated overnight at 4°C, and the secondary antibody during 2 hours at room temperature and with agitation. Immunoreactive bands were detected with enhanced chemiluminescence (ECL) reagents (Clarity Max, Bio-Rad, CA, US) according to the manufacturer's procedure. Images were acquired using ChemiDoc Imaging System (Bio-Rad, CA, US) and analyzed using Image Lab software (version 6.0.0., Bio-Rad, CA, US). The optical density (OD) values were expressed in arbitrary units.

2.7 LC-MS/MS of mitochondria-enriched fractions

Mitochondria-enriched fractions were incubated with 50 mM 4-ethylmorpholine buffer (pH 8.5) supplemented with 6 M guanidine-HCl and 15% acetonitrile in the ratio 1:5 (v/v). After sonication of 30 seconds and incubation on ice for 30 minutes, the samples were centrifuged at 10000 *g* for 5 minutes and the supernatant was collected. Total protein concentration was measured using the commercial kit BCA Protein Assay (Thermo Fisher Scientific, Massachusetts, US). An aliquot containing 10 µg of total protein was diluted with 10 mM 4-ethylmorpholide (pH 8.5) to a final volume of 20 µL. Samples were reduced and alkylated by adding 10 mM Tris(2-carboxyethyl)phosphine and 30 mM 2-chloroacetamide, followed by incubation at 70°C for 5 minutes. A two-step digestion was performed. Firstly, LysC (Promega, Wisconsin, US) was added to the samples in the ratio 1:10 and incubated at 37°C for 1 hour. Secondly, trypsin (Promega, Wisconsin, US) was added in the ratio 1:10 and incubated at 37°C for 2 hours. The digestion process was terminated by the addition of TFA to a final concentration of 0.5% (v/v).

For MS and MS/MS analyses, samples were desalted using the StageTip approach (Rappsilber et al., 2007). LC-MS/MS analyses were performed by using Vanquish Neo UHPLC system (Thermo Fisher Scientific, Massachusetts, US) coupled with timsTOF SCP (Bruker Daltonics, Massachusetts, US).

Proteins were identified using MaxQuant software (version 1.6.17, Max Planck Institute of Biochemistry, Planegg, DE). The MS proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (Perez-Riverol et al., 2022) partner repository with the dataset identifier PXD048802. MS data were analyzed for quantitative comparisons using the Perseus software (version 2.0.7, Max Planck Institute of Biochemistry, Planegg, DE). Principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA) were applied to examine proteome dynamics between groups (the raw data were log-transformed for these analyses). Volcano plots were used to annotate proteins that were present in significantly different amounts between groups. These analyses were performed using the MetaboAnalyst web portal (version 5.0, www.metaboanalyst.ca, accessed in July 2023). We also sought for specific PTMs that were searched in MaxQuant and then further confirmed by manual inspection of MS spectra. Oxidations at methionine (Met), histidine (His), phenylalanine (Phe), tyrosine (Tyr) and tryptophane (Trp) were searched, given that these amino acids are generally most prone to oxidation due to the high reactivity of their side chains (e.g., sulfur-containing side chain and aromatic rings) towards various reactive oxygen species (ROS) (Grassi & Cabrele, 2019). Acetylation at lysine (Lys) was also searched since it is the most reported (Ramazi & Zahiri, 2021).

2.8 Statistical analyses

Data normality was evaluated by the Shapiro-Wilk test, except for CSA data where the Kolmogorov-Smirnov test was employed. The significant differences among groups were determined by performing the two-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons post-hoc test, and the values are present as mean ± standard deviation (SD). When statistically significant, the results from the two-way ANOVA are given below the corresponding graphic or in

the last column in the table. Pearson correlations were also performed, when considered relevant. Results were considered significantly different when $p<0.05$. *Gastrocnemius* muscle mass values were normalized to body weight and tibia length. All the statistical analyses were performed using the GraphPad Prism software for Windows (version 6.01, California, US).

3. Results

3.1 Characterization of the sex-specific systemic alterations and muscle morphological remodeling in response to aging

Table 1 shows that the body weight increased with age in both old female and male rats with males having a higher body weight than females independently of age. To eliminate variations due to body weight changes with aging, the *gastrocnemius* muscle mass was normalized to body weight and a decrease in both old female and male rats was observed, indicative of age-related skeletal muscle wasting. To eliminate variations due to animal size changes with aging, the *gastrocnemius* muscle mass was normalized to tibia length and a sex-related increase of the ratio value in males compared to females was observed, which may be explained by the fact that male rats are usually larger than female rats due to a persistent difference in their growth curve (Slob & van der Werff Ten Bosch, 1975).

To explore the effect of sex hormones on the age-induced skeletal muscle wasting observed, the serum levels of 17β-estradiol, which is the most potent estrogen that can also be produced by aromatization in the skeletal muscle (Tian et al., 2023), and testosterone were evaluated (**Table 1**). Old female rats had an approximately 79% decrease of the 17β-estradiol levels compared to young female rats and unchanged testosterone levels. No differences were observed in old male rats for either hormone.

Table 1 Anthropometric data given by body weight (BW), tibia length (TL), and *gastrocnemius* (GS) muscle mass in young female (n=7), old female (n=13), young male (n=8), and old male (n=11) rats. The *gastrocnemius* muscle mass normalized to body weight and tibia length is also depicted. The serum levels of 17β-estradiol and testosterone of young female (n=5), old female (n=10), young male (n=5) and old male (n=10) rats analyzed by ELISA are also displayed.

	Young Female	Old Female	Young Male	Old Male	% of total variation
BW (g)	278.71 ± 10.47$^{****,\#}$	405.93 ± 38.60	328.75 ± 20.24	638.64 ± 33.01$^{****,\#\#\#\#}$	Inter: 10.07, p<0.0001 Sex: 24.12, p<0.0001 Age: 57.66, p<0.0001
TL (cm)	3.6 ± 0.2$^{***,\#\#\#\#}$	4.0 ± 0.1	4.3 ± 0.1	4.7 ± 0.1$^{****,\#\#\#\#}$	Sex: 66.02, p<0.0001 Age: 20.94, p<0.0001
GS mass (g)	3.21 ± 0.21$^{\#\#\#\#}$	3.30 ± 0.10	4.58 ± 0.31	5.23 ± 0.35$^{****,\#\#\#\#}$	Inter: 2.11, p<0.01 Sex: 73.97, p<0.0001 Age: 3.87, p<0.001
GS mass/BW (%)	1.15 ± 0.09$^{****,\#\#\#\#}$	0.82 ± 0.07	1.40 ± 0.08	0.81 ± 0.04$^{\#\#\#\#}$	Inter: 6.26, p<0.0001 Sex: 5.35, p<0.0001 Age: 80.24, p<0.0001
GS mass/TL (g.cm^{-1}%)	89.7 ± 3.1$^{\#\#\#\#}$	83.1 ± 2.0	108.8 ± 6.1	111.9 ± 8.2****	Inter: 2.71, p<0.05 Sex: 65.35, p<0.0001
17β-estradiol serum levels (pg.mL^{-1})	60.34 ± 1.74$^{****,\#\#\#\#}$	12.55 ± 7.89	6.11 ± 2.88	11.06 ± 5.93	Inter: 47.00, p<0.0001 Sex: 52.46, p<0.0001 Age: 31.00, p<0.0001
Testosterone serum levels (pg.mL^{-1})	0.06 ± 0.05	0.00(4) ± 0.00(1)	2.13 ± 4.17	0.05 ± 0.01	

**** p<0.0001 vs. old female, *** p<0.001 vs. old female, $^{\#\#\#\#}$ p<0.0001 vs. young male, and $^{\#}$ p<0.05 vs. young male; Abbreviations: Inter, interaction.

Conventional biochemical analyses of serum samples were performed, where a typical aging phenotype (Liu & Li, 2015) was observed with an age-induced increase of cholesterol and triglycerides levels in both sexes (**Supplementary Figure 1**). An age-induced increase in lactate serum levels was also present in both sexes (**Supplementary Figure 1**), which jointly with unchanged muscle PFKM levels and LDH activity with aging (**Supplementary Figure 2**) indicates an impaired lactate clearance due to, for instance, decreased hepatic gluconeogenesis or uptake into skeletal muscle, as suggested by the literature (Horn et al., 1997; Masuda et al., 2009).

The observed age-induced loss of skeletal muscle mass in both sexes was allied to a decrease of the CSA of the *gastrocnemius* muscle fibers, which was higher in female rats (age-induced decrease of approximately 19% and 15% in, respectively, females and males; **Figure 1A**). In accordance, old rats had a higher percentage of muscle fibers with inferior CSA, varying from 676-5357 μm^2 and 793-7117 μm^2 in, respectively, old female and male rats compared to 1082-7510 μm^2 and 1372-6996 μm^2 of, respectively, young female and male rats (**Figure 1B**). In contrast, old rats had increased fibrosis area, which was higher in females (approximately 3.4-fold and 3.0-fold age-induced increase in, respectively, females and males; **Figure 1C**). In female rats, the fibers CSA was positively correlated with the serum levels of 17β-estradiol and testosterone, whereas the fibrosis area was negatively correlated with the serum levels of 17β-estradiol (**Figure 1F**). For male animals, no correlation was found between histological findings and the circulating levels of sex hormones (**Supplementary Figure 3**)

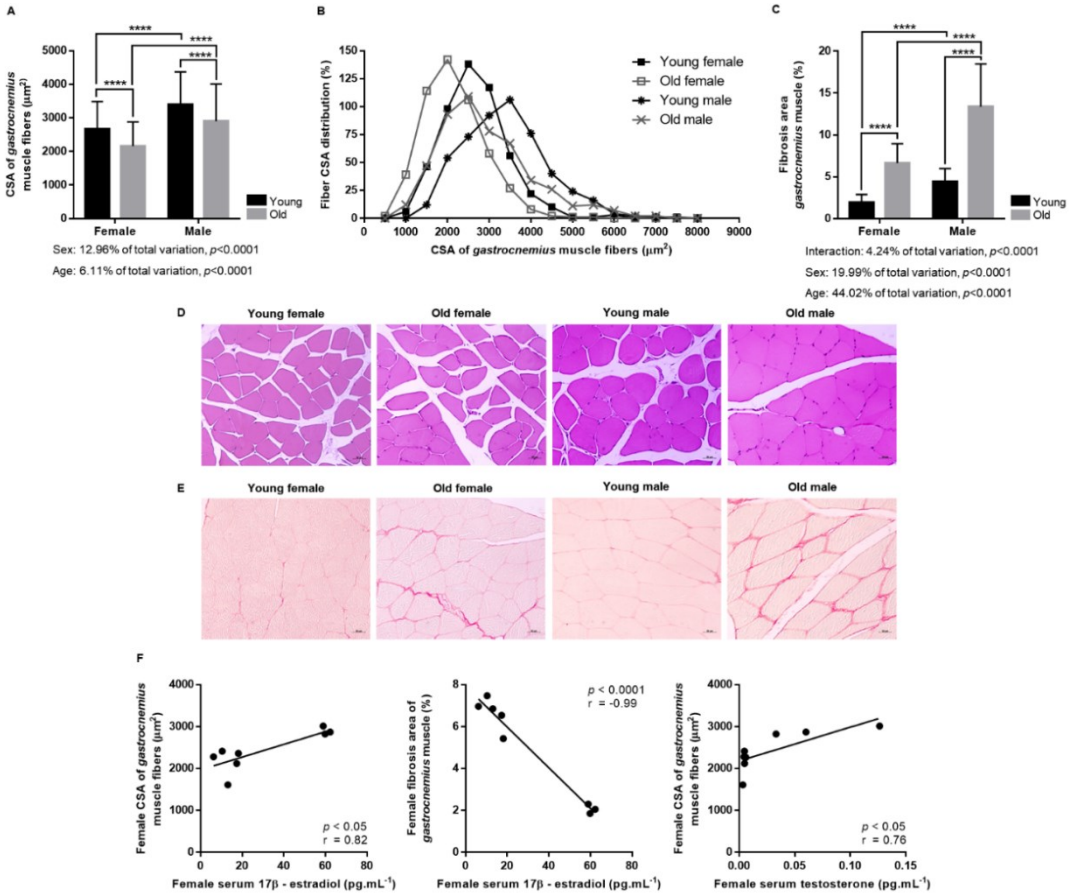

Figure 1 (A) Cross-sectional area (CSA) of the *gastrocnemius* muscle fibers, (B) fiber CSA distribution of the *gastrocnemius* muscle, and (C) fibrosis area of the

gastrocnemius muscle (n=5 *per* group in each analysis; **** $p<0.0001$). Representative photomicrographs of (D) H&E-stained and (E) Sirius Red-stained *gastrocnemius* muscle sections. The bar scale represents 50 μm. (F) Pearson correlations were performed for an in-deep understanding of the results.

3.2 Characterization of the sex-specific mitochondrial remodeling in response to aging

The assessment of the age-related mitochondrial remodeling was initiated by a general analysis in the total homogenate of common mitochondrial turnover-related proteins. The activity of CS was maintained throughout aging in both sexes but the levels of PGC1α increased with age in females, which was accompanied by decreased Parkin levels (**Figure 2A**).

Given the sex viewpoint of this study, the levels of ERα, ERRα (due to its possible contribution to mitochondrial function and skeletal muscle regeneration (Casaburi et al., 2018; Yoh et al., 2023)) and AR were analyzed. Aging had no effect on the levels of these receptors when total homogenate was evaluated (**Figure 2A**); however, aging affected the ERα mitochondrial pool of females, which was correlated with the decreased 17β-estradiol serum levels observed in these rats (**Figure 2B and 2C**). In addition, mitochondrial density (given by CS activity) was positively correlated to whole muscle AR levels in both sexes, indicating that AR mitochondrial pool impacts whole muscle levels (**Figure 2C**).

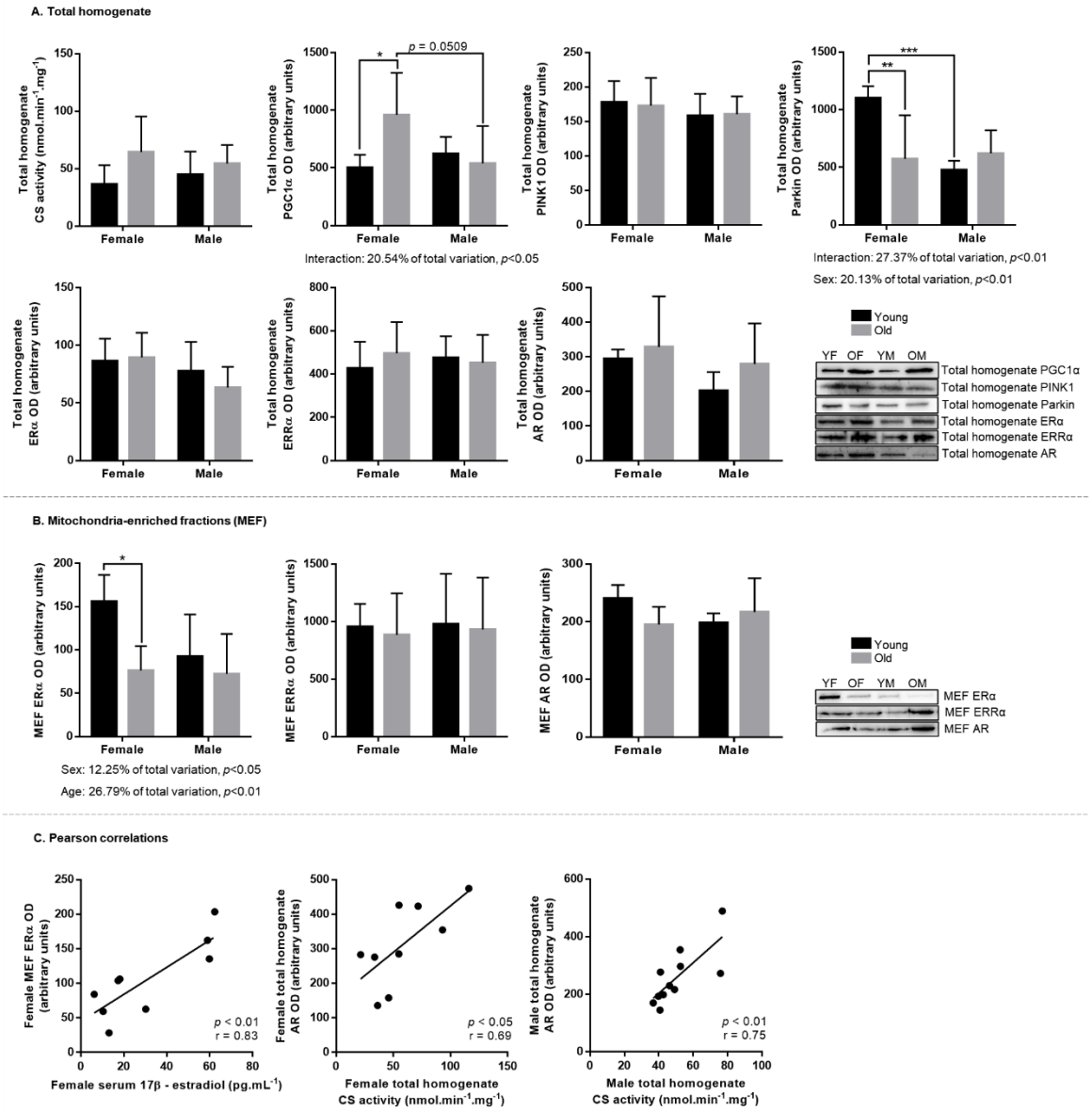

Figure 2 The effect of aging and sex on the levels of PGC1α, PINK1, Parkin, ERα, ERRα and AR levels evaluated by immunoblotting in young female (YF), old female (OF), young male (YM) and old male (OM) rats in the (A) total homogenate (YF n=6, OF n=7, YM n=7 and OM n=7) or (B) mitochondria-enriched fractions (MEF; YF n=6, OF n=7, YM n=6 and OM n=7). (A) CS activity was spectrophotometrically evaluated in the total homogenate (YF n=6, OF n=7, YM n=7 and OM n=7). Representative immunoblot images are displayed. (C) Pearson correlations were performed for an in-deep understanding of the results (refer to **Supplementary Figure 4** for non-significant correlations; *** p<0.001, ** p<0.01, and * p<0.05)

The mitochondrial proteome was then thoroughly explored by LC-MS/MS profiling of mitochondria-enriched fractions and consolidated with the analysis of some specific protein markers performed in mitochondria-enriched fractions, as follow.

LC-MS/MS identified 445 distinct proteins with at least two unique peptides and false discovery rate (FDR) of 1%. From these, 44% (196 proteins) were assigned as mitochondrial proteins according to MitoCarta3.0 (Rath et al., 2021). However, data from *Rattus norvegicus* is not included in this inventory, and so, the comparison was made with data from *Mus musculus*. In addition, some of the identified proteins may be moonlighting proteins, thus having more than one location not yet validated and considered in MitoCarta3.0. Among the biological processes identified, the most predominant were maintenance of mitochondria location, metabolism, and regulation of skeletal muscle contraction. From the identified proteins, 364 were common to all groups, as demonstrated in the Venn diagram (**Supplementary Figure 5**).

Unsupervised PCA and supervised PLS-DA were performed to visualize group separation based on proteome datasets by means of dimensionality reduction. PCA analyses showed no clustering of proteome data *per* group (**Figure 3A**), with a higher heterogeneity noticed in old male and young female rats. PLS-DA showed group separation being the age effect more prominent (**Figure 3B**). To gain a better insight into the *per se* effect of sex and age, pairwise PCA and PLS-DA were performed. While PCA analyses showed no clustering of the proteome data, PLS-DA analyses demonstrated a similar separation between ages and between sexes, in an independent manner (**Figure 3C to 3J**).

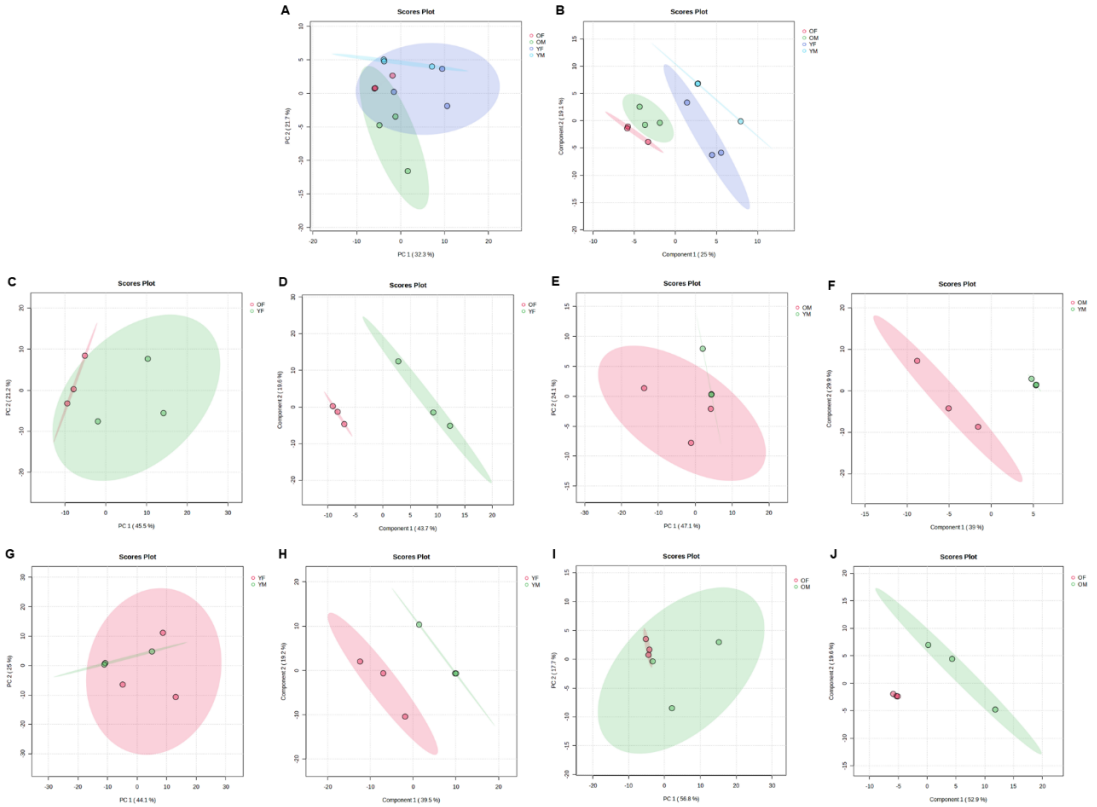

Figure 3 (A, C, E, G, and I) PCA and (B, D, F, H, and J) PLS-DA analyses of the mitochondrial proteome data (YF, young female; OF, old female; YM, young male; OM, old male)

To identify proteins with statistically significant changes between groups, volcano plot analyses, which show $\log_2$ of fold-change vs. $\log_{10}$ of the *p*-value, were conducted, which resulted in the identification of 41 proteins (**Supplementary Figure 6**). Approximately 80.5% (33 proteins) of these proteins are localized in the mitochondrion of *Rattus norvegicus* (according to UniProt Knowledgebase (UniProtKB) (Consortium, 2023) and Gene Ontology (GO)), and their details can be observed in **Table 2**. These proteins were included in a protein-protein interaction (PPI) analysis in STRING that demonstrated the interaction of trifunctional enzyme subunit beta (HADHB) with ERα and of CS with ERRα, whereas no interactions with AR were found (**Supplementary Figure 7**).

