



**Identificação de Moléculas de Comunicação e Interação Celular no
Músculo Esquelético em Resposta ao Exercício, Condições Catabólicas
e Envelhecimento Utilizando Dados Transcriptômicos**

Jakeline Santos Oliveira

Botucatu – São Paulo

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“Júlio de Mesquita Filho”
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**Identificação de Moléculas de Sinalização Celular em Resposta ao
Exercício, Condições Catabólicas e Envelhecimento do Músculo
Esquelético Utilizando Dados Transcriptômicos**

Discente: Me. Jakeline Santos Oliveira

Orientador: Prof. Dr. Robson Francisco Carvalho

Co-Orientador: Prof. Dr. Geysson Javier Garcia Fernandez

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AUTORA: JAKELINE SANTOS OLIVEIRA

ORIENTADOR: ROBSON FRANCISCO CARVALHO

COORIENTADOR: GEYSSON JAVIER FERNANDEZ GARCIA

Aprovada como parte das exigências para obtenção do Título de Doutora em Ciências Biológicas (Genética), pela Comissão Examinadora:

Prof. Dr. ROBSON FRANCISCO CARVALHO (Participação Presencial)
Departamento de Biologia Estrutural e Funcional / Instituto de Biociências de Botucatu UNESP

Prof. Dr. TIAGO FERNANDES (Participação Virtual)
Depto. de Biodinâmica do Movimento do Corpo Humano / Escola de Educação Física e Esporte - Universidade de São Paulo - USP - São Paulo

Profa. Dra. CAMILA RENATA CORREA CAMACHO (Participação Presencial)
Unidade de Pesquisa Experimental (UNIPEX) / Faculdade de Medicina de Botucatu - Unesp

Profa. Dra. FRANCIS LOPES PACAGNELLI (Participação Virtual)
Pesquisa e Extensão- Curso de Fisioterapia / Universidade do Oeste Paulista - UNOESTE

Profa. Dra. PAULA SCHMIDT AZEVEDO GAIOLLA (Participação Presencial)
Departamento de Clínica Médica / Faculdade de Medicina de Botucatu - Unesp

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FLÁVIA DANIELI MARTINS GODINHO
Técnico Administrativo Substituto da Seção Técnica de Pós-Graduação
Instituto de Biociências de Botucatu – UNESP

Instituto de Biociências - Câmpus de Botucatu -
Rua Doutor Antonio Celso Wagner Zanin, s/nº, 13051-860
<http://www.ibb.unesp.br/pos-graduacao/ciencias-biologicas/genetica/CNPJ-480219180002259>

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Epígrafe

*“A coisa mais indispensável para alguém é reconhecer o uso que deve
fazer do seu próprio conhecimento.”*

Platão

Resumo (Comunicação Científica)

Entender o músculo esquelético no contexto de exercício físico, doenças crônicas e envelhecimento é essencial porque esse tecido vai além da movimentação corporal — ele tem um papel central na saúde geral e na regulação do organismo. A comunicação entre o músculo e outros órgãos, mediada por moléculas específicas, é fundamental para processos vitais, como a vascularização, controle inflamatório e manutenção da massa muscular. No contexto do exercício físico, o músculo é ativado de forma a melhorar essas interações, fortalecendo não apenas os músculos, mas também o sistema cardiovascular e outros órgãos. Isso resulta em benefícios como maior força, resistência e melhor qualidade de vida. Nas doenças crônicas, como diabetes e câncer, o músculo sofre mudanças que afetam essa comunicação, levando a perda de massa muscular e, conseqüentemente, à piora da saúde geral. Na caquexia, por exemplo, há uma ativação inflamatória intensa, que acelera a degradação muscular, complicando ainda mais o quadro clínico. No envelhecimento, o músculo passa por um processo natural de perda de massa e força (sarcopenia), que impacta diretamente a mobilidade e aumenta o risco de quedas e lesões. A falta de estímulo por meio do exercício físico pode acelerar esse processo, levando a complicações que afetam a independência e a qualidade de vida. Compreender o funcionamento do músculo nesses três contextos oferece insights sobre como prevenir ou minimizar as conseqüências de doenças crônicas e do envelhecimento, e como o exercício pode ser uma ferramenta poderosa na preservação da saúde muscular e na prevenção de complicações.

Resumo

O músculo esquelético é responsivo a diversos estímulos e desempenha um papel crucial em diversos aspectos da saúde humana. O exercício físico exerce no músculo respostas adaptativas benéficas, como manutenção e aumento da massa muscular, aumento da capacidade e liberação de mioquinas. Condições catabólicas como caquexia tumoral, diabetes, desuso, envelhecimento e sarcopenia afetam negativamente o músculo esquelético, reduzindo a síntese proteica, aumento da degradação proteica, comprometimento do funcionamento das células musculares e redução da capacidade de regeneração. O objetivo deste trabalho foi investigar o papel do secretoma e do interactoma muscular no exercício físico e em condições catabólicas. O sequenciamento de RNA de conjuntos de dados de músculo esquelético em humanos foi usado para identificar e prever genes diferencialmente expressos (DEGS) presentes no secretoma humano. Nós caracterizamos a comunicação celular em conjuntos de dados de músculo esquelético de humanos e camundongos, por predição das interações entre ligantes e receptores. Utilizamos conjunto de dados de sequenciamento de células únicas (scRNA-Seq) de músculo esquelético para identificarmos os tipos celulares das respectivas interações (LxR). Os resultados demonstram diferenças no secretoma do exercício físico, envelhecimento e sarcopenia. A comunicação celular do músculo aponta para interações entre componentes MEC, componentes do endotélio e do sistema imunológico. O exercício aumenta a expressão de moléculas que estão envolvidas no crescimento e na reparação muscular, enquanto o envelhecimento diminui a expressão dessas moléculas. Em condições que geram a atrofia muscular a comunicação celular aponta para o papel de componentes do sistema imunológico na regeneração muscular. Esse estudo fornece novos *insights* sobre novas terapias que direcionem o secretoma e o interactoma muscular para prevenir e tratar a atrofia muscular.

Abstract

Skeletal muscle is responsive to various stimuli and plays a crucial role in various aspects of human health. Physical exercise elicits beneficial adaptive responses in the muscle, such as maintenance and increase of muscle mass, enhanced capacity, and release of myokines. Catabolic conditions like tumor cachexia, diabetes, disuse, aging, and sarcopenia negatively affect skeletal muscle, reducing protein synthesis, increasing protein degradation, impairing muscle cell function, and diminishing regenerative capacity. The aim of this study was to investigate the role of muscle secretome and interactome in physical exercise and catabolic conditions. Bulk RNA-Seq of human skeletal muscle datasets was utilized to identify and predict differentially expressed genes (DEGs) present in the human secretome. We characterized cell communication in human and mouse skeletal muscle datasets by predicting interactions between ligands and receptors. We used single-cell sequencing datasets (scRNA-Seq) of skeletal muscle to identify cell types involved in respective ligand-receptor interactions (LxR). The results demonstrate differences in the secretome of physical exercise, aging, and sarcopenia. Muscle cell communication highlights interactions between extracellular matrix components, endothelial components, and the immune system. Exercise enhances the expression of molecules involved in muscle growth and repair, while aging reduces the expression of these molecules. Under conditions leading to muscle atrophy, cell communication points to the role of immune system components in muscle regeneration. This study provides new insights into potential therapies targeting the muscle secretome and interactome to prevent and treat muscle atrophy.

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Introdução

Tecido Muscular Esquelético: Estrutura, Função e Respostas Adaptativas

O tecido muscular estriado esquelético é responsável pela locomoção, respiração, termogênese, postura e equilíbrio corpóreo. Ele representa cerca de 40-50% da massa total de um indivíduo com peso corpóreo saudável¹ e cerca de 50-75% das reservas totais corporais de proteínas, lipídeos e carboidratos^{2,3}. Deste modo, em condições extremas, como a fome ou jejum, o tecido muscular é capaz de sintetizar glicose, uma adaptação crucial para manter a homeostase energética e nutricional⁴. Do ponto de vista mecânico, a função primordial do músculo esquelético é transformar a energia química em energia mecânica, resultando na geração de força e impulso, que desempenham um papel central na biomecânica do organismo⁵.

O músculo esquelético é um tecido altamente organizado e composto por vasos sanguíneos, nervos, tecido conjuntivo e fibras musculares esqueléticas, conferindo-lhe uma arquitetura complexa e altamente funcional⁶. As fibras musculares, unidades fundamentais dessa estrutura, se destacam como células alongadas e cilíndricas de natureza heterogênea, especializadas na contração muscular⁷. As fibras musculares apresentam no seu citoplasma as miofibrilas - estruturas longas e finas compreendidas por miofilamentos de actina e miosina⁸. Os miofilamentos de actina e miosina são dispostos em uma estrutura regular que permite que a contração das fibras musculares^{7,9}. A miosina é uma proteína composta por duas proteínas pesadas (MHC, do inglês *Myosin Heavy Chains*) e duas cadeias leves (MLC, do inglês *Myosin Light Chains*), a qual determinam a natureza da mecânica ação executada¹⁰. A unidade funcional do músculo esquelético é caracterizada como a unidade motora. Cada unidade motora tem a capacidade de inervar um número variável de fibras musculares, sendo essa distribuição determinada de acordo com a sua dimensão¹¹. As fibras musculares de contração lenta

são inervadas por unidades motoras pequenas, enquanto as fibras musculares de contração rápida são inervadas por unidades motoras maiores¹⁰.

As fibras musculares apresentam amplo espectro da heterogeneidade devido ao tipo de contração (rápida vs. lenta) e metabolismo energético (aeróbico vs. anaeróbico)¹². São definidos três tipos principais de fibras musculares: (1) As fibras oxidativas são caracterizadas pela contração lenta, demonstram resistência à fadiga, apresentam baixa capacidade de geração mecânica e sustentam um metabolismo aeróbico altamente ativo⁷; (2) As fibras glicolíticas rápidas, em contraste, são facilmente fatigáveis, com alta taxa de produção mecânica e um metabolismo glicolítico predominante; (3) As fibras oxidativas intermediárias rápidas são caracterizadas por combinar a alta potência mecânica e notável resistência à fadiga⁷. Cada um desses três tipos de fibras musculares apresenta padrões específicos de ativação das unidades motoras, viabilizando, assim, a sua identificação¹³. Além disso, a proporção da composição de fibras lentas e/ou rápidas é variável quanto ao subtipo de músculo esquelético¹².

Os músculos esqueléticos vastos lateral, tibial, gastrocnêmio e sóleo são comumente utilizados em pesquisas em camundongos e seres humanos, para avaliar a composição e função dos tipos de fibra muscular em diferentes contextos fisiológicos^{14–16}. O vasto lateral cujo nome em latim "*Vastus lateralis*" significa “grande lateral”, é um dos músculos que compõem o quadríceps femoral, um grupo muscular localizado na região anterior da coxa. O vasto lateral é composto por fibras musculares do tipo de contração rápida e lenta¹⁷. O gastrocnêmio, denominado em latim "*Gastrocnemius*", que se traduz como “músculo da barriga da perna”, ocupa a região posterior da perna e é composto predominantemente fibras musculares rápidas¹⁸. Todos os músculos esqueléticos apresentam capacidade adaptativa, o que significa que são capazes de modificar suas estruturas em respostas a estímulos e condições externas¹⁰.

A plasticidade muscular está associada às alterações prolongadas e funcionais na estrutura das fibras musculares¹⁷. Além disso, inclui modificações nas proporções da população de fibras musculares em um músculo ou grupo muscular específico⁷. Nesse contexto, as respostas adaptativas das fibras musculares são reguladas por vias de sinalização subcelulares, as quais são influenciadas por estímulos mecânicos e bioquímicos¹⁹. Diante disso, estímulos e condições como exercício físico, desuso, diabetes, caquexia associada ao câncer, envelhecimento e sarcopenia, exercem no músculo esquelético respostas adaptativas na estrutura quanto nas propriedades funcionais das fibras musculares^{3,20-22}. Essas mudanças ocorrem com a participação de várias células que compõem o microambiente muscular.

Papel do Microambiente Muscular na Regeneração e Homeostase Muscular

O microambiente que envolve as fibras musculares é uma mistura complexa de moléculas, fibroblastos, células imunológicas, vasos sanguíneos e nervos, que sustentam a função e homeostase muscular²³. Esse microambiente muscular desempenha importantes papéis na fisiologia muscular, uma vez que é capaz de modular respostas moleculares adaptativas a estímulos específicos⁶. No músculo esquelético homeostático, as células-tronco musculares, denominadas de células satélites, estão quiescentes e em íntima associação com as fibras musculares, residindo entre o sarcolema das fibras musculares e a lâmina basal que circunda essas fibras²⁴.

Em resposta ao estresse ou lesão, as células satélites são capazes de proliferar, fundir e formar novas miofibras para reparar o tecido danificado⁶. As células satélites em seu estado quiescente são caracterizadas pela expressão dos fatores de transcrição *PAX7* (*Paired Box 7*) e *FOXO* (*forkhead box*)²⁵. Quando ativadas, durante o processo de regeneração muscular, as células satélites expressam fatores de transcrição denominados fatores reguladores miogênicos (MRFs) que incluem *MYOD1* (*Myogenic Differentiation*

1), *MYF5* (*Myogenic Factor 5*), *MRF6* (*Myogenic Factor 6*) e miogenina (*MYOG*)^{26,27}. O microambiente lesionado libera sinais para ativar células satélites para permitir a tradução de *MYF5* e *MYOD1*, e bloqueando o fator de transcrição *FOXO*²⁸. Em seguida, as células satélites ativadas (*PAX7*, *+MYF5+*, *MYOD+*) se expandem por divisão simétrica²⁸. A fase de diferenciação terminal dos mioblastos, caracterizada pela regulação positiva de *MYOG* e *MYF6* e perda da expressão de *PAX7* e *MYF5*, gera miócitos e, finalmente, fibras musculares, através da fusão para gerar mionúcleos suficientes para apoiar a síntese de proteínas, ou fusão de mioblastos para a formação de miotubos que evoluem para fibras musculares maduras^{27,29,30}.

O nicho em que as células satélites estão inseridas permitem que seja mantido seu estado quiescente, fornece a capacidade de proliferar, migrar, autorrenovar, progredir através da linhagem miogênica e se diferenciar para reparar o tecido danificado⁶. No processo de regeneração muscular é exigido a ação coordenada de células inflamatórias, fibroblastos, células mesenquimais e células satélites, para garantir a restauração completa da vasculatura, nervos e fibras musculares²⁹. As células satélites são o foco da maioria dos estudos em regeneração muscular, devido ao papel crucial nesse processo²³. No entanto, pesquisas atuais vêm mostrando o papel dos demais componentes presentes no microambiente muscular e o respectivo potencial de ativar as células satélites, bem como o auxílio orquestrado durante a regeneração e reparo do músculo lesionado⁶.

Atualmente, impulsionado pelo avanço de técnicas como o sequenciamento de RNA de células únicas (do inglês *Single-cell RNA sequencing* (scRNA-Seq)), a caracterização e função de células bem como potenciais identificação de moléculas sinalizadoras no microambiente muscular vem sendo detalhado durante processos como regeneração muscular em resposta ao exercício físico, doenças crônicas e envelhecimento^{31,32}.

Funções dos Componentes da Matriz Extracelular na Estrutura e Função Muscular

A matriz extracelular (MEC) consiste em uma malha de componentes colagenosos, bem como uma mistura de outras macromoléculas, como várias glicoproteínas e proteoglicanos³³. As fibras musculares estão envolvidas pela ECM no nicho muscular. Nos últimos anos, estudos têm evidenciado o papel da ECM no desenvolvimento³⁴, crescimento³⁵, reparo muscular³⁶, e na propagação de força muscular³⁷.

A MEC no músculo esquelético é uma malha complexa de colágenos, glicoproteínas, proteoglicanos e elastina³⁸. Os colágenos formam uma rede de tecido conjuntivo intramuscular. No músculo esquelético, o tecido conjuntivo intramuscular é organizado em três camadas: o endomísio, camada mais interna que envolve as fibras musculares; o perimísio a camada que circunda determinado agrupamento de fibras musculares e o epimísio, camada que envolve todo o tecido muscular³³. O endomísio que está estritamente associado às fibras musculares é composto principalmente por colágeno do tipo I, III e integrinas³³.

Recentemente, o papel de componentes da MEC, como moléculas de colágenos e integrinas, têm recebido considerável atenção nos processos de regeneração muscular^{39–41}. Especificamente, componentes da MEC, como colágeno do tipo V, apresentam respostas adaptativas na ativação das células satélites⁴². Enquanto colágenos do tipo I e III estão implicados na redução da força muscular no envelhecimento e no desuso⁴³. De fato, estudos têm evidenciado a importância dos componentes de MEC para o músculo esquelético, no entanto a maioria dos estudos são revisões sistemática ou meta-análises, que possuem como objetivo principal fornecer uma visão ampla sobre os aspectos dos componentes de ECM na remodelagem muscular, e poucos abordam as interações moleculares e comunicação celular^{6,33,39,42}.

Componentes do Sistema Imunológico na Regeneração e Homeostase Muscular

O microambiente muscular é um ambiente dinâmico e componentes do sistema imunológico desempenham papel fundamental na manutenção da integridade e resposta imunológica do tecido muscular⁴⁴. Os macrófagos, neutrófilos e linfócitos T e B desempenham importante papel na homeostase e regeneração muscular⁴⁵. Os macrófagos são células fagocitárias que desempenham ampla resposta a processos biológicos como na eliminação de patógenos, liberação de citocinas, reparo de lesões e regeneração tecidual²⁸. Podem ser classificados em macrófagos M1 (ativados classicamente) e macrófagos M2 (ativados alternativamente)⁴⁶. Os macrófagos M1, caracterizados como pró-inflamatórios, são ativados por diferentes moléculas, sendo a citocina interferon gama (IFN- γ) e o lipopolissacarídeo (LPS) da parede bacteriana as principais moléculas envolvidas nessa ativação²⁸. A ativação dos macrófagos M2, caracterizados como anti-inflamatórios, pró-regenerativos ou pró-fibróticos, é regulada por várias citocinas e fatores de crescimento, incluindo interleucina-4 (IL-4) e interleucina-13 (IL-13), que são citocinas anti-inflamatórias²⁸.

Durante a regeneração do músculo esquelético, os macrófagos, montam uma resposta inflamatória enquanto exercem funções tróficas nas células-tronco musculares e mesenquimais⁴⁷. Os macrófagos pró-inflamatórios passam a ser anti-inflamatórios, desencadeando a resolução da inflamação no músculo esquelético⁴⁷. Na fase de restauração, os macrófagos amenizam a inflamação, e ao mesmo tempo promove diferenciação das células-tronco, a angiogênese e a remodelação da matriz⁴⁷.

Assim, como os macrófagos, os neutrófilos também são relevantes no microambiente muscular⁴⁸. Os neutrófilos são células envolvidas nos mecanismos de defesa do organismo contra infecção bacteriana. Os neutrófilos apresentam papel significativo na regulação dos processos iniciais da inflamação muscular, especialmente

através da liberação de mediadores pro-inflamatórios e da interação com outras células imunes presentes no tecido muscular⁴⁸.

Durante a regeneração muscular, os neutrófilos são as primeiras células inflamatórias que infiltram o músculo lesionado⁴⁹. Os neutrófilos fagocitam detritos musculares e liberam espécies reativas de oxigênio (ROS), proteases e citocinas pro-inflamatórias^{50,51}. A ausência de neutrófilos durante a lesão aguda do músculo esquelético prejudica a fagocitose do tecido necrótico e retarda a sua regeneração⁵².

Conjunto de dados de scRNA-Seq do músculo esquelético de camundongo por integração, tem apontado o papel central de células imunes, como macrófagos e neutrófilos, bem como outros tipos celulares durante a regeneração muscular⁵³. Devido ao papel crucial e complexo das células do sistema imune inato e adaptativo no processo de regeneração muscular, além das interações das células imunes com as demais células presentes no microambiente muscular^{48,54}. Uma maior compreensão dessas interações celulares abrirá caminho para novas estratégias terapêuticas⁵⁴.

Interação entre Células Endoteliais e Células Satélites na Regeneração Muscular

O músculo esquelético é altamente vascularizado e as miofibras são entrelaçadas com uma densa microvasculatura⁵⁵. A microvasculatura é essencial para fornecer nutrientes e oxigênio às células musculares, bem como para remover produtos residuais do metabolismo celular⁵⁶.

As células endoteliais da microvasculatura e as células miogênicas compartilham a mesma origem embrionária, o dermomiótomo⁵⁷. Portanto, as relações e interações entre células satélites e as células endoteliais têm sido cada vez mais elucidadas⁵⁸. Evidências *in vitro* e *in vivo* têm mostrado que as células endoteliais e as

células precursoras miogênicas interagem, acoplando a miogênese e a angiogênese na regeneração do músculo esquelético⁵⁵.

Através de uma meta-análise de diversos conjuntos de dados publicados, foi identificado o fator VEGFA, derivado das células satélites, auxilia na manutenção da expressão de *DLL4* nas células endoteliais⁵⁸. A comunicação entre as células endoteliais e as células satélites também foi estudada em células humanas *in vitro*, onde foi observado que a co-cultura dessas células aumenta a proliferação das células satélites, bem como na formação de capilares⁵⁸. Recentemente, fatores secretados que possibilitam o acoplamento entre miogênese e angiogênese vem sendo identificados, incluindo apelina (*APLN*) das células endoteliais, periostina (*POSTN*) de células perivasculares (FAPs) e oncostatina M (*OSM*) de macrófagos anti-inflamatórios⁵⁸. Entretanto, várias lacunas sobre a relação entre as células satélites e endoteliais na regeneração muscular, ainda não estão elucidadas^{55,58}.

Principais Mecanismos Moleculares de Atrofia Muscular

A manutenção da homeostase muscular é vital para a saúde⁵. Em doenças crônicas, as mudanças metabólicas nos músculos esqueléticos podem afetar a massa, força, flexibilidade muscular e levar fadiga que reduz a qualidade de vida elevando o risco de fraturas e morte^{1,59}. Consequentemente, essas perturbações na homeostase muscular também constituem um importante preditor de mortalidade⁵⁹.

A atrofia muscular é definida como a perda de massa muscular⁶⁰. Em adultos, a massa e o desempenho muscular se adaptam às diferentes condições, como exercício físico e fisiopatológicas, por meio da ativação de vias de crescimento muscular e vias distintas que regulam o *turnover* proteico⁶¹. Em condições fisiopatológicas, estudos apontam para a redução da ativação das células satélites⁶², mudanças da composição das

fibras musculares⁶⁰ e perturbações na via de sinalização atrofica e do metabolismo de poliaminas para fibras glicólicas, que alteram a denervação muscular⁶³.

As condições fisiopatológicas apresentam variações nos mecanismos subjacentes à atrofia muscular, elucidando diferentes cenários de alterações musculares. Em modelos de diabetes do tipo 2, ocorre uma sensibilização à insulina pelas células, consequentemente, ocorre uma redução na captação de glicose mediada pela insulina reduzindo a quantidade de fibras musculares oxidativas⁶⁴. Além disso, camundongos *knockout* para o receptor de insulina muscular apresentam fibras musculares glicolíticas significativamente menores do que as dos camundongos selvagens⁶⁵. Na caquexia relacionada ao câncer, pacientes com câncer estão em um balanço energético negativo, o que faz com que ocorra um aumento da liberação de aminoácidos dos músculos⁶⁶. Além disso, ocorre uma cascata de liberação de proteínas pró-inflamatórias pelo tumor para os tecidos do hospedeiro, ativando vias de degradação de proteínas musculares⁶⁷. Já na atrofia muscular por desuso, ocorrem alterações em resposta a uma diminuição na atividade muscular, reduzindo a síntese de proteínas musculares⁶⁸. Essas diferentes condições fisiopatológicas ilustram os diversos caminhos que podem levar à atrofia muscular, com mecanismos específicos em cada caso.

Há mais de uma década, foi descoberto um conjunto de genes, cuja expressão ao nível do mRNA se alterava de forma semelhante em diferentes situações catabólicas⁶⁹. Os atrogenes, ou seja, genes relacionados com a atrofia, desempenham papel central nos mecanismos associados à perda de proteínas musculares⁵⁹, considerados como genes marcadores de atrofia muscular⁶⁹. A maioria dos atrogenes foram associados aos principais sistemas de degradação celular, a ubiquitina-proteassoma e a autofagia-lisossoma⁵⁹.

O sistema de ubiquitina-proteassoma é um mecanismo intracelular importante para a degradação de proteínas danificadas, desdobradas e inúteis⁷⁰. Desempenha um papel importante na mediação da perda de massa muscular⁷¹. Os atrogenes, os *Fbxo32* (Atrogin-1/MAFbx) e *Trim63* (MuRF1) são ligases do sistema do ubiquitina-proteossoma relacionadas com a perda de massa durante a atrofia muscular^{72,73}. A MuRF1 é responsável pela ubiquitinação de várias proteínas estruturais musculares, incluindo troponina I, proteínas de cadeias pesadas de miosina, actina, proteína C ligante de miosina e cadeias leves de miosina 1 e 2⁵⁹. Por outro lado, os substratos de Atrogin-1 parecem estar envolvidos em processos relacionados ao crescimento ou vias de sobrevivência⁵⁹.

A autofagia é um mecanismo homeostático altamente conservado usado para a degradação e reciclagem de proteínas de vida longa e organelas, por meio da maquinaria lisossômica⁷⁴. A falha na autofagia pode resultar em degeneração e fraqueza muscular⁷⁴. Isso porque, o sistema proteolítico da autofagia, é ativado em condições catabólicas, e é controlado pela transcrição de genes relacionados à autofagia, como os ATG (do inglês, *autophagy-related genes*). Alguns dos ATG, especialmente aqueles envolvidos no comprometimento de membranas e reconhecimento de carga, fazem parte da lista dos atrogenes⁵⁹. Estudos em camundongos *knockout* específicos para proteínas relacionadas à autofagia, como Atg5 (*Autophagy Related 5*) e ULK2 (*Unc-51 Like Autophagy Activating Kinase 2*) apresentaram fenótipo de degeneração e fraqueza muscular⁷⁵⁻⁷⁷. Em estudos que correlacionam a autofagia a condições como sarcopenia e envelhecimento, a deleção específica de Atg7 (*Autophagy Related 7*) ou a superexpressão de TSC2 (*TSC Complex Subunit 2*) recapitula características como aumento do estresse oxidativo, acúmulo de mitocôndrias disfuncionais e denervação de fibras musculares^{59,78,79}.

A compreensão dos mecanismos que geram a atrofia muscular em diferentes condições fisiopatológicas é crucial para a identificação de alvos específicos para o

desenvolvimento de tratamentos mais eficazes a perda muscular^{59,80}. Além disso, diante da importância da composição e homeostase do músculo esquelético, isso pode ajudar a prevenir ou retardar a progressão da atrofia muscular e melhorar a qualidade de vida das pessoas afetadas⁸⁰.

Sarcopenia: Impacto do Envelhecimento na Massa e Função Muscular

À medida que a sociedade envelhece, aumenta a incidência de limitações do desempenho físico⁸¹. Nas últimas décadas, a população idosa global tem aumentado significativamente; projetando-se uma estimativa de 2,1 bilhões de indivíduos acima de 60 anos até 2050⁸². Estima-se que cerca de 42% de pessoas com mais de 60 anos de idade têm dificuldades em realizar alguma atividade física diária⁸¹. A inatividade física está diretamente relacionada à diminuição do desempenho do músculo esquelético⁸¹.

O processo de envelhecimento, repouso absoluto, inatividade física, desnutrição, doenças crônicas e tratamentos farmacológicos são alguns dos fatores que contribuem para a perda de músculo esquelético⁸³. No processo de envelhecimento, há uma perda de cerca de 5% da massa muscular até à idade de 50 anos⁸⁴. Após os 50 anos de idade, há uma redução de 1-2% a cada ano na massa muscular⁸⁴. A partir dos 80 anos a perda da massa muscular chegará a 30 %⁸⁵. Aproximadamente 30% da população de indivíduos idosos é acometida com a sarcopenia⁸⁶.

O termo "sarcopenia" deriva do grego, onde "*sarx*" significa carne e "*penia*" se refere à perda, com consequente redução da qualidade de vida, aumento da incapacidade física, risco a doenças crônicas e morte⁸³. A sarcopenia apresenta origem complexa, envolvendo fatores hormonais, acúmulo de espécies reativas de oxigênio (ROS), citocinas pró-inflamatórias, alterações na autofagia e disfunção das células satélites⁸⁷. Mudanças dependentes da idade tanto no microambiente tecidual quanto na população de células progenitoras musculares, parecem estar envolvidas no início e progressão dessa

condição⁸⁸. Entretanto, as alterações do músculo esquelético na sarcopenia também podem ocorrer antes do declínio das células satélites⁸⁹. Essa perda de massa muscular na sarcopenia se manifesta pela atrofia das fibras musculares das fibras de contração rápida (tipo II)⁸⁷. Portanto, essas características da sarcopenia apontam para a importância de uma abordagem multifatorial na compreensão e tratamento da condição⁸⁵.

Vários fatores de risco estão associados à sarcopenia. Entre eles, o avanço da idade cronológica é um dos principais⁸⁵, afetando uma parte substancial da população idosa⁸⁶. À medida que a idade avança, o risco de desenvolver sarcopenia aumenta. Por exemplo, a prevalência de sarcopenia é estimada em cerca de 13% entre pessoas de 60-70 anos de idade, e esse número aumenta para aproximadamente 50% em indivíduos com mais de 80 anos⁸³. O gênero também desempenha um papel nessa condição, com uma prevalência mais alta em homens idosos em comparação com mulheres idosas⁸⁶. Aproximadamente 1,5% das mulheres idosas são afetadas, contrastando com uma taxa significativamente maior de cerca de 14,3% em homens idosos⁸⁶. Além disso, tabagismo, inatividade física e o índice de massa corporal (IMC), são fatores de risco como com resultados estatisticamente significativos ($p < 0,005$)⁹⁰.

A definição e critérios de diagnóstico da sarcopenia diferem entre os principais grupos de pesquisa em todo o mundo⁹¹. O *European Working Group on Sarcopenia in Older People* (EWGSOP) definiu em 2010 a sarcopenia como “uma síndrome caracterizada pela perda progressiva e generalizada de massa e força muscular esquelética com risco de resultados adversos, como incapacidade física, má qualidade de vida e morte”. Além disso, recomendam combinar baixa massa muscular com baixa função muscular como critério diagnóstico⁹². Em 2018, a EWGSOP2 revisou a definição da sarcopenia para ser uma doença com código de diagnóstico ICD-10-MC, e o critério diagnóstico foi atualizado para priorizar a baixa força muscular como principal indicador

de sarcopenia. Consequentemente, a força muscular é reconhecida atualmente como a medida mais confiável da função muscular⁹².

O *Asia Working Group for Sarcopenia* (AWGS) definiu a sarcopenia como "perda de massa muscular relacionada à idade, além de baixa força muscular e/ou baixo desempenho físico"⁹³. No entanto, o AWGS atualizou essa definição em 2019 atualização, incorporando novos conhecimentos e diretrizes. Embora a definição anterior de sarcopenia tenha sido mantida, foram revisados o algoritmo de diagnóstico, protocolos e alguns critérios de força e desempenho⁹³.

O *International Working Group on Sarcopenia* (IWGS) define a sarcopenia como “a perda involuntária de massa e funcionalidade do músculo esquelético relacionada à idade”, estabelecendo como critério diagnóstico o parâmetro de massa livre de gordura (MLG)⁹⁴. A *Society for Sarcopenia Cachexia and Wasting Disorders* (SCWD) apoia o consenso da sarcopenia como a “baixa de massa muscular junto com baixa da função muscular” e estabelece também como critério diagnóstico o questionário medição da força de prensão ou se levantar da cadeira junto com estimativa da Avaliação da Composição Corporal (DXA) da massa muscular apendicular⁹⁴.

Apesar dos esforços para uma definição e diagnóstico plausíveis, a sarcopenia continua pouco conhecida e subdiagnosticada⁹⁵. O tratamento da sarcopenia é abordado por meio de suplementação dietética, intervenções hormonais e exercícios físicos⁸⁷.

Exercício Físico na Saúde Humana: Benefícios, Adaptações Musculares e suas Vias de Sinalização

O exercício físico é um componente fundamental para a saúde humana⁹⁶. O *American College of Sports Medicine* (ACSM) define exercício físico como “movimento corporal planejado, estruturado e repetitivo feito para melhorar ou manter um ou mais

componentes da aptidão física" (*ACSM's Guidelines for Exercise Testing and Prescription, 11th Edition*). De acordo com Organização Mundial da Saúde (OMS) e o ACMS, é recomendado que indivíduos adultos façam pelo menos 150 minutos de exercício aeróbica de intensidade moderada ou 75 minutos de exercício aeróbica de alta intensidade⁹⁷.

