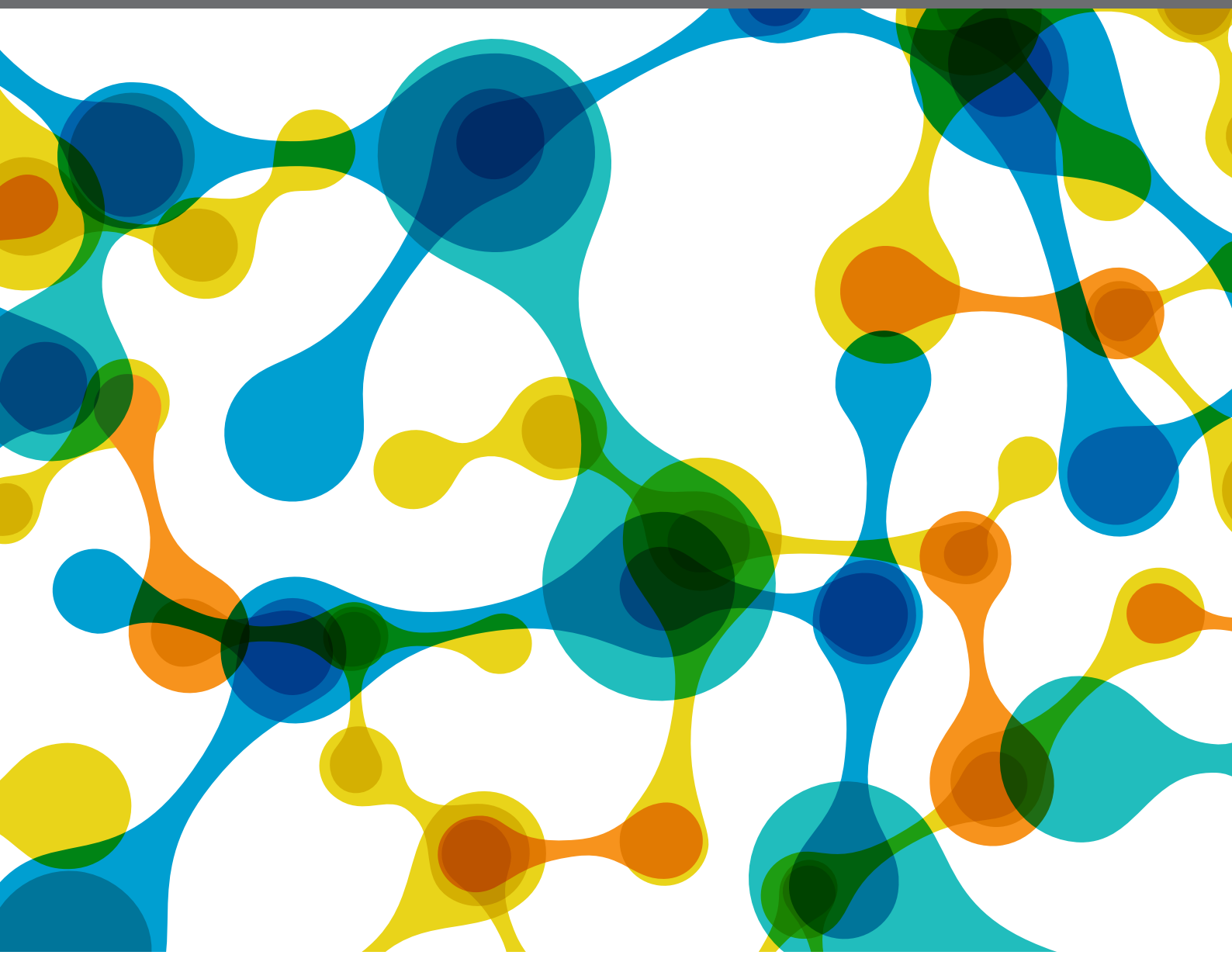
TRENDS IN MUSCLE AND TENDON MOLECULAR AND CELL BIOLOGY

EDITED BY: Rita de Cassia Marqueti, Michael Kjaer and Anselmo Sigari Moriscot

PUBLISHED IN: Frontiers in Physiology



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ISSN 1664-8714
ISBN 978-2-88974-583-8
DOI 10.3389/978-2-88974-583-8

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TRENDS IN MUSCLE AND TENDON MOLECULAR AND CELL BIOLOGY

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Citation: de Cassia Marqueti, R., Kjaer, M., Moriscot, A. S., eds. (2022). Trends in Muscle and Tendon Molecular and Cell Biology. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-88974-583-8

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frontiers in Physiology

EDITORIAL
published: 03 February 2022
doi: 10.3389/fphys.2021.832613

Editorial: Trends in Muscle and Tendon Molecular and Cell Biology

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Keywords: skeletal muscle, tendon, myotendinous junction, extracellular matrix, mechanosignaling, basic science

Editorial on the Research Topic

Trends in Muscle and Tendon Molecular and Cell Biology

INTRODUCTION

The musculoskeletal system performs a vital role over life, participating in structural support, delicate to complex movements involved in daily life activities, elite sporting events, and body protection/stabilization (Greising et al., 2020). The musculoskeletal system includes muscles and tendons, as well as ligaments and bones (Yamamoto and Abe, 2020). Muscles and tendons are fundamental for locomotion and posture maintenance, however, although they have been largely explored at the structural, functional, and cellular/molecular biology levels, their adaptive mechanisms are still largely unknown. The connection site between muscle and tendon is named the myotendinous junction (MTJ), which is a fundamental transitional structure for transferring muscle force from the contractile apparatus to the tendon, and ultimately to the final destination: bone, cartilage, skin, and eye (Charvet et al., 2012). Further, the MTJ is the most prominent place for traumatic muscle strain injury. This will put it into perspective also of sports injury. Considering this critical issue, the overall aim of this Research Topic was to investigate how muscle, tendon, and the myotendinous junction adapt under different stimuli conditions, such as mechanical unloading (atrophy) and loading (exercise), sepsis, inflammation, lesion, and aging, under the perspective of basic science. Another goal was to characterize the role of the extracellular matrix (ECM), matrix cell interactions with molecular pathways, and morphological tissue properties under these conditions. In this Research Topic we will present a collection of studies on skeletal muscle (seven articles) and tendon (two articles), and one study specifically addressing the muscle-tendon junction. Researchers in the area can benefit from the advancement in the science and increased chances of integrative insights into muscle and tendon.

MUSCLE

Skeletal muscle tissue contains immense, highly specialized cells with an exceedingly developed cytoskeleton, where myosin interacts with actin, generating mechanical energy. Furthermore, skeletal muscle is particularly abundant, comprising 40–60% of total body mass in humans, and is involved in body mobility and stands as an essential energy reservoir (Frontera and Ochala, 2015). It is now clear that decreased skeletal muscle mass is an independent predictor of mortality (Du et al., 2014). Therefore, deep understanding of the biology of muscle atrophy is essential for the future development of more efficient treatments, especially in older adults, which is one of the focuses of this Research

Edited and reviewed by:
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Specialty section:
This article was submitted to Striated Muscle Physiology, a section of the journal Frontiers in Physiology

Received: *10 December 2021*
Accepted: *30 December 2021*
Published: *03 February 2022*

Citation:
Marqueti RC, Kjaer M and Moriscot AS (2022) Editorial: Trends in Muscle and Tendon Molecular and Cell Biology. Front. Physiol. 12:832613. doi: 10.3389/fphys.2021.832613

Topic: He et al. revealed the circulating microRNAs involved in older adults with sarcopenia. In addition a comprehensive time course of the transcriptome in the clinically relevant rotator cuff tear model, including intense atrophy was reported by Vasquez-Bolanos et al. Diaphragm muscle is especially prone to develop atrophy and quick functional loss under stressful conditions such as sepsis and muscular dystrophies. In this collection, it was emphasized that diaphragm under sepsis undergoes functional loss through mitochondrial dysfunction (Oliveira et al.). Furthermore, it was showed that oxidative stress is a key player in diaphragm degeneration, which can be mitigated by anti-oxidant treatment (Silva et al.). Physical exercise is certainly one of the most powerful strategies to fight skeletal muscle atrophy. Physical activity could create an intergenerational drive, attenuating the harmful effects of a high fat diet (HFD) in skeletal muscle from offspring (Salomão et al.). As demonstrated by Rovina and coauthors, even a single bout of physical activity can boost the expression of Nr1d1, a nuclear factor known to decrease atrophy gene program and increase mitochondrial biogenesis (Rovina et al.).

TENDON

Of all consultations performed by primary care physicians, 20% are related to musculoskeletal diseases; 30% of these are associated with tendon injuries, called tendinopathies, which represent a highly prevalent problem in musculoskeletal medicine (Jordan et al., 2014). According to Burton, an important range of physical, genetic, and psychological individual factors has been recognized in patients affected by tendinopathy, resulting in a tremendous heterogeneous population and a challenge for rehabilitation (Burton). For this reason, understanding the basic science of tendons such as tendon homeostasis, maintenance, remodeling, and repair is an essential strategy for improving rehabilitation. Accordingly, we know that mechanical load is vital for tendon development and homeostasis to preserve all properties of the extracellular matrix (ECM). Tendons are mechanosensitive soft tissues able to transmit tensile forces between muscle and bone, acting in a catapult-like manner, also called a mechanical spring mechanism, by storing and releasing energy and then increasing the efficiency of the movement system (Nakajima et al., 2021). It is well-known that tendon fibroblasts adapt to the mechanical forces, transmitting forces across the extracellular matrix (ECM) without losing mechanical dynamism (Sawadkar et al., 2020). The specific ECM enables joint function and mediates mechanical signals to tendon cells, driving biological responses to exercise or injury. Interactions between the cells and the matrix (cell-matrix interactions) permit tenocytes (predominant cell type in the tendon) to sense and reply to mechanical signals with a catabolic or anabolic response (Kjaer, 2004; Chatterjee et al., 2021). Mechanotransduction is an essential mechanism by which mechanical stress acts upon a cell, initiating intracellular signaling, promoting cell growth and survival. As a tissue that efficiently transmits the mechanical forces, it is essential to comprehend the mechanisms of mechanotransduction. New insights have been gained in which several ion channels and receptors are identified as mechanosensors. The mechanosensitive ion channel PIEZO1 is responsible for different mechanotransduction processes in the lymphatic, cardiovascular, renal, and skeletal systems. Passini et al. (2021) analyzed the effect of reduced and elevated PIEZO1 mechanosignaling in tendons of rodents. *Piezo1* knockout mice (*Piezo1*cKO) showed reduced tail tendon stiffness, while *in vitro* pharmacological PIEZO1 stimulation and *in vivo* PIEZO1 overactivity improved tendon stiffness and strength (Passini et al., 2021). Mechanical properties allow tendons to respond and adapt to the load transmitted by the muscles. Collagen fibrils are tendon force-transmitting units distributed within the ECM and oriented parallel to the bone-muscle axis (Eekhoff et al., 2021). Tension across the muscle and tendon is essential for maintaining the integrity of both tissues. When one of them undergoes a change, both are affected. In this collection, it was reported that transcriptional changes in supraspinatus muscle after a rotator cuff tear model in rabbits. Consequently, the dysfunction led to muscle atrophy and fatty infiltration (Vasquez-Bolanos et al.). However, the transmitting force generated by the muscle to the tendon depends on the connection between these tissues, the MTJ.

MYOTENDINOUS JUNCTION

The MTJ is a specific anatomical region connecting skeletal muscle to tendon. The MTJ constitutes an integrated mechanical unit, the major site of force transmission. The muscle cell membrane is folded into finger-like extensions and invaginations to increase the area and allow the membrane to resist muscle contraction forces (Charvet et al., 2012; Subramanian and Schilling, 2015). The significant molecules of the tendon side are collagen I and tenascin-care, while the laminins and collagen IV are the major constituents of the muscle side (Subramanian and Schilling, 2015). Jakobsen and Krogsgaard, presented in this collection a review exploring the importance of MTJ in the sports field since the majority of strains are most dominantly located at the MTJ. The MTJ adapts to load by increasing the remodeling and junctional interface, while the opposite effect, shortening the folding in a smaller interface, occurs during inactivity. The authors also concluded that the limited knowledge about human MTJ is related to the lack of a standard method to obtain biopsies from this region. The MTJ is an essential source of information about structural proteins. However, investigations focusing on other molecules such as macrophages, satellite cells, fibroblasts, and adipocytes are necessary, since these molecules are essential for remodeling following exercise, injury, and inactivity.

CONCLUSION

This Research Topic mainly highlights the critical role of mechanical load in maintaining the integrity of muscle, tendon, and the connection site between these tissues (MTJ). Most of the studies published on this Research Topic referred to muscle tissue. However, forthcoming studies need to address additional

aspects, such as the mechanisms of molecular and cellular pathways in response to tendon and skeletal muscle in a wide-ranging situation such as injury; chronic and acute responses to loading, unloading, and aging; how the muscle-tendon unit adapts to inactivity and immobilization in combination with injury and aging, and with particular attention to the interactions of these mechanisms in MTJ.

AUTHOR CONTRIBUTIONS

RM, MK, and AM: study conception and design and draft manuscript preparation. All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

ACKNOWLEDGMENTS

The authors are grateful for the financial support provided by Fundação de Apoio a Pesquisa do Distrito Federal (FAPDF) and Fundação de Apoio a Pesquisa do Estado de São Paulo FAPESP, the Decanato de Pesquisa e Inovação e de Pós-Graduação (UnB), and Conselho Nacional de Desenvolvimento Científicoe Tecnológico (CNPq). We also thank Robin Hambly for reviewing the English.

REFERENCES

Charvet, B., Ruggiero, F., and Le Guellec, D. (2012). The development of the myotendinous junction. A review. *Muscles Ligaments Tendons J.* 2, 53–63.

Chatterjee, M., Muljadi, P. M., and Andarawis-Puri, N. (2021). The role of the tendon ECM in mechanotransduction: disruption and repair following overuse. *Connect. Tissue Res.* 63, 28–42. doi: 10.1080/03008207.2021.1925663

Du, Y., Karvellas, C. J., Baracos, V., Williams, D. C., and Khadaroo, R. G. (2014). Sarcopenia is a predictor of outcomes in very elderly patients undergoing emergency surgery. *Surgery* 156, 521–527. doi: 10.1016/j.surg.2014.04.027

Eekhoff, J. D., Iannucci, L. E., and Lake, S. P. (2021). Shear mechanosensing drives tendon adaptation. *Nat. Biomed. Eng.* 5:1409–1410. doi: 10.1038/s41551-021-00724-x

Frontera, W. R., and Ochala, J. (2015). Skeletal muscle: a brief review of structure and function. *Behav. Genet.* 45, 183–195. doi: 10.1007/s00223-014-9915-y

Greising, S. M., Corona, B. T., and Call, J. A. (2020). Musculoskeletal regeneration, rehabilitation, and plasticity following traumatic injury. *Int. J. Sports Med.* 41, 495–504. doi: 10.1055/a-1128-7128

Jordan, K. P., Jóud, A., Bergknut, C., Croft, P., Edwards, J. J., Peat, G., et al. (2014). International comparisons of the consultation prevalence of musculoskeletal conditions using population-based healthcare data from England and Sweden. *Ann. Rheum. Dis.* 73, 212–218. doi: 10.1136/annrheumdis-2012-202634

Kjaer, M. (2004). Role of extracellular matrix in adaptation of tendon and skeletal muscle to mechanical loading. *Physiol. Rev.* 84, 649–698. doi: 10.1152/physrev.00031.2003

Nakajima, T., Nakahata, A., Yamada, N., Yoshizawa, K., Kato, T. M., Iwasaki, M., et al. (2021). Grafting of iPS cell-derived tenocytes promotes motor function recovery after Achilles tendon rupture. *Nat. Commun.* 12, 1–12. doi: 10.1038/s41467-021-25328-6

Passini, F. S., Jaeger, P. K., Saab, A. S., Hanlon, S., Chittim, N. A., Arlt, M. J., et al. (2021). Shear-stress sensing by PIEZO1 regulates tendon stiffness in rodents and influences jumping performance in humans. *Nat. Biomed. Eng.* 5, 1–15. doi: 10.1038/s41551-021-00716-x

Sawadkar, P., Player, D., Bozec, L., and Mudera, V. (2020). The mechanobiology of tendon fibroblasts under static and uniaxial cyclic load in a 3D tissue engineered model mimicking native extracellular matrix. *J. Tissue Eng. Regen. Med.* 14, 135–146. doi: 10.1002/term.2975

Subramanian, A., and Schilling, T. F. (2015). Tendon development and musculoskeletal assembly: emerging roles for the extracellular matrix. *Devlopment* 142, 4191–4204. doi: 10.1242/dev.114777

Yamamoto, M., and Abe, S. (2020). Mechanism of muscle-tendon-bone complex development in the head. *Anat. Sci. Int.* 95, 165–173. doi: 10.1007/s12565-019-00523-0

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frontiers in Physiology

ORIGINAL RESEARCH
published: 01 February 2021
doi: 10.3389/fphys.2021.626096

One Bout of Aerobic Exercise Can Enhance the Expression of *Nr1d1* in Oxidative Skeletal Muscle Samples

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The nuclear receptor subfamily 1, group D member 1 (*Nr1d1),* plays a role in the skeletal muscle's oxidative capacity, mitochondrial biogenesis, atrophy genes, and muscle fiber size. In light of the effects of physical exercise, the present study investigates the acute response of *Nr1d1* and genes related to atrophy and mitochondrial biogenesis on endurance and resistance exercise protocols. In this investigation, we observed, after one bout of endurance exercise, an upregulation of *Nr1d1* in soleus muscle, but not in the gastrocnemius, and some genes related to mitochondrial biogenesis and atrophy were enhanced as well. Also, analysis of muscle transcripts from diverse isogenic BXD mice families revealed that the strains with higher *Nr1d1* gene expression displayed upregulation of AMPK signaling and mitochondrial-related genes. In summary, a single session of endurance exercise can enhance the *Nr1d1* mRNA levels in an oxidative muscle.

Keywords: *Nr1d1*, exercise, skeletal muscle, atrophy, mitochondrial biogenesis

OPEN ACCESS

Edited by:
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Reviewed by:
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Specialty section:
This article was submitted to Exercise Physiology, a section of the journal Frontiers in Physiology

Received: *04 November 2020*
Accepted: *04 January 2021*
Published: *01 February 2021*

Citation:
Rovina RL, da Rocha AL, Marafon BB, Pauli JR, de Moura LP, Cintra DE, Ropelle ER and da Silva ASR (2021) One Bout of Aerobic Exercise Can Enhance the Expression of Nr1d1 in Oxidative Skeletal Muscle Samples. Front. Physiol. 12:626096. doi: 10.3389/fphys.2021.626096

INTRODUCTION

Acute and chronic physical exercises can be used as a non-pharmacological strategy to prevent and treat several diseases, improve life quality, and maximize athletes' performance (Gleeson et al., 2011). The acute resistance exercise can stimulate the gain of muscle mass and strength through the activation of protein kinase B (Akt)/mammalian target of rapamycin (mTOR) pathway (Bodine et al., 2001). On the other hand, glucocorticoids can inhibit the mTOR pathway through Krüppel-like factor 15 (KLF15). This pathway has an important function in skeletal muscle catabolism due to the transcriptional upregulations of atrogin-1 and muscle RING-finger protein-1, which are related to muscle mass control. Also, branched-chain amino acid transaminase 2 (BCAT2) is a regulator of mTOR activity, but through the participation in the BCAA catabolism event (Liu et al., 2017; Shimizu et al., 2011).

The acute endurance exercise is related to the activation of the AMP-activated protein kinase (AMPK)/proliferator-activated receptor gamma coactivator 1-alpha (PGC1-α) pathway, increasing skeletal muscle oxidative capacity, mitochondrial content, and maximal oxygen uptake ($VO2_{max}$) (Hardie et al., 2012). The PGC1-α is classically known as a transcriptional coregulator of events like mitochondrial biogenesis and angiogenesis, playing a central role in endurance adaptations. However, as Ruas and colleagues (Woldt et al., 2013) described, the isoform named PGC1-α4 is linked to hypertrophy responses in skeletal muscle. This specific isoform of PGC-1

regulates the insulin-like growth factor 1 and myostatin pathways, having a different role from the other isoforms.

In an elegant investigation, Woldt et al. (2013) showed that the knocking down of the nuclear receptor subfamily 1, group D member 1 (*Nr1d1*), the codify gene of the protein Rev-erb-alpha, was linked to lower skeletal muscle oxidative capacity, mitochondrial content, and VO_2max. Interestingly, the Rev-erb-alpha can regulate the mitochondrial biogenesis through the Stk11 (Serine/threonine kinase 11)-Ampk-Sirt1-Ppargc1α pathway. The Stk11 plays a significant role in regulating the expression of Ampk and, consequently, of the Ppargc1α. On the other hand, a deficiency of Rev-erb-alpha leads to lower Ppargc1α expression in skeletal muscle, one of the central genes in the mitochondrial biogenesis pathway (Woldt et al., 2013). Also, Mayeuf-Louchart et al. (2017) demonstrated that the genetic ablation of *Nr1d1* was associated with the increased expression of the atrophy-related genes and reduced muscle mass and fiber size in skeletal muscle samples.

Knowing the pharmacological activation of Rev-erb-alpha enhanced mitochondrial content and respiration in skeletal muscle cells, as well as exercise capacity, and reverted the negative effects of dexamethasone treatment in skeletal muscle mass and atrophy-related genes (Woldt et al., 2013; Mayeuf-Louchart et al., 2017), it is relevant to investigate physiological strategies capable of increasing the expression of this gene in skeletal muscle. Therefore, we investigated the acute effects of resistance and endurance exercise protocols on the messenger ribonucleic acid (mRNA) levels of *Nr1d1*, *Prkaa1*, *Ppargc1a*, *Fbxo32*, *Trim63*, *Ubc*, and *Bcat2* in mice skeletal muscle samples.

MATERIALS AND METHODS

Experiment Animals

Eight-week-old C57BL/6 mice from the Central Animal Facility of the Ribeirão Preto campus from the University of São Paulo (USP) were used for the experiment. The animals were accommodated in sterile micro-insulators (three animals per cage) in a ventilated rack (INSIGHT®, Ribeirão Preto, Brazil) with controlled temperature (22 ± 2°C) on a 12:12-h light-dark inverted cycle. Food (Purina chow) and water were provided *ad libitum*. According to the Brazilian College of Animal Experimentation (COBEA), all experimental procedures were approved by the Ethics Committee of the University of São Paulo (I.D. 2017.5.33.9037).

Mice were divided into three experimental groups: Control (CT; sedentary), Resistance (RES; submitted to the resistance exercise protocol), and Endurance (END; submitted to the endurance exercise protocol). The sample size (*n*) for each experiment is available in the figure legends. Mice from the RES group were submitted to 5 days of adaptation on a ladder-climbing (INSIGHT^{TM}, Ribeirão Preto, Brazil) with and without external load (Wang et al., 2016). The ladder had 1,110 mm of height, 80° of inclination, and 85 steps with a distance of 6 mm between each. Also, mice from the END group were submitted to 5 days of adaptation on a treadmill (INSIGHT^{TM}, Ribeirão Preto, Brazil), 10 min/day, at a speed of 6 m.min^{-1}. The experimental design is described in **Figure 1A**.

Resistance Group

Mice from the RES group were submitted to 5 days of adaptation on a ladder-climbing (INSIGHT^{TM}, Ribeirão Preto, Brazil) without external load. The ladder had 1,110 mm of height, 80° of inclination, and 85 steps with a distance of 6 mm between each. On the day of the acute exercise protocol, the RES group first performed one climb without external load to warm-up. After that, an external load corresponding to 75% of body weight was applied at the base of each animal's tail, and mice performed ten climbs with a 2 min recovery between each. This protocol was adapted from the investigation of Wang and coworkers (Wang et al., 2015), which verified molecular signs of hypertrophy in rodents. The total duration of the acute resistance exercise protocol was approximately 20 min.

Endurance Group

Mice from the END group were submitted to 5 days of adaptation on a treadmill (INSIGHT^{TM}, Ribeirão Preto, SP, Brazil), 10 min/day, at a speed of 6 m.min^{-1}. After 48 h of the treadmill adaptation, the locomotor performance test started at an initial velocity of 6m/min, at 0% of inclination for the END group, with 3 m/min increments every 3 min until voluntary exhaustion. The exhaustion velocity (EV) was used to prescribe the exercise intensity for the END group. Mice ran at 60% of the EV at 0% inclination for 60 min (Ferreira et al., 2007). Previously, Ferreira et al. (2007) showed the intensity corresponding to 60% of EV obtained in the incremental load test was similar to the MLSS intensity, which can be defined as the highest exercise intensity in which balance between the production and removal of blood lactate occurs, and is used as the gold standard to determine exercise intensity (Da Silva et al., 2010; Ferreira et al., 2007).

Glucose Levels

The blood from the tail tip was collected, and glucose levels were measured before and immediately after the acute physical exercise protocols using a glycemic monitoring system (Accu-Chek^{TM} Active model, Roche, Santo André, Brazil).

Extraction of the Skeletal Muscle

Immediately after the acute physical exercise protocols, the animals were anesthetized by an intraperitoneal administration of xylazine (10 mg/kg of body weight) and ketamine (100 mg/kg of body weight). As soon as the loss of pedal reflexes confirmed the effect of anesthesia (i.e., about 5 min), the gastrocnemius and soleus samples were removed, washed with sterile saline, and stored for reverse transcription-quantitative polymerase chain reaction (RTq-PCR) technique [storage at −80°C with RNAlater (Ambion, Life Technologies, Grand Island, NY, United States)].

