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HELOÍZA PARANZINI BORDINI

**ANÁLISE DA PERDA DE FORÇA E DE MASSA MUSCULAR
NA SÍNDROME DA CAQUEXIA INDUZIDA PELA FORMA
SÓLIDA DO ADENOCARCINOMA DE EHRLICH**

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Tese apresentada ao Programa de Pós-Graduação em Patologia Experimental da Universidade Estadual de Londrina como requisito parcial para a obtenção do título de Doutora.

Orientadora: Profa. Dra. Flávia Alessandra Guarnier

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Londrina, 21 de julho de 2020.

Dedico aos meus pais,
meus maiores incentivadores, que sempre me
mostraram que o melhor caminho é a
educação.
À vocês, minha eterna e imensurável gratidão.

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“Por vezes sentimos que aquilo que fazemos não é senão uma gota de água no oceano, mas o oceano seria menor se lhe faltasse uma gota.”

Madre Teresa de Calcutá

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RESUMO

Caquexia é uma síndrome complexa que afeta pacientes com doenças crônicas, como o câncer e piora a qualidade de vida e o prognóstico da doença, pois faz com que o indivíduo se torne resistente à terapia anticâncer, culminando em um mau prognóstico em cerca de 70-80% dos pacientes com estágio avançado. A caquexia é diretamente responsável por 20-30% das mortes por câncer, devido à falência cardíaca e respiratória. Sabe-se que espécies reativas de oxigênio têm importante participação na caquexia, afetando principalmente o músculo esquelético, levando ao dano oxidativo e perda de massa muscular, o que provoca fraqueza e fadigabilidade, distúrbios no metabolismo de todo o corpo, recuperação tardia de doenças e lesões agudas, aumento da morbimortalidade por doenças crônicas, quedas, estadas hospitalares prolongadas e perda de vida independente. O objetivo deste estudo é avaliar a interferência do estresse oxidativo na atrofia e perda de força presentes na síndrome da caquexia em tempos experimentais diferentes (14 dias e 28 dias), em um novo modelo experimental de tumor de Ehrlich, comparando dois tipos de fibras musculares: fibras lentas e fibras rápidas. Malondialdeído (MDA) foi quantificado por TBARS, os hidroperóxidos lipídicos por quimiluminescência, e proteínas carbonílicas por ELISA. A área de secção transversa foi analisada para avaliar a área das fibras. As fibras rápidas mostram mais estresse oxidativo comparado às fibras lentas em 14 dias; no entanto, estas fibras mostram menos estresse oxidativo em 28 dias, sugerindo que este tipo de fibra tem ação de algum efeito protetor. Alguns parâmetros oxidativos, como a carbonilação de proteínas e a hidroperodidação lipídica não parecem ser danos importantes para as fibras rápidas, ao contrário do MDA, que parece ser o principal indutor de atrofia neste tipo de músculo, que apresenta maior diminuição na área das fibras, mesmo em 14 dias. Este trabalho tráz à luz a sugestão de que diferentes tipos de fibras musculares se comportam de maneira diferente, de acordo com o modelo experimental indutor de caquexia. Portanto, o estudo de diferentes modelos experimentais se faz necessário para que se possa compreender quais são as possíveis alterações que acontecem na síndrome da caquexia no câncer.

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ABSTRACT

Cachexia is a complex syndrome that affects patients with chronic diseases, such as cancer and worsens the quality of life and the prognosis of the disease, as it makes the individual resistant to anticancer therapy, culminating in a poor prognosis in about 70-80% of patients in advanced stage. Cachexia is directly responsible for 20-30% of cancer deaths due to heart and respiratory failure. It is known that reactive oxygen species play an important role in cachexia, mainly affecting skeletal muscle, leading to oxidative damage and loss of muscle mass, which causes weakness and fatigue, disturbances in the metabolism of the entire body, late recovery from diseases and acute injuries, increased morbidity and mortality due to chronic diseases, falls, prolonged hospital stays and loss of independent life. The aim of this study is to evaluate the interference of oxidative stress in the atrophy and loss of strength present in the cachexia syndrome at different experimental times (14 days and 28 days), in a new experimental model of Ehrlich tumor, comparing two types of muscle fibers: slow fibers and fast fibers. Malondialdehyde (MDA) was quantified by TBARS, lipid hydroperoxides by chemiluminescence, and carbonyl proteins by ELISA. The cross-sectional area was analyzed to assess the fiber area. Fast fibers show more oxidative stress compared to slow fibers in 14 days; however, these fibers show less oxidative stress in 28 days, suggesting that this type of fiber has some protective effect. Some oxidative parameters, such as protein carbonylation and lipid hydroperoxidation, do not appear to be important damages for fast fibers, unlike MDA, which seems to be the main atrophy inducer in this type of muscle, which presents a greater decrease in the area of fibers, even in 14 days. This work brings to light the suggestion that different types of muscle fibers behave differently, according to the cachexia-inducing experimental model. Therefore, the study of different experimental models is necessary in order to understand what are the possible changes that happen in the cachexia syndrome in cancer.

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LISTA DE ABREVIACOES E SIGLAS

UPS	Sistema ubiquitina-proteassoma
ROS	Espcies reativas de oxignio
NFkB	Fator nuclear kappa <i>b</i>
Nrf2	Fator nuclear eritroide 2-relacionado ao fator 2
ARE	<i>Antioxidant-Response Element</i>
MAPK	<i>Mitogen-activated kinases</i>
MuRF1	<i>Muscle ring-finger protein-1</i>
MAFbx	<i>Muscle atrophy F-box</i>
FOXO	<i>Forkhead box</i>
PIF	Fator indutor de protelise
LMF	Fator mobilizador de lipdeos
SNC	Sistema nervosa central
NYP	Neuropeptdeo Y
PBS	Soluo salina tamponada
TA	Tibial Anterior
EDL	Extensor longo dos dedos
GAST	Gastrocnemio
SOL	Sleo
HGT	<i>Hand Grip Test</i>
ELISA	Ensaio de imunoabsoro enzimtica
CSA	rea de seco transversa
MDA	Malondialdedo
BSA	Albumina srica bovina
GT	Glutaciona total
GSSG	Glutaciona Oxidada
CL	Quimiluminescncia
RLU	Unidades relativas de luz

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1 1 Introdução

3 1.1. Caquexia e Câncer

5 Caquexia é uma palavra de origem grega (*kakos hexis*) que significa
6 “má condição” (TISDALE, 2002). A caquexia é um evento complexo e de
7 grande importância clínica, porém seus mecanismos são pouco elucidados.
8 Recentemente, por meio de um Consenso Internacional, a caquexia foi definida
9 como uma síndrome multifatorial, caracterizada por perda contínua de massa
10 muscular esquelética, com ou sem perda de massa gorda, que não pode ser
11 completamente revertida por um suporte nutricional convencional e leva à
12 insuficiência funcional progressiva (FEARON *et al.*, 2011).

13 Essa síndrome acomete principalmente indivíduos com doenças
14 crônicas, como câncer, insuficiência cardíaca congestiva e diabetes.
15 Clinicamente, indivíduos com caquexia apresentam perda de peso, atrofia
16 muscular, fadiga, fraqueza, perda de apetite (anorexia), atrofia de órgãos
17 viscerais, miopatia e depressão (YOSHIDA & DELAFONTAINE, 2015).

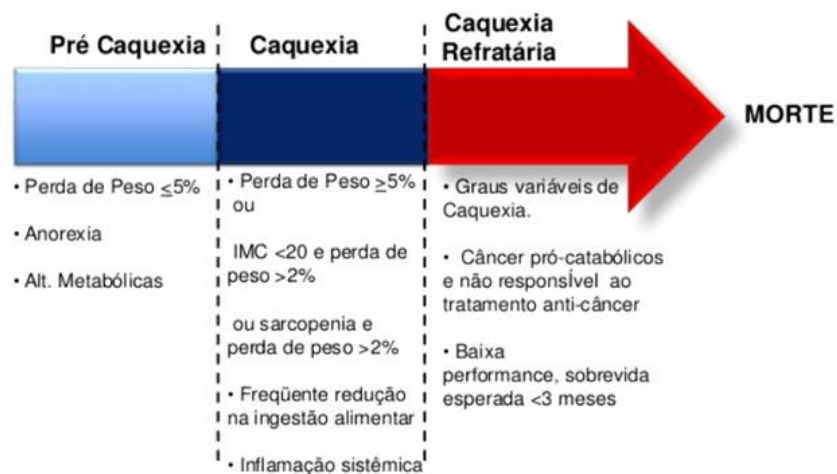
18 No câncer, a caquexia é de grande importância, pois não só a perda
19 de massa corpórea está relacionada à redução da função física e da qualidade
20 de vida, bem como faz com que o indivíduo se torne resistente à terapia
21 anticâncer (FEARON *et al.*, 2011), culminando em um mau prognóstico em
22 cerca de 70-80% dos pacientes com estágio avançado (ROELAND *et al.*,
23 2016). A caquexia é diretamente responsável por 20-30% das mortes por
24 câncer devido à falência cardíaca e respiratória (TISDALE, 2010; FEARON *et*
25 *al.*, 2013).

26 A incidência de caquexia varia entre os tipos de câncer devido aos
27 diferentes fenótipos tumorais (TISDALE, 2004), mas o tamanho do tumor não
28 tem relação direta com o grau de caquexia (NELSON, 2000). Segundo
29 BARACOS *et al.* (2018), metade das mortes de câncer no mundo (8,2 milhões
30 de pessoas por ano) está relacionada aos tipos de câncer que mais
31 desenvolvem caquexia, sendo estes o pancreático, esofágico, gástrico,
32 pulmonar, hepático e colorretal.

33 A classificação da caquexia pode dar-se em três estágios: (i) pré-
34 caquexia, na qual os primeiros sinais clínicos e metabólicos (como anorexia e

1 redução da tolerância à glicose) podem preceder a perda de peso $\leq 5\%$; (ii)
 2 caquexia, quando os doentes têm mais de 5% de perda de peso corporal em 6
 3 meses de doença, ou um índice de massa corporal (IMC) inferior a 20 kg/m² e
 4 perda de peso contínua superior a 2%, ou sarcopenia somada à perda de peso
 5 contínua de mais de mais de 2%, redução no consumo de alimentos e
 6 inflamação sistêmica; e (iii) caquexia refratária, que acontece em indivíduos em
 7 estágios avançados de câncer, com atividade altamente catabólica e que
 8 apresentam não-responsividade ao tratamento anticâncer. Neste último
 9 estágio, a expectativa de vida é baixa, estimada em menos de três meses
 10 (FEARON *et al.*, 2011) (Figura 1).

11



12

13 Figura 1. O esquema representa a progressão da caquexia em três fases e as alterações encontradas em
 14 cada uma delas. IMC = índice de massa corpórea. Figura adaptada de Fearon *et al* (2011).

15

16 Esta classificação é baseada na clínica, ou seja, em estudos que
 17 analisaram indivíduos com câncer que desenvolveram a síndrome da caquexia.
 18 No entanto, ainda não existe um padrão de diagnóstico para a caquexia, já que
 19 a classificação e a identificação da síndrome dependem de outros fatores,
 20 como a alteração na composição corporal, aceleração na degradação de
 21 proteínas e o aumento na toxicidade do tratamento (von HAELING & ANKER,
 22 2010), este, inclusive, frequentemente, faz com que pacientes tenham o
 23 planejamento das aplicações do tratamento anticâncer alterado, com limitação
 24 de dose e muitas vezes, o tratamento precisa ser interrompido, o que reduz a
 25 eficiência da droga e também a sobrevida do paciente (PIN *et al.*, 2018). Além
 26 disso, vale ressaltar que utilizar o IMC (índice de massa corpórea) sozinho

1 como fonte de diagnóstico não é recomendado, já que pacientes caquéticos
2 obesos podem apresentar IMC normal por causa da massa gorda, ou até
3 mesmo pela retenção de água, que pode mascarar a perda de massa muscular
4 (von HEALING *et al.*, 2010; PENNA *et al.*, 2019).

5 O músculo esquelético é num tecido heterogêneo e sua demanda
6 funcional depende da necessidade de cada espécie. A musculatura esquelética
7 representa cerca de 50% de toda a massa corpórea e tem papel importante no
8 metabolismo sistêmico (BASSEL-DUBY & OLSON, 2006). A perda de massa
9 muscular é a principal característica da caquexia e é a principal causa de
10 comprometimento de função, fadiga e complicações respiratórias (BOSSOLA *et*
11 *al.*, 2008). A perda de 30% de massa corpórea pode representar a perda de
12 cerca de 75% de massa muscular, o que piora o prognóstico do paciente e
13 pode levar a óbito (MUSCARITOLI *et al.*, 2006).

14 Na caquexia, ocorre desbalanço entre síntese e degradação de
15 proteínas no músculo estriado esquelético, por meio de aumento de
16 degradação e diminuição da síntese (YOSHIDA & DELAFONTAINE, 2015).
17 Esse estado altamente catabólico ocorre principalmente por meio do sistema
18 ATP-ubiquitina-proteassoma (UPS), uma via proteolítica dependente de ATP,
19 capaz de degradar proteínas marcadas à peptídeos (TISDALE, 2005).

20

21

22 **1.2. Estresse Oxidativo e Caquexia**

23

24 Dentre as proteínas alvo a serem degradadas pelo UPS, estão as
25 proteínas que sofreram ação das espécies reativas de oxigênio (ERO). Tais
26 proteínas oxidadas ficam severamente danificadas estrutural e funcionalmente.
27 Algumas células não têm facilidade para se desfazer destas proteínas, gerando
28 um acúmulo de proteínas danificadas no seu interior, as quais ficam com a
29 viabilidade comprometida (HÖHN *et al.*, 2013).

30

31 As espécies reativas de oxigênio são derivadas principalmente da
32 redução univalente do oxigênio na mitocôndria, durante o processo de geração
33 de energia. Algumas das espécies geradas são $O_2^{\cdot-}$ (ânion superóxido), OH^{\cdot}
34 (radical hidroxila) e H_2O_2 (peróxido de hidrogênio). É preciso ressaltar que H_2O_2
é uma espécie reativa, porém não é um radical livre - provoca danos ao entrar

1 nas células através dos canais aquaporinas, e então reagir com metais, como
2 por exemplo o ferro, gerando a reação de fenton, que tem como produto o
3 radical livre OH^{\bullet} (HALLIWELL & GUTTERIDGE, 2015). O radical livre com
4 maior capacidade reativa, e portanto, de provocar mais lesões é o OH^{\bullet} , capaz
5 de provocar modificação no DNA (com modificação das bases e quebras das
6 fitas), danos nas proteínas e inativação enzimática e peroxidação lipídica
7 (VASCONCELOS *et al.*, 2007). Os radicais livres possuem um elétron
8 desemparelhado na última camada de valência, o que lhes confere grande
9 instabilidade e reatividade (FERREIRA & MATSUBARA, 1997). Quando há um
10 desbalanço entre a produção dessas espécies e o sistema antioxidante,
11 instala-se um quadro de estresse oxidativo (PRYOR & GODBER, 1991).
12 Estresse oxidativo pode ser definido como desequilíbrio entre oxidantes e
13 antioxidantes em favor de oxidantes, levando a uma interrupção da sinalização
14 redox e controle e / ou dano molecular (JONES, 2006).

15 O estresse oxidativo pode apresentar cinco principais consequências
16 ao organismo: (i) aumento da proliferação – muitas células respondem a um
17 baixo nível de estresse oxidativo com o aumento de proliferação; (ii) adaptação
18 – faz com que células ou o organismo se adaptem através da regulação do
19 sistema de defesa; (iii) injúria celular – danos provocados à estruturas
20 celulares como lipídeos, DNA, proteínas e carboidratos; (iv) senescência – é
21 um estado permanente de não-divisão, ou seja, a célula sobrevive, mas não faz
22 divisão celular; (v) morte celular – caso a célula não consiga se adaptar após a
23 injúria, o dano oxidativo ou suas consequências podem levar a célula à necrose
24 ou apoptose, especialmente quando ocorrem danos no DNA (HALLIWELL &
25 GUTTERIDGE, 2015).

26 Porém, sob condições fisiológicas normais, o balanço redox é
27 finamente regulado. As ERO possuem importante função sobre a resposta
28 imunológica (fagocitose e defesa contra infecções), e também atuam como
29 fatores de transcrição na sinalização intracelular, induzindo a apoptose (ZIADA
30 *et al.*, 2020). Na musculatura, as espécies reativas estão envolvidas na
31 regulação do crescimento celular, proliferação e diferenciação, performance
32 contrátil durante o exercício, sinalização de cálcio, consumo de glicose,
33 biogênese de mitocôndrias (MOULIN & FERREIRO, 2017) ou mesmo
34 controlando a massa muscular durante processos de adaptação (NISS &

1 SIMON, 2007; POWERS *et al.*, 2010). Porém, quando há um desbalanço, estas
2 espécies reativas se tornam potencialmente tóxicas para o organismo, reagindo
3 com os ácidos graxos poli-insaturados que formam as membranas celulares
4 (peroxidação lipídica), proteínas celulares, enzimas e especialmente o DNA
5 (MANTOVANI *et al.*, 2012).

6 Além das ERO, também podem ser formadas espécies reativas de
7 nitrogênio (ERN), dentre elas o óxido nítrico (NO) e o peroxinitrito (ONOO⁻)
8 (CHANG *et al.*, 2015). Somente quando em níveis mais elevados, o NO reage
9 com o O₂^{•-}, formando o ONOO⁻ (ESPEY *et al.*, 2000), que é uma molécula de
10 grande potencial citotóxico com habilidade de destruir patógenos e contribuir
11 para a defesa imune (KONDO *et al.*, 2002). Porém, quando em concentrações
12 não fisiológicas, o ONOO⁻ em contato com as células, tecidos ou fluidos
13 corporais pode provocar a depleção de grupos -SH e outros antioxidantes,
14 também pode provocar danos irreversíveis em proteínas através da nitração,
15 lipídeos e bases de DNA (HALLIWELL & GUTTERIDGE, 2015).

16 Em indivíduos com caquexia, o estresse oxidativo pode resultar de
17 vários mecanismos, dentre eles a ingestão alimentar reduzida (devido a
18 sintomas como anorexia, náusea, vômito, suplementação inadequada de
19 nutrientes), alterações metabólicas, inflamação crônica e o uso de algumas
20 drogas antineoplásicas que produzem ERO em grandes quantidades
21 (MANTOVANI *et al.*, 2012).

22 Um nível moderado de estresse oxidativo é capaz de induzir uma
23 expressão elevada dos principais componentes do UPS no músculo
24 esquelético, contribuindo para a degradação proteica. Um mecanismo
25 conhecido que medeia a ação do estresse oxidativo no músculo é a ativação
26 do NFκB pelas ERO, regulando positivamente o UPS (GOMES-MARCONDES
27 & TISDALE, 2002). Semelhante às citocinas pró-inflamatórias, as ERO são
28 capazes de ativar diversas vias catabólicas no músculo esquelético, não só
29 como o NFκB, mas também a via da p38-MAPK (*mitogen-activated kinases*),
30 reduzindo, portanto, a síntese de proteínas (MOYLAN & REID, 2007).

31 Devido às proteínas serem abundantes no organismo, elas são o
32 principal alvo de dano oxidativo e nitrosativo em células, e modificações nessas
33 moléculas podem afetar inúmeras vias intracelulares, já que proteínas oxidadas
34 tendem a perder sua função bioquímica. Os principais produtos de oxidação

1 irreversível são a hidroxilação e a carbonilação de proteínas
2 (CHONDROGIANNI *et al.*, 2014). Com o processo oxidativo, as porções
3 hidrofóbicas das proteínas ficam expostas e interagem entre si, levando à
4 perda da estrutura tridimensional e desencadeia-se a agregação de proteínas
5 (HÖHN *et al.*, 2013).