Os benefícios do exercício físico são cumulativos à saúde corpórea⁹⁸. A prática regular de exercício físico poderá reduzir o risco de doenças crônicas, como diabetes do tipo 2, doenças cardíacas, derrames e alguns tipos de cânceres. Isso se deve ao controle da glicose circulante, fortalecimento do sistema cardiorrespiratório, redução da pressão arterial, manutenção do peso, redução de inflamação, melhoria do sono e manutenção da força e massa e muscular⁹⁹. Há evidências de que o exercício regular pode minimizar os efeitos fisiológicos do sedentarismo, aumentando a expectativa de vida ativa e limitando o desenvolvimento e a progressão de doenças crônicas^{100,101}. O exercício físico também favorece benefícios psicológicos e cognitivos significativos em adultos¹⁰⁰, reduzindo os sintomas de depressão e ansiedade, elevando o humor, a autoestima e reduzindo o estresse¹⁰². Dessa forma, profissionais da saúde reconhecem e recomendam o exercício físico como prevenção ao surgimento de diversas doenças crônicas e mentais.

Existem diferentes tipos de exercício físico. O exercício aeróbico está associado ao aumento da frequência cardíaca e respiratória. Caminhar, correr, andar, nadar são alguns exemplos de exercício aeróbico¹⁰³. O treinamento intervalado de alta intensidade (HIIT) é caracterizado por intercalações breves de atividades vigorosas, seguidas de intervalos de repouso ou atividade de baixa intensidade¹⁰⁴. O treino resistido, por sua vez, é definido pela sobrecarga progressiva aos músculos esqueléticos, visando o aumento de força e que, frequentemente, resulta em hipertrofia¹⁰⁵.

A resposta adaptativa do músculo esquelético é dependente do tipo de estímulo de exercício físico, magnitude do volume e intensidade do treinamento empregado¹⁰⁶. O exercício promove diversas mudanças nas propriedades moleculares e estruturais do músculo esquelético, incluindo alterações na biogênese mitocondrial, metabolismo e proteoma¹⁰⁷. Além de maior capilarização muscular e a modulação do conteúdo das fibras musculares¹⁰⁸.

Em exercício aeróbico, há o aumento da capacidade oxidativa no músculo esquelético. Consequentemente, o músculo esquelético apresenta maior resistência à fadiga devido às mudanças fisiológicas como aumento da biogênese mitocondrial, uma remodelação dinâmica e degradação controlada, conhecida como mitofagia^{106,109}. As mitocôndrias são organelas dinâmicas responsáveis pela síntese de moléculas de ATP (*adenosina trifosfato*). Para atender alterações na demanda de energia, as mitocôndrias se adaptam ativando inúmeras vias relacionadas ao estresse, que suportam alterações em processos biológicos relacionados ao metabolismo, crescimento e regeneração mitocondrial¹⁰⁹. No contexto do exercício resistido, são observadas alterações que levam ao aumento da massa muscular, devido a adaptação das fibras musculares, aumento da síntese de proteínas, aumento nos núcleos das miofibras e na extensão das unidades motoras¹¹⁰.

A remodelação muscular se inicia por estímulos extracelulares que atingem os receptores de membrana, ativando diversas vias de sinalização intracelulares¹⁰⁸. Esses sinais são propagados no interior da célula, com consequente alterações na transcrição de genes e modulação do proteoma muscular^{59,108}. A caracterização fundamental da hipertrofia do músculo esquelético reside no aumento da síntese proteica que, consequentemente, aumenta as fibras musculares¹¹¹. Também ocorre um aumento

concomitante do número de mionúcleos, bem como a geração de miofibras, os quais dependem das células satélites¹¹².

O exercício físico gera um estresse e impõe uma resposta sistêmica, gerando diversas alterações metabólicas e hormonais no organismo⁹⁸. Dentre essas alterações, destaca-se o aumento da liberação de hormônios, incluindo o fator de crescimento semelhante à insulina (IGF) e a insulina¹¹¹. Tanto a insulina quanto o IGF são potentes fatores anabólicos no músculo, ao se ligar aos seus receptores específicos (receptor de insulina e receptor de IGF1) ocorre uma ativação em cascata da fosforilação que, por sua vez, exerce uma influência regulatória sobre proteínas, enzimas e fatores de transcrição, modulando assim positiva ou negativamente processos essenciais^{113,114}.

A via de sinalização insulina/IGF1-AKT-mTOR é uma das principais vias de sinalização responsável pela promoção de hipertrofia muscular¹¹⁴. Essa via controla diversos processos celulares, incluindo a regulação da síntese proteica, degradação, proliferação celular e sobrevivência celular, bem como a captação de glicose e a produção de energia¹¹¹. Após a ligação de insulina ou IGF aos seus receptores, ocorre fosforilação e ativação de *Akt* (*Protein kinase B*) e consequente supressão do fator de transcrição *Foxo* (*Forkhead box*), inibindo assim as vias de degradação proteica ubiquitina-proteassoma e autofágica lisossomal¹¹⁵. Elemento central dessa via para a síntese e degradação de proteínas é a quinase mTOR⁵⁹. A mTOR integra múltiplos estímulos provenientes de hormônios, citocinas, nutrientes, relação ATP/AMP e sinais para a maquinaria de tradução via p70S6K1⁵⁹. Através desta via, a mTOR exerce controle sobre a proteína ribossomal S6 e a proteína 1 de ligação ao fator 4E (*4EBP1*), as quais regulam o fator iniciador de tradução eucariótica ribossômica 4E5¹¹⁶. Simultaneamente, a mTOR inibe a quebra de proteínas bloqueando a autofagia via ULK1 (*Unc-51 Like Autophagy Activating Kinase 1*)¹¹⁷. A mTOR interage com várias proteínas para formar dois complexos diferentes. O

complexo TORC1 (*the target of rapamycin kinase complex 1*) sensível à rapamicina e composto por Raptor, e o complexo TORC2 (*the evolutionarily conserved target of Rapamycin complex-2*) insensível à rapamicina, que contém Rictor⁵⁹.

Em adição, as vias de sinalização musculares se conectam às funções autócrinas, parácrinas e endócrinas desse tecido. A comunicação autócrina ocorre quando as células musculares liberam sinais que afetam diretamente as próprias células, regulando processos intracelulares¹¹⁸. A comunicação parácrina se refere à influência desses sinais em células vizinhas, permitindo a coordenação de atividades locais¹¹⁹. A sinalização endócrina, por sua vez, envolve a liberação de substâncias como miocinas na corrente sanguínea, alcançando órgãos distantes e desempenhando funções sistêmicas¹²⁰. Portanto, as vias de sinalização e as miocinas desempenham papéis interligados na coordenação das respostas musculares, metabólicas e fisiológicas, influenciando o equilíbrio entre crescimento, degradação e interações sistêmicas^{119,120}.

Músculo Esquelético como Órgão Secretor e suas Implicações Metabólicas

O músculo esquelético também tem sido reconhecido como um órgão endócrino, com a capacidade de liberar várias moléculas bioativas, conhecidas como miocinas, em resposta ao exercício físico¹²¹. Os papéis biológicos das miocinas incluem efeitos sobre cognição, metabolismo de lipídios e glicose, escurecimento da gordura branca, formação óssea, alteração da função da célula endotelial, hipertrofia, estrutura da pele e crescimento tumoral em humanos¹²². Dessa forma, as miocinas podem ser biomarcadores chaves em diversas doenças crônicas como por exemplo, câncer, diabetes, doenças degenerativas, fratura e sarcopenia¹²²⁻¹²⁴.

As miocinas apresentam regulação dependente ou independente da contração muscular¹²⁵. Isso porque, as miocinas podem ser liberadas devido a contração muscular em resposta ao estresse gerado pelo exercício físico¹²⁶, bem como as miocinas também

são secretadas em repouso, independentemente da contração muscular, como resultado da sinalização das células basais^{125,127}. Dentre as vias mais estudadas na liberação e ativação das miocinas, destaca-se a via AMPK (proteína quinase ativada por AMP) e a via p38 MAPK (proteína quinase ativada por mitógenos)¹²⁵.

A AMPK (Proteína quinase ativada por AMP) é uma molécula de sinalização crítica central para o metabolismo da glicose e lipídios, síntese de proteínas e autofagia^{128,129}. Encontra-se ativada quando os níveis de adenosina monofosfato (AMP) aumentam, o que ocorre quando a célula não tem energia suficiente¹³⁰. A AMPK então fosforila proteínas que regulam diferentes vias metabólicas, levando a uma série de efeitos benéficos, como aumento da sensibilidade à insulina, diminuição da inflamação e proteção contra doenças cardíacas^{131–133}. A IL-6 (*Interleukin 6*) é uma miocina amplamente investigada e conhecida como mediadora da homeostase energética, sendo sugerida a liberação da IL-6 pela ativação da AMPK por decorrência do exercício físico^{125,134}.

No entanto, são poucos os estudos que elucidam o envolvimento das miocinas no metabolismo energético em vias de sinalização que ativa a AMPK durante o exercício físico, bem como em outras vias metabólicas no músculo esquelético¹²⁵. Dessa forma, a investigação do secretoma muscular em resposta ao exercício físico poderá fornecer informações sobre possíveis caminhos de investigação em estudos de doenças e terapias¹²⁵.

O Secretoma Humano e a Comunicação Celular: *Insights* para a Biologia do Músculo

As proteínas secretadas pelas células em humanos são referidas como secretoma humano. São fundamentais para a compreensão básica da biologia humana, bem como identificação de alvos potenciais para futuros diagnósticos e terapias¹³⁵. O secretoma

humano é um conjunto de proteínas, lipídios, ácidos nucleicos e pequenas moléculas, que são ativamente transportadas dentro da via secretora para destinos fora do citoplasma e do núcleo da célula¹³⁵. O secretoma é extremamente dinâmico, e a alteração da expressão de proteínas ocorre durante o desenvolvimento do órgão, maturação, em várias doenças e estímulos ambientais¹³⁶.

O secretoma é transcolado parcial ou completo por meio da membrana do retículo endoplasmático em eucariotos¹³⁷. As proteínas direcionadas para a secreção recebem a sequência de sinal, chamado de peptídeo de sinal, que é uma sequência N-terminal hidrofóbica curta, inserida na membrana do retículo endoplasmático e subsequentemente separada da proteína¹³⁵. A translocação das proteínas ocorre pela formação de um complexo heterotrimérico de membrana-proteínas conservadas¹³⁷. Dependendo dos parceiros de ligação do canal, os polipeptídeos são movidos por reconhecimento realizado por proteínas específicas¹³⁷. As sequências de sinal do retículo endoplasmático são reconhecidas por proteínas chaperonas que guiam os ribossomos sintetizadores para o retículo endoplasmático rugoso, onde ocorre a translocação da sequência de proteína em um complexo denominado *translocon*¹³⁵. Dentro do retículo endoplasmático, as chaperonas garantem o dobramento, montagem correta das proteínas, além da adição de pontes de dissulfeto, carboidratos e clivagens proteolíticas no ambiente do retículo endoplasmático¹³⁵. Após a inspeção do controle de qualidade realizado no retículo endoplasmático, as proteínas são transportadas via vesículas para o aparelho de *Golgi*, onde são posteriormente modificadas em processos importantes como glicosilação e fosforilação, e então liberadas para o espaço extracelular¹³⁵.

As proteínas secretadas desempenham funções essenciais na sinalização célula-célula, intercomunicação e crescimento celular^{135,136}. Além disso, devido à sua capacidade de refletir diferentes estágios de condições patológicas, elas representam uma

fonte promissora para a investigação de biomarcadores e novas descobertas científicas¹³⁵. Isso porque, as proteínas secretadas pelas células em resposta a vários estímulos são provavelmente secretados no sangue, a identificação e pré-seleção de biomarcadores de proteínas candidatas a partir do secretoma celular com posterior validação de sua presença em níveis mais elevados no sangue é uma estratégia promissora na medicina regenerativa¹³⁸.

O *The Human Protein Atlas* (<https://www.proteinatlas.org/>), é uma plataforma atualizada com predição de proteínas presente no corpo humano em diferentes tecidos, facilitando para os pesquisadores o entendimento e caracterização do secretoma humano¹³⁵. O estudo de Uhlén e colaboradores (2015)¹³⁵ buscou identificar as proteínas secretadas ativamente em humanos com base em sua origem de expressão e localização final no corpo. As proteínas foram classificadas em três categorias principais: proteínas do sangue, proteínas secretadas localmente e proteínas intracelulares. Os resultados são apresentados em uma versão atualizada do *The Human Protein Atlas*, na qual cada gene que codifica uma proteína do secretoma é anotado para fornecer um recurso de conhecimento de acesso aberto do secretoma humano¹³⁵.

A análise integrada entre proteoma (secretoma), expansão de banco de dados de integração proteína-proteína e avanços em dados transcriptômicos, também pode fornecer informações relevantes sobre a sinalização intercelular¹³⁹. A comunicação celular coordena a atividade de vários tipos de células, como respostas imunológicas¹⁴⁰, crescimento celular¹⁴¹ e homeostase¹⁴², no entanto a interação entre ligantes e receptores se limitavam a ensaios laboratoriais. O estudo de Ramilowski *et al.*, (2015)¹⁴³ forneceu amplo *draft* sistemático sobre a interação ligante-receptor utilizando perfis de expressão de 642 ligantes e 589 receptores e suas respectivas interações, com suporte na literatura em um painel de 144 tipos de células primárias em humanos¹⁴³. A partir de então,

diferentes estudos têm visado responder questões sobre a comunicação celular em interação ligantes-receptores de tecidos^{144,145} e microambiente tumoral¹⁴⁶.

As interações célula-célula orquestram o desenvolvimento dos organismos, a homeostase tecidual e funções unicelulares¹³⁹. Estudos sobre as interações entre ligantes e receptores celulares avançaram bastante à medida que os dados de sequenciamento de RNA e sequenciamento de células únicas se tornaram comuns e bem caracterizados na literatura^{139,147,148}. Desta forma, as interações entre ligantes e receptores podem ser usados para inferir a comunicação intercelular a partir da expressão coordenada de seus genes cognatos em diferentes contextos como inferência da comunicação autócrina/parácrina do músculo esquelético em respostas adaptativas ao exercício físico e condições fisiopatológicas associadas a atrofia muscular em dados de sequenciamento de RNA e sequenciamento de células únicas¹³⁹. Plataformas *onlines* como o CellPhoneDB (<https://www.cellphonedb.org/>) fornecem o repositório de ligantes, receptores e suas respectivas interações, para a investigar do *crosstalk* de diferentes tipos de células em distintos contextos de processos fisiológicos¹⁴⁹. Além disso, essa plataforma fornece informações sobre interações entre ligantes e receptores presentes em diferentes organismos como camundongos e humanos¹⁴⁹.

Desta forma, essa estratégia de análise do secretoma e interação ligante e receptor “*in silico*” tem grande potencial para revelar mediadores, biomarcadores e alvos para tratamento da atrofia muscular durante diferentes condições catabólicas e no processo de envelhecimento do músculo esquelético. Desta forma, é fundamental compreendermos o papel da comunicação do secretoma do músculo esquelético em condições fisiológicas em resposta ao exercício físico, e contrapor com condições catabólicas e envelhecimento muscular, no intuito de buscar caracterizar as moléculas secretadas pelo músculo e compreensão da sinalização celular e antagonismo a atrofia muscular. Até o presente

momento, não existem estudos que se utilizam de informações de bancos de dados públicos que correlacionem dados do secretoma humano e interactoma de ligantes e receptores na sinalização autócrina/endócrina em dados transcriptômicos do músculo esquelético em respostas adaptativas ao exercício físico, condições fisiopatológicas e no envelhecimento.

A hipótese desse trabalho é de que a análise do perfil transcricional do músculo esquelético estriado em resposta adaptativa ao exercício físico, desuso, diabetes, caquexia associada ao câncer, envelhecimento e sarcopenia pode prever o perfil secretome e a composição de moléculas sinalizadoras autócrina/parácrina que atuam como mediadoras na regeneração muscular.

Objetivo Geral

Caracterizar o perfil transcricional do secretoma e comunicação celular do músculo esquelético em diferentes respostas adaptativas em humanos e camundongos.

Objetivos específicos

- ✓ Analisar dados transcriptômicos em sequenciamento de RNA e sequenciamento de células únicas do músculo esquelético em humanos e camundongos;
- ✓ Identificar os perfis transcricionais do secretoma em repostas adaptativas do músculo esquelético em humanos no exercício físico, envelhecimento e sarcopenia;
- ✓ Caracterizar a comunicação autócrina/parácrina do músculo esquelético em humanos em resposta adaptativa ao exercício físico, envelhecimento e sarcopenia;
- ✓ Caracterizar a comunicação autócrina/parácrina do músculo esquelético em repostas adaptativas do músculo esquelético em camundongos em condições fisiopatológicas;
- ✓ Definir os componentes celulares no microambiente muscular envolvidos na comunicação autócrina/parácrina do músculo esquelético.

Chapter 1: Extracellular matrix components are fundamental in cell-cell communication during muscle regeneration in response to physical exercise in young individuals.

Jakeline Santos Oliveira¹, Sarah Santiloni Cury¹, Diogo de Moraes², Jeferson Santos¹, Paula Paccielli Freire³, Maeli Dal Pai-Silva¹, Geysson Javier Fernandez⁴ & Robson Francisco Carvalho^{1#}.

1. Department of Structural and Functional Biology, Institute of Biosciences, São Paulo State University, UNESP, Botucatu, São Paulo, Brazil.
2. Department of Biochemistry and Tissue Biology, Institute of Biology, State University of Campinas, UNICAMP, Campinas, São Paulo, Brazil.
3. Department of Immunology, University of São Paulo, USP, São Paulo, Brazil.
4. Group Biología y Control de Enfermedades Infecciosas, Universidad de Antioquia, Medellín, Colombia.

Corresponding author

Professor Robson Francisco Carvalho
Department of Structural and Functional Biology
Institute of Biosciences, São Paulo State University - UNESP
CEP: 18.618-689, Botucatu, São Paulo – Brazil
Telephone number: +55 14 3880 0473
robson.carvalho@unesp.br

Abstract

Regular physical exercise is beneficial to health because it prevents the risk of developing chronic diseases. Skeletal muscles are highly responsive to external stimuli. During the adaptive response to physical exercise, skeletal muscle alters the proliferation of muscle satellite cells (MuSCs), mitochondrial function, metabolic regulation, vascularization, and remodeling of extracellular matrix (ECM) components. The function of specific extracellular matrix molecules in regulating angiogenesis during exercise is poorly understood. We globally profiled the interactions of muscle ligands and receptors based on the transcriptome of *vastus lateralis* muscle in response to 12 weeks of high-intensity aerobic interval (HIIT) and resistance (RT) training in young and old individuals. First, we analyzed the RNA-seq dataset GSE97084, available on the Gene Expression Omnibus, using the BioJupies platform. Next, we identified differentially expressed genes ($\text{Log2FC} > 1.5$; $\text{FDR} < 0.05$) between pre- and post-exercise in young (HIIT, $n=11$; RT, $n=10$) and old individuals (HIIT, $n=8$; RT, $n=8$). The upregulated genes were used to predict the interactions of the ligand (L) and receptor (R) pairs retrieved from literature. Positive L-R interactions (L or R with $\text{FDR} < 0.33$) were selected for functional enrichment analysis using EnrichR. Finally, we identified the cell types contributing to the gene expression of L-R interactions using single-cell RNA-Seq data (GSE214544). The genes with the highest L-R interaction scores (*COL1A1*, *COL1A2*, *COL3A1*, *COL4A1*, *COL5A1*, *ITGA1*, *ITGB1*, *DDR1*, and *DDR2*) were expressed in satellite cells, mesenchymal progeny cells, and vascular, arterial, and capillary endothelial cells. The prediction of genes in the secretome in young and elderly individuals in the HIIT exercise was higher when compared to the RT exercise in young and elderly individuals. For skeletal muscle autocrine communication, HIIT and RT exercises in young individuals increased the communication score compared to elderly individuals. Our findings establish an interaction between ECM molecules released by progenitor cells and satellite cells with vascular endothelial cell receptors. This finding suggests that communication between mesenchymal and vascular cells is fundamental for muscle remodeling after physical exercise. ECM components activate muscle satellite cells in addition to repair and angiogenesis. Additionally, our findings provide new insights into the relevance of muscle adaptation in response to physical exercise.

Keywords: Skeletal Muscle, Physical Exercise, Muscle remodeling, Secretome, Ligand-Receptors.

Introduction

Physical exercise causes widespread adaptive changes in different tissues and organs, and consequently promotes an increase in quality of life and a reduction in the risk of chronic diseases⁹⁸. Skeletal muscle tissue is highly adaptive to physical exercise, which induces changes in contractile proteins, mitochondrial function, metabolic regulation, intracellular signaling, and transcriptional responses¹⁵⁰.

Different exercise types, encompassing a spectrum of frequencies, intensities, and durations, lead to unique metabolic and molecular changes in the skeletal muscles¹⁵⁰. Notably, high-intensity interval training (HIIT) rapidly and robustly increases young people's aerobic capacity, mitochondrial respiration, and insulin sensitivity¹⁵¹. In parallel, the modalities of resistance training (RT) can modify gene transcription and muscle protein synthesis rates, consequently increasing muscle volume¹⁵¹. Physical exercise changes the physiological responses of different tissues and organs, thus generating different adaptive responses¹⁵². In skeletal muscle, the contraction of muscle fibers induces the secretion of cytokines and peptides, named "myokines," which exert autocrine, paracrine, or endocrine effects¹⁵³. Myokines play multiple roles ranging from regulating energy demand to the broadly beneficial effects of exercise on cardiovascular, metabolic, and mental health¹⁵⁴. However, signaling molecules released during skeletal muscle adaptation in response to exercise have not been fully explored.

Skeletal muscle aging is characterized by a progressive decline in muscle mass, strength, and regenerative capacity¹⁵⁵. Physical exercise is also well established to prevent muscle aging. Physical exercise attenuates muscle mass loss, increases muscle strength and regenerative capacity, and slows or prevents muscle metabolic deficiency¹⁵⁶. Moderate-intensity exercise can help to reverse the aging-related loss of muscle mass¹⁵⁷, and in old mice, accelerates muscle repair and improves muscle satellite cell (MuSC) function¹⁵⁸. During muscle repair and regeneration, MuSCs are activated to produce myogenic progenitor cells that can fully restore muscle structure and function¹⁵⁹. In addition, new blood vessels are formed during the regeneration process to aid tissue healing, and recent findings have shown that extracellular matrix (ECM) proteins promote muscle injury repair and regeneration¹⁶⁰. If the existing ECM scaffolding is not maintained, it becomes much more difficult to mount a response to resistance training in adult muscles¹⁶¹. ECM remodeling is an important process that can help regulate cell-based processes, including the establishment and maintenance of stem cell niches, angiogenesis, and wound repair¹⁶².

Research has shown the role of the ECM in revascularization during bone tissue regeneration¹⁶³. However, little is known about the role of ECM components in angiogenesis during the repair and remodeling of skeletal muscles in the adaptive response to physical exercise in young and elderly individuals. However, the understanding of molecular processes, as well as cell-cell communication that regulates the maintenance of muscle satellite cells and vascularization during muscle remodeling in aging, is still an expanding field for muscle physiology¹⁶⁴.

Here, we studied the skeletal muscle Bulk RNA-Seq of young and old individuals subjected to the physical exercise modalities HIIT and RT to characterize the secretome transcriptional profile and ligand-receptor pair interaction that potentially participates in skeletal muscle autocrine signaling. Moreover, the expression of selected genes was further investigated using single-cell RNA-Seq to identify the muscle cells that contribute to ligand-receptor communication within the muscle microenvironment. We identified an increased expression of secretome genes associated with ECM, collagen biosynthesis, protein digestion and absorption, and PI3k-AKT-mTOR signaling. Most of the genes predicted as ligands comprise the collagen gene family, which induces the proliferation and differentiation of mesenchymal satellite cells. Genes predicted as receptors mostly comprise families of integrins and tyrosine kinases that induce angiogenesis in capillary cells of the endothelium. Our data contribute to the elucidation of cell-cell communication and the importance of molecules in muscle remodeling in young and elderly individuals after physical exercise. In addition, these data may contribute to future studies to understand the reduction of muscle remodeling in aging and its association with metabolic and chronic diseases.

Material and methods

Differential expression analysis of RNA-Seq data for physical exercise modalities and inactivity

We included raw counts from RNA-Seq data of 52 muscle samples from vastus lateralis biopsies. The dataset comprised young individuals (N= 21) with a mean age of 25 years and elderly individuals (N = 16) with a mean age of 70 years. Male and female subjects were included in both groups. The dataset is publicly available in the Gene Expression Omnibus (GEO) under accession number GSE97084¹⁵¹. We used the Biojupies platform (<https://amp.pharm.mssm.edu/biojupies/>)¹⁶⁵ to identify differentially expressed genes (DEGs), comparing the biopsy samples of the skeletal muscle (*vasto lateralis*) of the

individuals before (pre-samples) and after (post-samples) submitting to the physical exercise protocols HIIT (5 days for 12 weeks) and RT (2 days for 12 weeks) (Figure 1A and Supplementary Table S1). HIIT was three days per week of cycling (4 3 4 min at >90% of peak oxygen consumption [VO₂ peak] with 3 min pedaling at no load) and two days per week of treadmill walking (45 min at 70% of VO₂ peak). RT consisted of lower and upper body exercises (4 sets of 8–12 repetitions) 2 days each per week. Genes with Log₂ of fold change $\geq |1.5|$ and false discovery rate (FDR) < 0.05 were considered DEGs (Supplementary Table S2).

Transcriptome-based secretome analysis

We filtered the DEGs coding for secreted and plasma proteins based on the lists in The Human Protein Atlas (<https://www.proteinatlas.org/humanproteome/secretome>)¹³⁵ (Supplementary Table S6-10). These proteins were predicted by a whole-proteome scan using at least two of the following three methods for signal peptide prediction: SignalP4.0, Phobius, and SPOCTOPUS.

Transcriptome-based cell-cell communication analysis

We obtained the set of potential receptor-ligand interactions from the IUPHAR-International Union of Pharmacology (<https://www.guidetopharmacology.org/>)¹⁶⁶ and connectome database (https://fantom.gsc.riken.jp/5/suppl/Ramilowski_et_al_2015/vis/#/hive), which include a total of 695 ligands, 652 receptors, and 2695 ligand-receptor interactions, as described by Ramilowski et al. (2015)¹⁴³ (Supplementary Table S11). We also used analysis strategies provided by Armingol and Col (2021)¹³⁹ by calculating the product of the expression thresholding of the ligand-receptor and the expression of receptor-ligand in the respective analyzed protocols (Calculating by the function Ligand x 0.33 and Receptor x 0.33 by Log₁₀Padj < 0.05 value, where 1 for score communication would be positive). We obtained Benjamini Hochberg's false discovery rate [FDR] < 0.33 by assessing each interaction score's statistical significance to reduce possible false negatives or false positives in our analyses. To increase the list of positive interactions, we considered transcriptome genes, even if they were not predicted to be secreted in that type of physical exercise (Supplementary Table S12-13).

Enrichment analysis

We used EnrichR (<https://maayanlab.cloud/Enrichr/>)¹⁶⁷ as a performance tool for functional enrichment analysis in the generated lists: 1) secretome predicted genes, 2) genes predicted as ligands and receptors, and 3) genes scored as promoting positive ligand-receptor and receptor-ligand interactions. We used the Wiki Human pathway, Reactome Human pathways, KEGG Human pathway, and Gene Ontology databases. We filtered the lists of each functional enrichment database using an Adjusted P-Value <0.05, considering only the functional enrichment routes with statistical significance. We classified the -Log10Padj <0.05 values from highest to lowest and considered the top 10 for each condition analyzed.

Single-Cell Transcriptome Analysis of Human skeletal muscle tissues

We reanalyzed the scRNA-Seq GSE214544³¹ dataset available in the GEO database. We analyzed *vastus lateralis* muscle tissue samples from three patients before and after physical exercise in the modality of sprint exercise on a mechanically braked cycle ergometer (3 x 30s in a single section). Sample one refers to female subjects aged 26 years. Sample two refers to a 50-year-old male individual. Sample three refers to a 26-year-old male individual. Count data were downloaded and analyzed using R version 4.2.0, using the Seurat package (version 4.0.6)¹⁶⁸. We pre-processed the data from each sample and filtered the cells with counts of less than 200. We filtered the cells with >5% mitochondrial gene counts. After these steps, the data from each pre- and post-exercise sample were normalized using the LogNormalize method, and we used the FindVariableFeatures function considering the "vst" method. The data from the pre- and post-exercise samples were then integrated using the IntegrateData function in Seurat. The clusters were named by identifying the marker genes of each cluster (FindConservedMarkers function). We used the Enrichr platform to define the clusters using Tabula Sapiens and Cell Markers 2021.

Data representation and analysis

Correlation analysis and bar plots were constructed using GraphPad Prism (GraphPad Software, v.8.0.1). Venn diagrams were plotted using the webserver (<http://bioinformatics.psb.ugent.be/webtools/Venn/>). Heatmaps and Principal Component Analysis (PCA) plots were created using the web tools ClustVis (<http://biit.cs.ut.ee/clustvis/>)¹⁶⁹ and Morpheus (<https://software.broadinstitute.org/morpheus>)¹⁷⁰. Euclidean distance was used for clustering analysis of genes expressed in the secretome and the clustering analysis of

Spearman's correlation for the selected genes contributing to cell-cell communication. We used protein-protein interaction through STRING (<https://string-db.org/>) to identify the main interacting proteins. We utilized the parameters experiments, databases, co-expression, neighborhood, and co-occurrence as sources of active interactions. The minimum required interaction score was set at 0.700 (medium confidence). We visualized protein-protein interaction by Cytoscape v3.8.2 software¹⁷¹. We used the Circos plot (<http://circos.ca/>)¹⁷² for visualizing the comparisons between Ligand-Receptor interactions.

Results

Exercise modalities in aged groups induce distinct transcriptional profiles of secretome genes.

The list of differentially expressed genes from HIIT and RT in young and elderly individuals was filtered using the list of secreted proteins from The Human Protein Atlas (Supplementary Table S6). This analysis revealed 209 out of 2,641 Human Protein Atlas secretome genes that were differentially expressed in response to exercise training in individuals ($\log_2$ fold change ≥ 1.5) and P-adjusted value $p < 0.05$) (Supplementary Table S7 and Table S8). We identified the genes *COL19A1*, *ELN*, *COL1A1*, *COL3A1*, *LOXL2*, *LOX*, *THBS4*, *ASPN*, *COL4A1*, and *COL4A2* predicted to be in the secretome that presented Fold Change (Log2) variation in relation to the total number of DEGs (Supplementary figure S1). Most of the genes secreted with the upregulated profile were genes of the collagen family (Supplementary Figure S1). The expression profiles of the predicted genes in the secretome were similar between the young and elderly individuals. However, there was a slight difference in the expression between HIIT and RT physical exercise when clustered by Euclidean distance in the heatmap (Figure 1B). We analyzed the expression variation of expression values (FoldChange > 1.5) corrected by the Z-score. HIIT showed the most significant variation in expression levels in young and elderly individuals (Figure 1C). A principal component analysis (PCA) identified the clustering of predicted genes in the secretome based on training and age range (Figure 1D). The RT presented in young and elderly individuals presented close clusters, whereas the HIIT among young and elderly individuals presented distinct clusters. We analyzed the amount of differentially expressed genes (DEGs) predicted as from the secretome. HIIT had the highest number of DEGs (202) among the young and elderly individuals. In

contrast, RT had a lower number of DEGs (63) among individuals, specifically among older adults (Figure 1E). Finally, we analyzed the Venn diagram of the predicted genes in the secretome during the HIIT and RT exercises in young and elderly individuals. In total, 11 secretome genes and all conditions were present (Figure 1F). Most HIIT genes are shared between young and older individuals. All predicted secretome genes were present in the RT of young and old individuals (Figure 1F).