Reverse Transcription-Quantitative Polymerase Chain Reaction

Total RNA from the whole gastrocnemius and soleus were extracted with TRIZOL (Invitrogen, Carlsbad, CA, United States). All procedures were performed under standard RNase-free conditions to avoid exogenous RNase contamination. The quantitative Real time-PCR technique was performed

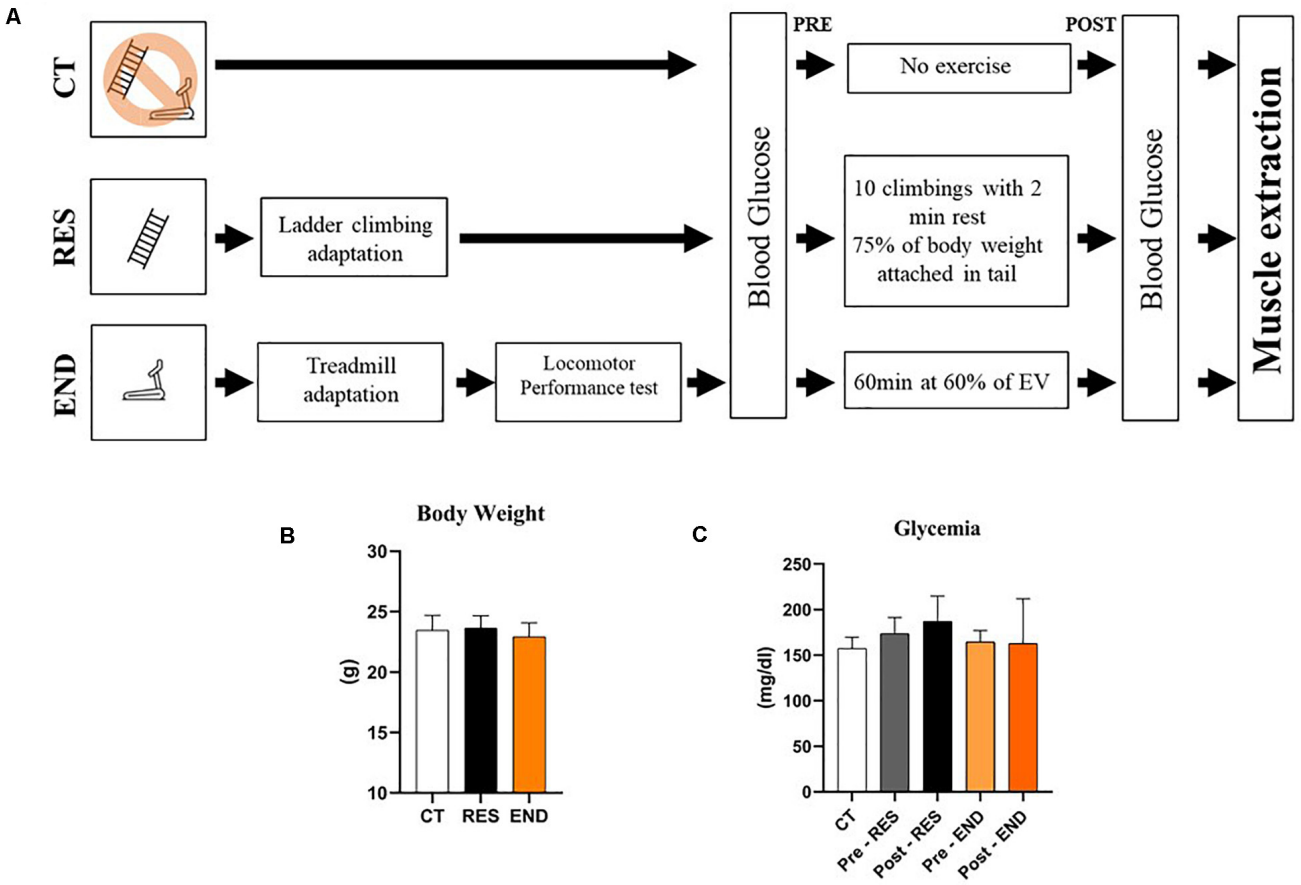

FIGURE 1 | Schematic representation of the experimental procedures **(A)**; Body weight of the CT, RES, and END groups **(B)**; Glycemia of the CT, RES, and END groups **(C)**. Data correspond to the mean ± SD of $n = 5$ mice/group. CT, sedentary mice; RES, mice submitted to the resistance protocol; END, mice submitted to the endurance protocol.

by the ViiA7 Real-Time PCR System (Applied Biosystems) for analysis of gene mRNA expression for *Nr1d1* (nuclear receptor subfamily 1, group D, member 1), *Prkaa1* (protein kinase, AMP-activated, alpha 1 catalytic subunit), *Ppargc1a* (peroxisome proliferative activated receptor, gamma, coactivator 1 alpha), *Mtor* (mechanistic target of rapamycin kinase), *Fbxo32* (F-box protein 32), *Trim63* (tripartite motif-containing 63), *Ubc* (ubiquitin C), and *Bcat2* (branched-chain aminotransferase 2, mitochondrial).

Reverse transcription-quantitative polymerase chain reaction was performed in duplicate with the following reagents: 5 μl HOT FIREPol EvaGreen qPCR SuperMix from Solis BioDyne (Tartu, Estonia), 1 μl primer forward, 1 μl primer reverse (both at a final concentration of 200 nM), 1 μl cDNA (10 ng), and 1 μl d H_2O. Each amplification reaction occurred with standard cycling with the following cycles: one cycle at 95°C for 12 min, 40 cycles of 15 s at 95°C, 25 s at 60°C, and 25 s at 72°C. Relative quantitation was calculated by the $2^{-\Delta\Delta CT}$ method using Thermo Fisher Cloud Software, RQ version 3.7 (Life Technologies Corporation, Carlsbad, CA, United States). All values were corrected by the value obtained for the *Gapdh* (Glyceraldehyde-3-phosphate dehydrogenase) amplification. Primer designs are described in **Table 1**.

Bioinformatic Analysis

Correlation analyses were performed using a data set from muscle *Nr1d1* [EPFL/LISP BXD CD Muscle Affy Mouse Gene 1.0 ST (Dec11) RMA] and muscle genes related to the AMPK signaling and mitochondria of genetically diverse BXD mice as previously published (Andreux et al., 2012). The four strains with the highest *Nr1d1* values and the four strains with the lowest *Nr1d1* values were selected to correlate with the other genes. All data are accessible on Genetwork[1]. The heatmap graph was obtained using the Gene-E software.

Statistical Analysis

Results are expressed as mean ± standard deviation (SD). The Shapiro–Wilk's W-test was used to verify data normality, and Levene's test was used to test the homogeneity of variances. One-way ANOVA and *post hoc* and Bonferroni were used

[1]http://www.genenetwork.org

TABLE 1 | The primers design.

Gene	Forward	Reverse
Nr1d1	AGAGAGGCCATCACAACCTC	TGTAGGTGATAACACCACCTGT
Prkaa1	CCAGGTCATCAGTACACCATCT	TTTCCTTTTCGTCCAACCTTCC
Ppargc1a	GAGTTGAAAAAGCTTGACTGGC	CAGCACACTCTATGTCACTCC
Mtor	CCACGTGGTTAGCCAGACT	TAGCGGATATCAGGGTCAGGA
Fbxo32	CAAAGGAAGTACGAAGGAGCG	TCAGCTCCAACAGCCTTACTA
Murf1	CAGGCTGCGAATCCCTACTG	GCCGGTCCATGATCACTTCA
Ubc	CGCGCTGATCCCTCCG	CTGCATCGTCTCTCTCACGG
Bcat2	TATGGACCCACTGTGGCTGT	CAGCTCCAGTACTCCGTCTTC
Gapdh	AAGAGGGATGCTGCCCTTAC	CGGGACGAGGAAACACTCTC

Nr1d1, Nuclear receptor subfamily 1, group D, member 1; Prkaa1, Protein kinase, AMP-activated, alpha 1 catalytic subunit; Ppargc1a, Peroxisome proliferative activated receptor, gamma, coactivator 1 alpha; Mtor, Mechanistic target of rapamycin kinase; Fbxo32, F-box protein 32; Trim63, Tripartite motif-containing 63; Ubc, Ubiquitin C; Bcat2, Branched-chain aminotransferase 2, mitochondrial; Gapdh, Glyceraldehyde-3-phosphate dehydrogenase.

to verify the effects of acute experimental protocols. When applicable, Pearson's correlation coefficient was used to test the association between the studied parameters. All statistical analyses were two-sided, and the significance level was set at $p \leq 0.05$. Statistical analyses were performed using the software SPSS v.20.0 for Windows (IBM, Chicago, IL, United States).

RESULTS

Metabolic Parameters and Gene Expression

Figures 1B,C show body weight and glycemia were not different between the experimental groups, respectively. For the gastrocnemius samples, the mRNA levels of *Nr1d1*, *Prkaa1*, *Fbxo32*, *Murf*, and *Ubc* were not different between the experimental groups (**Figure 2A**). The mRNA levels of *Ppargc1a* and *Mtor* were higher for the END group than the CT and RES groups (**Figure 2A**). Also, the mRNA levels of *Bcat2* were lower for the RES group compared to the CT group (**Figure 2A**).

For the soleus samples, the mRNA levels of *Nr1d1, Mtor, Fbxo32,* and *Trim63* were higher for the END group than the CT and RES groups (**Figure 2B**). Also, the mRNA levels of *Ppargc1a* were higher for the END group compared to the CT group (**Figure 2B**). The mRNA levels of *Prkaa1*, *Ubc*, and *Bcat2* were not different between the experimental groups (**Figure 2B**).

Figure 3 shows the correlation between the responses of *Nr1d1* gene in the soleus muscle for the END group, where *Mtor* ($p < 0.01$; $r = 0.95$), *Ppargc1a* ($p < 0.005$; $r = 0.97$) and *Bcat2* ($p < 0{,}01$; $r = 0.95$) displayed significant positive correlations. The *Nr1d1* responses for the RES group in the soleus muscle did not present significant correlations with the other genes. The *Nr1d1* responses in gastrocnemius for both RES and END groups were not significantly correlated with other genes.

Bioinformatic Results

To test our hypothesis in different mice strains, we resorted to a bioinformatics analysis using the BXD database, which is the largest and best-categorized family of isogenic strains and provides a broad set of data appropriate for investigations. First, we visualized the distribution of muscle *Nr1d1* mRNA levels in 42 strains of isogenic BXD mice, highlighting four strains with lower (BXD95, 98, 89, and 68) and four strains with higher (BXD45, DBA/2J, BXD60, and BXD90) levels of *Nr1d1* mRNA in the skeletal muscle (**Figures 4A,B**). The transcriptomic analysis demonstrated that the variations of *Nr1d1* influenced several genes related to AMPK signaling and mitochondria (**Figures 4C–E**).

DISCUSSION

The control of the muscular physiological state during rest or exercise requires fine adjustment between the oxidative capacity of fibers with the consequent use of energetic substrates and molecular signals dependent on regulated genomic orchestration. Therefore, the main findings of this study were: (1) Regardless of the skeletal muscle type, most of the significant changes were observed for the END group; (2) While the mRNA levels of *Ppargc1a* and *Mtor* were upregulated in both skeletal muscle samples after the END protocol, the mRNA levels of *Nr1d1*, *Fbxo32*, and *Trim63* were upregulated only in the predominant oxidative skeletal muscle; (3) The mRNA levels of *Bcat2* were downregulated in the gastrocnemius sample after the RES protocol. **Figure 5** summarizes the data of the present investigation.

BXD mice population has been extensively used to explore the causal and mechanistic links between genomes and several physiological or pathological conditions (Andreux et al., 2012; Wu et al., 2014; Williams et al., 2016). Here, we found a differential distribution of *Nr1d1* gene expression in skeletal muscle from 42 BXD strains. Four families with higher *Nr1d1* gene expression displayed upregulation of AMPK signaling and mitochondrial-related genes. These data reinforce previous results showing *Nr1d1* activation led to an increase of mitochondrial content and respiration, as well as exercise capacity (Woldt et al., 2013).

Except for the *Bcat2* mRNA levels, the RES protocol did not modulate the other genes in any of the skeletal muscle samples, which can be linked to the lower volume of the RES protocol (approximately 20 min) compared to the END protocol (60 min). The global deletion of *Bcat2* led to elevated gastrocnemius protein turnover in mice (Lynch et al., 2015). Our RES protocol reduced the mRNA levels of *Bcat2* in the gastrocnemius muscle. In contrast, Roberson et al. (2018) did not observe significant changes for the *Bcat2* in the vastus lateralis of subjects performing ten sets of five repetitions of a back squat exercise at 80% of one-repetition maximum. Possibly, the main differences between Roberson's investigation (Roberson et al., 2018) and ours are the experimental models (humans vs. rodents), tissue extraction times (2 h after vs. immediately after), and training state (trained subjects vs. sedentary mice).

The exercise intensity and volume may justify that most alterations were observed for the END group, which ran for 60 min at 60% of the EV that corresponds to the

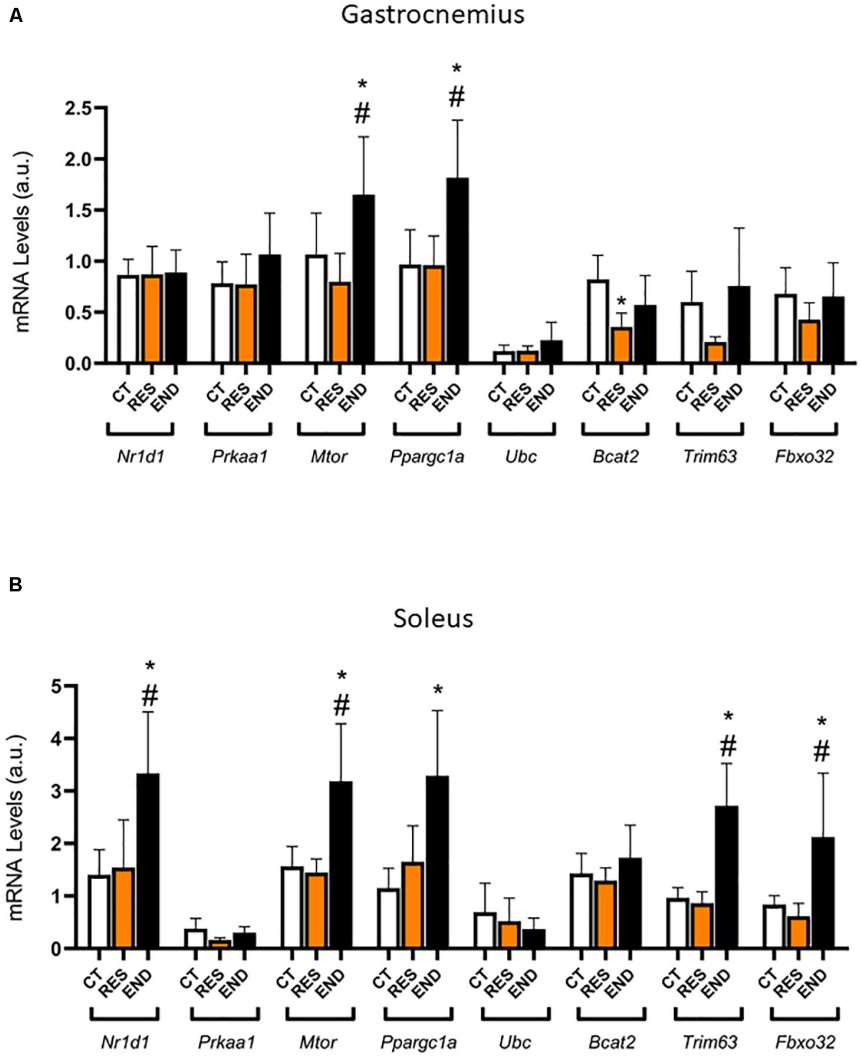

FIGURE 2 | (A) Gastrocnemius mRNA levels of *Nr1d1*, *Prkaa1*, *Ppargc1a*, *Mtor*, *Fbxo32*, *Trim63*, *Ubc*, and *Bcat2*. **(B)** Soleus mRNA levels of *Nr1d1*, *Prkaa1*, *Ppargc1a*, *Mtor*, *Fbxo32*, *Trim63*, *Ubc*, and *Bcat2*. Data correspond to the mean ± SD of $n = 5$ mice/group. $^{*}p \leq 0.05$ *vs.* END group; $^{\#}p \leq 0.05$ *vs.* RES group. CT, sedentary mice; RES, mice submitted to the resistance protocol; END, mice submitted to the endurance protocol.

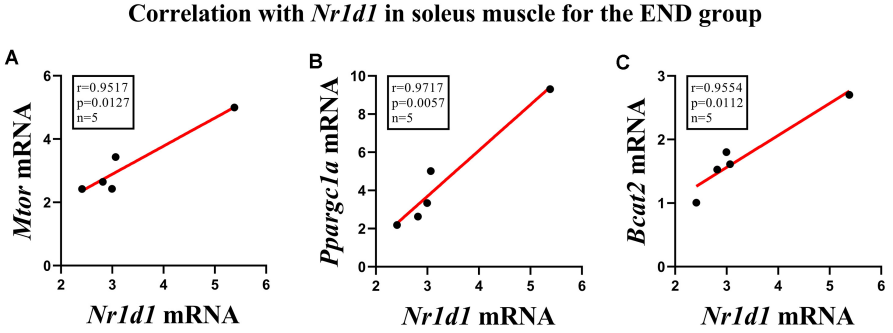

FIGURE 3 | Correlations of *Nr1d1* with **(A)** *Mtor*, **(B)** *Ppargc1a*, and **(C)** *Bcat2* in soleus muscle for the endurance group.

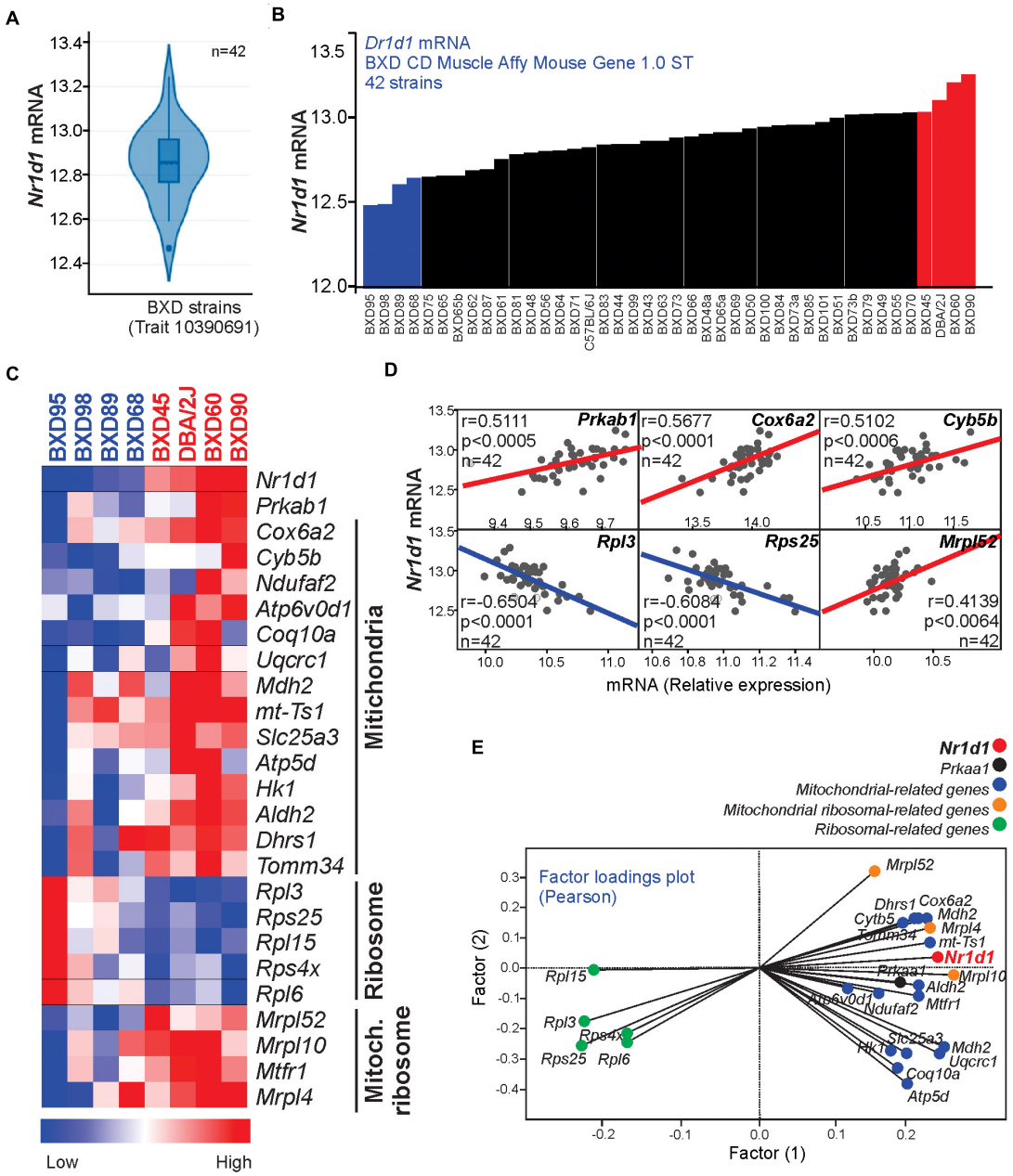

FIGURE 4 | (A) The violin plot shows the *Nr1d1* gene expression distribution in the muscle of 42 BXD mice strains **(B)** *Nr1d1* gene expression in each BXD strain muscle. **(C)** Heatmap graph highlighting the gene expression in 4 BXD strains with low (blue) and 4 BXD strains with high (red). **(D)** Pearson's correlation and **(E)** two factors analysis graphs show the correlation between *Nr1d1*gene expression and *Prkaa1* mRNA levels, mitochondrial-related, and ribosomal-related genes. Blue lines indicate negative and red lines indicate a positive correlation.

moderate-exercise intensity (Ferreira et al., 2007; Da Silva et al., 2010). Interestingly, the END protocol increased the mRNA levels of Ppargc*1a* and *Mtor* in both skeletal muscle samples. Transgenic mice overexpression *Ppargc1a* highlighted its fundamental role in regulating the mitochondrial biogenesis in skeletal muscles (Calvo et al., 2008). Also, the sensitivity of *Ppargc1a* to acute endurance exercise was recently reviewed (Popov, 2018).

On the other hand, mTOR complex 1 inhibition prevents growth and increases atrophy in skeletal muscles (Bentzinger et al., 2013). Corroborating our findings, Hayasaka et al. (2014) verified an increase of mTOR phosphorylation in serine 2,448 immediately after a treadmill running at 28 m/min for 60 min in gastrocnemius muscles of Sprague–Dawley rats. Woldt and coauthors (Woldt et al., 2013) demonstrated the higher expression of Rev-erb-alpha occurred in soleus (a predominant

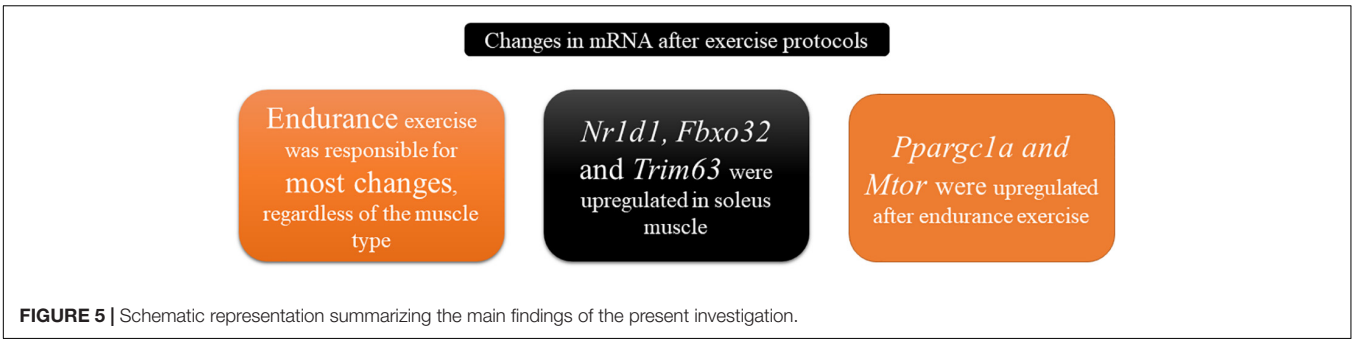

FIGURE 5 | Schematic representation summarizing the main findings of the present investigation.

oxidative muscle), and its global deletion impaired mitochondrial and exercise capacity. Also, these authors verified that 8 weeks of treadmill running at an initial intensity of 8 m/min, which increased to 16 m/min during the last 4 weeks, 5 days a week, 1–2 h per day, increased the protein contents of Rev-erb-alpha in gastrocnemius and soleus samples (Woldt et al., 2013).

Although Yasumoto et al. (2015) verified an increased expression of *Nr1d1* in mice's skeletal muscle in response to chronic wheel-running activity, to the best of our knowledge, this is the first investigation showing the *Nr1d1* was elevated immediately after one single bout of moderate-intensity endurance exercise. Interestingly, this result occurred only in the most oxidative skeletal muscle. Although we did not find significant changes after the RES protocol, future studies should evaluate different time-points since the mRNA expression alterations can occur up to 12 h after acute exercise (Egan and Zierath, 2013). Atrogin-1 and MuRF1 are encoded by the atrophy-related genes *Fbxo32* and *Trim63*, respectively (Dang et al., 2016). The Nr1d1 and the mRNA levels of *Fbxo32* and *Trim63* in soleus were elevated after the END protocol. These results are contrary to the investigation of Mayeuf-Louchart et al. (2017), who observed increased levels of *Atrogin* and *Murf1* mRNA levels in quadriceps muscle of Rev-erb-alpha knockout mice. Stefanetti et al. (2015) did not monitor significant changes for *Atrogin-1* mRNA levels in vastus lateralis muscle of subjects immediately after an endurance or resistance exercise session. Also, the mRNA levels of *Atrogin-1* were increased 2.5, 5.0, and 22 h after the acute endurance exercise.

Here, we showed positive associations between the *Nr1d1* gene with *Ppargc1a*, *Mtor,* and *Bcat2* genes in soleus muscles after a single bout of endurance exercise. Interestingly, most of the alterations of the END group occurred in the predominant oxidative phenotype muscle. It is essential to point out that the gastrocnemius and soleus present phenotypical differences between de muscle fiber composition. For instance, studying BALB/c mice, Dimauro et al. (2019) observed that the myosin heavy chain (MHC)-IIA was the most expressed isoform in gastrocnemius (40.25% ± 6.55) without significant differences among the other isoforms (MHC-IA, 18.6% ± 4.39; MHC-IIX, 6.75% ± 2.75; MHC-IIB, 15.8% ± 3.35). In soleus samples, MHC-IIA was also the isoform more expressed (50.75% ± 4.03), followed by MHC-I (42.20% ± 3.70). Regarding C57BL6J mice, Augusto et al. (2017) observed that the gastrocnemius muscle had (median ± semi amplitude) type IIB (54.42 ± 8.11%), IIDB (19.37 ± 2.98%), IID (2.26 ± 2.24%), IIAD (12.40 ± 2.34%), IIA (5.73 ± 3.24%) and I (5.74 ± 2.55%) fibers. In contrast, the soleus contained type I (37.42 ± 8.20%), IIA (38.62 ± 6.81%), IIAD (18.74 ± 6.95%), and IID (5.69 ± 3.09%) fibers. The authors concluded that the gastrocnemius presented IIB and IIDB fibers predominantly, while the soleus muscles displayed mostly type IIA fibers. Although these fiber type differences may influence the expression of the analyzed genes in response to acute exercise sessions, further investigations should evaluate these genes' expression in isolated skeletal muscle fibers to confirm this theory.

Altogether, these findings support our initial hypothesis that *Nr1d1* would be increased concomitantly with oxidative capacity-related genes in response to the acute endurance exercise session. In conclusion, an acute bout of endurance exercise elevated the mRNA levels of *Nr1d1* in oxidative skeletal muscle, which was accompanied by an increase of mitochondrial biogenesis and atrophy-related genes. These findings have health implications since the global genetic ablation of this gene was linked to negative adaptations such as the impairment of skeletal muscle oxidative capacity and exercise performance, as well as the increased expression of the atrophy-related genes and reduction of the skeletal muscle fiber size. Future studies should evaluate the circadian rhythm response of *Nr1d1* to different exercise models and the post-translational modifications for metabolism regulation central proteins. Moreover, new studies should assess if the muscle-specific knockout of Nr1d1 blunts these responses to confirm our hypothesis for a mechanistic approach.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by According to the Brazilian College of Animal Experimentation (COBEA), all experimental procedures were approved by the Ethics Committee of the University of São Paulo (I.D 2017.5.33.9037).

AUTHOR CONTRIBUTIONS

RR, AR, and AS designed the manuscript. RR and AS wrote the paper. RR, AR, and BM performed the experiments, data collection, and/or statistical analysis. RR designed the figures for the manuscript. AR, RR, BM, JP, DC, ER, and AS contributed to data analysis, discussion, and/or supported financial costs. All authors have read and approved this manuscript.