6

7

8 **1.3. Degradação de proteínas, atrofia muscular e perda de força**

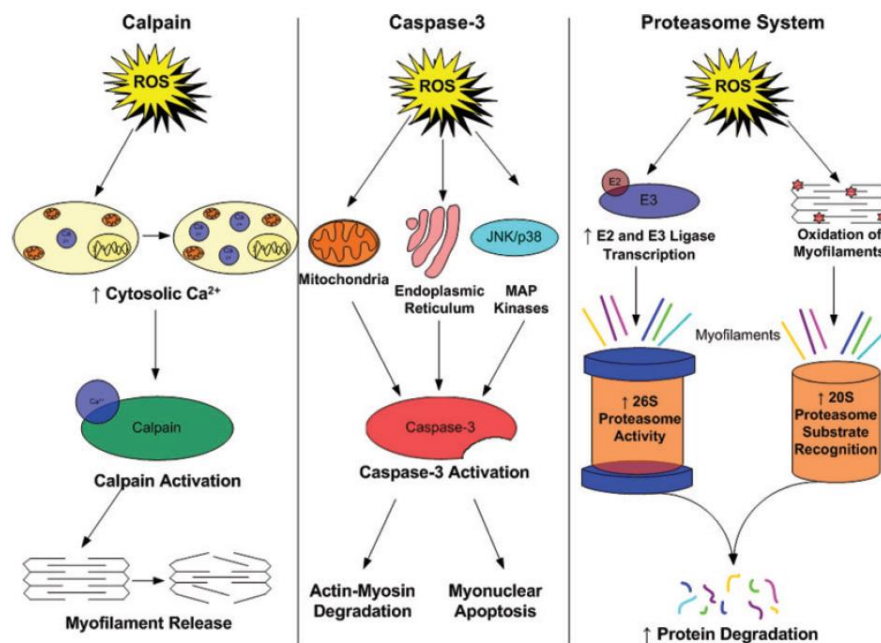
9

10 A musculatura esquelética, composta principalmente pelas proteínas
11 actina e miosina, é o tecido no qual há maior necessidade de reserva e
12 mobilização de proteínas (ATTAIX *et al.*, 1999). O UPS da musculatura
13 esquelética se mostra eficiente na remoção de proteínas oxidadas, mas a
14 consequência disso é a perda de massa muscular e sarcopenia, como o que
15 ocorre na caquexia (MOSONI *et al.*, 2004).

16 A maioria das proteínas oxidadas é eliminada através de
17 degradação, pois muitos danos são irreversíveis e provocam profundas
18 mudanças na função das proteínas, não sendo possível seu reparo
19 (CHONDROGIANNI *et al.*, 2014). A manutenção das proteínas pode ser feita
20 por diferentes vias de degradação, como (i) calpaína – são proteases
21 dependentes de cálcio, ativadas pela liberação do íon no citosol, durante a
22 atrofia muscular (GOLL *et al.*, 2003; MAES *et al.*, 2007); (ii) caspase-3 – é
23 ativada em resposta a condições catabólicas, com papel importante no início
24 da degradação proteica, capaz de clivar o complexo actomiosina (DU *et al.*,
25 2004); (iii) sistema ubiquitina-proteassoma (UPS) – é a principal via proteolítica
26 que rege a eliminação de proteínas modificadas, proteínas anormais,
27 desnaturadas e proteínas danificadas em geral, bem como o controle da
28 degradação de proteínas de vida curta (GOLDBERG, 2007) (Figura 2). Há
29 também o envolvimento da proteólise lisossomal, mais conhecido como
30 autofagia. É um processo fisiológico encarregado de degradar componentes
31 celulares, que pode ser maior ou menor, dependendo da falta de nutrientes ou
32 pela presença de organelas danificadas, como as mitocôndrias. No entanto,
33 esta via depende da quebra dos miofilamentos, que não pode ser feita pelos
34 lisossomos, ou pelo proteassoma, mas acontece pelas vias da calpaína e das

1 caspases (PENNA *et al.*, 2019). A desregulação do processo autofágico, tanto
 2 pelo excesso, quanto pela falta, pode afetar a musculatura esquelética,
 3 portanto, este processo também necessita de um fino regulamento, a fim de
 4 preservar a massa muscular (NEEL *et al.*, 2013).

5



6

7 Figura 2. Ilustração da interação de cinases e fosfatases na regulação de fatores de transcrição e
 8 subsequente síntese protéica no músculo esquelético (Adaptado de Powers *et al.*, 2010).

9

10 O sistema UPS é uma via extralissossomal, dependente de ATP e do
 11 polímero de ubiquitina. A degradação de proteínas ocorre através de uma
 12 cascata multi-enzimática composta das enzimas E1 (enzima ativadora de
 13 ubiquitina), E2 (enzima conjugadora de ubiquitina) e E3 (ubiquitina ligase). A
 14 ubiquitina se torna ativada ao ligar-se à enzima E1, a ubiquitina é então
 15 transferida para uma segunda enzima E2. A transferência final de ubiquitina
 16 para a proteína alvo é então mediada por uma terceira enzima E3, que é
 17 responsável pelo reconhecimento seletivo de proteínas alvo. Essas proteínas,
 18 então são levadas ao proteossoma 26S, uma estrutura cilíndrica, formada por
 19 dois subcomplexos terminais regulatórios (19S) e um núcleo catalítico central
 20 (20S), no qual ocorre efetivamente a degradação das proteínas através de
 21 enzimas proteolíticas que ficam alinhadas na cavidade interna do cilindro. As
 22 partículas 19S medeiam a ligação e desdobram o substrato proteico antes de
 23 ser transferido para o interior do núcleo 20S. O proteossoma 26S libera

1 oligopéptídeos, que serão então degradados a peptídeos (COOPER, 2000;
2 ADAMS, 2004; TISDALE, 2005).

3 As proteínas oxidadas podem ficar parcialmente desdobradas devido
4 à perda da estrutura secundária e terciária, expondo porções hidrofóbicas. Isso
5 faz com que a agregação proteica seja facilitada ou que tais sítios expostos
6 sejam reconhecidos para a proteólise (GRUNE *et al.*, 2003). O estresse
7 oxidativo estimula a conjugação de ubiquitina a proteínas musculares através
8 da regulação transcricional das enzimas E2 e E3, que conjugam ubiquitina a
9 proteínas musculares para promover a proteólise (LI *et al.*, 2003).

10 No entanto, algumas proteínas oxidadas podem ser degradadas
11 diretamente pelo núcleo catalítico 20S sem serem previamente ubiquitinadas
12 (GRUNE *et al.*, 2003), um indicativo de que o estresse oxidativo pode acelerar
13 a degradação de proteínas musculares pelo 20S e pelo proteossoma 26S
14 (POWERS *et al.*, 2010).

15 A perda de massa muscular e conseqüentemente, de sua função
16 (observada através da perda de força), é uma das principais características da
17 caquexia no câncer, pois compromete significativamente a qualidade de vida e
18 a sobrevivência do indivíduo acometido, já que está relacionada com a resistência
19 à terapia anticâncer (PENNA *et al.*, 2019).

20 Perda de massa e perda de força são os principais sinais que
21 caracterizam a sarcopenia – do grego “*sarx*” (carne) e “*penia*” (pobreza).
22 Estudos anteriores definem a sarcopenia como a perda de massa muscular,
23 com aumento do risco de incapacidade motora e menor qualidade de vida,
24 associados com o envelhecimento. No entanto, essa definição vem mudando,
25 já que a sarcopenia é um fenômeno associado à outras doenças, como a
26 própria síndrome da caquexia, sendo possível defini-la como qualquer perda de
27 massa muscular esquelética que ocorre em indivíduos de idade avançada e
28 como fator secundário em decorrência de outras doenças, como o câncer
29 (EVANS, 1999; MUSCARITOLI *et al.*, 2010; CRUZ-JENTOFT *et al.*, 2010).
30 Grande parte dos indivíduos caquéticos tem sarcopenia, no entanto a maioria
31 dos indivíduos com sarcopenia não são considerados caquéticos, tornando a
32 sarcopenia seja um componente da síndrome da caquexia (CRUZ-JENTOFT *et*
33 *al.*, 2010) (Figura 3).

1 A sarcopenia no envelhecimento está associada com um estado
 2 crônico de inflamação, com o aumento dos níveis plasmáticos de mediadores
 3 pró-inflamatórios, como TNF- α e IL-6, capazes de estimular a proteólise,
 4 principalmente pela via UPS, o que também é observado na caquexia (DALLE
 5 *et al.*, 2017). Portanto, as duas síndromes – caquexia e sarcopenia, podem
 6 acontecer de forma simultânea, em pacientes mais idosos e em indivíduos com
 7 caquexia, pois o potencial regenerativo desses indivíduos é comprometido
 8 devido à perda da capacidade das células satélites de se renovarem e
 9 diferenciarem-se em células musculares esqueléticas propriamente ditas
 10 (PARK *et al.*, 2017).

Parâmetro	Caquexia	Sarcopenia
Perda de peso	Sim	Não
Massa muscular	Reduzida	Reduzida
Massa gorda	Reduzida	Aumentada
Anorexia	++	+
Gasto energético em repouso	Aumentado	Reduzido
Inflamação sistêmica	+++	+
Doença de base	Sim	Sim/Não
Degradação de proteínas musculares	+	+++
Aumento da apoptose muscular	Sim	Sim
Diminuição na regeneração muscular	Sim	Sim

12
 13 Figura 3. Tabela adaptada de Argilés *et al.*, 2015. Parâmetros envolvidos na caquexia e na sarcopenia.

14
 15 Para esclarecer o diagnóstico de sarcopenia na clínica, inclusive na
 16 caquexia, são necessários dois critérios: (i) perda de massa muscular – baixa
 17 qualidade e quantidade de massa muscular, sendo calculada pela massa
 18 magra apendicular (MMA) ajustada pela altura: $MMA/altura^2 < 7,23$ ou
 19 $< 5,67\text{kg}/\text{m}^2$ para homens e mulheres, respectivamente; e (ii) perda de função
 20 muscular, caracterizada pela perda de força ou da performance, sendo a perda
 21 de força a medida mais importante, realizada pela força de preensão (*grip*
 22 *strength*), sendo considerado significativo quando $< 30\text{kg}$ ou $< 20\text{kg}$ para
 23 homens e mulheres, respectivamente. O teste de performance é um teste
 24 complementar, capaz de apontar a gravidade do quadro. Quando há resultado

1 positivo para estes dois critérios, pode-se confirmar o diagnóstico para
2 sarcopenia (CRUZ-JENTOFT *et al.*, 2019).

3 Além da perda de massa ser um fator de grande importância para a
4 caquexia, pacientes que apresentam sarcopenia tendem a apresentar maiores
5 índices de morbidades por doenças infecciosas, resistência à insulina, doenças
6 cardiovasculares e uma maior taxa de mortalidade, ou seja, a sarcopenia
7 reflete a fragilidade e o *status* geral de saúde do paciente (FUKUSHIMA *et al.*,
8 2019).

9 A degradação muscular na caquexia está relacionada principalmente
10 com o aumento da degradação proteica (COSTELLI & BACCINO, 2000) e com
11 a falta de diferenciação das células satélites (SAMBASIVAN & TAJBAKHSI,
12 2015). Proteínas são os componentes mais importantes e abundantes da
13 musculatura, e a regulação de seus níveis depende do balanço entre a síntese
14 e a degradação proteica. Desequilíbrios neste balanço podem ativar uma
15 resposta adaptativa que buscará uma nova homeostase que pode,
16 alternativamente, resultar em hipertrofia ou atrofia, dependendo da prevalência
17 da síntese ou da degradação, respectivamente (ARGILÉS *et al.*, 2014).

18 A atrofia é definida como a perda de massa muscular esquelética
19 associada com a perda da área de secção transversa e também do conteúdo
20 proteico muscular, que faz com que as miofibras “encolham”. Ocorre em casos
21 de doenças crônicas, inatividade (desuso) ou envelhecimento (SCHIAFFINO *et al.*,
22 2013). É uma condição séria que leva à redução da função muscular e da
23 qualidade de vida, causando fraqueza e fadigabilidade, distúrbios no
24 metabolismo de todo o corpo, recuperação tardia de doenças e lesões agudas,
25 aumento da morbimortalidade por doenças crônicas, quedas, estadas
26 hospitalares prolongadas e perda de vida independente (EBERT *et al.*, 2019).

27 A degradação de proteínas na musculatura esquelética, que levam à
28 atrofia, está relacionada com as vias de degradação citadas anteriormente
29 (Figura 2), estando relacionada diretamente com a ativação excessiva dessas
30 vias proteolíticas (PENNA *et al.*, 2014). Estudos sobre a atrofia muscular
31 trazem a responsabilidade da degradação proteica principalmente para a via
32 UPS, através da ativação das E3 ligases MuRF1 (*muscle ring finger 1*) e
33 MAFbx (*muscle atrophy F-box*, mais conhecida como atrogin-1) (EBERT *et al.*,
34 2019). A via Akt é conhecida por promover o crescimento das células

1 musculares e, simultaneamente, bloquear a degradação proteica (LAI *et al.*,
2 2004). Essa via regula tanto a via UPS como a via da autofagia, mediando esse
3 controle através dos fatores de transcrição FOxO (*forkhead box*). Os membros
4 da família FOxO mais importantes para a musculatura esquelética são as
5 isoformas FOxO1, FOxO3 e FOxO4. Akt fosforila todas essas FOxOs,
6 promovendo a exportação delas do núcleo para o citoplasma. Porém, com a
7 atividade reduzida da Akt, observada em vários modelos de atrofia muscular,
8 ocorre a diminuição da fosforilação de FOxO, que fica reduzido no citoplasma e
9 elevado no núcleo. A translocação e a atividade transcricional dos membros da
10 família FOxO são suficientes para promover a expressão de atrogin-1 e
11 MuRF1, e por consequência, a sinalização para a atrofia muscular (CALNAN &
12 BRUNET, 2008).

13 Diferentes tipos de fibras musculares respondem de maneira
14 diferente ao processo atrofico, sendo o músculo de metabolismo glicolítico mais
15 susceptível a atrofia do que o músculo de metabolismo oxidativo, em condições
16 de caquexia (SCHIAFFINO *et al.*, 2013). Em 2012, REED *et al.*, demonstraram
17 que em camundongos inoculados com células de carcinoma pulmonar de
18 Lewis (LLC), o processo atrofico se mostrou mais intenso no músculo tibial
19 anterior comparado ao músculo sóleo, no mesmo período experimental, o que
20 sugere que a ativação do processo atrofico pode levar mais tempo para se
21 desenvolver no músculo sóleo.

22 Os tipos de músculos utilizados neste trabalho são
23 predominantemente de fibras lentas (tipo 1 – ex.: músculo sóleo) e
24 predominantemente de fibras rápidas (tipo 2 – ex.: extensor longo dos dedos e
25 tibial anterior). As fibras do tipo 1 possuem alta densidade mitocondrial que
26 permite a alta capacidade oxidativa, capaz de suportar altas demandas de
27 energia por um maior período de tempo, já as fibras do tipo 2 são
28 predominantemente glicolíticas, mas podem ser subdivididas em oxidativas-
29 rápidas (tipo IIa) ou glicolíticas-rápidas (tipo IIb/x) (JULIEN *et al.*, 2018). As
30 fibras lentas contêm maior suprimento sanguíneo, portanto possuem maior
31 quantidade de mioglobina (que confere a cor avermelhada), que se combina
32 com o oxigênio e o armazena até ser necessário, o que acelera o transporte do
33 oxigênio para as mitocôndrias. Possuem fibras menores, comparado às do tipo
34 II, com motoneurônios que as inervam também de calibre menor, o que faz com

1 que haja menor quantidade de estímulos por unidade de tempo, ou seja, é um
2 tipo de fibra com menor capacidade de contração, baixa capacidade de gerar
3 força e baixa fatigabilidade. Já as fibras rápidas, possuem maior liberação de
4 cálcio e grande quantidade de enzimas glicolíticas, têm suprimento sanguíneo
5 menor, pois o metabolismo oxidativo é de importância secundária. Por se
6 utilizar do metabolismo glicolítico, ocorre a formação de menor quantidade de
7 ATP e produz ácido lático, que leva o músculo a fadiga, porém este tipo de
8 fibra tem maior capacidade e velocidade de contração e também de gerar força
9 (JUNQUEIRA *et al.*, 2017).

10 Sabe-se que espécies reativas de oxigênio podem agir através de
11 mecanismos de sinalização via sistema redox – como a via NFkB, alterando a
12 expressão miogênica, o que causa perda de massa muscular (LANGEN *et al.*,
13 2002). A via que pode contrabalancear o efeito provocado pelas ERO é através
14 da estimulação de Nrf2 (nuclear erythroid-2-p45-related factor-2) (FILOMENI *et*
15 *al.*, 2015).

16 Nrf2 é um fator de transcrição com papel importante na mediação da
17 resposta antioxidante intracelular, uma vez que é capaz de regular
18 positivamente a expressão de anticorpos e enzimas de detoxificação de fase II
19 em resposta ao aumento de estresse oxidativo (NGUYEN *et al.*, 2005), como
20 catalase, heme oxigenase e glutathione peroxidase (KITAOKA *et al.*, 2016). Para
21 ser ativado, é necessário que a porção Keap1, que fica constitutivamente
22 ligada ao Nrf2 – levando-o à degradação via UPS, seja liberada para que o
23 fator de transcrição chegue ao núcleo e se ligue especificamente ao ARE
24 (*antioxidant-response element*), o que sugere que a interação entre Keap1-Nrf2
25 constitui um sensor de estresse oxidativo envolvido no desencadeamento da
26 resposta ARE para restaurar o status de estresse fisiológico (NGUYEN *et al.*,
27 2005; CRILLY *et al.*, 2016). Porém, informações sobre como esta via age em
28 situações de atrofia muscular, principalmente vinculada à caquexia no câncer
29 ainda precisam ser melhor elucidadas.

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1 **1.4. Alterações metabólicas na caquexia**

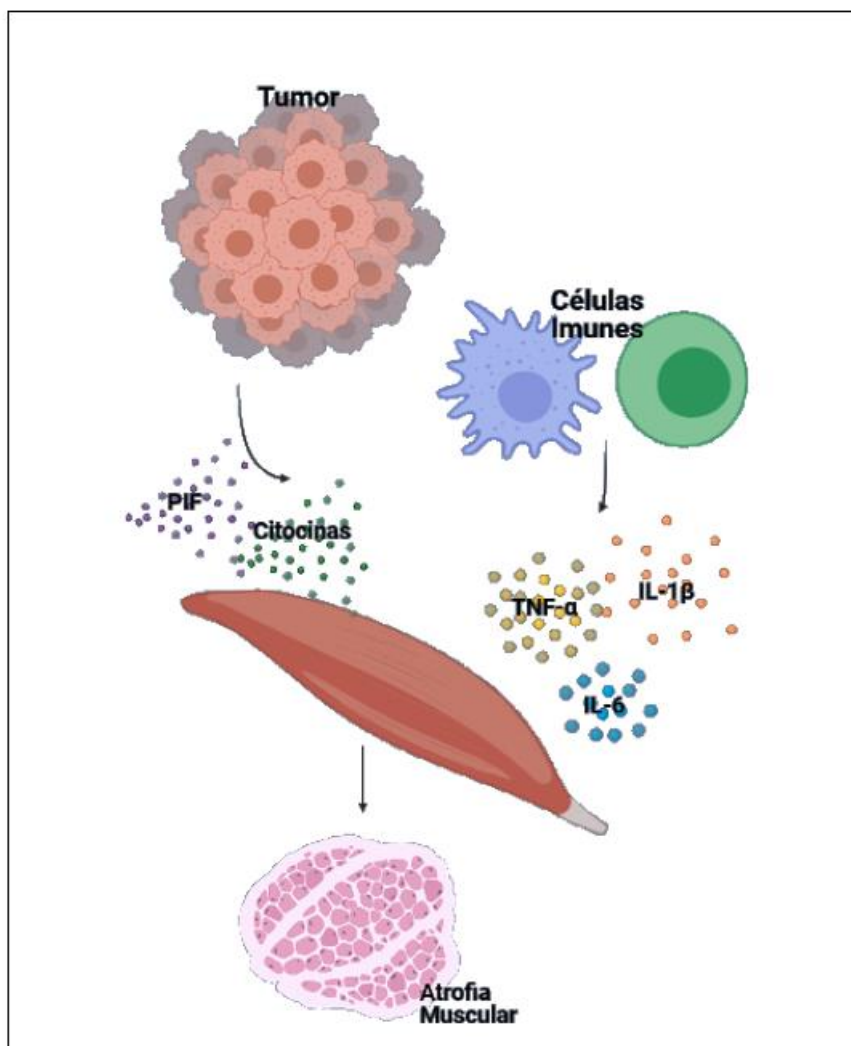
2
3 As alterações metabólicas observadas na caquexia do câncer são,
4 em maior parte, resultado da produção de mediadores catabólicos pelo próprio
5 tumor – como PIF (*proteolysis-inducing factor*) e LMF (*lipid-mobilising factor*),
6 que promovem a degradação de tecido muscular esquelético pelo aumento da
7 via UPS, e degradação de tecido adiposo através da via do cAMP (*cyclic*
8 *adenosine monophosphate*) (HIRAI *et al.*, 1998; TISDALE, 2002),
9 respectivamente, ou pelo próprio organismo em resposta ao tumor, como as
10 citocinas pró-inflamatórias (FEARON & MOSES, 2002) (Figura 4).