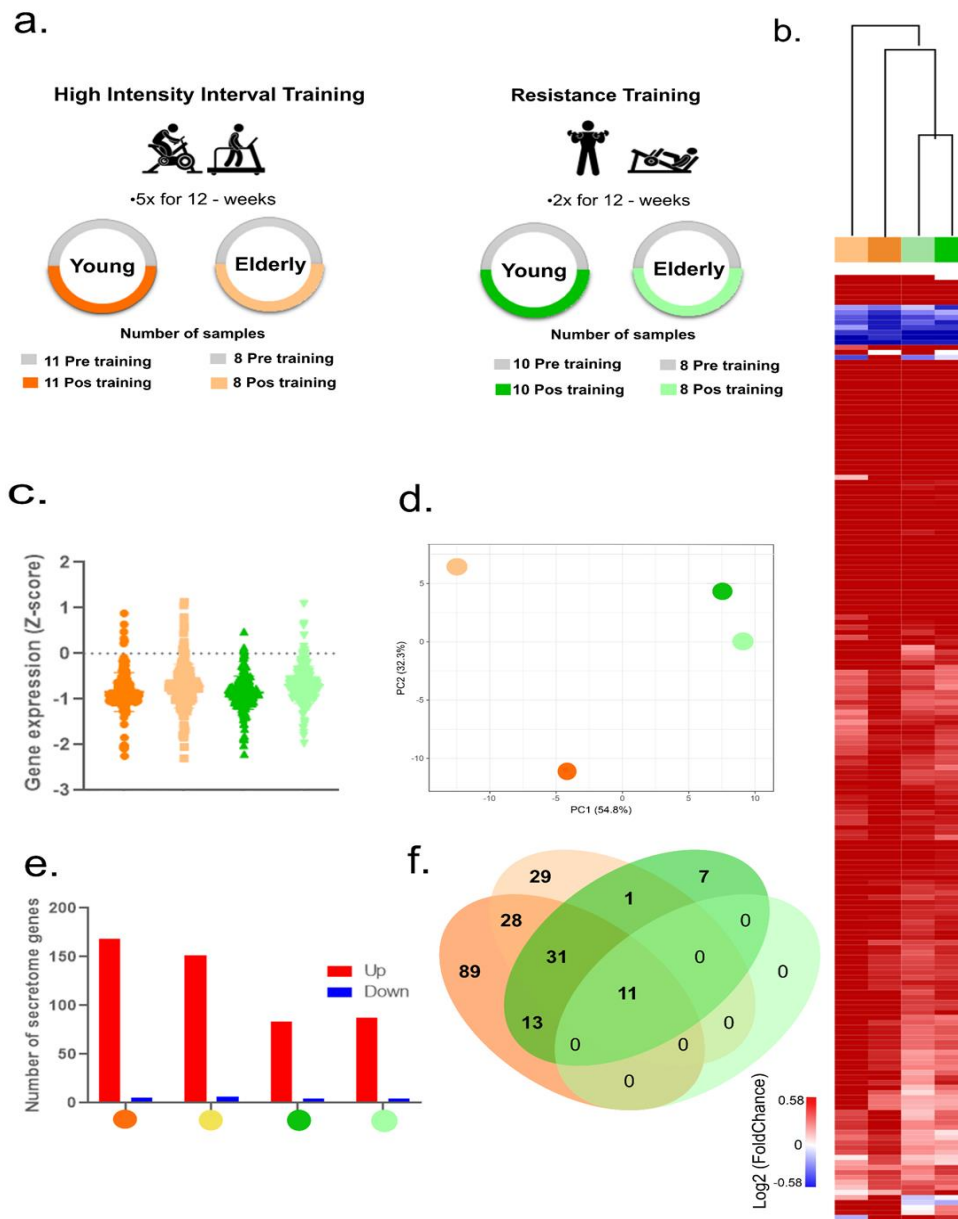


Figure 1. Gene expression analysis of the secretome components in the skeletal muscle from young and old individuals submitted to high-intensity interval or resistance training. (a) Characterization of vastus lateralis skeletal muscle samples (GSE97084) retrieved from Gene Expression Omnibus (GEO; <https://www.ncbi.nlm.nih.gov/geo/>). Young and elderly individuals following high-intensity interval training (HIIT) are represented in orange and light orange, respectively. Young and elderly individuals who

underwent resistance training (RT) are represented in green and light green, respectively. **(b)** Heatmap showing the effects of HIIT or RT on secretome gene expression ($\log_2 FC \geq 1.5$ and $FDR \leq 0.05$) in the vastus lateralis muscle of young and elderly individuals compared with controls. Rows (secretome genes) and columns (HIIT and RT in young and old individuals) were grouped using Euclidean distance. Differential expression levels were calculated using BioJupies (<https://amp.pharm.mssm.edu/biojupies/>). The resulting number of genes encoding secreted proteins was filtered based on the predicted secretome data available at the Human Protein Atlas (<https://www.proteinatlas.org/humanproteome/secretome>). **(c)** Dot plot of secretome gene expression levels (z-score-normalized). **(d)** Principal component analysis (PCA) of the secretome expression data. **(e)** Total number of differentially expressed secretome genes ($\log_2 FC \geq 1.5$, $FDR \leq 0.05$). The up- and down-regulated genes with absolute values of $\log_2 FC \geq 1.5$ and $FDR \leq 0.05$ (Statistics) are represented in red and blue, respectively. **(f)** Venn diagrams displaying the number of upregulated and downregulated genes shared by young and elderly individuals at HIIT and RT.

Secretome genes reveal the involvement of extracellular matrix components in muscle remodeling after exercise.

We evaluated the pathways enriched by the secretome that were common in all conditions and specific to HIIT and RT in young and elderly individuals. The pathways associated with secretome genes in all conditions were extracellular matrix organization, collagen extracellular matrix organization, collagen formation, collagen biosynthesis and modifying enzymes, assembly of collagen fibrils and other multimeric structures, protein digestion and absorption, miRNA targets in ECM and membrane receptors, ECM-receptor interaction, degradation of the extracellular matrix, focal Adhesion-PI3K-Akt-mTOR-signaling, ECM proteoglycans, and PI3K-Akt signaling (Figure 2). HIIT exercise enriched the highest number of pathways in young and old individuals. The axon guidance pathway was enriched only in elderly individuals who underwent HIIT. Pathway elastic fiber formation was present in young individuals, regardless of the exercise performed. The relaxin signaling pathway and diabetic cardiomyopathy were enriched during RT in young and elderly individuals. Pathways such as vesicle-mediated transport, senescence, Autophagy in Cancer, and signaling by PDGF were associated with RT exercise in elderly individuals (Figure 2). We investigated the genes *COL19A1* (Collagen Type XIX Alpha 1 Chain), *ELN* (Elastin), *COL1A1* (Collagen Type I Alpha 1 Chain), *COL1A3* (Collagen Type I Alpha 3 Chain), *COL3A1* (Collagen Type III Alpha 1 Chain), *LOXL2* (Lysyl Oxidase Like 2), *LOX* (Lysyl Oxidase), *THBS4* (Thrombospondin 4), *ASPN* (Asporin), *COL4A1* (Collagen Type IV Alpha 1 Chain) and *COL4A2* (Collagen Type IV Alpha 2 Chain) which participate in the most significant number of pathways.

HIIT training in young individuals resulted in the highest number of upregulated genes. Except for *LOXL2* and *COL4A1*, all these genes were predicted in the plasma (Supplementary Figure 2a). We investigated the protein-protein interaction network (PPI) of these genes, and the *COL3A1* gene presented the highest centrality (Betweenness Centrality=0.11), which allowed us to infer that this gene was in a central position in the interaction network (Supplementary Figure 2b). We investigated transcription factors (TFs) and kinases related to ECM gene regulation, considering $p < 0.05$ as statistically significant. The TFs *SUZ12*, *TRIM28*, and *EZH2* were predicted to regulate the most significant number of genes. Among the kinases, the most significant number of interactions was found for *CSMKZA1*, *MAPK1*, and *GSK38* (Supplementary Figure 2c).

The skeletal muscle interactome in response to physical exercise HIIT and RT induces interactions between extracellular matrix genes, integrins, and tyrosine kinase receptors.

We investigated the cellular communication of skeletal muscle in adaptive response to high-intensity interval training (HIIT) and resistance training (RT) in both young and elderly individuals. We selected the lists of 641 ligands and 589 receptors, and 1.894 pairs of ligand-receptor interactions reported by the study by Ramilowski et al., 2015¹⁴³ and compared them with the respective lists of the DEGs found in each condition analyzed herein (Supplementary Table S11). To infer cell–cell interactions and communication from gene expression, we filtered the p -adj value by Benjamini Hochberg false discovery rate [FDR] < 0.33 between predicted genes as ligands and receptors (Supplementary Table S12-13). The genes *COL1A1*, *COL1A2*, *COL3A2*, *COL4A1*, *COL5A1*, *COL6A1* (Collagen Type VI Alpha 1 Chain), *EFNA1* (Ephrin A1), *EFNB3* (Ephrin B3), and *IL34* (Interleukin 34) are up-regulated in all conditions. The *EPHB3* (EPH Receptor B3), *EPHB4* (EPH Receptor B4), *CSF1R* (Colony Stimulating Factor 1 receptor), and *NRP2* (neuropilin 2) receptor genes were upregulated under all conditions (Supplementary Figure 3a). Furthermore, most of the genes predicted to be ligands were also predicted to be located in the human secretome (Supplementary Figure 3a). Thirty-nine genes were predicted as ligands and 46 genes as receptors. Young individuals subjected to HIIT and RT had the highest number of expressed ligands and receptors (Supplementary Figure 3b). Ligand genes had higher FC values than receptor genes in our data (Supplementary Figure 3b). We then identified the functional enrichment of the predicted genes as ligands and receptors, considering the genes in common in the Biological Process and Molecular

Function ontology ($p_{adj} > 0.05$). The most common ontologies promoted by ligand-receptor interactions were extracellular matrix, axon guidance, positive regulation of epithelial cell proliferation, and leukocyte chemotaxis (Supplementary Figure 3c).

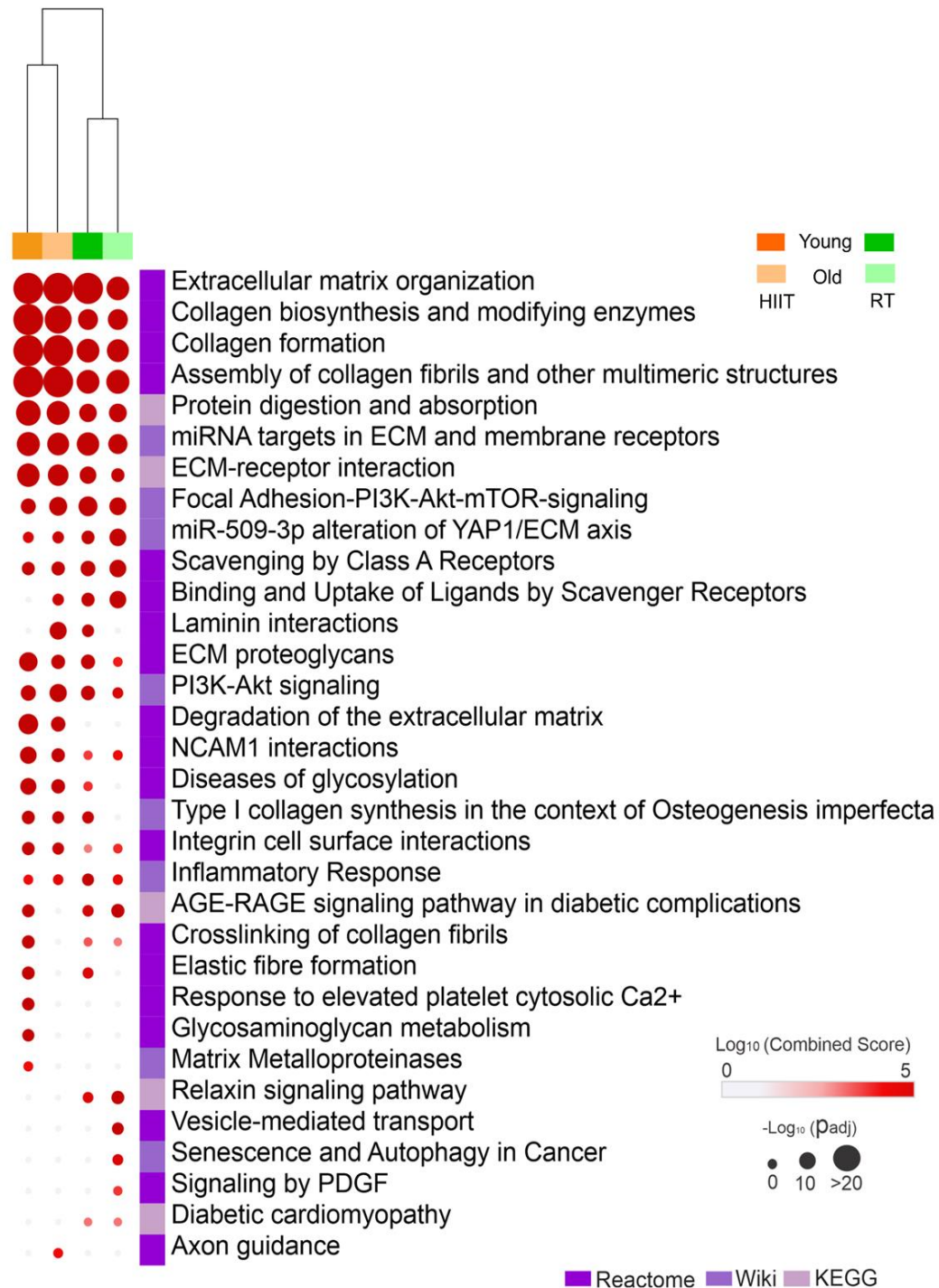


Figure 2. Pathway enrichment analysis of the differentially expressed secretome genes in the skeletal muscle of young and old individuals subjected to high-intensity interval or resistance training.

Functional Enrichment analysis was performed using the DEGs upregulated secretome genes ranked by the lowest adjusted P-value determined by the gene set enrichment analysis tool EnrichR

(<https://maayanlab.cloud/Enrichr/>). Pathways were included in the analysis when a $-\log_{10}(\text{Padj}) > 2$ score was presented in at least one condition. Heatmap rows and columns were grouped based on the Euclidean distance between $\log_2$ combined score values. The size of the dots corresponds to $-\log_{10}(\text{Padj})$, indicating the statistical significance of the pathways.

We calculated the LR interaction communication score ($\log_2$ FoldChange of the ligand vs. $\log_2$ FoldChange of the receiver) (Figure 3a). The HIIT and RT exercises in young individuals had the highest number of LR interactions with the highest communication score and P-adj significance (Figure 3a). These exercises also shared most of the LR interactions (Figure 3b). HIIT and RT exercises in older adults had the lowest number of LR interactions; however, HIIT demonstrated a more significant number of LR interactions and shared these interactions with young individuals (Figure 3a and b). Among the LR interactions, most ligands belong to the collagen gene family, including *COL1A1*, *COL1A2*, *COL4A1*, *COL18A1* (Collagen Type XVIII Alpha 1 chain), and *COL4A2* (Figure 3c). Among the LR interactions, most receptors belong to the alpha integrin genes families *ITGA1* (Integrin Subunit Alpha 1), *ITGB1* (Integrin Subunit Beta 1), and Integrin Subunit Alpha V (ITGAV), which interact with the tyrosine kinase receptor genes *DDR2* (Discoidin Domain Receptor Tyrosine Kinase 2), *DDR1* (Discoidin Domain Receptor Tyrosine Kinase 1), *FLT4* (Fms Related Receptor Tyrosine Kinase 4), and *EPHB3* (Figure 3c). We investigated the PPI of the genes predicted as ligands and receptors with higher significance values in LR interactions. Collagen genes primarily interact with *DDR1* and *DDR2* tyrosine kinase receptors and *ITGA1* and *ITGB1* integrin receptors (Figure 3d). We investigated the relationship between the genes that make up PPI and transcriptional factors. We sought to identify transcriptional factors that may alter gene expression of the genes that presented higher communication scores. The transcription factor *NFE2L2* modulates the transcription of most genes in the PPI network (Figure 3d).

***In silico* validation on scRNA-Seq data indicates ECM components produced by progenitor mesenchymal cells interacting with vascular endothelium cells**

To validate the LR interactions found in the bulk RNA-Seq data, we used scRNA-Seq from the vastus lateralis skeletal muscle in response to physical exercise in young and elderly subjects. Compared to bulk RNA-Seq analysis, scRNA-Seq analysis allows the identification of gene expression profiles in their respective cell types, even in less

abundant cell types. In the integration analysis, we identified 15.668 cells composing 21 clusters. Most clusters consisted of endothelial and mesenchymal cells. The main targets of the L-R interaction (*COL4A1*,

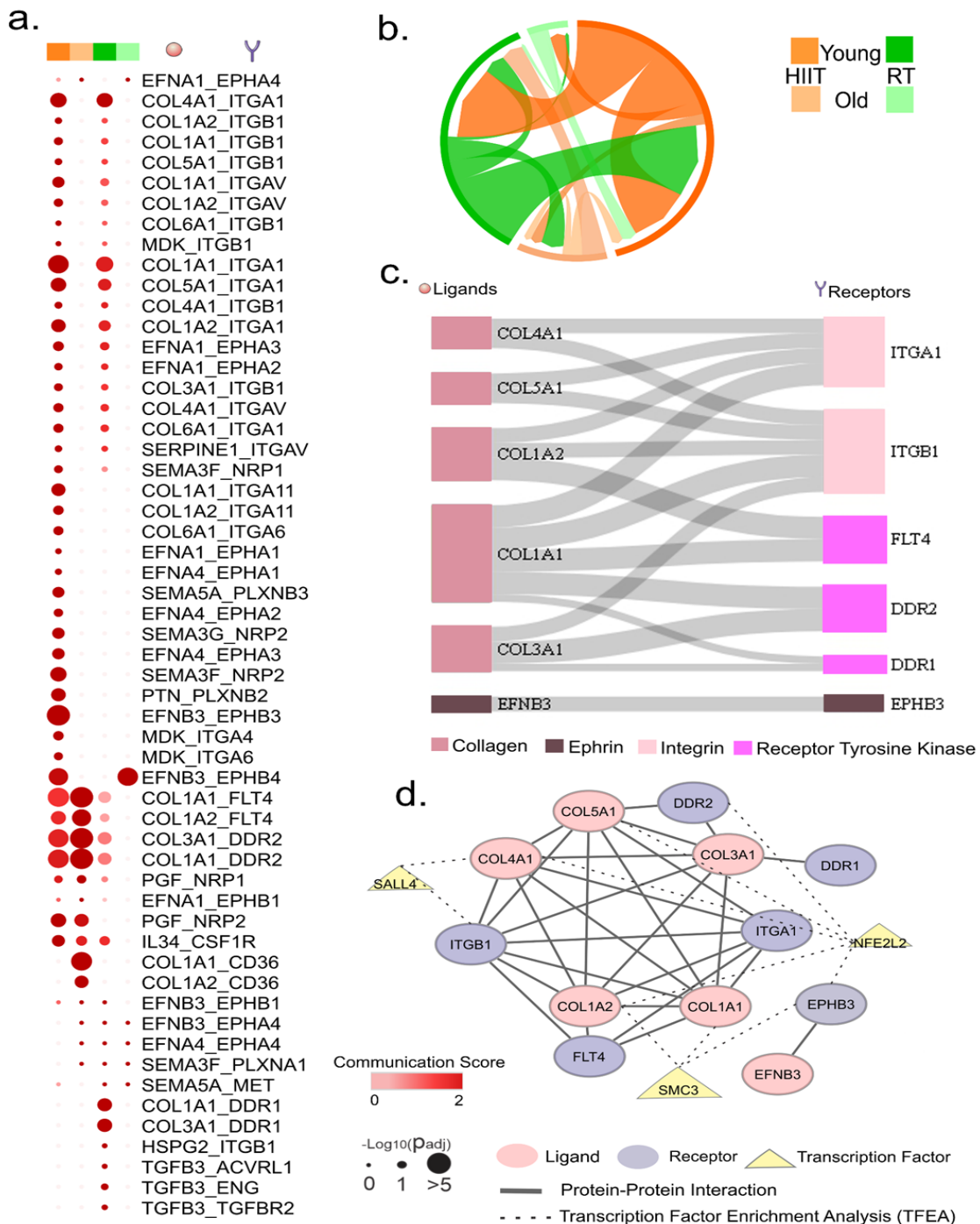


Figure 3. Expression thresholding of autocrine skeletal muscle interactions occurring in HIIT and RT. (a) Heatmap showing the Spearman correlation P_{adj} -value of selected interactions calculated as the expression thresholding by ligand ($L > 0.33$) and receptors ($R > 0.33$) in skeletal muscle among the genes associated with ligands. The size of the ball > 1 represents interactions that are significantly present in skeletal muscle (Benjamini Hochberg's false discovery rate [FDR] < 0.33). (b) Circus plot showing ligand-

receptor interactions in HIIT and RT exercise in young and elderly individuals. The outermost ring represents HIIT and RT exercises in young and elderly individuals, according to the color bar. The thickness of the lines represents the quantity of LxR interactions. **(c)** Representation of predicted genes as ligands and receptors with the highest number of LR (circle size) interactions. **(d)** Protein-Protein Interaction Network of the linkage between the 12 ligands and receptors that show more significant interactions. In yellow color, the main transcriptional factors predicted to modulate the identified genes.

COL1A2, *COL1A1*, *COL3A1*, *COL5A1*, *ITGA1*, *ITGB1*, and *DDR2*) were identified in clusters of mesenchymal satellite cells, progeny, vascular endothelium, and capillary cells. The interaction between *COL1A1* and *ITGA1* has been described in progenital mesenchymal and capillary endothelial cells (Figure 4a). Furthermore, the differential expression of these genes in these cell types was increased in the post-exercise samples (Figure 4a).

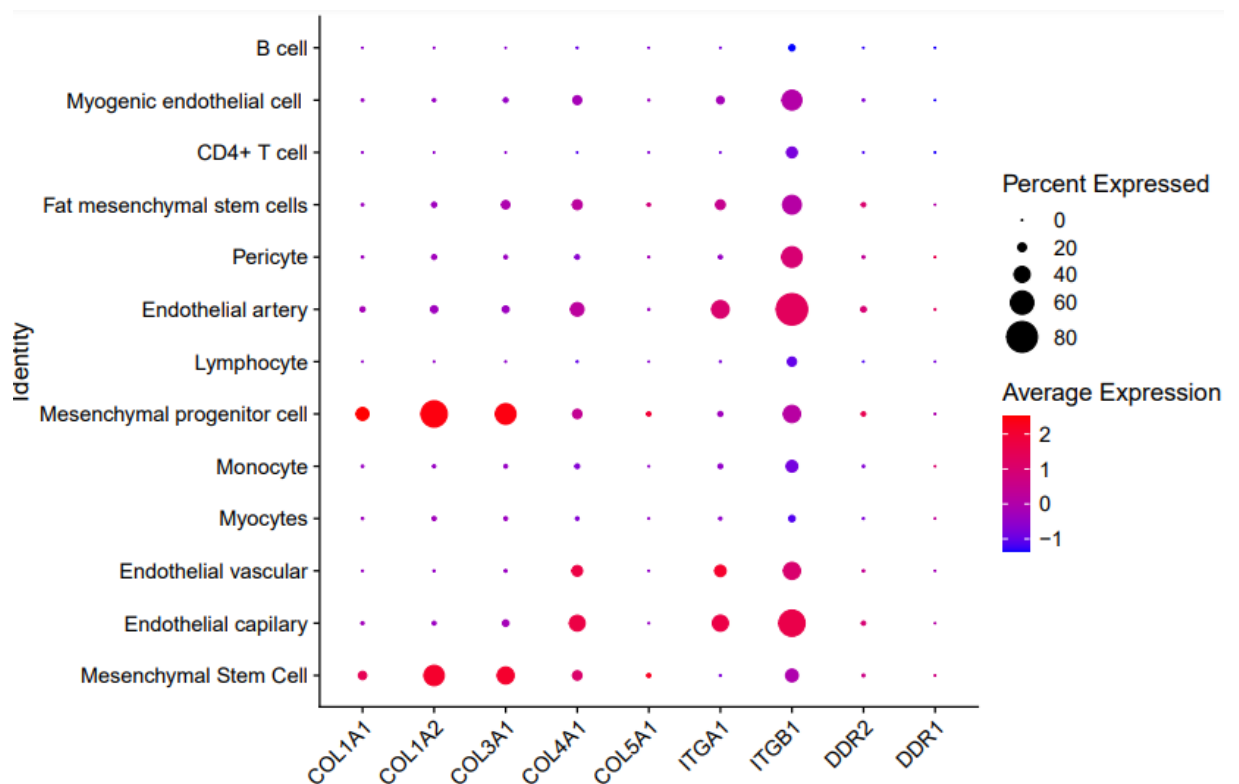


Figure 4. Single-cell RNA-Seq integration analysis reveals cellular communication between satellite, mesenchymal, and endothelial cells in skeletal muscle in response to physical exercise. Characterization of cell type and gene expression by single-cell sequencing analysis of vastus lateralis muscle samples. The “identify” refer to the cell types of skeletal muscle. “Features” refer to the genes that have been identified in cellular communication. The “Percent Expressed” is the quantity of cells. “Average expression” represents the gene expression profile.

Discussion

Although many studies have focused on cytokines, many other peptides¹⁷³, extracellular vesicles¹⁷⁴, and metabolites¹⁷³ are released in response to contraction muscle. Skeletal muscle signaling molecules are essential for tissue metabolism and other tissues and organs¹⁷⁵. Here, we characterized the secretome and autocrine signaling of skeletal muscle tissue in response to HIIT and RT exercise in young and elderly individuals using the bulk RNA-Seq public dataset. Our results demonstrate that exercise induces gene expression alterations in secretome genes associated with ECM in the muscle. Collagen molecules (COL1 and COL4) interact with integrin receptors (*ITGA1* and *ITGB1*) and tyrosine kinases (*DDR1* and *DDR2*). This signaling occurs between the satellite cells, mesenchymal progenitors, and endothelial cells. Here, we predict that ECM components play key roles in the muscle environment, mainly the activation of satellite cells in muscle fiber regeneration and capillary angiogenesis in muscle tissue. In addition, our results suggest a more significant signaling among young individuals than among elderly individuals in muscle regeneration in response to HIIT and RT physical exercise. Our strategy allowed us to infer the secreted molecules of skeletal muscle and autocrine/paracrine signaling. These findings are relevant to muscle physiology and provide a better understanding of the molecules associated with muscle hypertrophy and hyperemia.

In our muscle secretome prediction in response to HIIT and RT exercise in young and old individuals, we found the largest number of genes predicted to be in the human secretome in HIIT, regardless of age group. The original study of the dataset analyzed here revealed that HIIT has a more robust increase in gene transcripts than other exercise modalities, such as combined training and resistance training. Furthermore, HIIT reversed many age-related differences in the proteome, particularly by increasing mitochondrial protein synthesis. In addition, all types of training, including combined training, RT, and HIIT, induced similar extracellular matrix (ECM) transcriptional profiles in both aged groups¹⁵¹. Miyamoto-Mikami et al. (2018)¹⁷⁶ conducted a study that analyzed the transcriptome of the skeletal muscle (*vastus lateralis*) in young males using the HIIT protocol for nine weeks. The study found genes with increased expression in the ontology of glucose metabolism, ECM organization, angiogenesis, and the mitochondrial membrane.

We observed that regardless of the physical exercise protocol, most genes that increase their expression in skeletal muscles are implicated in ECM pathways. Exercise

with acute and long-term physical activity for more than 12 weeks promotes increased expression of several ECM component genes (specifically collagen types I, III, and IV) and proteoglycans in young and elderly individuals^{177,178}. Amar and colleagues (2021)¹⁷⁹ conducted a meta-analysis in 2021, using data from skeletal muscle or blood from 43 studies, including 739 individuals before and after exercise or training in different exercise protocols, which also describes increased gene expression in ECM ontology. Few studies have focused on the whole muscle secretome in response to physical exercise in humans^{127,180}. Most genes with increased expression predicted in the secretome in our study were genes associated with ECM signaling pathways. This suggests that the increase in expression of genes encoding ECM components is also reflected in the increase in molecules secreted by the muscle, regardless of the individuals' exercise protocol (HIIT or RT) and age.

Evidence has demonstrated that exercise training induces muscle remodeling, which is accompanied by ECM adaptation⁴². All exercise interventions promoted a widespread increase in ECM collagens, glycoproteins, and proteoglycan genes in younger and older participants⁴². The ECM is important for growth³⁵, muscle repair³⁶, as well as for the transmission of contractile force³⁷. In fact, ECM components play an important role in muscle satellite cell (MuSC) activation during muscle repair¹⁸¹, as well as in angiogenic processes in muscle³³. Our results indicate that most of the predicted ligand-receptor interactions occurred between collagen family genes (*COL1A1*, *COL3A1*, *COL4A1*, and *COL5A1*), integrin genes (*ITGA1* and *ITGB1*), and tyrosine kinase genes (*DDR1* and *DDR2*), independent of physical exercise HIIT and RT in young and elderly individuals. Collagen is the major structural protein in skeletal muscle ECM, and types I and III collagens represent 75% of the total muscle collagen¹⁸². Moreover, receptors and regulators, such as integrins and matrix metalloproteinases (MMP), are also present in the ECM¹⁸³. Here, we demonstrated the importance of ECM components in skeletal muscle angiogenesis in response to physical exercise. Angiogenesis induced by physical exercise in skeletal muscle is well recognized in the literature as an adaptive physiological response due to its benefits in improving the health of tissues such as cardiovascular and skeletal muscles¹⁸⁴. Capillary density in skeletal muscle is increased by physical exercise and this increase varies according to muscle fiber type¹⁸⁵. However, understanding the ECM molecules involved and their role in forming new capillaries in the muscle is an emerging field in muscle physiology in adaptation to exercise¹⁸⁶.

During exercise, there is a substantial increase in the blood flow to the muscles, which results in increased shear stress in the blood capillaries. This, in turn, stimulates the process of new blood capillary formation known as angiogenesis¹⁸⁶. Angiogenesis in physical exercise occurs by changing the gene expression pattern in endothelial cells¹⁸⁶. ECM components are crucial for all stages of angiogenesis¹⁸⁷. Integrins are cell adhesion receptor proteins located in the plasma membrane that bind to the cytoskeleton of ECM. They are responsible for transmitting biochemical and mechanical signals between cells and the surrounding environment and are important for the regulation of several cellular processes in health situations and diseases¹⁸⁸. In our findings, *ITGA1* and *ITGB1* integrins had the highest number of interactions with collagen ligands in an adaptive response to physical exercise. In addition, integrin genes showed increased expression in endothelial cells (capillary, vascular, and arterial) in scRNA-Seq integration data from young and elderly individuals after a physical exercise session (Figure 4). The study by Perruzzi et al (2003)¹⁸⁹ demonstrated that the interaction between type I collagen and $\alpha11$ and $\alpha2\beta1$ integrins, present in human dermal microvascular endothelial cells, as well as in mouse model, can activate the MAPkinase pathway and, consequently, promote angiogenesis, cell survival, and inhibit apoptosis. Indeed, deletion of $\beta1$ integrin (*Itgb1*) in mice reduced endothelial cell survival and showed the importance in adhesion and migration¹⁹⁰. However, despite the importance of integrins in muscle remodeling and angiogenesis in endothelial cells, few studies have investigated the role of integrins and their signaling in the muscle microenvironment in response to physical exercise. Our data point to important targets for the investigation of muscle angiogenesis in adaptive responses, in addition to providing our insights.

Another important point to be discussed is that the interaction between collagen components and integrin receptors may induce angiogenesis and the activation of satellite cells and cell proliferation and differentiation in muscle regeneration in response to physical exercise. However, the mediating factor (e.g., *IGF* and *VEGF*) signals the specific cellular response in the cells that comprise the muscular environment¹⁸⁶. The relationships and interactions between MuSCs and endothelial cells have been increasingly elucidated⁵⁸. *In vitro* and *in vivo* evidence has shown that endothelial cells and myogenic precursor cells interact with myogenesis and angiogenesis during skeletal muscle regeneration⁵⁵. In our findings, most interactions in HIIT and RT exercises occurred between ligands and collagens, present in mesenchymal cells (progenitor and

stem cells), and integrin receptors (*ITGA1* and *ITGB1*) present in endothelial cells (capillary, vascular, and arterial). These interactions, specifically *COL1A1* and the integrin receptor *ITGB1*, have already been described in 3D skeletal muscle organoid experiments on rapid adhesion cells (RACs) and skeletal muscle myogenic progenitor cells (MPCs)¹⁹¹. We hypothesized that integrins (*ITGA1* and *ITGB1*) are essential in muscle satellite and endothelial cells during muscle regeneration. Rozo et al. (2016)¹⁹² demonstrated the interactions between satellite cells and the muscle microenvironment in mice. The main findings of the study conclude that augmenting $\beta 1$ integrin activity with a monoclonal antibody restores FGF-2 sensitivity and improves regeneration after experimentally induced muscle injury. Knockout of the $\alpha 1$ integrin subunit in mice resulted in a decrease in the expression of collagen type I and III genes, TGF- $\beta 1$ and IGF-1 in the muscle or tendon¹⁹³. During skeletal muscle development, alpha 1 integrin is expressed in myogenic precursors, during myoblast migration, and in differentiating myotubes. These results indicate the potential involvement of alpha 1 integrin in the skeletal muscle repair process¹⁹⁴. In fact, with advances in skeletal muscle research, the description of new components that induce differentiation and proliferation of satellite cells and angiogenesis has been increasing in recent years. However, it is essential to point out that muscle development during embryogenesis occurs with the same molecules inducing different responses in each cell type and that this same pattern is repeated in adults¹⁸⁶.