FUNDING

The present work received financial support from the São Paulo Research Foundation (FAPESP; process numbers 2017/09038-1, 2017/12765-2, and 2018/12519-4) National Council for Scientific and Technological Development (CNPq; process number 301279/2019-5), and the Coordination for the Improvement of Higher Education Personnel (CAPES; finance code 001).

REFERENCES

Andreux, P. A., Williams, E. G., Koutnikova, H., Houtkooper, R. H., Champy, M.-F., Henry, H., et al. (2012). Systems genetics of metabolism: the use of the BXD murine reference panel for multiscalar integration of traits. *Cell* 150, 1287–1299. doi: 10.1016/j.cell.2012.08.012

Augusto, V., Padovani, C. R., and Campos, G. E. R. (2017). Skeletal muscle fiber types in C57BL6J mice. *J. Morphol. Sci.* 21:0.

Bentzinger, C. F., Lin, S., Romanino, K., Castets, P., Guridi, M., Summermatter, S., et al. (2013). Differential response of skeletal muscles to mTORC1 signaling during atrophy and hypertrophy. *Skelet Muscle* 3:6. doi: 10.1186/2044-5040-3-6

Bodine, S. C., Stitt, T. N., Gonzalez, M., Kline, W. O., Stover, G. L., Bauerlein, R., et al. (2001). Akt/mTOR pathway is a crucial regulator of skeletal muscle hypertrophy and can prevent muscle atrophy in vivo. *Nat. Cell Biol.* 3, 1014–1019. doi: 10.1038/ncb1101-1014

Calvo, J. A., Daniels, T. G., Wang, X., Paul, A., Lin, J., Spiegelman, B. M., et al. (2008). Muscle-specific expression of PPARgamma coactivator-1alpha improves exercise performance and increases peak oxygen uptake. *J. Appl. Physiol.* 104, 1304–1312. doi: 10.1152/japplphysiol.01231.2007

Da Silva, A. S., Pauli, J. R., Ropelle, E. R., Oliveira, A. G., Cintra, D. E., De Souza, C. T., et al. (2010). Exercise intensity, inflammatory signaling, and insulin resistance in obese rats. *Med. Sci. Sports Exerc.* 42, 2180–2188. doi: 10.1249/mss.0b013e3181e45d08

Dang, K., Li, Y. Z., Gong, L. C., Xue, W., Wang, H. P., Goswami, N., et al. (2016). Stable atrogin-1 (Fbxo32) and MuRF1 (Trim63) gene expression is involved in the protective mechanism in soleus muscle of hibernating Daurian ground squirrels (Spermophilus dauricus). *Biol Open* 5, 62–71. doi: 10.1242/bio.015776

Dimauro, I., Antonioni, A., Mercatelli, N., Grazioli, E., Fantini, C., Barone, R., et al. (2019). The early response of αB-crystallin to a single bout of aerobic exercise in mouse skeletal muscles depends upon fiber oxidative features. *Redox Biol.* 24:101183. doi: 10.1016/j.redox.2019.101183

Egan, B., and Zierath, J. R. (2013). Exercise metabolism and the molecular regulation of skeletal muscle adaptation. *Cell Metab.* 17, 162–184. doi: 10.1016/j.cmet.2012.12.012

Ferreira, J. C., Rolim, N. P., Bartholomeu, J. B., Gobatto, C. A., Kokubun, E., and Brum, P. C. (2007). Maximal lactate steady state in running mice: effect of exercise training. *Clin. Exp. Pharmacol. Physiol.* 34, 760–765. doi: 10.1111/j.1440-1681.2007.04635.x

Gleeson, M., Bishop, N. C., Stensel, D. J., Lindley, M. R., Mastana, S. S., and Nimmo, M. A. (2011). The anti-inflammatory effects of exercise: mechanisms and implications for the prevention and treatment of disease. *Nat. Rev. Immunol.* 11, 607–615. doi: 10.1038/nri3041

Hardie, D. G., Ross, F. A., and Hawley, S. A. (2012). AMPK: a nutrient and energy sensor that maintains energy homeostasis. *Nat. Rev. Mole. Cell Biol.* 13, 251–262. doi: 10.1038/nrm3311

Hayasaka, M., Tsunekawa, H., Yoshinaga, M., and Murakami, T. (2014). Endurance exercise induces REDD 1 expression and transiently decreases mTORC 1 signaling in rat skeletal muscle. *Physiol. Rep.* 2:e12254. doi: 10.14814/phy2.12254

Liu, Y., Dong, W., Shao, J., Wang, Y., Zhou, M., and Sun, H. (2017). Branched-chain amino acid negatively regulates KLF15 expression via PI3K-AKT pathway. *Front. Physiol.* 8:853. doi: 10.3389/fphys.2017.00853

Lynch, C. J., Kimball, S. R., Xu, Y., Salzberg, A. C., and Kawasawa, Y. I. (2015). Global deletion of BCATm increases expression of skeletal muscle genes associated with protein turnover. *Physiol. Genomics* 47, 569–580. doi: 10.1152/physiolgenomics.00055.2015

Mayeuf-Louchart, A., Thorel, Q., Delhaye, S., Beauchamp, J., Duhem, C., Danckaert, A., et al. (2017). Rev-erb-α regulates atrophy-related genes to control skeletal muscle mass. *Sci. Rep.* 7:14383.

Popov, D. V. (2018). Adaptation of Skeletal Muscles to Contractile Activity of Varying Duration and Intensity: The Role of PGC-1α. *Biochemistry* 83, 613–628. doi: 10.1134/s0006297918060019

Roberson, P. A., Haun, C. T., Mobley, C. B., Romero, M. A., Mumford, P. W., Martin, J. S., et al. (2018). Skeletal muscle amino acid transporter and BCAT2 expression prior to and following interval running or resistance exercise in mode-specific trained males. *Amino Acids* 50, 961–965. doi: 10.1007/s00726-018-2570-2

Shimizu, N., Yoshikawa, N., Ito, N., Maruyama, T., Suzuki, Y., Takeda, S., et al. (2011). Crosstalk between glucocorticoid receptor and nutritional sensor mTOR in skeletal muscle. *Cell Metab.* 13, 170–182. doi: 10.1016/j.cmet.2011.01.001

Stefanetti, R. J., Lamon, S., Wallace, M., Vendelbo, M. H., Russell, A. P., and Vissing, K. (2015). Regulation of ubiquitin proteasome pathway molecular markers in response to endurance and resistance exercise and training. *Pflugers Arch.* 467, 1523–1537. doi: 10.1007/s00424-014-1587-y

Wang, H., Sharma, N., Arias, E. B., and Cartee, G. D. (2016). Insulin Signaling and Glucose Uptake in the Soleus Muscle of 30-Month-Old Rats After Calorie Restriction With or Without Acute Exercise. *J.Gerontol. A Biol. Sci. Med. Sci.* 71, 323–332. doi: 10.1093/gerona/glv142

Wang, W., Choi, R. H., Solares, G. J., Tseng, H. M., Ding, Z., Kim, K., et al. (2015). L-Alanylglutamine inhibits signaling proteins that activate protein degradation, but does not affect proteins that activate protein synthesis after an acute resistance exercise. *Amino Acids* 47, 1389–1398. doi: 10.1007/s00726-015-1972-7

Williams, E. G., Wu, Y., Jha, P., Dubuis, S., Blattmann, P., Argmann, C. A., et al. (2016). Systems proteomics of liver mitochondria function. *Science* 352:aad0189. doi: 10.1126/science.aad0189

Woldt, E., Sebti, Y., Solt, L. A., Duhem, C., Lancel, S., Eeckhoute, J., et al. (2013). Rev-erb-[alpha] modulates skeletal muscle oxidative capacity by regulating mitochondrial biogenesis and autophagy. *Nat. Med.* 19, 1039–1046. doi: 10.1038/nm.3213

Wu, Y., Williams, E. G., Dubuis, S., Mottis, A., Jovaisaite, V., Houten, S. M., et al. (2014). Multilayered genetic and omics dissection of mitochondrial activity in a mouse reference population. *Cell* 158, 1415–1430. doi: 10.1016/j.cell.2014.07.039

Yasumoto, Y., Nakao, R., and Oishi, K. (2015). Free access to a running-wheel advances the phase of behavioral and physiological circadian rhythms and peripheral molecular clocks in mice. *PLoS One* 10:e0116476. doi: 10.1371/journal.pone.0125646

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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REVIEW
published: 26 March 2021
doi: 10.3389/fphys.2021.635561

The Myotendinous Junction—A Vulnerable Companion in Sports. A Narrative Review

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The incidence of strain injuries continues to be high in many popular sports, especially hamstring strain injuries in football, despite a documented important effect of eccentric exercise to prevent strains. Studies investigating the anatomical properties of these injuries in humans are sparse. The majority of strains are seen at the interface between muscle fibers and tendon: the myotendinous junction (MTJ). It has a unique morphology with a highly folded muscle membrane filled with invaginations of collagen fibrils from the tendon, establishing an increased area of force transmission between muscle and tendon. There is a very high rate of remodeling of the muscle cells approaching the MTJ, but little is known about how the tissue adapts to exercise and which structural changes heavy eccentric exercise may introduce. This review summarizes the current knowledge about the anatomy, composition and adaptability of the MTJ, and discusses reasons why strain injuries can be prevented by eccentric exercise.

Keywords: myotendinous junction, strain injury, Nordic Hamstring, Eccentric exercise, force transmission, injury prevention, hamstring strain injury

OPEN ACCESS

Edited by:
Rita de Cassia Marqueti, University of Brasilia, Brazil

Reviewed by:
Christina Karatzaferi, University of Thessaly, Greece
Ivo Neto, University of Brasilia, Brazil

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Specialty section:
This article was submitted to Striated Muscle Physiology, a section of the journal Frontiers in Physiology

Received: *30 November 2020*
Accepted: *15 February 2021*
Published: *26 March 2021*

Citation:
Jakobsen JR and Krogsgaard MR (2021) The Myotendinous Junction—A Vulnerable Companion in Sports. A Narrative Review. Front. Physiol. 12:635561. doi: 10.3389/fphys.2021.635561

INTRODUCTION

The transition zone between skeletal muscle and tendon, the myotendinous junction (MTJ), has a key role in being the structure where muscle fibers interact with tendon. During muscle activity and especially during high effort exercise, large forces are transmitted from muscle fibers to the tendon through the MTJ (Huijing, 1999). The ability to transmit force is optimized by a unique architecture of the muscle fibers at their termination at the MTJ (Knudsen et al., 2015). Despite this, force applied to the MTJ can be so extensive, especially during eccentric loading, that strain injuries occur (Garrett, 1996). These are among the most frequent injuries in many popular sports (Orchard and Seward, 2002). There is intense research to develop models to predict who are at risk to be afflicted by these injuries, how to prevent them and on what the best rehabilitation strategy is, once they have occurred. Strangely, there has been a negligible focus on the ultrastructure of the human MTJ, which is where many injuries occur, and how the MTJ adapts to training and inactivity (Mjolsnes et al., 2004; Petersen et al., 2011; Seagrave et al., 2014; Van Der Horst et al., 2015). Even though such basic science studies would be highly relevant to understand the clinical aspects of strain injuries, the human studies have mainly explored the ultrastructure of the MTJ. The plasticity of the MTJ in relation to loading and unloading which is of particular interest for clinicians working

with strain injuries, have been studied in animals exposed to running or weightlessness, and these activities are not easily transformable to human exercise situations.

The aim of this article is to review current knowledge about the ultrastructure and composition of the MTJ with focus on the human MTJ, strain injuries, with particular focus on hamstring strain injuries, and possible strategies for prevention. Also, the review suggests directions for research related to the MTJ in the future.

METHODOLOGY

The literature for this review was found by searching PubMed. To identify studies regarding the structure of the MTJ and strain injuries, the following strategy was used:

(Myotendinous junction OR Muscle-tendon interface) AND (ultrastructure OR morphology OR force transmission OR strain injury OR hamstring strain injury).

To identify studies addressing strain injuries and prevention of hamstring strain injuries the following strategy was used: [(Muscle strain injury) OR (Hamstring strain injury)] AND (Injury prevention) NOT (sprain) NOT (tendinopathy) NOT (ACL).

All studies identified with the search strategies were manually evaluated by reading either the title, abstract or full article. In addition to articles identified from the search strategy, we used articles that we knew from our research on the MTJ. Studies regarding the neonatal or developing MTJ was not included in this review, and it has been reviewed thoroughly by others (Charvet et al., 2011, 2012; Valdivia et al., 2017).

The identified literature describing the basic structures of the MTJ and the adaptations at the MTJ following exercise seemed slightly dated. In contrast, there are many recent clinical investigations describing the preventive effects of various training protocols on the incidence of strain injuries, and the Nordic hamstring protocol is by far the most documented.

ULTRASTRUCTURE

The ultrastructure of the MTJ in animals, show finger-like processes from tendon, protruding into muscle at the contact area between the two tissues (Trotter et al., 1985). By 3-D EM of the human MTJ these processes have been shown to be foldings of tendon, protruding into invaginations of the muscle membrane (**Figure 1**; Knudsen et al., 2015). This ultrastructure increases the contact area between muscle and tendon, making it possible for the contractile force from the myofibrils to be transmitted through a larger area. This results in a reduced amount of stress and higher breaking strength of the MTJ (Eisenberg and Milton, 1984; Tidball, 1991). In a model of frog semitendinosus MTJ it has been calculated that these protrusions increase the surface area by 13.2 times compared to a smooth cone-shaped junction (Tidball, 1984). Also, the folding structure increases the angle of force transmission, and consequently a high proportion of force is transmitted through shear stress (Tidball, 1983). For chicken latissimus dorsi muscles it has been reported that the surface area of the MTJ in type II muscle fibers is 40% larger compared to type I fibers (Trotter and Baca, 1987). Type II muscles are capable of producing higher forces than type I fibers, and it is good strategy that the transmission surface is larger in relation to type II fibers. However, whether this difference in surface area between muscle fiber types also exists in humans is not known. The correlation between muscle fiber type and risk of strain injury has been suggested previously (Garrett et al., 1984). In contrast to rats and mice, where many muscles are primarily composed of either type I or type II fibers, human muscles consist of varying proportions of both fiber types and in the most frequently injured muscles in humans, the hamstrings, rectus femoris and gastrocnemius, type II fibers are dominant (Polgar et al., 1973). Whether the surface area in the MTJ from these muscles is larger by development or can be increased through training, is unknown.

The plasticity (ability to adapt) of the MTJ to various loads has mainly been investigated in animals (summarized in **Table 1**) where most of the studies are slightly dated, and there is only one study investigating the human MTJ (Kannus et al., 1992; Tidball and Quan, 1992; Zamora et al., 1995; Roffino et al., 2006, 1998; Kojima et al., 2008; Palma et al., 2011; Curzi et al., 2012, 2013, 2016; Rissatto Sierra et al., 2018; Jacob et al., 2019; Pimentel et al., 2020). The protocols for the animal studies are very inhomogeneous, as the animals have been subjected to different regimens for loading and unloading, and as various species and muscles have been analyzed, which makes it impossible to draw one conclusion from these studies. In addition, the method to measure the area of the MTJ interface varies between studies (**Table 1**).

Overall, in the rat MTJ there is evidence of an increasing complexity of the foldings as response to a period of intensified loading (by running), with higher number of branches and longer foldings (Kojima et al., 2008; Curzi et al., 2013, 2016). This indicates that the MTJ adapts to load by increasing the junctional interface. Higher intensity of loading provided as fast running has shown significantly greater increases in the surface area of the MTJ compared to a lighter intensity running intervention (Curzi et al., 2016). This positive effect of load was not seen on the length and thickness of the evaginations when compared in a model of ladder climbing and loaded ladder climbing (Pimentel et al., 2020). It would be relevant in future studies to implement various training variables, such as intensity, and study the effect on the surface area of the MTJ.

There are varying results on the effect of unloading (**Table 1**). The rat MTJ shows a smaller interface between muscle and tendon in groups after 3 weeks of unloading compared to control groups, and this is effected by shortening of the foldings. The surface area of the MTJ is almost 50% in the unloading groups, independent of muscle fiber type (Kannus et al., 1992), and similar changes have been recorded after only 4 days of weightlessness during a spaceflight (Tidball and Quan, 1992). Contrary, the length of foldings in MTJs from rats that had been exposed to a 14 days spaceflight and weightlessness was 58% higher than in controls (Roffino et al., 1998).

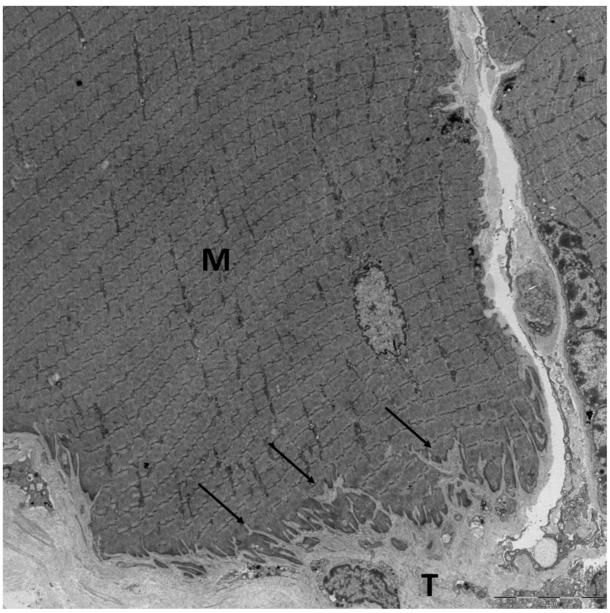

FIGURE 1 | The MTJ of a human semitendinosus muscle fiber viewed with EM. The protrusions (arrows) from the tendon (T) into the muscle fiber (M) increase the contact area between the muscle and tendon. Scale bar is 10 μm. Image by Andreas B. Knudsen, made at the Centre for Integrated Microscopy (CFIM) at the Panum Institute in Copenhagen, Denmark, used by permission.

These conflicting results may be explained by the method to calculate the surface area, namely as the ratio between the length of foldings at the MTJ and muscle fiber diameter. It is reasonable to assume that 14 days of weightlessness results in a higher degree of muscle fiber atrophy than 4 days, and if the length of the foldings remain constant, the calculated surface area increases with increasing length of loading. This may explain the surprising higher surface area of MTJ in rats after 14 days space flight.

The effect of unloading on the surface area of the MTJ in humans has been studied in 12 elderly patients who had a femoral amputation after being bed based for a long time period due to chronic disease. When compared to specimens from legs of 3 young persons who had acute amputations after accidents, there was a reduction in the surface area of the MTJs in the bed based patients (Palma et al., 2011). However, it is not known to which degree this was a result of unloading, the age difference or the chronic disease conditions.

Reduction of the surface area between muscle and tendon as a consequence of unloading can theoretically weaken the MTJ by making it less capable to transmit load – and thereby more susceptible to injuries. This may be the reason why elite soccer players, who had less than 10 training sessions before they participated in a competitive match after an injury period, had an increased risk of a subsequent injury (Bengtsson et al., 2019). For every additional training session before returning to match, the odds for an injury were 7% lower. This indicates that after a period of reduced activity, loading of the hamstrings MTJ should be implemented in training as soon as possible before full activity is resumed.

COMPOSITION OF THE MTJ

Through invagination of the muscle fiber membrane the cytoskeleton from the muscle fibers is in contact with the extracellular matrix (ECM) which connects muscle to tendon: the cytoskeletal connection—from actin to the ECM (Henderson et al., 2017). In brief, intramuscular bundles of actin filaments from the last A-band attaches at the cytoplasmic side of the sarcolemma to either dystrophin, which connects with the transmembrane dystroglycan complex, or the vinculin-talin-integrin complex (Shear and Bloch, 1985). These complexes penetrate the sarcolemma and attach actin on the intracellular side with laminin and collagen fibrils on the other, providing the path for force transmission from muscle fiber to tendon. Dystrophin and integrins therefore represent the physical structure through which force is transmitted. Knockout studies on mice have shown that absence of either dystrophin or integrin severely reduces the strength of muscle and increases its susceptibility to injuries (Guo et al., 2006). Interestingly, it has been observed, that by increasing the levels of integrin, the negative effects from the knockout of dystrophin are largely compensated (Hakim et al., 2013). This finding is clinically relevant for patients with Duchennes muscular dystrophy, since absence of dystrophin is the main pathogenesis of the disease. However, in addition to impairing the force transmission through the MTJ, called the linear force transmission pathway as it occurs in line with the sarcomeres, absence of dystrophin also affects lateral force transmission (Ramaswamy et al., 2011). Through this pathway force is transmitted from the contractile elements in the muscle fiber laterally through costameres to the endomysium (Pardo et al., 1983; Ervasti, 2003). There are junctions between the endomysium and perimysium, suggesting that lateral force transmission might occur through these junctions to the perimysium, which continues as endotenon in the tendon (Passerieux et al., 2006, 2007). It is not known if the lateral force transmission is changed by loading and unloading. However, an increase in lateral instead of linear force transmission through the MTJ would relieve the MTJ of some of the extra stress related to increasing training load.

It has been demonstrated by confocal imaging of MTJ that collagen type XXII is exclusively present in the foldings immediately on the tendon side of the muscle fiber membrane (**Figures 2**, **3**; Jakobsen et al., 2017). The specific function of this collagen is not known, but knockout studies in zebrafish have shown a reduced potential for force transmission as well as an increased risk of ruptures at the MTJ when collagen XXII is absent (Charvet et al., 2013). Collagen XXII is located at tissue junctions and is not found elsewhere in skeletal muscle (Koch et al., 2004). Therefore, it is a marker to identify the MTJ, which is helpful, for instance in wide field imaging. Next to collagen XXII on the tendon side of the MTJ is a variety of collagens, including the smaller FACIT (Fibril Associated Collagen with Interrupted Triple Helices) collagens XII and XIV

TABLE 1 | Studies describing the effects of loading and unloading on the surface area of the MTJ with a description of species used, muscles and muscle fiber types and the method used for measurements.

Studies describing the effect of increased load on the MTJ

Author, year,species	Sample Size pr Group	Muscles/dominant fiber type	Intervention	Method of measurement	Results
Kojima,2008,rat	5	Gastrocnemius/ I Tibialis Ant./II	Running	Branching of fingers Angle of branching	↑
Curzi. 2012,rat	6	Gastrocnemius/ I EDL/II	Running	Branching of fingers	↑
Curzi. 2016. rat	3	EDL/II	Running	Interface length/baseline(IL/B ratio)	↑
Sierra. 2018. rat	3	Soleus/I	Swimming	Length and thickness of invaginations and evaginations	↑
Jacob. 2019. rat	5	Sternomastoid/II	Swimming	IL/B	
Neto J, 2020 rat	10	Plantaris/II	Loaded ladder climb	Length and thickness of invaginations and evaginations	↑

Studies describing the effect of unloading the MTJ

Author, year,species	Sample Size pr Group	Muscles/dominant fiber type	Intervention	Method of measurement	Results
Kannus, 1992. rat	8	Gastrocnemius/II Soleus/I	Immobilization 1 and 3 weeks	Length of surface area in relation to muscle fiber diameter	↓
Tidball 1992. rat	6	Plantaris/II	Space flight -4 davs	Folding factor	↓
Zamora. 1995. rat	2 5	Soleus/I Plantaris/II	Limb suspension for 8, 18 and 29 davs Space flight 14 days	No quantification	Only minor changes observed in Plantar is MTJ's. thinning and lengthening of the foldings at MTJ of Soleus MTJ s
Roffino, 1998. rat	8	Soleus/I	Space flight 14 days	IL B ratio-named normalized interface values by the authors.	↑
Roffino, 2006.monkey	2	Soleus/I +II	Space flight 14 days	No qualification	-
de Palma, 2011,human	12 Unloaded 3 controls	Gastrocnemius/ I+II	Bed rest (mean 60 months) in chronic diseased patients	Length of surface area n relation to muscle fiber diameter	↓
Curzi,2013,rat	4	Plantaris/II	Unloading	IL/B ratio Length of primary digits Brancing of fingers	↓

In most studies the assessments have not been performed blinded to study group. Most studies provide information about the number of foldings/invaginations measured per group and not per animal and have not provided information about the number of slides from each animal. Because of this, the results should be interpreted with some caution. Arrows indicate whether an increase (↑) or a decrease (↓) in the measures was reported. EDL, Extensor Digitorum Longus.

(**Figure 3**; Jakobsen et al., 2017). Future studies must determine the functions of these various collagens.

EFFECTS OF EXERCISE ON THE COMPOSITION OF THE MTJ

There is incomplete knowledge about the effects of exercise on the composition and ultrastructure of the MTJ.

In human muscle the concentration of integrin type α7β1 increases following eccentric exercise (De Lisio et al., 2015). This integrin is also located at the MTJ, and because integrins are responsible for direct force transmission from muscle to tendon, increasing levels may be a way to prepare MTJ for increasing stress (Boppart et al., 2006). Similarly, in mice a single session of multiple eccentric contractions induces a significant increase of vinculin and talin in the soleus and plantaris muscles. Similar results are found in rats exposed to running with a higher expression of the mRNA for vinculin (Curzi et al., 2016). Although these proteins are found in the MTJ, they are not exclusively seen in this region, indicating that the increase in expression following exercise could arise from different regions of the muscle. Like the MTJ, both the neuromuscular junction and the costameres involved in lateral force transmission contain vinculin (Pardo et al., 1983; Ervasti, 2003). Both studies analyzed whole muscles and parts of tendon meaning that the increases in

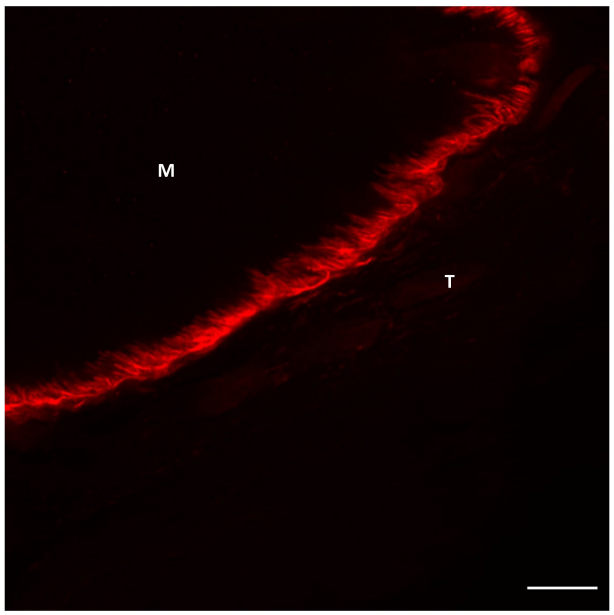

FIGURE 2 | Confocal image of human semitendinosus MTJ showing the highly folded muscle membrane at the interface between muscle (M) and tendon (T). Collagen XXII labeling (red) the foldings connecting the tendon (T) with the muscle (M) The nuclei are stained blue (DAPI). Scale bar is 10 μm. For staining protocol and details regarding image acquisition (see **Supplementary File**).

vinculin and talin are not necessarily seen at the MTJ. However, these two proteins are known to be present to a high extent at the MTJ but also neuromuscular junction, meaning that the increase following exercise is likely to arise from the MTJ where they are both elements in the chain of proteins that are responsible for force transmission through the junction (Frenette and Côté, 2000). Increase in expression of vinculin was also observed in rats exposed to a running intervention. However, it is not known if the increased levels of these proteins after exercise result in strengthening of the MTJ. It could be expected that the amount of larger collagens in the human MTJ would increase following heavy resistance exercise, but this has not been demonstrated for collagen I, III and VI (Jakobsen et al., 2018). However, the FACIT collagen type XIV increases in the endomysium surrounding the muscle fibers closest to the MTJ following exercise (Jakobsen et al., 2017). Like the MTJ, the endomysium contains a wide variety of collagens, and collagen XIV might be responsible for anchoring the larger collagen fibrils to form stronger networks.