The mitochondrial proteome of females was more susceptible to age-related alterations, which will be briefly described. Increased amounts of HADHB, hydroxyacyl-coenzyme A dehydrogenase (HADH), enoyl-CoA delta isomerase

(ECI1), and electron transfer flavoprotein subunit beta (ETFB) were observed, pointing towards an escalated fatty acid oxidation (FAO) in old female rats. Specific subunits of the OXPHOS complexes were also affected by aging in females, namely, decreased abundance of NADH dehydrogenase [ubiquinone] flavoprotein 2 (NDUFV2) from complex I, and increased and decreased abundance of, respectively, cytochrome b-c1 complex subunit 8 (UQCRQ) and cytochrome b-c1 complex subunit 1 (UQCRC1) of complex III. Following these results, immunoblot analyses of mitochondrial deoxyribonucleic acid (mtDNA)-encoded proteins, which are essential for the function of the OXPHOS system, were performed (**Figure 4**). While the levels of the subunit MT-ATP6 of ATP synthase were decreased in old female rats (vs. young females) and correlated with reduced ATP synthase activity, the levels of subunit MT-CYB of complex III were not modulated by age in either sex.

Regarding males, the age-related alterations of the mitochondrial proteome were circumscribed to an increased abundance of pyruvate dehydrogenase E1 component subunit beta (PDHB) and ETFB.

The effect of sex at young and old ages was also noticed with, for instance, females having a higher abundance of proteins involved in tricarboxylic acid (TCA) cycle and amino acid catabolism at young age, and decreased abundance of proteins involved in ion transport at old age, compared to males. Of note, the abundance of phosphoglycerate kinase 1 (PGK1), which under stress stimuli translocates to the mitochondrion, was increased in old females compared to old males, which might have contributed to the increased lactate serum levels in old females (vs. old males) due to the PGK1-mediated inhibition of the pyruvate metabolism (Li et al., 2016).

Table 2 List of mitochondrial proteins, identified by LC-MS/MS, whose abundance, given by the fold change (log_2), was significantly different ($p<0.05$) between groups in mitochondria-enriched fractions. Description of each protein including the accession number (AC) and the main biological process(es) are also present (according to UniProtKB database and GO for *Rattus norvegicus*).

			Fold change (log_2)				
AC UniProt	**Protein name**	**Gene name**	**OF vs. YF**	**OM vs. YM**	**YF vs. YM**	**OF vs. OM**	**Main biological process(es)**
Q60587	Trifunctional enzyme subunit beta, mitochondrial	*Hadhb*	1.40			1.31	Fatty acid metabolism
Q9WVK7	Hydroxyacyl-coenzyme A dehydrogenase, mitochondrial	*Hadh*	1.21				Fatty acid metabolism
P23965	Enoyl-CoA delta isomerase 1, mitochondrial	*Eci1*	1.49				Fatty acid metabolism
Q704S8	Carnitine O-acetyltransferase	*Crat*			1.38		Fatty acid metabolism
P08461	Dihydrolipoyllysine-residue acetyltransferase component of pyruvate dehydrogenase complex, mitochondrial	*Dlat*	1.30				Carbohydrate/glucose metabolism Tricarboxylic acid cycle
P49432	Pyruvate dehydrogenase E1 component subunit beta, mitochondrial	*Pdhb*		1.57		- 0.81	Carbohydrate/glucose metabolism Tricarboxylic acid cycle
P16617	Phosphoglycerate kinase 1	*Pgk1*				1.57	Glucose metabolism
Q01205	Dihydrolipoyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex, mitochondrial	*Dlst*				- 0.87	Tricarboxylic acid cycle
P13086	Succinate-CoA ligase [ADP/GDP-forming] subunit alpha, mitochondrial	*Suclg1*				1.69	Tricarboxylic acid cycle
P56574	Isocitrate dehydrogenase [NADP], mitochondrial	*Idh2*			0.98		Tricarboxylic acid cycle
Q8VHF5	Citrate synthase, mitochondrial	*Cs*			1.05		Tricarboxylic acid cycle
P10860	Glutamate dehydrogenase 1, mitochondrial	*Glud1*			1.14		Tricarboxylic acid cycle Regulation insulin secretion Glutamate catabolism
P12007	Isovaleryl-CoA dehydrogenase, mitochondrial	*Ivd*			1.23		Leucine catabolism
Q5I0C3	Methylcrotonoyl-CoA carboxylase subunit alpha, mitochondrial	*Mccc1*			1.49		

			Fold change (log_2)				
AC UniProt	**Protein name**	**Gene name**	**OF vs. YF**	**OM vs. YM**	**YF vs. YM**	**OF vs. OM**	**Main biological process(es)**
P07633	Propionyl-CoA carboxylase beta chain, mitochondrial	*Pccb*			1.58		Amino acid catabolism Fatty acid catabolism
P51650	Succinate-semialdehyde dehydrogenase, mitochondrial	*Aldh5a1*	1.21				Gamma-aminobutyric acid catabolism
Q02253	Methylmalonate-semialdehyde dehydrogenase [acylating], mitochondrial	*Aldh6a1*			2.13		Beta-alanine, thymine and valine catabolism
Q68FU3	Electron transfer flavoprotein subunit beta	*Etfb*	1.13	1.17			Electron transport
F7FFF0	Cytochrome b-c1 complex subunit 7	*LOC685596*	1.63				Electron transport
Q7TQ16	Cytochrome b-c1 complex subunit 8	*Uqcrq*	1.31				Electron transport
Q68FY0	Cytochrome b-c1 complex subunit 1, mitochondrial	*Uqcrc1*	- 1.00			- 0.98	Electron transport
P19234	NADH dehydrogenase [ubiquinone] flavoprotein 2, mitochondrial	*Ndufv2*	- 1.88				Electron transport
A9UMV9	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 7	*Ndufa7l*	2.36			1.96	Electron transport
B2RZD6	NDUFA4, mitochondrial complex associated	*Ndufa4*	1.61		-1.22		Positive regulation of cytochrome *c* oxidase activity
P29418	ATP synthase subunit epsilon, mitochondrial	*Atp5e*	3.39				ATP synthesis
P85834	Elongation factor Tu, mitochondrial	*Tufm*				1.01	Protein biosynthesis
P16036	Solute carrier family 25 member 3	*Slc25a3*	- 1.74				Phosphate ion transmembrane transport
F1LX07	Electrogenic aspartate/glutamate antiporter SLC25A12, mitochondrial	*Slc25a12*	- 1.19				Aspartate and L-glutamate transmembrane transport
P81155	Voltage-dependent anion-selective channel protein 2	*Vdac2*				- 0.83	Ion transport
Q9R1Z0	Voltage-dependent anion-selective channel protein 3	*Vdac3*				- 1.80	Ion transport
P09605	Creatine kinase S-type, mitochondrial	*Ckmt2*				1.12	Phosphocreatine biosynthesis
A0A8I5ZLF3	AFG3 like matrix AAA peptidase subunit 2	*Afg3l2*	1.19				Proteolysis
P48721	Stress-70 protein, mitochondrial	*Hspa9*	1.18				Protein refolding

			Fold change (log_2)				
AC UniProt	**Protein name**	**Gene name**	**OF vs. YF**	**OM vs. YM**	**YF vs. YM**	**OF vs. OM**	**Main biological process(es)**
P26772	10 kDa heat shock protein, mitochondrial	*Hspe1*	1.05				Protein refolding

Abbreviations: OF, old female; YF, young female; OM, old male; YM, young male

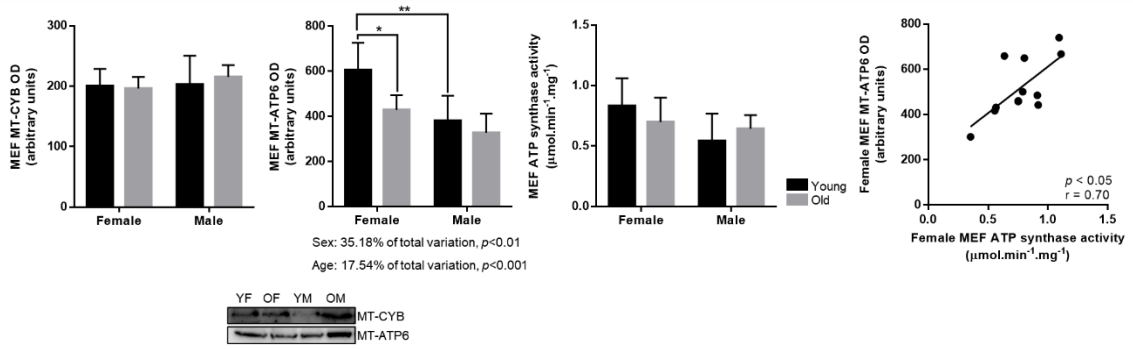

Figure 4 The effect of aging and sex in the levels of MT-CYB and MT-ATP6 evaluated by immunoblotting, and in the activity of ATP synthase evaluated spectrophotometrically in mitochondria-enriched fractions (MEF) of young female (YF, n=6), old female (OF, n=7), young male (YM, n=6) and old male (OM, n=7) rats. Representative immunoblot images are displayed. Pearson correlations were performed for an in-deep understanding of the results (refer to **Supplementary Figure 8** for the non-significant correlation in males; ** p<0.01 and * p<0.05)

To further understand the effect of age, sex, and their possible interplay on mitochondrial proteome remodeling, we sought for specific PTMs. **Table 3** lists the mitochondrial proteins identified as being more prone to oxidation and acetylation. Anew, the mitochondrial proteome of old female rats had a higher susceptibility to oxidation than the one of males. Indeed, only ATP synthase subunit alpha (ATP5A1) was found oxidized at Met51 in old male rats. In old female rats, the proteins more prone to oxidation are involved in TCA cycle, including isocitrate dehydrogenase [NADP] (IDH2) and isocitrate dehydrogenase [NAD] subunit alpha (IDH3A) at, respectively, Met411 and Met206, and in OXPHOS, namely subunits ATP5A1 and ATP5MPL from ATP synthase at, respectively, Met189 and Met1. The higher susceptibility of OXPHOS proteins to oxidation is well known given the proximity of the complexes to mitochondria-generated ROS (Liemburg-Apers et al., 2015). Some oxidative PTMs were found

in both young and old female rats or in both female and male young rats, but their levels were unchanged in response to aging or sex (data not shown). Malate dehydrogenase (MDH2), which is involved in TCA, was the only protein found to be acetylated. This PTM occurred at Lys307 in young female rats, and it is believed to enhance the enzyme activity by 3.9-fold without any other noteworthy effects reported (Venkat et al., 2017).

Table 3 List of mitochondrial proteins more prone to oxidization or acetylation in mitochondria-enriched fractions, including their accession number (AC). The amino acid (AA) and the position of the modification (PM) are depicted. The group(s) where the modification was found is(are) also shown (G). The main biological process(es) of each protein is(are) described (according to UniProtKB database and GO for *Rattus norvegicus*).

AC Uniprot	Protein name	Gene name	AA	PM	Sequence	G	Main biological process(es)
OXIDATION							
P56574	Isocitrate dehydrogenase [NADP], mitochondrial	*Idh2*	M	411	QTLEKVCVQTVESGAMTKDLAGCIHGLSNVK	OF	Tricarboxylic acid cycle
P56574	Isocitrate dehydrogenase [NADP], mitochondrial	*Idh2*	M	293	FDKNKIWYEHRLIDDMVAQVLKSSGGFVWAC	YF	Tricarboxylic acid cycle
Q99NA5	Isocitrate dehydrogenase [NAD] subunit alpha, mitochondrial	*Idh3a*	M	206	HRSNVTAVHKANIMRMSDGLFLQKCREVAEN	OF	Tricarboxylic acid cycle
P04636	Malate dehydrogenase, mitochondrial	*Mdh2*	M	266	MAYAGARFVFSLVDAMNGKEGVIECSFVQSK	YM	Tricarboxylic acid cycle
P21913	Succinate dehydrogenase [ubiquinone] iron-sulfur subunit, mitochondrial	*Sdhb*	M	73	PRMQTYKVDLNKCGPMVLDALIKIKNEIDST	YF YM	Tricarboxylic acid cycle Electron transport

AC Uniprot	Protein name	Gene name	AA	PM	Sequence	G	Main biological process(es)
D3ZS58	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 2	*Ndufa2*	M	89	NVSLNNLSAAEVTKAMENVLSGKA	OF YM	Electron transport
P32551	Cytochrome b-c1 complex subunit 2, mitochondrial	*Uqcrc2*	M	217	EELHYFVQNHFTSARMALVGLGVSHSILKEV	YF	Electron transport
P32551	Cytochrome b-c1 complex subunit 2, mitochondrial	*Uqcrc2*	M	437	DVVKAAKKFVSGKKSMTASGNLGHTPFLDEL	YM	Electron transport
P11240	Cytochrome c oxidase subunit 5A, mitochondrial	*Cox5a*	M	70	YFNKPDIDAWELRKGMNTLVGYDLVPEPKII	OF YF YM	Electron transport
P12075	Cytochrome c oxidase subunit 5B, mitochondrial	*Cox5b*	M	64	REIMIAAQRGLDPYNMLPPKAASGTKEDPNL	YM	Electron transport
P11951	Cytochrome c oxidase subunit 6C-2	*Cox6c2*	M	12	MSSGALLPKPQMRGLLAKRLRVHIVGA	OF YM	Electron transport
P35171	Cytochrome c oxidase subunit 7A2, mitochondrial	*Cox7a2*	M	41	NKVPEKQKLFQEDNGMPVHLKGGTSDALLYR	YF	Electron transport
A0A8I6AES7	NADH dehydrogenase [ubiquinone] iron-sulfur protein 7, mitochondrial	*Ndufs7*	F	115	HMAAPRYDMDRFGVVFRASPRQADVMIVAGT	YM	Electron transport
P15999	ATP synthase subunit alpha, mitochondrial	*Atp5a1*	M	189	LKAPGIIPRISVREPMQTGIKAVDSLVPIGR	OF	ATP synthesis
P15999	ATP synthase subunit alpha, mitochondrial	*Atp5a1*	M	324	IIYDDLSKQAVAYRQMSLLLRRPPGREAYPG	YF	ATP synthesis
P15999	ATP synthase subunit alpha, mitochondrial	*Atp5a1*	M	51	LHASNTRLQKTGTAEMSSILEERILGADTSV	OM YF	ATP synthesis
P15999	ATP synthase subunit alpha, mitochondrial	*Atp5a1*	Y	311	MGEYFRDNGKHALIIYDDLSKQAVAYRQMSL	YF YM	ATP synthesis
P10719	ATP synthase subunit beta, mitochondrial	*Atp5b*	M	145	APIKIPVGPETLGRIMNVIGEPIDERGPIKT	OF YF YM	ATP synthesis

AC Uniprot	Protein name	Gene name	AA	PM	Sequence	G	Main biological process(es)
P10719	ATP synthase subunit beta, mitochondrial	*Atp5b*	M	408	GIYPAVDPLDSTSRIMDPNIVGSEHYDVARG	YF YM	ATP synthesis
P10719	ATP synthase subunit beta, mitochondrial	*Atp5b*	M	113	EVAQHLGESTVRTIAMDGTEGLVRGQKVLDS	YF YM	ATP synthesis
D3Z9R8	ATP synthase subunit ATP5MPL, mitochondrial	*Atp5mpl*	M	1	MLQSFIKKVWVPMKPY	OF	ATP synthesis
Q6P9Y4	ADP/ATP translocase	*Slc25a4*	W	275	KIARDEGGKAFFKGAWSNVLRGMGGAFVLVL	OF	ADP/ATP transmembrane transport
P09605	Creatine kinase S-type, mitochondrial	*Ckmt2*	M	64	PPSADYPDLRKHNNCMAECLTPTIYAKLRNK	YM	Phosphocreatine biosynthesis
P09605	Creatine kinase S-type, mitochondrial	*Ckmt2*	M	213	GLKGDLAGRYYKLSEMTEQDQQRLIDDHFLF	YM	Phosphocreatine biosynthesis
ACETYLATION							
P04636	Malate dehydrogenase, mitochondrial	*Mdh2*	K	307	LLLGKKGLEKNLGIGKITPFEEKMIAEAIPE	YF	Tricarboxylic acid cycle

Abbreviations: YF, young female; OF, old female; YM, young male; OM, old male; M, methionine; F, phenylalanine; Y, tyrosine; W, tryptophane; K, lysine

Thenceforth the PTMs identified, ROS-induced carbonylation and the levels of total ubiquitination were examined by immunoblotting and no age-related alterations were noticed (**Figure 5**). In addition, no differences were observed in the bands profile between groups in the DNP blot. Concerning the analysis of protein ubiquitination, only one band between 50-75 kDa was detected in the blot, which may correspond to PINK1 or Parkin given their molecular weight, presence on mitochondria and known susceptibility to this PTM (Durcan et al., 2014; Liu et al., 2017). Ubiquitination of PINK1 ensures PINK1 homeostasis and function as a mitochondrial quality control factor (Liu et al., 2017) and ubiquitination of Parkin inhibits mitophagy (Durcan et al., 2014). The negative correlation between ubiquitinated and DNP-labeled proteins also indicates that low ubiquitin-dependent degradation contributes to the accretion of mitochondrial proteins damaged by carbonylation.

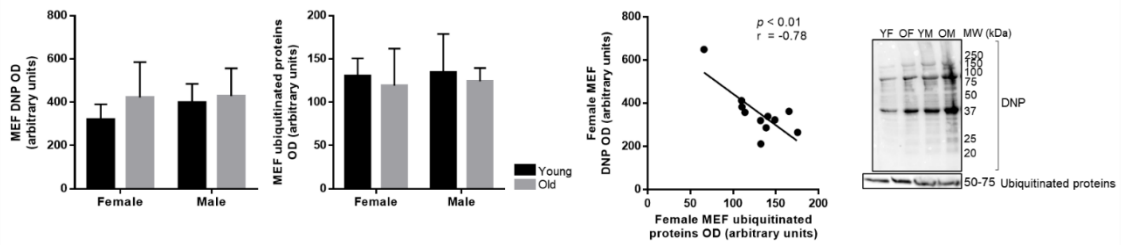

Figure 5 The effect of aging and sex on the levels of DNP and ubiquitinated proteins in young female (YF, n=6), old female (OF, n=7), young male (YM, n=6) and old male (OM, n=7) rats, evaluated in mitochondria-enriched fractions (MEF) by immunoblotting. Representative immunoblot images are displayed along with the molecular weight (MW). Pearson correlations were performed for an in-deep understanding of the results (refer to **Supplementary Figure 9** for the non-significant correlation in males).

4. Discussion

Data from the present study highlight the higher susceptibility of females to age-induced atrophy and fibrosis of the *gastrocnemius* muscle than males, which was associated with decreased 17β-estradiol levels. In agreement, the mitochondrial proteome of female rats was more prone to the effects of aging than the one of male rats and included a potential increase in FAO and impairment of OXPHOS function accompanied by a higher susceptibility to oxidative modifications. These data propose a sex-specificity in the process of aging of the skeletal muscle mitochondrial proteome that may support sex disparities in age-related skeletal muscle wasting, which will be now dissected.

Both old female and male rats presented atrophy of the g*astrocnemius* muscle (**Table 1**), substantiated by decreased relative *gastrocnemius* muscle mass and fibers CSA, which was accompanied by an increased fibrosis (**Figure 1**). Both histopathological modifications have been linked with impaired skeletal muscle functionality (Mahdy, 2019). This adverse age-induced remodeling of the muscle was more accentuated in females, which was associated to their diminished 17β-estradiol serum levels (**Figure 1F**; being 19 months old, these rats were expected to be in reproductive senescence, mimicking women menopause (Ferreira et al., 2012)). Low levels of this sex hormone may hamper skeletal muscle regeneration

given its role in the activation and proliferation of satellite cells and in the downregulation of inflammation (after muscle damage) and oxidative stress (La Colla et al., 2015). In agreement, the mitochondrial pool of ERα was reduced in old female rats (**Figure 2B**) and its correlation with the decreased 17β-estradiol levels (**Figure 2C**) insinuates an age-related dwindled signaling of this sex hormone through mitochondrial ERα. The idea of a modulation of mitochondrial functionality by this sex hormone was previously hypothesized (Chen et al., 2004). In skeletal muscle, a diminished ERα signaling might impair mitophagy and mtDNA replication and increase the production of ROS (Hevener et al., 2021). Our findings substantiate an impaired mitophagy, evidenced by diminished levels of Parkin (**Figure 2A**), and a decreased content of the ATP synthase subunit MT-ATP6 originated from mtDNA (**Figure 4**), in the muscle of old females compared to their young counterparts. Age-related skeletal muscle impairment is also vastly associated to reduced testosterone levels, particularly in men. Herein, testosterone levels, as well as AR levels, remained unchanged from young to old ages in male and female rats despite *gastrocnemius* muscle atrophy and fibrosis (**Figure 1**, **Figure 2A** and **Figure 2B**). These results emphasize the magnitude of the non-generalization of the association between reduced skeletal muscle mass and strength and low testosterone levels (Barone et al., 2022), which can ensue the non-diagnosis of more than 80% of men that preserve normal levels throughout life (Hackett et al., 2023). To reinforce with a clinical viewpoint, sarcopenia was formerly diagnosed in older men with normal testosterone levels (Yuki et al., 2013). Still, in female rats, low testosterone levels seem to contribute to *gastrocnemius* muscle atrophy given the significant correlation observed (**Figure 1F**).

Mitochondrial remodeling, the hub of this study, was initially perceived by the analysis of commonly analyzed markers of mitochondrial turnover. To start, age appeared to trigger mitochondrial biogenesis in female rats due to their elevated PGC1α levels (**Figure 2A**), which might reflect an endeavor to replace dysfunctional non-intact mitochondria, resulting in the unchanged CS activity observed. Nonetheless, an age-related decline in mitochondrial density due to a reduced biogenesis is pointed out by literature (Peterson et al., 2012).

Subsequently, the mitochondrial proteome was more exhaustively investigated by means of MS-based proteomics. The results revealed that females are more prone to age-induced mitochondrial alterations than males, with age simply increasing the abundance of PDHB and ETFB in male rats, vaguely suggesting an enhanced aerobic metabolism.