We conclude that ECM molecules are crucial in the muscle microenvironment, play a fundamental role in cell communication, and consequently act in the activation of MuSCs and vascularization during muscle regeneration. We highlight the importance of ECM molecules in communication in the muscle microenvironment for activating stem cells, differentiation, and proliferation of muscle fibers. Furthermore, we demonstrated communication between mesenchymal cells and endothelial cell integrin receptors. These targets are essential for elucidating muscle autocrine signaling during muscle regeneration in response to physical exercise. Finally, we demonstrate that muscle signaling decreases with age, and collagen and integrin molecules can be interesting targets for functional studies in response to pathological conditions.

Limitations

Our study could not predict the signaling pathways in mesenchymal and endothelial cells in the context of intracellular active mechanisms, and we were unable to validate these

findings in samples of humans in different modalities of physical exercise. Unfortunately, we could not compare transcriptome analysis by scRNA-Seq in response to chronic exercise in young and elderly individuals.

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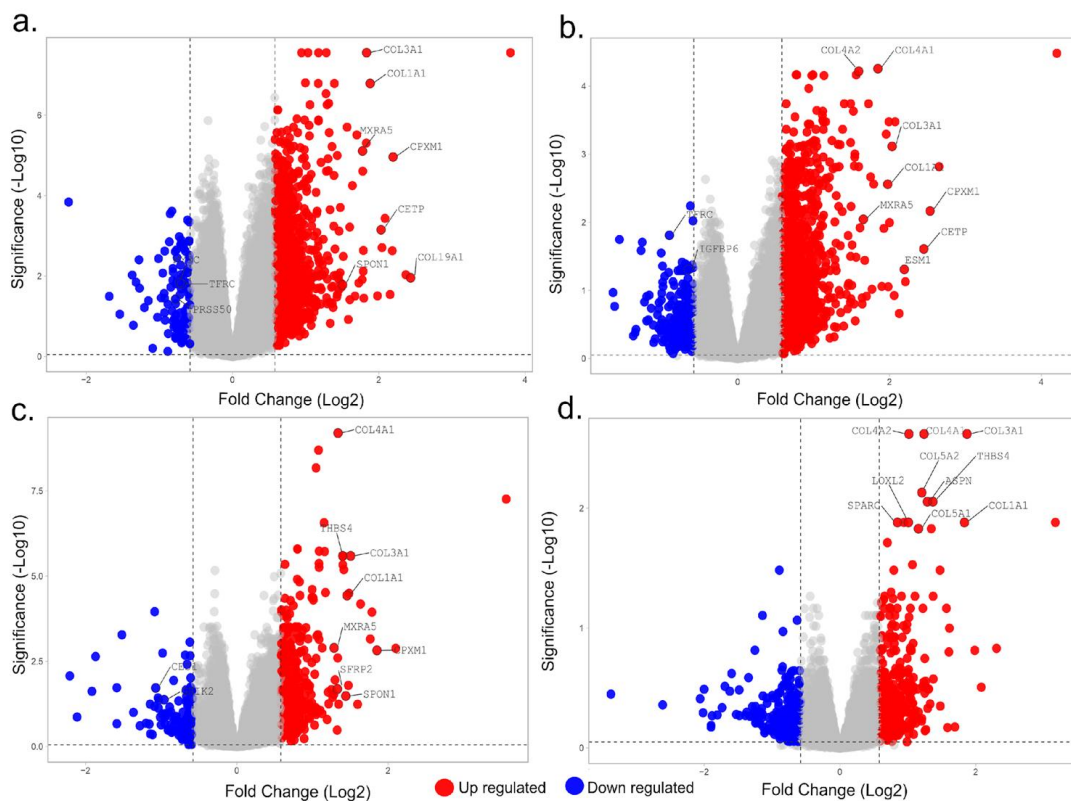
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Data Availability Statement: Publicly archived datasets analyzed herein are available at the Gene Expression Omnibus (GEO) under accession numbers GSE97084 and GSE214544.

Conflict of interests

The authors declare that they have no conflicts of interest.

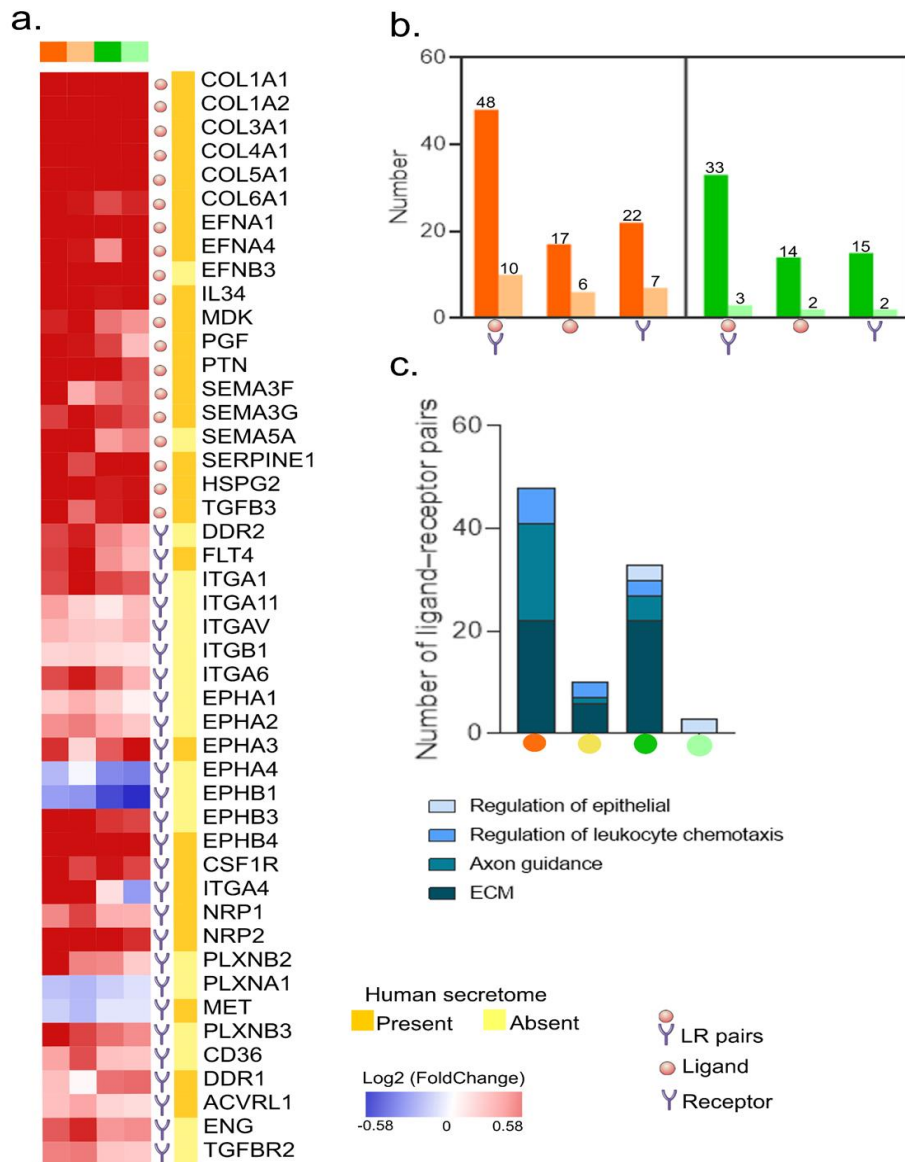
Supplementary Figures



Supplemental Figure 1. Differential expression analysis results of different physical exercises in young and old individuals in genes predicted in the secretome. The volcano plots in A-D visualize the top 10 secretome genes by values of the adjusted p-value and the corresponding log2-fold change of the differential expression analyses between **(a)** HIIT in young individuals. **(b)** HIIT in elderly individuals. **(c)** RT in young individuals. **(d)** RT in elderly individuals.

Supplemental Figure 2. Regulatory pathways and upstream regulatory networks from differentially expressed secretome genes in the skeletal muscle of young and old individuals following exercise high-intensity interval or resistance training. (a)

The heat-scatterplot presenting the log₂FCs and corresponding significance (false discovery rate [FDR]-adjusted p values) for the ten genes secreting making up the top present in most common pathways in the HIIT and Rt exercise in young and old. Heatmap rows and columns were clustered based on Euclidean distance between log₂ Fold Change values. **(b)** Cytoscape protein-protein interaction analyses *COL3A1* with the highest Betweenness Centrality value (0.117). **(c)** The eXpression2Kinases (X2K)¹⁹⁵ network of genes *COL19A1*, *ELN*, *COL1A1*, *COL1A3*, *COL3A1*, *LOXL2*, *LOX*, *THBS4*, *ASPN*, *COL4A1*, and *COL4A2* found in enriched pathways. The network includes kinases (blue), intermediate proteins (gray), and transcriptional factors (red) predicted to regulate the expression of the top 10 deregulated secretome genes found in Enrich pathways.



Supplemental Figure 3. Characterization of cell-cell communication of muscle transcriptome in HIIT and RT exercise in young and elderly individuals (a) Heatmap of the expression values of log₂FC associated as ligands or receptors in exercise HIIT and RT compared to their respective controls. **(b)** Bar graph representing the number of LR interactions, predicted genes as ligands and receptors. In orange is the HIIT exercise in young people, and light orange the HIIT exercise in older adults. In green, the RT exercise in young people, and light green the RT in older adults. **(c)** A 100% stacked column graph displaying the distribution of the number of LR interactions according to ontology (biological process and molecular function) filtered in the EnrichR database (<https://maayanlab.cloud/Enrichr/>).

Chapter 2: Interactome-transcriptomics-based analysis in multiple models of muscle atrophy

Jakeline Santos Oliveira¹, Sarah Santiloni Cury¹, Amanda Piveta Schnepfer¹, Ana Luiza Labbate Bonaldo¹, Victória Larissa Schimdt Camargo¹, Caio Fernando Ferreira Mussatto¹, Paula Paccielli Freire², Maeli Dal Pai-Silva¹, Geysson Javier Fernandez³ & Robson Francisco Carvalho^{1*}.

¹ Department of Structural and Functional Biology, Institute of Biosciences, São Paulo State University, UNESP, Botucatu, São Paulo, Brazil.

² Department of Immunology - University of São Paulo USP, São Paulo, Brazil.

³ Faculty of Medicine, University of Antioquia, UdeA, Medellín, Colombia.

***Correspondence:**

Professor Robson Francisco Carvalho
Department of Structural and Functional Biology
Institute of Biosciences, São Paulo State University - UNESP
CEP: 18.618-689, Botucatu, São Paulo – Brazil
Telephone number: +55 14 3880 0473
robson.carvalho@unesp.br

Abstract

The loss of skeletal muscle mass reduces quality of life and increases mortality risk in humans. Skeletal muscle autocrine communication in different conditions that lead to muscle wasting is crucial for clarifying genes and which pathways can demilitarize the muscle atrophy profile. Here, we investigate the interactome of conditions leading to muscle wasting in mice. Skeletal muscle autocrine communication performed using Bulk RNA-Seq from mice under atrophy conditions (cancer-associated cachexia, aging, diabetes, and disuse) reveals the interactions between ligand genes and receptors. We demonstrate that the *Selpg*, *Il1b*, *S100a8*, and *S100a9* genes interact with the *Cxcr2*, *Il1r1*, *Sell*, *Sele*, and *Tlr4* receptor genes, respectively. These interactions were more frequently identified in cancer-associated cachexia. These genes are in neutrophils, macrophages, NK cells, and monocytes in the skeletal muscle. The results provide novel insights into the adaptive cell-in-response communication of skeletal muscle to conditions of muscle atrophy. In addition, it directs toward the identification of potential therapeutic targets.

Keywords: interactome, mRNA, muscle atrophy, atrophy conditions, muscle cell communication.

Introduction

Skeletal muscle atrophy is a debilitating condition associated with different catabolic states such as aging, disuse, and chronic diseases⁷³. Consequently, there is a reduction in muscle mass and strength, which reduces the quality of life and increases the risk of death⁷³. Skeletal muscle tissue is responsible for locomotion, breathing, feeding, and energy expenditure, as well as for glucose, homeostasis of amino acids, lipids, and an essential reservoir of proteins⁵⁹. However, each catabolic condition differs in terms of susceptibility to muscle atrophy, and this is because muscle is composed of muscle different fiber types (slow and fast twitch) and metabolic properties⁶⁰. Therefore, investigating molecules that trigger muscle atrophy in chronic diseases, aging, and disuse is paramount for preventive medicine for chronic diseases, aging, and disuse^{59,60}.

The muscle microenvironment is composed of various cell and molecular types in which muscle fibers are wrapped²⁹. Immune cells, fibroblasts, blood vessels, and extracellular matrix molecules are crucial for muscle satellite cell activation and regeneration during muscle repair²⁹. Changes in muscle homeostasis due to changes in cell composition and changes in cell interactions activate compensatory responses to restore balance¹⁹⁶. If these compensatory responses occur exaggeratedly in the muscle microenvironment, it can lead to muscle atrophy¹⁹⁷. Inflammation is a typical process in many diseases. Long-term inflammatory changes alter the muscle microenvironment by activating muscle atrophy pathways in muscle cells. Pro-inflammatory cytokines, such as TNF- α , IL-6, interleukin-1 (IL-1), and interferon- γ (IFN- γ), have been correlated with cancer-associated cachexia¹⁹⁸. These molecules act as signaling ligands, directly and indirectly impacting muscle and adipose tissue metabolism and inducing muscle atrophy¹⁹⁶.

Furthermore, different immune cells, such as neutrophils and monocytes/macrophages in pathological conditions, can play dysfunctional responses in tissues¹⁹⁹. In this way, inflammatory cells and molecules play an essential role in activating muscle catabolism pathways; however, it is a complex process with specific mechanisms¹⁹⁷. Understanding molecules and molecular mechanisms of muscle atrophy by inflammation is fundamental for identifying pharmacological targets.

Large-scale transcriptomic data have been instrumental in describing and identifying the molecular factors involved in muscle adaptation²⁰⁰. However, most studies used gene expression data from microarray analysis. In recent years, the application of RNA-seq to

the analysis of muscle atrophy has provided novel insight into the transcriptional changes associated with atrophy and muscle homeostasis⁷³. Skeletal muscle is known to respond to an affinity of systemic signals vital for body homeostasis, and muscle fiber is responsive to externally received global styles²⁰¹. Skeletal muscle microenvironment interactions have established functions in muscle response to regeneration from injury, growth, aging, overload-induced hypertrophy, and exercise²⁰². However, the integration of bulk-RNA analysis for investigating skeletal muscle autocrine communication under different conditions of muscle atrophy is missing.

Here, we show that interactions between ligand genes *Selpg* and receptors genes *Cxcr2*, *Sell*, and *Sele* were more significant in the condition of cancer-associated cachexia. These interactions are present in the cell types of macrophages M2, NK cells, and endothelial cells in skeletal muscle. Furthermore, these genes are present in biological processes of muscle cell differentiation and inflammatory processes. Our results demonstrate that the interaction profile is different between muscle atrophy conditions. Our findings reveal new insights and potential therapeutic targets in muscle wasting.

Material and methods

Differential expression analysis of RNA-Seq data for pathological conditions in muscle wasting

We considered datasets that provided the raw RNA-seq data in counts of 22 muscle samples from gastrocnemius biopsies in C57BL/6J mice male. These datasets are available in Gene Expression Omnibus (GEO) under the accession numbers GSE114820 (protocol cancer-cachexia)²⁰³, GSE161693 (protocol diabetes mellitus and disuse)²⁰⁴, and GSE139204 (protocols aging)²⁰⁵ (Supplementary Table S1). We use the Biojupies platform (<https://amp.pharm.mssm.edu/biojupies/>)²⁰⁶ to identify differentially expressed genes (DEGs) before and after treatment of each protocol, regardless. Genes with Log2 of fold change $\geq |2.0|$ and false discovery rate (FDR) < 0.05 were considered DEGs (Supplementary Figure 1-4 and Supplementary Table S2). To validate our data reanalysis strategy, we compared genes that appeared in at least one condition (cancer-cachexia associated, aging, diabetes, and disuse) with the list of genes described as atrogenes in the studies by Taillandier et al. 2019⁶⁹ and Jagoe et al., 2002²⁰⁷ (Supplementary Table S3).

Bulk-RNA interactome analysis

We obtain the list of potential Ligand-Receptor interactions from the CellTalkDB database (<http://tcm.zju.edu.cn/celltalkdb/>)²⁰⁸, which includes a total of 651 ligands, 588 receptors, and 2033 ligand-receptor interactions, as described by Shao et al., 2020²⁰⁸ (Supplementary Table S4). We also used analysis strategies provided by the study by Armingol and Col (2021)¹³⁹ that provide a way to examine interactions and apply them in different contexts. We evaluated the interactions of each DEGs of the various conditions by calculating the product of the expression thresholding of the ligand-receptor and the expression of receptor-ligand in the respective analyzed protocols (Calculating by the function $\text{Ligand} \times 0.33$ and $\text{Receptor} \times 0.33$ by $\text{Log10Padj} < 0.05$ value, where 1 for score communication would be positive). We obtained Benjamini Hochberg's false discovery rate [FDR] < 0.33 by assessing each interaction score's statistical significance to reduce possible false negatives or false positives in our analyses (Supplementary Table S5 and S7).

Enrichment analysis

We used EnrichR (<https://maayanlab.cloud/Enrichr/>)¹⁶⁷ as a functional enrichment tool for the genes: 1) Differentially expressed between the analyzed conditions; 2) Of the genes predicted as ligands and receptors in cellular communication. We use the databases of Gene Ontology terms and The Molecular Signatures Database (MSigDB). We filtered the lists of each functional enrichment database using an adjusted P-Value < 0.05 , considering only the functional enrichment routes with statistical significance. We classified the $-\text{Log10Padj} < 0.05$ values from highest to lowest and considered the top 10 for each condition analyzed (Supplementary Table S6 and S8).

Single-Cell Transcriptome Analysis of *mus musculus* skeletal muscle

We performed a reanalysis of the scRNA-Seq GSE172410³² dataset available at GEO. The experiment design used a mix of muscle tissue mononuclear cells (C57BL/6) young (6 months, $n=3$) and old (24 months, $n=3$) in mice. For scRNA-seq, the Illumina HiSeq 4000 platform was used, and the raw data were processed with Cell Ranger (v3.0) (10X Genomics) and aligned to the mouse genome version mm10. Data analysis was performed using R version 4.2.0 using the Seurat package (version 4.0.6)¹⁶⁸. After completing the data integration, we normalize and identify variable features for each mouse sample independently. In clusters where naming was not possible, we used the

FindMarkers function in Seurat and filtered the top ten markers based on log-fold changing values and confirming the cell type in the Enrichr database in the Tabula Muris, CellMarker, and Mouse Gene Atlas libraries.

Protein-Protein Interaction Network

The proteins identified in the interactome were submitted to the STRING database (Search Tool for Retrieval of Interacting Genes, version 11.5)²⁰⁹ for the construction of a protein-protein interaction network of genes in common in at least three muscle wasting conditions. For constructing networks, we consider experiments, databases, co-expression, neighborhood, and co-occurrence as sources of active interaction. The minimum interaction score required was 0.400 (medium confidence), and nodes disconnected from the network were hidden to simplify the display. The PPI enrichment p-value indicates the statistical significance provided by STRING (accessed June 2022). We visualized protein-protein interaction by Cytoscape v3.8.2 software¹⁷¹.

Data representation and analysis

Correlation analysis and bar plots were constructed with GraphPad Prism (GraphPad Software, v.8.0.1). Venn diagrams were plotted using the webserver <http://bioinformatics.psb.ugent.be/webtools/Venn/>. Heatmaps and Principal Component Analysis (PCA) plots were created using the web tools ClustVis (<http://biit.cs.ut.ee/clustvis/>)¹⁶⁹ and Morpheus (<https://software.broadinstitute.org/morpheus>)¹⁷⁰. Clustering Euclidean distance analysis for grouping the profile of genes expressed in the secretome and the clustering analysis of Spearman's correlation for the genes expressed in the secretome in cell-cell communication. We used the Circos plot (<http://circos.ca/>) for visualizing the comparisons between Ligand-Receptor interactions¹⁷².

Results

The gene expression profile is different between conditions associated with muscle atrophy

To examine how conditions (cancer-associated cachexia, aging, diabetes, and disuse) can induce muscle atrophy, we performed a reanalysis of RNA sequencing (RNA-seq) datasets from the gastrocnemius muscle in male mice (Supplementary Table S1). We found 2,216 differentially expressed genes (DEGs) ($\log_2 \text{FC} \leq -1$ or ≥ 1 and $\text{FDR} \leq 0.05$) present in at least one of the four conditions analyzed (Supplementary Table S2).

Clustering by Euclidean distance grouped two groups with an inverse gene expression profile and the conditions of cancer-associated cachexia and aging with a similar expression profile. Diabetes and disuse conditions also have similar gene regulation profiles (Figure 1a). Principal component analysis (PCA) revealed differences in gene expression profiles of cancer-associated cachexia (PCA-2) and aging conditions. Diabetes and disuse conditions showed clustering similarities (Figure 1b). We identified 295, 76, 775, and 768 up-regulated and 434, 30, 351, and 409 down-regulated in muscle atrophy conditions caused by cancer-associated cachexia, aging, diabetes, and disuse (Figure 1c and Supplementary Table S2). Notably, transcriptome changes in muscle atrophy conditions were more drastic in cancer-associated cachexia, diabetes, and disuse than in aging. The portion of DEGs in common among all conditions was considerably small; only the genes *Col1a1*, *Neto2*, *Kera*, *Rmi2*, and *Amd1* are present in common (Figure 1d).

Comparison of conditions with muscle atrophy marker genes (atrogenes)

We found 221 genes described as atrogenes grouped into four clusters (Figure 2a). When we considered statistical significance ($\text{Log2FC} < 2$; $\text{FDR} > 0.05$), although most genes in common from the conditions and atrogene literature were in cluster two, few genes with statistical significance were found in our data (Figure 2b). Most genes with statistical significance were in clusters one, three, and four (Figure 2b). In cluster one, the enriched pathways were muscle contraction, myogenesis, and actin-myosin filament (Figure 2c). The *Myl1*, *Myl3*, and *Mylpf* genes have been described in these pathways, and, in our findings, they were down-regulated in the condition of cancer-associated cachexia (Figure 2d). Metabolic processes such as carbohydrate, pyruvate, and glycolytic catabolism were also present in this cluster (Figure 2c); The *Pgam1*, *Pgam2*, and *Eno3* genes were up-regulated in the conditions of cancer-associated cachexia, diabetes, and disuse (Figure 2d). Cluster three presents' genes involved in protein ubiquitination pathways, proteasome mediators, cell cycle, intrinsic apoptotic signaling, and TNF-alpha signaling via NF-kB and mTORC1 signaling (Figure 2c). These processes are enriched with genes *Cul1*, *Cul3*, *Fbxo32*, *Gadd45a*, *Cdkn1a*, *Ppp1r15a*, *Eif2s3x* and *Eif2s3y* that form two distinct networks of protein-protein interactions (Figure 2d). In our data, these genes are upregulated in most conditions analyzed (Figures 2a and 2b). Cluster four shows the enrichment of extracellular structure organization, collagen fibril organization, epithelial-mesenchymal transition, and angiogenesis pathways (Figure 2c). *Col1a1*, *Col3a1*, *Col5a1*, *Col6a1*, *Fn1*, and *Fbn1* genes are present in these pathways, in addition

to protein-protein interaction (Figure 2d). Our data shows these genes are downregulated in aging, diabetes, and disuse (Figures 2a and 2b).

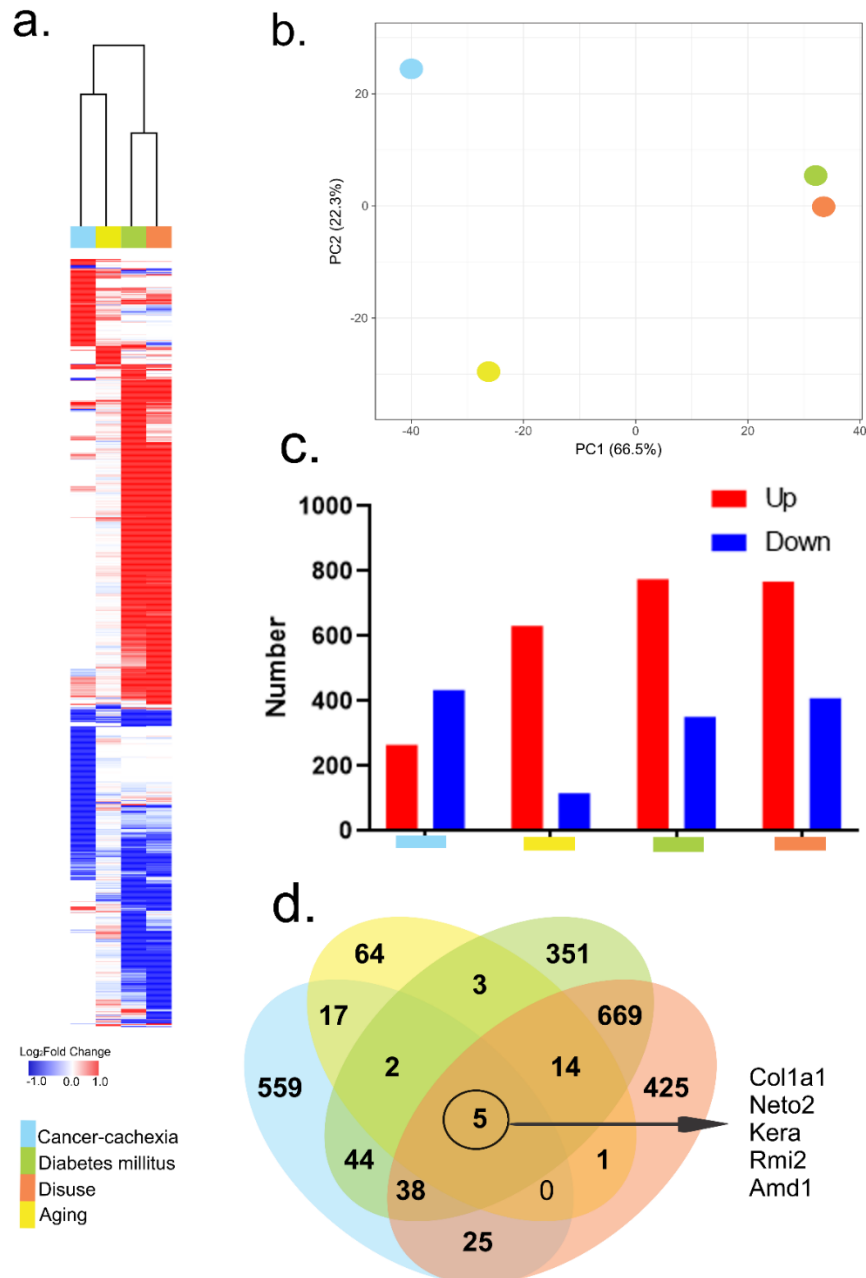


Figure 1. Alteration of the transcriptome profile in skeletal muscle atrophy conditions. Hierarchical clustering analysis of differentially expressed genes after the induction of each atrophy model. (b) Principal component (PC) analysis of conditions that cause muscle atrophy. (c) The number of up-regulated (red) and down-regulated (down) DEGs in each muscle atrophy condition, respectively. (d) Venn diagrams show the number of common and exclusive DEGs in each muscle atrophy condition. Highlighted in the black arrow are the genes *Col1a1*, *Neto2*, *Kera*, *Rmi2*, and *Amd1*, in common in all conditions of muscle atrophy in mice.

We analyzed the PPI network for the 59 DEGs shared by at least three studied muscle atrophy conditions. We cluster the DEGs in three clusters (cluster 1 - red, cluster 2 - green, and cluster 3 - blue) and then analyze each cluster's functional enrichment (Supplementary Figure 5a). In addition, we also used The Molecular Signatures Database (MSigDB) for performing gene set enrichment analysis. In cluster one, the genes *Tlr4*,

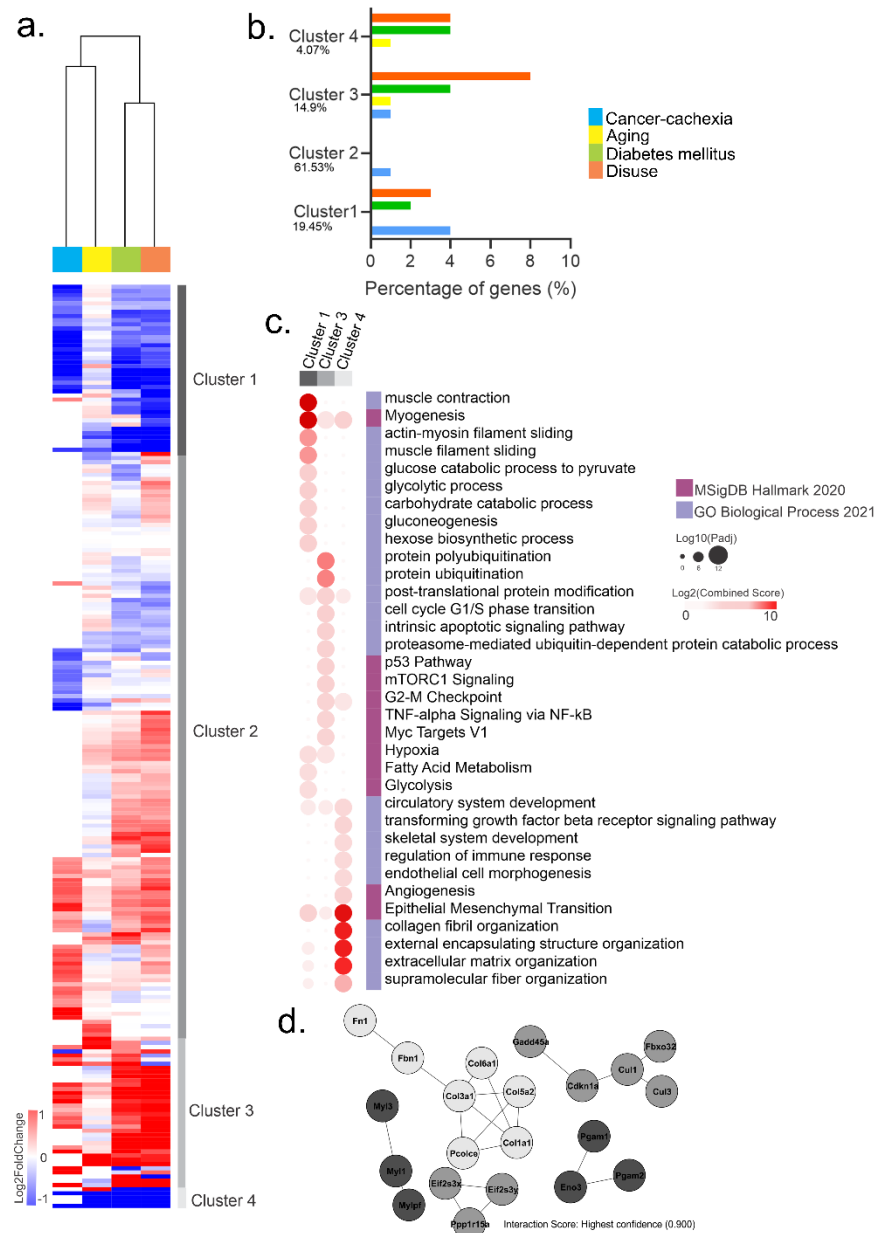


Figure 2. Atrogenes present in muscle atrophy conditions in mice. (a) Heatmap of altered atrogenes expression in conditions of cachexia associated with cancer, aging, diabetes, and disuse. Clustering by K-means (1-4) between rows (FoldChange<1; FDR>0.05). (b) Percentage of genes described as atrogenes in the literature separated by clustering. Clusters 1, 3, and 4 showed greater significance for p-adj-value <0.05 in genes differentially expressed in the conditions analyzed in mice. (c) Functional enrichment using

MSigDB databases Hallmark 2020 (purple color) and Go Biological Process 2021 (lilac color). Ball size refers to significance per Padj-value per Log10. The red color refers to the combined score by Log2. (d) Protein-protein interaction (PPI) of cluster 1 (dark grey), cluster 3 (grey), and cluster 4 (light grey). High Confidence Interaction Score (0.900).