The effects of various exercises on the length of the distal sarcomere in rat MTJs have been studied. Whether these lengths are important for the MTJ and risk of injury is unknown. Theoretically shorter sarcomere length, not to be confused with fascicle length, would be beneficial at the MTJ since they are more resistant to stretch during heavy loading, due to more overlapping cross-bridges between actin and myosin (Morgan et al., 1991; Rassier et al., 2020). In rats exposed to swimming exercise the distal sarcomeres of the sternomastoid MTJ are significantly shorter than in resting control rats (Jacob et al., 2019). However, the opposite is found following the same exercise intervention in rats of similar age, in the soleus muscle (Rissatto Sierra et al., 2018). So, there is conflicting evidence.

Following loaded and unloaded ladder exercise the distal sarcomere of rat soleus muscle is found to be shorter than in control rats. In contrast, the distal sarcomere from the plantaris muscle from the same animal is longer compared to control rats (Rocha et al., 2020). Whether the inhomogeneous effects of exercise on the length of the distal sarcomere at the MTJ is due to differences in the exercise regimens used, or that different muscles are loaded differently during the exercises, is not known. Future research is needed, preferably with the use of eccentric exercise as the chosen exercise modality since this is what has been shown to have a positive effect on the risk of strain injury (as discussed in detail later). Results from each animal or person should be pooled before statistical analysis of group values (instead of pooling all results in the groups). Finally, the person analyzing data should be blinded to the intervention. In a number of earlier studies (Kannus et al., 1992; Tidball and Quan, 1992; Zamora et al., 1995; Kojima et al., 2008; Curzi et al., 2012, 2013, 2016; Rissatto Sierra et al., 2018; Jacob et al., 2019; Pimentel et al., 2020) there is no certainty that data have been acquired after such basic principles.

CELL TURNOVER

The MTJ is not a resting structure. There is a very high rate of remodeling in the muscle fibers near the MTJ (Jakobsen et al., 2018). Approximately half of the fibers adjacent to the MTJ contain one or more centralized nuclei and stain positive for the Neural Cell Adhesion Molecule (NCAM), indicating remodeling or formation of new muscle fibers (**Figure 4A**; Jamali et al., 2000; Chargé and Rudnicki, 2004). Contrary, in the muscle belly only a small percentage of the muscle fibers contain centralized nuclei (Dreyer et al., 2006). Even in muscles that have not exercised, almost 50% of the fibers at the MTJ contain centralized nuclei, indicating extensive remodeling in this area. On cross-section images the increased proportion of fibers with centralized nuclei normalizes just few fibers from the MTJ. Similarly, longitudinal sections have shown that the same muscle fiber has a higher expression of NCAM at its distal end near the MTJ compared to further from the MTJ, suggesting regional differences in protein expression across the same muscle fiber (**Figure 4B**). This remodeling involves different cell types, including satellite cells, fibroblasts and macrophages (Murphy et al., 2011; Bentzinger et al., 2013).

Satellite cells surround the periphery of resting muscle fibers, ready to be activated. Upon activation, they proliferate into daughter cells that can be donated as new myonuclei or fuse to form new myotubes. In skeletal muscles, satellite cells proliferate when muscles are exposed to resistance exercise (Kadi and Thornell, 2000), and even a single exercise session is enough to induce this (Bellamy et al., 2014). However, the high rate of remodeling at the MTJ is not associated with a concomitant increase in concentrations of satellite cells. In fact, the number of satellite cells near MTJ is the same as in muscle tissue far from

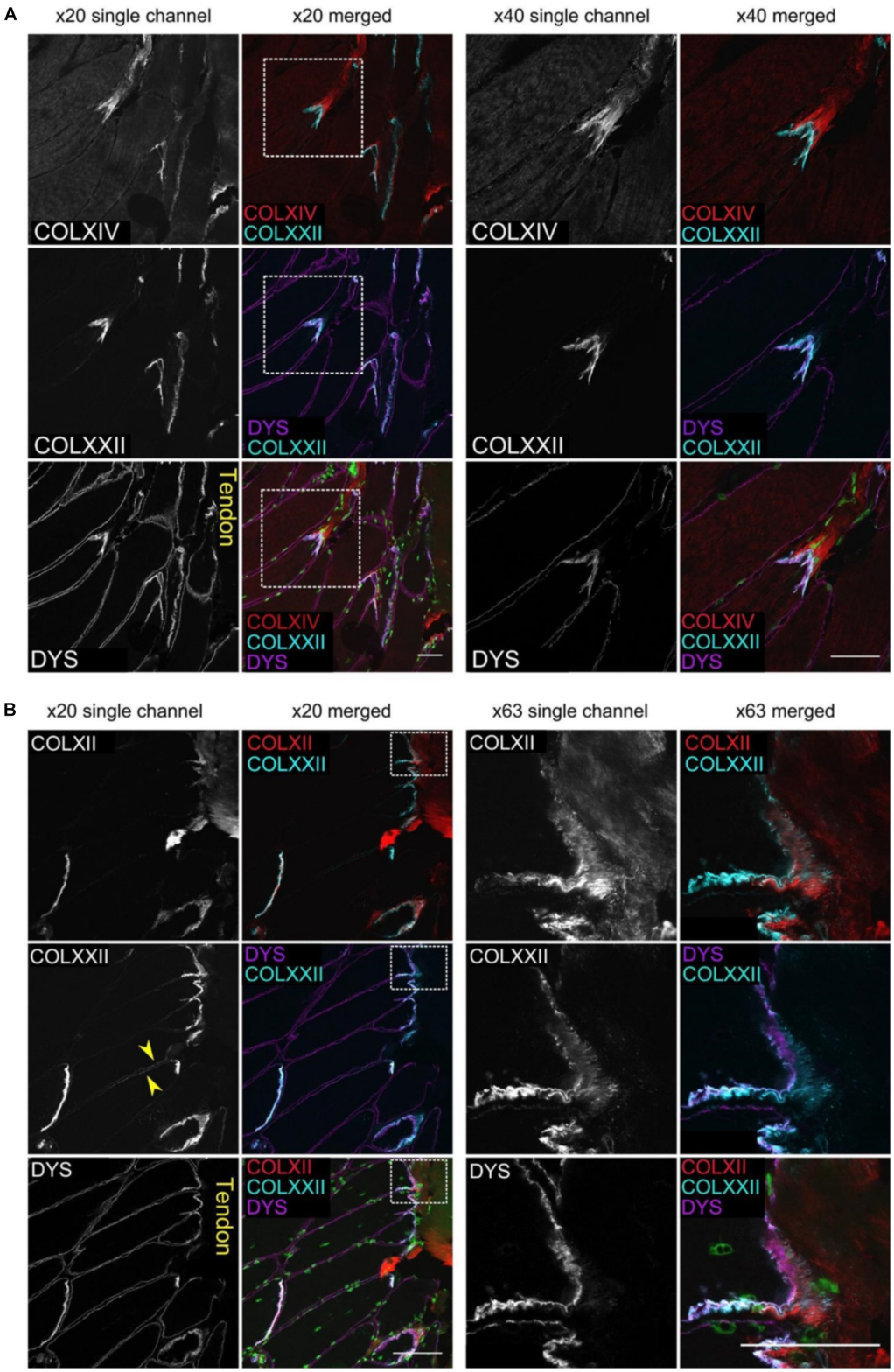

FIGURE 3 | The figure shows multiple images of human gracilis MTJ stained with immunofluorescent antibodies against collagen XXII, dystrophin and either collagen XIV **(A)** or XII **(B)**. The muscle fibers show to the left and the tendon to the right. Dystrophin is located closest to the muscle fiber with collagen XXII immediately outside the muscle membrane. Collagen XIV and XII are present next to collagen XXII on the outside of the muscle fiber. Scale bars are 50 μm. The figure is from Jakobsen et al. (2017), used by permission.

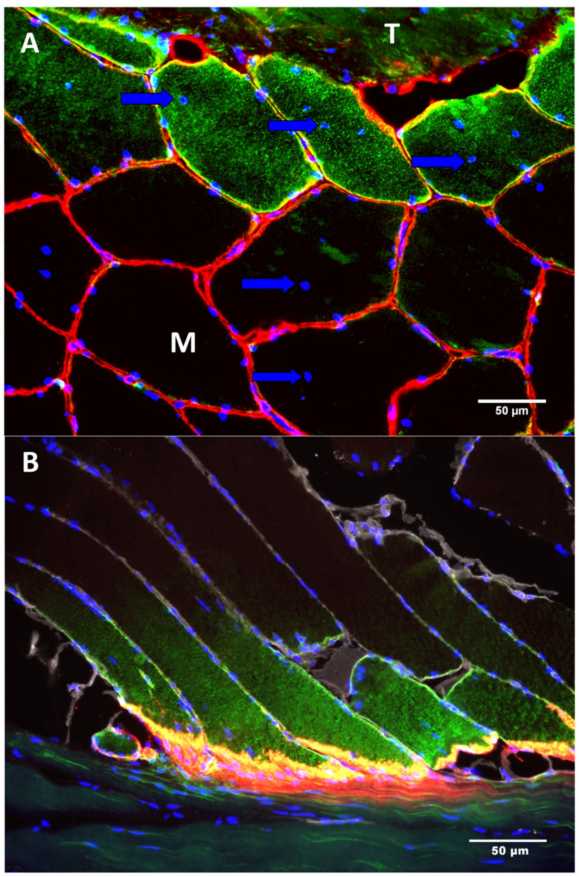

FIGURE 4 | The figure shows two sections **(A,B)** of a human semitendinosus muscle stained for NCAM (green), collagen IV (red) and DAPI (Blue—indicating the nuclei). **(A)** In the top of the image there is tendon (T) and below is the muscle (M). The muscle fibers located closest to the tendon express signs of remodeling by containing centralized myonuclei (arrow) as well as NCAM(CD5S6) staining of the cytoplasm. **(B)** As the muscle fibers approach the MTJ the immunoreactivity of NCAM increases whereas no staining is seen in the fibers in the upper part of the image, farthest from the MTJ. Scale bar is 50 μm. Images are reprinted with permission from Jakobsen et al. (2018).

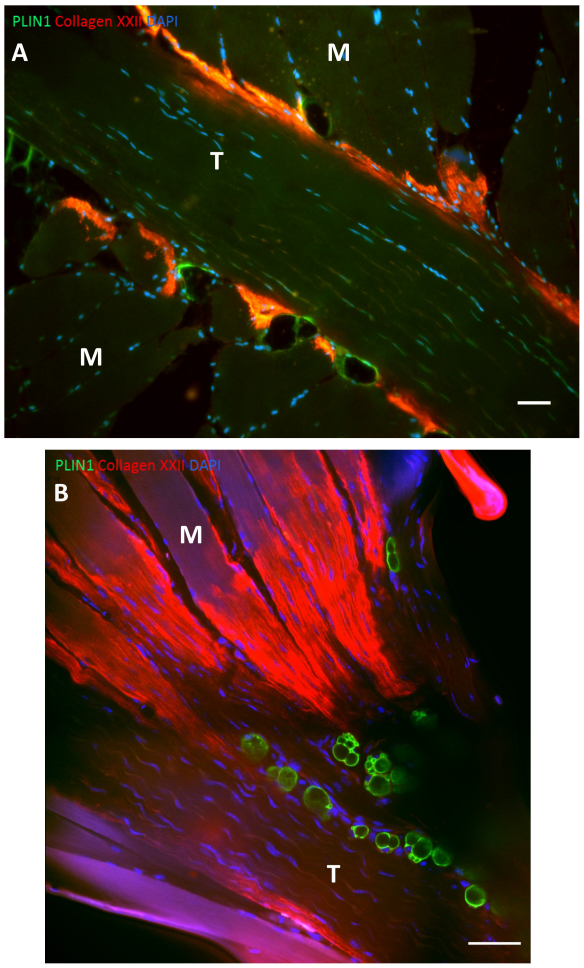

FIGURE 5 | Adipocytes at human and mouse MTJ's. **(A)** The figure shows a section from human semitendinosus muscle with the tendon (T) in the middle and the muscle fibers (M) approaching from both sides. The MTJ is identified by collagen XXII immunofluorescent staining (red) and the adipocytes are stained with PLIN1 (green), and nuclei are stained with DAPI (blue). Adipocytes are located very close to the MTJ. Scale bar is 50 μm. **(B)** Mouse soleus MTJ in confocal microscopy showing adipocytes (PLIN = green) and collagen XXII indicating the MTJ (red). Scale bar is 50 μm. Images are reprinted with permission from Jakobsen et al. (2020).

the MTJ, and the concentration of satellite cells is unaffected by 4 weeks resistance exercise (Jakobsen et al., 2018). This indicates that satellite cells at the MTJ respond differently than those in muscle. However, it could be a dynamic phenomenon: the extremely high rate of remodeling at the MTJ could initiate a high demand for satellite cells, and the resulting, newly formed daughter cells could fuse immediately with a muscle fiber. If this is the case, the increase in the satellite cell pool will not show in a static cell count. However, additional studies are needed to fully understand the dynamics of satellite cells at the MTJ. While satellite cells have been shown to be detrimental for optimal muscle regeneration and repair they are not the only cell type involved (Joe et al., 2010; Sambasivan et al., 2011; Wang and Rudnicki, 2012). Inflammatory cells, such as macrophages and neutrophils, participate in this complex process, as well as fibroblasts (Toumi et al., 2006; Tidball and Villalta, 2010). Interestingly, fibro-adipogenic cells (FAPs) which are the precursor cells for fibroblasts and adipocytes, have been shown to be important for myogenic proliferation following muscle injury (Joe et al., 2010; Heredia et al., 2013; Biferali et al., 2019).

It has recently been demonstrated that adipocytes exist in high numbers in close relation to the MTJ in humans and mice (**Figure 5**; Jakobsen et al., 2020). Adipocytes in skeletal muscle is normally a sign of poor muscle function and insulin resistance (Vettor et al., 2009), but at the MTJ adipocytes are present in healthy humans and animals. The MTJ has previously been reported to contain a high mitochondrial content indicating an increased oxidative phosphorylation but since the adipocytes are located extracellularly, they might not be involved in the energy metabolism of the muscle fibers (Eisenberg and Milton, 1984).

However, since adipocytes are known to be capable of producing various cytokines involved in numerous processes throughout the body (Ronti et al., 2006), they may play a role in the highly active remodeling process at the MTJ or in the regeneration following injury.

The regeneration and repair following muscle strain injury is a complex process which is not fully understood yet. However, following injury there is a rapid increase in inflammatory cells, macrophages and neutrophils (Butterfield et al., 2006). Macrophages initially enter the injured region as pro-inflammatory M1 macrophages, capable of phagocytosis and secretion of pro-inflammatory cytokines. As they decline in concentration, the anti-inflammatory M2 macrophages increase to aid in the repair of the injured tissue (St. Pierre and Tidball, 1994). By secreting cytokines, e.g., platelet derived growth factor (PDGF) macrophages activate fibroblasts, thereby ensuring a restoration of the ECM and formation of fibrosis (Kovacs and Dipietro, 1994). Without proper coordination between the macrophages, fibroblasts and satellite cells, optimal restoration of the tissue cannot be accomplished resulting in excessive formation of fibrosis and impaired muscle function. In skin lesions the interplay between adipocytes and fibroblasts is important for optimal healing (Schmidt and Horsley, 2013). This has not been confirmed in muscle yet, but the presence of adipocytes at the MTJ and the finding of cytokines involved in adipogenesis in the exudate of human MTJ following injury, suggests a prominent role for this cell-type in the repair of the MTJ (Bayer et al., 2019).

STRAIN INJURIES

Muscle injuries can be classified as either direct or indirect. The most common indirect type is the strain injury, defined as a muscular trauma affecting the tissue structure with various degree of tear (Mueller-Wohlfahrt et al., 2013). Strain injuries are among the most common injuries in a wide range of sports affecting various muscles (Orchard and Seward, 2002; Ekstrand et al., 2011; Opar et al., 2014; Ishøi et al., 2019). They are most dominantly located at the MTJ and show as small disruptions and edema between the muscle and tendon on ultrasound and MRI imaging (De Smet et al., 1990; De Smet and Best, 2000).

Muscles which are often exposed to rapid stretch during powerful movements, such as the hamstrings, rectus femoris, hip-adductor and calf muscles, are most susceptible to strain injury (Ekstrand et al., 2011; Eckard et al., 2017; Green and Pizzari, 2017; Green et al., 2020; Hultman et al., 2020). Generally, these injuries occur during activities where the muscle is either passively over-stretched or during fast eccentric contractions where the MTJ is exposed to high loads during lengthening (Askling et al., 2008; Danielsson et al., 2020; Huygaerts et al., 2020).

In the hamstring muscles two types of injury mechanisms have been identified: the stretch-type injury and sprinting-type injury. The stretch-type commonly occurs during kicking activities or when the hip is flexed while the leg is stretched, for example during pick-up of a ball in American type football (Hagel, 2005). The sprint-type injury is named after its occurrence during sprinting, and it mostly affects the proximal part of the biceps femoris long head (Ekstrand et al., 2011; Fiorentino et al., 2014; Huygaerts et al., 2020). It is suggested that the late swing phase and the early stance phase is when the hamstrings are subjected to the highest loads during running (Chumanov et al., 2011; Schache et al., 2012; Fiorentino et al., 2014). During these phases the hamstring muscles are stretched while contracting rapidly.

Similar to the hamstring strain injuries both rectus femoris and adductor longus injuries are frequently seen during kicking and sprinting activities where the muscles are exposed to rapid stretch while contracting affecting athletes in multiple disciplines where kicking and sprinting activities are involved (Mendiguchia et al., 2013; Serner et al., 2015, 2019). Strain injury to the calf muscles, "Tennis-Leg," is seen in the medial head of gastrocnemius as well as the soleus, probably depending on injury mechanism (Delgado et al., 2002; Green et al., 2020). The mechanism of tennis leg has not been fully elucidated, but it is thought to occur during the late stance phase in sprinting activities where the knee is extended while a strong plantar flexion is performed by the gastrocnemius (Orchard et al., 2002).

Eccentric contractions can potentially cause muscle damage due to stretching of the sarcomeres, leading to fewer connections between actin and myosin and a suboptimal length-tension relationship (Gordon et al., 1966; Morgan, 1990; Dueweke et al., 2017). However, a large force production itself is not sufficient to induce strain injury in otherwise healthy MTJ (Lieber and Friden, 1993; Garrett, 1996). This indicates that in addition to a strong contraction of the sarcomeres, a forceful pull on the muscle provided by the tendon is needed to create a strain injury where the strength of the adhesion between these two tissues is exceeded. This is also the case in the previously described injury mechanisms for the most frequent strain injuries where a rapid stretch is seen together with a strong muscle contraction. Experimentally induced strain injuries in animal muscles, where the muscles are pulled while stimulated to contract, have shown that a disruption occurs either in the distal part of the muscle fibers or at the basal lamina between the muscle fiber and tendon (Tidball, 1984; Tidball and Chan, 1989), confirming that the connection between the muscle fibers and the tendon is the "weak-spot." Unfortunately, a histologic description of a strain injury has not yet been published for humans.

PREVENTION OF HAMSTRING STRAIN INJURIES

A recent review has summarized the current knowledge regarding diagnosis, prevention and treatment of the most common strain injuries (Ishøi et al., 2019). Therefore, the current review will focus on prevention of hamstring strain injuries, for which there is the best evidence.

Hamstring strain injuries are among the most frequent non-contact injuries in sports involving high speed running such as football, Australian rules football and athletics (Orchard and Seward, 2002; Ekstrand et al., 2011; Opar et al., 2014). It is the most common reason for inability to participate in sports. The pathogenesis of these injuries, the risk factors and possible

prevention strategies have been subject for several recent studies (Mjolsnes et al., 2004; Petersen et al., 2011; Seagrave et al., 2014; Van Der Horst et al., 2015; Bourne et al., 2017). Low eccentric strength has been proposed as a risk factor for hamstring strain injury as well as short fascicle length, low hamstring to quadriceps strength ratio, hamstring tightness, muscle fatigue, poor warm-up, previous hamstring strain and age among others (Mair et al., 1996; Petersen, 2005; Opar et al., 2015; Timmins et al., 2016).

The role of muscle fatigue and fascicle length in relation to strain injury has not been fully elucidated. In a mechanistic study of strain injury, in which rabbit muscles were stretched while either being stimulated to induce contraction or left unstimulated, the stimulated muscles could produce 15% more force at failure, while the absorbed energy was 100% greater than in the unstimulated state. This suggests that muscle contractions affect the ability of the passive components to absorb force in the muscle fibers, i.e., the connective tissue (Garrett, 1996). Therefore, any condition that affects the muscle ability of the fibers to activate optimally, such as fatigue or weakness, would tend to increase the risk of injury (Mair et al., 1996). This might also explain why greater fascicle lengths are protective against strain injury since longer fascicles are capable of providing maximum force at a greater muscle length and higher velocity, meaning they are more resistant to rapid stretch (Bodine et al., 1982; Lieber and Fridén, 2000). This resistance to stretch means that fewer sarcomeres are disrupted during eccentric contractions leading to an unaffected force production and thereby an increased energy absorption from the passive structures surrounding the muscle fibers. Eccentric exercise training has been shown to increase fascicle length by adding sarcomeres in series (Lynn and Morgan, 1994), however, this has been questioned in a very recent study where there was no addition of sarcomeres in human biceps femoris muscle following 3 weeks of Nordic Hamstring exercise (Pincheira et al., 2021). Therefore, it is not clear what the mechanism behind the lengthening of the fascicles is. In addition, to induce lengthening of fascicles eccentric exercise also increases eccentric hamstring strength. Many exercise interventions have focused on eccentric exercise in the prevention of strain injuries with the Nordic Hamstring protocol being the most widely used (Askling et al., 2003; Mjolsnes et al., 2004; Arnason et al., 2008; Petersen et al., 2011; Van Der Horst et al., 2015; Bourne et al., 2018).

The original Nordic Hamstring protocol consisted of the eccentric exercise, Nordic Hamstring, periodized with increasing volume over 10 weeks (Mjolsnes et al., 2004). The Nordic Hamstring protocol reduce the incidence of hamstring strain injuries by up to 51% (Al Attar et al., 2017). The clinical effect of this type of exercise is probably due to its effects on many of the previously mentioned risk factors for strain injury. Besides increasing hamstring strength (and thereby also improving hamstring to quadriceps strength ratio) it increases the length of fascicles and probably also eccentric hamstring strength capacity (Mjolsnes et al., 2004; Bourne et al., 2017; Ishøi et al., 2018; Presland et al., 2018; Ribeiro-Alvares et al., 2018; Duhig et al., 2019; Pollard et al., 2019; Siddle et al., 2019; Whyte et al., 2019; Medeiros et al., 2020).

Future studies looking at hamstring strain injury prevention should focus on the implementation of the Nordic Hamstring protocol. Among the 32 best football clubs in Europe and 18 clubs from the Norwegian Tippeliga (where the Nordic Hamstring protocol was developed) only 10% of the clubs were compliant in 2015 with the Nordic Hamstring protocol (Bahr et al., 2015). A recent study described that athletes didn't want to do Nordic Hamstring due to fear that they would be too sore thereby affecting their other training (Chesterton et al., 2020). This is important to address in future studies. In on our previous research we have used the Nordic Hamstring exercise on patients with anterior cruciate ligament rupture in the study of the MTJ (Jakobsen et al., 2017). Our clinical experience is that the Nordic Hamstring protocol is easily implemented, even in subjects with a low activity level, and the Delayed Onset Muscle Soreness (DOMS) rarely occur after the first couple of sessions. Therefore, the strategy to implement the Nordic Hamstring protocol in an athletic setting (Arnason et al., 2008; Petersen et al., 2011), where it is used more intensively off-season to build up strength and tolerance and less intensively in-season, seems logical.

In addition to the clinical focus on the implementation of prophylactic training, future human studies can increase our understanding of the tissue mechanisms, which besides changes in fascicle length include for instance alterations in the surface area of the MTJ and changes in the concentration of specific proteins involved in adaption of the MTJ following eccentric training. This has been explored to some extent in rats and mice, showing increases in the surface area of MTJ and concentrations of structural proteins (vinculin, talin and collagen XIV) at the MTJ following exercise (Frenette and Côté, 2000; Roffino et al., 2006; Kojima et al., 2008; Curzi et al., 2012), but it has only been sparsely studied in humans (Jakobsen et al., 2017). A deeper understanding of the effects of eccentric exercise on the MTJ at the molecular and ultrastructural level is a necessary evidence base for introducing new effective methods to prevent strain injuries.

PERSPECTIVES

Knowledge about the human MTJ is limited. There is no standard method to obtain percutaneous biopsies from MTJ. Since the MTJ consists of two different tissues, muscle and tendon, it is necessary technically to separate samples into muscle, MTJ and tendon to investigate the metabolism in this region, and currently there is no reliable method to do this. Once these methodological challenges have been overcome, structured research in the human MTJ can be performed as basis for an understanding of strain injuries and their prevention. With the current knowledge candidate explanations of how MTJ may adapt to eccentric training are: by increasing fascicle length, increasing the surface area between muscle and tendon through enlargement of foldings, increasing concentration of structural

proteins of importance for force transmission between tissues, such as dystrophin, integrins, and collagens, and increasing the lateral force transmission through the costameres. Also, in the future the interplay between macrophages, satellite cells, fibroblasts and adipocytes in the coordination of remodeling following exercise, injury and inactivity should be studied.

WHAT IS ALREADY KNOWN

The myotendinous junction (MTJ) is the interface between muscle and tendon and where force is transmitted between the two tissues. It is also a common location for strain injuries in sports. Most of these can be prevented by heavy eccentric exercise.

WHAT ARE THE NEW FINDINGS

The surface area between muscle and tendon is increased by foldings of tendon into muscle, and this reduces stress between the tissues. In animals, the size and number of foldings are increased as a response to heavy training and reduced during inactivity. In humans, the muscle fibers near the MTJ show very high rates of remodeling compared to other regions of the muscle. Adipocytes are seen in high number at MTJ and might be important for the adaption of MTJ to various loading conditions.

AUTHOR CONTRIBUTIONS

Both authors contributed to the planning, writing, and editing of the manuscript.

FUNDING

We were supported by Copenhagen University Hospital, Bispebjerg and Frederiksberg, the International Olympic Committee as well as the Aase and Einar Danielsens foundation.