In contrast, categorical processes were found altered in old female rats, which will be now looked over. An age-induced escalated FAO was discerned in females that presented higher abundance of HADHB (also known as 3-ketoacyl-CoA thiolase, specific for long-chain fatty acid intermediates), HADH (also known as short-chain (S)-3-hydroxyacyl-CoA dehydrogenase, SCHAD, highest specificity to short-chain fatty acid intermediates) and ECI1 (degradation of mono- and poly-unsaturated fatty acids) (**Table 2**) (Houten et al., 2016). It is worth noting that HADHB is believed to be negatively modulated by 17β-estradiol and tamoxifen (which can also function as an agonist) (Zhou et al., 2012), possibly enlightening, at least in part, why HADHB levels were elevated in old female rats that had low 17β-estradiol levels. This age-related higher dependance on FAO of females may reflect the age-related remodeling of the muscle phenotype that comprises an increase in the number of slow-twitch fibers (Ohlendieck, 2011). Data also uncovered a likely age-induced dysregulation of the OXPHOS system in females noticed by, for example, the downregulation of the core subunit NDUFV2 from complex I as well of the subunit 1 (UQCRC1) from complex III, which was accompanied by an upregulation of subunits 7 (LOC685596) and 8 (UQCRQ) also from complex III (**Table 2**). Since complexes I and III constitute the main sites of superoxide generation, their faulty function may ensue ROS production (Bae et al., 2011). With regard to ATP synthase, an age-related increase in its subunit epsilon (ATP5E) was perceived in females (**Table 2**), as well as an age-related decrease in its mtDNA-encoded subunit 6 (MT-ATP6) that was correlated with low ATP synthase activity (**Figure 4**), which can contribute to a weakened skeletal muscle contractility. Of note, most of disease-causing mutations in ATP synthase are located in the MT-ATP6 gene, reflecting the importance of this subunit in proton translocation, and have muscle weakness as one of the clinical manifestations (Xu et al., 2015). The levels of MT-ATP6 appear to be positively

modulated by mitochondrial ERα given the correlation noticed (data not shown; $p<0.05$ and r=0.71), which is in line with previous findings (Chen et al., 2004). Decreased abundance of solute carrier family 25 member 3 (SLC25A3, **Table 2**) might also contribute to a deficient OXPHOS-mediated ATP production in old female rats given its function in the transport of inorganic phosphate across the mitochondrial membrane for the final step of OXPHOS. As a matter of a fact, reduced SLC25A3 levels were previously associated with muscle weakness, exercise intolerance and lactic acidosis (also noticed in old females), and the downregulation of *SLC25A3* gene was reported in old sarcopenic women compared to old non-sarcopenic ones (Bhoj et al., 2015; Kang et al., 2021). The decreased abundance of the electrogenic aspartate/glutamate antiporter SLC25A12 (SLC25A12) present in old females (**Table 2**) might also adversely influence ATP production due to its role in the malate-aspartate shuttle (Amoedo et al., 2016). Its gene was found downregulated in the skeletal muscle of old women (vs. young women) (Liu et al., 2013), and gene mutations that cause reduced activity of the protein were associated with decreased ATP production (Palmieri et al., 2020).

The outcomes of age-related mitochondrial dysfunction ramify to an exacerbated production of ROS, primarily by the organelle itself, which can culminate in oxidative stress and severe damage to the cell, organelle membranes, DNA, lipids and proteins (Maldonado et al., 2023). The evaluation of the susceptibility to oxidation, which is frequently a protein inactivating PTM (Reeg & Grune, 2015), of skeletal muscle mitochondrial proteins revealed a higher proneness of the proteome of old females than the one of old males (**Table 3**), as suggested by the literature (Colom et al., 2015; Gorni & Finco, 2020). The opposite, however, was also reported (Fanò et al., 2001). Case in point, the subunits ATP5MPL and ATP5A1 of ATP synthase were found oxidized at, respectively, Met1 and Met189 in old females. The subunit ATP5A1 experienced dissimilar oxidations in all groups, but no significant differences between them were observed. Oxidation of the TCA cycle enzymes IDH2 and IDH3A at, respectively, Met411 and Met206 was also noticed in old females (**Table 3**), and IDH3A was previously found to be carbonylated in the *gastrocnemius* muscle of old female rats (Feng et al., 2008).

By being oxidatively modified, proteins can partially unfold, which is in line with the upregulation of the chaperones stress-70 protein (HSPA9) and 10 kDa heat shock protein (HSPE1), and of the protease AFG3 like matrix AAA peptidase subunit 2 (AFG3L2) in old females (**Table 2**). These proteins are involved in the mechanisms of protein quality control of mitochondria, namely the mitochondrial unfolded protein response (mtUPR), with the main aim of maintain proper folding and remove damaged proteins (Szczepanowska & Trifunovic, 2022). In the particular context of the data obtained in this study, the protease AFG3L2 may modulate MT-AP6 processing, as previously reported (Richter et al., 2015), and the overexpression of PGC1α was reported to stimulate the mtUPR in the form of an adaptative response of the skeletal muscle to acute exercise and in the context of Parkinson's disease (Cai et al., 2020; Wu et al., 2011) in a non-sustained form. However, when persistently activated during adulthood, the mtUPR is believed to contribute to age-related muscle diseases like sarcopenia (Wang et al., 2023; Wodrich et al., 2022). One alleged trigger of this activation is a defective mitophagy (Wang et al., 2023), which was implied to be present in old females given the reduced Parkin levels observed (**Figure 2A**). This weakened Parkin signaling in old females may vindicate the unchanged levels of ubiquitinated proteins in response to aging that, and in agreement with the negative correlation observed (**Figure 5**), might contribute to the age-related accumulation of oxidatively modified mitochondrial proteins in female rats. Data concerning the age-related alterations in the content of ubiquitinated proteins is still open to questions given the higher or unchanged levels noticed in age-related studies as reviewed elsewhere (Ventura-Clapier et al., 2017). A compromised mitophagy has being mentioned as one of the main culprits of the age-related accumulation of dysfunctional mitochondria, a famed hallmark of aging believed to be involved in skeletal muscle wasting and functional impairment (Bellanti et al., 2021; Hughes et al., 2022; Leduc-Gaudet et al., 2021; López-Otín et al., 2013).

5. Conclusions

Overall, this study endorses that skeletal muscle aging ensues in a sex-dependent manner. The skeletal muscle of females encompasses an age-

induced accentuated mitochondrial proteome remodeling characterized by an increased FAO, dysfunctional OXPHOS and higher proneness to oxidative modifications, possibly prompted by an age-related decline in 17β-estradiol signaling through mitochondrial ERα. This adverse females-specific remodeling contributes to their exacerbated skeletal muscle atrophy and fibrosis, whereas mitochondrion of males is faintly affected by aging. The data from this study potencies the foundations of the need of a sex-personalized diagnostic and management of skeletal muscle diseases such as sarcopenia, and the inevitability of mitochondrial therapeutics exploration.

Acknowledgments

This work was supported by CIAFEL (UIDB/00617/2020), ITR (LA/P/0064/2020), LAQV (UIDB/50006/2020), CITAB (UIDB/04033/2020, https://doi.org/10.54499/UIDB/04033/2020), UnIC (UIDB/IC/00051/2020 and UIDP/00051/2020), and RISE (LA/P/0053/2020) research units projects through national founds by the Portuguese Foundation for Science and Technology (FCT)/Ministry of Science, Technology and Higher Education (MCTES), and by EPIC-XS (project number 0000475), funded by the Horizon 2020 program of the European Union. We also thank Doctor Germana Meroni for the kind supply of the anti-ubiquitin antibody.

Sources of funding

A.M.-P. thanks the Portuguese Foundation for Science and Technology (FCT)/Ministry of Science, Technology and Higher Education (MCTES) and the European Social Fund (ESF) through PT2020 for her grant (SFRH/BD/144396/2019).

R.N.-F. acknowledges FCT for the research contract CEECIND/03935/2021 (https://doi.org/10.54499/2021.03935.CEECIND/CP1685/CT0001) under the CEEC Individual 2021.

Author contribution statement

Conceptualization, R.F. and J.A.D.; Methodology, A.M.-P., R.V., C.S.-M., P.A.O., P.N., and D.L; Validation, A.M.-P., R.V., and J.A.D.; Formal Analysis, A.M.-P., R.F.,

and R.V.; Investigation, A.M.-P., R.N.-F., M.J.N., C.S.-M., A.N., P.N., and D.L.; Writing – Original Draft Preparation, A.M.-P.; Writing – Review & Editing, R.F., J.A.D., P.A.O., A.L.-M., C.L., and A.A.; Visualization, A.M.-P. and R.F.; Supervision, R.F., P.A.O., and J.A.D.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request. Some data may not be made available because of privacy or ethical restrictions.

Declaration

Conflict of interest

The authors declare no conflict of interest.

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Chapter III

Subchapter IIIB

Age and cancer in skeletal muscle wasting: which one to blame?

Moreira-Pais, A., Ferreira, R., Aires, I., Oliveira, P. A., & Duarte, J. A. (2024). Age and cancer in skeletal muscle wasting: which one to blame? (**in preparation**)

Age and cancer in skeletal muscle wasting: which one to blame?

Alexandra Moreira-Pais[a,b,c], Rita Ferreira[b], Inês Aires[b], Paula A. Oliveira[c], José A. Duarte[a,d]

[a] Research Center in Physical Activity, Health and Leisure (CIAFEL), Faculty of Sport, University of Porto (FADEUP) and Laboratory for Integrative and Translational Research in Population Health (ITR), 4200-450 Porto, Portugal

[b] LAQV-REQUIMTE, Department of Chemistry, University of Aveiro, 3810-193 Aveiro, Portugal

[c] Centre for Research and Technology of Agro Environmental and Biological Sciences (CITAB), Inov4Agro, University of Trás-os-Montes and Alto Douro (UTAD), Quinta de Prados, 5000-801 Vila Real, Portugal

[d] TOXRUN – Toxicology Research Unit, University Institute of Health Sciences, CESPU, CRL, 4585-116 Gandra, Portugal

ORCIDs and emails:

Alexandra Moreira-Pais: 0000-0003-2582-1481; alexandrapais@ua.pt

Rita Ferreira: 0000-0002-6872-4051; ritaferreira@ua.pt

Inês Aires: 0009-0005-1411-9146; iaa@ua.pt

Paula A. Oliveira: 0000-0001-9519-4044; pamo@utad.pt

José A. Duarte: 0000-0003-4756-5917; jose.duarte@iucs.cespu.pt

Corresponding author:

Alexandra Moreira-Pais

Departamento de Química

Universidade de Aveiro

Campus Universitário de Santiago

3810-193 Aveiro

Portugal

e-mail: alexandrapais@ua.pt

Abstract

Sarcopenia and cancer cachexia are two life-threatening conditions often misdiagnosed. The skeletal muscle is one of the organs most adversely affected by these conditions, culminating in poor quality of life and low survival chances. Herein, we sought to investigate markers of inflammation and neuromuscular junction (NMJ) remodeling during aging and in response to cancer. For that, female young, adult and old rats were used, as well as a chemically-induced animal model of breast cancer (BCa) implemented in adult female rats. The wasting of the g*astrocnemius* muscle was observed in old and adult BCa rats compared to adult ones. No tumor-induced inflammation or NMJ impairment was observed. In contrast, aging led to high serum levels of interleukin (IL)-6, brain-derived neurotrophic factor (BDNF) and calcitonin gene-related peptide (CGRP). IL-6 and BDNF were negatively correlated with muscle wasting in aging. In the *gastrocnemius* muscle, BDNF levels were decreased in old rats and correlated with muscle wasting, suggestive of an impaired skeletal muscle regeneration upon age-induced damage. Hence, this work highlights BDNF as a putative specific biomarker of age-induced skeletal muscle wasting, at least, in the differential diagnosis against cancer-induced skeletal muscle wasting.

Keywords: cachexia, inflammation, muscle loss, neuromuscular junction, sarcopenia

1. Introduction

Sarcopenia and cachexia are frequently interchanged, though they represent different conditions. Sarcopenia is a muscle disease defined by low levels of muscle strength, muscle quantity or quality and physical performance that affects 10-27% of people aged 60 years or older and it develops gradually throughout lifetime in response to aging (Cruz-Jentoft et al., 2019; Petermann-Rocha et al., 2022). In turn, cachexia is a syndrome defined by the loss of body weight (more than 5% in six months or over 2% in individuals with a body mass index below 20 $kg.m^2$) and skeletal muscle mass with or without adipose tissue loss that cannot be entirely reversed by conventional nutritional support and is a negative outcome of an underlying illness, such as cancer (termed cancer cachexia, the focus of this work) (Cruz-Jentoft et al., 2019; Setiawan et al., 2023). Cancer cachexia affects up to 85% of cancer patients and is responsible for 20-30% of all cancer-related deaths (Ferrara et al., 2022). As in sarcopenia, skeletal muscle wasting is a significant feature of cancer cachexia, with cachectic cancer patients having a 10-33% reduction in the *quadriceps* muscle (vs. healthy control individuals) and 4-13% reduction in the skeletal muscle mass index (vs. non-cachectic cancer patients) (Martin & Freyssenet, 2021). Data from animal models of cancer cachexia also consistently report a decrease in skeletal muscle mass (vs. control animals or non-cachectic cancer animals), as reviewed elsewhere (Martin & Freyssenet, 2021). Both sarcopenia and cancer cachexia are debilitating and life-threatening conditions that profoundly affect the patient's quality of life by reducing physical function, which results in loss of autonomy, and by increasing surgical risks and the susceptibility to the adverse effects of chemotherapy, culminating in decreased survival rates and premature mortality (Coletta & Phillips, 2023; Martin & Freyssenet, 2021). While the development of sarcopenia is an insidious lifelong process, cancer cachexia develops more rapidly with many patients being cachectic at time of cancer diagnosis (Maccio et al., 2021). The pathophysiology of sarcopenia and cancer cachexia is multifactorial, with the two conditions sharing impaired mechanisms. Nonetheless, while age-related perturbations of the neuromuscular environment seem to have a higher

contribution to sarcopenia development, augmented inflammation appears to be the main culprit of cancer cachexia (Moreira-Pais et al., 2021).

Sarcopenia and cancer cachexia are under- and misdiagnosed, possibly due to the complexity of their differential diagnosis and the inexistence of a worldwide diagnostic procedure. The current diagnostic criteria for sarcopenia and cancer cachexia are mainly based on the loss of skeletal muscle strength and mass, and on the loss of body mass and muscle function, respectively (Ebner et al., 2020). These criteria are rather limiting, and in time when muscle or weight loss is noticeable, it may be late for its management, weakening the hypothesis of success. Furthermore, therapeutic options to sarcopenia or cancer cachexia remain rare, and no drugs have been yet approved. This might be one of the outcomes of the limited number of studies that simultaneously investigate sarcopenia and cancer cachexia. Often, studies delve into each condition separately, leading to the analysis of distinct mechanisms and markers, hampering drawing conclusions. Moreover, preclinical models of cancer cachexia usually harbor rodents aged from four to twelve weeks, which corresponds approximately to adolescent humans, not mimicking the majority of human cancers that predominantly develop in more older individuals (Penna et al., 2016). Hence, it is imperative to unravel a panel of condition-specific biomarkers to pave the way for a precise and early clinical diagnosis and management of sarcopenia and cancer cachexia.

Considering the aforementioned facts, we sought to investigate the differences between age- and cancer-induced skeletal muscle remodeling focusing on inflammation and neuromuscular environment. For that, rats were left aging until old age, as a sarcopenia model, and a chemically-induced breast cancer (BCa) protocol was implemented in adult rats, as a cancer cachexia model. Young and adult rats were also considered. A chemically-induced model of BCa was used since it was previously reported that these rats develop cachexia (Faustino-Rocha et al., 2015), and cancer cachexia has become increasingly common in BCa patients in recent years (Liu et al., 2021). The use of rats in this study was a well thought-through choice based on the chronic nature of sarcopenia that takes years to develop in humans and the consequent extended time course and ethical

concerns surrounding the invasive acquisition of skeletal muscle biopsies from healthy and potentially ill individuals, which is also valid for cancer cachexia. In addition, confounding factors, such as physical status and diet, can be controlled using animals. Anthropometric data, mainly absolute and relative *gastrocnemius* muscle mass, were related with promising markers of inflammation and neuromuscular junction (NMJ) remodeling. The *gastrocnemius* muscle was selected because it is an important muscle in lower limb activities (Xie et al., 2021), and for comparative proposes with another studies (Börsch et al., 2021; Martin & Freyssenet, 2021; Xie et al., 2021).

2. Materials and methods

2.1 Animal protocol

Female Wistar rats were obtained from the Charles River Laboratories (FR) at four (young and adult groups) or twelve (old group) weeks. Before the initiation of the experiments all rats were acclimatized to the handler and the experimental protocol for one week and then randomly distributed (four to three rats *per* cage; 1500U Eurostandard Type IV S cages, Tecniplast, Varese, IT) into four experimental groups: young (n=7), old (n=13), adult (n=8) and adult BCa (n=8). The rats were housed at controlled temperature (22 ± 2°C) and relative humidity (50 ± 5%) with a 12:12h light-dark cycle with *ad libitum* food (standard diet 4RF21, Mucedola, Milan, IT) and water access.

The rats from the adult BCa group received one intraperitoneal injection (50 $mg.kg^{-1}$) of the carcinogen agent *N*-methyl-*N*-nitrosourea (MNU, Sigma Chemical Co., Madrid, ES) at seven weeks to induce mammary tumors. Adult rats received a single administration of the vehicle (saline solution at 0.9%). A chemically-induced model was implemented since it has a short period of latency and higher reproducibility when compared to other animal models, as well as a similar carcinogenesis process to the one that occurs in humans (Faustino-Rocha et al., 2015). In addition, MNU-induced mammary tumors in rodents is a well-established animal model for BCa research that resembles woman mammary tumors and can be used as a model of cancer cachexia (Faustino-Rocha et al., 2015).

The rats from the young, adult and adult BCa, and old groups were weighted and sacrificed at 6 months (corresponds to approximately 18 years in humans (Sengupta, 2013)), 10 months (corresponds to approximately 26 years in humans (Sengupta, 2013)) and 19 months (corresponds to approximately 45-50 years in humans (Sengupta, 2013)), respectively, by an intraperitoneal overdose of ketamine (75 $mg.kg^{-1}$, Imalgene 1000, Merial SAS, Lyon, FR) and xylazine (10 $mg.kg^{-1}$, Rompun 2 %, Bayer, Kiel, DE) followed by exsanguination as indicated by the Federation of European Laboratory Animal Science Associations (Forbes et al., 2007). The blood was collected from the inferior vena cava, and the two *gastrocnemius* muscles of each rat were collected and weighed (*vide infra* for sample-specific processing). The right and left tibiae were dissected to determine tibia length, which is an indicator of animal body size that is independent of alterations in muscle and fat masses (Yin et al., 1982). The skin was evaluated under a light and the number and localization of mammary tumors were recorded.

All the procedures were approved by the University of Trás-os-Montes and Alto Douro Ethics Review Body ORBEA (Orgão Responsável pelo Bem-Estar e Ética Animal, reference 424-e-DCV-2016) and by the Portuguese Competent Authority DGAV (Direção Geral de Alimentação e Veterinária, license number 004583 and 004584), according to the European Guidelines, and following the Portuguese law (decree-law number 113/2013) on animal protection for scientific purposes.

2.2 Serum samples preparation and immunoblot analyses

The blood collected from the heart at necropsy was allowed to clot and then centrifuged at 4°C for 15 minutes at 3000 *g*. Samples were diluted 1:50 in Tris-buffered saline (TBS) and then blotted under vacuum onto a nitrocellulose membrane (Amersham™, Protan®, GE Healthcare, Illinois, US). For comparison purposes each performed immunoblot contained samples from all groups. Nonspecific binding was blocked by the incubation of the membranes in a 5% (w/v) solution of non-fat dry milk in TBS with Tween 20 (TBST) for 1 hour at room temperature and with agitation. Then, each membrane was incubated with the corresponding primary antibody: anti-brain-derived neurotrophic factor (BDNF, ab226843), anti-C reactive protein (CRP, ab32412), and anti-interleukin 6 (IL-6,

ab6672) from Abcam (Cambridge, UK); and anti-calcitonin gene-related peptide (CGRP, PA5-114931) from Invitrogen (Massachusetts, US). This incubation was followed by three 10-minute washes with TBST, incubation with the secondary antibody (anti-rabbit (NA934V) from GE Healthcare, Illinois, US) and new washes. Both primary and secondary antibodies were diluted 1:1000 in 5% (w/v) non-fat dry milk in TBST. The primary and secondary antibodies were incubated during 1 hour at room temperature with agitation. Immunoreactive bands were detected with enhanced chemiluminescence (ECL) reagents (Clarity Max, Bio-Rad, California, US) according to the manufacturer's procedure. Images were acquired using the ChemiDoc Imaging System (Bio-Rad, California, US) and analyzed using the Image Lab software (v6.0.0., Bio-Rad, California, US). The optical density (OD) values were expressed in arbitrary units.

2.3 Preparation of gastrocnemius muscle homogenates and immunoblot analyses

Approximately 50 mg of one *gastrocnemius* muscle was homogenized on a Teflon® pestle on a motor-driven Potter-Elvehjem glass homogenizer at 0-4°C using a lysis buffer (0.5 mM EGTA, 10 mM HEPES, NaOH pH 7.4, 0.1% Triton X-100, 1:1000 PMSF and 1:400 protease inhibitor cocktail (P8340, Sigma-Aldrich, Missouri, US)) in the proportion of 50 mg of muscle to 1 mL of lysis buffer. The protein content of the muscle homogenates was assessed using the commercial kit DC™ Protein Assay (Bio-Rad, CA, US), according to the manufacturer's instructions and using bovine serum albumin as standard. Then, the total homogenates were preserved at -80°C for the biochemical analyses that follow.