Gadd45a, *Map2k6*, and *Aurka* are present in biological processes of regulation of NF-kappaB transcription factor activity, regulation of reactive oxygen species metabolic processes, PI3K/AKT/mTOR signaling, apoptosis, TNF-alpha signaling via NF-kB, glycolysis, and mTORC1 signaling (Supplementary Figure 5b). In cluster two, the genes *Col1a1*, *Col1a2*, *Col3a1*, *Fbxo32*, and *Actc1* enrich extracellular matrix organization (ECM), response to denervation involved in the regulation of muscle adaptation, skeletal muscle thin filament assembly, myogenesis, KRAS signaling and angiogenesis (Supplementary Figure 5b). In cluster three, the genes *Smox*, *Amd1*, *Ckmt2*, and *Gamt* enrich the polyamine biosynthetic process, creatine metabolic process, polyamine metabolic process, muscle contraction, myogenesis, and KRAS signaling. In our findings, most genes show expression alterations, and these genes have already been described in the literature related to muscle atrophy (Supplementary Figure 5b).

Skeletal muscle interactome under atrophy conditions indicates distinct L-R interactions between cancer-associated cachexia conditions, aging, diabetes, and disuse

We analyzed the genes predicted as ligands and receptors for genes with increased expression and $FDR < 0.05$ in each condition associated with muscle atrophy. The 126 positive L-R interactions were found, of these 28, 10, 15, and 11 in muscle atrophy conditions caused by cancer-associated cachexia, aging, diabetes, and disuse (Supplementary Table S5). Although positive L-R interactions were calculated by $FDR < 0.05$, we filter these interactions by communication score > 1 (Ligand expression x Receptors expression). Reduced the list of positive L-R interactions to 48 (Supplementary Table S5), between these 43, 13, 38, and 33 in muscle atrophy conditions caused by cancer-associated cachexia, aging, diabetes, and disuse (Supplementary Table S5). Most positive L-R interactions were unique to cancer-associated cachexia and aging conditions (Figure 3a). About 16 positive L-R interactions were shared between diabetes and disuse (Figure 3a). We analyzed for Euclidean distance clustering by selecting the high L-R interactions in the communication score (Figure 3b). Of the incoming interactions *Selplg-*

Cxcr2, *Selplg-Selp*, *Selplg-Sell*, *Selplg-Sele*, *Il1b-Il1r*, *Il1b-Il1r2*, *S100a8-Tlr4*, and *S100a9-Tlr4* were highly represented in cancer-associated cachexia (Figure 3b). The interactions between *Ccl8-Ccr1* and *Ccl8-Ccr2* showed high interactions in aging conditions (Figure 3b). In diabetes and disuse, the interactions *Hsp90b1-Tlr4*, *Lin7c-Abca1*, *Adam10-Il6ra*, *Hmgb1-Tlr4*, *Mstn-Acvr2a*, and *Inhbb-Acvr2a* were more significant in our analyzes (Figure 3b).

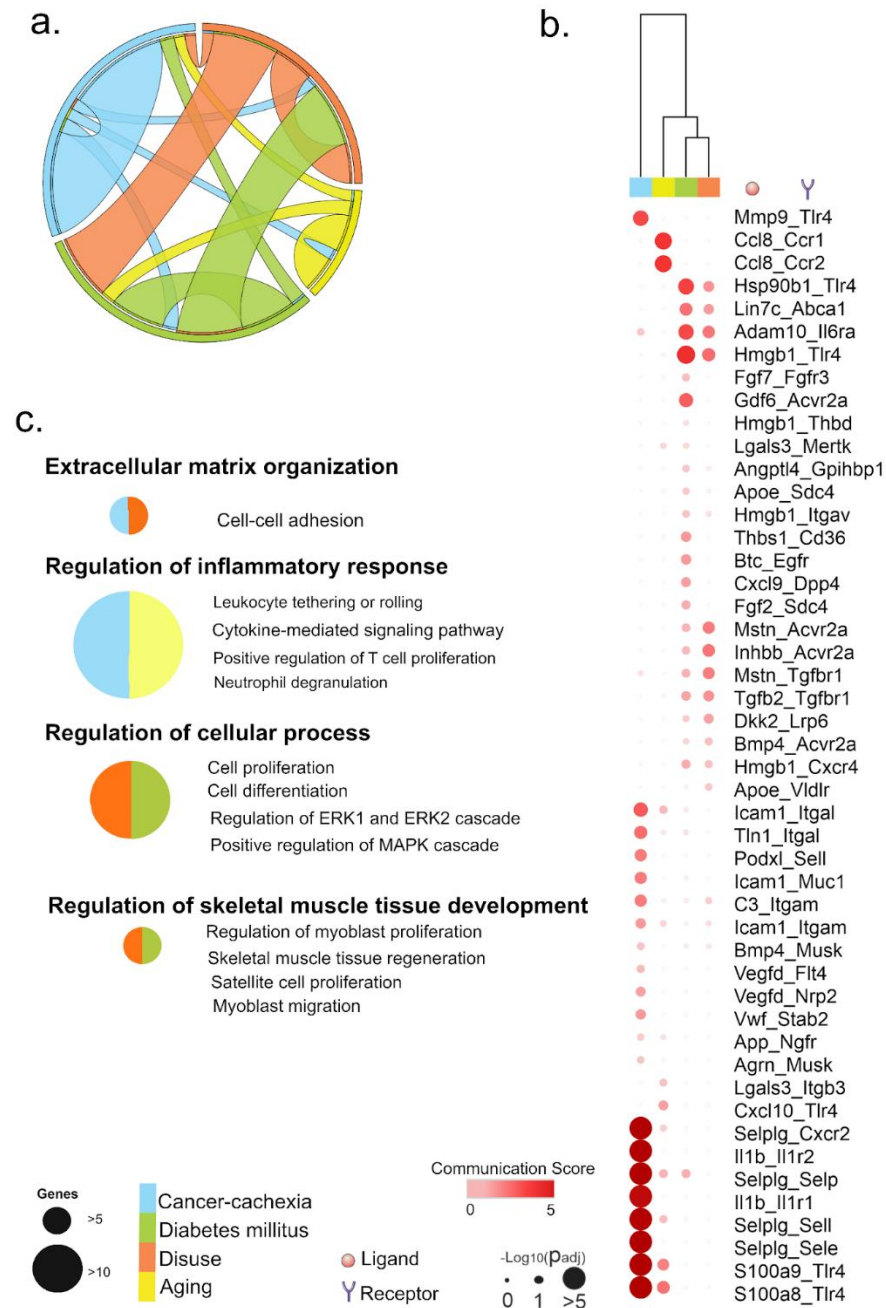


Figure 3. Cellular communication of gastrocnemius skeletal muscle in mice under conditions of muscle atrophy. The circos plot shows ligand-receptor interaction in conditions associated with cancer cachexia (blue color), aging (yellow color), diabetes (green color) and disuse (orange color) in mouse

muscle atrophy. Line thickness refers to the number of LigandXReceptor interactions. (b) Heatmap showing the Spearman correlation of selected interactions calculated as expression thresholding by ligand ($L > 0.33$) and receptors ($R > 0.33$) in skeletal muscle among the genes associated as ligands. Ball size refers to the significance of the p-adj value by Log10. The red color refers to the communication score (0-5). (c) Go Biological process of predicted genes as ligands and receptors. The size of the ball refers to the number of genes in each term. The color refers to the associated conditions.

We analyzed functional enrichment considering the Biological Process of ligand-receptor interactions (Figure 3c). In cancer cachexia and aging, most genes are associated with biological processes of inflammation and cellular responses to inflammation. Specifically, in the regulation of cytokine inflammatory response and positive regulation of T cell proliferation (Figure 3c). In diabetes and disuse, there is enrichment of biological processes such as proliferation, differentiation, regulation of ERK1 and ERK2 cascades. These conditions also enrich genes involved in the regulation and proliferation of myoblasts and muscle regeneration (Figure 3c).

Interactions between ligands and receptors are more significant in NK cells, M2 macrophages, and capillary endothelial cells of skeletal muscle tissue

To assess the cell populations in which L-R interactions were significant in aging and cancer-associated cachexia, we reanalyzed scRNA-Seq data and integrated (Seurat v.3.0) skeletal muscle aging datasets in mice. We detected 5,469 cells in common in the three samples of aged mouse muscle. After clustering, twenty cell populations were identified. Endothelial capillary, Myonuclei (Type IIb), Endothelial vein, FAPs (Stem), FAPs (pro-remodeling), FAPs (Adipogenic), Myonuclei (Type IIx), Endothelial artery, M2 macro, Others, Smooth muscle, MuSCs, Schwann cells, CD4+, Endothelial, Quiescent MuSCs, Mesenchymal cells, Granulocytes Mac1, Erythrocyte, NK cells and Neural (Figure 4a). We identified that the genes *Selplg*, *Cxcr2*, and *Sell* are present in NK cells and M2 macrophages (Figure 4a). The genes *Selp*, and *Sele* are present in capillary endothelium cell type, FAPs (adipogenic), and FAPs (pro-remodeling) (Figure 4a). We performed linear regression analysis between ligands and receptors considering the expression values in TMM between the conditions and their respective controls. Our findings indicate a positive correlation between the interactions *Selplg_Selp* (p-value=0.0029), *Selplg_Sell* (p-value=0.0017), and *Selplg_Cxcr2* (p-value=0.0001) (Figure 4b-c).

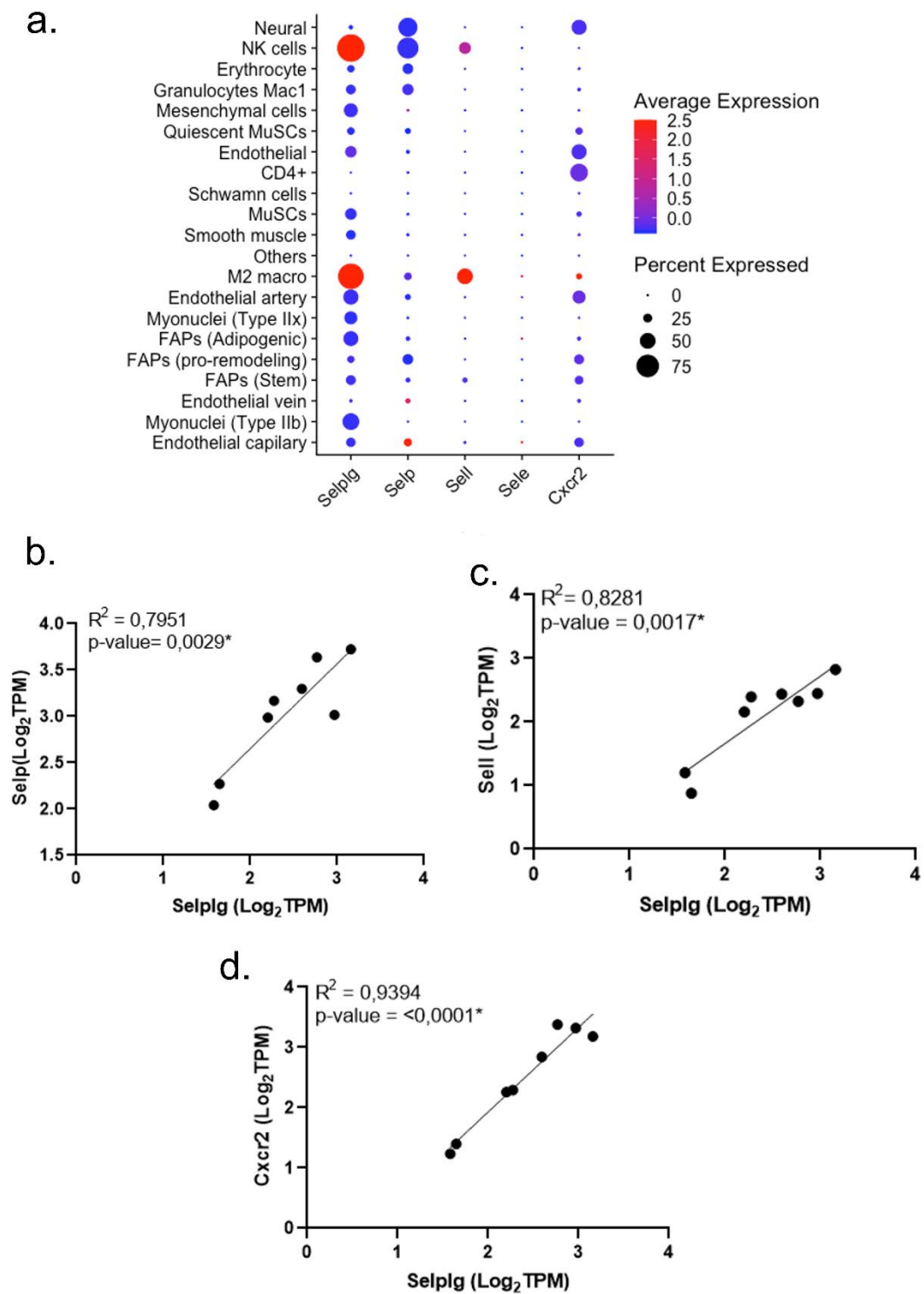


Figure 4. Interactions between Selpg ligand and Selp, Sele, and Cxcr2 muscle receptors occur in type II macrophage cells and endothelial cells in skeletal muscle. Dotplot showing the average expression

levels for 21 cell-type specific genes differentially expressed in skeletal muscle of mice. The dot size represents the ratio of cells with expressed genes out of all cells. Genes with higher expression levels in the cells were displayed on a red background. (b-d) Simple linear regression between *Selplg* ligand (x-axis) and *Selp*, *Sele*, and *Cxcr2* receptors (y-axis) with transcript per million (TPM) gene expression values in eight samples of mice inoculated with CLL for four weeks. Significance was considered by p-value < 0.005(*).

Discussion

Muscle atrophy is a decrease in muscle mass generated by the imbalance between protein synthesis and degradation⁶⁰. Different stimuli act as triggers in muscle atrophy, and unraveling the stimuli as well as signaling pathways and effectors that contribute to muscle depletion is critical to developing therapeutic interventions^{60,210}. Here, we analyzed autocrine communication in skeletal muscle under conditions of muscle atrophy in mice. The cellular communication among the genes predicted as ligands and receptors points to the interaction among *Selplg_Selp*, *Selplg_Sell*, and *Selplg_Cxcr2*. These interactions exhibit a higher communication score in the cancer-associated cachexia condition. The ligand-receptor interactions are present in cell types such as M2 macrophages, NK cells, and endothelial cells in the skeletal muscle of muscular mice. These findings indicate the distinct profile of muscle autocrine signaling in different conditions leading to muscle atrophy.

We compared the differentially expressed genes of cancer-associated cachexia, aging, diabetes, and disuse with genes known as atrogenes in the literature^{69,207}. In our findings, the genes *Gadd45a*, *Fbxo32*, *Eif4ebp1*, *Mylpf*, *Ckmt2*, and *Col1a1* were significant in mice's list of genes described as atrogenes (Figure 2). The *Gadd45a* gene is essential in regulating critical cellular processes, such as DNA repair, cell proliferation, and programmed cell death, in response to cellular stressors. The study by Ebert et al. (2012)²¹¹ demonstrates that growth arrest and DNA damage-inducible protein 45a (*Gadd45a*) is a critical mediator of muscle atrophy. This is because *Gadd45a* is a nuclear protein that stimulates myonuclear remodeling and generalized changes in skeletal muscle gene expression²¹¹. Furthermore, *Gadd45a* reduces multiple barriers to atrophy and stimulates multiple atrophy mechanisms²¹¹. In our findings, the *Gadd45a* gene shows increased expression in conditions of aging, diabetes, and disuse. These data agree with the literature that presents the *Gadd45a* altering gene increased expression in conditions such as denervation, fasting, hindlimb unweighting, and immobilization⁶⁹.

Increased expression of the *Fbxo32* genes (atrogen-1/MAFbx) occurs in muscle atrophy due to proteasomal degradation of target proteins⁶⁰. In our findings, the *Fbxo32* gene is increasing in conditions of cancer-associated cachexia, diabetes, and disuse. Studies in the literature point to increased expression of these genes in aging conditions, diabetes, denervation, and inflammatory cytokines⁶⁹. Finally, our findings demonstrate that the *Col1a1* gene (Collagen, type I, alpha 1) presents reduced gene expression in all conditions analyzed here. The *Col1a1* gene was described in the study by Sacke et al. (2007)²¹² with reduced gene expression in conditions of fatigue, tumor-bearing, uremia, diabetes, and disuse. Recently, administering collagen peptides in mice with muscle atrophy proved effective for treating atrophy²¹³. In addition, the central pathway for synthesizing muscle proteins AKT/mTOR was increased in the groups that received collagen peptides. However, the role of the *Col1a1* gene in muscle atrophy has not yet been fully elucidated. Thus, the study of atrogen genes in different disease conditions may help to understand the molecular mechanisms involved in muscle atrophy and, therefore, develop strategies to prevent or treat this condition²¹³.

Inflammation plays a vital role in muscle homeostasis; under normal conditions, inflammation is essential during muscle tissue repair and adaptation¹⁹⁷. We performed skeletal muscle autocrine communication under different catabolic conditions that induce muscle atrophy. Our results point to interactions between genes involved in inflammatory processes (*Selplg_Cxcr2/Selp/Sell/Sele*; *Il1b_Il1r1/Il1r2*; *S100a8/S100a9_Tlr4*) with a higher communication score and significance in the condition of cachexia associated with cancer. Chronic inflammation may play a negative role in muscle homeostasis, leading to muscle mass loss⁵⁹. We found the predicted gene as *Selplg* ligand (P-selectin glycoprotein ligand 1) interacting with *Selp* (Selenoprotein P), *Sell* (L-selectin), and *Sele* (E-selectin) receptors.

Selectins are carbohydrate-binding molecules that bind fucosylated and sialylated glycoprotein ligands and are found in endothelial cells, leukocytes, and platelets²¹⁴. Selectins are involved in acute and chronic inflammatory processes because selectins participate in the steps of capture, scrolling, and slow scrolling of leukocytes²¹⁴. Furthermore, leukocyte rolling and interaction with vascular endothelium are mediated by selectins during inflammation²¹⁵. The study by Tan et al. (2012)²¹⁶ identified that individuals carrying the C allele in the SNP rs6136 (*SELP* gene) were associated with weight loss > 10%. It was also found that the expression of the *Selp* (P-selectin) gene was

significantly increased in animal models of cancer cachexia. These findings provide evidence for the probable involvement of P-selectin in muscle wasting²¹⁶. The reanalysis of the Lewis lung carcinoma cells (LLC) cancer-cachexia model study in a time course of up to 4 weeks points out the increase in the expression of the *Selp* gene in LLC mice from three weeks of inoculation with the tumor compared to control mice²¹⁶. Furthermore, the *Selplg* gene predicted as a ligand increases gene expression from the first week of inoculation with the LLC cells²¹⁶. In identifying the cell types that most express the *Selplg* and *Selp* genes, our data point to increased expression of the *Selplg* gene in NK cells and type 2 macrophages and the *Selp* receptor in capillary and vein cells.

Our findings of autocrine communication in muscle demonstrate that selectins play an essential role in cell signaling, in addition to acting in the recruitment of macrophages by the endothelium to muscle tissue in response to inflammation generated by cancer-associated cachexia. Knockout of P-selectins by gene or use of specific inhibitors points to the role of selectins in the recruitment of neutrophils and macrophages in the inflammatory response^{217,218}. Liou et al. (2013)²¹⁹ demonstrated that P-sel^{-/-} mice revealed less neutrophil and macrophage infiltration, significantly different from P-sel^{+/+} mice in early and later partial sciatic nerve ligation (PSNL) stages. Thus, P-selectin is one of the primary mediators for the recruitment of monocytes and neutrophils after peripheral nerve injury and is responsible for the development of neuropathic pain²¹⁹. We hypothesize that under catabolic conditions in skeletal muscle, such as cachexia associated with cancer, aging, and diabetes, selectins recruit macrophages through the endothelium, increasing the flow of inflammatory molecules into skeletal muscle. The *Selplg*, *Selp*, and *Sele* genes selectin response is more exacerbated in cancer-associated cachexia. Thus, we highlight the vital role of selectins in muscle atrophy, highlighting these molecules as targets for cancer cachexia therapy.

The *Tlr4* is essential in modulating innate immunity²²⁰. The *TLR4*, a receptor located on the cells' surface, starts signaling pathways involving the activation of kinases and transcription factors, leading to the generation of innate immune responses against pathogens²²¹. Thus, *Tlr4* activation can slow insulin action by activating pro-inflammatory kinases and indirectly by activating cytokine signaling cascades and systemic release of pro-inflammatory and insulin desensitizing factors²²¹. We observed that the *Tlr4* receptor gene (Toll-like receptor 4) is significant in cell communication in conditions such as diabetes, disuse, and cancer-associated cachexia. In the model of

diabetes, the *Tlr4* receptor interaction occurs between *Hsp90b1* and *Hmgb1* ligands. Increased expression of the *Tlr4* gene has been associated with type 2 diabetes mellitus²²⁰. The study by Alibashe-Ahmed and collaborators (2019)²²² determined the role of TLR4 in the activation and proliferation of T lymphocytes in the onset of autoimmune diabetes using the non-obese diabetic mouse model (NOD) and *Tlr4* signaling inhibitor. The results indicate that TLR4 signaling contributes to developing and maintaining autoimmune diabetes²²². In addition, protein box 1 of the high mobility group (*Hmgb1*) also plays a vital role in insulin resistance in diabetes. The *Hgmb1* gene has also been implicated in the autophagy process, and autophagy processes have been correlated with insulin resistance²²³. Thus, diabetes ends up altering the response of innate inflammatory response receptors, which consequently leads to interaction with binding molecules of muscle catabolism pathways, such as autophagy and increased insulin sensitivity, which may be implicated in muscle atrophy in diabetes due to lack of signaling of muscle hypertrophy pathways such as IGF/Akt/mTOR.

This study provides information on the interactions between ligands and receptors in different conditions of muscle catabolism and indicates potential molecules associated with muscle atrophy in mice. Cellular communication in muscle has different profiles in conditions such as cachexia associated with cancer, diabetes, and disuse. We address the relationship of molecules such as selectins with chronic inflammation and muscle endothelium, highlighting the role of *Selplg* genes and *Selp* receptors. The identified ligands and receptors will help us raise molecular targets directed to molecular therapy to develop interventions for stimulus-specific atrophies.

Limitations of the study

A limitation of this study is that we did not analyze protein expression associated with genes predicted as *Selplg* ligands and *Selp*, *Sele*, and *Cxcr2* receptors. It was impossible to obtain scRNA-Seq data on cancer-associated cachexia in LLC model mice to validate our findings *in silico*.

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To study collaborators and CAPES.

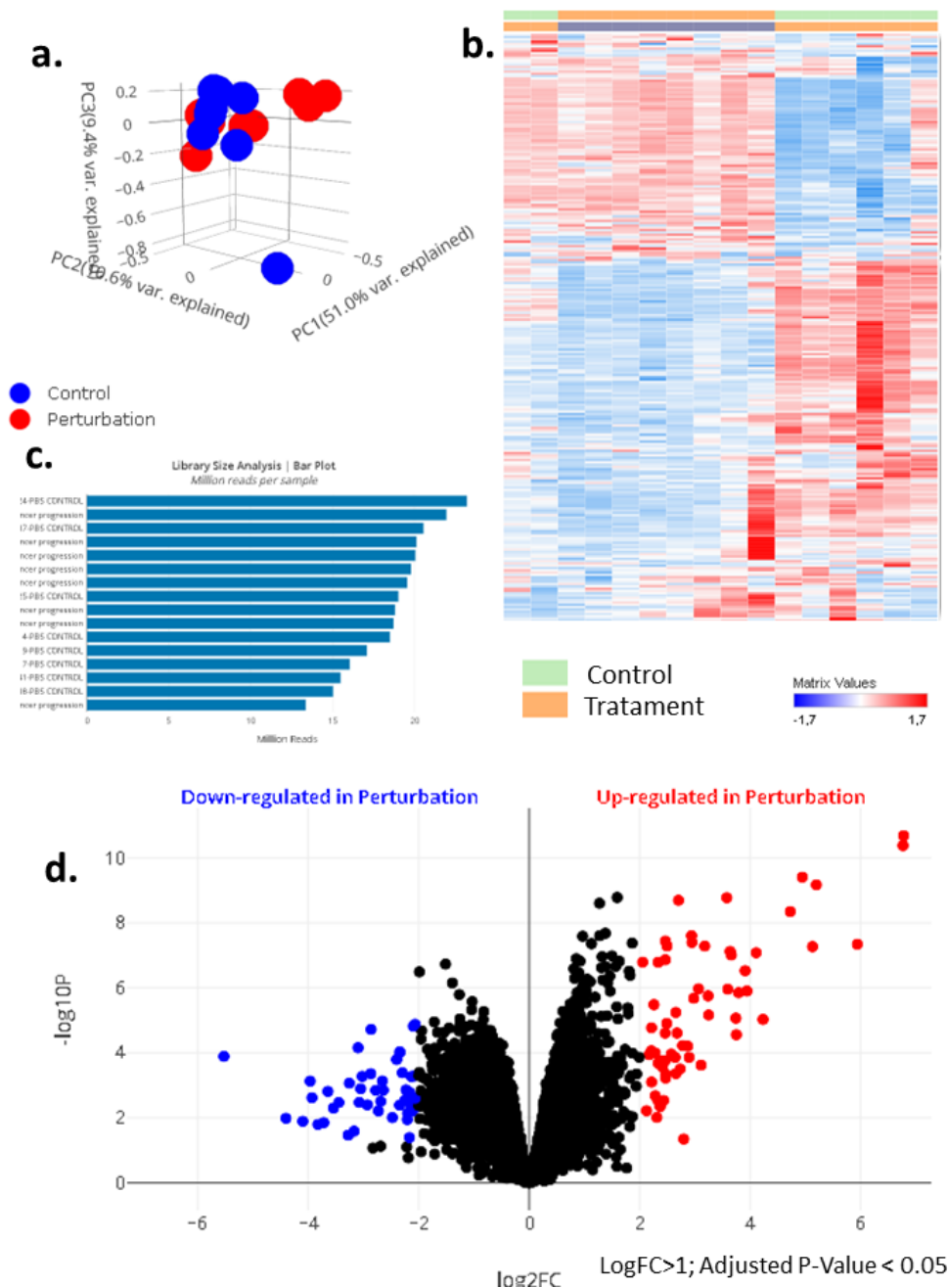
Data Availability Statement: Publicly archived datasets analyzed herein are available at the Gene Expression Omnibus (GEO) under accession numbers GSE114820, GSE161693, GSE139204 and GSE172410.

Conflict of interests

The authors declare that they have no conflicts of interest.

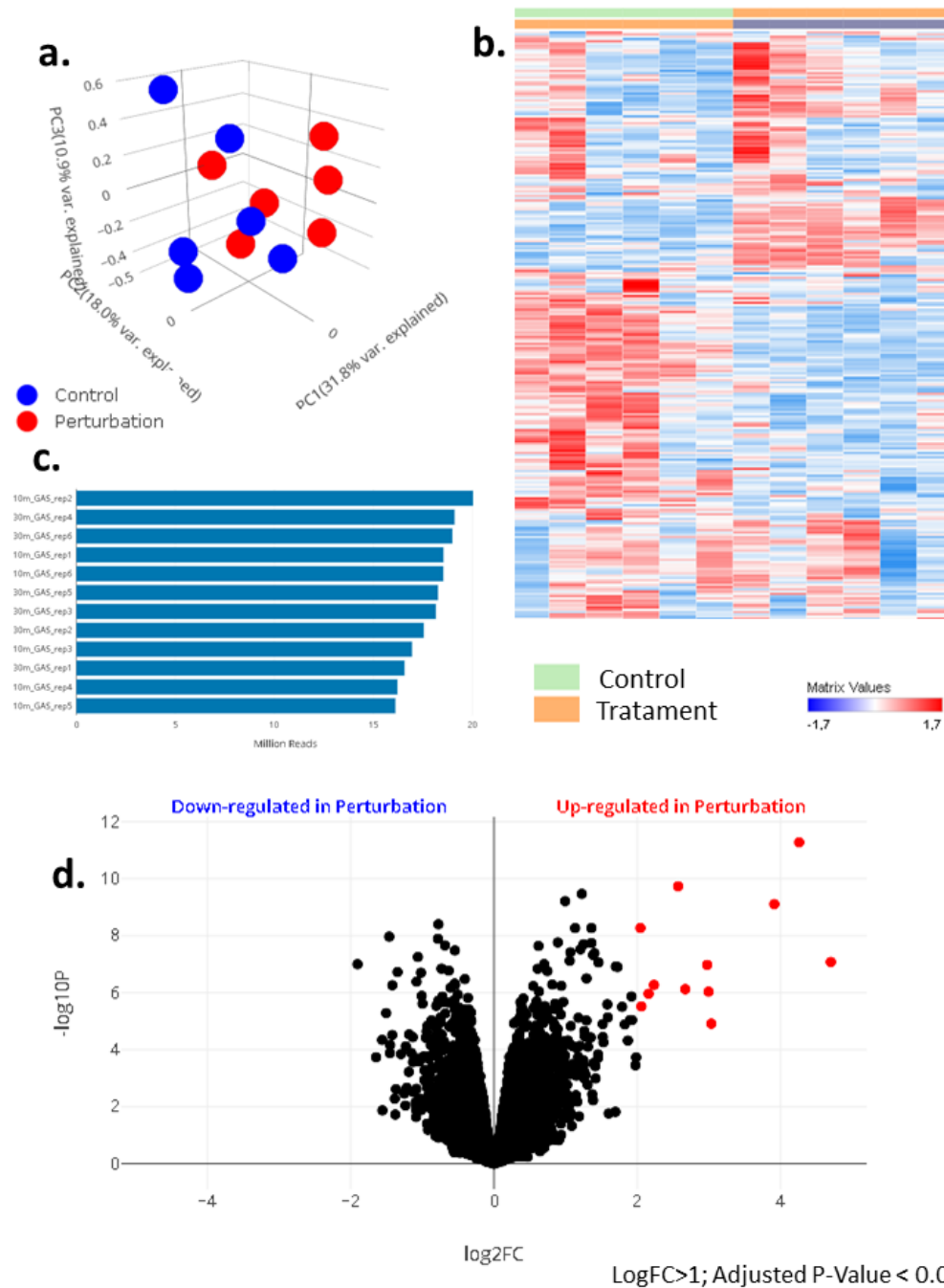
Supplementary figures

Protocol: Cancer-Cachexia GSE114820



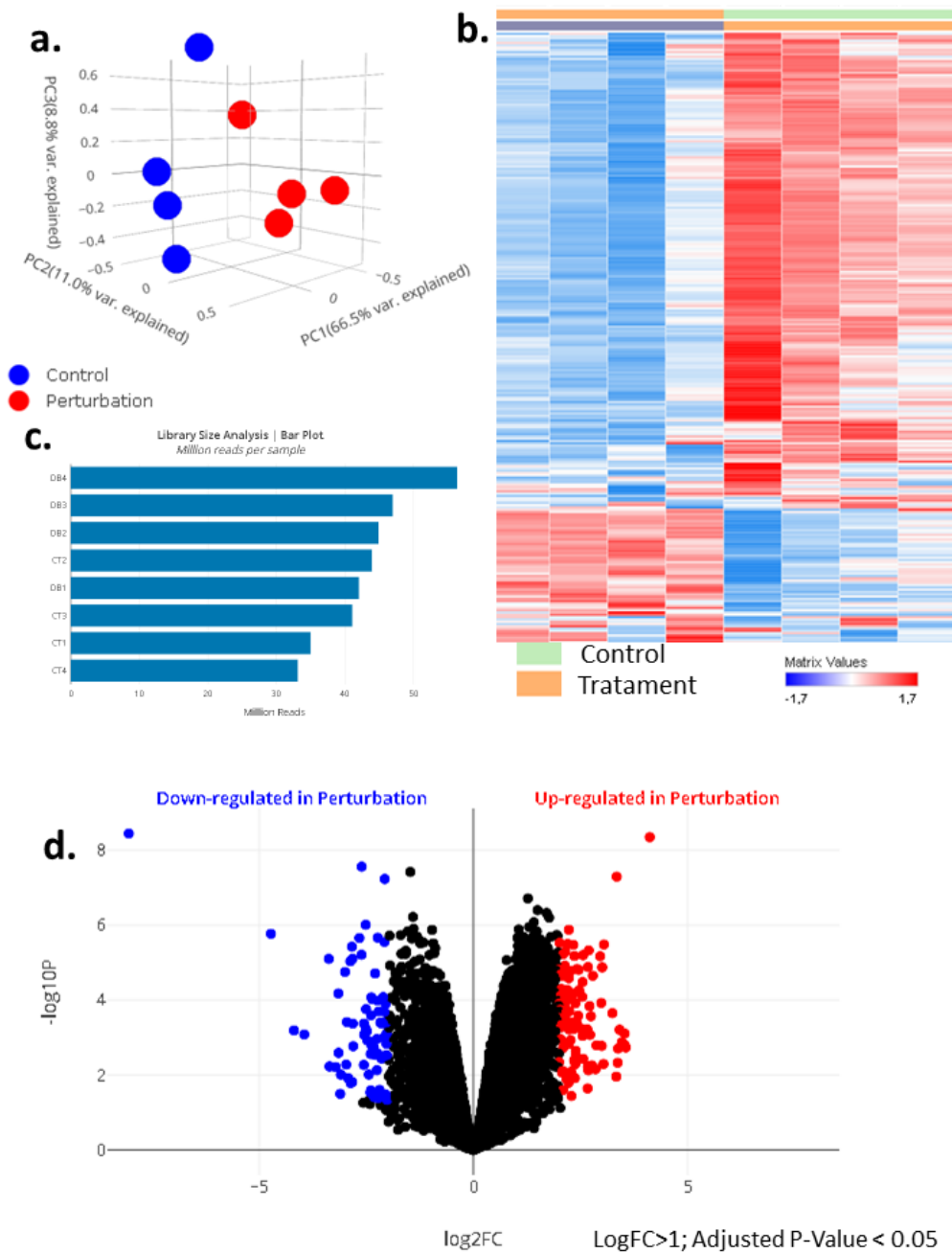
Supplementary Figure 1. Differentially expression analysis on cancer-associated cachexia in CLL mouse model. (a) Principal component analysis (PCA) in Control mice vs. LLC mice four weeks. (b) Heatmap of differentially expressed genes. (c) RNA-Seq library size by plot bar samples. (d) Volcano plot demonstrating differentially expressed genes in control samples versus CLL treatment at four weeks.