ACKNOWLEDGMENTS

A special thanks to Abigail Mackey and Michael Kjaer for valuable inputs and discussions about the human MTJ. We also acknowledge the Core Facility for Integrated Microscopy, Faculty of Health and Medical Sciences, and University of Copenhage where the confocal and electron microscopy images are captured.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2021.635561/full#supplementary-material

REFERENCES

Al Attar, W. S. A., Soomro, N., Sinclair, P. J., Pappas, E., and Sanders, R. H. (2017). Effect of Injury Prevention Programs that Include the Nordic Hamstring Exercise on Hamstring Injury Rates in Soccer Players: A Systematic Review and Meta-Analysis. *Sport. Med.* 47, 907–916. doi: 10.1007/s40279-016-0638-2

Arnason, A., Andersen, T. E., Holme, I., Engebretsen, L., and Bahr, R. (2008). Prevention of hamstring strains in elite soccer: an intervention study. *Scand J. Med. Sci. Sport* 18, 40–48. doi: 10.1111/j.1600-0838.2006.00634.x

Askling, C., Karlsson, J., and Thorstensson, A. (2003). Hamstring injury occurrence in elite soccer players after preseason strength training with eccentric overload. *Scand. J. Med. Sci. Sport* 13, 244–250. doi: 10.1034/j.1600-0838.2003.00312.x

Askling, C. M., Tengvar, M., Saartok, T., and Thorstensson, A. (2008). Proximal hamstring strains of stretching type in different sports: Injury situations, clinical and magnetic resonance imaging characteristics, and return to sport. *Am. J. Sports Med.* 36, 1799–1804. doi: 10.1177/0363546508315892

Bahr, R., Thorborg, K., and Ekstrand, J. (2015). Evidence-based hamstring injury prevention is not adopted by the majority of Champions League or Norwegian Premier League football teams: The Nordic Hamstring survey. *Br. J. Sports Med.* 49, 1466–1471. doi: 10.1136/bjsports-2015-094826

Bayer, M. L., Bang, L., Hoegberget-Kalisz, M., Svensson, R. B., Olesen, J. L., Karlsson, M. M., et al. (2019). Muscle-strain injury exudate favors acute tissue healing and prolonged connective tissue formation in humans. *FASEB J.* 33, 10369–10382. doi: 10.1096/fj.201900542R

Bellamy, L. M., Joanisse, S., Grubb, A., Mitchell, C. J., McKay, B. R., Phillips, S. M., et al. (2014). The Acute Satellite Cell Response and Skeletal Muscle Hypertrophy following Resistance Training. *PLoS One* 9:e109739. doi: 10.1371/journal.pone.0109739

Bengtsson, H., Ekstrand, J., Waldén, M., and Hägglund, M. (2019). Few training sessions between return to play and first match appearance are associated with an increased propensity for injury: a prospective cohort study of male professional football players during 16 consecutive seasons. *Br. J. Sports Med.* 2019:100655. doi: 10.1136/bjsports-2019-100655

Bentzinger, C. F., Wang, Y. X., Dumont, N. A., and Rudnicki, M. A. (2013). Cellular dynamics in the muscle satellite cell niche. *EMBO Rep.* 14, 1062–1072. doi: 10.1038/embor.2013.182

Biferali, B., Proietti, D., Mozzetta, C., and Madaro, L. (2019). Fibro–Adipogenic Progenitors Cross-Talk in Skeletal Muscle: The Social Network. *Front. Physiol.* 10:01074. doi: 10.3389/fphys.2019.01074

Bodine, S. C., Roy, R. R., Meadows, D. A., Zernicke, R. F., Sacks, R. D., Fournier, M., et al. (1982). Architectural, histochemical, and contractile characteristics of a unique biarticular muscle: The cat semitendinosus. *J. Neurophysiol.* 48, 192–201. doi: 10.1152/jn.1982.48.1.192

Boppart, M. D., Burkin, D. J., and Kaufman, S. J. (2006). Alpha7beta1-integrin regulates mechanotransduction and prevents skeletal muscle injury. *Am. J. Physiol. Cell Physiol.* 290, C1660–C1665. doi: 10.1152/ajpcell.00317.2005

Bourne, M. N., Duhig, S. J., Timmins, R. G., Williams, M. D., Opar, D. A., Al Najjar, A., et al. (2017). Impact of the Nordic hamstring and hip extension exercises on hamstring architecture and morphology: Implications for injury prevention. *Br. J. Sports Med.* 51, 469–477. doi: 10.1136/bjsports-2016-096130

Bourne, M. N., Timmins, R. G., Opar, D. A., Pizzari, T., Ruddy, J. D., Sims, C., et al. (2018). An Evidence-Based Framework for Strengthening Exercises to Prevent Hamstring Injury. *Sport. Med.* 48, 251–267. doi: 10.1007/s40279-017-0796-x

Butterfield, T. A., Best, T. M., and Merrick, M. A. (2006). The dual roles of neutrophils and macrophages in inflammation: A critical balance between tissue damage and repair. *J. Athl. Train.* 41, 457–465. doi: 10.1016/s0162-0908(08)79217-1

Chargé, S. B. P., and Rudnicki, M. A. (2004). Cellular and molecular regulation of muscle regeneration. *Physiol. Rev.* 84, 209–238. doi: 10.1152/physrev.00019.2003

Charvet, B., Guiraud, A., Malbouyres, M., Zwolanek, D., Guillon, E., Bretaud, S., et al. (2013). Knockdown of col22a1 gene in zebrafish induces a muscular dystrophy by disruption of the myotendinous junction. *Development* 140, 4602–4613. doi: 10.1242/dev.096024

Charvet, B., Malbouyres, M., Pagnon-Minot, A., Ruggiero, F., and Le Guellec, D. (2011). Development of the zebrafish myoseptum with emphasis on the

myotendinous junction. *Cell Tissue Res.* 346, 439–449. doi: 10.1007/s00441-011-1266-7

Charvet, B., Ruggiero, F., and Le Guellec, D. (2012). The development of the myotendinous junction. *A Rev. Muscles Ligaments Tendons J.* 2, 53–63.

Chesterton, P., Tears, C., Wright, M., and Portas, M. (2020). Hamstring injury prevention practices and compliance of the Nordic hamstring program in English professional football. *Transl. Sport. Med.* 2020:tsm2.209. doi: 10.1002/tsm2.209

Chumanov, E. S., Heiderscheit, B. C., and Thelen, D. G. (2011). Hamstring musculotendon dynamics during stance and swing phases of high-speed running. *Med. Sci. Sports Exerc.* 43, 525–532. doi: 10.1249/MSS.0b013e3181f23fe8

Curzi, D., Lattanzi, D., Ciuffoli, S., Burattini, S., Grindeland, R. E., Edgerton, V. R., et al. (2013). Growth hormone plus resistance exercise attenuate structural changes in rat myotendinous junctions resulting from chronic unloading. *Eur. J. Histochem.* 57:e37. doi: 10.4081/ejh.2013.e37

Curzi, D., Salucci, S., Marini, M., Esposito, F., Agnello, L., Veicsteinas, A., et al. (2012). How physical exercise changes rat myotendinous junctions: an ultrastructural study. *Eur. J. Histochem.* 56:e19. doi: 10.4081/ejh.2012.19

Curzi, D., Sartini, S., Guescini, M., Lattanzi, D., Di Palma, M., Ambrogini, P., et al. (2016). Effect of Different Exercise Intensities on the Myotendinous Junction Plasticity. *PLoS One* 11:e0158059. doi: 10.1371/journal.pone.0158059

Danielsson, A., Horvath, A., Senorski, C., Alentorn-Geli, E., Garrett, W. E., Cugat, R., et al. (2020). The mechanism of hamstring injuries- A systematic review. *BMC Musculoskelet. Disord* 21, 3658–3658. doi: 10.1186/s12891-020-03658-8

De Lisio, M., Farup, J., Sukiennik, R. A., Clevenger, N., Nallabelli, J., Nelson, B., et al. (2015). The acute response of pericytes to muscle-damaging eccentric contraction and protein supplementation in human skeletal muscle. *J. Appl. Physiol.* 119, 900–907. doi: 10.1152/japplphysiol.01112.2014

De Smet, A. A., and Best, T. M. (2000). MR imaging of the distribution and location of acute hamstring injuries in athletes. *AJR. Am. J. Roentgenol.* 174, 393–399. doi: 10.2214/ajr.174.2.1740393

De Smet, A. A., Fisher, D. R., Heiner, J. P., and Keene, J. S. (1990). Magnetic resonance imaging of muscle tears. *Skeletal Radiol.* 19, 283–286. doi: 10.1007/BF00191673

Delgado, G. J., Chung, C. B., Lektrakul, N., Azocar, P., Botte, M. J., Coria, D., et al. (2002). Tennis leg: Clinical US study of 141 patients and anatomic investigation of four cadavers with MR imaging and US. *Radiology* 224, 112–119. doi: 10.1148/radiol.2241011067

Dreyer, H. C., Blanco, C. E., Sattler, F. R., Schroeder, E. T., and Wiswell, R. A. (2006). Satellite cell numbers in young and older men 24 hours after eccentric exercise. *Muscle Nerve* 33, 242–253. doi: 10.1002/mus.20461

Dueweke, J. J., Awan, T. M., and Mendias, C. L. (2017). Regeneration of skeletal muscle after eccentric injury. *J. Sport Rehabil.* 26, 171–179. doi: 10.1123/jsr.2016-0107

Duhig, S. J., Bourne, M. N., Buhmann, R. L., Williams, M. D., Minett, G. M., Roberts, L. A., et al. (2019). Effect of concentric and eccentric hamstring training on sprint recovery, strength and muscle architecture in inexperienced athletes. *J. Sci. Med. Sport* 22, 769–774. doi: 10.1016/j.jsams.2019.01.010

Eckard, T. G., Padua, D. A., Dompier, T. P., Dalton, S. L., Thorborg, K., and Kerr, Z. Y. (2017). Epidemiology of Hip Flexor and Hip Adductor Strains in National Collegiate Athletic Association Athletes, 2009/2010-2014/2015. *Am. J. Sports Med.* 45, 2713–2722. doi: 10.1177/0363546517716179

Eisenberg, B. R., and Milton, R. L. (1984). Muscle fiber termination at the tendon in the frog's sartorius: A stereological study. *Am. J. Anat.* 171, 273–284. doi: 10.1002/aja.1001710304

Ekstrand, J., Hägglund, M., and Waldén, M. (2011). Injury incidence and injury patterns in professional football: The UEFA injury study. *Br. J. Sports Med.* 45, 553–558. doi: 10.1136/bjsm.2009.060582

Ervasti, J. M. (2003). Costameres: The Achilles' heel of Herculean muscle. *J. Biol. Chem.* 278, 13591–13594. doi: 10.1074/jbc.R200021200

Fiorentino, N. M., Rehorn, M. R., Chumanov, E. S., Thelen, D. G., and Blemker, S. S. (2014). Computational models predict larger muscle tissue strains at faster sprinting speeds. *Med. Sci. Sports Exerc.* 46, 776–786. doi: 10.1249/MSS.0000000000000172

Frenette, J., and Côté, C. H. (2000). Modulation of structural protein content of the myotendinous junction following eccentric contractions. *Int. J. Sports Med.* 21, 313–320. doi: 10.1055/s-2000-3774

Garrett, W. Jr. (1996). Muscle strain injuries. *Am. J. Sport Med.* 1996, 2–8.

Garrett, W. E., Califf, J. C., and Bassett, F. H. (1984). Histochemical correlates of hamstring injuries. *Am. J. Sports Med.* 12, 98–103. doi: 10.1177/036354658401200202

Gordon, A. M., Huxley, A. F., and Julian, F. J. (1966). The variation in isometric tension with sarcomere length in vertebrate muscle fibres. *J. Physiol.* 184, 170–192. doi: 10.1113/jphysiol.1966.sp007909

Green, B., Lin, M., Schache, A. G., McClelland, J. A., Semciw, A. I., Rotstein, A., et al. (2020). Calf muscle strain injuries in elite Australian Football players: A descriptive epidemiological evaluation. *Scand. J. Med. Sci. Sport.* 30, 174–184. doi: 10.1111/sms.13552

Green, B., and Pizzari, T. (2017). Calf muscle strain injuries in sport: A systematic review of risk factors for injury. *Br. J. Sports Med.* 51, 1189–1194. doi: 10.1136/bjsports-2016-097177

Guo, C., Willem, M., Werner, A., Raivich, G., Emerson, M., Neyses, L., et al. (2006). Absence of alpha 7 integrin in dystrophin-deficient mice causes a myopathy similar to Duchenne muscular dystrophy. *Hum. Mol. Genet.* 15, 989–998. doi: 10.1093/hmg/ddl018

Hagel, B. (2005). Hamstring injuries in Australian football. *Clin. J. Sport Med.* 15, 400. doi: 10.1097/01.jsm.0000179227.01404.d3

Hakim, C. H., Burkin, D. J., and Duan, D. (2013). Alpha 7 integrin preserves the function of the extensor digitorum longus muscle in dystrophin-null mice. *J. Appl. Physiol.* 115, 1388–1392. doi: 10.1152/japplphysiol.00602.2013

Henderson, C. A., Gomez, C. G., Novak, S. M., Mi-Mi, L., and Gregorio, C. C. (2017). Overview of the muscle cytoskeleton. *Compr. Physiol.* 7, 891–944. doi: 10.1002/cphy.c160033

Heredia, J. E., Mukundan, L., Chen, F. M., Mueller, A. A., Deo, R. C., Locksley, R. M., et al. (2013). Type 2 innate signals stimulate fibro/adipogenic progenitors to facilitate muscle regeneration. *Cell* 153, 376–388. doi: 10.1016/j.cell.2013.02.053

Huijing, P. A. (1999). Muscle as a collagen fiber reinforced composite: a review of force transmission in muscle and whole limb. *J Biomech.* 32, 329–345.

Hultman, K., Szukics, P. F., Grzenda, A., Curriero, F. C., and Cohen, S. B. (2020). Gastrocnemius Injuries in Professional Baseball Players: An Epidemiological Study. *Am. J. Sports Med.* 48, 2489–2498. doi: 10.1177/0363546520938759

Huygaerts, S., Cos, F., Cohen, D. D., Calleja-González, J., Guitart, M., Blazevich, A. J., et al. (2020). Mechanisms of Hamstring Strain Injury: Interactions between Fatigue. *Muscle Activ. Funct. Sports* 8:65. doi: 10.3390/sports8050065

Ishøi, L., Hölmich, P., Aagaard, P., Thorborg, K., Bandholm, T., and Serner, A. (2018). Effects of the Nordic Hamstring exercise on sprint capacity in male football players: a randomized controlled trial. *J. Sports Sci.* 36:1409609. doi: 10.1080/02640414.2017.1409609

Ishøi, L., Krommes, K., Husted, R. S., Juhl, C. B., and Thorborg, K. (2019). Diagnosis, prevention and treatment of common lower extremity muscle injuries in sport-grading the evidence: A statement paper commissioned by the Danish Society of Sports Physical Therapy (DSSF). *Br. J. Sports Med.* 54:528. doi: 10.1136/bjsports-2019-101228

Jacob, C. D. S., Rocha, L. C., Neto, J. P., Watanabe, I. S., and Ciena, A. P. (2019). Effects of physical training on sarcomere lengths and muscle-tendon interface of the cervical region in an experimental model of menopause. *Eur. J. Histochem.* 63, 131–135. doi: 10.4081/ejh.2019.3038

Jakobsen, J. R., Jakobsen, N. R., Mackey, A. L., Knudsen, A. B., Hannibal, J., Koch, M., et al. (2020). Adipocytes are present at human and murine myotendinous junctions. *Transl. Sport. Med.* 2020:tsm2.212. doi: 10.1002/tsm2.212

Jakobsen, J. R., Jakobsen, N. R., Mackey, A. L., Koch, M., Kjaer, M., and Krogsgaard, M. R. (2018). Remodeling of muscle fibers approaching the human myotendinous junction. *Scand. J. Med. Sci. Sports* 2018:sms.13196. doi: 10.1111/sms.13196

Jakobsen, J. R., Mackey, A. L., Knudsen, A. B., Koch, M., Kjær, M., and Krogsgaard, M. R. (2017). Composition and adaptation of human myotendinous junction and neighboring muscle fibers to heavy resistance training. *Scand. J. Med. Sci. Sport* 27:sms.12794. doi: 10.1111/sms.12794

Jamali, A. A., Afshar, P., Abrams, R. A., and Lieber, R. L. (2000). Skeletal muscle response to tenotomy. *Muscle Nerve* 23, 851–862.

Joe, A. W. B., Yi, L., Natarajan, A., Le Grand, F., So, L., Wang, J., et al. (2010). Muscle injury activates resident fibro/adipogenic progenitors that facilitate myogenesis. *Nat. Cell Biol.* 12, 153–163. doi: 10.1038/ncb2015

Kadi, F., and Thornell, L. E. (2000). Concomitant increases in myonuclear and satellite cell content in female trapezius muscle following strength training. *Histochem. Cell Biol.* 113, 99–103.

Kannus, P., Jozsa, L., Kvist, M., Lehto, M., and Järvinen, M. (1992). The effect of immobilization on myotendinous junction: an ultrastructural, histochemical and immunohistochemical study. *Acta Physiol. Scand.* 144, 387–394. doi: 10.1111/j.1748-1716.1992.tb09309.x

Knudsen, A. B., Larsen, M., Mackey, A. L., Hjort, M., Hansen, K. K., Qvortrup, K., et al. (2015). The human myotendinous junction: an ultrastructural and 3D analysis study. *Scand. J. Med. Sci. Sport.* 25, e116–e123. doi: 10.1111/sms.12221

Koch, M., Schulze, J., Hansen, U., Ashwodt, T., Keene, D. R., Brunken, W. J., et al. (2004). A novel marker of tissue junctions, collagen XXII. *J. Biol. Chem.* 279, 22514–22521. doi: 10.1074/jbc.M400536200

Kojima, H., Sakuma, E., Mabuchi, Y., Mizutani, J., Horiuchi, O., Wada, I., et al. (2008). Ultrastructural changes at the myotendinous junction induced by exercise. *J. Orthop. Sci.* 13, 233–239. doi: 10.1007/s00776-008-1211-0

Kovacs, E. J., and Dipietro, L. A. (1994). Fibrogenic cytokines and connective tissue production. *FASEB J.* 8, 854–861. doi: 10.1096/fasebj.8.11.7520879

Lieber, R. L., and Friden, J. (1993). Muscle damage is not a function of muscle force but active muscle strain. *J. Appl. Physiol.* 74, 520–526. doi: 10.1152/jappl.1993.74.2.520

Lieber, R. L., and Fridén, J. (2000). Functional and clinical significance of skeletal muscle architecture. *Muscle Nerve* 23, 1647–1666. doi: 10.1002/1097-4598(200011)23:11<1647::AID-MUS1<3.0.CO;2-M

Lynn, R., and Morgan, D. L. (1994). Decline running produces more sarcomeres in rat vastus intermedius muscle fibers than does incline running. *J. Appl. Physiol.* 77, 1439–1444. doi: 10.1152/jappl.1994.77.3.1439

Mair, S. D., Seaber, A. V., Glisson, R. R., and Garrett, W. E. (1996). The role of fatigue in susceptibility to acute muscle strain injury. *Am. J. Sports Med.* 24, 137–143. doi: 10.1177/036354659602400203

Medeiros, D. M., Marchiori, C., and Baroni, B. M. (2020). Effect of Nordic Hamstring Exercise Training on Knee Flexors Eccentric Strength and Fascicle Length: A Systematic Review and Meta-Analysis. *J. Sport Rehabil.* 2020, 1–10. doi: 10.1123/jsr.2019-0388

Mendiguchia, J., Alentorn-Geli, E., Idoate, F., and Myer, G. D. (2013). Rectus femoris muscle injuries in football: A clinically relevant review of mechanisms of injury, risk factors and preventive strategies. *Br. J. Sports Med.* 47, 359–366. doi: 10.1136/bjsports-2012-091250

Mjolsnes, R., Arnason, A., Osthagen, T., Raastad, T., and Bahr, R. (2004). A 10-week randomized trial comparing eccentric vs. concentric hamstring strength training in well-trained soccer players. *Scand J Med. Sci. Sport.* 14, 311–317. doi: 10.1046/j.1600-0838.2003.367.x

Morgan, D. L. (1990). New insights into the behavior of muscle during active lengthening. *Biophys. J.* 57, 209–221. doi: 10.1016/S0006-3495(90)82524-8

Morgan, D. L., Claflin, D. R., and Julian, F. J. (1991). Tension as a function of sarcomere length and velocity of shortening in single skeletal muscle fibres of the frog. *J. Physiol.* 441, 719–732. doi: 10.1113/jphysiol.1991.sp018775

Mueller-Wohlfahrt, H. W., Haensel, L., Mithoefer, K., Ekstrand, J., English, B., McNally, S., et al. (2013). Terminology and classification of muscle injuries in sport: The Munich consensus statement. *Br. J. Sports Med.* 47, 342–350. doi: 10.1136/bjsports-2012-091448

Murphy, M. M., Lawson, J. A., Mathew, S. J., Hutcheson, D. A., and Kardon, G. (2011). Satellite cells, connective tissue fibroblasts and their interactions are crucial for muscle regeneration. *Development* 138, 3625–3637. doi: 10.1242/dev.064162

Opar, D. A., Drezner, J., Shield, A., Williams, M., Webner, D., Sennett, B., et al. (2014). Acute hamstring strain injury in track-and-field athletes: A 3-year observational study at the Penn Relay Carnival. *Scand. J. Med. Sci. Sports* 24, e254–e259. doi: 10.1111/sms.12159

Opar, D. A., Williams, M. D., Timmins, R. G., Hickey, J., Duhig, S. J., and Shield, A. J. (2015). Eccentric hamstring strength and hamstring injury risk in Australian footballers. *Med. Sci. Sports Exerc.* 47, 857–865. doi: 10.1249/MSS.0000000000000465

Orchard, J., and Seward, H. (2002). Epidemiology of injuries in the Australian Football League, seasons 1997-2000. *Br. J. Sports Med.* 36, 39–44. doi: 10.1136/bjsm.36.1.39

Orchard, J. W., Alcott, E., James, T., Farhart, P., Portus, M., and Waugh, S. R. (2002). Exact moment of a gastrocnemius muscle strain captured on video. *Br. J. Sports Med.* 36, 222–223. doi: 10.1136/bjsm.36.3.222

Palma, L. D., Marinelli, M., Pavan, M., and Bertoni-Freddari, C. (2011). Involvement of the muscle-tendon junction in skeletal muscle atrophy: An ultrastructural study. *Rom. J. Morphol. Embryol.* 52, 105–109.

Pardo, J. V., D'Angelo Siliciano, J., and Craig, S. W. (1983). A vinculin-containing cortical lattice in skeletal muscle: Transverse lattice elements ('costameres') mark sites of attachment between myofibrils and sarcolemma. *Proc. Natl. Acad. Sci. U. S. A.* 80, 1008–1012. doi: 10.1073/pnas.80.4.1008

Passerieux, E., Rossignol, R., Chopard, A., Carnino, A., Marini, J. F., Letellier, T., et al. (2006). Structural organization of the perimysium in bovine skeletal muscle: Junctional plates and associated intracellular subdomains. *J. Struct. Biol.* 154, 206–216. doi: 10.1016/j.jsb.2006.01.002

Passerieux, E., Rossignol, R., Letellier, T., and Delage, J. P. (2007). Physical continuity of the perimysium from myofibers to tendons: involvement in lateral force transmission in skeletal muscle. *J. Struct. Biol.* 159, 19–28. doi: 10.1016/j.jsb.2007.01.022

Petersen, J. (2005). Evidence based prevention of hamstring injuries in sport. *Br. J. Sport. Med.* 39, 319–323. doi: 10.1136/bjsm.2005.018549

Petersen, J., Thorborg, K., Nielsen, M. B., Budtz-Jorgensen, E., and Holmich, P. (2011). Preventive effect of eccentric training on acute hamstring injuries in men's soccer: a cluster-randomized controlled trial. *Am. J. Sport Med.* 39, 2296–2303. doi: 10.1177/0363546511419277

Pimentel, N. J., Rocha, L. C., Barbosa, G. K., Jacob, C., dos, S., Krause, N. W., et al. (2020). Myotendinous junction adaptations to ladder-based resistance training: identification of a new telocyte niche. *Sci. Rep.* 10, 70971–70976. doi: 10.1038/s41598-020-70971-6

Pincheira, P. A., Boswell, M. A., Franchi, M. V., Delp, S. L., and Lichtwark, G. A. (2021). Biceps femoris long head sarcomere and fascicle length adaptations after three weeks of eccentric exercise training. *bioRxiv* 2021:427202. doi: 10.1101/2021.01.18.427202

Polgar, J., Johnson, M. A., Weightman, D., and Appleton, D. (1973). Data on fibre size in thirty-six human muscles. An autopsy study. *J. Neurol. Sci.* 19, 307–318. doi: 10.1016/0022-510X(73)90094-4

Pollard, C. W., Opar, D. A., Williams, M. D., Bourne, M. N., and Timmins, R. G. (2019). Razor hamstring curl and Nordic hamstring exercise architectural adaptations: Impact of exercise selection and intensity. *Scand. J. Med. Sci. Sports* 29, 706–715. doi: 10.1111/sms.13381

Presland, J. D., Timmins, R. G., Bourne, M. N., Williams, M. D., and Opar, D. A. (2018). The effect of Nordic hamstring exercise training volume on biceps femoris long head architectural adaptation. *Scand. J. Med. Sci. Sport.* 28, 1775–1783. doi: 10.1111/sms.13085

Ramaswamy, K. S., Palmer, M. L., van der Meulen, J. H., Renoux, A., Kostrominova, T. Y., Michele, D. E., et al. (2011). Lateral transmission of force is impaired in skeletal muscles of dystrophic mice and very old rats. *J. Physiol.* 589, 1195–1208. doi: 10.1113/jphysiol.2010.201921

Rassier, D. E., Haeger, R., De, F., and Leite, S. (2020). *Sarcomere length non-uniformities dictate force production along the descending limb of the force-length relation*. London: Royal Society. doi: 10.1098/rspb.2020.2133

Ribeiro-Alvares, J. B., Marques, V. B., Vaz, M. A., and Baroni, B. M. (2018). Four Weeks of Nordic Hamstring Exercise Reduce Muscle Injury Risk Factors in Young Adults. *J. Strength Cond. Res.* 32, 1254–1262. doi: 10.1519/JSC.0000000000001975

Rissatto Sierra, L., Fávaro, G., Cerri, B. R., Rocha, L. C., de Yokomizo, de Almeida, S. R., et al. (2018). Myotendinous junction plasticity in aged ovariectomized rats submitted to aquatic training. *Microsc. Res. Tech.* 81, 816–822. doi: 10.1002/jemt.23040

Rocha, L. C., Pimentel Neto, J., de Sant'Ana, J. S., Jacob, C., dos, S., Barbosa, G. K., et al. (2020). Repercussions on sarcomeres of the myotendinous junction and the myofibrillar type adaptations in response to different trainings on vertical ladder. *Microsc. Res. Tech.* 83, 1190–1197. doi: 10.1002/jemt.23510

Roffino, S., Carnino, A., Charpiot, P., and Marini, J. F. (1998). Increase in rat soleus myotendinous interface after a 14-d spaceflight. *C. R. Acad. Sci. III.* 321, 557–564. doi: 10.1016/s0764-4469(98)80457-5

Roffino, S., Carnino, A., Chopard, A., Mutin, M., and Marini, J. F. (2006). Structural remodeling of unweighted soleus myotendinous junction in monkey. *C R Biol.* 329, 172–179. doi: 10.1016/j.crvi.2006.01.008

Ronti, T., Lupattelli, G., and Mannarino, E. (2006). The endocrine function of adipose tissue: An update. *Clin. Endocrinol.* 64, 355–365. doi: 10.1111/j.1365-2265.2006.02474.x

Sambasivan, R., Yao, R., Kissenpfennig, A., van Wittenberghe, L., Paldi, A., Gayraud-Morel, B., et al. (2011). Pax7-expressing satellite cells are indispensable for adult skeletal muscle regeneration. *Development* 138, 3647–3656. doi: 10.1242/dev.067587

Schache, A. G., Dorn, T. W., Blanch, P. D., Brown, N. A. T., and Pandy, M. G. (2012). Mechanics of the human hamstring muscles during sprinting. *Med. Sci. Sports Exerc.* 44, 647–658. doi: 10.1249/MSS.0b013e318236a3d2

Schmidt, B. A., and Horsley, V. (2013). Intradermal adipocytes mediate fibroblast recruitment during skin wound healing. *Dev.* 140, 1517–1527. doi: 10.1242/dev.087593

Seagrave, R. A., Perez, L., McQueeney, S., Toby, E. B., Key, V., and Nelson, J. D. (2014). Preventive Effects of Eccentric Training on Acute Hamstring Muscle Injury in Professional Baseball. *Orthop. J. Sport. Med.* 2:2325967114535351. doi: 10.1177/2325967114535351

Serner, A., Mosler, A. B., Tol, J. L., Bahr, R., and Weir, A. (2019). Mechanisms of acute adductor longus injuries in male football players: A systematic visual video analysis. *Br. J. Sports Med.* 53, 158–164. doi: 10.1136/bjsports-2018-099246

Serner, A., Tol, J. L., Jomaah, N., Weir, A., Whiteley, R., Thorborg, K., et al. (2015). Diagnosis of Acute Groin Injuries: A Prospective Study of 110 Athletes. *Am. J. Sports Med.* 43, 1857–1864. doi: 10.1177/0363546515585123

Shear, C. R., and Bloch, R. J. (1985). Vinculin in subsarcolemmal densities in chicken skeletal muscle: localization and relationship to intracellular and extracellular structures. *J. Cell Biol.* 101, 240–256. doi: 10.1083/jcb.101.1.240

Siddle, J., Greig, M., Weaver, K., Page, R. M., Harper, D., and Brogden, C. M. (2019). Acute adaptations and subsequent preservation of strength and speed measures following a Nordic hamstring curl intervention: a randomised controlled trial. *J. Sports Sci.* 37, 911–920. doi: 10.1080/02640414.2018.1535786

St. Pierre, B. A., and Tidball, J. G. (1994). Differential response of macrophage subpopulations to soleus muscle reloading after rat hindlimb suspension. *J. Appl. Physiol.* 77, 290–297. doi: 10.1152/jappl.1994.77.1.290

Tidball, J. G. (1983). The geometry of actin filament-membrane associations can modify adhesive strength of the myotendinous junction. *Cell Motil.* 3, 439–447. doi: 10.1002/cm.970030512

Tidball, J. G. (1984). Myotendinous junction: Morphological changes and mechanical failure associated with muscle cell atrophy. *Exp. Mol. Pathol.* 40, 1–12. doi: 10.1016/0014-4800(84)90060-1

Tidball, J. G. (1991). Force transmission across muscle cell membranes. *J. Biomech.* 24(Suppl. 1), 43–52. doi: 10.1016/0021-9290(91)90376-x

Tidball, J. G., and Chan, M. (1989). Adhesive strength of single muscle cells to basement membrane at myotendinous junctions. *J. Appl. Physiol.* 67, 1063–1069. doi: 10.1152/jappl.1989.67.3.1063

Tidball, J. G., and Quan, D. M. (1992). Reduction in myotendinous junction surface area of rats subjected to 4-day spaceflight. *J. Appl. Physiol.* 73, 59–64.