11 O metabolismo tumoral provoca consequências no metabolismo do
12 indivíduo por provocar alterações no metabolismo de lipídeos, carboidratos e
13 proteínas, e da glicose, amplamente requerida pelo tumor, o que reduz a
14 eficiência do metabolismo energético, já que o tumor se usa dessas alterações
15 para o fornecimento de substratos que garantam seu suporte nutricional e de
16 crescimento. Essas alterações metabólicas contribuem para um elevado gasto
17 energético em repouso, o que pode ser um dos principais fatores que levam ao
18 desenvolvimento da caquexia (FRIESEN *et al.*, 2015).

19 Nos humanos, a estocagem de carboidratos é feita no fígado, na
20 forma de glicogênio. Esta reserva é suficiente para aproximadamente dois dias,
21 quando toda essa reserva é consumida, as proteínas e os lipídeos sofrem
22 catabolismo para liberar produtos que são utilizados como fonte de energia.
23 Uma das consequências mais prejudiciais desse catabolismo é o grande gasto
24 energético do indivíduo, levando em consideração que a fonte de energia pode
25 vir de órgãos importantes para a vida, como coração e músculos como o
26 diafragma (BARTON, 2001). Os primeiros órgãos afetados pela proteólise e
27 lipólise provocados pela presença do tumor são, nesta ordem: músculo
28 esquelético, tecido adiposo e o coração (MURPHY, 2016). Ao mesmo tempo, o
29 tumor é capaz de diminuir o ganho energético, provocado, por exemplo, pela
30 perda de apetite (FRIESEN *et al.*, 2015).

31 Outra alteração metabólica importante é a liberação de citocinas pró-
32 inflamatórias em resposta ao tumor, pois sabe-se que a inflamação participa na
33 geração de fatores catabólicos que controlam o SNC (sistema nervoso central)
34 estimulando sinais para anorexia e fadiga (BARACOS *et al.*, 2018) (Figura 5).

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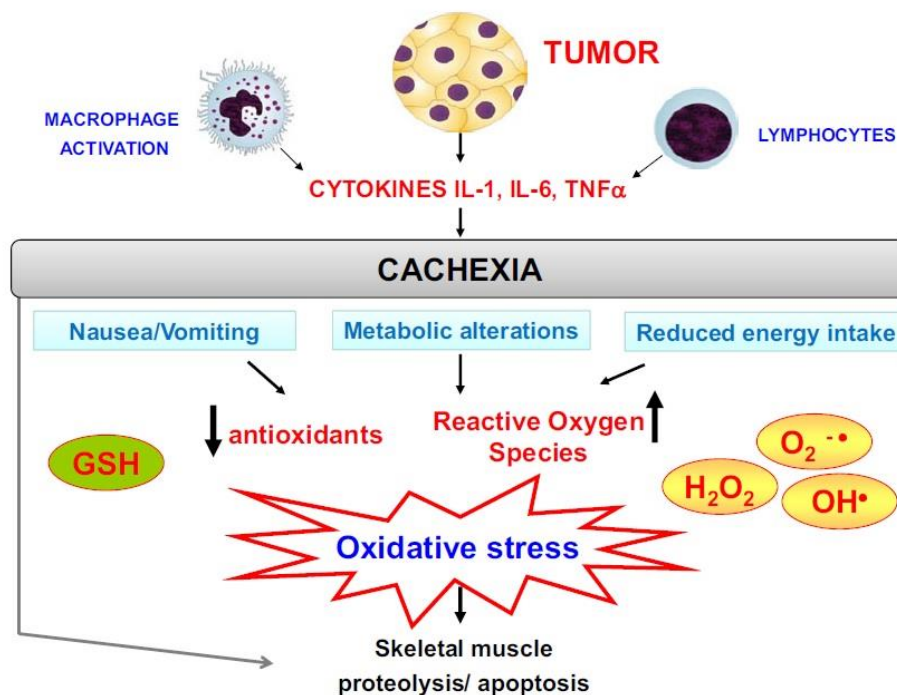
3 Figura 4. Esquema representativo dos mediadores liberados pelo tumor e pelo organismo em resposta ao
 4 tumor, culminando na degradação e atrofia da musculatura esquelética.

5

6 Citocinas inflamatórias, como TNF- α (fator de necrose tumoral alfa),
 7 IL-1 (interleucina 1) e IL-6 (interleucina 6) têm sido observadas como
 8 participantes importantes na redução da ingestão alimentar e aumento do gasto
 9 energético em situações associadas à síndrome anorexia-caquexia
 10 (ENGINEER & GARCIA, 2012). Cada uma destas citocinas desempenha um
 11 papel importante na síndrome da caquexia, porém o bloqueio de apenas uma
 12 delas não reverte o quadro, o que sugere que suas atividades acontecem de
 13 forma simultânea, como se formassem uma rede de citocinas, na qual podem
 14 ativar umas às outras (TIJERINA, 2004). TNF- α , inicialmente chamada de
 15 “caquetina”, é responsável por aumentar a gliconeogênese, lipólise e

1 proteólise, pode ativar vias catabólicas através do NFkB e ativar IL-1 (HAN *et*
 2 *al.*, 1999; TIJERINA, 2004). IL-1, por sua vez, induz anorexia em pacientes
 3 caquéticos por provocar saciedade precoce e supressão da fome, e ativa IL-6
 4 (TIJERINA, 2004; PORPORATO, 2016). IL-6, apesar de apresentar um papel
 5 contraditório, têm mostrado um papel importante na conversão de gordura
 6 branca para gordura marrom, o que custa um alto gasto energético para o
 7 indivíduo e também está relacionada com a perda de proteína da musculatura
 8 esquelética (CARSON & BATGALVIS, 2010; HAN *et al.*, 2018) (Figura 6).

9 A resposta inflamatória é uma das principais responsáveis pelas
 10 alterações metabólicas observadas no câncer. Quando o hipotálamo é exposto
 11 a estímulos inflamatórios, como as citocinas IL-1 β e TNF, desencadeiam uma
 12 resposta aguda que leva à anorexia, perda de peso e atrofia da musculatura
 13 esquelética (BARACOS *et al.*, 2018). Os mediadores inflamatórios que são
 14 produzidos pelo tumor, por células do sistema imune que secretam citocinas,
 15 quimiocinas e outros mediadores (ARGILÉS *et al.*, 2014) têm participação em
 16 vias metabólicas presentes na perda de músculo esquelético, como aumento
 17 da proteólise, apoptose de miócitos ou diminuição do transporte e regeneração
 18 de aminoácidos (ARGILÉS *et al.*, 2015).



20
 21
 22

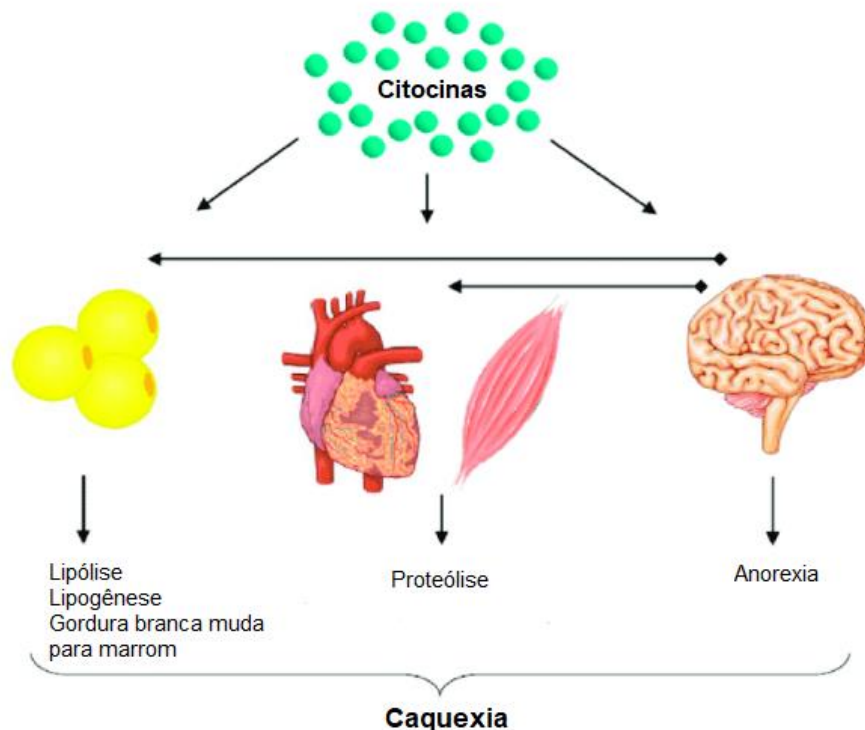
Figura 5. Representação da interação entre caquexia e estresse oxidativo. Figura retirada de Mantovani & Macciò (2012).

1 Uma das alterações mais frequentemente observadas está
2 relacionada ao controle da ingestão alimentar. É notável em indivíduos com
3 caquexia o desenvolvimento da anorexia, provocada por sinalizações
4 previamente descritas acima, mas também pelo próprio tratamento
5 quimioterápico, que provoca alterações no paladar, o que leva à aversão por
6 comida, agravando o quadro de anorexia na síndrome (VAUGHAN *et al.*,
7 2013). A diminuição no apetite e saciedade precoce, em humanos, têm
8 prevalência em estágios avançados, com diminuição do apetite em mais de
9 50% dos casos (SKIPWORTH *et al.*, 2007) e saciedade precoce em 51% dos
10 indivíduos (STRASSER, 2006). Estas alterações na alimentação sugerem que
11 haja um comprometimento da sinalização central e periférica do
12 comportamento alimentar dos indivíduos com caquexia (VAUGHAN *et al.*,
13 2013).

14 Foi observado em animais com câncer que o balanço entre
15 neuropeptídeos orexígenos e anorexígenos é desregulado, e que a liberação do
16 neuropeptídeo Y (NYP) que estimula a ingestão alimentar, pelo hipotálamo,
17 está reduzida em animais com câncer em relação a animais saudáveis (INUI,
18 2002). Um peptídeo produzido pelas células adiposas, a leptina, tem sido
19 apontada como a responsável por afetar o balanço de tais neuropeptídeos, pois
20 aumenta a expressão de neurônios anorexígenos, antagonizando a ação dos
21 neurônios orexígenos, o que levaria os animais inoculados com tumor a terem
22 menos apetite, e por consequência, apresentarem menor consumo de ração,
23 comparado aos animais sem tumor (ENGINEER & GARCIA, 2012).

24 Modelos experimentais que estudam a caquexia se fazem
25 necessários para compreender os mecanismos da caquexia e identificar
26 possíveis terapias. Um modelo muito utilizado atualmente é o tumor de Walker
27 256, o qual apresenta dois subtipos: tipo metastizante, que leva a caquexia
28 terminal dos ratos portadores em aproximadamente 27 dias; e o tipo que não
29 forma metástases e induz a caquexia precoce do portador, levando a morte em
30 15 dias (LUSHNIKOVA *et al.*, 2011; SIMPKINS *et al.*, 1991). Além do tumor de
31 Walker 256, em ratos, outro modelo experimental frequentemente utilizado
32 inclui o hepatoma de Yoshida, utilizado na sua forma ascítica (BALLARÒ *et al.*,
33 2016). Em camundongos, os modelos experimentais mais frequentemente

1 utilizados incluem o carcinoma C26 de cólon, o carcinoma pulmonar de Lewis,
 2 o adenocarcinoma MAC 16, além do melanoma B16 (BALLARÒ *et al.*, 2016).
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 5 Figura 6. Adaptação de Duval *et al.*, 2018. Mecanismos envolvidos na caquexia. Citocinas pró-
 6 inflamatórias agem em diferentes tecidos, provocando catabolismo em excesso.

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8 A necessidade de se estudar diferentes modelos experimentais de
 9 caquexia se baseia na justificativa de que os diferentes fenótipos tumorais
 10 desenvolvem a síndrome da caquexia em diferentes graus de agressividade,
 11 sendo possível avaliar as diferentes alterações existentes nesta síndrome.
 12 (TISDALE, 1999).

13 O tumor de Ehrlich é uma neoplasia experimental transplantável de
 14 origem epitelial maligna, correspondente ao adenocarcinoma mamário em
 15 camundongos fêmeas (SILVA *et al.*, 2006). Pode apresentar-se de duas
 16 formas, a ascítica, através de passagens intraperitoneais utilizada para a
 17 manutenção do tumor, e a forma sólida, que evolui através de inoculação
 18 subcutânea (FRAJACOMO *et al.*, 2016). A vantagem deste tumor é que há o
 19 conhecimento prévio da quantidade e das características iniciais das células
 20 tumorais a serem inoculadas, rápido desenvolvimento de neoplasia, eficiência
 21 na produção de células neoplásicas livres e precisão em termos do tempo de

1 sobrevivência. Além disso, o custo de manutenção é baixo, o repique do tumor
2 é extremamente fácil e o tumor tem rápido crescimento, aparecendo sete dias
3 após a inoculação (SILVA *et al.*, 2006; FRAJACOMO *et al.*, 2016).

4 Levando em consideração que os parâmetros utilizados para
5 caracterização da caquexia em humanos não podem ser extrapolados de forma
6 idêntica em animais, a caracterização da síndrome em questão será avaliada
7 pelos principais sinais observados: perda de peso, perda de força, atrofia
8 muscular e redução da ingestão alimentar. Desta forma, o objetivo deste
9 trabalho é, uma vez caracterizada a caquexia através desses parâmetros,
10 avaliar as consequências do envolvimento do estresse oxidativo na caquexia
11 induzida pelo tumor de Ehrlich, em tempos diferentes, a fim de acompanhar e
12 observar as possíveis alterações no desenvolvimento da síndrome.

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2 JUSTIFICATIVA

A caquexia é uma síndrome complexa e multifatorial, de grande importância clínica, uma vez que a quantidade de massa muscular e de massa gordurosa presentes no indivíduo podem interferir diretamente, tanto no tratamento como na qualidade e expectativa de vida de indivíduos com câncer. Como em pacientes, diferentes tipos de tumor possuem crescimentos diferenciados e comportamentos metabólicos diversos, o estudo de um novo modelo experimental para estudo da caquexia no câncer contribuiria de maneira fundamental para a elaboração de mecanismos ainda não completamente elucidados, principalmente em aspectos relacionados à perda de massa muscular, utilizando a linhagem *Swiss* de camundongos. Um trabalho recentemente publicado juntamente com colaboradores (FRAJACOMO *et al.*, 2016), trouxe à luz a possibilidade de uso do modelo no estudo da perda de massa muscular e desenvolvimento da síndrome. Até o momento, não há estudos experimentais que tragam a caracterização da evolução da caquexia em tempos diferentes, a fim de esclarecer como as alterações dessa síndrome acontecem ao longo do tempo.

Este trabalho se propõe a esclarecer os possíveis mecanismos envolvidos na perda de massa e força muscular principalmente através da análise de testes de estresse oxidativo e processo inflamatório em animais inoculados com a forma sólida do adenocarcinoma de Ehrlich, sendo estruturado da seguinte maneira: objetivos gerais e específicos, referências bibliográficas da introdução (citadas em seguida) e dois artigos científicos confeccionados a partir dos experimentos realizados (anexos A e B).

3 OBJETIVOS GERAIS

Este trabalho tem como objetivo diferenciar o efeito da caquexia na musculatura esquelética em tempos experimentais diferentes, assim como o envolvimento do estresse oxidativo no desenvolvimento da síndrome.

3.1.OBJETIVOS ESPECÍFICOS

- Avaliar se o tumor de Ehrlich é capaz de induzir caquexia cumprindo os parâmetros, como perda de massa corpórea, perda de função muscular, diminuição no apetite e alterações metabólicas, permitindo a sua classificação no tempo;

- Verificar a participação do estresse oxidativo e os possíveis danos provocados nos tempos experimentais estudados;

- Verificar ativação das vias NFkB e Hsp70 para analisar a relação com o processo inflamatório;

- Verificar ativação da via Nrf2 para análise da atividade antioxidante nos animais inoculados com tumor;

- Avaliação de processo inflamatório sistêmico através da dosagem de citocinas no plasma.

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ANEXO A

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How Oxidative Stress Interferes In Muscle Loss and Strength During Cachexia After Ehrlich Carcinosarcoma Inoculation

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1 **How Oxidative Stress Interferes In Muscle Loss And Strength During**
2 **Cachexia After Ehrlich Carcinosarcoma Inoculation**

3

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10

11 **Abstract**

12 Cachexia is a complex syndrome that affects cancer patients and worsens life
13 quality and prognosis. Reactive oxygen species are known to participate in
14 cachexia, affecting mainly the skeletal muscle, leading to oxidative damage and
15 muscle loss (atrophy). The aim of this study was to evaluate the interference of
16 oxidative stress in different periods of time (14 days and 28 days), in a new
17 experimental model of Ehrlich tumor, comparing two types of muscle fiber: slow-
18 twitch and fast-twitch. Malondialdehyde was measured by TBARS,
19 hydroperoxides by chemiluminescence, carbonyl proteins by ELISA, and cross
20 sectional area was evaluated to analyse the muscle loss by reduction of muscle
21 fiber area. Fast-twitch fibers shows more oxidative stress than slow-twitch
22 fibers, yet, somehow, the muscle shows reduced oxidative stress in 28 days
23 compared to 14 days, suggesting there may be a protective effect in this type of
24 fiber. Slow-twitch fiber, interestingly do not show elevated oxidative stress as
25 the fast ones, yet, they end up having a more elevated muscle loss, even in 14

1 days, suggesting that different muscle fiber types may behave differently
2 according to the experimental model of cancer cachexia, showing the
3 importance to study different experimental models. The aim of this study is to
4 comprehend the damages that oxidative stress brings to different types of
5 skeletal muscle fibers in cancer cachexia syndrome in different periods.