Protocol: Aging GSE139204



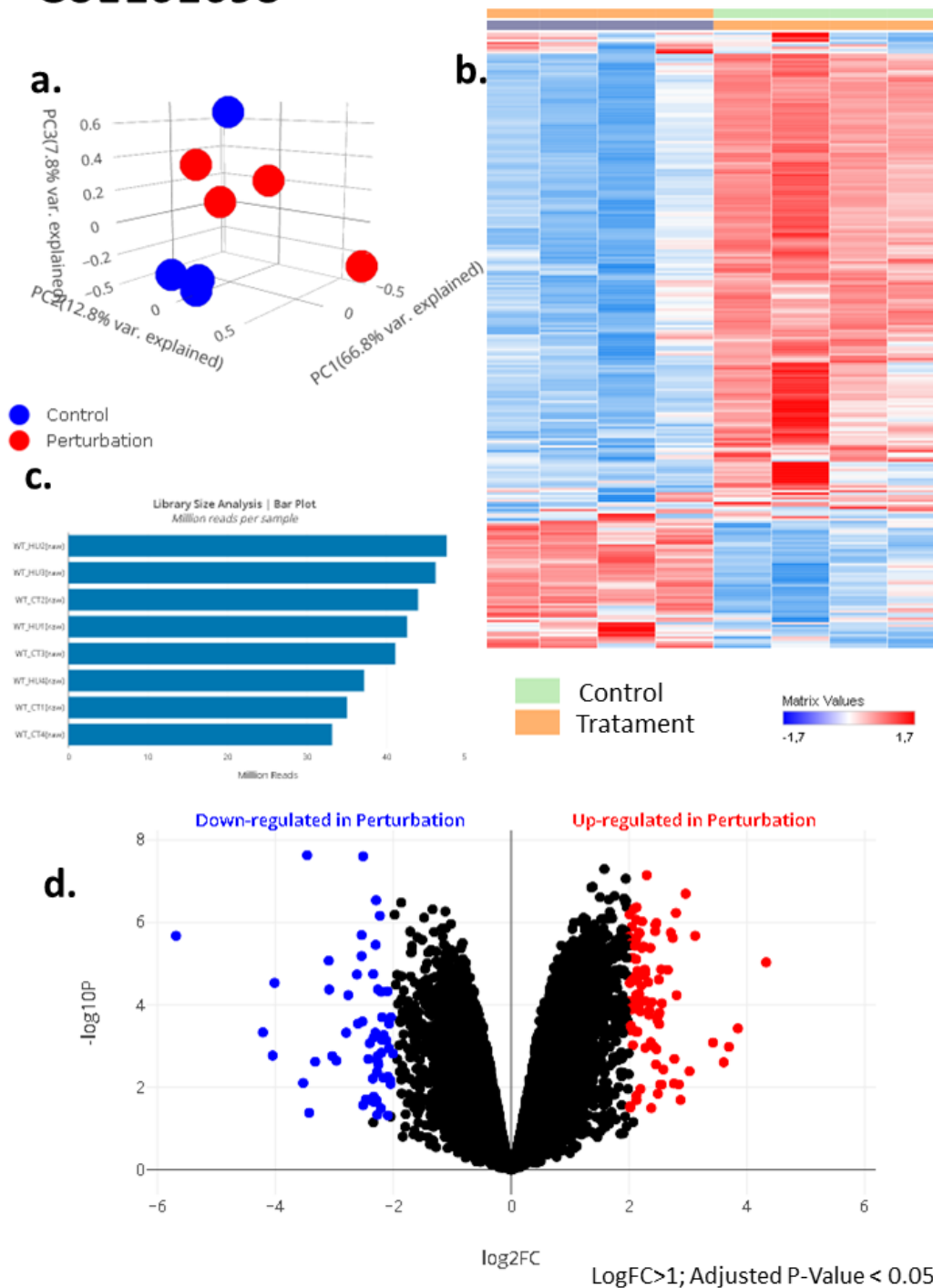
Supplementary Figure 2. Differentially expression analysis in aged mice. (a) Principal component analysis (PCA) in control of young mice versus aged mice. (b) Heatmap of differentially expressed genes. (c) RNA-Seq library size by plot bar samples. (d) Volcano plot demonstrating differentially expressed genes in control samples versus aged mice.

Protocol: Diabetes mellitus GSE161693

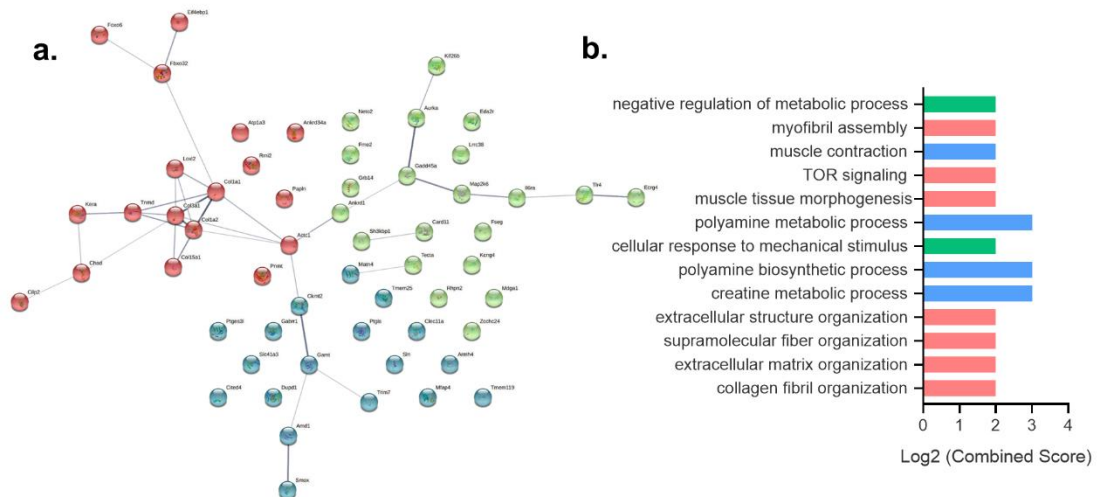


Supplementary Figure 3. Differentially expression analysis mice with diabetes. (a) Principal component analysis (PCA) in control non-diabetic mice versus Diabetes Mellitus mice. (b) Heatmap of differentially expressed genes. (c) RNA-Seq library size by plot bar samples. (d) Volcano plot demonstrating differentially expressed genes in control samples versus Diabetes Mellitus mice.

Protocol: Unloading (disuse) GSE161693



Supplementary Figure 4. Differential expression analysis mice with disuse. (a) Principal component analysis (PCA) in control mice versus mice in a disuse model. (b) Heatmap of differentially expressed genes. (c) RNA-Seq library size by plot bar samples. (d) Volcano plot demonstrating differentially expressed genes in control samples versus disuse model.



Supplementary Figure 5. Protein-protein interaction (PPI) analysis and functional enrichment of atrogenes in cancer cachexia, disuse, aging, and diabetes. (a) PPI analysis of common DEGs with the list of atrogenes. Three clusters were identified. (b) Biological Process of PPIs by clusters.

Chapter 3: Transcriptomic profiling of the secretome and interactome of skeletal muscles during aging and sarcopenia

Jakeline Santos Oliveira¹, Victória Larissa Schmidt Camargo¹, Amanda Piveta Schnepfer¹, Caio Fernando Ferreira Mussatto¹, Ana Luiza Labbate Bonaldo¹, Paula Paccielli Freire², Maeli Dal Pai-Silva¹, Sarah Santiloni Cury¹, Geysson Javier Fernandez³ & Robson Francisco Carvalho^{1*}.

1. Department of Structural and Functional Biology, Institute of Biosciences, São Paulo State University, UNESP, Botucatu, São Paulo, Brazil.
2. Department of Biochemistry and Tissue Biology, Institute of Biology, State University of Campinas, UNICAMP, Campinas, São Paulo, Brazil.
3. Department of Immunology, University of São Paulo, USP, São Paulo, Brazil.
4. Faculty of Medicine, University of Antioquia, UdeA, Medellín, Colombia.

Corresponding author

Professor Robson Francisco Carvalho
Department of Structural and Functional Biology
Institute of Biosciences, São Paulo State University - UNESP
CEP: 18.618-689, Botucatu, São Paulo – Brazil
Telephone number: +55 14 3880 0473
robson.carvalho@unesp.br

Abstract

Skeletal muscle aging is characterized by loss of muscle mass and strength. Sarcopenia is associated with loss of muscle mass and strength, increasing the risk of failure, fracture, physical inactivity, and mortality in elderly individuals. It is not clear what molecular changes occur in the muscle microenvironment during sarcopenia. Therefore, investigating secretome and muscle signaling molecules is paramount for identifying potential biomarker targets for sarcopenia. This study aimed to predict changes in the skeletal muscle secretome and interactome genes. We utilized bulk RNA-Sequencing data from the vastus lateralis skeletal muscle of healthy and sarcopenic elderly male individuals. The secretome was filtered using *The Human Protein Atlas*, and the interactome was filtered using a list of predicted ligands and receptors from the *International Union of Pharmacology* and Connectome database. Our results indicate differences in the transcriptional profiles of the secretome and interactome of skeletal muscle tissue in elderly and sarcopenic individuals. We identified markers of the autophagy pathway that are highly associated with muscle loss and advanced age in individuals with sarcopenia. Furthermore, the secretome exhibited a downregulated expression profile of genes related to Extracellular Matrix (ECM) components and endothelial growth factors, especially in groups of individuals over 80 years of age. A similar profile of altered molecules was observed in the skeletal muscle interactome, indicating reduced muscle communication during aging and sarcopenia. Our data suggest interactions between collagen ligands such as *COL1A1* and *COL5A1* and receptors for endothelial growth factors (*VEGFB*) and tyrosine kinase (*RET*). These findings provide insights into alterations in muscle microenvironment signaling and reduced communication between fibroadipogenic progenitors (FAPs) and endothelial cells. Thus, our data underscores the importance of ECM components and endothelial elements in activating skeletal muscle regeneration. These results point to potential targets for investigating muscle biomarkers of sarcopenia in elderly individuals. We showed that the aging process of skeletal muscle alters the expression profile of genes involved in different pathways. Understanding cell-cell communication in the skeletal muscle during aging and sarcopenia may reveal muscle atrophy mediators and facilitate the future development of therapies.

Keywords: Skeletal Muscle, Aging, Sarcopenia, Secretome, Ligand-Receptors, Cell-Cell Communication

Introduction

Sarcopenia is a progressive and generalized condition of the skeletal muscle associated with an increased risk of adverse outcomes, including falls, fractures, physical disability, and mortality^{90,224}. Advanced age is a leading risk factor for sarcopenia⁹⁰. From age 50, there is a yearly decline of 1-2% in muscle mass; after age 80, this reduction can reach up to 30%⁸⁴.

Skeletal muscle regulates systemic aging²²⁵. Aged skeletal muscle presents structural changes, such as a reduction in the regenerative capacity and potential of satellite cells²²⁶, and neurological and vascular changes that further compromise muscle function²²⁷. Muscles from sarcopenic individuals exhibit distinct characteristics that include atrophy of type 2 muscle fibers (fast twitch), heterogeneity in fiber size, accumulation of intramuscular connective tissue and fat, as well as decreased oxidative capacity²²⁸. In addition, increased inflammation and protein catabolism characterize the sarcopenic muscle from other typical features of aging²²⁸. The age-related decline in skeletal muscle and sarcopenia is not well-defined due to its complex, multifactorial nature, with its underlying cellular mechanisms not fully elucidated²²⁴.

In aged muscle tissue, satellite cells are maintained in a quiescent state, retaining the ability to activate proliferation, differentiation, and self-renewal of satellite cells in response to injury during the repair of damaged muscle tissue²²⁹. The muscle microenvironment plays a regulatory role in satellite cells. Numerous non-myogenic cells, such as fibroadipogenic progenitors (FAPs), macrophages, and endothelial cells, among others, are essential in regulating satellite cells function^{230,231}. Furthermore, alterations in systemic factors from the bloodstream, as well as in structural components such as myofibers and the composition of the extracellular matrix (ECM), also appear to be relevant for the regulation of satellite cells and other components of the muscle

microenvironment²³². Parabiosis experiments have shown that exposing aged muscle to young circulating factors can rejuvenate the regenerative capacity of aged muscle²³³.

The secretome and autocrine signaling are critical mechanisms that regulate muscle growth and function²³⁴. Studies have shown that the secretome of skeletal muscle cells contains a variety of proteins that promote muscle growth and regeneration^{234–236}. These proteins can act in an autocrine fashion, meaning they can signal back to the same cell that produces them²³⁷. In sarcopenia, the decrease in the number and size of muscle fibers is thought to be due to a reduction in the production of myokines, which are proteins secreted by muscle fibers that promote muscle growth and regeneration²³⁸. Therefore, studying secretome and autocrine signaling in sarcopenia is relevant because it could help identify new targets for treatment and potential sarcopenia biomarkers. We could potentially increase muscle growth and regeneration in patients with sarcopenia by targeting proteins in the secretome or autocrine signaling pathways. Secretome and autocrine signaling can also be biomarkers for diagnosing sarcopenia and tracking its progression in elderly individuals^{85,92,224}. Due to the complexity of sarcopenia, the development of a panel of potential biomarkers would be necessary, including indicators of the neuromuscular junction, muscle protein turnover, pathways associated with inflammation, factors related to oxidative stress, as well as hormones or other anabolic factors²³⁹.

In the present study, we used Bulk RNA-Seq data from healthy elderly individuals and sarcopenic elderly individuals and compared them to young individuals. We aimed to characterize skeletal muscle genes involved in secretome and autocrine communication during aging and sarcopenia. We demonstrated that autocrine communication is higher in sarcopenia than in aging, mainly in proteasome and autophagy pathways related to muscle atrophy. Furthermore, the correlation with the reduction in muscle mass in sarcopenia

was higher in the Ligand-Receptor Interactions (LxR). We used scRNA-Seq data from healthy elderly and young individuals to characterize the autocrine communication of skeletal muscle in the cell populations of the muscle tissue. Additionally, we used scRNA-Seq analysis to validate the LxR interactions found in Bulk RNA-Seq data from healthy elderly and sarcopenic individuals. Many populations altered during aging and sarcopenia comprise endothelial cells and fibroadipogenic progenitors (FAPs). These cells are essential for skeletal muscle remodeling, and during the aging process and sarcopenia, there is a reduction in this signaling. These data indicate fundamental interactions and target cells for treating sarcopenia in the elderly population.

Methods

Data source

We searched the Gene Expression Omnibus (GEO, <https://www.ncbi.nlm.nih.gov/geo/>) dataset using the keywords " Skeletal muscle and aging, skeletal muscle and sarcopenia." To characterize the skeletal muscle transcriptional profiles in aging healthy and sarcopenic elderly individuals, we used the GSE167186 dataset²⁴⁰. This study included 72 male subjects, 19 young individuals with a mean age of 20 years, and 53 elderly individuals aged 60, 70, and 80 years. Of these, 29 were healthy elderly individuals, and 24 were characterized as sarcopenic (Supplementary Table 1). The clinical aspects of the individuals, such as age, functional capacity assessment test, muscle assessment tests, and physical rehabilitation, were available and are provided in Supplementary Table 2. We considered sarcopenia and healthy elderly classifications according to the original study by Perez et al. (2022)²⁴⁰, which they used as the functional analysis criteria for the classification of The Foundation for the National Institutes of Health Sarcopenia Project (The FNIH Sarcopenia Project)²⁴¹.

Data processing

Raw count data from GSE167186 were normalized using the vst method in the DESeq2 package. Differentially expressed genes (DEGs) were identified between the healthy young group (18-30 years old) and healthy elderly individuals divided into three age groups (60, 70, and 80 years old), as well as between healthy elderly and sarcopenic elderly individuals within the same age groups. Statistical parameters used were an adjusted p-value < 0.05 and $\log_2$ (Fold Change (FC) ≥ 0.58). Genes with an increased expression profile (FC >1.5) and reduced expression profile (FC <1.5) were considered (Supplementary Table 3). Data and figure processing were performed using R software (v.4.2.2) and the "ggplot2" package.

Transcriptome-based secretome and plasma analysis

The DEGs encoding secreted and plasma proteins were filtered based on the lists available in The Human Protein Atlas (<https://www.proteinatlas.org/humanproteome/secretome>)¹³⁵. These proteins were predicted by a whole-proteome scan using at least two of the following three methods for signal peptide prediction: SignalP4.0, Phobius, and SPOCTOPUS.

Transcriptome-based cell-cell communication analysis

We used a set of potential receptor-ligand interactions from the IUPHAR-International Union of Pharmacology (<https://www.guidetopharmacology.org/>)¹⁶⁶ and connectome database (https://fantom.gsc.riken.jp/5/suppl/Ramilowski_et_al_2015/vis/#/hive), which includes a total of 695 ligands, 652 receptors, and 2695 ligand-receptor interactions, as described by Ramilowski et al. (2015)¹⁴³. These authors carried out the first large-scale map of cell-to-cell communication between 144 human primary cell types, supported by

the literature. We also used the analysis strategies provided by Armingol et al. (2021)¹³⁹, which provide a way to examine interactions and apply them in different contexts.

Gene-set enrichment analysis

To investigate the biological role of the selected genes, we used the EnrichR platform (<https://maayanlab.cloud/Enrichr/>)¹⁶⁷ as a performance tool for functional enrichment analysis. We used the Wiki Human pathway, Reactome Human pathways, KEGG Human pathway, and Gene Ontology databases. We filtered the lists of each functional enrichment database using an Adjusted P-Value <0.05, considering only the functional enrichment routes with statistical significance. We classified the values of Log10 (Combined Score) from highest to lowest and considered the top 10 for each condition analyzed.

Protein-Protein Interaction Network

The proteins identified in the interactome were submitted to the Search Tool for Retrieval of Interacting Genes STRING, version 11.5; (protein-protein interaction networks with increased coverage and integration)²⁰⁹ for the construction of a protein-protein interaction network based on genes shared by healthy aging and sarcopenic individuals. To construct networks, we considered experiments, databases, co-expression, neighborhood, and co-occurrence as sources of active interactions. The minimum interaction score required was 0.700 (medium confidence), and the nodes disconnected from the network were hidden to simplify the display. The PPI enrichment p-value indicates the statistical significance provided by STRING. Accessed in June 2023.

Single-cell sequencing analysis in skeletal muscle samples from young and old individuals

We reanalyzed the dataset GSE167186, which contains single nuclei profiling of aged skeletal muscle from 17 vastus lateralis skeletal muscle samples (elderly = 11 samples and young = 6 samples)²⁴⁰ (Supplementary Table 1). For scRNA-seq, the Illumina HiSeq 4000 platform was used, and reads were mapped to the human genome using Cell Ranger (10× Genomics) and GRCh38 genome reference. Data analysis was performed using R version 4.2.0 using the Seurat package (version 4.0.6). We independently normalized and identified variable features for each sample after we completed the data integration of the young and old samples separately. We used a list from Perez et al. (2022)²⁴⁰ to identify the marker genes of cell types. We used the *FindMarkers* function in Seurat in clusters in which naming was impossible. We filtered the top ten markers based on log-fold changing values and confirmed the cell type in the Enrich database in the Tabula Sapiens, CellMarker, and Gene Atlas libraries.

Data representation and analysis

Correlation analysis and bar plots were constructed using GraphPad Prism software (GraphPad Software v.8.0.1). We used the Geneshot platform (<https://maayanlab.cloud/geneshot/>) to retrieve literature-associated genes for the conditions of muscle aging and sarcopenia. Venn diagrams were plotted using the webserver <http://bioinformatics.psb.ugent.be/webtools/Venn/>. Principal Component Analysis (PCA) plots were created using the web tools ClustVis²⁴² (<http://biit.cs.ut.ee/clustvis/>) and Morpheus²⁴³ (<https://software.broadinstitute.org/morpheus>). Clustering Euclidean distance analysis was used for grouping the profile of genes expressed in the transcriptome, interactome, and functional enrichment. Correlation analyses between the gene expression matrix (normalized by vst) and clinical information for each sample were performed using Morpheus software (<https://software.broadinstitute.org/morpheus>)¹⁷⁰. The top 100

positively and negatively marked genes in the sarcopenia group compared to the healthy elderly and young individual groups were considered. We utilized the ontology genes in the autophagy, AMPK, oxidative phosphorylation, and proteasome pathways, available on the Kyoto Encyclopedia of Genes and Genomes (KEGG) platform (<https://www.genome.jp/kegg/>). Our aim was to analyze the expression profile of these genes across samples from young individuals, healthy elderly individuals, and elderly individuals with sarcopenia (Supplementary Table S4). We used the SarcoAtlas platform (<https://sarcoatlas.scicore.unibas.ch/>) for the analysis of gene expression profiles in time course or neuromuscular junction in mice during muscular aging²⁰⁵.

Results

The clinical parameters Biodex (kg) and Timed Up and Go (s) were correlated with advanced age and sarcopenia

We subdivided the healthy elderly and sarcopenic individuals according to the age groups of 60, 70, and 80 years (Supplementary Tables 1 and 2). We calculated the mean and standard deviation of the quantitative clinical information. The results point to a variation in Biodex (kg), time Up and Go (s), SPPB, and Leg press (kg) in the group of older adults over 80 years of age with sarcopenia (Supplementary Table 2). We conducted the unpaired ANOVA statistical test of the clinical parameters Biodex (kg), Leg press (kg), Time up and Go (s), and SPPB among the conditions of sarcopenia, healthy elderly individuals, and young individuals. Our results indicated statistical significance only between Biodex (kg) (P-value) and time Up and Go (s) (Supplementary Figure 1a and b). We performed linear regression analysis between the exact ages of the study participants and clinical parameters. Biodex (kg) and age exhibit a significant linear regression with a positive correlation (Supplementary Figure 1c). Time Up and Go (s) and age were

negatively correlated (Supplementary Figure 1d). These findings suggest a reduction in muscle mass and function in individuals of advanced age.

The transcriptional profile of skeletal muscle during aging and in sarcopenia

The gene expression profile of the *vastus lateralis* skeletal muscle was compared among young males and healthy individuals, as well as sarcopenic elderly individuals categorized into age groups of 60, 70, and 80 years (Supplementary Table S3). We maintained the classification of older adults with sarcopenia based on Perez et al. (2022)²⁴⁰. Only up-or downregulated genes (adjP-value <0.05 ; fold change ≥ 1.5) present in at least one age group were considered Differentially Expressed Genes (DEGs). Eight genes were upregulated and 28 were downregulated in healthy elderly individuals in the 60-year-old age group. In the age group of 70 years, 63 genes were upregulated and 107 genes were downregulated. In the age range of 80 years, 22 genes were upregulated, and 28 genes were downregulated (Figure 1a). Among the sarcopenic elderly individuals, the 60-year-old age group had 40 upregulated and 37 downregulated genes. In the age group of 70 years old, 129 genes were upregulated and 213 genes were downregulated. In the 80-year age group, 230 genes were upregulated and 292 were downregulated (Figure 1b).

We performed a Principal Component Analysis (PCA) of DEGs from the transcriptome. Our results demonstrate a similarity in the gene expression profiles of healthy elderly individuals. However, the profile differed within the sarcopenic group, with the age of 80 years showing a distinct profile compared with the other groups (Figure 1c). The gene expression profiles of healthy elderly individuals aged 60–80 years were highly similar. However, in healthy elderly individuals aged 70 years, the profile resembles that of sarcopenic elderly individuals aged 60 years. The ages of 70 and 80 years were the ones that most significantly altered the gene expression profile in sarcopenic elderly

individuals (Figure 1d). We filtered the (DEGs) using a heatmap and lists of genes associated with sarcopenia and muscular aging available in the GeneShot database (<https://maayanlab.cloud/geneshot/>)²⁴⁴. Sixteen genes were present in all the lists (Figure 1e). These genes enriched for pathways related to mitophagy, insulin resistance, neurotrophin signaling, thyroid hormone signaling, insulin signaling, and cGMP-PKG signaling. In terms of Gene Ontology Biological Process, these genes enriched terms such as negative regulation of insulin secretion, negative regulation of peptide hormone secretion, skeletal muscle contraction, ventricular cardiac muscle tissue development, and positive regulation of cellular catabolic process (Figure 1f). The genes *IRS1* (*Insulin Receptor Substrate 1*), *PPARGC1A* (*PPARG Coactivator 1 Alpha*), *FOXO1* (*Forkhead Box O1*), *FOXO3* (*Forkhead Box O3*), *JUN* (*Jun Proto-Oncogene, AP-1 Transcription Factor Subunit*), *MYH3* (*Myosin Heavy Chain 3*), *MYH6* (*Myosin Heavy Chain 6*), and *MYH7* (*Myosin Heavy Chain 7*) were present in the majority of enriched functional terms (Figure 1f).

The expression of autophagy pathway genes correlates with sarcopenia

We used a gene list comprising the autophagy, AMPK, oxidative phosphorylation and proteasome pathways available on the Kyoto Encyclopedia of Genes and Genomes (KEGG) platform (<https://www.genome.jp/kegg/>)²⁴⁵. These pathways are known to be altered in muscle atrophy during aging and sarcopenia; however, it is not clear in the literature whether there is a stronger correlation between sarcopenia and the consequent exacerbated loss of muscle mass and function⁵⁹. In our results, many pathways showed a correlation of gene expression in the sarcopenia group compared with the groups of healthy young and elderly individuals (Figure 2, Supplementary Figure 3 and Supplementary Table S4).

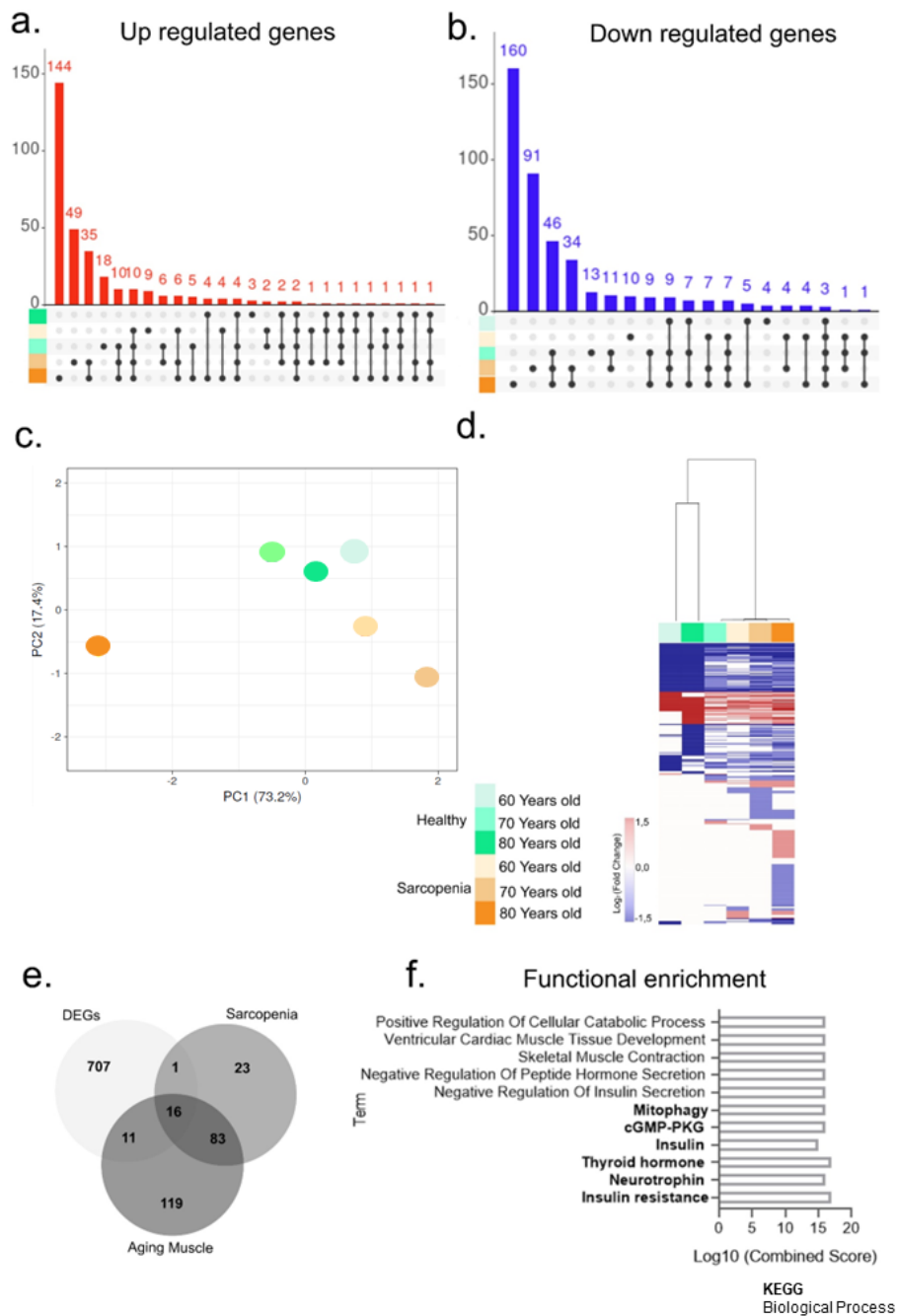


Figure 1: Transcriptional profile of the vastus lateralis muscle in different age groups under conditions of healthy aging and sarcopenia. a-b) UpSet plot of the intersection (<https://asntech.shinyapps.io/intervene/>) in healthy elderly and sarcopenic individuals in age groups of 60, 70, and 80 years old. Red represents the number of upregulated genes, and blue represents the number of downregulated genes. Black indicates gene intersections between age groups. c) Principal Component Analysis (PCA) grouping for the Differentially Expressed Genes (DEGs) under different conditions (<https://biit.cs.ut.ee/clustvis/>). d) Heatmap of the DEGs [$\log_2$ (Fold Change) ≥ 1.5 ; Adjusted p-value < 0.05] clustered by Euclidean distance. e) Intersection analysis (<https://jvenn.toulouse.inrae.fr/app/index.html>) between the total DEGs and between genes

associated with aging and sarcopenia on the GeneShot platform (<https://maayanlab.cloud/geneshot/>). f) Functional enrichment of the 16 common genes, where the bars represent log10 values of the Combined Score.