Equivalent volumes to 50 µg of protein of each muscle homogenate were electrophoresed on a 12.5% SDS-PAGE gel according to Laemmli (Laemmli, 1970) and then blotted onto a nitrocellulose membrane (Amersham™, Protan®, GE Healthcare, Illinois, US) during 2 hours at 200 mA. For comparison purposes each performed gel contained samples from all groups. Protein loading was controlled by Ponceau S staining, since most of the housekeeping markers used can be affected by stressful stimuli in skeletal muscle (Vigelsø et al., 2015). Nonspecific binding was blocked by the incubation of the membranes in a 5%

(w/v) solution of non-fat dry milk in TBST for 1 hour at room temperature and with agitation. Then, each membrane was incubated with the corresponding primary antibody: anti-BDNF (ab226843) from Abcam (Cambridge, UK); anti-CGRP (PA5-114931) from Invitrogen (Massachusetts, US); and anti-nicotinic acetylcholine receptor alpha 1 (nAChRα1, sc-65829) and anti-signal transducer and activator of transcription 3 (STAT3, sc-8019) from Santa Cruz Biotechnology (Texas, US). This incubation was followed by three 10-minute washes with TBST, incubation with the corresponding secondary antibody (anti-rabbit (NA934V) or anti-mouse (NA931V) from GE Healthcare, Illinois, US) and new washes. Both primary and secondary antibodies were diluted 1:1000 in 5% (w/v) non-fat dry milk in TBST. The primary antibody was incubated overnight at 4°C and the secondary antibody during 1 hour at room temperature with agitation. Immunoreactive bands were detected with ECL reagents (Clarity Max, Bio-Rad, California, US) according to the manufacturer's procedure. Images were acquired using the ChemiDoc Imaging System (Bio-Rad, California, US) and analyzed using the Image Lab software (v6.0.0., Bio-Rad, California, US). The optical density (OD) values were expressed in arbitrary units.

2.4 Statistical analyses

Data normality was evaluated by the Shapiro-Wilk test. The significant differences among groups were determined by performing the one-way analysis of variance (ANOVA) test to compare young vs. adult vs. old groups to evaluate the effect of aging, and the t-test to compare adult vs. adult BCa groups to evaluate the effect of cancer. The values are present as mean ± standard deviation (SD). Pearson correlations were also performed, when considered relevant, for an in-deep understanding of the results. Results were considered significantly different when $p<0.05$. *Gastrocnemius* muscle mass values were also normalized to body weight and tibia length. All the statistical analyses were performed using the GraphPad Prism software for windows (v6.01, California, US).

3. Results

Table 1 shows that body weight increased from adult into old age and no differences were found between adult and adult BCa rats. Nonetheless,

approximately 63% of adult BCa rats presented a decrement in body weight gain of more than 5% (vs. adult rats), suggesting the presence of body wasting in the majority of adult BCa rats. The percentage of rats that developed mammary tumors was of 100% in the adult BCa group and of 0% in the other groups (data not shown). The absolute *gastrocnemius* muscle mass decreased approximately 20% in adult BCa rats comparatively to adult ones, indicating cancer-induced muscle wasting. To eliminate variations due to body weight changes with aging, the *gastrocnemius* muscle mass was normalized to body weight and old rats had a decrease of approximately 29% of the ratio comparatively to young and adult ones. To eliminate variations due to animal size changes with aging, the *gastrocnemius* muscle mass was normalized to tibia length and no differences were observed between groups.

Table 1 The effect of aging or breast cancer (BCa) on the body weight (BW), tibia length (TL) and *gastrocnemius* (GS) muscle mass of young (n=7), old (n=13), adult (n=8) and adult BCa (n=8) rats. The *gastrocnemius* muscle mass normalized to body weight and tibia length is also depicted.

	Young	Old	Adult	Adult BCa
BW (g)	278.71 ± 10.47****	405.93 ± 38.60	294.98 ± 25.16****	285.29 ± 36.24
TL (cm)	3.6 ± 0.2***	4.0 ± 0.1	4.2 ± 0.1^{++++}	4.1 ± 0.1
GS muscle mass (g)	3.21 ± 0.20	3.30 ± 0.10	3.50 ± 0.25$^{\#,+,\$}$	2.81 ± 0.72
GS muscle mass/BW (%)	1.15 ± 0.09****	0.82 ± 0.07	1.15 ± 0.08****	0.99 ± 0.24
GS muscle mass/TL (g.cm^{-1} %)	87.9 ± 5.2	83.0 ± 3.5	82.7 ± 8.5	70.2 ± 17.9

**** p<0.0001 vs. Old; *** p<0.001 vs. Old; * p<0.05 vs. Old; $^{++++}$ p< 0.0001 vs. Young; $^{+}$ p<0.05 vs. Young; $^{\#}$ p<0.05 vs. Adult BCa; $^{\$}$ p=0.0731 vs. Old

Inflammation and NMJ remodeling seem to be differentially modulated by aging and cancer (Moreira-Pais et al., 2021). Thus, specific markers of inflammation and NMJ remodeling were analyzed in the serum (**Figure 1**). The levels of the widely studied acute-phase protein CRP were not modulated by aging or cancer. In contrast, IL-6 serum levels were higher in old rats compared to young and adult rats. No differences were found between young and adult rats, suggesting that chronic low-grade inflammation starts from adulthood into old age. No differences

were observed between adult and adult BCa rats, indicating that IL-6 did not increase in response to the development of mammary tumors. A correlation between relative *gastrocnemius* muscle mass and IL-6 serum levels in young, adult and old rats was performed to elucidate about IL-6 role in skeletal muscle aging. A strong negative correlation was found, showing the involvement of IL-6 as a pro-inflammatory cytokine that contributes to age-induced skeletal muscle wasting.

Serum BDNF and CGRP had similar behaviors, with their levels being higher in old rats compared to young ones (statistical tendency regarding CGRP). Since adult rats had higher serum levels of BDNF than young ones and no differences were found between adult and old rats, the levels of BDNF in serum seem to start to increase from young ages into adulthood remaining then stable. The limited knowledge about the source(s) of circulating BDNF and CGRP makes it difficult to theorize the significance of these results; however, it is believed that BDNF levels in circulation might derive from inflammatory mediators such as IL-6. Hence, a correlation between IL-6 and BDNF serum levels in young, adult and old rats was performed and a positive correlation was obtained, indicating an association between inflammation in aging and circulating neurotrophins. No correlation was found for CGRP (**Supplementary Figure 1**). This IL-6-induced BDNF upregulation with aging was correlated with the decrease of relative *gastrocnemius* muscle mass, indicating the possibility of circulating BDNF being involved in skeletal muscle wasting. No differences were found in the serum levels of BDNF and CGRP in adult BCa rats (vs. adult rats), denoting that they are not involved in the cancer-induced skeletal muscle wasting observed, as indicated by the non-significant correlations obtained (**Supplementary Figure 1**).

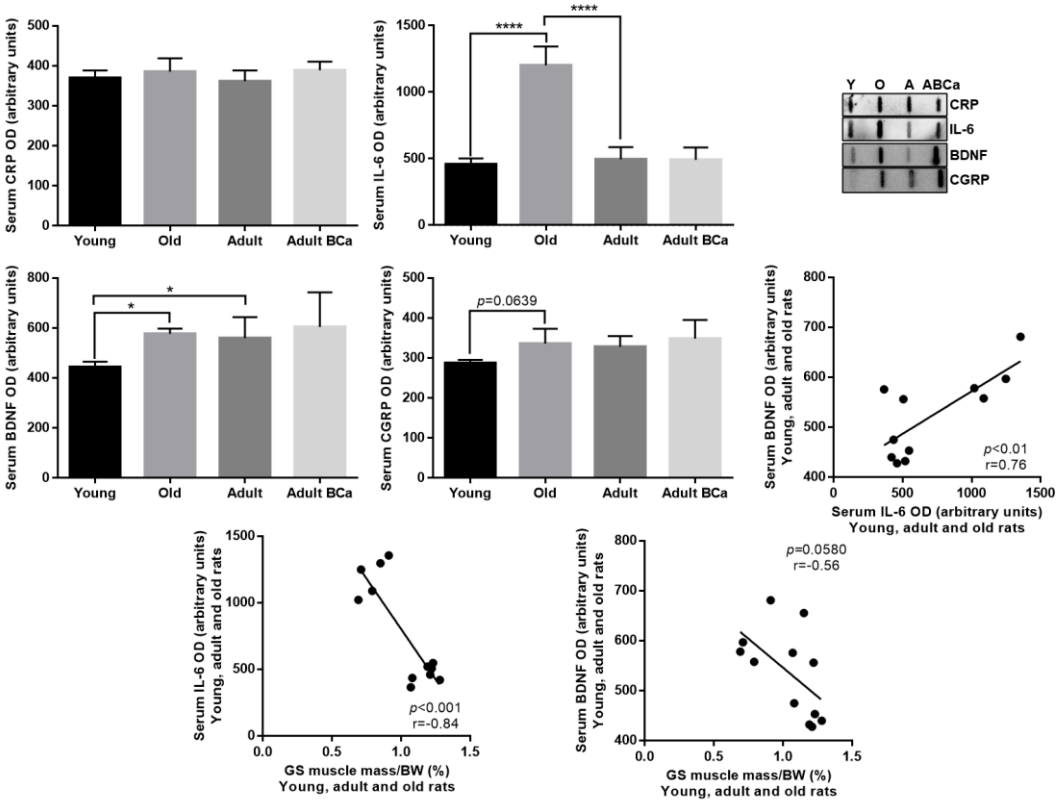

Figure 1 The effect of aging or breast cancer (BCa) in the serum levels of CRP, IL-6, BDNF and CGRP evaluated by immunoblotting [young (Y) n=4, old (O) n=5, adult (A) n=4 and adult BCa (ABCa) n=5]. Pearson correlations were performed for an in-deep understanding of the results (**** p<0.0001, *** p<0.001, * p<0.05).

Following the systemic response of the rats, the levels of the IL-6 downstream transcription factor STAT3, and of the NMJ-related markers nAChRα1, BDNF and CGRP were analyzed in the *gastrocnemius* muscle (**Figure 2**). No aging-related differences were found in STAT3, nAChRα1 or CGRP muscle levels, nor significant correlations to relative *gastrocnemius* muscle mass (**Supplementary Figure 1**). Likewise, no tumor-related differences were found in STAT3, nAChRα1, BDNF or CGRP muscle levels, nor significant correlations to absolute *gastrocnemius* muscle mass (**Supplementary Figure 1**). However, BDNF levels in muscle were decreased in old rats compared to adult ones. These results suggest that at adult ages, BDNF levels start to decrease in the muscle, which was correlated with the decreased relative *gastrocnemius* muscle mass, pointing to a role of muscle BDNF in age-induced skeletal muscle wasting.

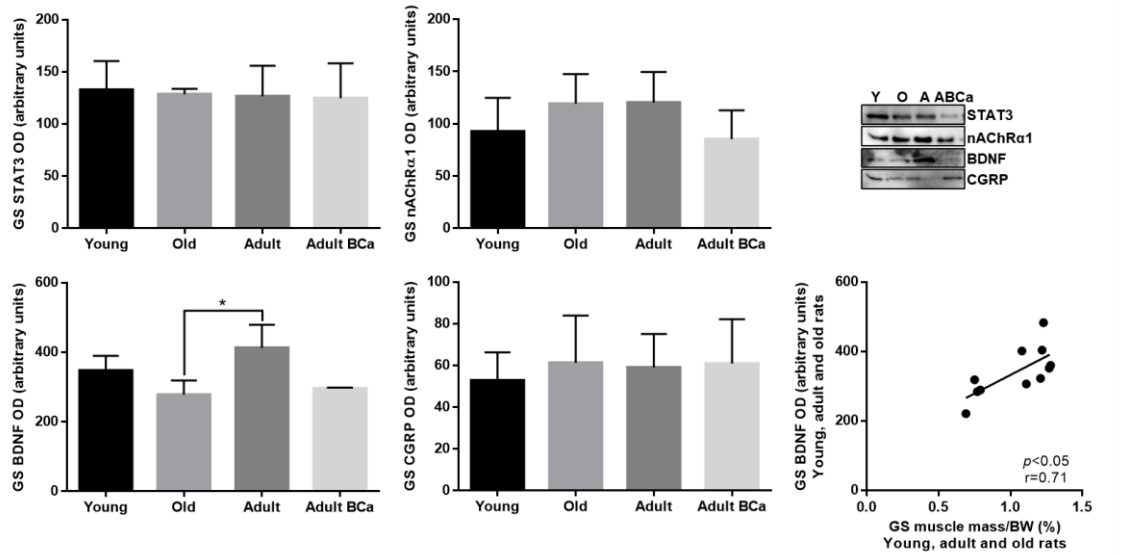

Figure 2 The effect of aging or breast cancer (BCa) in the *gastrocnemius* muscle levels of STAT3, nAChRα1, BDNF and CGRP evaluated by immunoblotting [young (Y) n=5, old (O) n=6, adult (A) n=5 and adult BCa (ABCa) n=6]. Pearson correlations were performed for an in-deep understanding of the results (* $p<0.05$).

4. Discussion

This study reinforces the loss of skeletal muscle mass as an adverse effect of aging or BCa, which in the aging setting was accompanied by an age-related increase in body weight in contrast with the body wasting perceived in the cancer scenery. Age-induced *gastrocnemius* muscle mass loss was associated with increased serum levels of IL-6 and decreased muscle levels of BDNF, pointing towards the presence of a sustained inflammatory environment and impaired skeletal muscle regeneration at NMJ level. None of these mediators seem to be involved in cancer-induced *gastrocnemius* muscle mass loss.

The loss of *gastrocnemius* muscle mass was evident in both old and adult BCa rats compared to adult ones, indicating muscle wasting in response to aging and tumor development. Specifically, aging induced a decrease of approximately 29% of the relative *gastrocnemius* muscle mass, whereas BCa induced a decrease of approximately 20% of the absolute *gastrocnemius* muscle mass. In adult BCa rats, no body weight decrease was observed compared to the adult group.

Notwithstanding, approximately 63% of the adult BCa rats had a reduced body weight gain (vs. adult rats), indicating the possible development of body wasting.

Inflammation seems to be present in both sarcopenia and cancer cachexia (Lena et al., 2021); however, literature suggests that the inflammatory response triggered in cancer cachexia is highly prominent with a major contribution to cachexia development, whereas in sarcopenia is of low-grade and not the main culprit (Ali & Garcia, 2014). In cancer setting, tumor-produced cytokines as IL-6 might induce apoptosis and proteolysis of muscle cells and inhibit anabolic mammalian target of rapamycin complex 1 (mTORC1), contributing to cancer-induced skeletal muscle wasting (Shivnani et al., 2023). Particularly in BCa, IL-6 was associated with tumor growth and metastasis and promotion of CRP production by the liver (Chen et al., 2022). Herein, no changes in IL-6 serum levels were found in adult BCa rats, which can justify why no differences were detected in CRP serum levels. In contrast, IL-6 serum levels were found elevated in old rats when compared to young and adult rats, indicating that the augmented inflammation starts in adulthood into old ages. Despite being of low-grade, inflammation has been implicated in sarcopenia pathogenesis (Nelke et al., 2019), with sarcopenic humans having higher IL-6 serum levels than non-sarcopenic ones (Rong et al., 2018). Indeed, IL-6 serum levels of young, adult and old rats correlated negatively with their relative *gastrocnemius* muscle mass, redefining the contribution of circulating IL-6 involvement in age-induced skeletal muscle wasting. One mechanism believed to be responsible for inflammaging is the senescence-associated secretory phenotype (He et al., 2022). Senescent cells can secrete a wide range of molecules, in which are included IL-6 and CRP, that possibly bolster sarcopenia development (He et al., 2022). Nevertheless, no differences or correlations were found for CRP levels, and a recent meta-analysis suggested no association between high CRP levels and muscle mass (Shokri-mashhadi et al., 2021), despite CRP serum levels have been found increased in sarcopenic subjects (Hida et al., 2018). One of the downstream transcription factors of IL-6 signaling is STAT3 that upon phosphorylation and dimerization, it is translocated to the nucleus where it regulates gene transcription. STAT3 chronic activation in myofibers induces protein degradation and promotes

skeletal muscle atrophy (Sala & Sacco, 2016). Unphosphorylated STAT3 can also regulate gene transcription by interacting with other transcription factors, including nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), forkhead box (Fox)O1 and FoxO3. Herein, the levels of STAT3 in *gastrocnemius* muscle were not affected by aging or cancer, which might suggest that IL-6 is acting through another mechanism to induce muscle atrophy in old rats.

Along with inflammation, markers of NMJ remodeling seem to hold a promising clinical value for the differential diagnosis, since the remodeling of the neuromuscular environment appears to be a major focal point of aged-related skeletal muscle perturbations (Deschenes et al., 2022). Nonetheless, this evidence must be cautiously pondered, since the studies on this field in cancer cachexia are very scarce and contradictory, reporting both stable and impaired NMJ (Boehm et al., 2020; Daou et al., 2020). Skeletal muscle contraction is triggered by the efficient binding of acetylcholine (ACh) to its receptor localized at the endplate (Bao et al., 2020). Literature has conflicted results regarding the content of AChR subunits in aged muscle, but the overall function of AChRs seems to be reduced during aging as reviewed elsewhere (Bao et al., 2020). Likewise, in cancer cachexia evidence is not clear (Brown et al., 2018). The subunit α1 of AChR was evaluated in the present study, but no differences were observed between groups nor a correlation to relative or absolute *gastrocnemius* muscle mass, suggesting that the content of, at least, nAChRα1 is not related to skeletal muscle wasting. BDNF is a neurotrophin synthetized and released from neurons as well as from the skeletal muscle (Leßmann & Brigadski, 2009). It is involved in the regulation of motoneuron survival, the presynaptic release of neurotransmitters and the maintenance of the postsynaptic region in skeletal myofibers (Kalinkovich & Livshits, 2015). Low levels of BDNF are constitutively expressed in skeletal muscle satellite cells, and increase in response to injury to, possibly, promote muscle regeneration (Mousavi & Jasmin, 2006). The loss of endogenous BDNF and subsequent signaling is believed to be present in aging muscle and to contribute to skeletal muscle impairment (Greising et al., 2015). In contrast, BDNF seems not to be involved in cancer cachexia development (de Castro et al., 2020). Herein, BDNF muscle levels were decreased in old rats

compared to adult ones. No differences were found between young and adult rats, which can be indicative that BDNF levels start to decrease in adulthood given fuel to sarcopenia development. In addition, this aging-related decrease of BDNF muscle levels was positively correlated with the relative *gastrocnemius* muscle mass, strongly suggesting a dysfunctional BDNF response to age-induced skeletal muscle damage, hindering muscle repair. BDNF expression also increases in response to muscle contraction, which may have also contributed to the decreased levels in old rats. Skeletal muscle-derived BDNF appears not to be released into circulation (Matthews et al., 2009); however, the knowledge is very limited. Instead, inflammation itself was suggested to induce BDNF production, elevating its circulating levels (Papathanassoglou et al., 2015). This might explain why BDNF levels were elevated in the serum of old rats, and in fact, a strong positive correlation was found between IL-6 and BDNF serum levels during aging. Indeed, BDNF plasma levels were already suggested to be associated with sarcopenia severity among older women (da Costa Teixeira et al., 2023). Hence, more studies are urgently needed to delve into the relationship between NMJ impairment and BNDF serum levels, given the attractiveness of having a sarcopenia-specific mediator measurable in a minimally invasive form. Another player involved in NMJ stabilization is the neuropeptide CGRP, which in skeletal muscle seems to potentiate muscle contraction, increase the rate of AChR desensitization, increase the number of AChRs and decrease the levels of acetylcholinesterase (Rossi et al., 2003). CGRP levels are markedly decreased in mature NMJs, being upregulated during reinnervation and nerve sprouting to, possibly, maintain NMJ stability by exerting antiproteolytic effects in atrophic denervated muscles (Blasco et al., 2020; Machado et al., 2019). In fact, the CGRP content in motoneurons of old mice were previously found increased comparatively to adult ones, which presented very low levels (Blasco et al., 2020). In the present study, the absence of increased CGRP muscle levels in old rats with skeletal muscle wasting might indicate a blunted response of this molecule to skeletal muscle denervation, which can lead to the accumulation of denervated fibers and might contribute to sarcopenia development and/or aggravation. Indeed, a failed reinnervation was pre-clinically observed with aging (Aare et al.,

2016). CGRP can also be measured in circulation as BNDF. It was observed that CGRP plasma levels in healthy humans are low due, in part, to its rapid metabolic clearance (Kee et al., 2018), and its levels were already found increased in the plasma of old rodents (Wimalawansa, 1992), as in the present study. The main source of circulating CGRP is thought to be a “spillover” from its release from the perivascular nerve endings or even from thyroid (Kee et al., 2018; Wimalawansa, 1991), but it is currently unclear. Anew, it is vital to disclose the association between muscle and circulating levels of CGRP. Since no differences were found for the levels of BDNF or CGRP in serum or skeletal muscle of adult BCa rats, these two molecules are promising biomarkers of age-induced muscle wasting and, perhaps, therapeutic targets. For that, further studies are urgently needed to elucidate the role of BDNF and CGRP in skeletal muscle aging.

5. Conclusions

This work sought to delve into markers of inflammation and NMJ remodeling with putative clinical differential value for the diagnosis of age- and cancer-induced skeletal muscle wasting. Wasting of the *gastrocnemius* muscle was observed in response to aging and cancer. The skeletal muscle wasting induced by aging was correlated with increased circulating levels of the pro-inflammatory cytokine IL-6 and of the NMJ-related marker BDNF. In skeletal muscle itself, however, decreased levels of BDNF seem to contribute to the development of skeletal muscle wasting. No tumor-induced changes were observed in the levels of the analyzed mediators. Hence, this work highlights BDNF as a conceivable biomarker for age-induced skeletal muscle wasting that deserves to be further explored, namely the association between circulating levels and skeletal muscle impairment.

Acknowledgments

This work was supported by CIAFEL (UIDB/00617/2020), ITR (LA/P/0064/2020), LAQV (UIDB/50006/2020) and CITAB (UIDB/04033/2020, https://doi.org/10.54499/UIDB/04033/2020) research units projects through national funds by the Portuguese Foundation for Science and Technology (FCT)/Ministry of Science, Technology and Higher Education (MCTES).

Sources of funding

A.M.-P. thanks the Portuguese Foundation for Science and Technology (FCT)/Ministry of Science, Technology and Higher Education (MCTES) and the European Social Fund (ESF) through PT2020 for her grant (SFRH/BD/144396/2019).

Author contribution statement

Conceptualization, R.F. and J.A.D.; Methodology, A.M.-P., R.F., and P.A.O.; Validation, A.M.-P., R.F., P.A.O., and J.A.D.; Formal Analysis, A.M.-P.; Investigation, A.M.-P., and I.A.; Writing – Original Draft Preparation, A.M.-P.; Writing – Review & Editing, R.F., J.A.D., and P.A.O.; Visualization, A.M.-P. and R.F.; Supervision, R.F., P.A.O., and J.A.D.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request. Some data may not be made available because of privacy or ethical restrictions.

Declaration

Conflict of interest

The authors declare no conflict of interest.