Tidball, J. G., and Villalta, S. A. (2010). Regulatory interactions between muscle and the immune system during muscle regeneration. *Am. J. Physiol.* 298, R1173–R1187. doi: 10.1152/ajpregu.00735.2009

Timmins, R. G., Bourne, M. N., Shield, A. J., Williams, M. D., Lorenzen, C., and Opar, D. A. (2016). Short biceps femoris fascicles and eccentric knee flexor weakness increase the risk of hamstring injury in elite football (soccer): A prospective cohort study. *Br. J. Sports Med.* 50, 1524–1535. doi: 10.1136/bjsports-2015-095362

Toumi, H., F'guyer, S., and Best, T. M. (2006). The role of neutrophils in injury and repair following muscle stretch. *J. Anat.* 208, 459–470. doi: 10.1111/j.1469-7580.2006.00543.x

Trotter, J. A., and Baca, J. M. (1987). A stereological comparison of the muscle-tendon junctions of fast and slow fibers in the chicken. *Anat. Rec.* 218, 256–266. doi: 10.1002/ar.1092180306

Trotter, J. A., Samora, A., and Baca, J. (1985). Three-dimensional structure of the murine muscle-tendon junction. *Anat. Rec.* 213, 16–25. doi: 10.1002/ar.1092130104

Valdivia, M., Vega-Macaya, F., and Olguín, P. (2017). Mechanical control of myotendinous junction formation and tendon differentiation during development. *Front. Cell Dev. Biol.* 5:00026. doi: 10.3389/fcell.2017.00026

Van Der Horst, N., Smits, D. W., Petersen, J., Goedhart, E. A., and Backx, F. J. G. (2015). The Preventive Effect of the Nordic Hamstring Exercise on Hamstring Injuries in Amateur Soccer Players: A Randomized Controlled Trial. *Am. J. Sports Med.* 43, 1316–1323. doi: 10.1177/0363546515574057

Vettor, R., Milan, G., Franzin, C., Sanna, M., De Coppi, P., Rizzuto, R., et al. (2009). The origin of intermuscular adipose tissue and its pathophysiological implications. *Am. J. Physiol. Metab.* 297, E987–E998. doi: 10.1152/ajpendo.00229.2009

Wang, Y. X., and Rudnicki, M. A. (2012). Satellite cells, the engines of muscle repair. *Nat. Rev. Mol. Cell Biol.* 13, 127–133. doi: 10.1038/nrm3265

Whyte, E. F., Heneghan, B., Feely, K., Moran, K. A., and O'Connor, S. (2019). The Effect of Hip Extension and Nordic Hamstring Exercise Protocols on Hamstring Strength. *J. Strength Cond. Res.* 1, jsc.0000000000003220. doi: 10.1519/jsc.0000000000003220

Zamora, A. J., Carnino, A., Roffino, S., and Marini, J. F. (1995). Respective effects of hindlimb suspension, confinement and spaceflight on myotendinous junction ultrastructure. *Acta Astronaut.* 36, 693–706. doi: 10.1016/0094-5765(95)00159-X

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Physiology

ORIGINAL RESEARCH
published: 26 April 2021
doi: 10.3389/fphys.2021.649793

Oxidative Stress, Inflammation, and Activators of Mitochondrial Biogenesis: Tempol Targets in the Diaphragm Muscle of Exercise Trained-*mdx* Mice

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OPEN ACCESS

Edited by:
Anselmo Sigari Moriscot, University of São Paulo, Brazil

Reviewed by:
Jose R. Lopez, Mount Sinai Medical Center, United States
Edmar Zanoteli, University of São Paulo, Brazil

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Specialty section:
This article was submitted to Striated Muscle Physiology, a section of the journal Frontiers in Physiology

Received: *05 January 2021*
Accepted: *17 March 2021*
Published: *26 April 2021*

Citation:
Silva HNM, Covatti C, Rocha GL, Mizobuti DS, Mâncio RD, Hermes TA, Kido LA, Cagnon VHA, Pereira ECL and Minatel E (2021) Oxidative Stress, Inflammation, and Activators of Mitochondrial Biogenesis: Tempol Targets in the Diaphragm Muscle of Exercise Trained-mdx Mice. Front. Physiol. 12:649793. doi: 10.3389/fphys.2021.649793

The *mdx* mouse phenotype aggravated by chronic exercise on a treadmill makes this murine model more reliable for the study of muscular dystrophy. Thus, to better assess the Tempol effect on dystrophic pathways, the analyses in this study were performed in the blood samples and diaphragm muscle from treadmill trained adult (7–11-weeks old) *mdx* animals. The m*dx* mice were divided into three groups: *mdx*Sed, sedentary controls ($n = 28$); *mdx*Ex, exercise-trained animals ($n = 28$); and *mdx*Ex+T, exercise-trained animals with the Tempol treatment ($n = 28$). The results demonstrated that the Tempol treatment promoted muscle strength gain, prevented muscle damage, reduced the inflammatory process, oxidative stress, and angiogenesis regulator, and up regulated the activators of mitochondrial biogenesis. The main new findings of this study are that Tempol reduced the NF-κB and increased the PGC1-α and PPARδ levels in the exercise-trained-*mdx* mice, which are probably related to the ability of this antioxidant to scavenge excessive ROS. These results reinforce the use of Tempol as a potential therapeutic strategy in DMD.

Keywords: tempol, dystrophic muscles, exercise, oxidative stress, inflammatory process

INTRODUCTION

Duchenne muscular dystrophy (DMD) represents one of the most devastating types of muscular dystrophies, which affects one in 3,000–6,000 male children (Choi et al., 2016). DMD is an X-linked recessive genetic disease caused by a mutation in the dystrophin gene (Andrews and Wahl, 2018), for which to date, there is no cure.

Previous work showed that DMD is a multifactorial disease, whereby inflammation, mitochondrial dysfunction, altered angiogenesis, and oxidative stress are among the main promoters of the dystrophic features (Guiraud and Davies, 2017). Regarding oxidative stress, our research group has evaluated the effects of some antioxidant drugs on the dystrophic muscle of *mdx* mice, which was the experimental DMD model (Tonon et al., 2012; De Senzi et al., 2013;

Mâncio et al., 2017a). Recently, we demonstrated that Tempol (4-hydroxy-2,2,6,6-tetramethylpiperidine-N-oxyl), a synthetic antioxidant that mimics the role of superoxide dismutase (SOD), improved the dystrophic phenotype (reducing myonecrosis and the inflammatory process) (Hermes et al., 2019) and contributed to the normalization of the redox homeostasis (Hermes et al., 2020) in young *mdx* mice during the acute dystrophic disease phase (about three weeks post-natal age). In addition, Burns et al. (2017) also reported that Tempol supplementation restores the diaphragm force and metabolic enzyme activities in adult *mdx* mice.

The present study further addresses the potential benefit of Tempol for dystrophy by evaluating the effects of this antioxidant in the diaphragm muscle in treadmill trained adult (7–11-weeks-old) *mdx* animals. Although the *mdx* mice lack dystrophin, similar to dystrophic DMD patients, these animals develop a milder form of dystrophy (Yucel et al., 2018). Thus, to get a more reliable animal model of the human dystrophy, the murine phenotype has been worsened by chronic exercise on a treadmill (Granchelli et al., 2000; De Luca et al., 2003). We carried out one of the most used protocols to worsen the murine phenotype based on the Treat-NMD Neuromuscular Network UK, 2018 for exercise in *mdx* mice. This consists of the *mdx* mice undergoing a 12 m/min exercise for 30 minutes twice a week. Under these conditions, the *mdx* muscle has often shown functional impairment indicated by the decline in grip strength, histopathological features of a worsening of the injury, and even a decrease of genes usually expressed in response to exercise (Camerino et al., 2014; Hyzewicz et al., 2015) enabling potential therapeutic interventions to be evaluated more rigorously throughout the *in vivo* treatment.

Consistent with the above actions of Tempol on the dystrophic muscle, we verified its potential pathways and effects on muscle damage, oxidative stress, inflammatory process, angiogenesis, and mitochondrial biogenesis in the blood samples and diaphragm muscle from adult exercised *mdx* mice.

MATERIALS AND METHODS

Animals

Male m*dx* (C57BL/10-Dmdmdx/PasUnib) mice were used in all the procedures, which were previously approved by the Ethics Committee on the Use of Animals (CEUA) of the State University of Campinas (UNICAMP; #4847-1). The animals were housed and cared for following the guidelines of the Brazilian College for Animal Experimentation (COBEA). Mice chow and water were provided *ad libitum* and the animals were kept in a temperature-controlled room (25°C ± 0.5) with relative humidity (55% ± 1) and 12-h light/dark cycles.

Experimental Design

Male m*dx* mice (seven weeks old; the age chosen was based on the previous exercise protocols) (Radley-Crabb et al., 2012; Hyzewicz et al., 2015) were randomly assigned into three groups: *mdxSed*, sedentary controls (n = 28); *mdx*Ex, exercise-trained animals (n = 28); and *mdx*Ex+T, exercise-trained animals with the Tempol treatment (n = 28).

The exercise protocol was based on the previous research (Radley-Crabb et al., 2012; Hyzewicz et al., 2015) using the TREAT-NMD protocols for exercise in *mdx* mice. After the five days of acclimatization, the *mdx* mice were trained for four weeks with progressively increasing loads (**Figure 1**). The protocol consisted of a treadmill exercise regimen of 30 min of treadmill running at a speed of 12 m/min twice a week for four weeks (keeping a constant interval of 2–3 days between each trial).

Tempol treatment began after the acclimatization period and continued daily until the day of euthanasia (**Figure 1**). The Tempol dose (100 mg/kg) dissolved in 100 μL of saline solution was administered intraperitoneally, according to our previous studies (Hermes et al., 2019; Hermes et al., 2020).

Twenty-four hours after the last training session, the animals were euthanized using a mixture of ketamine hydrochloride (130 mg/kg; Franco tar, Virbac, Fort Worth, TX, United States) and xylazine hydrochloride (6.8 mg/kg, 2% Virbaxil; Virbac). The blood samples were collected by cardiac puncture and the diaphragm (DIA) muscle was removed.

Grip Strength Evaluation (n = 7 Per Group)

Forelimb muscle strength was evaluated with a grip strength meter (New Primer, São Paulo, Brazil), as previously described (Hermes et al., 2019). The measurements were obtained for each animal at the beginning and at the end of the experimental period and the absolute strength was normalized to body weight.

Blood Samples for the Biochemical Assessment of Muscle Fiber Degeneration

For the creatine kinase (CK) assay, the blood samples (n = 7 per group) were micro-centrifuged in conical microtubes at 3,000 rpm for 10 min, and the supernatant (serum) was removed and used for analysis after incubation at room temperature for one to two hours to allow clotting. The CK assay was carried out using a commercially available kit (CK Cinetico Crystal, BioClin, Ireland) and a BioTek Spectrophotometer (BioTek Instruments Inc., Winooski, VT, United States). Values are reported as international units per liter.

Histopathological Analysis (n = 7 Per Group)

Regenerated Muscle Fiber and Diameter Analyses

For the morphological analysis of the regenerated muscle fibers (central nucleated fibers) and normal fibers (peripheral nucleated fibers), cryosections of the DIA muscles were stained with hematoxylin/eosin. Slides were examined under a Nikon Eclipse TS100 microscope which is connected to a computer and a video camera (Nikon DS-Ri1). Non overlapping images of the entire cross-section were taken and tiled together using the NIS-elements AR Advances Research software (Nikon Instruments Inc., Melville, NY, United States) as described

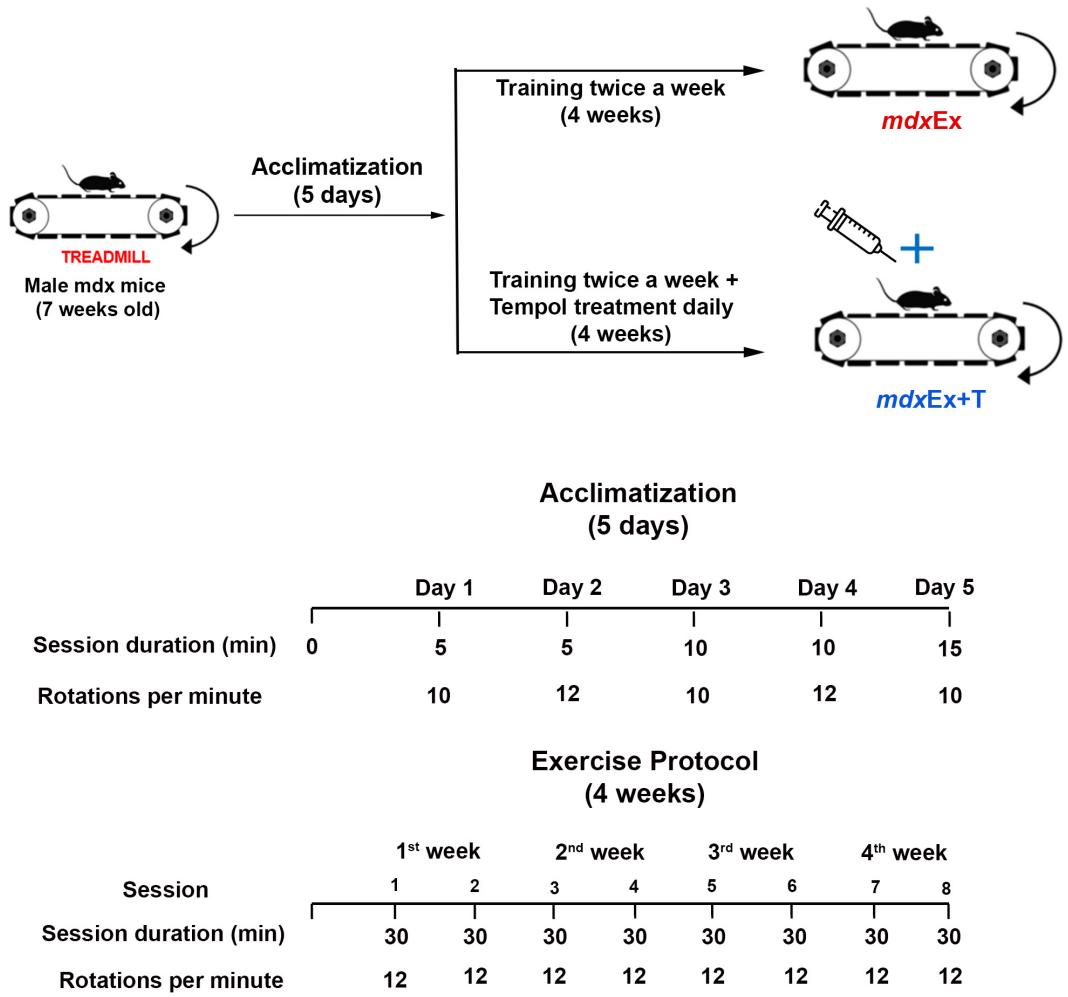

FIGURE 1 | Exercise protocol and Tempol treatment.

previously (Mâncio et al., 2017b). All the fibers of the cuts (normal and regenerated fibers) were counted to estimate the total population of the DIA muscle fibers. This allowed the percentage of normal and regenerated fibers of the studied animals to be obtained.

Minimum "Feret's Diameter" Analyses

Feret's diameter, the minimum distance of parallel tangents at opposing borders of the muscle fiber (Briguet et al., 2004), was analyzed by quantifying a total of a hundred muscle fiber diameter from 10 random fields at 20X magnification using the Image-Pro Express software (Media Cybernetic, Silver Spring, MD, United States). The results are shown as the mean ± SD.

Inflammatory Area

Inflammatory areas were characterized in hematoxylin/eosin stained serial cryosections (8 μm) of the DIA muscle based on the nucleus morphology and cell size and quantified as described previously (Hermes et al., 2019). Inflammatory cells are characterized by a poor cytoplasm and basophilic nuclear staining. Areas of inflammation and total muscle area were delimited. To limit these areas, the images were captured by light microscopy (Nikon Eclipse TS100) which is connected to a Nikon camcorder at 20X magnification and to a computer with a NIS-elements software AR Advances Researches. Areas of inflammation were expressed as a percentage of the total area. A blinded observer carried out the counts and measurements.

Macrophage Infiltration

F4/80 staining was used for macrophage infiltration quantification. Serial cryosections (8 μm) of the DIA muscle were fixed in acetone for 10 min, air-dried for 20 min, and washed with PBS. Sections were blocked for one hour at room temperature with 3% BSA in PBS. The histological slides were incubated overnight at 4°C with a primary antibody against F4/80, (1:200, monoclonal antibody; AbD Serotec, Raleigh, NC, United States). After washing with PBS, the slides were incubated with an anti-rat secondary antibody (1:250, Texas Red® Anti-rat IgG; Vector Laboratories, Burlingame, CA, United States) for one hour at room temperature. After

another washing with PBS, the muscle sections were mounted in 1,4-diazabicyclo[2.2.2]octane (DABCO; Sigma) mounting medium for fluorescence microscopy. The images of F4/80 staining were captured by light microscopy (Nikon Eclipse TS100) which is connected to a Nikon camcorder at 20X magnification and to a computer with a NIS-elements software AR Advances Researches. Macrophage infiltration areas (macrophages grouped in areas or dispersed by tissue) and total muscle area were delimited. Areas of macrophage infiltration were expressed as a percentage of the total area. A blinded observer carried out the counts and measurements.

Histopathological Oxidative Stress

For the quantification of the number of autofluorescent granules of lipofuscin, muscle samples were analyzed using serial cryosections unfixed (8 μm) of the DIA muscle. Quantification was performed in a fluorescent inverted microscope (Nikon, Eclipse TS100/TS100F) using the NIS-elements software AR Advances Researches in each cross-section (five cross-sectional areas per muscle).

Serial DIA cryosections (8 μm) were incubated with DHE (5 μl) to the quantification of the ROS levels. DHE staining presents a bright red emission in fluorescence microscopy. The intensity of reactive DHE by muscle area was quantified in a fluorescent inverted microscope (Nikon, Eclipse TS100) by measuring the pixels in a specific range (70 ± 255 wavelength). The equipment was adjusted to eliminate interference from background fluorescence.

Western Blot Analysis (n = 7 Per Group)

Muscle samples were homogenized immediately after removal in 2 ml of lysis buffer (Tris–HCl, 100 mM, pH 7.5; EDTA, 10 mM, pH 8.0; sodium pyrophosphate, 10 mM; sodium fluoride, 0.1 mM; sodium orthovanadate, 10 mM; PMSF, 2 mM; and aprotinin, 10 μg/ml) using the Polytron PTA 20S homogenizer (model PT 10/35; Kinematica Ag) operated at maximum speed for 30 seconds. Muscle samples detritus were removed by centrifugation at 11,000 rpm for 20 min at 4°C and the cleared lysate was subjected to SDS-Page gel electrophoresis. The Bradford method was used to determine the total protein content. Total protein from the muscle sample lysate (30 μg) was stacked on 12% SDS-polyacrylamide gels.

The proteins were transferred from gels to nitrocellulose membranes by electrophoresis (Mini Trans-Blot® electrophoretic transfer cell - Bio-Rad). A blocking buffer was used in all membranes for two hours at room temperature. Membranes were incubated with appropriate primary antibodies overnight at 4°C with gentle shaking. The following primary antibodies were used for Western blotting: 4-HNE (1:3,000, AHP1251, goat polyclonal, Bio-RAD, Hercules, CA, United States), anti-catalase (1:1,000, C0979, mouse monoclonal, Sigma-Aldrich, St. Louis, MO, United States), anti-SOD-2 (0,1:1,000, SAB 2501676, goat polyclonal, Sigma-Aldrich, St. Louis, MO, United States), anti-GSR (1:1,000, SAB2108147, rabbit, Sigma-Aldrich, St. Louis, MO, United States), anti-GPx1 (1:1,000, SAB2502102, goat polyclonal, Sigma-Aldrich, St. Louis, MO, United States), TNF-α (0,2:1,000, AAM19GA, rabbit anti-mouse polyclonal antibody; BIO-RAD, Hercules, CA, United States), NFkB p65 (1:1,000, AHP1342, rabbit polyclonal, BIO-RAD, Hercules, CA, United States), VEGF (1:200, sc53462, mouse monoclonal antibody; Santa Cruz Biotechnology), PGC-1α (1:1,000, ST1202, mouse monoclonal, Cambridge, United Kingdom), PPARδ (1:250, PA1-823A, rabbit polyclonal antibody, Invitrogen, CA, United States), Oxphos (6:1,000, ab110413, antibody cocktail, Abcam, Cambridge, United Kingdom), and β-actin (1:1,000, A1978, mouse monoclonal, Sigma-Aldrich, St. Louis, MO, United States).

Peroxidase-conjugated secondary antibodies from mice (1:2,500, 04-18-06, KPL, United States), goats (1:1,000, 14-13-06, KPL, United States), or rabbits (1:2,500, 04-15-06, KPL, United States) were used to incubate the membranes for two hours at room temperature. Membranes were washed 3 × 10 min with TBST after both incubations. Anti-β-Actin antibody was used as a control protein loading. All membranes were revealed using the Clarity Western ECL Substrate (Bio-Rad). Gene Tools from Syngene was used for bands intensity quantification.

Glutathione (GSH) Content (n = 7 Per Group)

Total GSH content was determined by Ellman's reaction using 5′5′-dithio-bis-2-nitrobenzoic acid (DTNB) as described by Anderson (1985). The intensity of the yellow color was read at 412 nm. The results were expressed as nmol per mg of protein.

TABLE 1 | Body weights, forelimb muscle strength, and CK levels.