6

7 **Key-Words:** cachexia, cancer, oxidative stress, muscle atrophy, muscle loss

8

9 **Introduction**

10 Cachexia is a complex event, defined as a multifactorial syndrome in which
11 occurs loss of weight by intense degradation of skeletal muscle, with or without
12 loss of adipose tissue (1). This syndrome affects patients with chronic diseases,
13 such as cancer, congestive heart failure and diabetes. Of the clinical signs
14 present in this syndrome, muscle atrophy is one of the most important
15 complications of this syndrome, because it not only reduces physical function
16 and quality of life, but also the chemotherapy treatment, making the patients
17 resistant to the treatment, therefore worsening the prognosis and increasing
18 mortality (1,2). Importantly, due to cardiac and respiratory failure, cachexia is
19 directly responsible for 20-30% of cancer deaths (3, 4). In this condition, the
20 intense muscle loss comes from an imbalance between protein synthesis and
21 degradation, being the degradation significantly increased (2). This high
22 catabolic state occurs mainly through the ubiquitin-proteasome system, a
23 proteolytic pathway-ATP dependent, able to degrade targeted proteins to
24 peptides (4). The first sign to be noticed, even before the actual diagnosis, is
25 the weight loss, that worsens prognosis. It occurs mainly due to decreased food

1 intake, a condition known as anorexia - as a response to chemotherapy, the
2 products of tumor signaling itself or as a response of the body to the tumor 6,
3 7). It has been noticed that the balance between orexigenic and anorexigenic
4 neuropeptides in cachectic mice is unregulated (8), and Leptin, a hormone
5 secreted mostly by adipose tissue (9) has been proposed to be responsible to
6 affect this neuropeptide balance (10). Yet, there are other metabolic changes
7 that influence weight loss, such as fat metabolism, that uses fat reserves as a
8 source of energy (11). Together, weight loss and atrophy may indicate a status
9 of sarcopenia, a component of cachexia syndrome (12). Cachectic patients that
10 develop sarcopenia tend to have higher rates of morbidity due to infectious and
11 cardiovascular diseases, insulin resistance, and a higher mortality rate, which
12 makes the identification of sarcopenia important, once it may reflect the fragility
13 and general health status of the patient (13). Protein degradation in skeletal
14 muscle by UPS is stimulated by ROS (reactive oxygen species) generated by
15 the cachexia syndrome (14). Oxidation by ROS damages and modifies the
16 structure and function of proteins, even irreversibly, causing these proteins to
17 be labeled and sent for degradation. In cachectic muscle, this happens
18 intensely, leading to degradation at a high level (15). In muscle mass, ROS
19 have important roles in cell growth, proliferation, differentiation, contractile
20 performance during physical activity, calcium signaling, glucose consumption,
21 mitochondria biogenesis (16-18) or even by controlling muscle mass during
22 adaptation processes (17). However, when there is an imbalance and this
23 espécies are elevated, they become toxic to our organism, and react with
24 polyunsaturated fatty acids that form cell membranes, enzymes and DNA (19).
25 Different types of muscle fiber may behave differently to atrophic process. In a

1 LLC cachexia model, glycolytic fibers showed to be more susceptible to atrophy
2 compared to muscles with oxidative fiber predominance (20). However, how
3 oxidative stress interferes in different types of muscle fiber in cachectic
4 condition has yet to be clarified, since different experimental models may show
5 different alterations. In this study, we propose the new model of cachexia
6 induced by Ehrlich tumor (21) as an experimental model in which is possible to
7 compare the effects of oxidative stress in muscle atrophy in different muscle
8 fibers, in different periods of time. Here, we show that this model behaves
9 differently in some aspects from the pre-existing experimental models, bringing
10 out the attention to the variety of possible alterations that may occur in cachexia
11 syndrome.

12

13 **Materials and Methods**

14 **Animals**

15 Male *Swiss* mice with 7 week old were obtained from the animal house of the
16 Biological Sciences Center at Universidade Estadual de Londrina (UEL). We
17 used 120 animals, divided in 30 animals/group. A pilot study was conducted in
18 order to establish the period which Ehrlich tumor induced pre cachexia and
19 cachexia. The animals were divided in 4 groups: (i-ii) control 14 days and
20 control 28 days (C14d; C28d), (iii) tumor 14 days – inoculation with Ehrlich
21 tumor cells and maintained for 14 days (T14d), (iv) tumor 28 days – inoculation
22 with Ehrlich tumor cells and maintained for 28 days (T28d). Animals were fed
23 with commercial food (Nuvital®) and water *ad libitum*. Mice were treated
24 according to national resolutions, and the experiment was approved by the
25 Ethics Committee on Animal Use (CEUA-UEL).

1 **Tumor Inoculation**

2 According to the experimental protocols described above, animals from T14d
3 and T28d groups received 1.0×10^6 cells in a final volume of 250 μ L phosphate
4 buffer saline (PBS), and animals of control groups received the correspondent
5 volume of PBS. The implantation was performed through a single subcutaneous
6 injection in the back region of each animal. Tumor Ehrlich cells are maintained
7 *in vivo* in Swiss mice, as an ascitic intraperitoneal tumor through passages
8 every 10 days. The ascitic fluid of the host animals was collected, washed in
9 PBS, centrifuged at 200 x g for 10 minutes. The viable cells were determined by
10 trypan dye exclusion, using a Neubauer's chamber (21). On the 14th and 28th
11 days after tumor inoculation, animals were sedated intramuscularly with ketamin
12 and xilazin (100mg/Kg and 160mg/Kg, respectively) and euthanized.

13

14 **Cachexia Parameters**

15 **Body Weight**

16 Animals were weighed before the beginning of the experiment and on the last
17 day. Weight gain was calculated by $[(\text{Final Weight} - \text{Tumor Weight}) * 100 -$
18 $\text{Inicial Weight}]$ (g). To analyze the behavior of the lean mass, the muscles
19 anterior tibial (TA), extensor digitorum longus (EDL), gastrocnemius (GAST)
20 and soleus (SOL) were collected and weighted, all together. The fat collected
21 from the retroperitoneum and the perigonadal regions were also weighed
22 together.

23

24

25

1 **Tumor Size and Weight**

2 Tumor size was measured with a digital caliper each two days. After
3 euthanasia, tumors were collected and weighed.

4

5 **Hand Grip Test**

6 The strength of the forelimb were evaluated through Hand Grip Test (HGT), with
7 a digital dynamometer (EEF 305 Grip StrengthMeter, Insight), that registers the
8 peak of strength the animals grips to the equipment. Only after one week of
9 adaptation, animals were exposed to the test every day, at the same time, light
10 conditions, bar diameter and the same person (22). A mean was calculated with
11 the peak of each day of test.

12

13 **Food Intake**

14 As reduction in food intake is a symptom of cachexia (2), feeding was monitored
15 daily, by weighing the food of each experimental group. Food intake was
16 calculated by food consumption / group / animal / day (g).

17

18 **Temperature**

19 Significant variation in body temperature is a sign of metabolic alteration, which
20 is observed in initial stages of cancer cachexia in humans. Temperature was
21 measured using a digital thermometer via rectal. Measurements were realized
22 once a day, each three days, at the same time, light condition and the same
23 person.

24

25

1 **Blood samples**

2 At the last day of experiment, animals were sedated, and the blood was
3 collected by cardiac puncture. To obtain the plasma, blood was centrifuged
4 1500 x rpm for 5 minutes at 4°C, the plasma was collected and stocked at -
5 20°C, until the moment of use.

6

7 **Leptin Quantification**

8 Leptin is an important hormone produced and secreted by adipose tissue that
9 reflects and participates in the energy balance in our body (9). Leptin was
10 determine by ELISA, according to the manufacturer's instructions (Sigma-
11 Aldrich®, USA). The quantification was performed using a microplate reader
12 (Thermo Scientific) at 450 nm. Results are expressed in pg leptin/mL plasma.

13

14 **Glucose**

15 Plasma glucose levels were measured in a glucometer (Accu-Check), using
16 20µL of plasma for each animal. Three measurements were made and then the
17 media was calculated and used for statistics. Results are expressed in mg/dL.

18

19 **Cross Sectional Area (CSA)**

20 Samples of SOL and EDL were included in paraffin, and were made semi-
21 seriated cuts of 5 µm (5 cuts/slide; gap of 25 µm/cut). Next, the slides were
22 stained with hematoxylin and eosin and the CSA was analyzed. It was recorded
23 3 fields/cut/animal, totalizing 300 fibers/animal, using a photomicroscope
24 (Olympus BX43) (400x). To determine the area of each fiber in µm², the

1 program Image-Pro Plus (4.0; Media Cybernetics, Silver Spring, MD, USA) was
2 used.

3

4 **Muscle Proteolytic Activity**

5 Chymotrypsin-like proteolytic activity was quantified using a Proteasome Glo™
6 Chymotrypsin-like Cell Based Assay kit (Promega, Madison, WI, USA). This kit
7 estimates the activity of the proteasome; the final objective is to evaluate
8 whether proteolytic activity is present in skeletal muscle. For the assay, 25 mg
9 of GAST muscle powder was added to 1 ml 10 mM KH₂PO₄, pH 7.4, in 0.9%
10 NaCl and gently homogenized. Fifty microlitres of the resulting muscle
11 homogenate was pipetted in duplicate on to 96-well microplates, and the final
12 reagent mixture with luminescent substrate was added to the medium. After 10
13 min, under light protection, the luminescent signal at the plate was detected with
14 a Glo-Runner microplate reader luminometer (Turner Designs), and the results
15 were expressed as RLU per milligram total protein (23).

16

17 **Western Blotting**

18 SOL and EDL muscles of tumor bearing animals and healthy animals were
19 homogenized in lysis buffer containing 0.1 M TRIS-HCl (pH 7.2), 0.2% SDS,
20 0.1% sodium deoxycolate, and 0.3M NaCl, and centrifuged for 15 min at 900×g
21 at RT. Total protein quantification was performed by using Bradford method. 20
22 µg of total protein were separated in 12% sodium dodecyl sulphate-
23 polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to
24 nitrocellulose membrane. Membranes were probed using primary antibodies
25 against SERCA (sarcoplasmic/endoplasmic reticulum calcium ATPase) (mouse

1 monoclonal, dilution 1:500; Thermo Fischer Scientific, MA, USA) and against
2 GAPDH (mouse monoclonal, dilution 1:2000; Thermo Fischer Scientific, MA,
3 USA), diluted in 10% nonfat dry milk in TBS-T overnight at 4 °C. Horseradish
4 peroxidase (HRP)-conjugated antimouse (dilution 1:10000, Merck Millipore,
5 Darmstadt, Germany) were used as secondary antibodies. Peroxidase activity
6 was detected using an Enhanced chemiluminescence (ECL) kit (Perkin Elmer,
7 Waltham, MA). The bands were visualized using a gel documentation system
8 (UVItec, Cambridge, UK), and the band densitometric quantification of signals
9 was performed using Image J software (National Institute of Health, Bethesda,
10 MA). The values obtained were then averaged and normalized to those of
11 GAPDH used as a loading control.

12

13 **Oxidative Stress Parameters**

14 **TBARS**

15 Malondialdehyde (MDA) is a product of lipid peroxidation and is a golden
16 pattern used for oxidative stress. We used the muscles SOL and TA (20mg/mL
17 each). It was determined according to Jentzsch *et al.* (24), in a
18 spectrophotometer (Shimadzu UV1650 PC, Nakagyo-ku, Kyoto, Japan) in
19 535nm and 572nm and the results are expressed in nmol/20mg tissue.

20

21 **Carbonyl Protein**

22 Carbonyl protein content was measured by ELISA method as described by
23 Alamdari *et al* (15), using the muscles SOL and EDL. The method consists in
24 use of BSA oxidized as a standard and a small amount of sample (5µg of

1 protein). The absorbance was corrected by total protein and results are
2 expressed in nmol/mg of protein.

3

4 **Glutathione Assay**

5 Determination of total and oxidized glutathione (GT and GSSG, respectively)
6 was performed by the recycling method, as described by TIETZE (25). For GT
7 we used homogenates concentration of 20mg/mL for both SOL and TA, and for
8 GSSG we used 20mg/mL and 50mg/mL, respectively. Absorbance was
9 detected at 412nm, and results are expressed in μ L of GT/mg protein and μ L of
10 GSSG/mg protein.

11

12 **Chemiluminescence stimulated by Tert-Butyl hydroperoxide**

13 Reaction mixtures were placed in 2-ml luminescence tubes containing the
14 following: total muscle homogenate from SOL and EDL muscles (20mg/mL and
15 10 mg/mL, respectively); 10 mM KH₂PO₄/K₂HPO₄ buffer (with 120mM KCl, pH
16 7.4); and 3 mM tert-butyl hydroperoxide, in a final volume of 1 ml. The tert-butyl
17 hydroperoxide-initiated chemiluminescence (CL) reaction was assessed by
18 luminometer (TD/20 20; Turner Designs) with a response range of 300–650 nm.
19 The tubes were kept in the dark until the moment of assay, which was carried
20 out at a room temperature of 30°C (26, 27). For each animal was performed a
21 40 min curve, where each point represented the differential smoothing of 300
22 readings, was obtained by interpolation. The results were expressed in relative
23 light units per gram of tissue (RLU/g tissue), and after the final calculation a final
24 curve was determined by Gaussian fit. The entire curve and area, extracted by

1 integral calculus of each animal curve, were used to determine the lipid
2 hydroperoxides present in the sample.

3

4 **Protein Concentration**

5 Protein was determined by the method of Lowry *et al* (28), modified by Miller
6 (29) except for protein carbonyl contents, where absorbance at 280 nm was
7 used for each sample to determine total protein content. Both methods used
8 bovine serum albumin BSA as standard.

9

10 **Statistical Analysis**

11 When passed Shapiro-Wilk normality test, results are shown as mean \pm SD
12 (standard deviation), when data did not pass normality test, results are shown
13 as mean \pm standard error of mean (SEM). For parametric test we used one-way
14 analysis of variance (ANOVA) followed by Tukey's post hoc test. For non-
15 parametric test we used Mann-Whitney followed by Dunn's post hoc test. CL
16 curves were compared by two way analysis of variance (ANOVA) followed by
17 Tukey's post hoc test to determine differences in each time point. Statistical
18 significance was considered when $P < 0,05$.

19

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1 **Results**

2 **Body Weight**

3 Control groups 14d and 28d had similar body weight gain ($28.67 \pm 2.50\text{g}$; 24.97
4 $\pm 2.76\text{g}$, respectively). T14d group showed a significant reduced body weight
5 ($12.62 \pm 1.54\text{g}$) compared to C14d, and T28d differed significantly ($21.85 \pm$
6 2.44g) compared to C28d. The fat collected did not reduce significantly in T14d
7 ($0.89 \pm 0.33\text{g}$) and T28d ($0.90 \pm 0.34\text{g}$), compared to C14d ($1.25 \pm 0.50\text{g}$) and
8 C28d ($1.33 \pm 0.73\text{g}$), respectively. Muscle loss was significantly in T14d ($0.26 \pm$
9 0.03g) compared to C14d ($0.31 \pm 0.07\text{g}$), but T28d ($0.30 \pm 0.06\text{g}$) was not
10 significantly compared C28d ($0.31 \pm 0.03\text{g}$). Tumor weight differed significantly
11 between inoculated groups, being smaller in T14d ($0.49 \pm 0.29\text{g}$) compared to
12 T28d ($1.25 \pm 0.54\text{g}$) ($P < 0.005$) ($n = 20$ animals/group).

13

14 **Hand Grip Test**

15 Hand grip strength test demonstrated the inoculated animals had a progressive
16 strength loss along the experimental protocols, being significantly reduced in
17 T14d ($80.93 \pm 3.05\text{g}$) compared to C14d ($93.99 \pm 1.19\text{g}$) ($P < 0.0025$) (Figure
18 1b), and in T28d ($78.56 \pm 2.43\text{g}$) compared to C28d group ($96.33 \pm 0.73\text{g}$)
19 (Figure 1c). $P < 0.0001$.

20

21 **Food Intake**

22 Animals inoculated with Ehrlich tumor presented a significantly reduced food
23 consumption in T14d and T28d groups (156.10 ± 3.46 ; $197.5 \pm 3.41\text{g}$,
24 respectively) compared to C14d ($189.10 \pm 3.41\text{g}$) and C28d group ($328.40 \pm$
25 4.69g) (Figure 1a) $P < 0.0001$.

1 **Temperature**

2 Temperature did not show any significant variation in T14d and T28d groups
3 (35.42 ± 0.30 ; 32.35 ± 2.95 °C, respectively) compared to C14d (35.49 ± 0.23
4 °C) and C28d (32.35 ± 2.95 °C) (Figure 1e).

5

6 **Leptin Quantification**

7 Leptin level was significantly increased in T28d group (3.01 ± 0.27 pg/mL)
8 differed statistically from C14d (1.47 ± 0.29 pg/mL) ($P < 0.0091$). T28d group
9 (2.60 ± 0.34 pg/mL) did not differ statistically compared to C28d (2.64 ± 0.75
10 pg/mL) (Figure 1d).

11

12 **Glucose**

13 Plasma glucose levels did not differ between C14d and C28d (149.4 ± 21.08
14 mg/dL; 146.7 ± 18.06 mg/dL, respectively) and the tumor inoculated groups
15 T14d (145.1 ± 7.86 mg/dL) and T28d (137.2 ± 18.29 mg/dL) (Figure 1f).

16

17 **Cross Sectional Area**

18 The frequency distribution of fibre CSA differed significantly between groups. In
19 the histogram, it is possible to observe that T14d and T28d groups have a
20 retreatment to the left, indicating muscle atrophy compared to their respective
21 control groups. For SOL, group C14d had a 50th percentile area of $9.06 \mu\text{m}^2$
22 (minimum = $0.16 \mu\text{m}^2$; maximum = $23.23 \mu\text{m}^2$), T14d had a 50th percentile area
23 of $6.55 \mu\text{m}^2$ (minimum = $2.07 \mu\text{m}^2$; maximum = $29.67 \mu\text{m}^2$), C28d had a 50th
24 percentile area of $10.55 \mu\text{m}^2$ (minimum = $4.03 \mu\text{m}^2$; maximum = $116.80 \mu\text{m}^2$)
25 and T28d had a 50th percentile area of $5.44 \mu\text{m}^2$ (minimum = $1.67 \mu\text{m}^2$;

1 maximum = 26.26 μm^2). For EDL, group C14d had a 50th percentile area of 9.05
2 μm^2 (minimum = 0.55 μm^2 ; maximum = 20.54 μm^2), T14d had a 50th percentile
3 area of 4.94 μm^2 (minimum = 0.32 μm^2 ; maximum = 12.12 μm^2), C28d had a
4 50th percentile area of 11.72 μm^2 (minimum = 1.23 μm^2 ; maximum = 23.04 μm^2)
5 and T28d had a 50th percentile area of 4.85 μm^2 (minimum = 0.35 μm^2 ;
6 maximum = 14.10 μm^2) (Figure 2).

7

8 **Muscle Proteolytic Activity**

9 Inoculated groups T14d (29.91 ± 32.61 RLU/mg protein) and T28d ($31.50 \pm$
10 15.87 RLU/mg protein) did not show a significantly higher proteolytic activity
11 compared to their respective controls C14d (19.19 ± 9.54 RLU/mg protein) and
12 C28d (11.57 ± 7.53 RLU/mg protein) (Figure 3).

13

14 **Western Blotting**

15 SERCA protein quantification by western blotting showed that there was no
16 significant difference between control and inoculated groups in SOL muscle
17 (data not shown). As for EDL muscle no significant difference was showed
18 between C14d and T14d groups, or between C28d and T28d groups, but is it
19 possible to observe that there was a statistically significant reduction from T14d
20 to T28d. Relative values were normalized on GAPDH levels (Figure 3).

21

22 **Malondialdehyde**

23 In SOL muscle, inoculated groups T14d and T28d present a significantly higher
24 quantification of MDA (32.08 ± 15.52 nmol/20mg tissue; 26.52 ± 14.34
25 nmol/20mg tissue, respectively), compared to C14d (10.45 ± 1.47 nmol/20mg

1 tissue) and C28d (9.21 ± 2.02 nmol/20mg tissue). In TA muscle, we observe the
2 same pattern showed in SOL muscle, being T14d and T28d groups significantly
3 higher (28.44 ± 3.17 nmol/20mg tissue; 42.61 ± 6.72 nmol/20mg tissue,
4 respectively) than their respective control groups C14d (8.69 ± 1.12 nmol/20mg
5 tissue) and C28d (12.47 ± 2.31 nmol/20mg tissue) (Figure 4) $P < 0.0001$.