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Chapter III

Subchapter IIIC

Long-term impact of doxorubicin on skeletal muscle remodeling in aged mice

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Moreira-Pais, A., Ferreira, R., Baltazar, T., Neuparth, M. J., Vitorino, R., Reis-Mendes, A., Costa, V. M., Oliveira, P. A., & Duarte, J. A. (2024). Long-term impact of doxorubicin on skeletal muscle remodeling in aged mice. (**submitted**)

Long-term impact of doxorubicin on skeletal muscle remodeling in aged mice

Alexandra Moreira-Pais[a,b,c], Rita Ferreira[b], Telmo Baltazar[b], Maria João Neuparth[a,d], Rui Vitorino[e], Ana Reis-Mendes[f,g], Vera Marisa Costa[f,g] Paula A. Oliveira[c], José A. Duarte[a,d]

[a] Research Center in Physical Activity, Health and Leisure (CIAFEL), Faculty of Sports, University of Porto (FADEUP) and Laboratory for Integrative and Translational Research in Population Health (ITR), 4200-450 Porto, Portugal

[b] LAQV-REQUIMTE, Department of Chemistry, University of Aveiro, 3810-193 Aveiro, Portugal

[c] Centre for Research and Technology of Agro Environmental and Biological Sciences (CITAB), Inov4Agro, University of Trás-os-Montes and Alto Douro (UTAD), Quinta de Prados, 5000-801 Vila Real, Portugal

[d] TOXRUN – Toxicology Research Unit, University Institute of Health Sciences, CESPU, CRL, 4585-116 Gandra, Portugal

[e] iBiMED - Institute of Biomedicine, Department of Medical Sciences, University of Aveiro, 3810-193 Aveiro, Portugal

[f] Associate Laboratory i4HB - Institute for Health and Bioeconomy, Faculty of Pharmacy, University of Porto, 4050-313 Porto, Portugal

[g] UCIBIO - Applied Molecular Biosciences Unit, Laboratory of Toxicology, Department of Biological Sciences, Faculty of Pharmacy, University of Porto, 4050-313 Porto, Portugal

ORCIDs and emails:

Alexandra Moreira-Pais: 0000-0003-2582-1481; alexandrapais@ua.pt

Rita Ferreira: 0000-0002-6872-4051; ritaferreira@ua.pt

Telmo Baltazar: 0000-0003-1848-7294; baltazar.telmo@ua.pt

Maria João Neuparth: 0000-0002-1464-8700; mneuparth@hotmail.com

Rui Vitorino: 0000-0003-3636-5805; rvitorino@ua.pt

Ana Reis-Mendes: 0000-0002-2775-3147; afreis.mendes@gmail.com

Vera Marisa Costa: 0000-0002-0471-2756; veramcosta@ff.up.pt

Paula A. Oliveira: 0000-0001-9519-4044; pamo@utad.pt

José A. Duarte: 0000-0003-4756-5917; jose.duarte@iucs.cespu.pt

Corresponding author:

Alexandra Moreira-Pais

Departamento de Química

Universidade de Aveiro

Campus Universitário de Santiago

3810-193 Aveiro

Portugal

e-mail: alexandrapais@ua.pt

Abstract

Doxorubicin (DOX), a widely used chemotherapeutic drug, is associated with body wasting and skeletal muscle weakness and fatigue. Nonetheless, the molecular mechanisms are yet to be completely acknowledged. The observed body wasting may ensue from the loss of skeletal muscle mass. These symptoms can persist for years after the end of the treatment and may be exacerbated in older patients who already present skeletal muscle impairment. With the purpose of investigate the DOX-induced long-term effects on aged *soleus* muscle, a cumulative dose of 9 $mg.kg^{-1}$ of DOX was administered to 19-month-old male mice, which were sacrificed two months after the last administration. Data demonstrated that mice presented body wasting and atrophy of the *soleus* muscle, given the significant decrease of the cross-sectional area of muscle fibers. No DOX-related changes were observed in systemic inflammation, skeletal muscle energetic metabolism or mitochondria dynamics. Instead, our results suggest that long-term skeletal muscle atrophy in response to the chronic administration of DOX may develop from elevated myostatin levels and an excessive production of reactive oxygen species (ROS), which may trigger caspase-3-mediated apoptosis, and may impair skeletal muscle regeneration. Therefore, targeting the heightened production of ROS, apoptosis or satellite cells functionality may counteract the DOX-induced skeletal muscle atrophy, improving the quality of life and survival rates of cancer patients.

Keywords: aging, cachexia, chemotherapy, oxidative stress, muscle wasting, sarcopenia

1. Introduction

Chemotherapy is still one of the most successful therapeutic approaches against cancer. Doxorubicin (DOX) is a broadly used anthracycline in cancer treatment (van der Zanden et al., 2020). DOX acts on cancer cells by intercalating into deoxyribonucleic acid (DNA) and inhibiting topoisomerase-II, and by exacerbating reactive oxygen species (ROS) production, which leads to oxidative stress, lipid peroxidation, damage to cellular membranes and DNA, and activation of cell death pathways (Thorn et al., 2011). The cumulative total lifetime dose of DOX is currently set at 450-500 $mg.m^{-2}$ of body surface for adults (according to the Food and Drug Administration (FDA) and the European Medicines Agency (EMA)) as a result of an increased risk of cardiotoxicity above this dose. However, its use has been associated with other less studied dose-dependent acute and chronic adverse effects, such as myotoxicity (Hiensch et al., 2020). DOX-induced myotoxicity ensues a rapid atrophy of both limb and respiratory muscles, exercise intolerance, and skeletal muscle fatigue and weakness (Powers et al., 2019; Smuder, 2019). Skeletal muscle weakness and fatigue often persist after cessation of the treatment and can occur acutely or within the years after the therapy is over (Huertas et al., 2020). Skeletal muscle weakness might occur as a consequence of alterations at or distal to the neuromuscular junction (NMJ), a highly specialized synapse that ensures the efficient transmission of electric impulses from the presynaptic innervating motoneuron to the postsynaptic innervated muscle fibers to stimulate contraction (Huertas et al., 2020). In older patients, the DOX-induced loss of skeletal muscle mass and function may exacerbate the preexisting impairment of skeletal muscle induced by aging. Hitherto, DOX-induced cardiotoxicity has been receiving all the attention from the scientific community. Hence, the molecular foundations underlying DOX-induced skeletal muscle toxicity remain to be fully understood, especially the ones that contribute to the long-term effects of DOX in this organ. Indeed, according to a recent meta-analysis and systematic review, only six preclinical studies assessed the long-term effects of DOX treatment on the skeletal muscle (Hiensch et al., 2020). If we analyze the studies harboring older rodents, the number is even lower. In addition, in most of the preclinical studies that tested the effects of DOX

on the skeletal muscle, the treatment protocol consisted of a single dose of DOX, which does not mimic the regimen used in humans, biasing the extrapolation of the outcomes to the clinical setting.

Considering the aforementioned facts, the aim of this study was to investigate the impact of a cumulative dose of DOX, administered in cycles as in human therapy, on the *soleus* muscle of aged mice, two months after the last administration (which corresponds to about 4 years in humans (Sengupta, 2013)). Specifically, we explored the *soleus* muscle mass and fiber cross-sectional area (CSA), and its association with mitochondrial density and dynamics, and markers of systemic inflammation, oxidative stress, proteolysis, apoptosis, and NMJ. The *soleus* muscle was chosen for analysis due to its higher percentage of slow fibers, which seem to be particularly susceptible to DOX-induced skeletal muscle fatigue (Powers et al., 2019), and due to previous research that demonstrated that the functional impairment, i.e. maximal twitch force and fatigue, induced by DOX was superior in the *soleus* muscle compared to other limb muscles (Hydock et al., 2011).

2. Materials and methods

2.1 Animal protocol

Male CD-1 mice were obtained from the Charles River Laboratories (FR) and maintained in the School of Medicine and Biomedical Sciences Abel Salazar (ICBAS, Porto, PT) animal facilities until reaching 19 months (approximately 63 years in humans; lifespan of laboratory mice is of about 24 months (Dutta & Sengupta, 2016)). CD-1 male mice were used since they are the most used in toxicology studies and to avoid the need of cycle synchronization regarding the use of female mice, which leads to additional stress to the animals (at this age the mice were no longer in fertile age but this study was included in a wider study harboring younger ages) (Festing, 2007). Before the initiation of the experiments, mice were acclimatized to the handler and the experimental protocol for one week and then randomly distributed (maximum of three mice *per* cage; IVC Sealsafe Plus Green Mouse 500 cages, Tecniplast, London, UK) into two experimental groups: control group (CONT, n=8) and DOX group (DOX, n=9). To mimic human

DOX therapy, the mice from the DOX group received six intraperitoneal (i.p.) administrations of 1.5 $mg.kg^{-1}$ of DOX (cumulative dose of 9 $mg.kg^{-1}$; dissolved in 0.9% NaCl), two times *per* week for three consecutive weeks. This cumulative dose corresponds to approximately 27 $mg.m^{-2}$ in adult humans, using the formula based on body surface area (Reagan-Shaw et al., 2007), which is lower than the 450-500 $mg.m^{-2}$ recommended cumulative lifetime dose for adults (according to FDA and EMA). The use of subclinical doses allows the investigation of the adverse effects of the drug and the gathering of valuable data without subjecting the animals to excessive toxicity and/or harm, fulfilling the reduction and refinement objectives from the 3Rs principle (Russell & Burch, 1959). The i.p. route of administration of the drug was used, instead of intravenously, to avoid severe perivasculitis and necrosis resulting from the intravenous injection in the tail vein (Olson & Capen, 1978). All mice from the CONT group received six i.p. administrations of 0.9% NaCl, in the same volume and conditions as the mice from the DOX group. The mice were housed at controlled temperature (22 ± 2°C) and relative humidity (50 ± 5%) with a 12:12h light-dark cycle with *ad libitum* food (Mucedola 4RF21, Milan, IT) and water access. Two months after the last administration of DOX, the mice were weighted, anesthetized by isoflurane inhalation, and sacrificed by exsanguination in the inferior vena cava and the blood was collected. The presence of visible abnormalities, such as ascites, was performed by gentle palpation of the abdomen and examination of the abdominal cavity to assess the presence of fluid. The two *soleus* muscles of all mice were also collected and weighed. Sample-specific processing is explained in the following subsections. The right and left tibiae were dissected to determine tibia length, which is an indicator of animal body size that is independent of alterations in muscle and fat masses (Yin et al., 1982). All the procedures were performed in agreement with the European Council Directive (2010/63/EU) and approved by the local Committee Responsible for animal welfare (ORBEA) of ICBAS (project number 140/2015) and the General Directorate of Food and Veterinary Medicine (reference number 0421/000/000/2016).

2.2 Serum samples preparation and analyses

The blood collected from the inferior vena cava was transferred to a collecting tube with an inert clotting agent (Vetlab ZT, Plain (serum) + GEL, Vetlab Supplies, West Sussex, UK) and left at room temperature for at least 45 minutes to form the clot, followed by a room temperature centrifugation for 10 minutes at 900 *g*. The obtained serum was stored at -80°C for posterior biochemical analyses.

Total protein, albumin, cholesterol and non-fasting glucose levels in serum were measured on an AutoAnalyzer (PRESTIGE 24i, Cormay PZ, Diamond Diagnostics, Massachusetts, US). The levels of C-reactive protein (CRP), interleukin-6 (IL-6), myostatin, dinitrophenol (DNP), 3-nitrotyrosine (3-NT), brain-derived neurotrophic factor (BDNF) and calcitonin gene-related peptide (CGRP) were assessed by immunoblotting as described in subsection 2.6.

2.3 Histological analysis of the soleus muscle

One of the *soleus* muscles of each mice was fixed in 4% paraformaldehyde and embedded in paraffin to prepare paraffin blocks, which were sectioned (5 µm of thickness) using a manual microtome. After deparaffinization with xylol and hydration with decreasing concentrations of alcohol (100%, 95% and 75%, v/v), slides were stained with hematoxylin and eosin (H&E). Skeletal muscle slides were examined in the optical microscope Zeiss Axio Imager Z1 (Carl Zeiss, Jena, DE). The CSA was measured for, at least, 600 muscle fibers *per* group with the ZEN Lite software (ZEN v3.2 (blue edition), Carl Zeiss, Jena, DE).

2.4 Preparation of soleus muscle homogenates

The other muscle of each mice was homogenized on a Teflon® pestle on a motor-driven Potter-Elvehjem glass homogenizer at 0-4°C in a phosphate buffer (0.1 M KH_2PO_4 pH 7.4, 0.5% Triton X-100 and 1:1000 PMSF) in the proportion of 50 mg of muscle to 1 mL of buffer. The protein content of the muscle homogenates was assessed using the commercial kit DC™ Protein Assay (Bio-Rad, California, US), according to the manufacturer's instructions and using bovine serum albumin as standard. The *soleus* homogenates were then preserved at -80°C for the posterior biochemical analyses described in the subsections 2.5 and 2.6.

2.5 Enzymatic assays in the soleus muscle homogenates

Citrate synthase (CS) activity was assessed in *soleus* muscle homogenates based on the method described by Coore and collaborators (Coore et al., 1971). Briefly, 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) reacted with the thiol groups (-SH) of coenzyme A (CoA), previously released by the reaction of acetyl-CoA with oxaloacetate, which was spectrophotometrically measured at 412 nm (molar extinction coefficient of 13.6 $mM^{-1}.cm^{-1}$). The activity of CS was expressed in µmol *per* minute *per* milligram of *soleus* total protein.

Caspase-3 activity was spectrophotometrically assessed in *soleus* muscle homogenates based on the method previously described (Simões et al., 2013). Briefly, 25-30 µg of protein were incubated in assay buffer (25 mM HEPES pH 7.5, 0.1% CHAPS, 10% sucrose and 10 mM DTT) with 100 µM of caspase-3 substrate (Ac-DEVD-p-nitroanilide (p-NA), J65878 from Thermo Fisher Scientific, Massachusetts, US) for 2 hours at 37°C. The caspase-3 activity was determined by the detection of the chromophore p-NA measured at 405 nm and using p-NA as standard. The obtained activity values were expressed in µmol of p-NA *per* mg of *soleus* total protein *per* minute.

2.6 Immunoblot analyses

To determine the content of protein carbonyls of the *soleus* muscle and serum, the samples were derivatized before the slot blot analysis, as previously described (Padrão et al., 2012). Briefly, a certain volume (V) correspondent to 20 µg of protein of muscle homogenate or 5 µL of serum was mixed with 1V of SDS 12% and 2V of 10 mM dinitrophenylhydrazine (DNPH) in 10% trifluoroacetic acid (TFA), followed by 30 minutes of dark incubation. After this, 1.5V of a solution constituted by 18% of β-mercaptoethanol in Tris 2M was added. The derivatized samples were analyzed by slot blot and therefore were diluted 1:50 (*soleus* homogenate) or 1:150 (serum) in Tris-buffered saline (TBS) and blotted under vacuum onto a nitrocellulose membrane (Amersham™, Protan®, GE Healthcare, Illinois, US). The immunodetection was made using an anti-DNP antibody as explained below.

For the slot blot analysis of the other serum markers, samples were diluted 1:50 in TBS and then blotted under vacuum onto a nitrocellulose membrane (Amersham™, Protan®, GE Healthcare, Illinois, US).

Regarding the western blot analyses of the *soleus* muscle, equivalent volumes to 50 µg of protein of each muscle homogenate were electrophoresed on a 12.5% SDS-PAGE gel according to Laemmli (Laemmli, 1970) and then blotted onto a nitrocellulose membrane (Amersham™, Protan®, GE Healthcare, Illinois, US) during 2 hours at 200 mA. Protein loading was controlled by Ponceau S staining, since most of the housekeeping markers (cytoskeletal and metabolic proteins) used can be modulated by stressful stimuli in the skeletal muscle (De Beer et al., 1992; Vigelsø et al., 2015).

Nonspecific binding was blocked by the incubation of the membranes in a 5% (w/v) solution of non-fat dry milk in TBS with Tween 20 (TBST) for 1 hour at room temperature and with agitation. The membranes were then incubated with the corresponding primary antibody: anti-adenosine triphosphate synthase subunit beta mitochondrial precursor (ATPB, ab14730), anti-BDNF (ab226843), anti-CRP (ab32412), anti-electron transfer flavoprotein-ubiquinone oxidoreductase mitochondrial (ETFDH, ab91508), anti-IL-6 (ab6672), anti-manganese superoxide dismutase (MnSOD, ab13533), anti-myostatin (ab98337), anti-ATP-dependent 6-phosphofructokinase muscle type (PFKM, ab154804), anti-peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1α, ab191838), and anti-phosphorylated mothers against decapentaplegic homolog 3 (pSMAD3, ab51451) from Abcam (Cambridge, UK); anti-CGRP (PA5-114931), and anti-muscle RING-finger protein-1 (MuRF1, PA5-76695) from Invitrogen (Massachusetts, US); anti-3-NT (MAB5404), and anti-DNP (MAB2223) from Sigma-Aldrich (Missouri, US); anti-atrogin-1 (AP2041) from ECM Biosciences (Versailles, US); and anti-myoblast determination protein 1 (MyoD, sc-760) from Santa Cruz Biotechnology (Texas, US). This incubation was followed by three 10-minute washes with TBST, incubation with the corresponding secondary antibody (anti-rabbit (NA934V) or anti-mouse (NA931V) from GE Healthcare, Illinois, US) and new washes. Both primary and secondary antibodies were diluted 1:1000 in 5% (w/v) non-fat dry milk in TBST. The primary antibody was

incubated during 2 hours at room temperature with agitation or overnight at 4°C, and the secondary antibody during 2 hours at room temperature with agitation. Immunoreactive bands were detected with enhanced chemiluminescence (ECL) reagents (ECL Clarity, Bio-Rad, California, US) according to the manufacturer's procedure. Images were acquired using the ChemiDoc Imaging System (Bio-Rad, California, US) and analyzed using the Image Lab software (v6.0.0., Bio-Rad, California, US). The optical density (OD) values were expressed in arbitrary units.

2.7 Statistical analyses

Data normality was evaluated by the Shapiro-Wilk test, except for CSA data that was evaluated by the Kolmogorov-Smirnov test. The significant differences among groups were determined by performing the unpaired t-test for normally distributed data and the Mann-Whitney U test for non-normally distributed data, and the values are present as mean ± standard deviation (SD) or median ± quartiles, respectively. Spearman correlations were also performed for an in-deep understanding of the results, when considered relevant. Results were considered significantly different when $p<0.05$. The body weight gain of each mice was calculated using the formula body weight gain (%) = [(final body weight - initial body weight) / final body weight] ×100 (Oliveira et al., 2017). *Soleus* muscle mass values were also normalized to body weight and tibia length. All the statistical analyses were performed using the GraphPad Prism software for windows (v6.01, California, US).

3. Results

3.1 Characterization of the long-term response of mice to DOX administration

The long-term impact of DOX administration on body weight and *soleus* muscle mass can be observed in **Table 1**. No differences were found in any of these parameters, suggesting that the body weight and the mass of the *soleus* muscle were not changed two months after the end of DOX administration. Nevertheless, body weight might have been overrated due to the ascites observed in DOX mice (22% vs. 0% in CONT group), which may also have contributed to the absence

of significant differences in the body weight gain (**Table 1**). The decrease in weight gain in both groups can be justified by the natural growth of the mice. Nonetheless, a decrement in body weight gain of more than 5% was observed in approximately 56% of the mice that received DOX (vs. CONT mice), which is indicative of the presence of body wasting in the majority of DOX mice.

Table 1 Body weight, body weight gain and *soleus* muscle mass of control (CONT, n=8) and doxorubicin (DOX, n=9) mice. *Soleus* muscle mass normalized to body weight or tibia length is also shown. Values are present as mean ± SD. The percentage of mice with ascites is also demonstrated.

	CONT	**DOX**
Body weight (g)	47.190 ± 6.308	46.018 ± 5.243
Body weight gain (%)	-6.548 ± 25.260	-17.010 ± 20.140
***Soleus* muscle mass (g)**	0.010 ± 0.007	0.011 ± 0.003
***Soleus* muscle mass / body weight (%)**	0.020 ± 0.014	0.023 ± 0.006
***Soleus* muscle mass / tibia length ($g.cm^{-1}$ %)**	0.48 ± 0.38	0.52 ± 0.11
Percentage of mice with ascites	0%	22%

The levels of some general serum markers were measured to investigate the long-term systemic response of the mice after the end of the administration of DOX (**Figure 1**). No differences between CONT and DOX mice were seen in the levels of total protein, albumin, CRP and IL-6, suggesting that two months after the end of DOX administration inflammation is not present, as observed acutely (Renu et al., 2022). Circulating non-fasting glucose levels did not reach statistical significance but showed a tendency to increased levels in DOX mice (p=0.0808). In addition, myostatin levels were increased in DOX mice, indicating the presence of an imbalance between protein synthesis and degradation towards increased proteolysis. To evaluate whole body susceptibility to ROS- and reactive nitrogen species (RNS)-induced oxidative stress, the serum levels of DNP and 3-NT were assessed, and no differences were found, indicating that two months after the end of DOX administration, an exacerbated systemic oxidative stress state is not present.

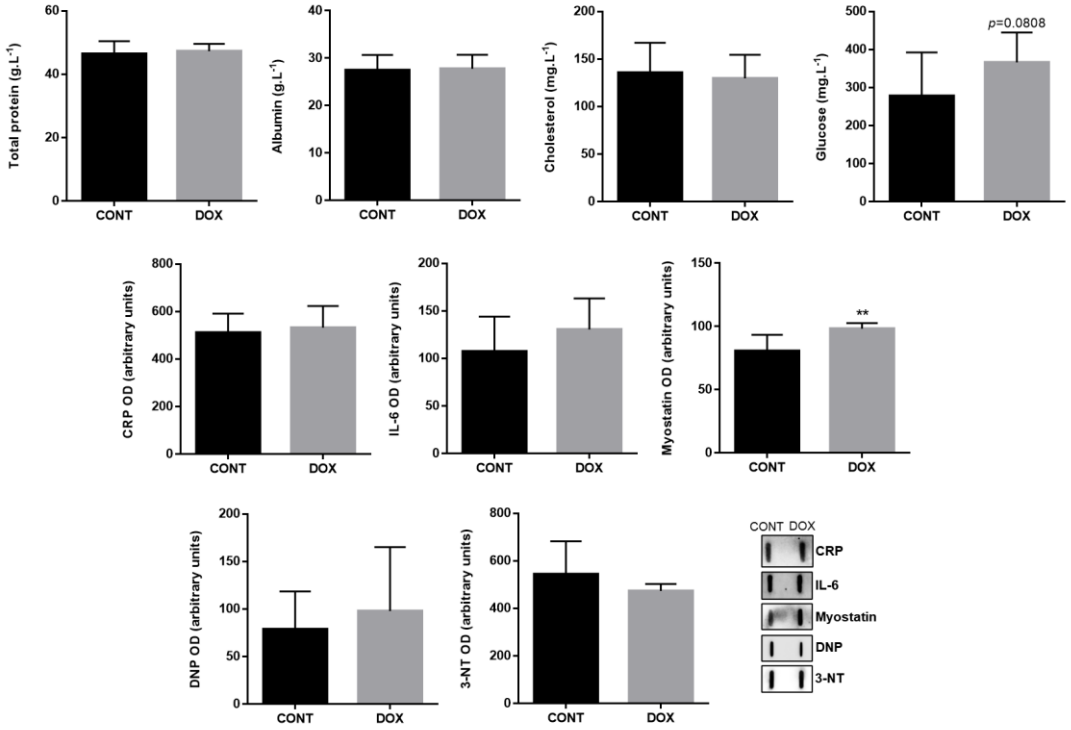

Figure 1 Serum markers in control (CONT, n=8) and doxorubicin (DOX, n=9) mice. Total protein, albumin, cholesterol and glucose levels were measured in an autoanalyzer, and CRP, IL-6, myostatin, DNP and 3-NT levels were assessed by immunoblotting. Representative immunoblots are displayed. Values are present as mean ± SD (** p<0.01).