In the autophagy pathway, the genes *AMBRA1* (*Autophagy and Beclin 1 Regulator 1*), *MTMR3* (*Myotubularin Related Protein 3*), *ATG7* (*Autophagy Related 7*), *LAMP2* (*Lysosomal Associated Membrane Protein 2*), *CTSD* (*Cathepsin D*), and *SMCR8* (*SMCR8-C9orf72 Complex Subunit*) show a positive correlation with sarcopenia (Figure 2a). In the protein-protein interaction analysis, positive sarcopenia marker genes were present in three distinct interaction networks (Figure 2b-c). These genes enrich biological processes such as Negative Regulation of Macroautophagy, Cellular Response to Glucose Starvation, Autophagosome Maturation, and Positive Regulation of Protein Modification Process (Figure 2d). On the other hand, the genes *ATG12* (*Autophagy Related 12*), *BNIP3* (*BCL2 Interacting Protein 3*), *RAB7B* (*RAB7B, Member RAS Oncogene Family*), *ITPR1* (*Inositol 1,4,5-Trisphosphate Receptor Type 1*), and *VMP1* (*Vacuole Membrane Protein 1*) were negatively correlated with sarcopenia (Figure 2a). These genes are enriched in biological processes such as Phagosome-Lysosome Fusion, Protein Catabolic Process, Organelle Assembly, and Apoptotic Mitochondrial Changes (Figure 2d). We analyzed the top 5 positive and negative marker genes in sarcopenia related to autophagy using mouse data, specifically mRNA profiles of synaptic (NMJ) and extra-synaptic (xNMJ) regions of the Tibialis anterior (TA) muscle from 10 (10m) and 30 (30m) months old mice. We utilized the SarcoAtlas platform: plot and analyze gene expression in skeletal muscles during aging. The results indicate changes in the expression of genes *Mttr3*, *Atg12*, and *Rab7b* in aged mice over 30 months old (Supplementary Figure 2).

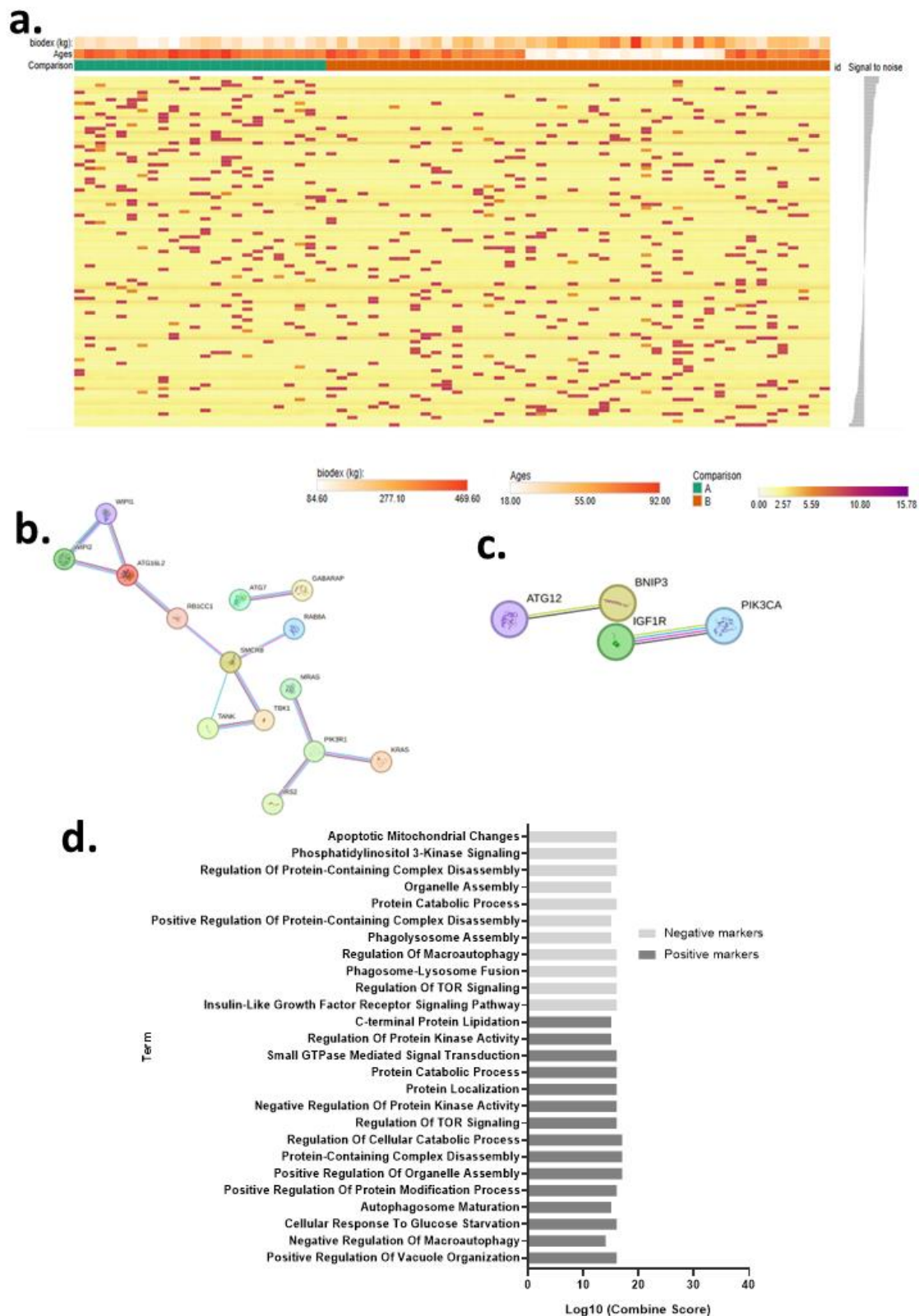


Figure 2: Sarcopenia is related to genes marking the Autophagy Pathway. a) Heatmap of the correlation among samples from the vastus lateralis muscle of elderly individuals with sarcopenia, healthy elderly individuals, and young individuals (expression values normalized by $\log_2(\text{vst})$). The top 100 positive and negative markers among elderly individuals with sarcopenia, healthy elderly individuals, and

young individuals were considered. Clinical information such as Biodex and the exact age of the individuals were considered. b) Protein-protein interaction analysis performed using STRING (<https://string-db.org/>) among the top 20 genes of positively correlated markers in sarcopenic individuals. A high-reliability threshold of 0.70 was considered in the analysis. c) Protein-protein interaction analysis was performed using STRING (<https://string-db.org/>) among the top 20 genes of negatively correlated markers in sarcopenic individuals. A high-reliability threshold of 0.70 was considered in the analysis. d) Ontology analysis considering Biological Processes of the top 20 positive (dark gray) and negative (light gray) markers.

In the AMPK pathway, genes that showed a positive correlation with sarcopenia were *PFKFB4* (6-Phosphofructo-2-Kinase/Fructose-2,6-Biphosphatase 4), *CREB5* (CAMP Responsive Element Binding Protein 5), *CIDEA* (Cell Death Inducing DFFA Like Effector A), *PFKFB1* (6-Phosphofructo-2-Kinase/Fructose-2,6-Biphosphatase 1), *LEP* (leptin), *ADRA1A* (Adrenoceptor Alpha 1A), *IRS2* (Insulin Receptor Substrate 2), *Leptin Receptor (LEPR)*, and *IGF1* (Insulin-Like Growth Factor 1) (Supplementary Figure 3a). The enriched biological processes included positive regulation of carbohydrate metabolic processes, energy derivation by oxidation of organic compounds, regulation of protein phosphorylation, T cell differentiation, and negative regulation of tumor necrosis factor production. In contrast, the genes *INSR* (Insulin Receptor), *IGF1R* (Insulin-Like Growth Factor 1 Receptor), *PIK3CA* (Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha), and *RAB2A* (RAB2A, Member RAS Oncogene Family), which are negatively correlated with sarcopenia, are involved in biological processes such as Positive Regulation of Phosphorylation, Phosphatidylinositol Phosphate Biosynthetic Process, Positive Regulation of Cell Migration, and Phosphatidylinositol-Mediated Signaling.

In the oxidative pathway, the genes *UQCRC1* (Ubiquinol-Cytochrome C Reductase, Rieske Iron-Sulfur Polypeptide 1), *COX6C* (Cytochrome C Oxidase Subunit 6C), *NDUFS7* (NADH: Ubiquinone Oxidoreductase Core Subunit S7), *NDUFA8* (NADH:

Ubiquinone Oxidoreductase Subunit A8), and *CYC1* (*Cytochrome C1*) were positively correlated with sarcopenia. These genes are involved in biological processes, such as Respiratory Chain Complex III Assembly, Mitochondrial Respiratory Chain Complex III Assembly, Mitochondrial Electron Transport, Ubiquinol to Cytochrome C, Respiratory Electron Transport Chain, and Electron Transport Chain (Supplementary Figure 3b). On the other hand, the genes *COX17* (*Cytochrome C Oxidase Copper Chaperone COX17*), *NDUFA11* (*NADH: Ubiquinone Oxidoreductase Subunit A11*), *NDUFA2* (*NADH: Ubiquinone Oxidoreductase Subunit A2*), *ATP12A* (*ATPase H⁺/K⁺ Transporting Non-Gastric Alpha2 Subunit*), and *NDUFB6* (*NADH: Ubiquinone Oxidoreductase Subunit B6*) exhibited a negative correlation with sarcopenia. Most of these genes are involved in biological processes such as ATP synthesis-coupled electron transport, copper ion transport, mitochondrial cytochrome C oxidase assembly, and positive regulation of ion transmembrane transporter activity.

In the proteasome, the genes *PSMB11* (*Proteasome Subunit Beta 11*), *PSMD12* (*proteasome 26S Subunit, Non-ATPase 12*), *PSMD2* (*proteasome 26S subunit ubiquitin-receptor, non-ATPase 2*), *PSME1* (*Proteasome Activator Subunit 1*), *PSMD13* (*proteasome 26S Subunit, Non-ATPase 13*), and *PSMB1* (*proteasome 20S Subunit Beta 1*) were positively correlated with sarcopenia (Supplementary Figure 3c). The genes *PSMD2* (*Proteasome 26S Subunit Ubiquitin Receptor, Non-ATPase 2*) and *PSME1* (*Proteasome Activator Subunit 1*) are enriched in biological processes such as protein N-linked glycosylation via asparagine, regulation of proteolysis involved in protein catabolic processes, positive regulation of peptidase activity, and positive regulation of endopeptidase activity. On the other hand, the genes *PSMA6* (*Proteasome 20S Subunit Alpha 6*), *PSMC1* (*Proteasome 26S Subunit, ATPase 1*), *PSMD6* (*Proteasome 26S Subunit, Non-ATPase 6*), *PSMB2* (*Proteasome 26S Subunit, Non-ATPase 6*), *PSMB5*

(*Proteasome 20S Subunit Beta 5*), *PSME4* (*Proteasome Activator Subunit 4*), and *PSMB9* (*Proteasome 20S Subunit Beta 9*), which exhibit a negative correlation with sarcopenia, are involved in biological processes such as Proteolysis and Regulation Of Cysteine-Type Endopeptidase Activity.

Sarcopenic individuals exhibit downregulated secretome genes associated with inflammatory and extracellular matrix components

In total, 75 DEGs were predicted in the human secretome. Among healthy elderly individuals, eight, 21, and eight secretome genes were described at the ages of 60, 70, and 80 years, respectively. In contrast, among sarcopenic individuals, two, 42, and 43 secretome genes were present at the ages of 60, 70, and 80 years, respectively (Supplementary Table S5). The majority of predicted secretome genes have been described at the ages of 70 and 80 years in sarcopenic individuals. The expression profile of the predicted secretome genes was downregulated with advancing chronological age, specifically in the groups of elderly sarcopenic individuals (Figure 3a).

The predicted secretome genes *COL15A1* (Collagen Type XV Alpha 1 Chain), *COL5A1* (Collagen Type V Alpha 1 Chain), and *COL6A6* (Collagen Type VI Alpha 6 Chain) were identified as upregulated and enriched pathways related to Protein Digestion and Absorption. In contrast, *VEGFB* (Vascular Endothelial Growth Factor) and *HBEGF* (Heparin Binding EGF Like Growth Factor) are upregulated in the 70-year-old sarcopenic group, and these genes are associated with biological functions such as Growth Factor Activity. The genes *ANGPT2* (Angiopoietin 2), *TGFB1* (Transforming Growth Factor Beta 1), *ERBB3* (Erb-B2 Receptor Tyrosine Kinase 3), *TGFB3* (Transforming Growth Factor Beta 3), *PDGFC* (Platelet Derived Growth Factor C), *IGF2* (Insulin-Like Growth Factor 2), *IL18* (Interleukin 18), *C3* (Complement C3), *C1S* (Complement C1s), *PLAT*

(Plasminogen Activator, Tissue Type), WNT5A (Wnt Family Member 5A), COL12A1 (Collagen Type XII Alpha 1 Chain), COL21A1 (Collagen Type XXI Alpha 1 Chain), COL19A1, INHBB (Inhibin Subunit Beta B), and INHBA (Inhibin Subunit Beta A) are predicted in the secretome and exhibit a downregulated expression profile. The majority of these genes enriched pathways such as MAPK signaling, inflammatory pathways, complement and coagulation cascades, protein digestion and absorption, signaling pathways regulating the pluripotency of stem cells, and cellular senescence.

We performed a correlation analysis between the 75 genes of the human secretome and the normalized gene expression matrix from the transcriptome in each sample of the young, healthy elderly, and sarcopenic elderly groups. We included clinical information, such as the exact age of individuals, body composition information by Biodex (kg), and functional information, such as time Up and Go (s). We selected the top 100 positively and negatively correlated genes with sarcopenia compared to the other conditions (healthy elderly and young individuals). The body composition information obtained by Biodex (kg) was more associated with the sarcopenic group (Figure 3c). Among the positive markers for sarcopenia are the genes *FCMR* (Fc Mu Receptor), *SAA1* (Serum Amyloid A1), *MTRNR2L8* (MT-RNR2 Like 8 (Pseudogene)), *SAA2* (Serum Amyloid A2), and *EDIL3* (EGF Like Repeats and Discoidin Domains 3). In the protein-protein interaction analysis, the genes *SAA1* and *SAA2* showed an interaction (Figure 3d). In the Biological Process ontology, these genes were associated with inflammation, enriching terms for interleukins 1, 4, and 13 (Figure 3e). In the time course transcriptional profile analysis in mice, the *FCMR* and *EDIL3* genes showed a transcriptional reduction in older mice (Supplementary Figure 4a-d).

Among the negative marker genes for sarcopenia samples, the most correlated were *SPOCK2* (SPARC (Osteonectin), Cwcv and Kazal Like Domains Proteoglycan 2), *IGF2*

(Insulin-Like Growth Factor 2), *ITIH4* (Inter-Alpha-Trypsin Inhibitor Heavy Chain 4), *POMC* (Proopiomelanocortin), and *SLIT2* (Slit Guidance Ligand 2). In the protein-protein interaction analysis, *IGF2* interacts with *IGFBP3* (Insulin-Like Growth Factor Binding Protein 3) (Figure 3d). These negative marker genes were present in the ontology of growth factor regulation, c-AMP-mediated signaling regulation, and IGF regulation (Figure 3e).

Eleven plasma secretome genes are expressed in both healthy aging and sarcopenia

We also filtered the DEG list using the human plasma list from The Human Protein Atlas. In total, 188 genes were predicted in the human plasma. Approximately 10, 49, and 14 plasma-predicted genes are present at the ages of 60, 70, and 80 years in healthy elderly individuals, respectively. Eighteen, 87, and 133 plasma-predicted genes were present in elderly sarcopenic individuals aged 60, 70, and 80 years, respectively. Approximately 115 genes are predicted in human plasma were downregulated specifically at the ages of 70 and 80 years. Thirty-six genes were shared between the ages of 70 and 80 in the sarcopenic elderly group (Supplementary Table 7). These genes enriched pathways such as calcium signaling, HIF-1 signaling, AMPK signaling, pentose phosphate, nicotinate and nicotinamide metabolism, adipocytokine signaling, inositol phosphate metabolism, cytoskeleton organization, apoptotic process, sarcoplasmic reticulum calcium ion transport, striated muscle contraction, and muscle contraction. *RET* (Ret Proto-Oncogene), *TPI1* (Triosephosphate Isomerase 1), and *GAPDH* (Glyceraldehyde-3-Phosphate Dehydrogenase) are essential in several signaling pathways and exhibit significant protein-protein interactions (PPI).

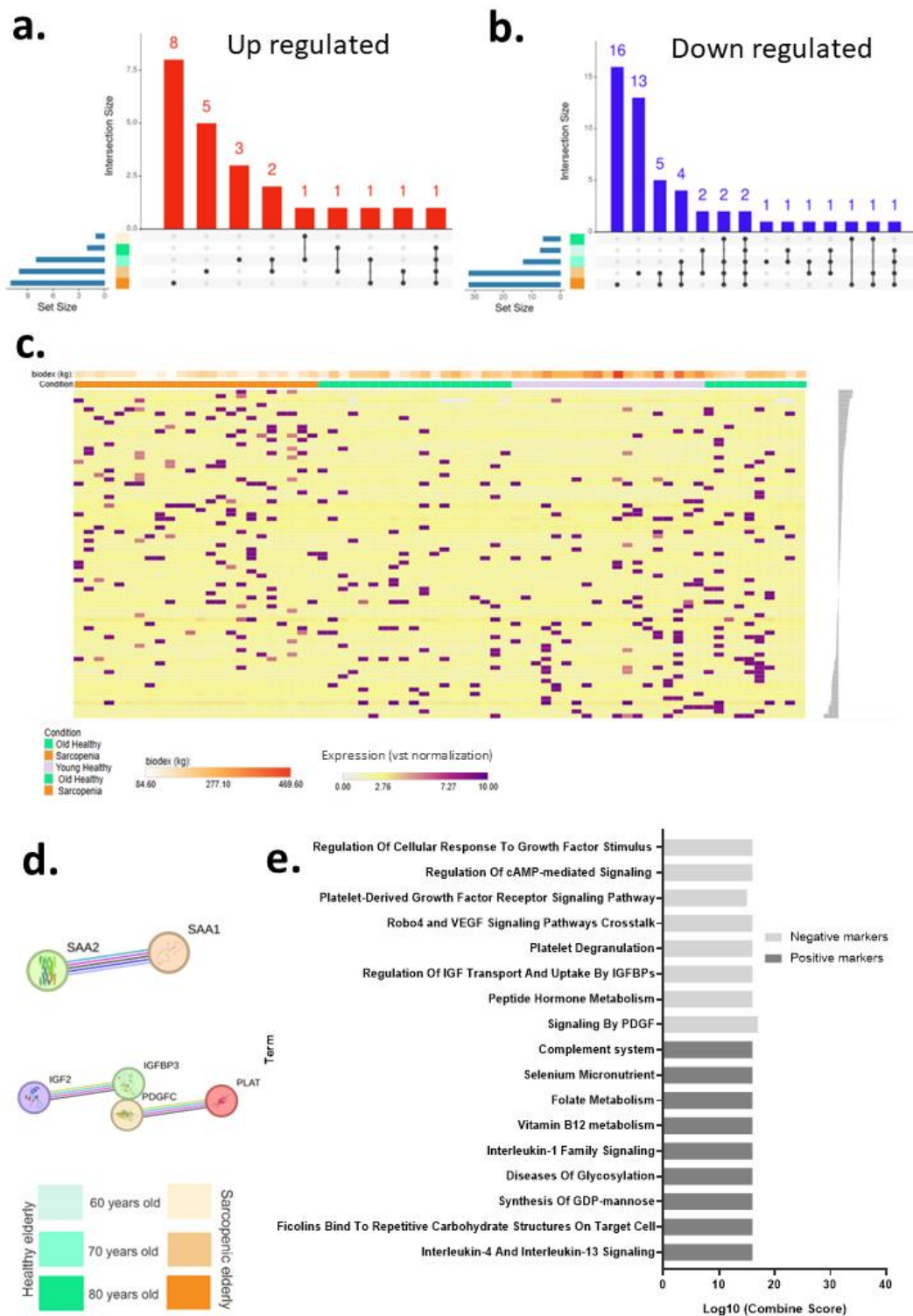


Figure 3: Predicted genes in the secretome exhibit distinct correlations between genes and groups of healthy and sarcopenic elderly individuals. a-b) UpSet plot of the intersection (<https://asntech.shinyapps.io/intervene/>) in healthy and sarcopenic elderly individuals aged 60, 70, and 80 years, focusing on DEGs predicted in the human secretome. Red represents the number of upregulated genes, and blue represents the number of downregulated genes. c) Heatmap depicting the correlation between predicted secretome genes and samples from the vastus lateralis muscle of male individuals in the

elderly sarcopenic, healthy elderly, and young groups (expression values normalized by $\log_2(\text{vst})$). The top 100 positive and negative markers among the elderly individuals with sarcopenia, healthy elderly individuals, and young individuals were considered. d) Protein-protein interaction analysis was performed using STRING (<https://string-db.org/>) among the top 20 genes of positively and negatively correlated markers related to sarcopenic individuals. A high-reliability threshold of 0.70 was considered in the analysis. f) Ontology analysis considering Biological Processes of the top 20 positive (dark gray) and negative (light gray) markers.

We compared the lists of genes predicted in the secretome and human plasma, and ten genes were present in both (Figure 4a). We conducted protein-protein interaction analysis of the common genes in the secretome and plasma, with most proteins interacting with epidermal growth factor components (Figure 4b). We performed statistical analysis using the Turkey test for the 11 genes, where genes *COL19A 1* (Collagen Type XIX Alpha 1 Chain), *INHBA* (Inhibin Subunit Beta A), and *POSTN* (Periostin) showed variation between the groups of healthy elderly individuals and sarcopenic individuals (Figure 4c). Among these genes predicted in the secretome and human plasma, *POSTN*, *COL19A1*, *SLPI* (Secretory Leukocyte Peptidase Inhibitor), *ANGPT2* (Angiopoietin 2), and *INHBA* are present in most biological processes. Only *INHBA* was upregulated in sarcopenic elderly individuals aged 70 and 80 years. These genes enriched biological processes and molecular functions related to external encapsulating structure organization, response to lipids, glomerulus vasculature development, regulation of collagen fibril organization, peptidase inhibitor activity, and endopeptidase regulator activity (Figure 4d).

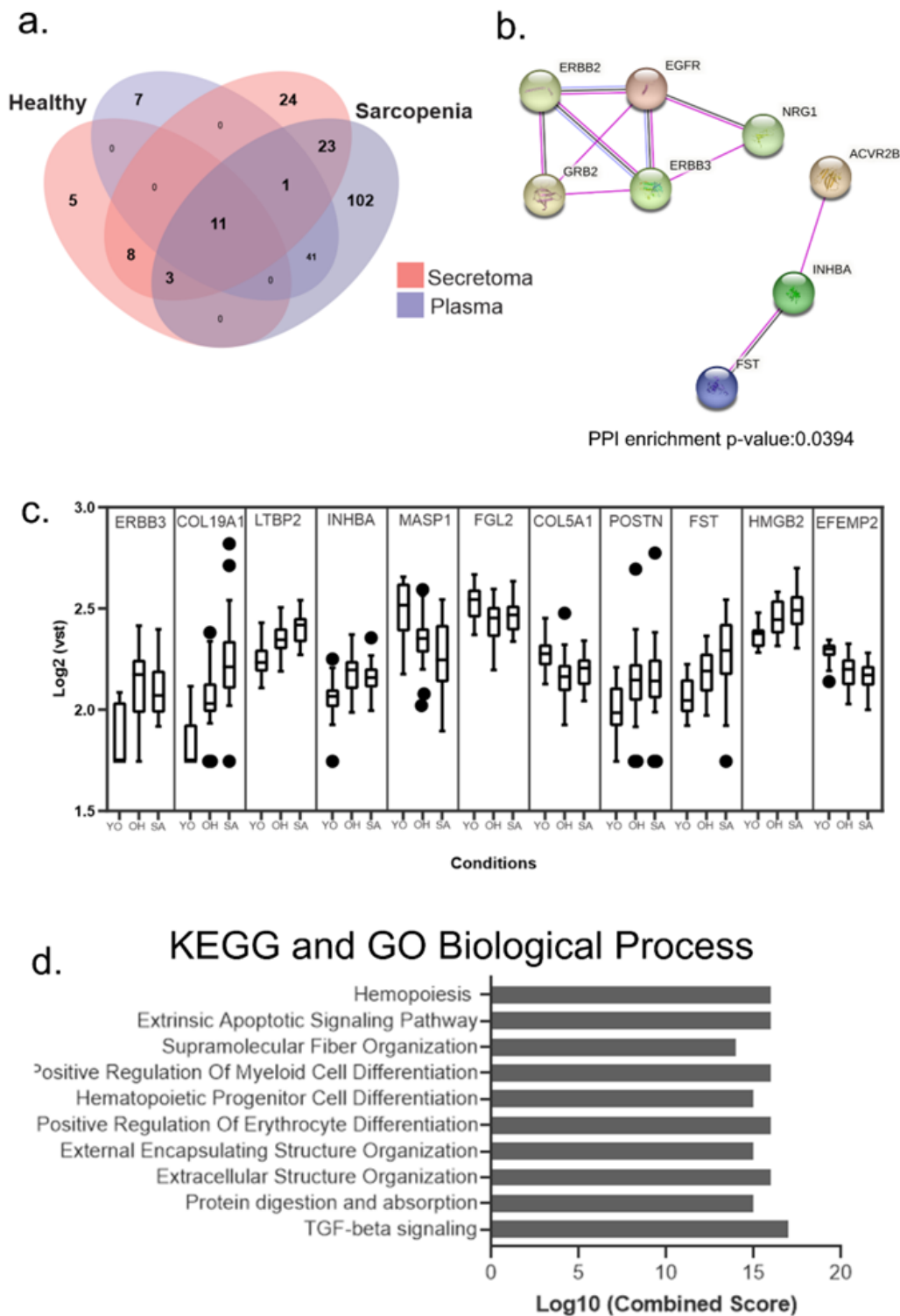


Figure 4: Eleven genes are common in the secretome and plasma of healthy elderly and sarcopenic individuals. a) Analysis of the intersection between the predicted secretome and plasma genes under the conditions of healthy aging and sarcopenia revealed 11 common genes. b) Protein-protein interaction analysis of these genes demonstrated their interaction. A high-reliability threshold of 0.70 and PPI enrichment with a P-value of 0.0394 were considered. c) Statistical analysis using the Tukey test for the

expression of shared genes among conditions. The black dot represents the statistical variation among young, healthy elderly, and sarcopenic conditions. d) Functional enrichment analysis using KEGG and Biological Process libraries.

Increased ligand-receptor interactions in sarcopenic individuals compared to healthy elderly individuals

We aimed to investigate the autocrine communication of Bulk RNA-Seq data from groups of 60-, 70-, and 80-year-old healthy elderly individuals and sarcopenic individuals. We selected a list of 641 ligands and 589 receptors, along with 1894 pairs of ligand-receptor interactions reported by Ramilowski et al. (2015)¹⁴³ (Supplementary Table S8). We filtered this list based on the differentially expressed genes (DEGs) for each analyzed condition to identify genes that predict ligand or receptor proteins and their respective interactions. We applied statistical filters, including an Adj p-value > 0.33, and the sum of the communication score of the fold-change expression value for the ligand and receptor. In total, 48 L × R interactions were described in the 70- and 80-year-old age groups of both healthy elderly and sarcopenic individuals (Supplementary Table S9-10). Among the groups of healthy elderly individuals, the 70-year-old age group showed 12 unique LxR interactions specific to that group, whereas the 70- and 80-year-old age groups of sarcopenic individuals had 13 and 12 LxR interactions, respectively. The 80 years old age group in the sarcopenic elderly group exhibited many interactions. However, the communication scores for these interactions were low (Figure 5a). This allowed us to infer that autocrine communication among cells in skeletal muscle is reduced in sarcopenia. Among the significant interactions, most genes predicted as ligands and receptors were associated with extracellular matrix (ECM) molecules such as collagen, fibulin, and integrins (*COL1A1_ITGA1*, *COL5A1_ITGA1*, *TGFB1_ITGB6*), growth factors, tyrosine kinase receptors, Serpin peptidase inhibitors, alpha-2-macroglobulin receptor, and hormonal receptors (*VEGFB_RET*, *EFEMP2_AQP1*,

SERPINE2_LRP1, *NCAM1_ROBO1*, *OSTN_NPR3*) (Figure 5b). We analyzed protein-protein interactions of the genes predicted as ligands and receptors, and three clusters were identified: ITGB6 and TGFB1 (green), COL5A1 and COL1A1 (red), and GDNF and RET (blue) (Figure 5c).

Validating Ligand-Receptor Interactions in Elderly and Sarcopenic Individuals using Integrated scRNA-Seq and Bulk RNA-Seq Data

To validate the ligand-receptor interactions found in our bulk RNA-Seq data from elderly and sarcopenic individuals in silico, we integrated scRNA-Seq data from the vastus lateralis skeletal muscles of elderly individuals. Most of the ligand-receptor interactions (*COL1A1_ITGA1*, *COL5A1_ITGA1*, *EFEMP2_AQP1*, *VEGFB_RET*, *GDNF_RET*, *SERPINE2_LRP1*, and *TGFB1_ITGB6*) occur with FAPS, endothelial cells, slow skeletal muscle and fast skeletal muscle (Figure 5d).

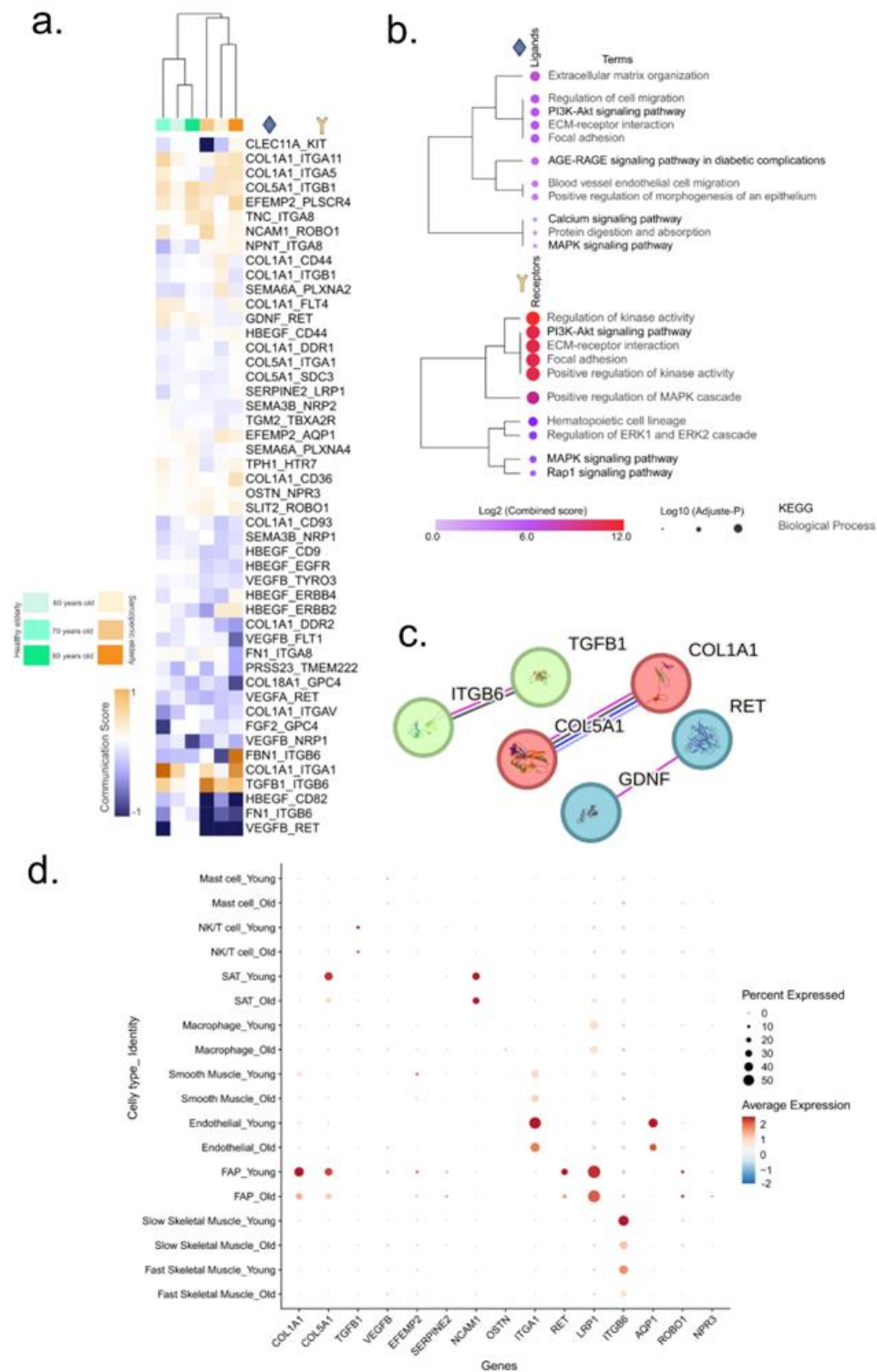


Figure 5: Skeletal Muscle Interactome in Sarcopenia Differs from Healthy Aging. a) Heatmap of interactions between healthy aging and sarcopenia conditions clustered by Euclidean Distance. Color refers to the Communication Score between the ligand's log2FoldChange multiplied by the receptor. b) Functional enrichment of genes predicted as ligands and genes predicted as receptors. c) Protein-protein interactions between ligands and receptors with the highest number of interactions. d) Characterization of cell type and

gene expression by single-cell sequencing analysis of vastus lateralis muscle samples from young males (N=5) and elderly individuals (N=12).