6

7 **Carbonyl Protein**

8 Carbonilated protein content in T14d was statistically higher than C14d (567.40
9 ± 47.18 nmol/mg protein; 421.50 ± 64.22 nmol/mg protein, respectively), and
10 T28d group also differed significantly from C28d (567.60 ± 54.31 nmol/mg
11 protein; 462.70 ± 64.16 nmol/mg protein). $P < 0.0015$. For EDL muscle,
12 inoculated groups T14d and T28d (537.00 ± 42.18 nmol/mg protein; $542.90 \pm$
13 41.78 nmol/mg protein, respectively) did not differ statistically from C14d
14 (547.70 ± 97.61 nmol/mg protein) and C28d (502.90 ± 76.77 nmol/mg protein),
15 respectively (Figure 4).

16

17 **Glutathione Assay**

18 The reduced form of GSH were statistically different in tumor inoculated groups
19 T14d (0.29 ± 0.03 μ L/mg protein) and T28d (0.51 ± 0.07 μ L/mg protein),
20 compared C14d (0.28 ± 0.05 μ L/mg protein) and C28d (0.17 ± 0.04 μ L/mg
21 protein). For the oxidized form GSSH in SOL muscle, T14d (0.11 ± 0.12 μ L/mg
22 protein) and T28d (0.35 ± 0.12 μ L/mg protein) statistically different from each
23 other compared to the control groups C14d (0.08 ± 0.03 μ L/mg protein) and
24 C28d (0.09 ± 0.05 μ L/mg protein). $P < 0.0001$. There was no statistical
25 significance for GSH in TA muscle between inoculated T14d (0.08 ± 0.01 μ L/mg

1 protein) and T28d ($0.07 \pm 0.02 \mu\text{L}/\text{mg}$ protein) compared to C14d (0.09 ± 0.01
2 $\mu\text{L}/\text{mg}$ protein) and C28d ($0.05 \pm 0.02 \mu\text{L}/\text{mg}$ protein). But, in GSSG, C28d
3 ($0.21 \pm 0.09 \mu\text{L}/\text{mg}$ protein), showed statistical difference from C14d and T14d
4 ($0.05 \pm 0.03 \mu\text{L}/\text{mg}$ protein; $0.04 \pm 0.02 \mu\text{L}/\text{mg}$ protein, respectively), and
5 showed no difference from T28d group ($0.08 \pm 0.09 \mu\text{L}/\text{mg}$ protein). $P < 0.0106$
6 (Table 1).

7

8 **Chemiluminescence**

9 Measurement of lipid hydroperoxides by CL, showed that in SOL muscle T14d
10 group differed significantly only at the peak of the curve from C14d ($P < 0.0001$),
11 and then the tail of the curve was maintained statistically similar between this
12 two groups, but in T28d group, the entire curve differed statistically from C28d.
13 In EDL muscle, T14d group also differed significantly only at the peak of the
14 curve from C14d ($P < 0.0001$), but the curve of T28d group did not differ
15 statistically from C28 at any moment (Figure 4).

16

17 **Discussion**

18 In this paper, we emphasize that Ehrlich tumor is a reliable model to
19 study cancer cachexia (21), once it fits some of the main parameters of this
20 condition observed in humans, as weight loss, anorexia and sarcopenia (1).
21 Also, this model brings out the possibility to study cachexia in different phases,
22 as observed in a pilot study performed in our lab, in which maximal survival
23 reached ~ 28 days after inoculation (data not shown). This model is potentially
24 useful in the study of cancer cachexia syndrome, because it develops the main
25 cachexia-related parameters, but also because its maintenance is easy and

1 cost effective, and the tumor develops fast, starting within seven days after
2 inoculation. A previous paper from our group (30) showed that cachexia
3 syndrome makes the gastrocnemius muscle susceptible to protein degradation
4 through influence of oxidative stress starting from 5 days after Walker-256 solid
5 tumor s.c. inoculation, in rats. Since it is important to understand the variety of
6 the cachectic process, as the severity of cachexia changes according to the
7 different tumor phenotypes, this work brings the possibility to help understand
8 how muscle loss develops within oxidative stress influence, in a new
9 experimental model. In addition, to our knowledge, although the experimental
10 model is widely used in the study of cancer, Ehrlich tumor is still little explored in
11 cachexia field, and, so far, there are no publications differentiating the effect of
12 oxidative stress and protein degradation on different types of muscle fibers,
13 especially in different experimental times.

14

15 *Anorexia and Muscle atrophy are precocious signals of cachexia in Ehrlich*
16 *tumor:*

17 Reduced food intake is one of the first and most frequently signals to be
18 observed in cachexia syndrome, and is related to limited food intake, increased
19 morbidity and mortality and reduced quality of life (31). The anorexia present in
20 cachexia is named “secondary anorexia”, caused mainly by impairment of the
21 neuronal pathways that regulate eating behavior (32). Leptin is a hormone,
22 produced and secreted mainly by the adipose tissue that is related to fat
23 reserves and the energy intake balance, through binding on the hypothalamus
24 to activate or inactivate anorexigenic and orexigenic neuropeptides, depending
25 on the caloric demand of an individual (33). Studies had observed that tumor

1 bearing animals show an imbalance between these neuropeptides, being the
2 orexigenic one (NYP - responsible for appetite stimulation) downregulated (8).
3 Our leptin results corroborates with this findings, once it is elevated in the early
4 stage of cachexia progression, decreasing during time. As leptin suppresses
5 NYP, the hormone can stimulate satiety with a small amount of calories, then
6 promoting the maintenance of its secretion until the final period of 28 days of
7 tumor progression. These data corroborates with total food consumption found
8 among groups.

9 In literature, both mice and rat have different composition of skeletal
10 muscles, which affects their structure, metabolism, contractile and physiological
11 properties (34). Here, we analyze two types of muscle fiber: (i) slow-twitch (type
12 I), with high density of mitochondria, thus with a high oxidative capacity, and (ii)
13 fast-twitch (type II), with high sarcoplasmic reticulum density and has a glycolytic
14 metabolism (35-38). As in mice or rat, oxidative muscle fibers have shown to be
15 more resistant to damages, such as aging or oxidative stress (38-40). This
16 protection may be due to a higher rate in mitochondrial turnover in oxidative
17 muscle that preserves mitochondrial function (41), at least during aging. Here,
18 when CSA was analyzed individually, both muscles presented muscle atrophy,
19 by a reduction in the CSA already at 14 days, being the reduction of EDL cross
20 sectional area more intense when compared to SOL. Apparently, the
21 differences found in EDL are kept in time, while CSA of SOL is significantly
22 different in the same comparison, showing that SOL keep developing atrophy in
23 time. When analyzed together with the tumor evolution characteristics, these
24 data reveal that, despite the fact that the general weight loss is already
25 extensive 14 days after the tumor inoculation, muscle mass do not show gross

1 structural changes (reflected by the weight of the muscle), but the signaling for
2 the atrophy is already started, being reflected by the fine modifications
3 (detected by the CSA). Besides proteolysis analysis did not present statistical
4 difference between control and tumor inoculated groups, results presented in
5 CSA and SERCA blotting shows us that there is atrophy observed since 14
6 days after inoculation. This may be due to damages in SERCA units, which
7 contributes to calcium in excess in the cytoplasm, facilitating the proteolysis
8 activation.

9 Interestingly, diminished muscle strength (Figures 1B and 1C)
10 matches the precocious modifications found at 14 days. The fiber size reduction
11 can explain why tumor bearing mice loose strength significantly - and early -
12 when compared to control mice (42). Strength test, performed by HGT, is one of
13 the most used parameters to evaluate strength in patients. It is known to be
14 cheaper than isokinetic testing and is a portable, valid and reliable method (43).
15 In this sense, the initial steps of skeletal muscle loss appear to be a very
16 precocious signal of cachexia development in Ehrlich model, as leptin secretion
17 and decreased food consumption. Sarcopenia (muscle atrophy + strength loss)
18 is a very important parameter to be analyzed, once it compromises the patients
19 survival, by interfering negatively in the anticancer therapy (44), raising the
20 mortality rates, thus, sarcopenia may reflect the fragility and the health status of
21 the patient (13).

22

23

24

1 *Oxidative modifications are increased in both glycolytic and oxidative*
2 *fibers:*

3 It is known that oxidative stress can accelerate proteolysis by: 1)
4 stimulating gene expression and increasing the abundance of key proteins
5 involved in proteolytic systems (i.e., autophagy, calpain, and proteasome); 2)
6 increasing the activity of the 20S proteasome and activation of both calpain and
7 caspase-3; and 3) oxidizing proteins and increasing their susceptibility to
8 proteolytic breakdown (42). Moreover, reactive oxygen species and nitric oxide
9 have important roles in the force and contractile process of skeletal muscle,
10 when in small doses (45) leading to cell adaptation, preventing future oxidative
11 damage (42). In the present study, MDA, that is considered the main molecular
12 mechanism involved in oxidative damage to cells and is a classic marker of lipid
13 peroxidation (46), arises largely from peroxidation of PUFA with more than two
14 double binds, such as linoleic, arachidonic and docosahexaenoic acids, but
15 some is formed enzymatically during eicosanoid metabolism. MDA is the final
16 product of lipid peroxidation, which reacts with several residues, modifying them
17 (47). Also, MDA form adducts that produces biological damages, such as DNA
18 mutations, and has been related with diseases as Alzheimer's, cancer and
19 cardiovascular diseases. Quantifying MDA is important because its excessive
20 production may act as a biomarker for an advanced oxidative status (48). Here,
21 our results of MDA are in accordance to previous studies that also observed
22 elevation of this oxidative product in muscle of animals inoculated with other
23 types of cancers that induce cachexia, as Yoshida hepatoma tumor and Walker-
24 256 (30, 49-52). We observed that predominant glycolytic fibers (TA muscle)
25 presents a progressive elevation of MDA already in T14d group, suggesting that

1 lipid peroxidation of polyunsaturated membrane of TA appears to be an early
2 and continuing event during the muscle wasting process. Interestingly, SOL
3 muscle also showed significantly elevated MDA levels in T14d when compared
4 to C14d, but reduce within time. This may suggest that muscles with oxidative
5 metabolism may have a potential protective effect from the lipidperoxidation
6 damages caused by MDA, and that lipid peroxidation seems to be the main
7 source of membrane damage in glycolytic fibers, leading to atrophy.

8 However, we can also detect initial lipid peroxide products, the
9 hydroperoxides that can react with other amino acids leading to a propagation
10 chain that forms multiple hydroperoxides (47). To detect them, CL is a very
11 sensitive technique that is widely used, based on the fact that photons are
12 produced by the reaction with free radicals (27). Hydroperoxides can be used
13 as biomarkers that predict oxidative stress. Thus, together, these two lipid
14 peroxidation products may show the progression and the oxidative stress level
15 in an organism or a tissue (48).

16 Here, in SOL muscle, the hydroperoxides reduced in time, showing that
17 hydroperoxides can be detected precociously in this cachexia model, but
18 reduce within time. It appears that glycolytic fibers behave on a different
19 manner, as EDL showed no statistical differences in any of the cachexia
20 experimental times. The reduction on SOL muscle may be due to protective
21 effect – antioxidant or due to its metabolism, but also, it seems important to
22 remind that sarcoplasmic reticulum is the largest skeletal muscle cell membrane
23 system, constituting ~50% of the total volume of its membranes (53), so it is
24 important to consider that the decreased volume of membranes can be the
25 cause for the absence of statistical differences. These results differ from the CL

1 profile shown in Walker-256, in which hydroperoxide levels are elevated in
2 tumor bearing animals in a 14 days protocol (30), suggesting that different
3 tumor types may present different muscle alterations.

4 In addition, oxidative stress damages muscle proteins in an irreversible
5 manner, producing hydroxylated and carbonylated proteins (14), causing
6 activation of the UPS, leading to protein degradation (54). Carbonylated
7 proteins were elevated only in SOL of inoculated animals, but EDL muscle
8 showed no difference in carbonyl protein content at any moment of cachexia
9 evolution. This may show us that this protein damage may not be as important
10 for glycolytic muscle as it appears to be to oxidative fibers. This result for EDL is
11 in accordance with a previous study with Yoshida hepatoma tumor, that did not
12 show difference in carbonyl protein levels in tumor bearing rats, compared to
13 control group. But, in the same study, is it possible to see that our SOL results
14 for carbonyl protein disagree, since in our study SOL muscle of inoculated
15 animals show statistical difference compared to control, and in the compared
16 study it does not (51), reinforcing that different tumor phenotypes present
17 different behavior. For SOL muscle, we suggest that it adapts to oxidation
18 damages through the glutathione antioxidant pathway. In Ehrlich tumor
19 inoculated mice, GSH levels are elevated, and even more expressively in T28d
20 group, what shows that glutathione may be able to detoxify the hydroperoxides
21 generated by the oxidative species during cancer cachexia progression, thus
22 preventing SOL muscle to atrophy compared to EDL muscle.

23 In this study we show that muscles with different metabolisms behave
24 differently in response oxidative stress, being the oxidative muscle more
25 susceptible to oxidative stress damages in the initial steps of the syndrome,

1 thus, protected later by an adaptation process that has still to be better studied.
2 The glycolytic fiber, on the other hand, does not seem to be as affected as
3 oxidative fiber by the redox imbalance installed, but by the MDA, that appears to
4 be the main cause of this muscle's atrophy.

5

6 **Conclusion**

7 In this study we show the importance of studying different models of
8 cancer cachexia syndrome, in order to comprehend the variety of alterations
9 that different muscle fiber types may present in the presence of elevated
10 oxidative stress levels. Oxidative and glycolytic fibers don't seem to behave as it
11 would be expected in previous cancer-cachexia models, as in Ehrlich tumor,
12 glycolytic fibers do not seem to be more susceptible to the oxidative stress
13 parameters tested. In addition, strength loss appeared as a good parameter to
14 evaluate and correlate with muscle loss. It still is also important to comprehend
15 that oxidative muscle is capable of adaptation between the experimental times
16 used in this protocol, which leaves us a gap to study the right time and the right
17 way to possibly intervene in cachexia.

18

19 **Conflict of Interest statement**

20 The authors declare that there are no conflicts of interest.

21

22 **Acknowledgments**

23 We thanks Capes for financial support.

24

25

1 **Abbreviations**

- 2 - UPS: ubiquitin-proteasome system
- 3 - ROS: reactive oxygen species
- 4 - DNA: deoxyribonucleic acid
- 5 - LLC: Lewis lung carcinoma
- 6 - PBS: Phosphate Buffered Saline
- 7 - TA: tibial anterior
- 8 - EDL: extensor digitorum longus
- 9 - GAST: gastrocnemius
- 10 - SOL: soleus
- 11 - HGT: hand grip test
- 12 - ELISA: enzyme-linked immunosorbent assay
- 13 - CSA: cross sectional area
- 14 - RLU: relative light unit
- 15 - MDA: malondialdehyde
- 16 - BSA: bovine serum albumin
- 17 - GT: glutathione total
- 18 - GSSG: oxidized glutathione
- 19 - CL: chemiluminescence
- 20 - NYP: neuropeptide y
- 21 - PUFA: Polyunsaturated fatty acid
- 22 - SERCA: sarcoplasmic/endoplasmic reticulum calcium ATPase
- 23
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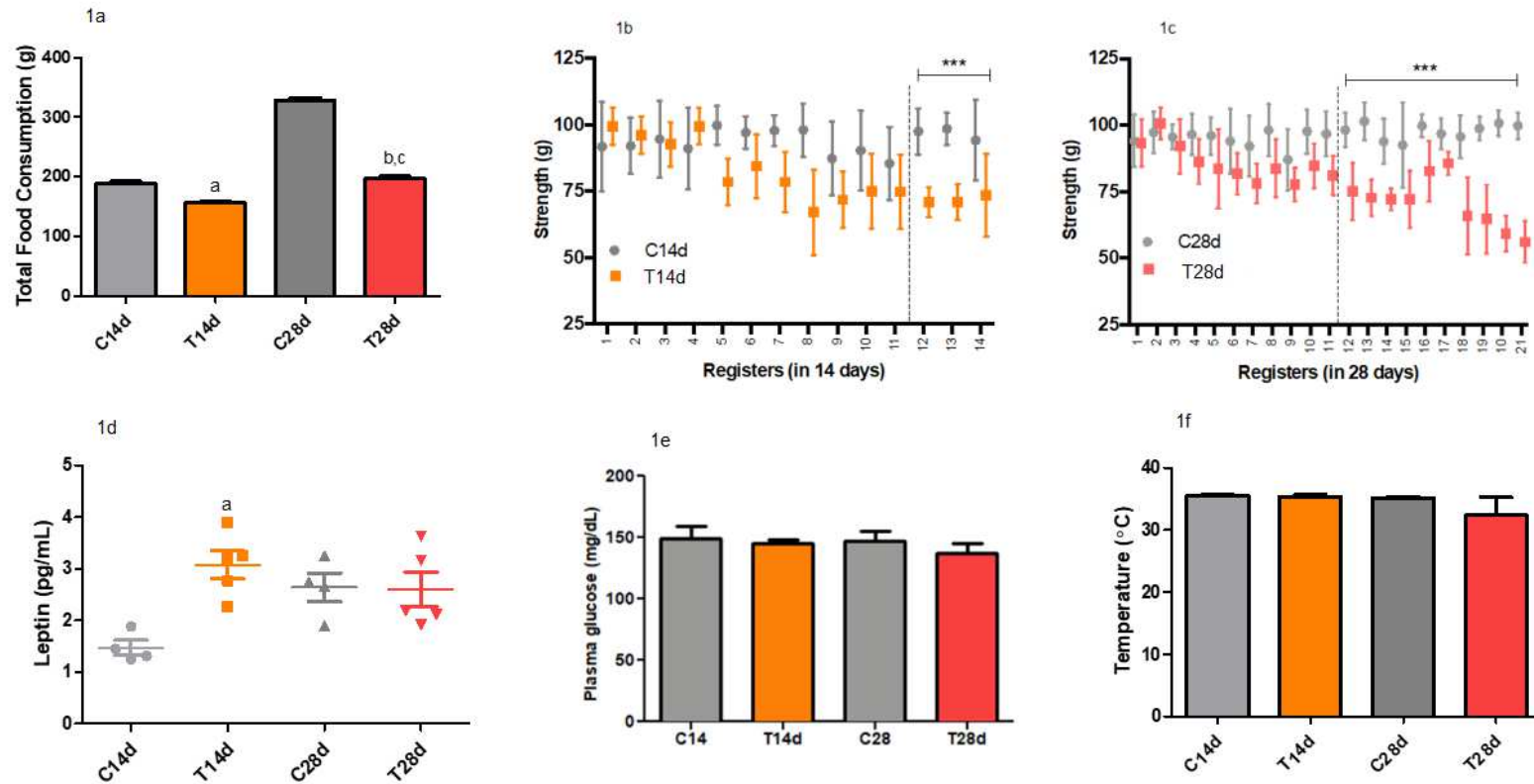
Figures and Tables

Table 1. Quantification of total and oxidized glutathione in cachexia progression in Ehrlich tumor.