The catabolic state implied by the increased myostatin levels was coherent with the fiber CSA measurements, which showed a significant decrease of fiber size in DOX mice (approximately 42% decrease vs. CONT mice), indicating the presence of fiber atrophy in the *soleus* muscle two months after the last DOX administration (**Figure 2a**). The histogram of fiber CSA distribution confirms these data by showing that DOX mice had a higher percentage of smaller *soleus* fibers compared with CONT mice (**Figure 2b**). Specifically, the fibers CSA of DOX mice varied from approximately 160-1997 μm^2 compared with 283-3275 μm^2 of CONT mice. While the fibers of CONT mice were grouped tightly together in fascicles, fibers from DOX mice were separated by an apparent widening of the interstitial space, especially of the endomysium, which agrees with the presence of muscle atrophy in these mice (**Figure 2c**). In addition, numerous angular atrophied muscle fibers were exclusively observed in the *soleus* of DOX mice (**Figure 2c**).

This histopathological finding can also be observed in aged muscle and represents denervation without reinnervation, prompting myofibers to activate apoptosis (Engel, 2015). Placing these facts together, it is implied that DOX may exacerbate the adverse effects of biological aging on the skeletal muscle.

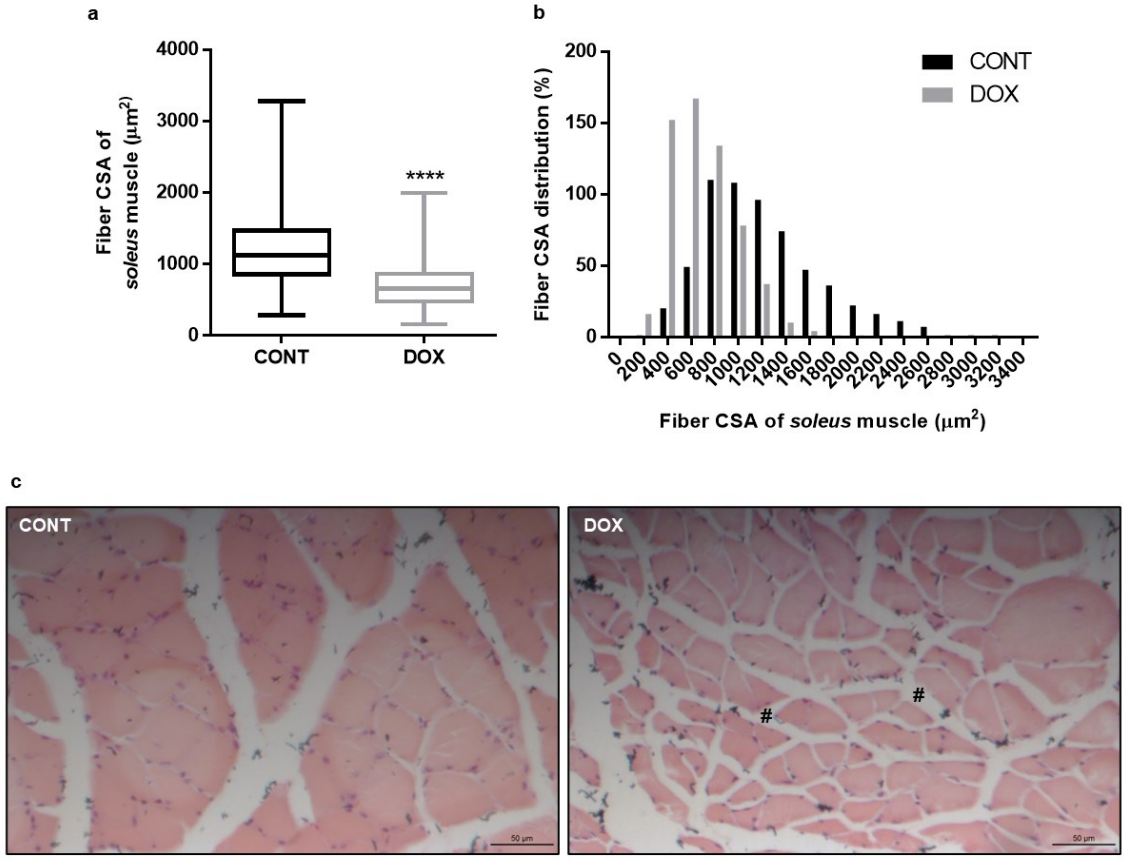

Figure 2 The long-term effects of doxorubicin (DOX) administration on fiber cross-sectional area (CSA). (a) Boxplot of fibers CSA of the *soleus* muscle. Values are present as median ± quartiles. (b) Fiber CSA distribution of the *soleus* muscle. (c) Representative photomicrographs of H&E-stained *soleus* muscle sections, where most of the muscle fibers from DOX mice were angulated (i.e., angular fibers; hash indicates the widened interstitial space). The reference bars represent 50 μm (**** p<0.0001).

3.2 Long-term impact of DOX administration in the soleus muscle energetic metabolic profile, and susceptibility to proteolysis, apoptosis and oxidative stress

Markers from energetic metabolism were analyzed (**Figure 3**). No differences in ATPB, PFKM and ETFDH levels or PFKM/ATPB and ETFDH/ATPB ratios were

noticed. Mitochondrial remodeling was also investigated by the analysis of the levels of the master regulator of mitochondrial biogenesis PGC1α (Kang & Ji, 2012) and CS activity, which is a valid biomarker for mitochondrial density in skeletal muscle (Vigelsø et al., 2014). No differences in PGC1α levels or CS activity were observed. These data suggest that the *soleus* energetic metabolism was unaffected or was recovered two months after the end of DOX administration.

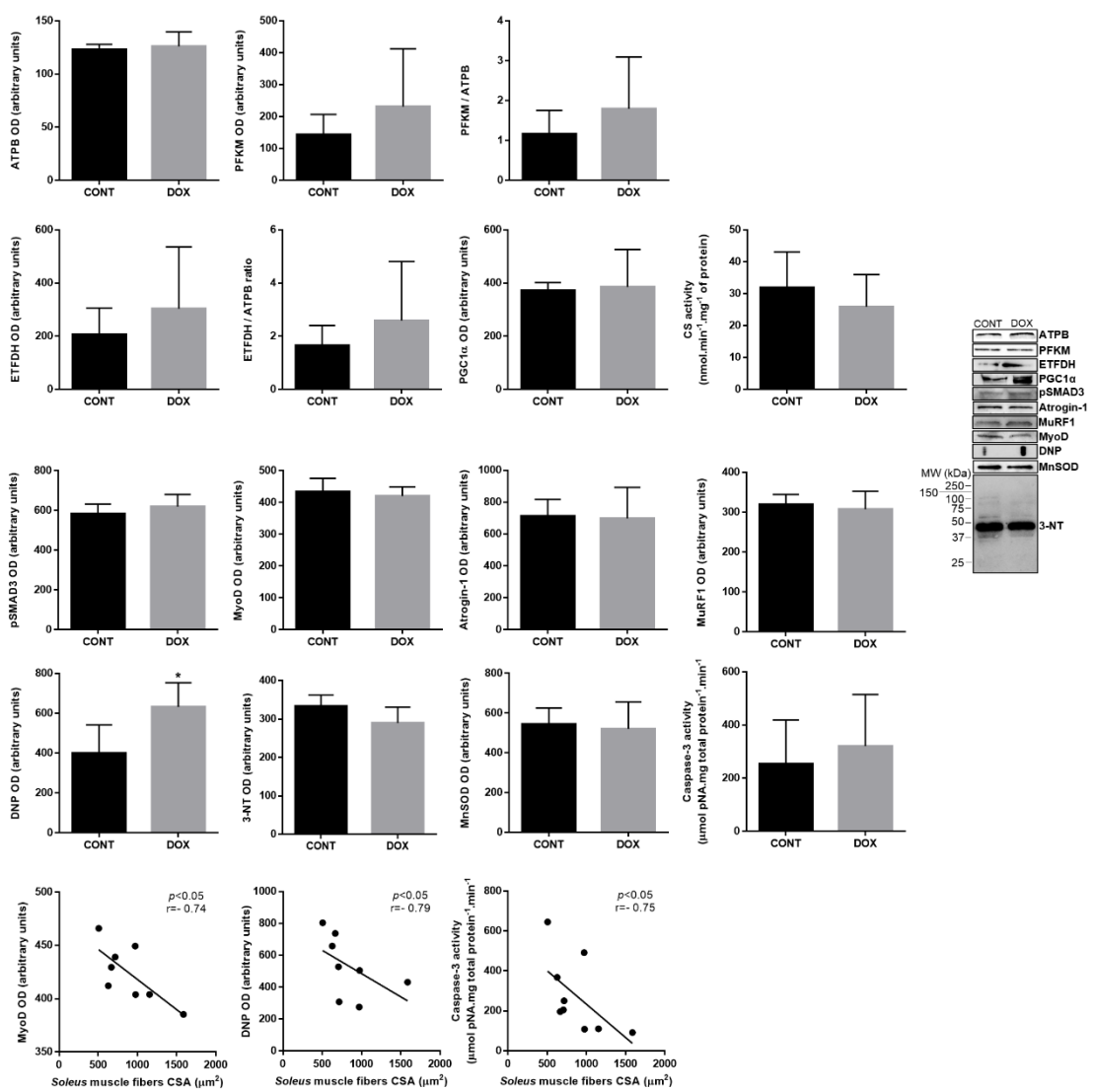

Figure 3 The levels of mediators of energetic metabolism and proteolytic pathways of *soleus* muscle in control (CONT, n=5) and doxorubicin (DOX, n=6) mice, evaluated by immunoblotting with exception of CS and caspase-3 activities that were spectrophotometrically measured. Representative immunoblots are displayed along with the molecular weight (MW) of the ladder when necessary.

Spearman correlations were performed for an in-deep understanding of the results. Values are present as mean ± SD (* p<0.05).

Given the increased serum levels of the catabolic myokine myostatin, its downstream players were evaluated (**Figure 3**) to ascertain its putative contribution to the skeletal muscle fiber atrophy observed in DOX mice. No differences were found in the levels of the regulatory pSMAD3 nor of the muscle-specific E3 ubiquitin ligases, atrogin-1 and MuRF1. It is also known that myostatin is a negative regulator of myogenesis through the deregulation of MyoD. Despite no differences in the *soleus* muscle levels, MyoD levels negatively correlated with the fibers CSA values (**Figure 3**), suggesting a higher myogenesis in DOX mice.

Protein carbonylation and nitration were also assessed in the *soleus* muscle to evaluate the direct effect of oxidative stress in the skeletal muscle (**Figure 3**). DOX mice had higher levels of DNP, which is indicative of higher protein carbonylation, and therefore of higher ROS-induced damage in the *soleus* muscle of these mice, two months after the end of DOX administration. Moreover, these DNP levels negatively correlated with the fibers CSA values (**Figure 3**), suggesting a role in the *soleus* muscle atrophy observed. Protein carbonylation is the most common ROS-induced protein modification (Lourenço dos Santos et al., 2015). It occurs when proteins directly interact with ROS, resulting in the introduction of carbonyl groups (aldehydes and ketones; C=O) in the side chains of certain amino acids, such as arginine, lysine, threonine, and proline (Lourenço dos Santos et al., 2015). Since protein carbonylation is irreversible and causes irreparable damages, leading to the loss of protein function, it has been considered an indicator of protein oxidative damage (Lourenço dos Santos et al., 2015). No differences, however, were found in the muscle levels of 3-NT (**Figure 3**), which is a biomarker of RNS-mediated protein damage. 3-NT is formed by the reaction of peroxynitrite ($ONOO^-$) with tyrosine residues, and $ONOO^-$ is, in turn, the product of nitric oxide ($^{\bullet}NO$) with superoxide anion ($O_2^{\bullet-}$) (Kehm et al., 2021). So, these results indicate that two months after the last DOX administration, oxidative stress was still exacerbated in the *soleus* muscle, which appears to be caused by a specific ROS production, with no significant changes in the muscle levels of RNS and, possibly, of $^{\bullet}NO$. The levels of MnSOD, one of the main

mitochondrial antioxidant enzymes, were also analyzed in the *soleus* muscle but no differences were discovered between groups (**Figure 3**).

Taking into consideration the increased oxidative protein damage and the angular fibers present in the muscle of DOX mice, we analyzed the activity of caspase-3 (**Figure 3**), but no differences were observed between groups. Still, caspase-3 activity negatively correlated with the fibers CSA values (**Figure 3**), indicating an association between the apoptotic pathway and the skeletal muscle atrophy observed in DOX mice.

3.3 The long-term effect of DOX administration on neuromuscular junction-related markers

We assessed the circulating levels of two NMJ-related mediators, BNDF and CGRP, by immunoblotting to evaluate the NMJ long-term remodeling in response to DOX administration. BDNF is involved in motoneuron survival, presynaptic release of acetylcholine (ACh), maintenance of the postsynaptic region, and regulation of skeletal muscle metabolism and regeneration, and satellite cell (SC) differentiation (Kalinkovich & Livshits, 2015). Moreover, it is secreted in an activity-dependent manner (Hurtado et al., 2017). CGRP is thought to be involved in the processes of reinnervation and nerve sprouting upon denervation (Blasco et al., 2020). No differences were found in the serum levels of these mediators (**Figure 4**), nor any significant correlation with the fiber CSA values (**Supplementary Figure 1**), suggesting that these NMJ-related mediators are not involved in the *soleus* muscle atrophy observed. However, the relation between skeletal muscle atrophy and changes in the circulating levels of BDNF and CGRP, particularly in older subjects, is not yet clarified.

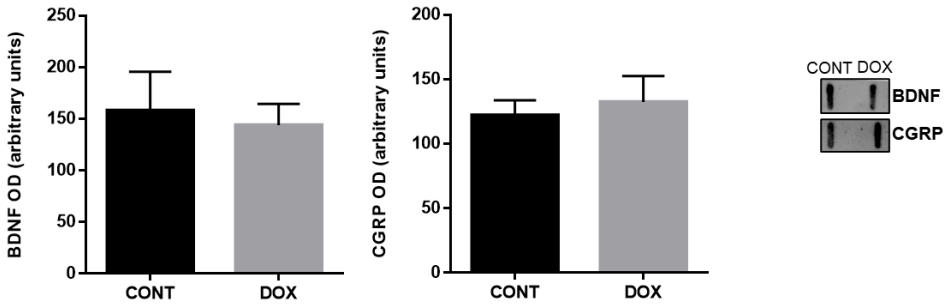

Figure 4 Markers of neuromuscular junction remodeling in control (CONT, n=5) and doxorubicin (DOX, n=6) mice. BDNF and CGRP levels were analyzed in serum by immunoblotting. Representative immunoblots are displayed. Values are present as mean ± SD.

4. Discussion

This study demonstrated that the atrophy of the aged *soleus* muscle constitutes a long-term effect of the administration of DOX, possibly triggered by a sustained exacerbation of ROS generation and myostatin that prompts caspase-3-mediated apoptosis and impairs skeletal muscle regeneration upon acute DOX-mediated damage. These study outcomes will be now discussed in detail.

Data obtained revealed a decrement in body weight gain of more than 5% (vs. CONT mice) in approximately 56% of the DOX mice, indicating body wasting as a long-term effect of DOX administration, despite the use of a cumulative dose lower than the recommended levels for cancer treatment in humans. This was accompanied by a decrease of approximately 42% of the *soleus* fibers CSA of DOX mice, suggesting skeletal muscle atrophy as a long-term effect of DOX. The coexistence of reduced CSA without decreased *soleus* muscle mass in DOX mice may be explained, at least in part, by the presence of a generalized enlarged interstitial space in these mice, which aligns with the occurrence of fiber atrophy. The enlargement of the interstitial space may also indicate the presence of interstitial edema, as previously observed in the heart (Abdelatty et al., 2021). The presence of skeletal muscle atrophy and body wasting in DOX mice led us to hypothesize the development of DOX-induced cachexia, but no signs of a long-term enhanced inflammation, given by unchanged serum levels of CRP, albumin and IL-6 levels, were noticed. This result strengthens the idea that the skeletal

muscle atrophy observed in DOX mice is a direct effect of the drug in the skeletal muscle and not an effect mediated by the action of systemic inflammatory factors. In contrast, myostatin serum levels were found elevated in DOX mice, which agrees with the presence of muscle atrophy in these animals (**Figure 5**), despite no differences in its downstream mediators, namely in the levels of the E3 ligases from the ubiquitin-proteasome system. Myostatin is also a known negative regulator of myogenesis, being able to debilitate skeletal muscle regeneration (Chen et al., 2021). However, the myogenic regulatory factor MyoD was negatively associated to *soleus* muscle atrophy, implying activation of myoblast differentiation in the skeletal muscle two months after DOX was administered. These data symbolize either a desensitization of the skeletal muscle to SCs or a DOX-induced repression of endogenous MyoD function as previously proposed (Kurabayashi et al., 1994). Besides, aging itself is also suggested to reduce the SC pool and function (Switching, 2022), turning skeletal muscle regeneration challenging.

Changes in fiber CSA are usually accompanied by metabolic adaptations, and by being a slow-twitch muscle, *soleus* is highly reliant on mitochondrion. It has been proposed that DOX induces skeletal muscle atrophy by triggering mitochondrial dysfunction in this organ, which might culminate in an exacerbated production of ROS by the organelle itself (Hiensch et al., 2020). In the present study no differences were found in CS activity nor in the levels of PGC1α, suggesting that a reduced mitochondrial density or biogenesis does not constitute a long-term effect of DOX exposure. Another hypothesis that might be raised is that DOX did not aggravate the already existing age-induced mitochondrial dysfunction, a famed hallmark of skeletal muscle aging (Dai et al., 2014).

DOX mice had higher levels of carbonylated muscle proteins, which were correlated with the *soleus* muscle atrophy, indicating a sustained production of ROS that contributes to skeletal muscle damage (**Figure 5**). In fact, ROS in skeletal muscle increase the susceptible of proteins to proteolysis, fueling skeletal muscle atrophy (Hiensch et al., 2020; Huertas et al., 2020). Since DOX binds specifically to cardiolipin located on the inner mitochondrial membrane, where it can be reduced by complex I of the electron transport chain causing the

formation of $O_2^{\bullet-}$, mitochondria are advocated as the main source of ROS following DOX administration (Montalvo et al., 2020). Therefore, the muscle levels of the mitochondrial antioxidant enzyme MnSOD were evaluated and no changes were noticed in response to DOX, pointing towards a blunted MnSOD signaling or a cytosolic generation of ROS. The latter can be provoked by myostatin-induced stimulation of nicotinamide adenine dinucleotide phosphate oxidase (NOX) in the absence of SMAD3 (Chen et al., 2021), which is in accordance with the unaffected levels of phosphorylated SMAD3 in DOX mice. Similar results were reported in cardiomyocytes (Zheng et al., 2022). The oxidative modifications induced by ROS can increase the susceptibility of myofibrillar proteins to degradation *via* caspase-3 (Smuder et al., 2010). An enhanced activity of caspase-3 in the *soleus* muscle was correlated with *soleus* muscle atrophy, denoting a role of apoptosis in long-term DOX-induced fiber atrophy (**Figure 5**). The histopathological finding of angular fibers in DOX mice strongly suggest the presence of denervated fibers that without reinnervation might trigger apoptosis (Engel, 2015), reinforcing the culprit role of apoptosis in skeletal muscle atrophy.

Following skeletal muscle damage, muscle fibers have the ability to regenerate by activating myogenesis, which may explain the negative correlation between MyoD levels and the fibers CSA (**Figure 5**). However, DOX mice still presented atrophy of the *soleus* muscle, implying a DOX-induced chronic skeletal muscle desensitization to SCs (elimination half-life of DOX is between 20 to 48 hours, according to the FDA), besides the myostatin-mediated effect on MyoD as discussed above, hampering skeletal muscle regeneration. A recent study reported that a cumulative dose of 12 $mg.kg^{-1}$ of DOX increased myogenic factor 5 (MYF5) mRNA expression, which is involved in SCs proliferation and self-renewal, but reduced SCs density in the *soleus* muscle of young animals, five days following the last administration (D'Lugos et al., 2019). This effect of DOX-induced decrease in SCs number may be exacerbated in aged muscle, since SCs pool diminishes with aging (Switching, 2022). In addition, the SCs fraction that survives throughout the aging process may present functional defects that influence their regenerative capacity, which is accompanied by a loss of SCs

capacity to maintain in quiescence (Parker, 2015; Switching, 2022). Future investigations should delve into the acute and long-term effects of DOX on the mechanisms of skeletal muscle regeneration, principally in aged skeletal muscle.

The NMJ impairment can reduce the contractile function and manifest as skeletal muscle weakness, which can occur acutely or within years following DOX exposure (Huertas et al., 2020). It is suggested that an excessive ROS generation is capable of induce NMJ abnormalities, including increased NMJ fragmentation, a typical signal of denervation (Ahn et al., 2019; Rudolf et al., 2014). The evidence on DOX effects on NMJ remodeling is very scarce. Therefore, we studied the levels of BDNF and CGRP in the serum due to skeletal muscle sample limitations. Still, serum levels of BDNF were previously associated with skeletal muscle function (Nakano et al., 2020). No differences were found in the circulating levels of these proteins nor significant correlations with fibers CSA, inferring that they are not involved in the atrophy of the *soleus* muscle or that DOX did not magnified the age-related effects. However, the main source and functional significance (for whole-body and skeletal muscle) of serum BDNF and CGRP are still controversial and unexplored. It is highlighted, however, that inflammation may induced BDNF production, elevating its circulating levels (Papathanassoglou et al., 2015), which might be the reason behind the results obtained. The main source of circulating CGRP is thought to be a “spillover” from its release from the perivascular nerve endings or even from thyroid (Kee et al., 2018; Wimalawansa, 1991), but it is currently unclear. Future studies should explore the effects of chemotherapeutic agents on the NMJ and how it impacts the circulating levels of NMJ-related mediators.