	Body weight (g)		Body weight/período	Force/Body weight (g/g)		Force gain/period	Creatine Kinase levels
	Start	End	(%)	Start	End	(%)	(U/L)
mdxSed	24.77 ± 1.43	28.16 ± 2.45	13.68	0.75 ± 0.05	0.77 ± 0.06	2.59	4,184 ± 3,442
*mdx*Ex	24.69 ± 1.24	27.67 ± 1.48	12.06	0.78 ± 0.17	0.55 ± 0.09***	−41.81	11,460 ± 1,149**
*mdx*Ex+T	24.24 ± 1.54	27.23 ± 2.18	12.33	0.72 ± 0.13	0.77 ± 0.11###	6.49	4,855 ± 1,756##

*Body weight (g) was measured at the beginning (Start) and after four weeks (End) of the exercise and Tempol treatment. Forelimb muscle strength was assessed by taking measurements of force at time points Start and End, norm alized by body weight (g/g). Gain force/period: percentage of muscular force gain during the treatment period. Creatine kinase levels were measured at the end of the experiment. Experimental groups: sedentary mdx mice (mdxSed), exercise trained mdx mice (mdxEx), and exercise trained mdx mice treated with Tempol (mdxEx+T). All values are shown as the mean ± standard deviation (SD) for 10 animals. **P ≤ 0.001 compared with the mdxSed group, ***P ≤ 0.0001 compared with the mdxSed group, ##P ≤ 0.001 compared with the mdxEx group, ###P ≤ 0.0001 compared with the mdxEx group (one-way .ANOVA with Tukey's post hoc test).*

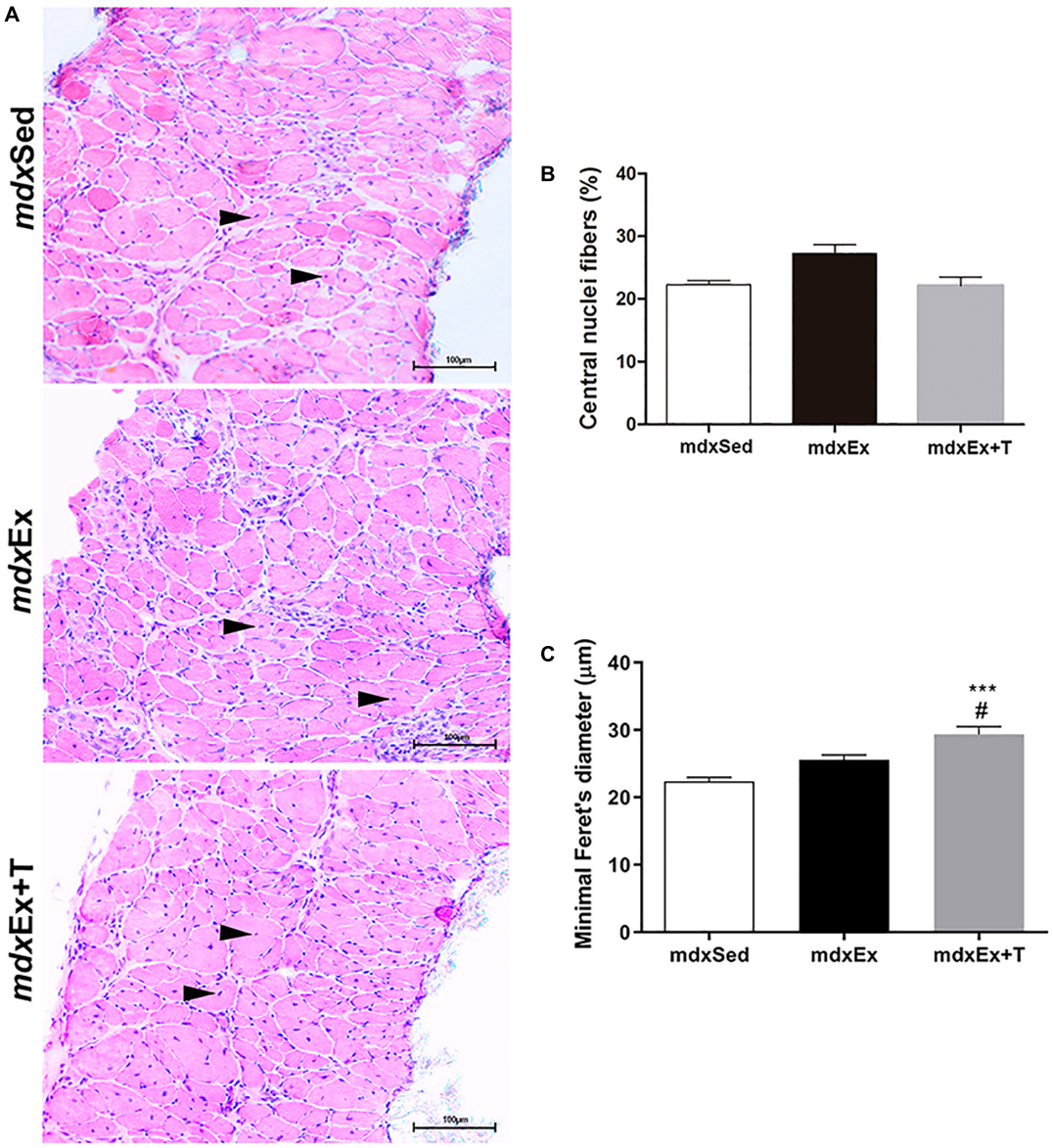

FIGURE 2 | Tempol effects on the regenerative muscular process. **(A)** Diaphragm (DIA) cross-sections showing fibers with central nuclei (black arrowheads) in the sedentary *mdx* mice *(mdx*Sed), exercise trained *mdx* mice (*mdx*Ex), and exercise trained *mdx* mice treated with Tempol (*mdx*Ex+T). **(B)** Graphs show the nuclei central fibers (%) in the DIA muscles of the *mdx*Sed, *mdx*Ex, and *mdx*Ex+T groups. **(C)** Graphs show the cross-sectional size (Minimum "Feret's diameter; μm) of the fibers with central nuclei in the DIA muscles of the *mdx*Sed, *mdx*Ex, and *mdx*Ex+T groups. All values expressed as mean ± standard deviation (SD). $^{***}P \leq 0.0001$ compared with the *mdx*Sed group, $^{\#}P \leq 0.05$ compared with the *mdx*Ex group (one-way ANOVA with Tukey's *post hoc* test).

Gene Expression (mRNA) by Real-Time qPCR (*n* = 7 Per Group)

Total RNA from the *mdx* muscular tissue was extracted using the Trizol reagent according to the manufacturer's instruction. RNA quantification was performed by spectrophotometry (260/280 nm). Synthesis of complementary DNA (cDNA) was performed from total RNA (2 μg) using the High-Capacity cDNA Kit (ThermoFisher). Gene expression was quantified by quantitative real-time PCR (ABI 7500 One Step quantitative PCR system–Applied Biosystems, CA, United States). The reactions were carried out in a mixture (20 μL) containing a sample of cDNA (2 μL), primers (300 nM), DEPC water, and SYBR Green PCR Master Mix (10 μL, Invitrogen). Gene expression levels were analyzed according to the ΔΔCT method (Livak and Schmittgen, 2001). qPCR analysis was performed in duplicate per sample on a plate. RPL39 was used as a reference gene. We used primers (Exxtend, Oligo Solutions, SP, Brazil) for Manganese Superoxide Dismutase (SOD 1; F: 5′-GCGGTGAACCAGTTGTGTTG-3′; R: 5′-CTGCACTGGTACAGCCTTGT′3′), Catalase (CAT; F: 5′-CTCGCAGAGACCTGATGTCC-3′; R: 5′-GACCCCGCGGTCA

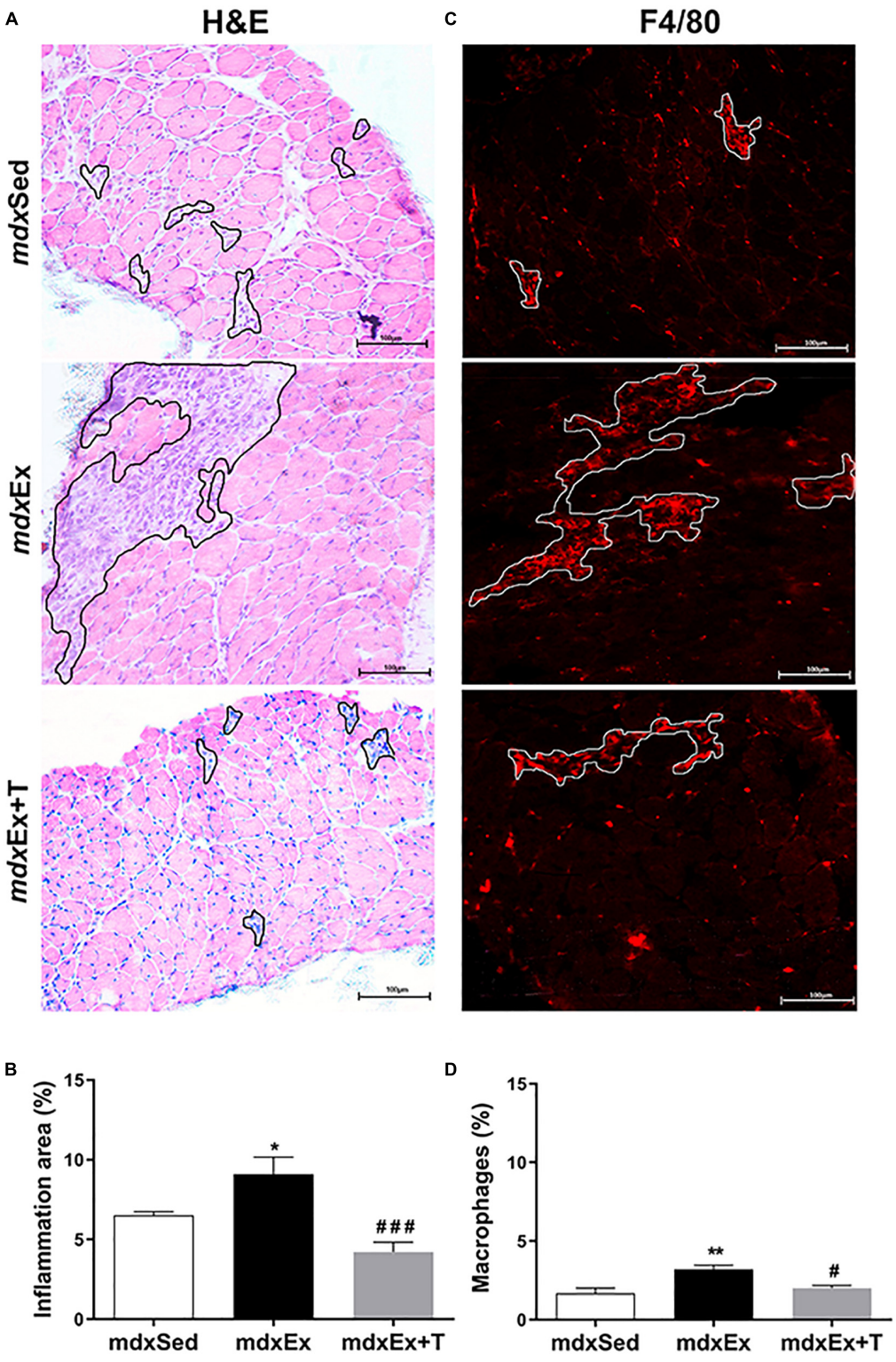

FIGURE 3 | Tempol effects on the inflammatory response morphology. **(A)** The outline indicates the representative area of inflammation in the diaphragm (DIA) of the sedentary *mdx* mice *(mdx*Sed), exercise trained *mdx* mice (*mdx*Ex), and exercise trained *mdx* mice treated with Tempol (*mdx*Ex+T). **(B)** Graphs show the inflammatory area (%) in the DIA muscles of the *mdx*Sed, *mdx*Ex, and *mdx*Ex+T groups. **(C)** The outline indicates the representative area of macrophage infiltration determined by F4/80 immunohistochemistry. **(D)** Graphs show the macrophage infiltration area (%) in the DIA muscles of the *mdx*Sed, *mdx*Ex, and *mdx*Ex+T groups. All values expressed as mean ± standard deviation (SD). $^{*}P \leq 0.05$ compared with the *mdx*Sed group, $^{**}P \leq 0.001$ compared with the *mdx*Sed group, $^{\#}P \leq 0.05$ compared with the *mdx*Ex group, $^{\#\#\#}P \leq 0.0001$ compared with the *mdx*Ex group (one-way ANOVA with Tukey's *post hoc* test).

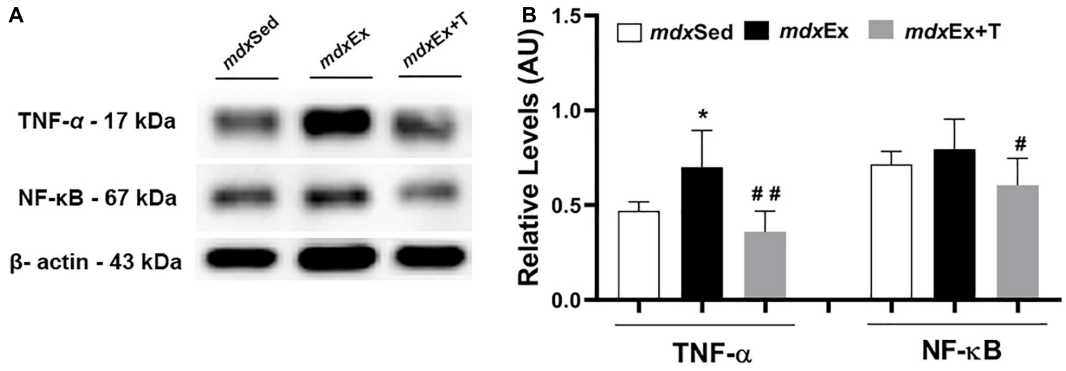

FIGURE 4 | Tempol effects on the inflammatory markers. **(A)** Western blotting analysis of tumor necrosis factor alpha (TNF-α) and nuclear factor kappa B (NF-κB). The blots of the proteins (top row) and β-actin (loading control, bottom row) are shown. **(B)** The graphs show the protein levels in the crude extracts of diaphragm (DIA) from the sedentary *mdx* mice *(mdx*Sed), exercise trained *mdx* mice (*mdx*Ex), and exercise trained *mdx* mice treated with Tempol (*mdx*Ex+T). The intensities of each band were quantified and normalized to those of the corresponding control. Relative values are expressed as mean ± standard deviation (SD). $^{*}P \leq 0.05$ compared with the *mdx*Sed group, $^{\#}P \leq 0.05$ compared with the *mdx*Ex group, $^{\#\#}P \leq 0.001$ compared with the *mdx*Ex group (one-way ANOVA with Tukey's *post hoc* test).

TGATATT-3′), Glutathione Peroxidase 1 (GPx1; F: 5′-TCCA GTATGTGTGCTGCTCAT-3′; R: 5′-TTCATCTCGGTGTAGTC CCG-3′), Glutathione-Disulfide Reductase (GSR, F: 5′-GG GGCTCACTGAAGACGAAG-3′; R: 5′-TCACAGCGTGATA CATCGGG-3′), and Ribosomal Protein L39 (RPL39, F: 5′-C AAAATCGCCCTATTCCTCA-3′; R: 5′-AGACCCAGCTT CGTTCTCCT-3′).

Statistical Analysis

All data are expressed as mean ± standard deviation (SD). Statistical analysis for direct comparison between the means of groups was performed by ANOVA, followed by the Tukey test which was used for multiple statistical comparisons between groups. The normal distribution of the data was assessed by the Shapiro-Wilk normality test. A $p \leq 0.05$ was considered statistically significant. We used the GraphPad Prims5 software package (GraphPad Software, CA, United States).

RESULTS

Tempol Effects on Body Weight, Forelimb Muscle Strength, and Degeneration/Regeneration Muscular Process

There was no statistical difference in the weight of the animals during the experiment (**Table 1**). The *mdx*Ex group showed a significant reduction in muscle strength over the study period (**Table 1**). The *mdx*Ex+T group presented strength gain in the period, which was significantly different from the *mdx*Ex group (**Table 1**).

The biochemical evaluation of the blood samples for CK levels showed a significant increase of this enzyme in the *mdx*Ex group (by 63.5%) compared to the *mdx*Sed group (**Table 1**). Concomitantly, there is a reduction of CK levels in the *mdx*Ex+T group (by 57.6%) compared to the *mdx*Ex group (**Table 1**).

The morphological evaluation of muscle fiber regeneration in the DIA muscles, based on the number of fibers with central nuclei, did not present a significant statistical difference between the analyzed groups (**Figures 2A,B**). Feret's diameter analyses showed a significant increase in the diameter of the fibers with central nuclei in the *mdx*Ex+T group compared to the *mdx*Ex group (**Figure 2C**).

Tempol Effects on the Inflammatory Process

The inflammatory process in the DIA muscle was evaluated by determining the inflammatory area, macrophage infiltration, NF-κB, and TNF-α content.

The inflammatory area showed conspicuous regions containing inflammatory cells, which were densely packed among the muscle fibers (**Figure 3A**). The *mdx*Ex group showed an expressive inflammatory area (by 29.2%) compared to the *mdx*Sed group (**Figure 2B**). The diaphragm muscles of the *mdx*Ex+T group presented a significant reduction in the inflammatory area (by 54.7%) compared to the *mdx*Ex group (**Figure 3B**).

In terms of macrophage infiltration, the training-*mdx* mice showed a higher proportion (by 47.7%) compared to the *mdx*Sed group mice (**Figures 3C,D**). The *mdx*Ex+T group showed a significant reduction in macrophage infiltration (by 37.8%) compared to the *mdx*Ex group (**Figures 3C,D**).

Immunoblotting revealed a significant increase in the TNF-α levels in the *mdx*Ex group (by 33 and 32.8%, respectively) compared to the *mdx*Sed group (**Figures 4A,B**). The *mdx*Ex+T group presented a significant decrease in the TNF-α and NF-κB levels (by 49, 24, and 41%, respectively) compared to the *mdx*Ex group (**Figures 4A,B**).

Tempol Effects on Oxidative Stress

The DIA muscles of the *mdx*Ex group showed a significant increase in the oxidative stress markers, such as the DHE

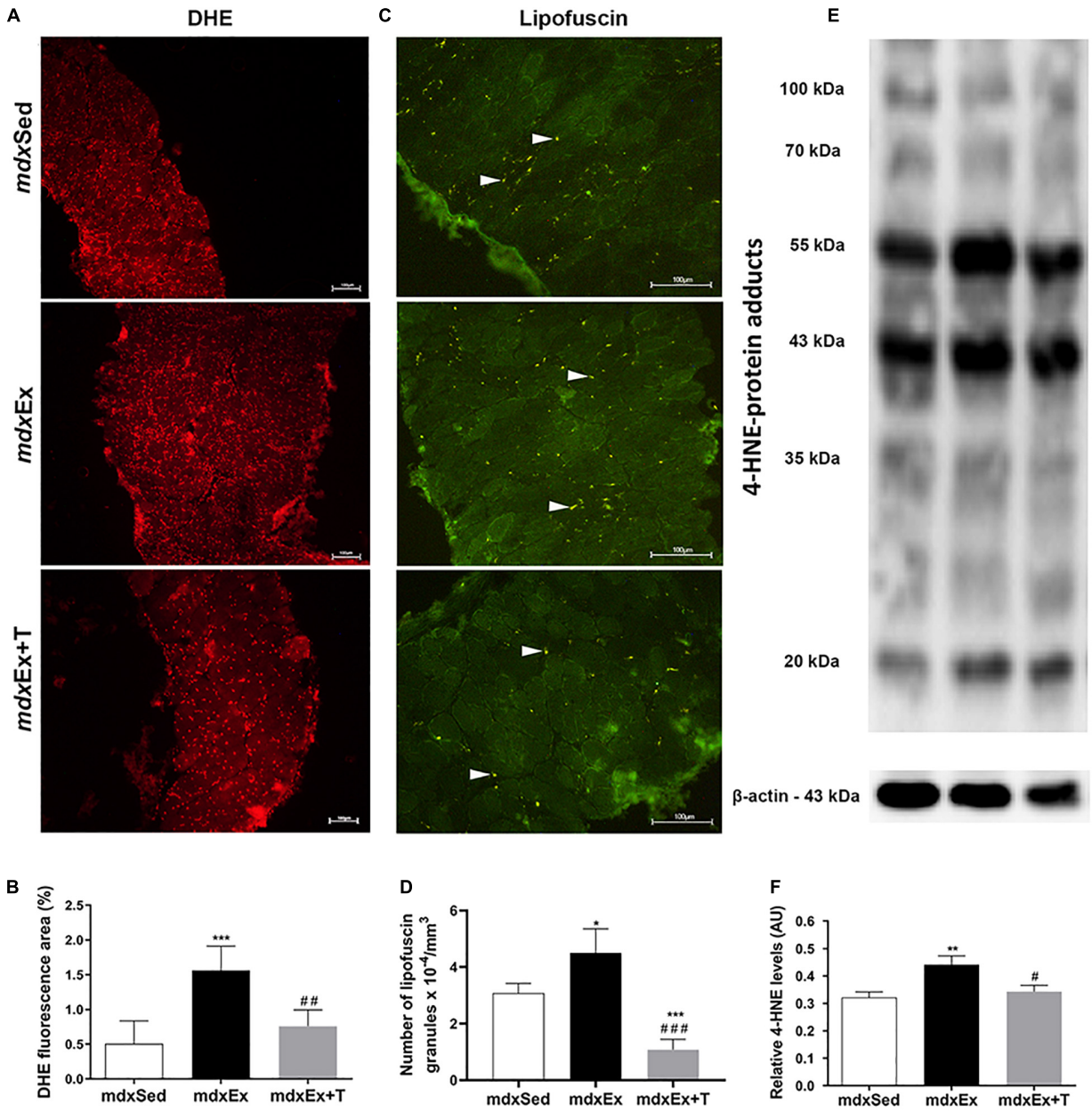

FIGURE 5 | Tempol effects on the oxidative stress markers. **(A)** Diaphragm (DIA) cross-sections showing dihydroethidium (DHE) fluorescence in sedentary *mdx* mice (*mdx*Sed), exercise trained *mdx* mice (*mdx*Ex), and exercise trained *mdx* mice treated with Tempol (*mdx*Ex+T). **(B)** The graphs show the DHE staining area (%) in DIA muscles of the *mdx*Sed, *mdx*Ex, and *mdx*Ex+T groups. **(C)** DIA cross-sections showing autofluorescent lipofuscin granules (white arrowheads) in DIA muscles of the *mdx*Sed, *mdx*Ex, and *mdx*Ex+T groups. **(D)** The graphs show the number of lipofuscin granules x 10^{-4}/mm^3 in DIA muscles of the *mdx*Sed, *mdx*Ex, and *mdx*Ex+T groups. **(E)** Western blotting analysis of 4-hydroxynonenal (4-HNE)-protein adducts. Bands corresponding to protein (top row) and β-actin (used as loading control) (bottom row) are shown. **(F)** The graphs show the protein levels in the crude extracts of DIA muscle from the *mdx*Sed, *mdx*Ex, and *mdx*Ex+T groups. All values expressed as mean ± standard deviation (SD). $^{*}P \leq 0.05$ compared with the *mdx*Sed group, $^{**}P \leq 0.001$ compared with the *mdx*Sed group, $^{***}P \leq 0.0001$ compared with the *mdx*Sed group, $^{\#}P \leq 0.05$ compared with the *mdx*Ex group, $^{\#\#}P \leq 0.001$ compared with the *mdx*Ex group, $^{\#\#\#}P \leq 0.0001$ compared with the *mdx*Ex group (one-way ANOVA with Tukey's *post hoc* test).

area, autofluorescent lipofuscin granules, and 4-HNE protein adduct levels (by 67.6, 29, and 27%, respectively) compared to the *mdx*Sed group (**Figures 5A–F**). The Tempol treated group presented a reduction of the oxidative stress markers by significantly decreasing the DHE area (51.5%), autofluorescent lipofuscin granules (69.4%), and 4-HNE protein adduct levels (22.4%) in the diaphragm muscle of the *mdx*Ex+T group (**Figures 5A–F**).

The effect of the Tempol treatment on catalase, SOD2, GPx1, GR, and GSH immunoblots levels is shown in **Figures 6A–C**.

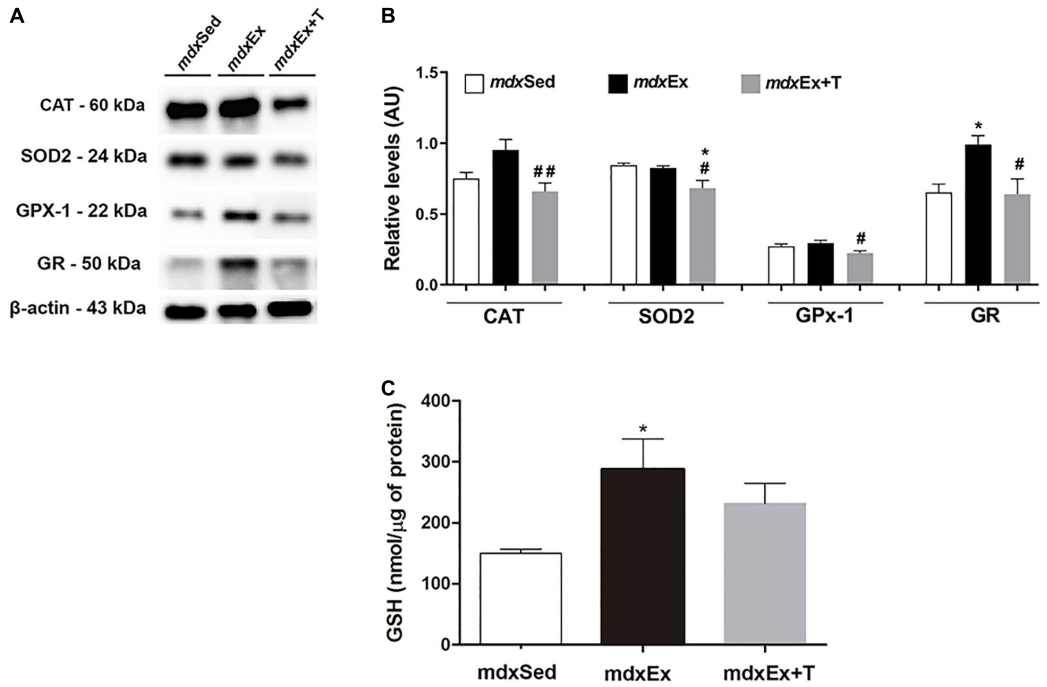

FIGURE 6 | Tempol effects on the antioxidant system levels. **(A)** Western blotting analysis of catalase (CAT), manganese-superoxide dismutase (SOD2), glutathione peroxidase (GPx1), and glutathione reductase (GR) content in the diaphragm (DIA) muscle from the sedentary *mdx* mice *(mdx*Sed), exercise trained *mdx* mice (*mdx*Ex), and exercise trained *mdx* mice treated with Tempol (*mdx*Ex+T). The blots of the proteins (top row) and β-actin (loading control, bottom row) are shown. **(B)** The graphs show the protein levels in the crude extracts of DIA from the *mdx*Sed, *mdx*Ex, and *mdx*Ex+T groups. The intensities of each band were quantified and normalized to those of the corresponding control. **(C)** glutathione content of the DIA muscle from the *mdx*Sed, *mdx*Ex, and *mdx*Ex+T groups. Relative values are expressed as mean ± standard deviation (SD). $^{*}P \leq 0.05$ compared with the *mdx*Sed group, $^{\#}P \leq 0.05$ compared with the *mdx*Ex group, $^{\#\#}P \leq 0.001$ compared with the *mdx*Ex group (one-way ANOVA with Tukey's *post hoc* test).

The reduction of catalase, SOD2, and GPx1 levels (by 31.6, 15.6, and 24.9%, respectively) in the *mdx*Ex+T group was found to be significant when compared with the *mdx*Ex group (**Figures 6A,B**). The GR and GSH levels significantly increased (by 37.8 and 47.8%, respectively) in the *mdx*Ex group (**Figures 6B,C**) compared to the *mdx*Sed group. The *mdx*Ex+T group showed a significant decrease in the GR levels (by 35.2%) compared to the *mdx*Ex group (**Figures 6A,B**).

The effect of the Tempol treatment on the enzymatic antioxidant defense system gene expression is shown in **Figures 7A–D**. A significant increase in the gene expression of SOD1, CAT, and GPx was observed (by 72.5, 49.6%, and 70%, respectively) in the *mdx*Ex group when compared to the *mdx*Sed group (**Figures 7A–C**). In the *mdx*Ex+T group, there was a significant decrease in the SOD1, CAT, and GPx gene expression (by 50.1, 54%, and 49.9%, respectively) compared to the *mdx*Ex group (**Figures 7A–C**). There was no change in the GR gene expression between the experimental groups (**Figure 7D**).

Tempol Effects on the VEGF Levels

The DIA muscles of the *mdx*Ex group showed a significant increase in the VEGF levels (by 14.1%) compared to the *mdx*Sed group (**Figure 8**). A decrease of the VEGF levels (by 27.1%) were observed in the *mdx*Ex+T group compared to the *mdx*Ex group (**Figure 8**).

Tempol Effects on the Activators of Mitochondrial Biogenesis and Oxidative Phosphorylation

An increase in the PPARδ and PGC-1α levels was observed in the *mdx*Ex+T group compared to the *mdx*Sed (by 29.1 and 42.3%, respectively) and *mdx*Ex groups (by 25.8 and 40.0%, respectively) (**Figures 9A,B**).

The DIA muscles of the *mdx*Ex group showed a significant decrease in the OXPHOS levels (complex V, III, II, and I) compared to the *mdx*Sed group (**Figures 9C,D**). An increase in the OXPHOS levels (complex V, III, II, and I) was observed in the *mdx*Ex+T group compared to the *mdx*Ex group (by 12.6, 11.01, 16.1, and 13.9%, respectively) (**Figures 9C,D**).