Group	GSH ($\mu\text{L}/\text{mg}$ protein)	GSSG ($\mu\text{L}/\text{mg}$ protein)
SOL		
C14d	0.28 ± 0.05	0.08 ± 0.03
T14d	0.29 ± 0.03 <u>c,d</u>	0.11 ± 0.12 d
C28d	0.17 ± 0.04	0.09 ± 0.05
T28d	0.51 ± 0.07 <u>a,b,c</u>	0.35 ± 0.12 c
TA		
C14d	0.09 ± 0.01	0.05 ± 0.03
T14d	0.08 ± 0.01	0.04 ± 0.02 c
C28d	0.05 ± 0.02	0.21 ± 0.09 a
T28d	0.07 ± 0.02	0.08 ± 0.09

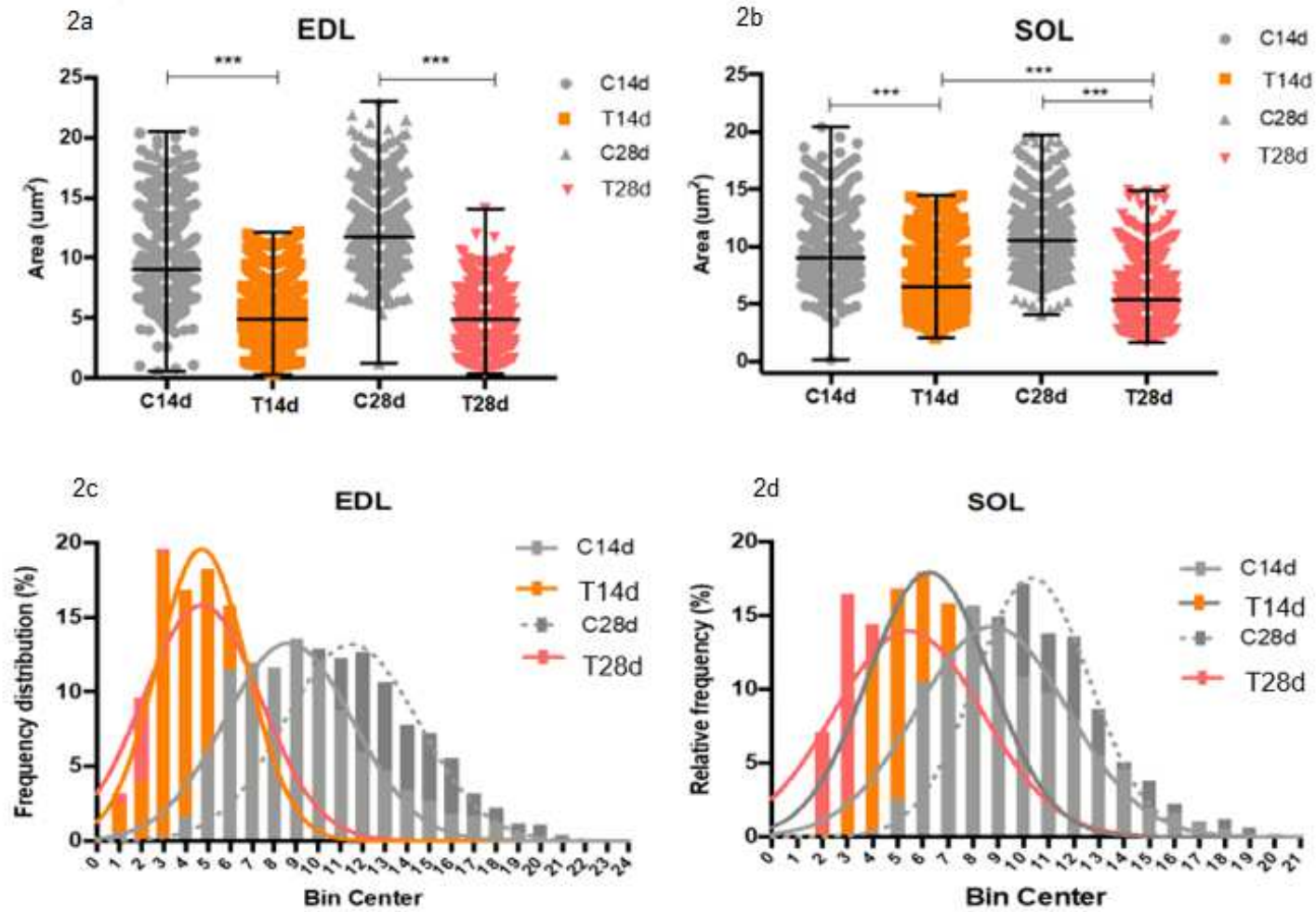
Groups represent GSH (reduced glutathione) and GSSG (oxidized glutathione) quantification in muscle of animals after subcutaneous injection of Ehrlich tumor (n=5/group). C14d – Control 14 days; T14d – Tumor 14 days; C28d – Control 28 days; T28d – Tumor 28 days. GSH – ($\mu\text{L}/\text{mg}$ protein) - $M \pm \text{SE}$; GSSG – oxidized glutathione ($\mu\text{L}/\text{mg}$ protein) - $M \pm \text{SD}$. a - significant difference compared to C14d; b – significant difference compared to T14d; c – significant difference compared to C28d; d – significant difference compared to T28d. $P < 0,05$.

Figure 1. Panel of cancer cachexia parameters.



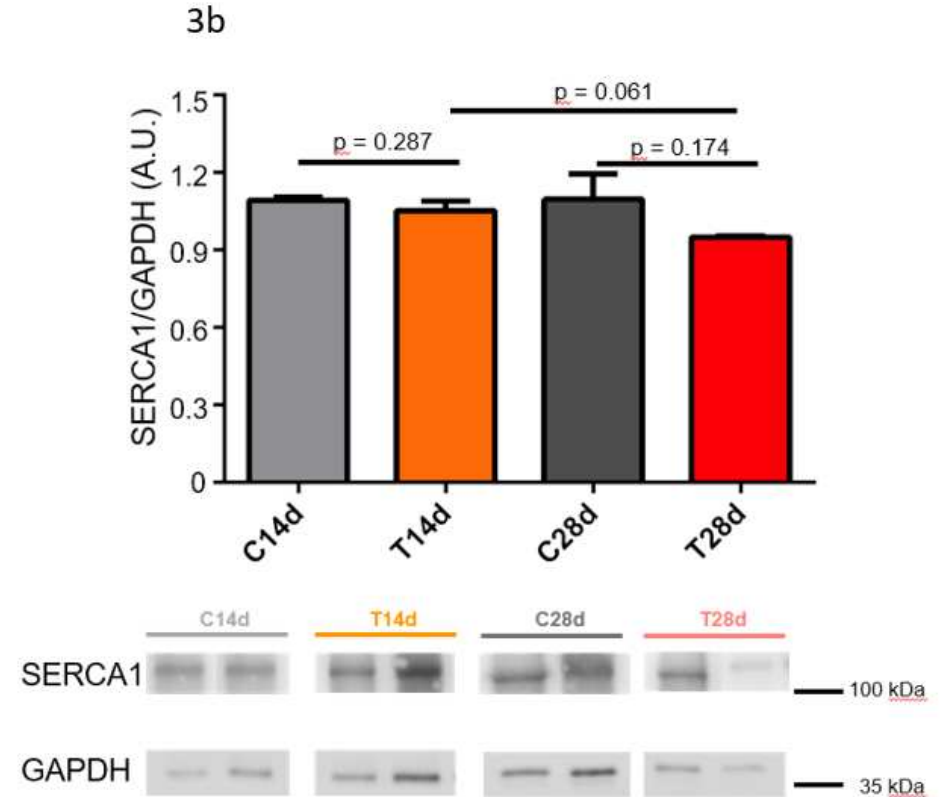
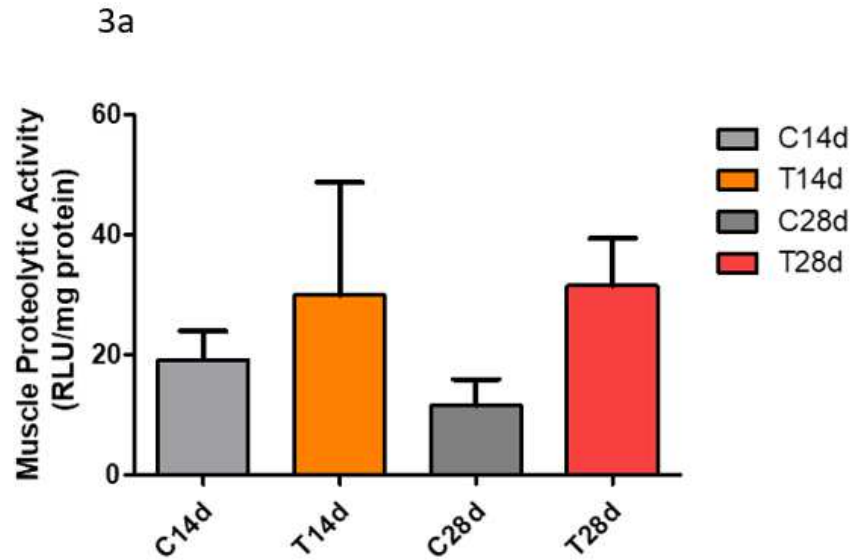
Graphs represent cachexia parameters of animals after subcutaneous injection of 1.0×10^6 Ehrlich tumor cells. Controls received an injection of phosphate-buffered saline (PBS). C14d – Control 14 days; T14d – Tumor 14 days; C28d – Control 28 days; T28d – Tumor 28 days. Total Food Consumption (g) – $M \pm SE$ ($n=20$ animals/group); Strength (measured by hand grip test – g) – $M \pm SD$ ($n=20$ animals/group); Temperature ($^{\circ}C$) – $M \pm SE$ ($n=20$ animals/group); Leptin (pg/mL) – $M \pm SD$ ($n=5$ animals/group); Plasma Glucose (pg/dL) – $M \pm SD$ ($n=5$ animals/group). a – significant difference from C14d; b – significant difference from T14d; c – significant difference compared to C28d; d – significant difference compared to T28d. $P < 0.05$.

Figure 2. Morphometric differences in skeletal muscles of mice inoculated with Ehrlich tumor.



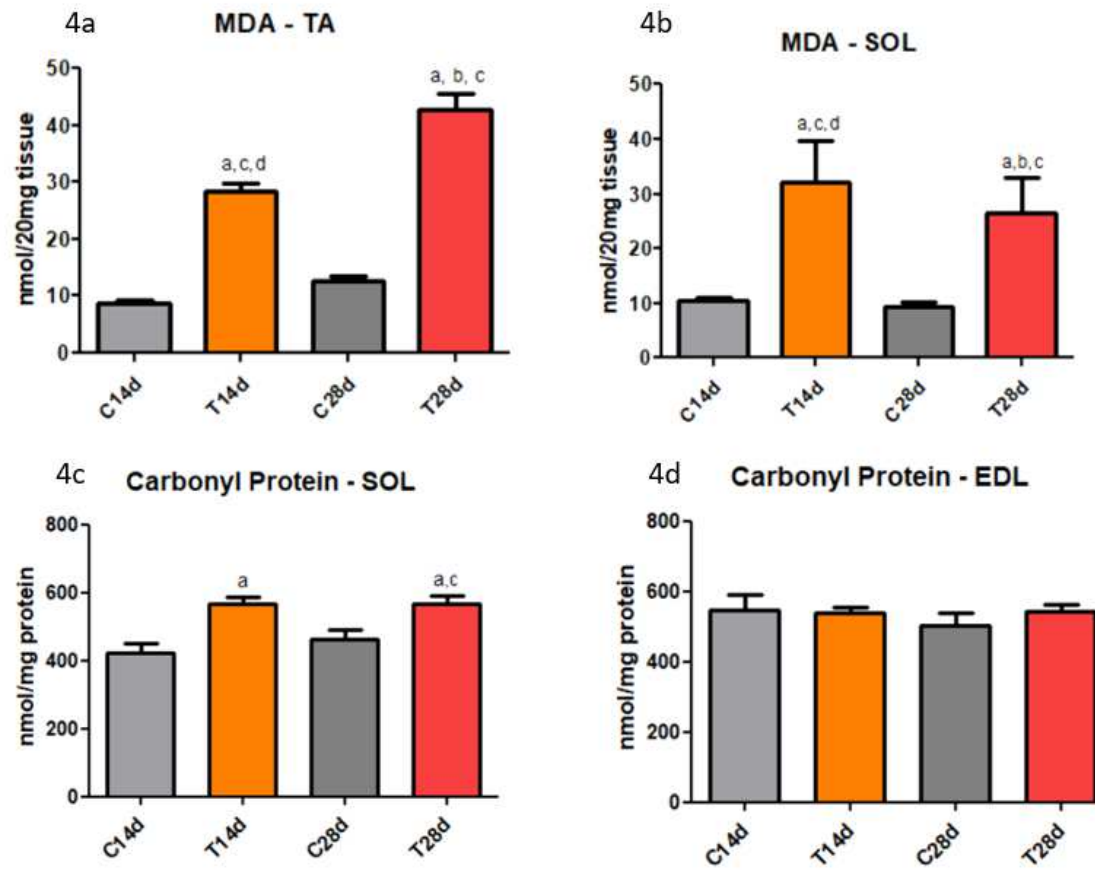
Graphs represent cross sectional area differences among groups control and groups inoculated with Ehrlich tumor cells. C14d – Control 14 days; T14d – Tumor 14 days; C28d – Control 28 days; T28d – Tumor 28 days (n = 3 animals/group). The graphics showed in figures 2a and 2b represent the statistical difference in fiber muscle area observed in inoculated groups with Ehrlich tumor (T14d and T28d) compared to the control groups C14d and C28d. Graphics showed in figures 2c and 2d represent the cross sectional area (CSA – frequency distribution %) difference between the inoculated groups with Ehrlich tumor (T14d and T28d) and the control groups C14d and C28d. *** significant difference compared to each pair of groups (respective inoculated and control groups – C14d and T14d; C28d and T28d). * significant difference between groups. $P < 0.05$

Figure 3. Proteolytic Activity in tumor bearing animals.

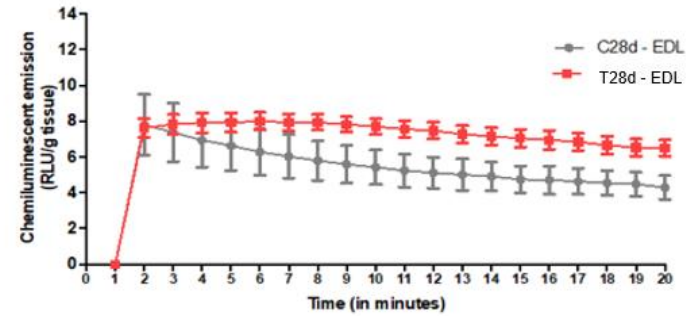
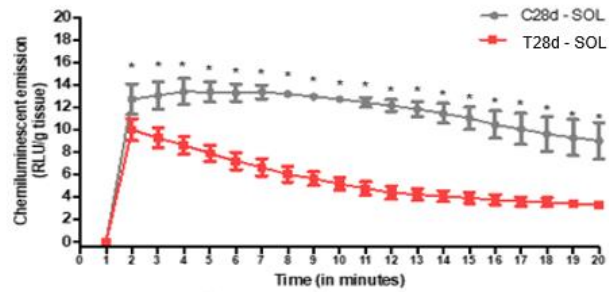
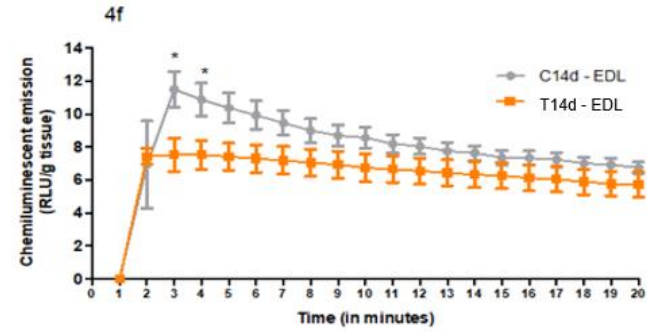
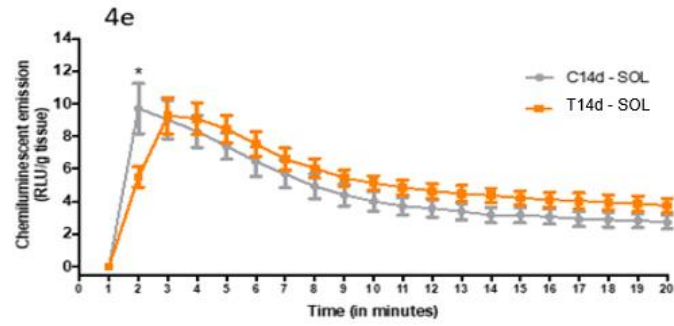


3a - Proteolytic activity performed with Proteasome GloTM Chymotrypsin-like Cell Based Assay kit (Promega, Madison, WI, USA). No statistical difference was observed between control groups and tumor groups. n = 5 animals/group. 3b - SERCA performed by western blotting. n = 3 animals/group. C14d - Control 14 days; T14d - Tumor 14 days; C28d - Control 28 days; T28d - Tumor 28 days. $P > 0.05$.

Figure 4. Panel of oxidative stress parameters and the alterations it caused in skeletal muscle of mice inoculated with Ehrlich tumor.



Graphs represent malondialdehyde (MDA – nmol/20mg tissue) levels of animals from control and groups inoculated with Ehrlich tumor cells. C14d – Control 14 days; T14d – Tumor 14 days; C28d – Control 28 days; T28d – Tumor 28 days. The graphic in figure 4a shows MDA levels in tibial anterior muscle (TA) in animals of control and inoculated animals. 4b - MDA levels in soleus muscle (SOL) in animals of control and inoculated groups. 4c - shows carbonyl protein levels in muscle extensor longus digitorium (EDL) in animals of control and inoculated animals. 4d – carbonyl protein levels in soleus muscle (SOL) in animals of control and inoculated groups. a – significant difference from C14d; c – significant difference from C28d. $P < 0.05$.



Graphs represent hydroperoxide levels by chemiluminescence (CL – RLU/g tissue) of animals from control and groups inoculated with Ehrlich tumor cells. C14d – Control 14 days; T14d – Tumor 14 days; C28d – Control 28 days; T28d – Tumor 28 days (n = 5 animals/group). The graphics in 4e shows hydroperoxide levels in muscle extensor longus digitorum (EDL) in animals of control and inoculated animals. 4f – hydroperoxide levels in soleus muscle (SOL) in animals of control and inoculated groups. * – significant difference between groups. $P < 0.05$.

ANEXO B

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Evaluation of cachexia induced by Ehrlich's carcinosarcoma considering ACASCO parameters: inflammation is critical in all tumor models?

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Short communication

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13
14
15 **Abstract**

16
17 One of the most important complications of cancer is the cachexia syndrome,
18 which can be observed in ~60% of all cancer patients, being directly
19 responsible for 20-30% of cancer deaths, but mainly due to lack of biomarkers
20 or even lack of a well-defined criteria to properly diagnose the syndrome, only
21 2.4% of patients receive the diagnose. Recently, a new way to classify cachexia
22 in experimental models was proposed – ACASCO (Animal Cachexia SCOrE),
23 making it possible to classify the levels of severity of the syndrome by
24 quantifying it in animal models, which bring the possibility to study cachexia in
25 separated stages. Inflammation is considered one of the most common
26 metabolic alterations observed in cancer cachexia and they are supposed to
27 work together in a robust network. But is this a reality to all experimental
28 models? What we see in this paper is that Ehrlich cachexia model develops
29 cachexia syndrome without presenting an intense inflammation status as other
30 animals models may present. Using ACASCO we could see that animals
31 inoculated with Ehrlich tumor for 14 days (T14d) had a lower score compared to
32 animals inoculated for 28 days (T28d), what tells us that cachexia progresses

1 and worsens within time; ELISA for IL-1, IL-6 and TNF- α showed that not all this
2 cytokines together are critical to show an altered metabolism in cancer cachexia
3 and Nrf2 immunohistochemistry showed that antioxidant defense is impaired in
4 tumor bearing animals in T14d and continues to reduce as the syndrome
5 progresses, but differently for predominant glycolytic and oxidative muscle
6 fibers. In this paper, we show the importance of classifying cachexia using
7 ACASCO to understand better how it progresses between the different stages
8 and that this may help identify when and how is the best way no intervene and
9 propose more specific treatments for cachexia, and that different cachexia
10 experimental models will have different alterations and thus, a different
11 cachexia score. Considering that, in some models, inflammation may not be of
12 such great importance, and strength, together with functional variables and loss
13 of muscle mass, may be more sensitive parameters to detect cachexia early
14 and provide more efficient interventions.