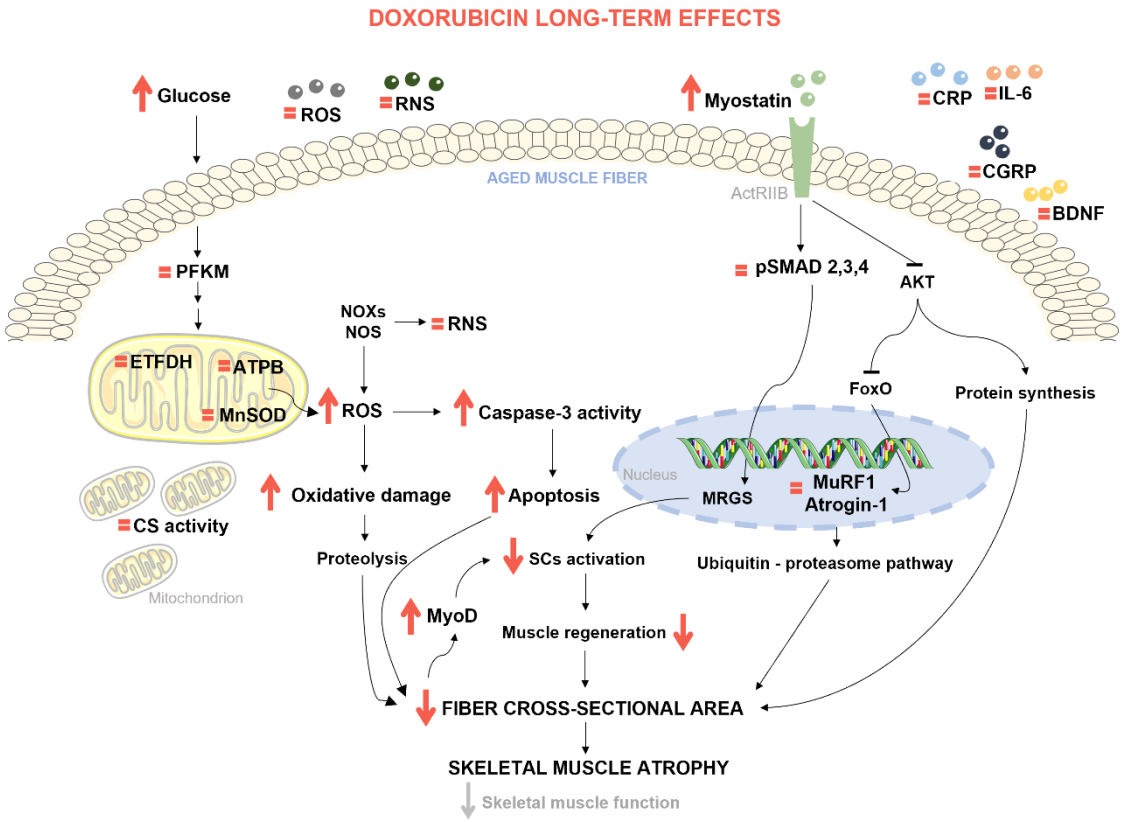

Figure 5 Proposed signaling pathways underlying the long-term adverse effects of doxorubicin in the skeletal muscle. This figure was partly generated using *Servier Medical Art*, provided by Servier, licensed under a Creative Commons Attribution 3.0 Unported License. Legend: ActRIIB: activin receptor type-2B; ATPB: adenosine triphosphate synthase subunit beta, mitochondrial precursor; BDNF: brain-derived neurotrophic factor; CGRP: calcitonin gene-related peptide; CRP: C-reactive protein; CS: citrate synthase; ETFDH: electron transfer flavoprotein-ubiquinone oxidoreductase, mitochondrial; FoxO: forkhead box, class O; IL-6: interleukin-6; MnSOD: manganese superoxide dismutase; MRGs: myogenic regulatory genes; MyoD: myoblast determination protein 1; MuRF1: muscle RING-finger protein-1; NOS: nitric oxide synthase; NOXs: nicotinamide adenine dinucleotide phosphate oxidases; PFKM: ATP-dependent 6-phosphofructokinase, muscle type; pSMAD: phosphorylated mothers against decapentaplegic homolog 3; RNS: reactive nitrogen species; ROS: reactive oxygen species; SCs: satellite cells

5. Conclusion

In summary, our findings indicate that DOX administration induces long-term body wasting and skeletal muscle atrophy, as observed in the *soleus* muscle, in aged mice. DOX induces ROS production and increases myostatin circulating levels in a chronic manner, which may contribute to the atrophy of the skeletal muscle. In this long-term atrophy, a ROS-induced apoptosis through caspase-3 activation, and the impairment of skeletal muscle regeneration possibly due to muscle desensitization to SCs may be involved. This study highlights that an aged muscle is unable to regenerate following the end of chemotherapy administration, which jeopardizes the quality of life and survival rates of the patients. The results from this study give support to future investigations targeting ROS production, apoptosis or SCs function as potential therapeutic strategies to mitigate DOX-induced skeletal muscle atrophy and weakness, particularly in older subjects.

Acknowledgements

This work was supported by CIAFEL (UIDB/00617/2020), ITR (LA/P/0064/2020), LAQV (UIDB/50006/2020) and CITAB (UIDB/04033/2020, https://doi.org/10.54499/UIDB/04033/2020) research units projects through national founds by the Portuguese Foundation for Science and Technology (FCT)/Ministry of Science, Technology and Higher Education (MCTES). The authors would like to thank Celeste Resende for her assistance in sample preparation for the morphological analyses.

Funding

A.M.-P. (SFRH/BD/144396/2019), A.R.-M. (SFRH/BD/129359/2017), and V.M.C (SFRH/BPD/110001/2015 under the transitional rule DL57/2016/CP1334/CT0006) thank the Portuguese Foundation for Science and Technology (FCT)/Ministry of Science, Technology and Higher Education (MCTES) and the European Social Fund (ESF) through PT2020 for their grants.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request. Some data may not be made available because of privacy or ethical restrictions.

Author contribution statement

Conceptualization, R.F. and J.A.D.; Methodology, A.M.-P., R.V., V.M.C.; Validation, A.M.-P. and J.A.D.; Formal Analysis, A.M.-P.; Investigation, A.M.-P., A.R.-M., M.J.N. and T.B.; Writing – Original Draft Preparation, A.M.-P.; Writing – Review & Editing, R.F., J.A.D., P.A.O. and V.M.C.; Visualization, A.M.-P. and R.F.; Supervision, J.A.D., P.A.O. and R.F.

Conflict of interest disclosure

The authors declare no conflict of interest.

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Chapter IV

General discussion

Skeletal muscle wasting stands out as a significant health concern among older adults and is commonly linked to the age-related condition known as sarcopenia (Moreira-Pais et al., 2022). Mitochondrial dysfunction is recognized among the key conceivable culprits of sarcopenia (Marzetti et al., 2013). Recently, the mitochondrion has emerged as a putative hub of conspicuous sex-specific behaviors, reflecting distinct metabolic capacities, calcium handling and resistance to oxidative stress (Ventura-Clapier et al., 2017). This evidence, however, is scarce and to what extent sex modulates mitochondrion is largely unknown with, sometimes, paradoxical data being reported (Junker et al., 2021, 2022). Shedding some light on the mediators involved in age-induced mitochondrial remodeling in the skeletal muscle will certainly abridge the pathway to the sex-personalized management of sarcopenia, and even, other skeletal muscle-related diseases towards superior survival rates and quality of life. Hence, the age-induced effects on mitochondrial remodeling were appraised with a sex viewpoint using young and old female and male rats. Both female and male rats presented *gastrocnemius* muscle atrophy at old ages, given by anthropometric and histological data (cross-sectional area), which was complemented with an increased fibrosis (**Figure 1**). Both histopathological modifications are known to be related with impaired skeletal muscle functionality (Mahdy, 2019). In a sex perspective, skeletal muscle atrophy and fibrosis were more accentuated in female rats, which identifies a higher susceptibility of females to the adverse effects of age on skeletal muscle remodeling. These outcomes were associated to the diminished 17beta(β)-estradiol serum levels in old female rats (**Figure 1**). The decline of the levels of this sex steroid hormone might impair satellite cells activation and proliferation, and promote inflammatory processes and oxidative stress, hampering an efficient muscle regeneration (Geraci et al., 2021; La Colla et al., 2015). Besides, 17β-estradiol serum levels were also related with the estrogen receptor (ER) alpha (ERα) levels in mitochondria (**Figure 1**), implying a management by the sex hormone of mitochondrial functionality. Actually, this conceivable modulation was previously reported (Chen et al., 2004). In contrast, in male rats, testosterone was not involved in age-induced *gastrocnemius* muscle atrophy or fibrosis (**Figure 1**),

which is in line with preceding findings averring the diagnosis of sarcopenia in older men with normal testosterone levels (Yuki et al., 2013). These data recalls the magnitude of the non-generalization of the association between sarcopenia and low testosterone levels in men, which can promote the non-diagnosis of more than 80% of men that maintain normal testosterone levels throughout life (Hackett et al., 2023).

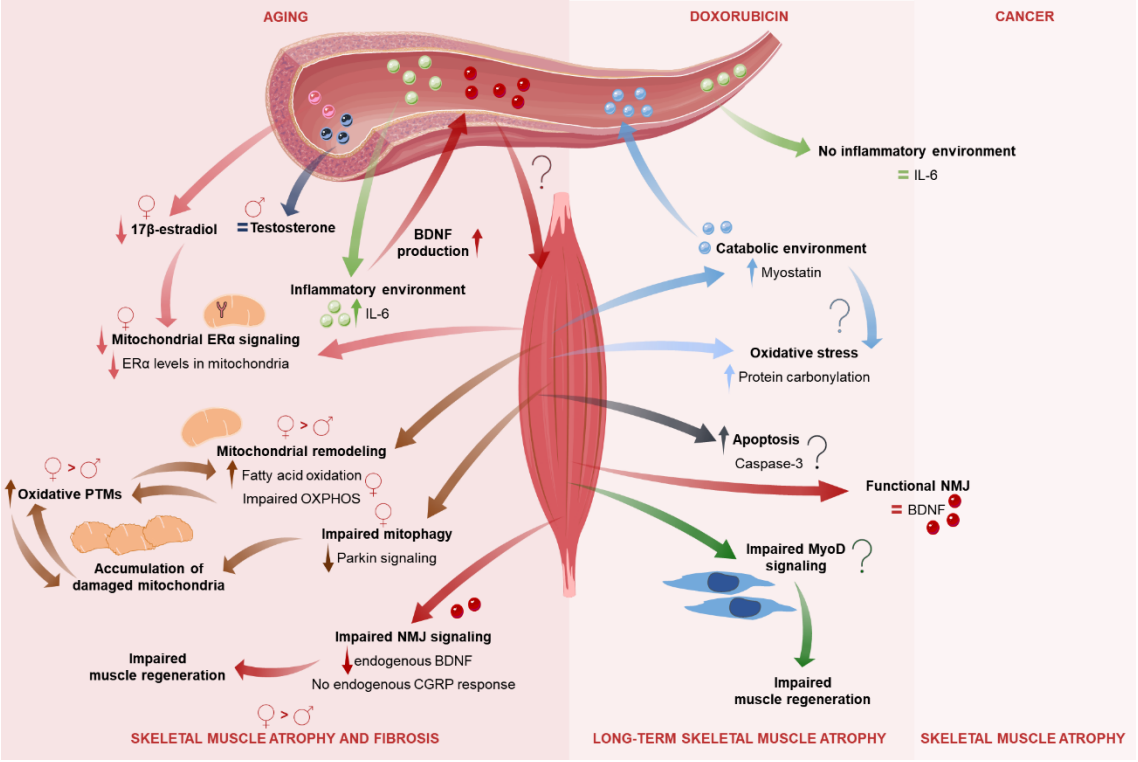

Figure 1 The distinct processes modulated by aging, doxorubicin, or cancer in the pathogenesis of skeletal muscle wasting based on the data obtained in this thesis. The sex-specific alterations in the aging setting are also depicted by the female (♀) and male (♂) symbols. It can be observed a decrease in 17beta(β)-estradiol serum levels and estrogen receptor alpha (ERα) signaling in mitochondrion in females and unchanged testosterone levels in males in response to aging. Whereas age induces mitochondrial remodeling, oxidative modifications, an inflammatory environment and neuromuscular junction (NMJ) signaling impairment, particularly in females, doxorubicin induces a catabolic environment and protein carbonylation as long-term effects. In both doxorubicin- or cancer-induced skeletal muscle wasting the NMJ signaling mediated by brain-derived neurotrophic factor (BDNF) appeared functional and no signs of an

inflammatory environment were observed. This figure was partly generated using *Servier Medical Art*, provided by Servier, licensed under a Creative Commons Attribution 3.0 Unported License. Abbreviations: CGRP, calcitonin gene-related peptide; IL-6, Interleukin-6; MyoD, myoblast determination protein 1; OXPHOS, oxidative phosphorylation; PTMs, posttranslational modifications

To unveil the age-induced effects on mitochondrial remodeling, the mitochondrial proteome was thoroughly examined by mass spectrometry profiling of mitochondria-enriched fractions. The more prominent feature observed was that females are strongly more prone to mitochondrial alterations in response to age than males, which harmonizes with the histopathological findings. The remodeling pattern in females included an increased fatty acid oxidation (FAO) and a compromised oxidative phosphorylation (OXPHOS, **Figure 1**), whereas in males, data denoted that no specific biological process was impaired by age, except for weak evidence towards an age-related induction of the aerobic metabolism. An increased reliance of females *gastrocnemius* muscle on FAO might reflect the increase of the number of slow-twitch muscle fibers that ensues during aging (Baskin et al., 2015; Ohlendieck, 2011). The impaired OXPHOS in females might lead to a deficient production of adenosine triphosphate (ATP) in skeletal muscle, since, for instance, mitochondrial DNA (mtDNA)-encoded subunit 6 (MT-ATP6) levels were decreased and positively correlated with ATP synthase activity. A modulation of MT-ATP6 levels by mitochondrial ERα was also pointed out in the study, establishing a role for the mitochondrial pool of ERs in ATP production, which is in line with the theory revolving around the regulation of mtDNA-encoded genes by mitochondrial ERs (Chen et al., 2004). Still concerning the OXPHOS system, the abundance of particular subunits of complexes I and III was altered in old females, implicating a deficient functionality of these complexes, which can prompt the mitochondrial production of reactive oxygen species (ROS) (Bae et al., 2011). An enhanced production might generate oxidative stress, culminating in skeletal muscle atrophy and impaired contractility (Chen et al., 2022). In point of fact, mitochondrial production of ROS and the consequential redox state has been closely allied to sarcopenia development (Foreman et al., 2021). A higher proclivity for age-related oxidative damage of

mitochondrial proteins was observed in females, particularly of proteins involved in energy production, which is in consensus with their higher skeletal muscle atrophy (**Figure 1**). These facts are in harmony with the increased abundance of the chaperones stress-70 protein (HSPA9) and 10 kDa heat shock protein (HSPE1) and of the protease AFG3 like matrix AAA peptidase subunit 2 (AFG3L2) in old female rats, probably striving mitochondrial homeostasis, giving their involvement in the protein quality control mechanisms of mitochondria, namely the mitochondrial unfolded protein response (mtUPR) (Szczepanowska & Trifunovic, 2022). Withal, it might come to a point when the capacity of the mitochondria protein quality control is outdone, occasioning protein aggregation, and further accrual of oxidized proteins and ROS formation (Reeg & Grune, 2015). In addition, when persistently activated during adulthood, the mtUPR is believed to contribute to age-related skeletal muscle diseases like sarcopenia, possibly when triggered as a result of a defective mitophagy (Wang et al., 2023; Wodrich et al., 2022). In fact, an age-induced dwindled Parkin signaling was observed in females, which can be indicative of a compromised mitophagy efficacy, giving rise to the accumulation of damaged mitochondria (**Figure 1**), a famed hallmark of aging associated with skeletal muscle wasting and functional impairment (Bellanti et al., 2021; Hughes et al., 2022; López-Otín et al., 2013). Indeed, an impaired mitophagy has been heralded as the culprit of such accumulation (Leduc-Gaudet et al., 2021). These observations accentuate the worth of the crosstalk between posttranslational modifications (PTMs) and skeletal muscle aging, which is currently slightly explored and accompanied by incongruous outcomes towards higher or lower susceptibility of females to oxidatively-modification of proteins than males (Colom et al., 2015; Fanò et al., 2001; Gorni & Finco, 2020).

Skeletal muscle wasting is not exclusive of aging since it can also be attributable to chronic diseases such as cancer. Many of the foundations inherent to sarcopenia and cancer cachexia are conjoint but the weight of their impact to the development of each condition might differ. Despite this being an illustrious hypothesis, the investigations delved into the simultaneous comparison of age- and cancer-induced skeletal muscle remodeling are infrequent. Herein,

mediators of inflammation and neuromuscular junction (NMJ) remodeling, two processes plausibly labelled as holding a high clinical differential power, were addressed in rats throughout young, adult and old ages as a model of sarcopenia, in tandem with adult rats with chemically-induced breast cancer (BCa) as a model of cancer cachexia. The implementation of this cancer model for the study of cancer cachexia considered the body and skeletal muscle wasting previously observed in this BCa preclinical model (Padrão et al., 2017). Although not as prevalent in BCa as in other cancer types, cancer cachexia incidence and prevalence (about 25%) is rising in BCa patients (Mallard et al., 2021). Both age and cancer *per se* induced *gastrocnemius* muscle wasting (**Figure 1**). Specifically in the aging scenery, the loss of skeletal muscle mass was evident from adulthood into old age. The loss of skeletal muscle mass in old rats was associated with an amplified inflammation in view of the increased interleukin-6 (IL-6) levels in serum, which anew was apparent from adulthood into old age, indicating a sustained inflammatory environment (**Figure 1**). It is thought that the IL-6 mechanism of action involves a prolonged activation of signal transducer and activator of transcription 3 (STAT3) signaling (Moresi et al., 2019), which was not supported by this study. The rise in IL-6 serum levels emerged as a potential inducer of brain-derived neurotrophic factor (BDNF) production, substantiating its increased levels in circulation in old rats, which were linked to skeletal muscle mass loss (**Figure 1**). The circumstance that increased BDNF plasma levels were associated with sarcopenia severity among older women (da Costa Teixeira et al., 2023), lay emphasis on its value as a differential diagnostic marker. Parallelly, BDNF levels in *gastrocnemius* muscle declined from adulthood into old age, which was associated with age-induced skeletal muscle mass loss (**Figure 1**). This dulled signaling of endogenous BDNF to damaged aged skeletal muscle contributes to further skeletal muscle impairment, giving its role in skeletal muscle regeneration (Mousavi & Jasmin, 2006), as alluded in literature (Greising et al., 2015). Concomitantly, calcitonin gene-related peptide (CGRP) levels were unchanged throughout aging in the skeletal muscle, which further contributes to muscle atrophy (**Figure 1**), given its role in NMJ maintenance *via* antiproteolytic actions upon fiber denervation (Gustafsson & Ulfhake, 2021; Machado et al.,

2019). No inflammation was perceived in response to tumor development (**Figure 1**), which stands out against what literature advocates (Chen et al., 2022), though it may reflect the disease stage. The neuromuscular environment was also not affected by tumor development (**Figure 1**), reinforcing BDNF as a biomarker of sarcopenia.

In the cancer setting, chemotherapy might also retain some liability on skeletal muscle impairment. The anticancer drug doxorubicin (DOX), which is extensively employed in the management of BCa, has been associated with skeletal muscle weakness and fatigue that frequently endure after treatment cessation (Huertas et al., 2020). Regardless of this robust evidence, DOX-induced myotoxicity remains an unheeded adverse effect of DOX, particularly in aged skeletal muscle. Thence, the DOX-induced long-term effects on the *soleus* muscle were explored in old mice. *Soleus* muscle atrophy was still evident two months following the termination of DOX administration, revealing an incapacity of the muscle to regenerate upon DOX-induced damage, even when administered in a subclinical dose. No signs of enhanced inflammation were apparent, withdrawing a sustained inflammatory environment as a pivotal factor accountable for long-term skeletal muscle atrophy (**Figure 1**). Instead, DOX engendered long-term *soleus* muscle atrophy through the upregulation of the myokine myostatin in unison with an amplified oxidative damage of skeletal muscle proteins through carbonylation (**Figure 1**). Myostatin is a negative regulator of myogenesis, debilitating skeletal muscle regeneration (Chen et al., 2021), and despite no differences in the levels of myoblast determination protein 1 (MyoD), this myogenic regulatory factor was negatively associated to *soleus* muscle atrophy, implying activation of myoblast differentiation in skeletal muscle two months after DOX was administered (**Figure 1**). These data symbolize either a desensitization of the skeletal muscle to satellite cells or a DOX-induced repression of endogenous MyoD function as previously proposed (Kurabayashi et al., 1994). Besides, aging itself reduces the satellite cell pool and weakens their function (Switching, 2022), turning skeletal muscle regeneration challenging. A proposed outcome of DOX is oxidative stress, principally by triggering ROS production in mitochondrion (Andreou & Matsakas, 2022). Notwithstanding with the elevated content of carbonylated proteins in the

soleus muscle of DOX mice, no response on manganese-dependent superoxide dismutase (MnSOD) side was observed, insinuating a debilitated MnSOD signaling or a cytosolic production of ROS. The latter can be provoked by myostatin-induced stimulation of nicotinamide adenine dinucleotide phosphate oxidase (NOX) in the absence of mothers against decapentaplegic homolog 3 (SMAD3) (Chen et al., 2021), which is in accordance with the unaffected levels of phosphorylated SMAD3 in DOX mice. The oxidative modification of myofibrillar proteins predispose them to degradation *via*, for instance, caspase-3 (Smuder et al., 2010). Despite unchanged activity of caspase-3 in the *soleus* muscle, a higher activity was correlated with skeletal muscle atrophy, revealing the involvement of apoptosis in DOX-induced long-term skeletal muscle wasting (**Figure 1**), as evinced in short-term effects (Hiensch et al., 2020). The existence of angular fibers in the *soleus* muscle of DOX mice fortifies the participation of apoptosis in DOX-induced long-term skeletal muscle atrophy, given that this histopathological feature characterizes denervated fibers, which without reinnervation prompt apoptosis (Engel, 2015). In addition, no DOX-related mitochondrial remodeling was observed, disavowing mitochondrial dysfunction as responsible for DOX-induced long-term skeletal muscle atrophy, at least, with regard to mitochondrial biogenesis and density. Likewise cancer, DOX *per se* did not change BDNF levels (**Figure 1**); however, due to skeletal muscle sample limitations, BDNF levels were examined in serum, and as enlightened formerly, BDNF levels in circulation constitute probably a reflection of an exacerbated inflammatory state, which was not observed in DOX mice. Even so, serum levels of BDNF were beforehand associated with skeletal muscle function (Nakano et al., 2020), inferring that DOX-induced long-term skeletal muscle atrophy does not implicate BDNF, as suggested by the non-significant correlation with fibers cross-sectional area, or does not magnifies the age effect on this neurotrophin, as well as on CGRP.