Discussion

Sarcopenia is defined as loss of muscle mass and function. The main risk factor for the development of sarcopenia is advanced age⁹⁰. Sarcopenia is associated with a higher risk of falls, fractures, functional impairment, and increased mortality rates⁹⁰. Molecular mechanisms, such as hormonal function (e.g., IGF-1 and insulin), muscle fiber composition, neuromuscular signaling, satellite cell potential for differentiation and proliferation, inflammatory pathways, and intracellular mechanisms in proteostasis and mitochondrial function processes, have been described as etiological factors of sarcopenia²⁴⁶. Here, we analyzed Bulk RNA-Seq of the *vastus lateralis* skeletal muscle from healthy elderly and sarcopenic individuals and compared it to the tissue from young males. Our results demonstrated an increase and decrease in the expression of genes involved in muscle atrophy pathways in sarcopenic individuals. The analysis of autocrine/paracrine communication in skeletal muscle showed alterations in signaling pathways related to the proteasome, ubiquitination, and mitophagy in skeletal muscle. These changes occur in fibroblast and muscle endothelial cell types. These findings demonstrate that sarcopenia differs from age-related muscle alterations owing to a significant increase in muscle atrophy pathways. Our work contributes to the understanding of alterations in autocrine communication in skeletal muscle, facilitating the identification of potential biomarkers for muscular sarcopenia associated with advancing chronological age.

Autophagy is the central intracellular degradation system through which cytoplasmic materials are delivered and degraded in the lysosome²⁴⁷. Moreover, autophagy is a dynamic recycling system that produces new building blocks and energy for cellular renewal and homeostasis²⁴⁷. Although autophagy is reduced with age²⁴⁸, recent studies

have pointed out that in many age-related diseases, there is an increase in autophagy to favor the degradation of defective proteins and consequent accumulation of misfolded proteins and dysfunctional organelles²⁴⁹. However, the role of autophagy in mammalian sarcopenia remains controversial. Both impaired autophagy and excessive autophagy have been linked to age-related sarcopenia²⁴⁹. We found a positive correlation between the genes *AMBRA1*, *ATG7*, *LAMP2*, and *CTSD* and samples from sarcopenic individuals. These genes appear to be related to the loss of muscle function due to dysregulation of autophagy^{78,250,251}.

Ambra1 is an intrinsically disordered protein that acts as a scaffold and is involved in cell cycle, proliferation, and autophagy²⁵⁰. Studies in mice have indicated that Ambra1-deficient muscles in both 3- and 6-month-old animals display a significant decrease in myofiber cross-sectional area and a 52% reduction in oxidative fibers²⁵⁰. The study by Carnio et al. (2014) points to a decline in the protein ATG7 and, concomitantly, LC3 lipidation in elderly animals when compared to young ones⁷⁸. In humans, there is a significant decrease in the protein levels of ATG7 and LC3II in the muscle biopsies of sedentary elderly individuals (sarcopenic). Morphological analyses of aged mice with *Atg7*^{-/-} knockout showed higher levels of atrophy, centrally nucleated fibers, and inflammation in muscles compared to age-matched controls⁷⁸. In a proteomic analysis, the lysosomal-associated membrane protein (LAMP2), considered one of the most abundant lysosomal proteins, was upregulated after denervation in mouse samples with muscular atrophy²⁵¹. A study by L'hôte et al. (2021)²⁵² showed that Cathepsin D is a diagnostic biomarker of sarcopenia. Memme et al. (2022)²⁵³ suggested a relationship with muscle denervation due to lysosomal dysfunction. These findings highlight the importance of autophagic pathways in sarcopenia, both in terms of the functional state of

the autophagic system and the quality control associated with autophagy during the pathogenesis of sarcopenia²⁵⁴.

Autocrine signaling is essential for skeletal muscles. Several factors, including nutrients, metabolites, ions, and cytokines, play crucial roles in cellular signaling and the maintenance of muscle mass²⁵⁵. During muscle aging, there is a decline in communication between muscle stem cells and other cells that constitute the muscle environment²⁵⁶. In our data, although there was an increase in the number of predicted genes in the human secretome during sarcopenia, there was a decline in the regulatory profile of these genes. Most genes were downregulated in sarcopenia. This decline in muscle communication capacity also persists during autocrine signaling. We found a reduction in the communication score between the predicted ligands and receptors in the skeletal muscle during the aging of healthy individuals and sarcopenic individuals. The secretome of the skeletal muscle has been commonly studied in response to exercise adaptation²⁵⁷. Despite the importance of the secretome for muscle during sarcopenia, few studies have investigated the muscle secretome during aging²⁵⁸.

Our data on autocrine signaling indicate communication between components of the extracellular matrix (ECM) and molecules of the capillaries and vascular endothelium. The ECM in skeletal muscle provides a stable microenvironment that supports cell adhesion, migration, proliferation, and differentiation¹⁸². Despite their profound importance, ECM biomarkers are underutilized in different contexts, such as the effects of exercise, disuse, and aging on muscle function, growth, and structure³⁹. Aging is associated with dense deposition of ECM and loss of muscle stem cell function, resulting in reduced muscle regenerative capacity and strength²⁵⁹. The relationship between the reduction of ECM components, such as collagens and muscle fibers, during aging is well established in the literature³⁹. Rodrigues et al. (1996)²⁶⁰ demonstrated that the diaphragm

and rectus abdominal muscles in aged mice showed a low amount of collagen in type I and II muscle fibers. In studies of skeletal muscle in older men in response to exercise stimuli, there was an increase in the expression of genes associated with ECM remodeling, specifically *MMP9* and *COL1A1* genes²⁶¹. Therefore, ECM components are crucial for muscle homeostasis. Furthermore, Baumert et al. (2018)²⁶² investigated polymorphisms in the *COL1A1* and *COL5A1* genes. These results indicate that alterations in these genes may negatively influence muscle strength and repair, possibly through a dysregulated collagen network affecting the mechanical properties of the muscle. In our study, the expression levels of the collagen genes *COL1A1* and *COL5A1* remained unchanged in the sarcopenic groups. Although cell-cell signaling factors that direct muscle development and regeneration have received considerable attention in recent years^{31,263}, the understanding of how ECM components and their interactions with cells of the muscle microenvironment continue to be poorly described in the literature.

During aging, many changes occur in the vasculature, hemostasis, and endothelium, including changes in platelets, coagulation, and fibrinolytic factors²⁶⁴. These changes imply a high risk of mortality and increased risk of functional dependence and geriatric syndromes. Aging induces specific impairment of vascular function in the skeletal muscle of aged mice²⁶⁵. In conditions such as osteoarthritis and sarcopenia, endothelial cells exhibit different responses depending on the tissue²⁶⁶. In aged skeletal muscle, the low-grade systemic inflammation associated with sarcopenia results in impaired angiogenesis, potentially highlighting inflammation as a trigger for vascular alteration in sarcopenia²⁶⁶. Furthermore, Wang et al. (2014)²⁶⁷ suggested an association between sarcopenia and satellite cell apoptosis and impairment of capillary functions, which likely contributes to the decline in muscle mass and functionality during aging. Most of the ligand-receptor interactions we found occurred between fibroadipogenic progenitors (FAPs) and

endothelial cells in the sarcopenic elderly group. With the ligand genes *VEGFB*, *EFEMP2*, and *GDNF* and the receptors *RET* and *AQP1*, the majority of interactions exhibited a reduction in communication score.

Insufficiency of vascular endothelial growth factor (VEGF) signaling forms the basis of vascular insufficiency in aged mice and contributes to the notion that age-related decline in vascular function is a driver of aging²⁶⁸. In mouse models of mdx mice transplanted with muscle-derived stem cells (MDSCs) overexpressing *VEGF* during muscle regeneration, there was an improvement in skeletal muscle repair through the modulation of angiogenesis, regeneration, and fibrosis in injected mdx skeletal muscle²⁶⁹. *RET* is dynamically expressed during myogenic progression in mouse and human myoblasts²⁷⁰. Constitutive *RET* expression increased myoblast proliferation, whereas siRNA-mediated knockdown of *RET* induced myogenic differentiation²⁷⁰. These data indicate the critical role of the interaction between *VEGF* and *RET* during muscle regeneration in terms of muscle cell differentiation and vascularization^{269,270}. Therefore, investigating these genes in sarcopenia is of utmost importance to understand the reduction in muscle mass and strength.

In conclusion, through a comprehensive analysis of the muscle secretome and interactome using transcriptomic profiling, we identified significant differences in gene expression profiles, mainly related to the autophagy pathway and the regulation of Extracellular Matrix (ECM) components and endothelial growth factors. Our results suggest that these alterations may contribute to the loss of muscle mass and strength observed in the elderly individuals with sarcopenia. The identification of collagen ligands and receptors for endothelial growth factors highlights the interaction between mesenchymal muscle cells and the vascular and capillary endothelium, underscoring the importance of cellular communication for healthy muscle function. These findings provide new insights into the

underlying mechanisms of sarcopenia and deepen our understanding of the molecular changes involved in age-related decline in muscle function. However, further investigations are needed to elucidate the specific molecular mechanisms and validate the pathways identified as promising therapeutic targets.

Limitation of the study

Due to the scarcity of literature providing further studies on Sarcopenia, we couldn't locate RNA sequencing datasets containing sarcopenia information in women. It was not feasible to assess the expression changes and cellular types occurring in skeletal muscle in Sarcopenia using scRNA-Seq datasets. Ligand-receptor interactions lacked *in vitro* or *in vivo* validation.

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Acknowledgments

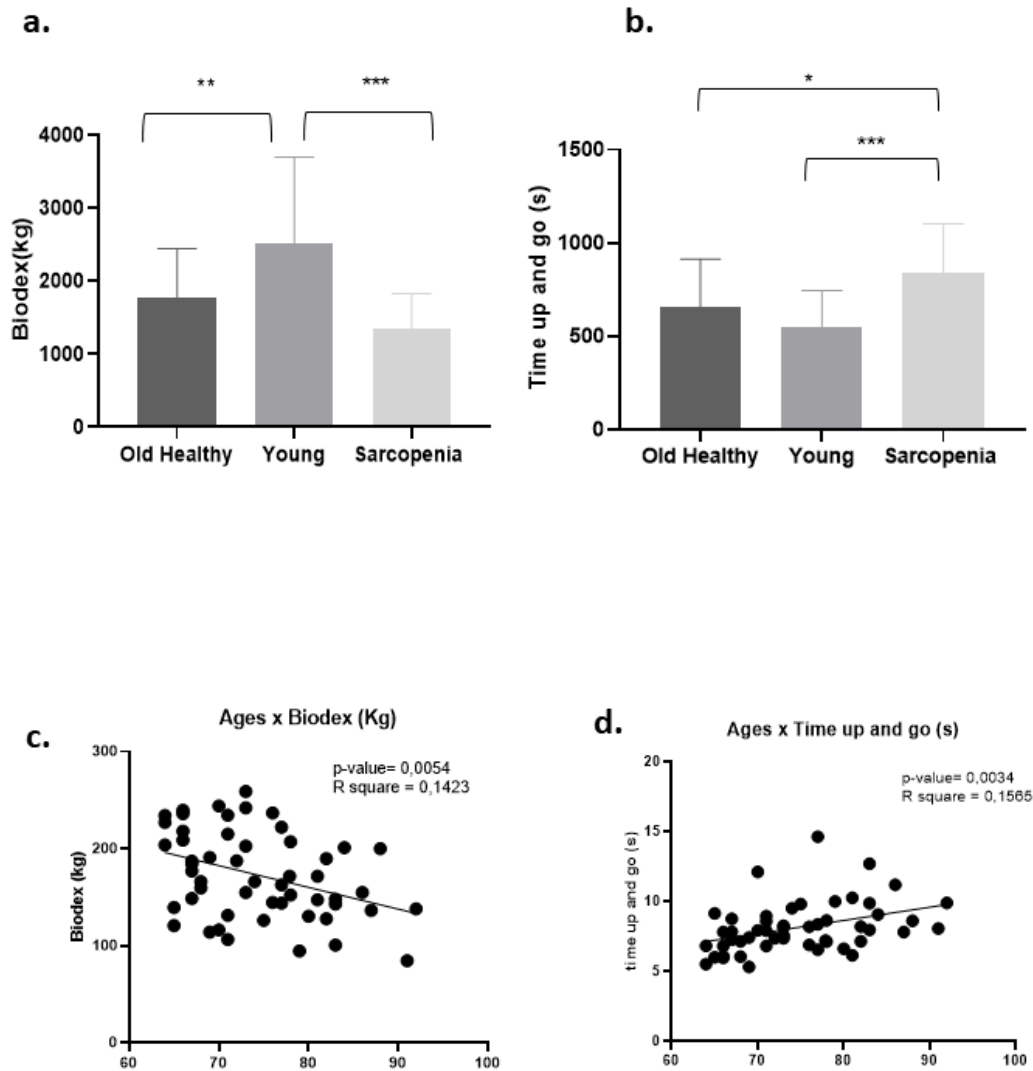
To study collaborators and CAPES.

Data Availability Statement: Publicly archived datasets analyzed herein are available at the Gene Expression Omnibus (GEO) under accession numbers GSE167186.

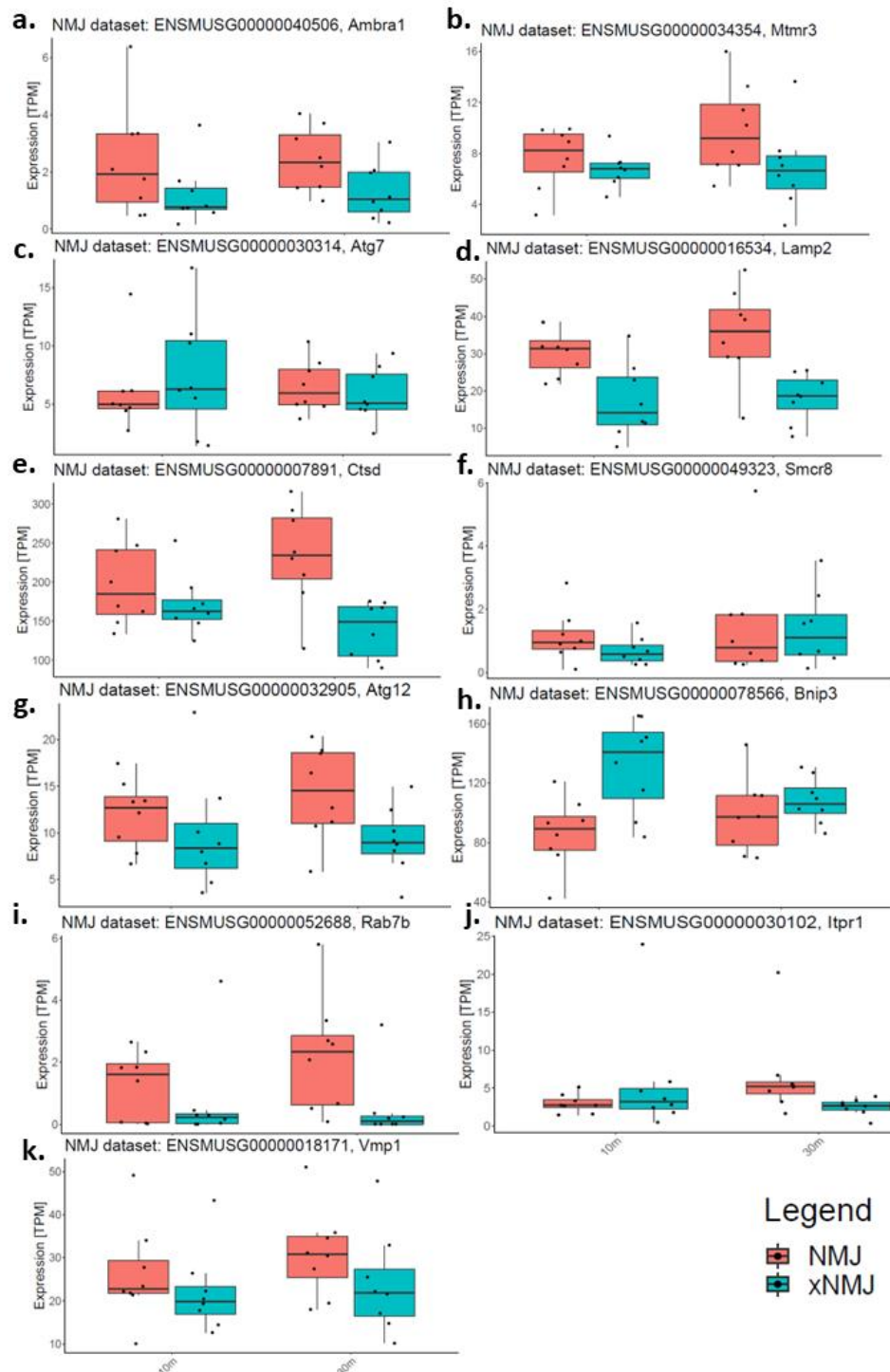
Conflict of interests

The authors declare that they have no conflicts of interest.

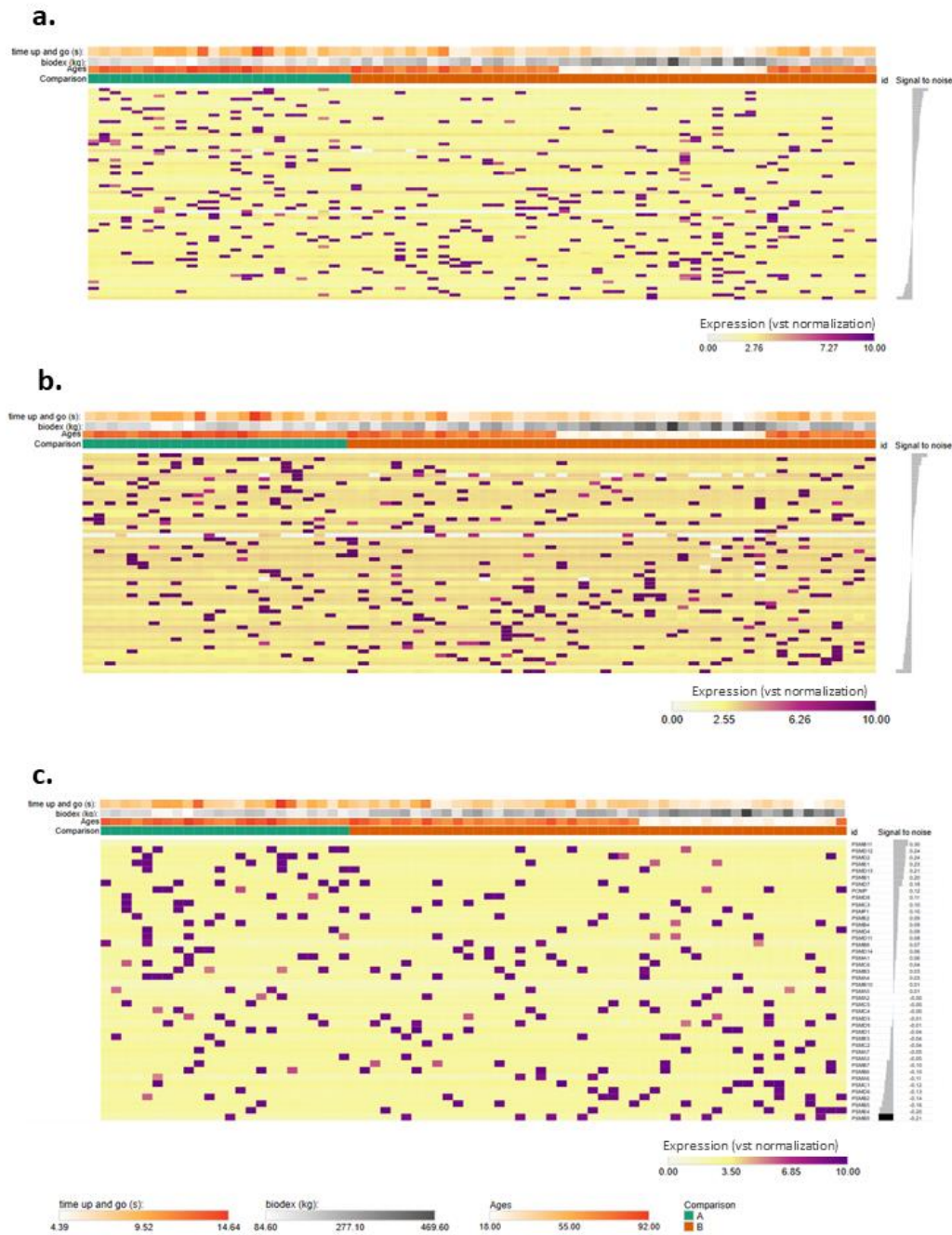
Supplementary Figure legends



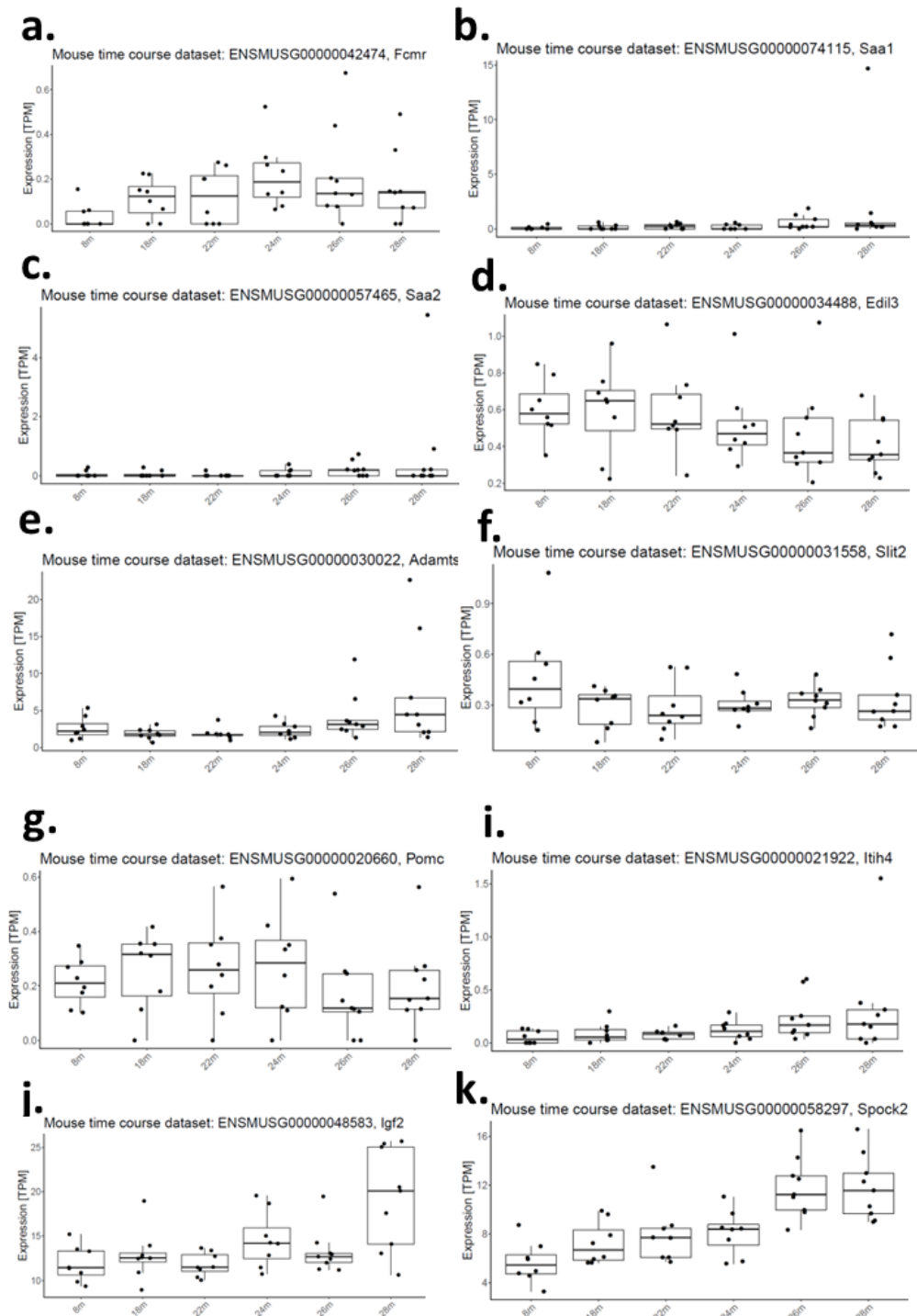
Supplementary Figure 1: Statistical Analysis of Clinical Data from Samples of Young, Healthy Elderly, and Sarcopenic Individuals. Unpaired ANOVA test among conditions (young, healthy elderly, and sarcopenic) considering Biodex clinical conditions (kg) and Time Up and Go test (s) One-way ANOVA analysis with Tukey's multiple comparisons test: **p-adj= 0.0060; ***p-adj < 0.0001. b) One-way ANOVA with Tukey's multiple comparison test: *p-adj= 0.0184; ***p-adj= 0.0006. c-d) Simple linear regression analysis considering the exact age of young, healthy elderly, and sarcopenic individuals with Biodex clinical conditions (kg) and the time Up and Go test (s).



Supplementary Figure 2: Gene expression analysis in mouse skeletal muscles during aging. The analysis was performed in SarcoAtlas (<https://sarcoatlas.scicore.unibas.ch/>), considering the mRNA profiles of synaptic (NMJ) and extra-synaptic (xNMJ) regions of the tibialis anterior muscle from 10 and 30-month-old wild-type mice. a-k) Genes present in the autophagy pathway correlated with positive markers for sarcopenia were considered.



Supplementary Figure 3: Heatmap of the correlation between sarcopenia and healthy elderly and young individuals. The matrix considers the gene expression values normalized by vst. Predicted genes in the pathways were extracted from the KEGG database (<https://www.genome.jp/kegg/kegg1b.html>). a) Genes present in the AMPK pathway. b) Genes present in the oxidative phosphorylation pathway. c) Genes present in the proteasome pathway.



Supplementary Figure 4: Gene expression analysis of skeletal muscles during aging in mice. The analysis was conducted using SarcoAtlas (<https://sarcoatlas.scicore.unibas.ch/>), considering mRNA profiles of the gastrocnemius muscle from 8-, 18-, 22-, 24-, 26-, and 28-month-old wild-type mice. a-e) Genes identified as positive markers of sarcopenia. f-k) Genes identified as negative markers for sarcopenia.

Considerações finais

O músculo esquelético é um tecido altamente adaptativo. O perfil transcricional do músculo esquelético em diferentes condições como exercício físico, desuso, diabetes, caquexia associada ao câncer, envelhecimento e sarcopenia nos fornecem novos *insights* sobre a versatilidade muscular. Neste trabalho, evidenciamos as diferentes respostas transcricionais do músculo, bem como as diferenças nos perfis de genes preditos no secretoma humano e a caracterização da sinalização autócrina/parácrina. No capítulo 1, caracterizamos o perfil do secretoma e comunicação celular pelo transcriptoma em exercício físico do tipo HIIT e RT em indivíduos jovens e idosos. Demostramos as alterações nos perfis do secretoma em diferentes modalidades de exercício físico. Evidenciamos as mudanças da comunicação celular em amostras de indivíduos idosos. Além disso, apontamos o papel crucial de componentes de matriz extracelular no microambiente muscular e na comunicação de células satélites e do endotélio. No capítulo 2, mostramos as diferenças entre o perfil de expressão de genes em condições catabólicas no músculo esquelético em camundongos. Evidenciamos o perfil transcricional de genes relacionados a marcadores de atrofia muscular, os atrogenes. Demostramos a comunicação celular nas condições de desuso, envelhecimento, diabetes e na caquexia associada ao câncer. Evidenciamos as mudanças da comunicação celular entre as condições catabólicas. Além de apontarmos o papel das moléculas e células do sistema imune inato e especializado na caquexia associada ao câncer. No capítulo 3, analisamos o perfil transcricional de genes do sistema da autofagia com o envelhecimento e sarcopenia correlacionado a informações clínicas como idade, Biodex (kg) e teste de desempenho funcional (Time Up e Go (s)). Apontamos para as mudanças nos perfis de genes preditos no secretoma, especificamente em idosos sarcopênicos com mais de 70 anos de idade. Elucidamos a comunicação celular no envelhecimento e na sarcopenia,

apontando mudanças na sinalização entre componentes de matriz extracelular e fatores de crescimento do endotélio vascular. Esses achados fornecem uma caracterização sobre moléculas que podem desempenhar importante papel no microambiente muscular durante a regeneração muscular em diferentes contextos. Como perspectivas futuras, esses resultados poderão ser utilizados como base para estudos que buscam desenvolver respostas terapêuticas para o declínio da sinalização bem como atrofia muscular em doenças crônicas.

Conclusões

A caracterização do secretoma e comunicação celular pelo transcriptoma do músculo esquelético foi diferente entre as condições de exercício físico, envelhecimento e sarcopenia em humanos. Bem como em condições como desuso, diabetes, caquexia associada ao câncer e envelhecimento em músculo esquelético de camundongos.

No conjunto de dados de sequenciamento de RNA e sequenciamento de células únicas há diferenças entre os perfis de expressão de genes do transcriptoma nas diferentes modalidades de exercício físico e nos grupos de indivíduos jovens e idosos. Essas alterações estavam presentes na caracterização da comunicação celular assim como no secretoma. Independente da prática de exercício físico, em indivíduos idosos, ocorre uma redução da comunicação celular no músculo esquelético.

No conjunto de dados de músculo esquelético em camundongos em condições catabólicas como o desuso, diabetes, envelhecimento e caquexia associada ao câncer. Apontamos para as diferenças nos perfis de expressão gênica. Além disso, filtramos esses genes com atrogenes descritos na literatura. Esses achados contribuem com a literatura que aponta diferenças nos perfis e genes envolvidos em mecanismos de atrofia muscular. Na análise da comunicação celular nossos achados apontam para a inflamação aguda que ocorre no músculo, especificamente na caquexia associada ao câncer. Apontamos para a redução da sinalização do músculo esquelético. Além de apontar a relação de componentes do sistema imune com células como macrófagos.

No conjunto de dados de indivíduos idosos saudáveis e sarcopenicos encontramos diferenças no perfil transcricional de genes e relacionamos marcadores positivos para a sarcopenia em via do sistema da autofagia. Apontamos relevância estatística entre a idade, biodex (kg) e teste de desempenho (Time Up and Go (s)) com amostras de indivíduos

idosos com sarcopenia. A análise dos genes diferencialmente expressos (DEGs) a redução de genes regulados no secretoma em indivíduos idosos com mais de 70 anos, especificamente nos indivíduos sarcopenicos. A maioria dos genes do secretoma estão associados a via de matriz extracelular, apontando a importância dos componentes de matriz para a comunicação celular. A análise da comunicação celular demonstrou a redução da comunicação entre componentes do endotélio e comunicação de componentes de matriz extracelular, especificamente colágenos do tipo I e V. Além disso, está clara a importância dos FAPs e células endoteliais para a sinalização muscular e regeneração.

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Materiais suplementares

As tabelas suplementares dos três capítulos desse trabalho estão disponíveis do drive, no seguinte link de acesso:

https://drive.google.com/drive/folders/1OV7SpCeRSrRJdXF3H0nCE69EwbR9HV3l?usp=drive_link