15
16 **Key-words:** cancer cachexia, inflammation, antioxidant, cachexia classification

17
18
19
20

21 **Introduction**

22 Noncommunicable diseases (NCD) such as heart disease, cancer, respiratory
23 diseases and diabetes, are increasing over the years, leading to 40.7 million
24 deaths in 2016. Of these, 9 million are attributed to cancer, being the second
25 major cause of deaths worldwide (1). One of the most important complications
26 of cancer is the cachexia syndrome, which can be observed in ~60% of all
27 cancer patients (2), being directly responsible for 20-30% of cancer deaths
28 (3,4), but mainly due to lack of biomarkers or even lack of a well-defined criteria

1 to properly diagnose the syndrome, only 2.4% of patients receive the diagnose
2 (5). According to Fearon *et al* (2011) (6), cachexia can be defined as a
3 “multifactorial syndrome characterized by an ongoing loss of skeletal muscle
4 mass (with or without loss of fat mass) that cannot be fully reversed by
5 conventional nutritional support and leads to progressive functional impairment”.
6 Recently, a new way to classify cachexia in experimental models was proposed
7 – ACASCO (Animal Cachexia SCOrE), making it possible to classify the levels
8 of severity of the syndrome by quantifying it in animal models, which bring the
9 possibility to study cachexia in separated stages (7).

10 Cachexia has a very important impact in cancer because patients may exhibit
11 muscular atrophy, weakness, fatigue and loss of appetite (8), which can affect
12 the chemotherapy treatment, thus worsening the prognosis and increasing
13 mortality (6). The syndrome also compromises the immune system, wound
14 repair, heart, lung, liver and kidney functions, which worsens the prognosis in
15 time (9).

16 In cancer cachexia, several metabolic alterations occur, either by the organism
17 in response to the tumor or by the tumor itself (10). Studies have demonstrated
18 that the tumor microenvironment produces proinflammatory cytokines capable
19 to modify the metabolism of an individual, including the energy expenditure,
20 adipose tissue and skeletal muscle metabolism (11-13). One of the first
21 probable metabolic alterations in cachexia syndrome is chronic inflammation,
22 through release of the cytokines IL-1 β , IL-6 and TNF- α , that have been
23 proposed as responsible for promoting proteolysis, anorexia, fatigue and
24 muscle and fat wasting (14-17). IL-1 β itself promotes anorexia by submitting the
25 hypothalamus to an inflammatory medium (18), TNF- α promotes tissue

1 proteolysis mainly by activation of the NFkB pathway (19) and IL-6, still has a
2 contradictory role in cancer cachexia, yet, it is related to lipolysis and muscle
3 protein loss (20,21). As this cytokines have been found circulating
4 simultaneously in high levels, these pro-inflammatory cytokines may work with
5 each other in a robust network resulting in cachexia (22). These cytokines act
6 as stimuli to activate NFkB through phosphorylation followed by ubiquitination of
7 Ikb α portion, which frees NFkB to translocate to the nucleus and bind to its
8 specific target genes (23). Once activated, NFkB alters myogenic expression,
9 which causes loss of muscle mass (24) mainly because it induces production of
10 oxidative stress that leads to muscle proteolysis by UPS (ubiquitin proteasome
11 system) (25) and also, these same ROS (reactive oxygen species) being
12 produced may activate p38-MAPK pathway, leading to reduced protein
13 synthesis (26).

14 But how are these cytokines released in cancer cachexia? TLR4 has shown to
15 recognize other endogenous ligands such as heat-shock proteins (HSP) (27),
16 and the activation of TLR4 (Toll-Like Receptor 4) receptor system by LPS
17 (lipopolysaccharide) produces the same proinflammatory cytokines that mediate
18 inflammation in cancer cachexia (28). Knowing this and that skeletal muscle
19 express TLR4, Cannon *et al* (29) hypothesized that the ability to activate the
20 TLR pathway in muscle would lead to production of inflammatory mediators that
21 could contribute to cancer cachexia. Using TLR4^{d/d} mice, the researchers
22 showed that intact TLR4 is required for muscle wasting in a model of cancer
23 cachexia. In normal operating muscle cells, HSP70 (*Heat shock protein 70*)
24 protects against damage, but under pathological conditions it can be released in

1 the extracellular space and act as a DAMP (Damage-Associated Molecular
2 Patterns).

3 As a counterpoint for the deleterious effects of ROS, there is Nrf2, an important
4 pathway in mediating the intracellular antioxidant response, being able to
5 positively regulate the expression of phase II detoxification enzymes in
6 response to increased oxidative stress (30). The absence or the deficiency of
7 Nrf2 in elderly mice that suffer from sarcopenia and/or muscle atrophy has been
8 related to worsen their condition, and it seems to be related to mitochondrial
9 dysfunction (31-33). Yet, how this pathway may counterpoint atrophy caused by
10 ROS in experimental models of cancer cachexia needs to be further elucidated.
11 In this paper, we analyze the possible relation of NFkB and Nrf2 in tumor
12 bearing animals that developed cachexia, taking in consideration the
13 inflammatory response caused by pro-inflammatory cytokines and the activation
14 of Hsp70 in a new experimental model of cancer cachexia induced by Ehrlich
15 tumor.

16

17 **Methods**

18 **Animals**

19 Male *Swiss* mice with 7 week old were obtained from the animal house of the
20 Biological Sciences Center at Universidade Estadual de Londrina (UEL). We
21 used 120 animals, divided in 30 animals/group. The animals were divided in 4
22 groups: (i-ii) control 14 days and control 28 days (C14d; C28d), (iii) tumor 14
23 days – inoculation with Ehrlich tumor cells and maintained for 14 days (T14d),
24 (iv) tumor 28 days – inoculation with Ehrlich tumor cells and maintained for 28
25 days (T28d). Animals were fed with commercial food (Nuvital®) and water *ad*

1 *libitum*. Mice were treated according to national resolutions, and the experiment
2 was approved by the Ethics Committee on Animal Use (CEUA-UEL).

3

4 **Tumor Inoculation**

5 According to the experimental protocols described above, animals from T14d
6 and T28d groups received 1.0×10^6 cells in a final volume of 250 μ L phosphate
7 buffer saline (PBS), and animals of control groups received the correspondent
8 volume of PBS. The inoculation was performed through a single subcutaneous
9 injection in the back region of each animal. Tumor Ehrlich cells are maintained
10 *in vivo* in Swiss mice, as an ascitic intraperitoneal tumor through passages
11 every 10 days. The ascitic fluid of the host animals was collected, washed in
12 PBS, centrifuged at 200 x g for 10 minutes. The viable cells were determined by
13 trypan dye exclusion, using a Neubauer's chamber (34). On the 14th and 28th
14 days after tumor inoculation, animals were sedated intramuscularly with ketamin
15 and xilazin (100mg/Kg and 160mg/Kg, respectively) and euthanized.

16

17 **Cachexia Parameters**

18 **Body Weight**

19 Animals were weighed before the beginning of the experiment and on the last
20 day. To analyze the behavior of the lean mass, the muscles anterior tibial (TA),
21 extensor digitorum longus (EDL), gastrocnemius (GAST) and soleus (SOL)
22 were weighted. The fat collected from the retroperitoneum and the perigonadal
23 regions were also weighed.

24

25 **Tumor Size and Weight**

1 Tumor size was measured with a digital caliper each two days. After
2 euthanasia, tumors were collected and weighed.

3

4 **Hand Grip Test**

5 The strength of the forelimb were evaluated through Hand Grip Test (HGT), with
6 a digital dynamometer (EEF 305 Grip StrengthMeter, Insight), that registers the
7 peak of strength the animals grips to the equipment. Only after one week of
8 adaptation, animals were exposed to the test every day, at the same time, light
9 conditions, bar diameter and the same person (35). A mean was calculated with
10 the peak of each day of test.

11

12 **Food Intake**

13 As reduction in food intake is a symptom of cachexia (8), feeding was monitored
14 daily, by weighing the food of each experimental group.

15

16 **ACASCO**

17 ACASCO is the Animal CAchexia SCORe proposed by Betancourt (7) that takes
18 in consideration the following components of scoring: (i) body and muscle
19 weight loss; (ii) inflammation and metabolic disturbances; (iii) anorexia; (iv)
20 quality of life measured using discomfort symptoms and behavioral tests (not
21 used in the paper). ACASCO shows that cachexia can be measured
22 quantitatively, and that different levels of cachexia may present different scores,
23 making it possible to properly classify cachexia in levels of severity.

24

25 **Blood samples**

1 At the last day of experiment, animals were sedated, and the blood was
2 collected by cardiac puncture. To obtain the plasma, blood was centrifuged
3 1500 x rpm for 5 minutes at 4°C, the plasma was collected and stocked at -
4 20°C, until the moment of use.

5

6 **Cytokine Quantification**

7 Interleukins IL-1 β , IL-6 were determined by ELISA (*enzyme-linked*
8 *immunosorbent assay*), according to the manufacturer's instructions
9 (eBioscience®, USA) and TNF- α was also determined by using a kit
10 (BioLegend®, USA). The quantification was performed using a microplate
11 reader (Thermo Scientific) at 450 nm. Results were expressed in pg
12 cytokine/mL plasma.

13

14 **Immunohistochemistry**

15 Slides were deparaffinized and blocked for endogenous peroxidase with 1%
16 hydrogen peroxide for 20 min, followed by immersion in FBS 1% for 30 min.
17 Specific antibodies were used for detection of Nrf2 (Thermo Fischer Scientific).
18 The capture antibody were applied overnight in humid medium under
19 refrigeration. Then, detection antibody was applied for 1 h. Diaminobenzidine
20 (DAB) was used as chromogen for 5 min at room temperature (Sima Aldrich).
21 Slides were counterstained with Harris hematoxylin, and cover slipped. Slides
22 were visualized in a photomicroscope (Olympus BX43), and the
23 photomicrographs were analyzed at MatLab in a routine constructed to detect
24 color intensity.

25 **Statistical Analysis**

1 After Shapiro-Wilk normality test, results are shown as mean \pm SD. When data
2 were considered non parametric, results were shown as mean \pm standard error
3 of the mean (SEM). For parametric test we used one-way analysis of variance
4 (ANOVA) followed by Tukey's post hoc test. For non-parametric test we used
5 Mann-Whitney followed by Dunn's post hoc test. Statistical significance was
6 considered when $P < 0.05$.

7

8 **Results**

9 **Body Weight**

10 Control groups 14d and 28d had similar body weight gain ($28.67 \pm 2.50\text{g}$; 24.97
11 $\pm 2.76\text{g}$, respectively). T14d group showed a significant reduced body weight
12 ($12.62 \pm 1.54\text{g}$) compared to C14d, and T28d differed significantly ($21.85 \pm$
13 2.44g) compared to C28d. The fat collected did not reduce significantly in T14d
14 ($0.89 \pm 0.33\text{g}$) and T28d ($0.90 \pm 0.34\text{g}$), compared to C14d ($1.25 \pm 0.50\text{g}$) and
15 C28d ($1.33 \pm 0.73\text{g}$), respectively. Muscle loss was significantly in T14d ($0.26 \pm$
16 0.03g) compared to C14d ($0.31 \pm 0.07\text{g}$), but T28d ($0.30 \pm 0.06\text{g}$) was not
17 significantly compared C28d ($0.31 \pm 0.03\text{g}$). Tumor weight differed significantly
18 between inoculated groups, being smaller in T14d ($0.49 \pm 0.29 \text{ g}$) compared to
19 T28d ($1.25 \pm 0.54 \text{ g}$) ($P < 0.005$) ($n = 20$ animals/group).

20

21 **Hand Grip Test**

22 Hand grip strength test demonstrated the inoculated animals had a progressive
23 strength loss along the experimental protocols, being significantly reduced in
24 T14d ($80.93 \pm 3.05 \text{ g}$) compared to C14d ($93.99 \pm 1.19 \text{ g}$) ($P < 0.0025$) (Figure

1 1b), and in T28d (78.56 ± 2.43 g) compared to C28d group (96.33 ± 0.73 g)
2 (Figure 1c). $P < 0.0001$.

3

4 **Food Intake**

5 Animals inoculated with Ehrlich tumor presented a significantly reduced food
6 consumption in T14d and T28d groups (156.10 ± 3.46 ; 197.5 ± 3.41 g,
7 respectively) compared to C14d (189.10 ± 3.41 g) and C28d group ($328.40 \pm$
8 4.69 g) (Figure 1a) $P < 0.0001$.

9

10 **ACASCO**

11 Table 1 shows that loss of body weight is the major responsible for ACASCO,
12 scoring 51% of the total score, followed by anorexia with 32%. It is also possible
13 to observe that T14d had a smaller score compared to T28d (with 100 more
14 points in just 14 days), what shows that cachexia severity worsens within time.

15

16 **Cytokine Quantification**

17 Although cytokines IL-1 β , IL-6 and TNF- α did not differ significantly between
18 PreCdq (3.30 ± 0.73 ; 0.76 ± 0.35 ; 25.84 ± 8.43 pg/mL) and Cdq groups ($2.59 \pm$
19 0.54 ; 0.56 ± 0.26 ; 35.00 ± 13.36 pg/mL) compared to C14d (1.17 ± 0.23 ; $2.36 \pm$
20 0.29 ; 19.97 ± 3.53 pg/mL), and C28d (1.55 ± 0.51 pg/mL; 1.76 ± 0.94 pg/mL),
21 they follow a pattern observed in cancer cachexia (Figure 2).

22

23 **Nrf2**

24 Immunohistochemistry for Nrf2 showed that SOL of tumor bearing animals had
25 a significantly reduced percentual of staining in T14d (31.82 ± 3.29 % of marked

1 area) and T28d (44.08 ± 8.23 % of marked area) compared to their respective
2 controls C14d (53.22 ± 3.29 % of marked area) and C28d (68.57 ± 1.66 % of
3 marked area), and also for the mean intensity, being T14d (18.14 ± 0.59 mean
4 of color intensity) and T28d (12.51 ± 0.64 mean of color intensity) with lower
5 color intensity compared to their respective control groups C14d (22.93 ± 1.08
6 mean of color intensity) and C28d (36.31 ± 0.64 mean of color intensity), and
7 T28d has a significant reduction on the mean color intensity compared to T14d
8 (Figure 3).

9 For EDL muscle, tumor bearing animals in T14d (67.66 ± 1.95 % of marked
10 area) had a significantly reduced percentual of staining compared to C14d
11 (77.36 ± 1.81 % of marked area), and T28d (48.57 ± 6.53 % of marked area)
12 did not differ from C28d (54.78 ± 7.57 % of marked area), but was significantly
13 reduced compared to T14d. As for the mean of color intensity, this patten was
14 repeated, being the T14d (30.73 ± 3.07 mean of color intensity) lower compared
15 to C14d (39.79 ± 2.29 mean of color intensity), and T28d (17.58 ± 5.13 mean of
16 color intensity) not different from C28d (21.00 ± 3.81 mean of color intensity),
17 but lower compared to T14d (Figure 3).

18

19

20 **Discussion**

21 Ehrlich tumor is a new experimental model to study cancer cachexia, first
22 proposed by Frajacomo *et al* (34). This model is potentially useful in the study of
23 cancer cachexia syndrome, because it develops the main cachexia-related
24 parameters (reduced food intake, loss of strength and muscle loss) (Figure 1

1 and Table 1), but also because its maintenance is easy and cost effective, and
2 the tumor develops fast, starting within seven days after inoculation.

3 Cachexia is one of the most important complications observed in cancer
4 patients, being responsible for several impacts on the individual's condition, as
5 muscle atrophy, weakness, fatigue, loss of appetite (8) and resistance in
6 chemotherapy treatment (6), which worsens the prognosis and ends up to be
7 directly responsible for 20-30% of all cancer deaths (3,4). Although the
8 syndrome causes important deleterious effects, only 2.4% of cancer patients
9 receiving the diagnosis (5), which makes the syndrome highly neglected, once it
10 can be observed in ~60% of all cancer patients (2).

11 In the last decades, several experimental models have been employed on the
12 studies of cachexia (36-38). However, literature has also demonstrated that not
13 all tumors grow in the same way or induce cachexia in equal intensities or
14 speed (39). In addition, in patients, a quite recent consensus established
15 degrees of cachexia severity (2) that are not used or rarely considered when
16 animal models are assayed. In 2019, Betancourt *et al* (7) published a study
17 proposing a score to classify cachexia in experimental tumor models. ACASCO
18 is the Animal CAchexia SCORe and is a scoring method that allows the
19 quantification and thereby, the classification of cachexia severity in degrees. It
20 takes in consideration: (i) body and muscle weight loss; (ii) inflammation and
21 metabolic disturbances; (iii) physical performance; (iv) anorexia; (v) quality of
22 life measured using discomfort symptoms and behavioral tests. This
23 classification is important to understand the differences that may occur in the
24 existing experimental models and also to study cachexia in its different stages
25 to propose more specific treatments. This scoring method for animals was

1 developed based in CASCO (CAchexia SCORe – used for humans) (40), which
2 takes in consideration: (i) body weight loss and composition; (ii) inflammation
3 and metabolic disturbances; (iii) immunosuppression; (iv) physical performance;
4 (v) anorexia and (vi) quality of life.

5 Analyzing our score results, it is possible to observe that body weight loss and
6 reduction in food consumption are really important measurements to determine
7 and quantify cachexia, which is in accordance to ACASCO (7), also T28d
8 animals had quantitatively more cachexia than T14d, what shows that cachexia
9 is a syndrome that progresses and worsens within time.

10 Loss of strength, measured by hand grip test (HGT) added to muscle loss is an
11 important indicator of impaired muscle function, known as sarcopenia, that
12 brings several complications such as cardiovascular diseases, susceptibility to
13 infectious diseases and thus a higher rate of mortality (41). In a previous study
14 of our group (submitted to publication) we observed that oxidative stress has a
15 really important role in causing sarcopenia in Ehrlich inoculated animals. These
16 animals developed muscle loss and atrophy in early stages of cachexia (14
17 days after tumor) together with early presence of oxidative stress markers, as
18 MDA (malondialdehyde), hydroperoxides and carbonylated proteins. Oxidative
19 stress is able to induce an elevated expression of the main components of UPS
20 (ubiquitin-proteasome system) in skeletal muscle, which contributes to protein
21 degradation. A known mechanism triggered by oxidative stress is NFκB that
22 regulates positively the UPS (42). Like the pro-inflammatory cytokines, oxidative
23 species are capable to activate not only NFκB pathway, but also, activation of
24 p38-MAPK (mitogen activated kinases), reducing the protein synthesis (26).

1 Inflammation is one of the most important metabolic alterations observed in
2 cancer cachexia and they work all together, forming a network in which one can
3 activate another (44). TNF- α , initially called “cachectin”, is responsible for
4 increasing gluconeogenesis, lipolysis and proteolysis and can activate catabolic
5 pathways through NF κ B and activate IL-1 (19, 43). IL-1, in turn, induces
6 anorexia in cachectic patients by causing early satiety and suppression of
7 hunger, and activates IL-6 (16, 43). IL-6, despite having a contradictory role,
8 have shown an important role in the conversion of white fat to brown fat, which
9 costs a high energy expenditure for the individual and is also related to the loss
10 of skeletal muscle protein (18, 44). Our TNF- α results are in accordance to this,
11 but unlike what we have seen in this paper, IL-1 and IL-6 does not seem to have
12 such an important role in Ehrlich model, and despite that, animals inoculated
13 with Ehrlich tumor did develop cachexia, as one can see through anorexia, loss
14 of body weight and strength and loss of muscle reflected by atrophy (paper
15 submitted to publication). Thus, in the Ehrlich cachexia model, we suggest that
16 it is not necessary to have all the known cytokines involved in cachexia working
17 together, as only TNF- α activation was sufficient to contribute to the syndrome
18 development. Considering this, the question of how much the activation of IL-6,
19 IL-1 and TNF all together is necessary/critical to determine whether or not there
20 is cachexia.