Overall, though skeletal muscle wasting is commonly observed in aging, cancer and chemotherapy treatment, distinct molecular mechanisms seem to contribute to its development. This intricate process involves both systemic and local factors, emphasizing the multifaceted nature of skeletal muscle wasting in these settings.

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Chapter V

Conclusions and future perspectives

This doctoral thesis endorses skeletal muscle atrophy as an adverse consequence of aging, cancer, or doxorubicin (DOX) chronic administration through the modulation of dissimilar biological processes.

The presence of a systemic inflammatory environment and reduced 17beta-estradiol serum levels appear to contribute to age-induced skeletal muscle wasting. Locally in the skeletal muscle, an adverse sex-specific remodeling of the mitochondrial proteome, comprising an escalated fatty acid oxidation, a dysfunctional oxidative phosphorylation, and a higher susceptibility to oxidative modifications, seems to be involved in age-induced skeletal muscle atrophy but only in females, possibly linked to an inadequate signaling of the mitochondrial estrogen receptor alpha. In addition, an impaired skeletal muscle regeneration was suggested by the age-related decreased levels of endogenous brain-derived neurotrophic factor (BDNF), a neuromuscular junction (NMJ)-related mediator.

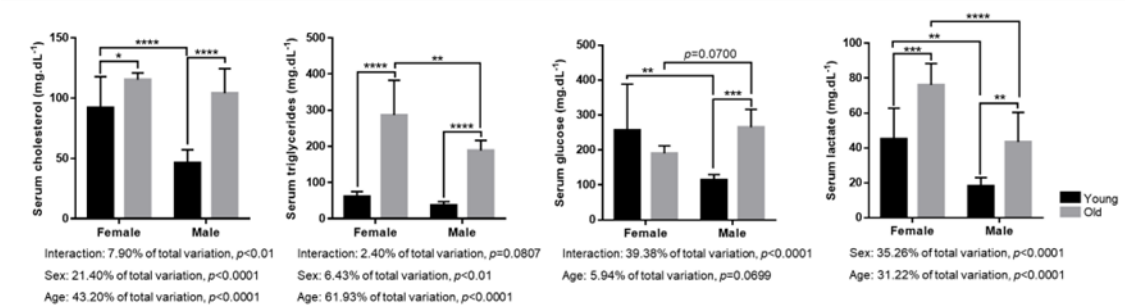
In contrast, in the cancer setting, systemic inflammation appears to not contribute to skeletal muscle wasting; however, this may reflect the analyzed stage of the disease. Moreover, cancer seems to not affect the levels of NMJ-related mediators, namely BDNF.

In turn, the chronic administration of DOX appears to induce skeletal muscle atrophy at older ages by enhancing proteolytic processes through myostatin and reactive oxygen species generation in a long-term manner, jointly with an impaired skeletal muscle regeneration as a result of a skeletal muscle desensitization to satellite cells or a defective function of endogenous myoblast determination protein 1, apparently without the involvement of NMJ-related mediators.

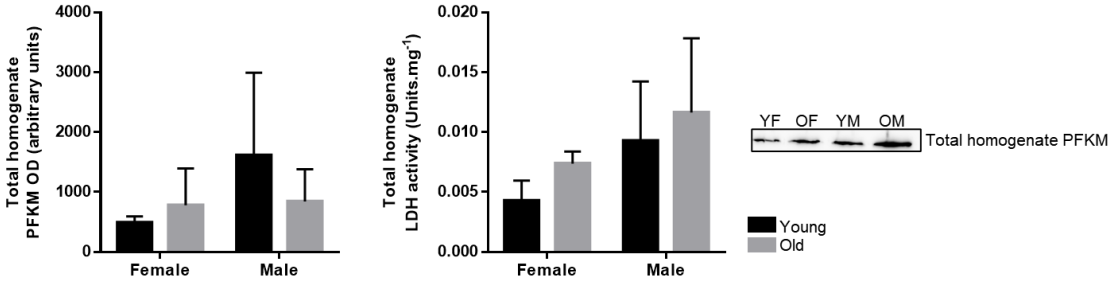
Despite the results herein gathered, there are still a collection of questions that need to be addressed. For instance, the application of BDNF as a specific biomarker for sarcopenia diagnosis can only be clinically doable if the relation between skeletal muscle function and circulating BDNF (and other NMJ-related mediators) is discerned. In parallel, the mitochondrion revealed to be a conceivable target to mitigate age-related skeletal muscle wasting, which should be further explored. However, these mitochondria-related therapeutics might

have to be approached in a sex-specific manner, and therefore, sex needs to be included in future investigations. As herein established, DOX chronically administered at older ages might induce skeletal muscle atrophy that persists throughout life, and so this devalued field of study needs to receive pressing attention from the scientific and medical communities, with studies focusing on, for instance, how DOX might affect skeletal muscle regeneration in terms of myogenesis and the function of satellite cells. Generally speaking, it is hoped that the observations of this thesis lay the foundations to future investigations in skeletal muscle wasting science that should be powered to harbor sex and age considerations to endow a framework in the direction of the mapping of the diversity athwart the human population.

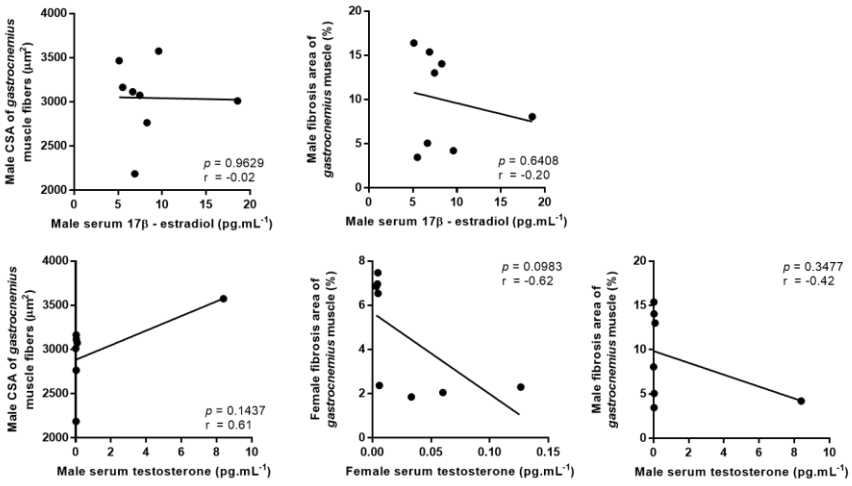
Annexes



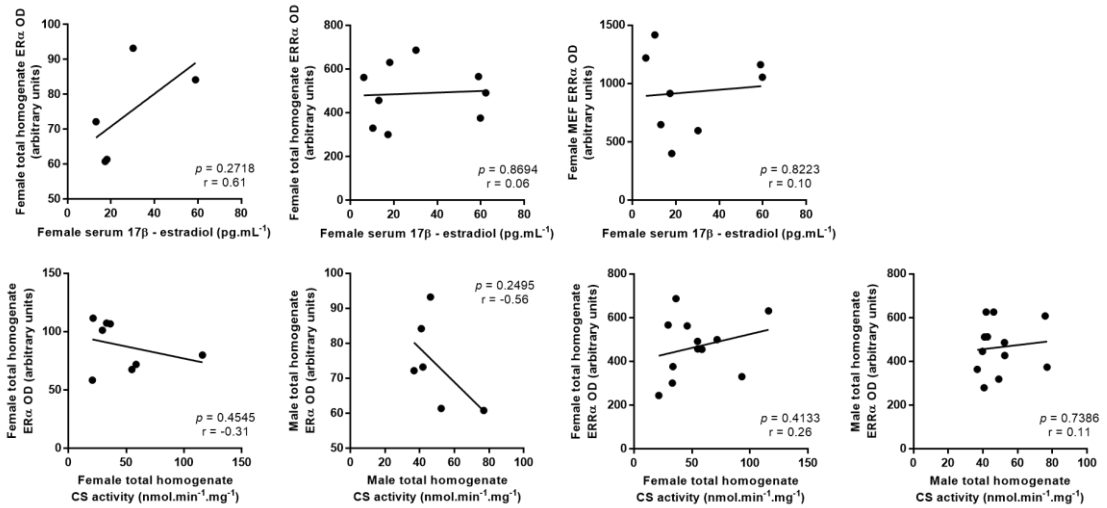
Supplementary Figure 1 Serum levels of cholesterol, triglycerides, glucose, and lactate of young female (n=7), old female (n=12), young male (n=8) and old male (n=10) rats analyzed in an autoanalyzer (****p<0.0001, ***p<0.001, ** p<0.01, and * p<0.05).



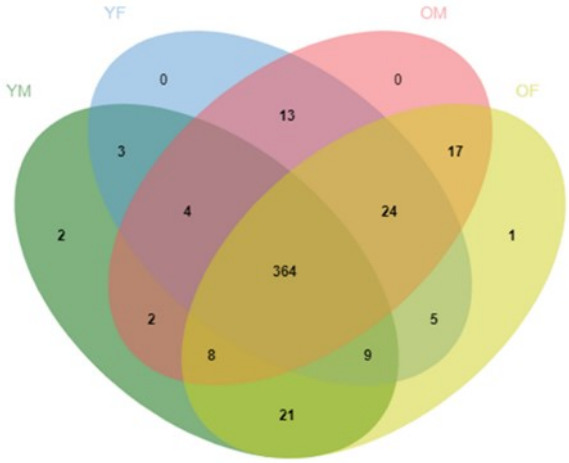
Supplementary Figure 2 Total homogenate PFKM levels and LDH activity in young female (YF, n=6), old female (OF, n=7), young male (YM, n=7) and old male (OM, n=7) rats analyzed by immunoblotting and spectrophotometrically, respectively. A representative immunoblot image is displayed.



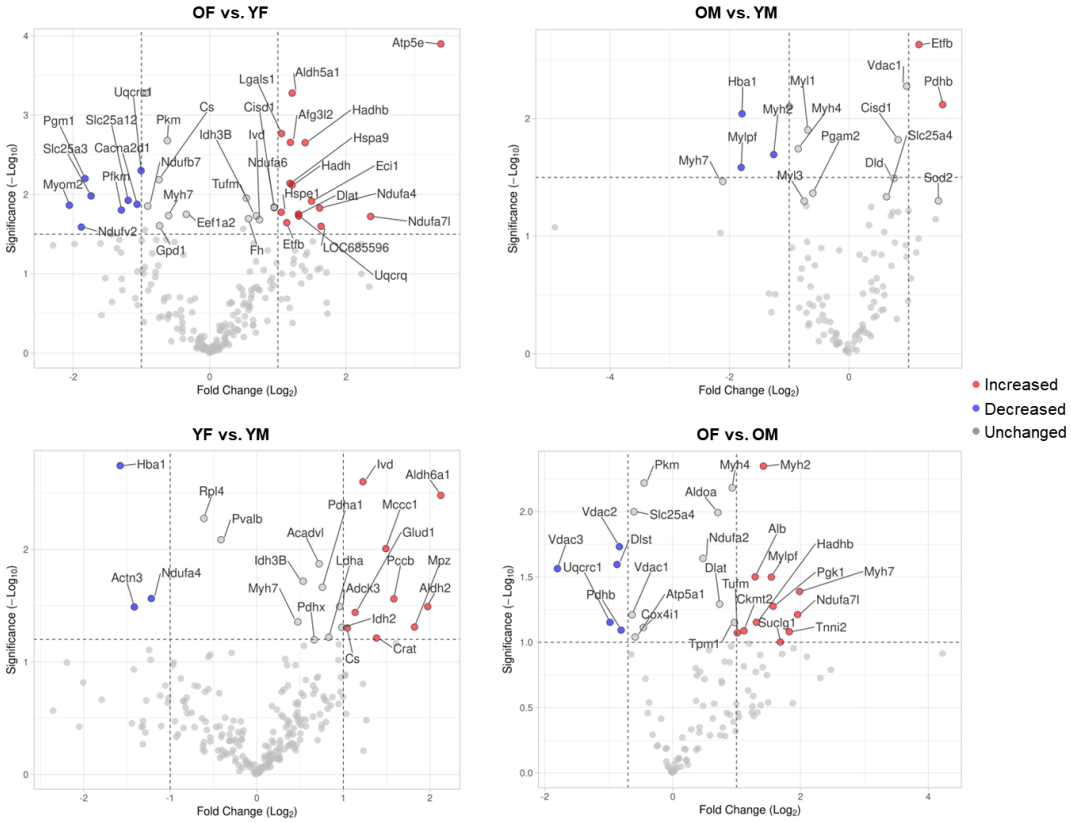
Supplementary Figure 3 Pearson correlations for an in-deep understating of the effect of 17β-estradiol and testosterone on skeletal muscle remodeling.



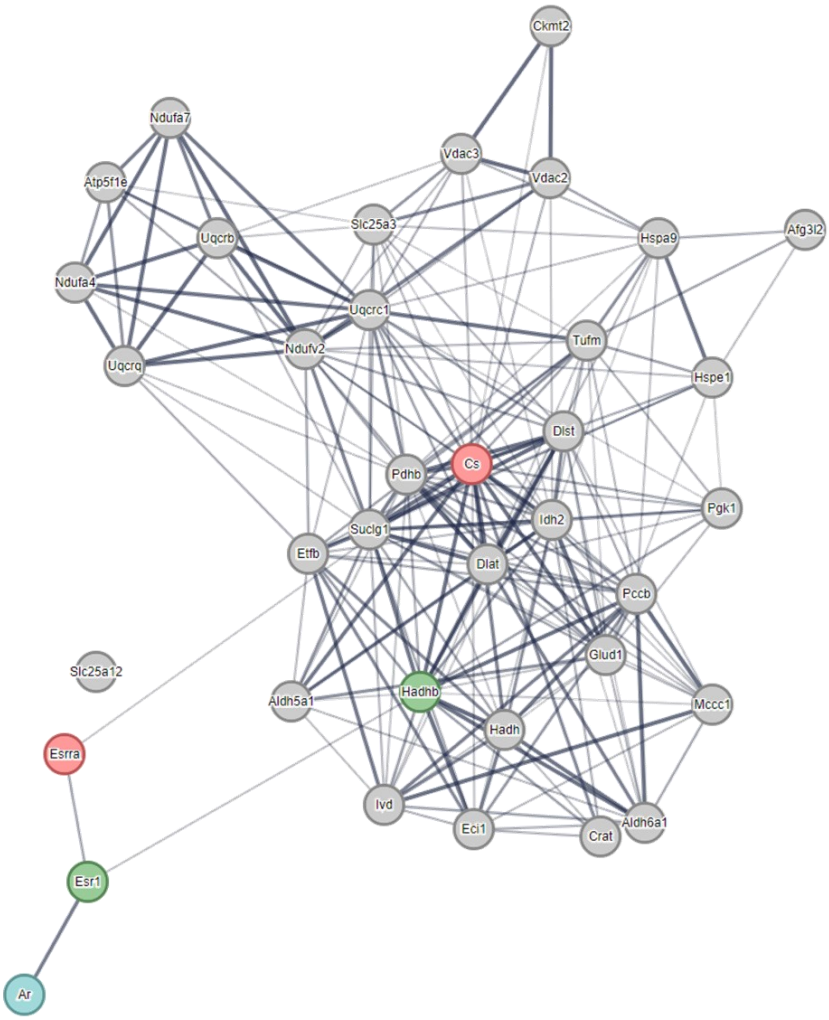
Supplementary Figure 4 Pearson correlations to evaluate the relation between 17β-estradiol serum levels and ERα and ERRα levels in females and between mitochondrial density (given by CS activity) and the receptors levels in total homogenate.



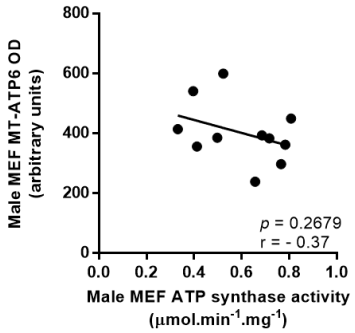
Supplementary Figure 5 Venn diagram demonstrating a total of 364 proteins common to all groups (YF, young female; OF, old female; YM, young male; OM, old male) in mitochondria-enriched fractions analyzed by LC-MS/MS.



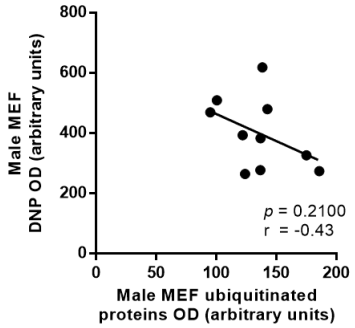
Supplementary Figure 6 Volcano plot analyses demonstrating the proteins with significant different abundances between groups in mitochondria-enriched fractions (YF, young female; OF, old female; YM, young male; OM, old male).



Supplementary Figure 7 Protein-protein interaction network demonstrating the proteins identified by LC-MS/MS in mitochondria-enriched fractions that are known to interact with estrogen receptor alpha (Esr1, interaction in green), estrogen-related receptor alpha (Esrra, interaction in red), and the androgen receptor (Ar in blue; no interaction was found). The figure was produced with the use of the STRING database.

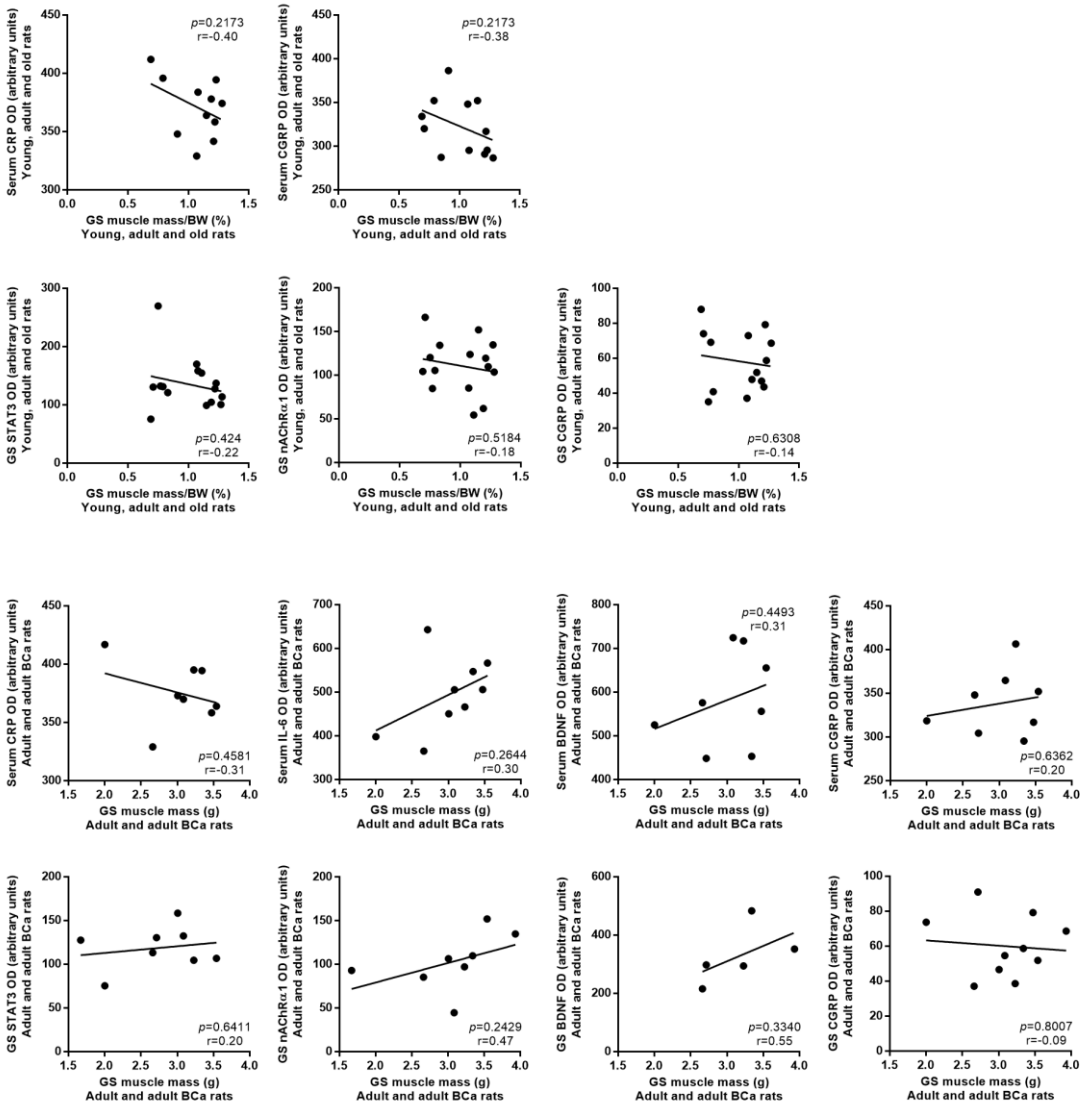


Supplementary Figure 8 Pearson correlation between male MT-ATP6 levels and ATP synthase activity.



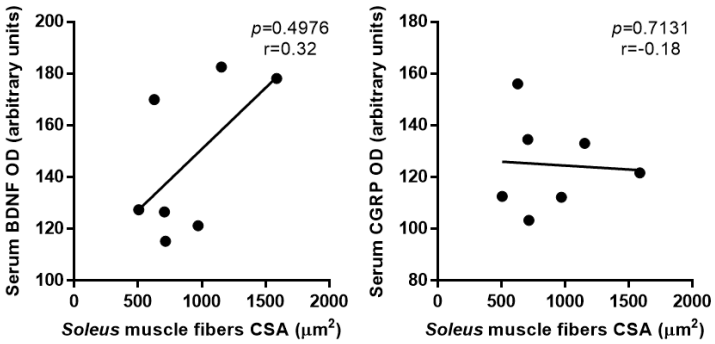
Supplementary Figure 9 Pearson correlation between male DNP levels and the levels of ubiquitinated proteins.

Chapter IIIB



Supplementary Figure 1. Pearson correlations for an in-deep understanding of the results regarding the effect of aging or tumor development on relative or absolute *gastrocnemius* muscle mass, respectively.

Chapter IIIC



Supplementary Figure 1 Spearman correlations between *soleus* muscle fibers cross-sectional area (CSA) values and BDNF and CGRP serum levels assessed by immunoblotting.

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