21 It is known that reactive oxygen species (ROS) can act through signaling
22 mechanisms via the redox system - such as the NF κ B pathway, altering
23 myogenic expression, which causes loss of muscle mass (24). The pathway
24 that can counterbalance the effect caused by ROS is through the stimulation of
25 Nrf2 (nuclear erythroid-2-p45-related factor-2) (45), which is able to positively

1 regulate the expression of antibodies and phase II detoxification enzymes in
2 response to increased oxidative stress (30), such as catalase and glutathione
3 peroxidase (33). Our results show that inoculated animals had lower
4 quantification of Nrf2 compared to their control groups, for both muscle fibers
5 oxidative and glycolytic in just 14 days after inoculation and continues to reduce
6 until 28 days, but, as one can observe, SOL (predominant oxidative fiber) has
7 even less Nrf2 than EDL (predominant glycolytic fiber) in T28d. This may
8 explain our previous results (paper submitted to publication) in which glycolytic
9 fibers did not present other oxidative stress damage than malondialdehyde
10 (MDA) compared to SOL muscle, because it might have more antioxidante
11 defense than SOL muscle had, and that the observed SOL protection to the
12 oxidative damages in T28d might as well come from another mechanism.

13 In this study we show that ACASCO is an important tool to classify cachexia
14 severity in animal models, not just to help understanding cachexia syndrome in
15 different stages, but because each experimental model may behave differently
16 in some aspects, as inflammation. Also, the better understanding of cachexia
17 progression may help identify when and how is the best way no intervene and
18 propose more specific treatments for cachexia. We suggest that inflammation,
19 especially as a network of pro-inflammatory cytokines dos not seem to be as
20 critical for all cachexia experimental model, and that they can develop the
21 syndrome besides that, and that TNF- α by itself seem to be sufficient to activate
22 pathways as NFkB and activate skeletal muscle proteolysis.

23

24

25

1 **Conclusion**

2 In this paper, we show the importance of classifying cachexia using ACASCO to
3 understand better how it progresses between the different stages and that this
4 may help identifying when and how is the best way no intervene and propose
5 more specific treatments for cachexia, and that different cachexia experimental
6 models will have different alterations and thus, a different cachexia score.
7 Considering that, in some models, inflammation may not be of such great
8 importance, and strength, together with functional variables and loss of muscle
9 mass, may be more sensitive parameters to detect cachexia early and provide
10 more efficient interventions.

11

12

13 **Conflict of Interest**

14 The authors declare that there are no conflicts of interest.

15

16 **Acknowledgments**

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18

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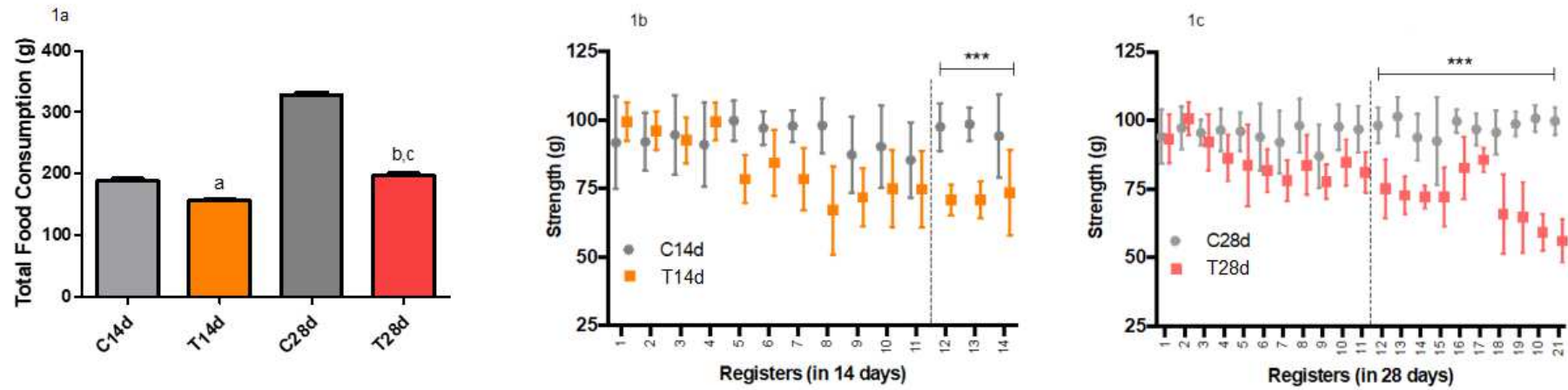
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Figure 1. Panel of cancer cachexia parameters.



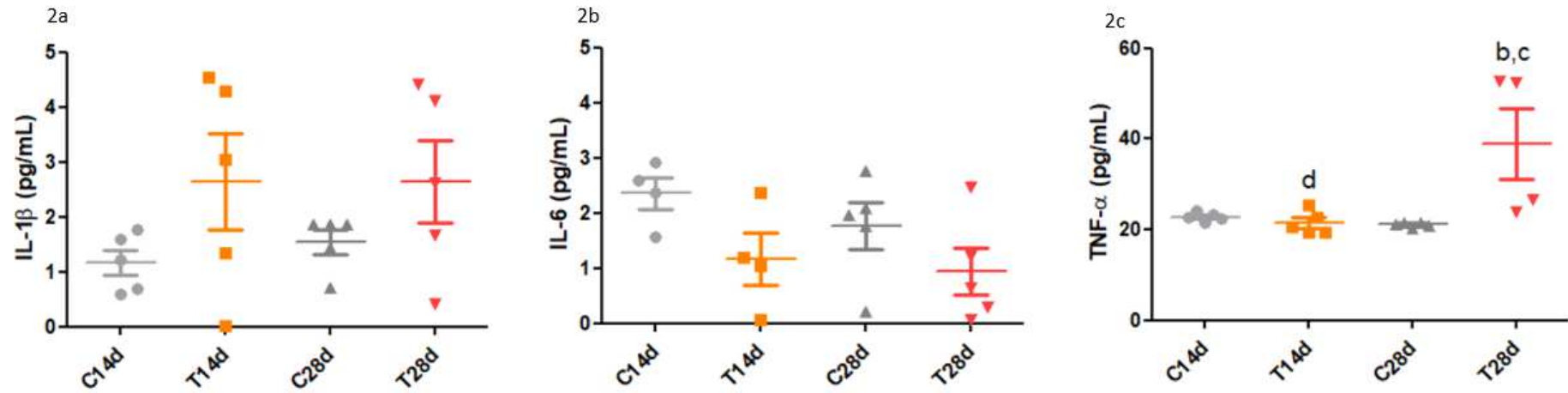
Graphs represent cachexia parameters of animals after subcutaneous injection of 1.0×10^6 Ehrlich tumor cells. Controls received an injection of phosphate-buffered saline (PBS). C14d – Control 14 days; T14d – Tumor 14 days; C28d – Control 28 days; T28d – Tumor 28 days. 1a - Total Food Consumption (g) – $M \pm SE$ ($n= 20$ animals/group); 1b/1c - Strength (measured by hand grip test – g) – $M \pm SD$ ($n= 20$ animals/group). a – significant difference from C14d; b – significant difference from T14d; c – significant difference compared to C28d; d – significant difference compared to T28d. $P<0.05$.

Table 1. ACASCO in animals inoculated with solid Ehrlich tumor in 14 days and 28 days.

Symptom	%	Measurement	Criteria	Score	N° animals 14d	Total Score 14d	N° animals 28d	Total Score 28d
Body Weight Loss	51	Body weight compared to control group	<2%	0	0	426,5	0	528
			≥2%	8	4		5	
			≥12%	16	3		0	
			≥15%	24	5		5	
			≥25%	32	2		2	
Muscle weight loss	3	Weight compared to control group	<10%	0	4		5	
			>10%	1	10	4		
			>20%	1.25	4	4		
Inflammation	4	TNF-a	Difference vs Control	8	0		5	
Metabolic Disturbances	2	Leptin	Difference vs Control	3.5	5		0	
Pshysical Activity	6	HGT	0 - 4.99	0	2		0	
			5 - 20.00	1	2	0		
			21 - 30.00	2	3	0		
			≥31	4	6	10		
Anorexia	32	Total Food Consumption	0 - 4.99%	0	5		0	
			5 - 19.99%	5	5	0		
			20 - 29.99	7	5	0		
			≥30%	10	5	20		

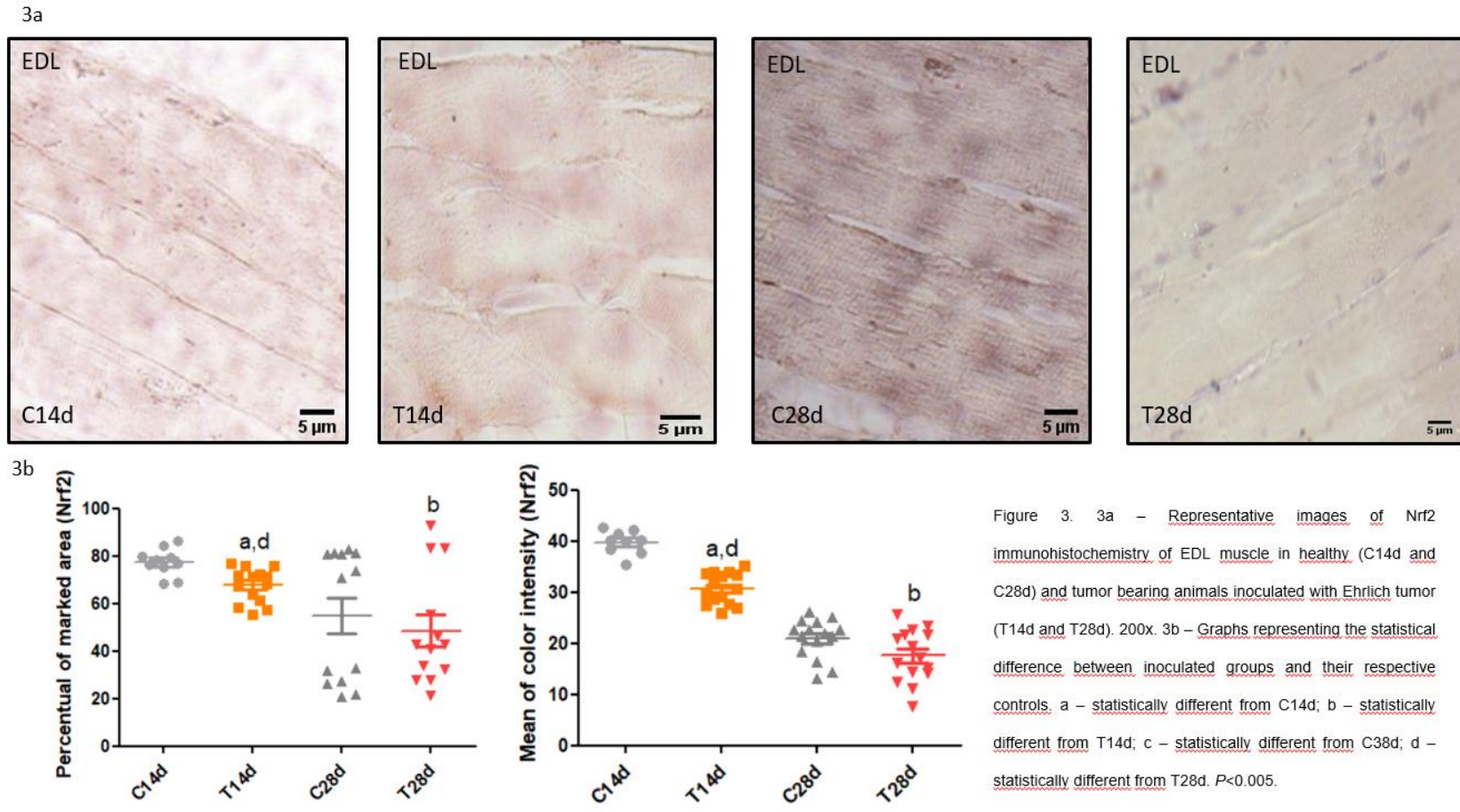
Table 1. Body Weight Loss = Final Weight – Tumor Weight (represented as percentage of control group); Muscle Weight Loss: GAST loss = (Weight GAST tumor / Weight GAST control) x 100 (used gastrocnemius muscle – GAST); Inflammation: statistical difference compared to control; Leptin: statistical difference compared to control; Physical Activity: HGT = (HGT tumor / Mean HGT control) x 100; Anorexia: Total Food Intake = (Food Intake tumor / Total Food Intake control) x 100. N° animals: number of animals consistent with each criteria.

Figure 2. Panel of plasma cytokines involved in cachexia induced by Ehrlich tumor inoculation.

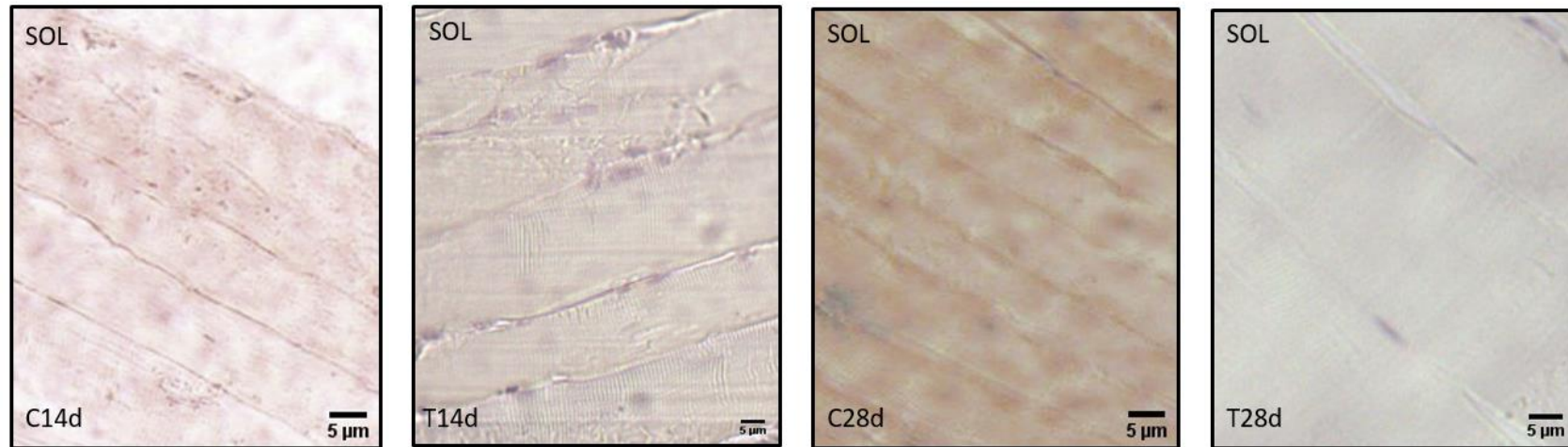


C14d – Control 14 days; T14d – Tumor 14 days group; C28d – Control 28 days; T28d – Tumor 28 days group. 2a – Interleukin 1- β (IL-1 β); 2b – Interleukin 6 (IL-6); 2c – Tumor necrosis factor alpha (TNF- α). Quantifications were made by ELISA. b – statistically different from T14d; c – statistically different from C28d; d – statistically different from T28d. $P > 0.05$.

Figure 3. Immunohistochemistry staining for Nrf2 as a marker of antioxidant defense system.



3c



3d

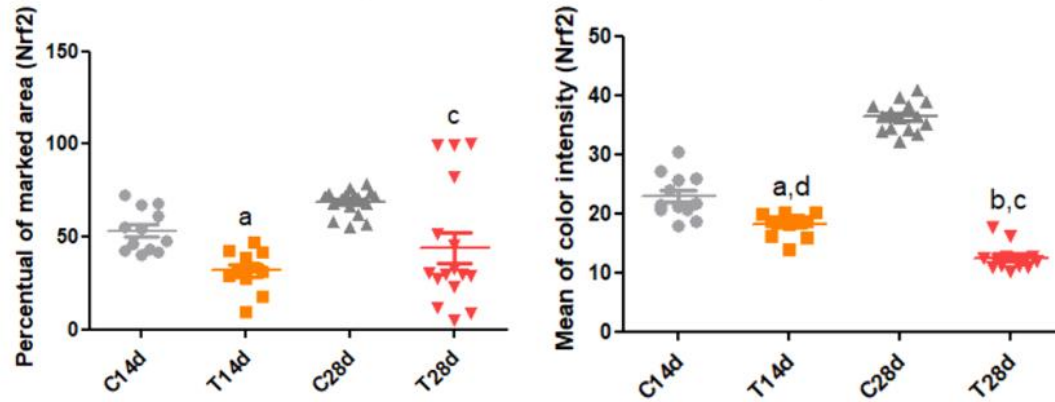


Figure 3. 3c - Representative images of Nrf2 immunohistochemistry of SOL muscle in healthy (C14d and C28d) and tumor bearing animals inoculated with Ehrlich tumor (T14d and T28d), 200x. 3b - Graphs representing the statistical difference between inoculated groups and their respective controls. a - statistically different from C14d; b - statistically different from T14d; c - statistically different from C38d; d - statistically different from T28d. $P < 0.005$.

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2
3

Anexo C



UNIVERSIDADE
ESTADUAL DE LONDRINA

COMISSÃO DE ÉTICA NO USO DE ANIMAIS

OF. CIRC. CEUA Nº 107/2017

Londrina, 25 de Agosto de 2017.

Prezado (a) professor (a)

Certificamos que o adendo ao projeto intitulado: "Avaliação da musculatura esquelética nos estágios de pré-caquexia e caquexia em modelo de tumor de Ehrlich." protocolo CEUA nº12549.2017.35 sob a responsabilidade de Flávia Alessandra Guarnier, que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem) para fins de pesquisa científica (ou ensino), encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), foi **aprovado** pela Comissão de Ética no Uso de Animais da Universidade Estadual de Londrina (CEUA/UEL), em reunião realizada em **22/08/2017**.

O objetivo é estabelecer o tumor de Ehrlich como um modelo confiável para se estudar a caquexia, e diferenciar o efeito da caquexia na musculatura esquelética nas fases de pré-caquexia e caquexia, assim como o envolvimento do estresse oxidativo no desenvolvimento da síndrome. Grau de invasividade=3

Vigência do Projeto	22/08/2017 a 22/08/2020
Espécie/linhagem	Camundongo heterogênico / Swiss
Nº de animais	80
Peso/Idade	20-25g /
Sexo	Machos.
Origem	Biotério Central - Universidade Estadual de Londrina.
Amostras a serem coletadas	Sangue; músculos tibial anterior, extensor longo dos dedos, sóleo e gastrocnêmio; tumor, gordura retroperitoneal e perigonadal

Cumpra orientar que caso pretendam-se quaisquer alterações no protocolo experimental aprovado, deve-se submeter o novo protocolo à apreciação da CEUA/UEL anteriormente à execução das modificações.

Coloco-me à disposição para quaisquer esclarecimentos que se fizerem necessária. Sem mais para o momento, subscrevo, cordialmente.

Profa. Dra. Glaucia Scantamburlo Alves Fernandes
Coordenadora da CEUA/UEL

Ilmo.(a) Sr.(a)

Prof. (a) Dr (a). Flávia Alessandra Guarnier

Responsável pelo projeto
Departamento de Ciências Patológicas

C/C para o Biotério Central do CCB
C/C para a Chefia do Depto. de Ciências Patológicas
C/C para a Direção de Centro do CCB

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