DISCUSSION

M*dx* mice are the most widely used model to study DMD and test potential therapies, but their phenotype is milder than that of the dystrophic patient, thus, limiting the range of histopathological parameters and molecular changes that can be measured in pre-clinical drug tests (Grounds et al., 2008). In agreement with previous studies (Camerino et al., 2014; for review Hyzewicz et al., 2015), the present study found that exercise training substantially affects the

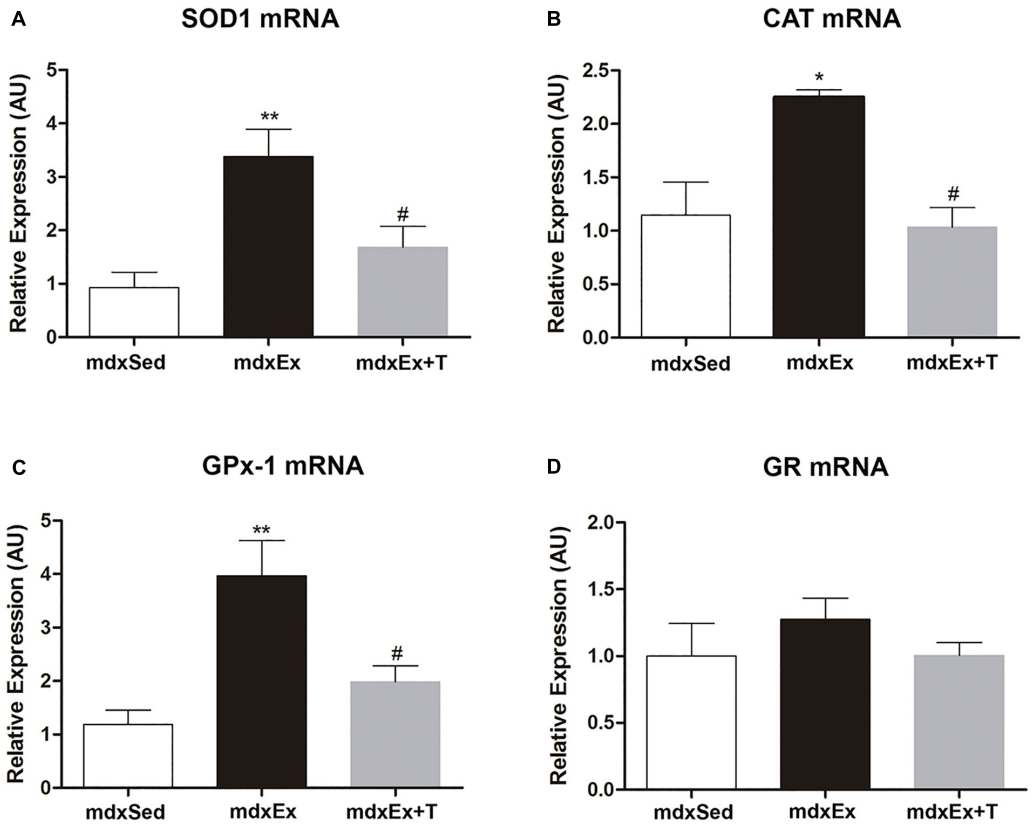

FIGURE 7 | Tempol effects on the enzymatic antioxidant system expression. Gene expression of **(A)** manganese-superoxide dismutase (SOD), **(B)** catalase, **(C)** glutathione peroxidase (GPx), and **(D)** glutathione reductase (GR) measured by qRT-PCR in the diaphragm (DIA) muscle from the sedentary *mdx* mice *(mdx*Sed), exercise trained *mdx* mice (*mdx*Ex), and exercise trained *mdx* mice treated with Tempol (*mdx*Ex+T). All values expressed as mean ± standard deviation (SD). $^{*}P \leq 0.05$ compared with the *mdx*Sed group, $^{**}P \leq 0.001$ compared with the *mdx*Sed group, $^{\#}P \leq 0.05$ compared with the *mdx*Ex group (one-way ANOVA with Tukey's *post hoc* test).

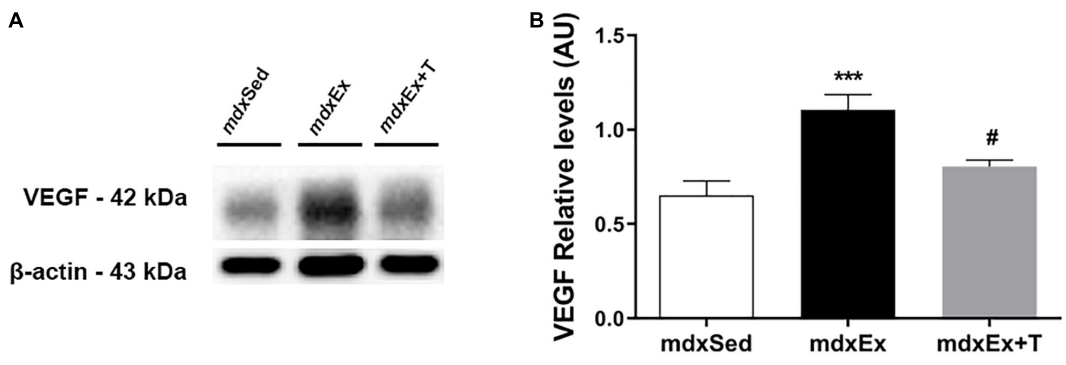

FIGURE 8 | Tempol effects on the VEGF factor. **(A)** Western blotting analysis of the vascular endothelial growth factor (VEGF) content in the diaphragm (DIA) muscle from the sedentary *mdx* mice *(mdx*Sed), exercise trained *mdx* mice (*mdx*Ex), and exercise trained *mdx* mice treated with Tempol (*mdx*Ex+T). The blots of the proteins (top row) and β-actin (loading control, bottom row) are shown. **(B)** The graphs show the protein levels in the crude extracts of DIA from the *mdx*Sed, *mdx*Ex, and *mdx*Ex+T groups. The intensities of each band were quantified and normalized to those of the corresponding control. All values expressed as mean ± standard deviation (SD). $^{***}P \leq 0.0001$ compared with the *mdx*Sed group, $^{\#}P \leq 0.05$ compared with the *mdx*Ex group (one-way ANOVA with Tukey's *post hoc* test).

dystrophic features of adult *mdx* mice by making them more closely resemble DMD.

Although muscle damage was found due to the high CK levels, the number of regenerated muscle fibers were not altered after the exercise. It has been demonstrated in literature that there is no absolute correlation between the extent of dystropathology in an individual *mdx* mouse and CK activity (Grounds et al., 2008). The increase in the CK serum levels indicates that the exercise

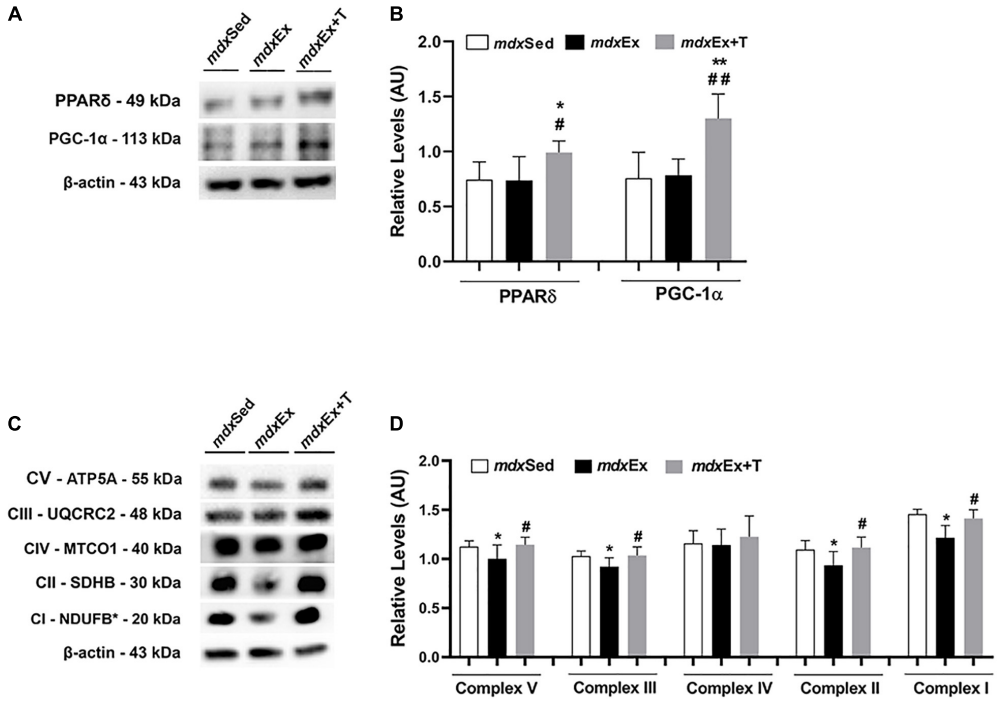

FIGURE 9 | Tempol effects on the activators of mitochondrial biogenesis and oxidative phosphorylation. **(A)** Western blotting analysis of peroxisome proliferator-activated receptor δ (PPARδ) and peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) in the diaphragm (DIA) muscle from the sedentary *mdx* mice *(mdx*Sed), exercise trained *mdx* mice (*mdx*Ex), and exercise trained *mdx* mice treated with Tempol (*mdx*Ex+T). **(B)** The graphs show the PPARδ and PGC-1α levels in the crude extracts of DIA from the *mdx*Sed, *mdx*Ex, and *mdx*Ex+T groups. **(C)** Western blotting analysis of the OXPHOS complexes (complex V, III, IV, II, and I) in the DIA muscle from the *mdx*Sed, *mdx*Ex, and *mdx*Ex+T groups. **(D)** The graphs show the OXPHOS protein in the crude extracts of DIA from the *mdx*Sed, *mdx*Ex, and *mdx*Ex+T groups. The blots of the proteins (top row) and β-actin (loading control, bottom row) are shown. The intensities of each band were quantified and normalized to those of the corresponding control. All values expressed as mean ± standard deviation (SD). $^{*}P \leq 0.05$ compared with the *mdx*Sed group. $^{**}P \leq 0.001$ compared with the *mdx*Sed group. $^{\#}P \leq 0.05$ compared with the *mdx*Ex group. $^{\#\#}P \leq 0.001$ compared with the *mdx*Ex group (one-way ANOVA with Tukey's *post hoc* test).

was sufficient to render the muscular membrane as leaky and allow the release of CK into the circulation (Kobayashi et al., 2012). CK serological levels have long been used to aid DMD diagnosis and remain an important laboratory test for diagnosis (Van Ruiten et al., 2014). In addition, the exercise protocol used in the present study was able to promote an expressive reduction of muscle strength and the worsening of the inflammatory process and oxidative stress on the diaphragm muscle of *mdx* mice.

The exercised *mdx* mice showed that multiple dystrophic pathological events are targeted by Tempol, which is the focus of our study. Among several antioxidants currently available, Tempol is a redox-cycling, membrane-permeable antioxidant, and is particularly interesting for promoting O_2 metabolism at rates that are similar to SOD (Batinic-Haberle et al., 2010; Wilcox, 2010). In addition, this antioxidant also facilitates the metabolism of a wide range of ROS and reactive nitrogen species and exhibits catalase activity (Francischetti et al., 2014). Particularly regarding DMD, previous work presented advantageous Tempol effects in the dystrophic skeletal muscle of *mdx* mice, which were partly associated with its anti-inflammatory and antioxidant actions (Hermes et al., 2019, 2020).

In this study, we observed that the Tempol treatment induced an improvement in terms of muscular regeneration, with an increased muscle strength gain and a good correlation with the increase in muscle fiber diameter and CK reduction in the exercise trained-*mdx* mice. Variations in the muscle fiber diameter are normally observed in dystrophic muscles as a result of the different levels of muscle fiber regeneration and recovery of functional innervation (Bulfield et al., 1984). In addition, in agreement with our functional results, previous studies showed that Tempol supplementation increased the dystrophic diaphragm force generation (Burns et al., 2017) and promoted gain in muscle strength in non-exercised *mdx* mice (Hermes et al., 2019).

Regarding the inflammatory process, our data showed that the Tempol treated group presented a decrease in the nuclear factor kappa B (NF-κB) levels. Although the reduction in the NF-κB activation by Tempol has already been shown in another pathology (Cuzzocrea et al., 2001), this is the first study presenting the Tempol action on the NF-κB pathway in the dystrophic muscle. This is a meaningful finding because NF-κB is an important transcription factor that regulates the expression of pro-inflammatory cytokines, such as TNF-α and IL-1β, which were found to have increased in the *mdx* muscle before the period of muscle fiber necrosis (Kumar and Boriek, 2003; Whitehead et al., 2006). Our results showed

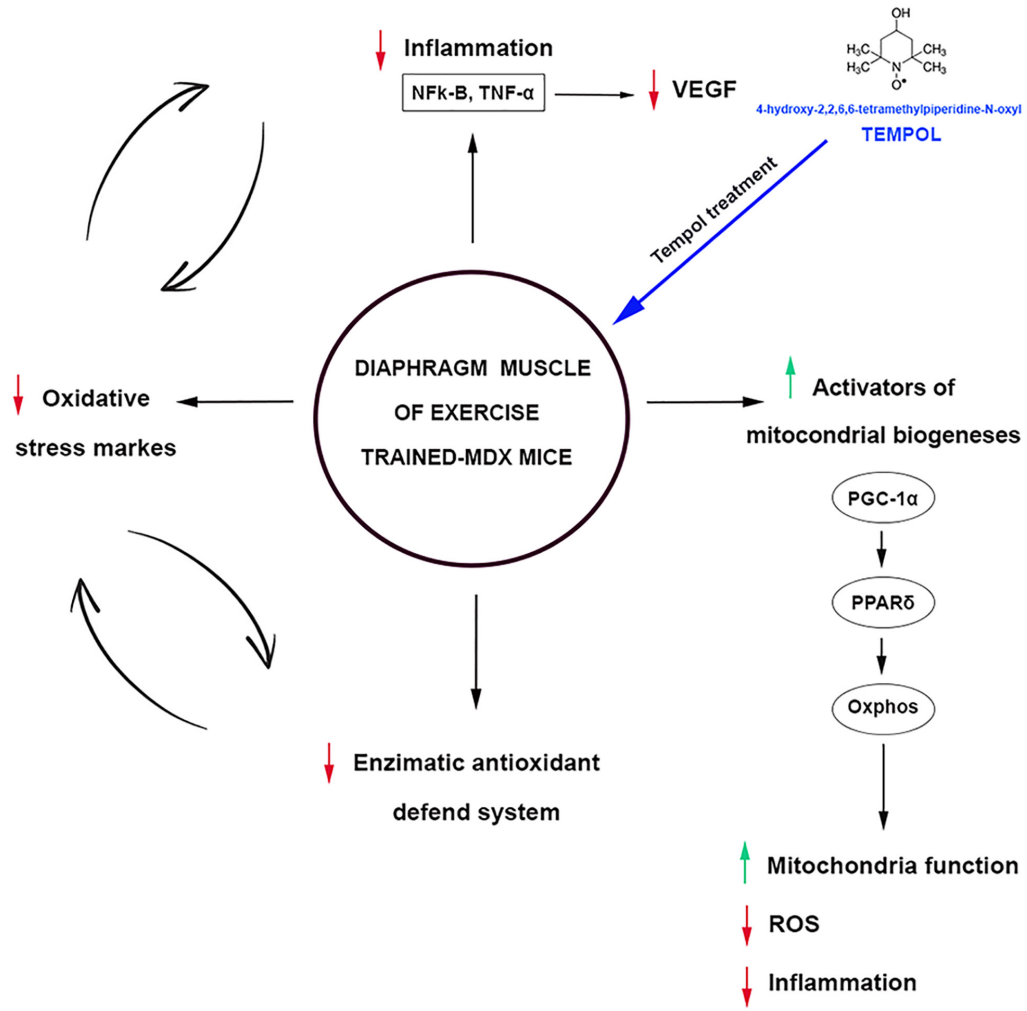

FIGURE 10 | Oxidative stress, inflammation, and activators of mitochondrial biogenesis: targets of Tempol treatment in the diaphragm muscle of exercised trained-*mdx* ↑ mice. ↓ Tempol down-regulated; ⟲ vicious cycle.

that the reduction of the NF-κB levels was accompanied by a decrease of the TNF-α content. In agreement with our results, other studies have also demonstrated the effects of Tempol in reducing the inflammatory markers (Gomez-Pinilla et al., 2010; Afjal et al., 2019).

Additionally, the NF-κB levels have also been associated with the vascular endothelial growth factor (VEGF), which is a key regulator of physiological angiogenesis (Neufeld et al., 1999). Kiriakidis et al. (2003) showed that the VEGF production in human macrophages is NF-κB dependent. This close relationship between NF-κB and VEGF may perhaps justify our data regarding this angiogenesis regulator in our experiments. Similar to the NF-κB results, we found that the Tempol treatment also promoted the reduction of the VEGF levels on exercised *mdx* mice, making them closer to the values of the sedentary *mdx* mice. Despite dysfunctional angiogenesis having a significant impact on the dystrophic phenotype and vascular-targeted therapy being proposed as a treatment option for DMD (Podkalicka et al., 2019; Verma et al., 2019), our results suggest that the beneficial effects of tempol observed on the dystrophic muscle are probably not correlated with the angiogenesis pathway.

The NF-κB activation has also been implicated in the amplification of ROS production and vice versa in the dystrophic muscle (Kumar and Boriek, 2003). For instance, in the *mdx* diaphragm muscle, high levels of the oxidative stress markers, such as DHE, lipofuscin granules, and 4-HNE protein adduct, and the NF-κB were shown (Hermes et al., 2016; Mâncio et al., 2017a,b). Similar to a previous study (Mâncio et al., 2017a), we also verified herein that antioxidant treatment promotes the reduction of oxidative stress markers concomitantly with the decrease of the NF-κ B levels.

In addition, recently, NF-κB has also been associated in a vicious cycle with peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α), where oxidative stress plays an essential role (Rius-Pérez et al., 2020). In our study, we also

observed this correlation between the increase of PGC-1α with the concomitant reduction of NF-κB and ROS in exercise-trained-*mdx* mice treated with Tempol. PGC-1α is highlighted as a master regulator of mitochondrial biogenesis and function, including oxidative phosphorylation (OXPHOS) and ROS detoxification (Mastropasqua et al., 2018). A previous study showed that the overexpression of PGC1-α may protect *mdx* muscles by stimulating mitochondrial biogenesis and by providing a calcium sink to limit calcium-related cellular abnormalities, as well as by preventing the activation of cell death pathways associated with mitochondrial permeabilization (Godin et al., 2012). It was also reported that the restoration of the miR-499 expression (which conducts a PGC-1-α dependent mitochondrial oxidative metabolism program) prevented the hallmarks of muscular dystrophy, including the reduction of CK serum release and the improvement of the exercise capacity in *mdx* mice (Liu et al., 2016).

Concomitantly with the PGC-1α results, we also found an increase in the Peroxisome proliferator-activated receptor δ (PPARδ) levels in exercise-trained-*mdx* mice treated with Tempol. PGC-1α is reported to be a direct target of PPARδ in the skeletal muscle (Phua et al., 2018). Recently, Bell et al. (2019) showed that PPARδ improves mitochondrial function in the *mdx* mice. We also investigated the mitochondrial respiratory complexes (I, II, III, IV, and V), and similar to the PGC-1α and PPARδ results, the levels of these complexes were up regulated in exercise-trained-*mdx* mice treated with Tempol. Therefore, these results support that modulating PPARδ and PGC-1α can represent an interesting strategy in dystrophy muscular disease, since they are implicated in mitochondria function, oxidative metabolism, ROS detoxification, and regulation of inflammatory cytokines (**Figure 10**).

Tempol can also act directly in scavenging excessive ROS. It was reported that the increased diaphragm functional capacity in *mdx* mice by Tempol was related to the ability of this antioxidant to scavenge excessive ROS (Burns et al., 2017). Recently, our research group also showed that Tempol improves the redox status in the diaphragm muscle of young *mdx* mice (Hermes et al., 2020). Some studies showed a beneficial Tempol action against oxidative damage in cells by the catalase-like activity, generating H_2O and O_2 from H_2O_2 and preventing OH production (Wilcox, 2010; Abouzied et al., 2016). This Tempol action may also be applied to our oxidative stress results, justifying the reduction found in oxidative stress markers and enzymatic antioxidant defense system components in the DIA muscle in exercise-trained-*mdx* mice treated with Tempol.

Despite the novelty of our study, some limitations must be recognized. Future studies with wild mice exposed to the same treatment with Tempol and exercise protocol will be useful to understand how changes in the physical activity affect the dystrophic pathology providing further validation for the innovative findings in the current study. In addition, seeing that muscular dystrophy in mice is notoriously variable, a large number of animals should be used when performing experiments with *mdx* mice. Also, although a difference in the inflammatory process morphology was observed between the experimental groups, additional histological methods, such as acid phosphatase, could be useful to complement and strengthen the findings.

To summarize, the striking finding of this work is that the Tempol treated group presented a decrease in the NF-κB in the exercise-trained-*mdx* mice, which is probably related to the ability of this antioxidant to scavenge excessive ROS. Our data also imply that there was an increase in the PGC1-α and PPARδ levels, however, further investigations are required to determine the mechanism by which Tempol modulates these activators of mitochondrial biogenesis. In addition, the present data also reinforce the Tempol as a potential therapeutic for the treatment of DMD.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The animal study was reviewed and approved by Ethics Committee on the Use of Animals (CEUA) of the State University of Campinas (UNICAMP; #4847-1).

AUTHOR CONTRIBUTIONS

HS conducted the study. CC, GR, DM, RM, TA, EP, and LK contributed substantially to the acquisition, analysis, and interpretation of data. EM and VC participated in the design of the study and were responsible for the management of grant and coordination. EM, HS, and EP helped in drafting the manuscript. All authors revised it critically for important intellectual content and gave their final approval of the version to be submitted.

FUNDING

This work was supported by the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP, grant 17/01638-0) and by the Coordenação de Pessoal de Nível Superior-Brasil (CAPES) – Finance Code 001. HS, DM, and GR were the recipients of a CAPES fellowship. CC and TA were the recipients of the CNPq and CAPES fellowship.

ACKNOWLEDGMENTS

We thank Mrs. Deirdre Jane Donovan Giraldo for the English revision of the manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2021.649793/full#supplementary-material

REFERENCES

Abouzied, M. M., Eltahir, H. M., Taye, A., and Abdelrahman, M. S. (2016). Experimental evidence for the therapeutic potential of tempol in the treatment of acute liver injury. *Mol. Cell. Biochem*. 411, 107–115. doi: 10.1007/s11010-015-2572-2

Afjal, M. A., Abdi, S. H., Sharma, S., Ahmad, S., Fatima, M., Dabeer, S., et al. (2019). Anti-inflammatory role of tempol (4-hydroxy-2, 2, 6, 6-tetramethylpiperidin-1-oxyl) in nephroprotection. *Hum. Exp. Toxicol.* 38, 713–723. doi: 10.1177/0960327119836203

Anderson, M. E. (1985). Determination of glutathione and glutathione disulfide in biological samples. *Methods Enzymol.* 113, 548–555. doi: 10.1016/s0076-6879(85)13073-9

Andrews, J. G., and Wahl, R. A. (2018). Duchenne and Becker muscular dystrophy in adolescents: current perspectives. *Adolesc. Health Med. Ther*. 9:53. doi: 10.2147/AHMT.S125739

Batinic-Haberle, I., Reboucas, J. S., and Spasojevic, I. (2010). Superoxide dismutase mimics: chemistry, pharmacology, and therapeutic potential. *Antioxid. Redox Signal*. 13, 877–918. doi: 10.1089/ars.2009.2876

Bell, E. L., Shine, R. W., Dwyer, P., Olson, L., Truong, J., Fredenburg, R., et al. (2019). PPARδ modulation rescues mitochondrial fatty acid oxidation defects in the MDX model of muscular dystrophy. *Mitochondrion* 46, 51–58. doi: 10.1016/j.mito.2018.02.006

Briguet, A., Courdier-Fruh, I., Foster, M., Meier, T., and Magyar, J. P. (2004). Histological parameters for the quantitative assessment of muscular dystrophy in the MDX-mouse. *Neuromusc. Disord.* 14, 675–682. doi: 10.1016/j.nmd.2004.06.008

Bulfield, G., Siller, W. G., Wight, P. A., and Moore, K. J. (1984). X chromosome-linked muscular dystrophy (MDX) in the mouse. *Proc. Natl. Acad. Sci. U.S.A.* 81, 1189–1192. doi: 10.1073/pnas.81.4.1189

Burns, D. P., Ali, I., Rieux, C., Healy, J., Jasionek, G., and O'Halloran, K. D. (2017). Tempol supplementation restores diaphragm force and metabolic enzyme activities in MDX mice. *Antioxidants* 6:101. doi: 10.3390/antiox6040101

Camerino, G. M., Cannone, M., Giustino, A., Massari, A. M., Capogrosso, R. F., Cozzoli, A., et al. (2014). Gene expression in MDX mouse muscle in relation to age and exercise: aberrant mechanical-metabolic coupling and implications for preclinical studies in Duchenne Muscular Dystrophy. *Hum. Mol. Genet*. 23, 5720–5732. doi: 10.1093/hmg/ddu287

Choi, I., Lim, H., Estrellas, K., Mula, J., Cohen, T. V., Zhang, Y., et al. (2016). Concordant but varied phenotypes among Duchenne muscular dystrophy patient-specific myoblasts derived using a human iPSC-based model. *Cell Rep*. 15, 2301–2312. doi: 10.1016/j.celrep.2016.05.016

Cuzzocrea, S., McDonald, M. C., Mazzon, E., Filipe, H. M., Costantino, G., Caputi, A. P., et al. (2001). Beneficial effects of tempol, a membrane−permeable radical scavenger, in a rodent model of splanchnic artery occlusion and reperfusion. *Shock* 14, 150–156. doi: 10.1097/00024382-200014020-00013

De Luca, A., Pierno, S., Liantonio, A., Cetrone, M., Camerino, C., Fraysse, B., et al. (2003). Enhanced dystrophic progression in MDX mice by exercise and beneficial effects of taurine and insulin-like growth factor-1. *J. Pharmacol. Exp. Ther.* 304, 453–463. doi: 10.1124/jpet.102.041343

De Senzi, M. P. R., Ferretti, R., Moraes, L. H. R., Neto, H. S., Marques, M. J., and Minatel, E. (2013). N-Acetylcysteine treatment reduces TNF-α levels and myonecrosis in diaphragm muscle of MDX mice. *Clin. Nut.* 32, 472–475. doi: 10.1016/j.clnu.2012.06.001

Francischetti, I. M., Gordon, E., Bizzarro, B., Gera, N., Andrade, B. B., Oliveira, F., et al. (2014). Tempol, an intracellular antioxidant, inhibits tissue factor expression, attenuates dendritic cell function, and is partially protective in a murine model of cerebral malaria. *PLoS One* 9:e87140. doi: 10.1371/journal.pone.0087140

Godin, R., Daussin, F., Matecki, S., Li, T., Petrof, B. J., and Burelle, Y. (2012). Peroxisome proliferator-activated receptor γ coactivator1- gene α transfer restores mitochondrial biomass and improves mitochondrial calcium handling in post-necrotic MDX mouse skeletal muscle. *J. Physiol.* 590, 5487–5502. doi: 10.1113/jphysiol.2012.240390

Gomez-Pinilla, P. J., Camello, P. J., Tresguerres, J. A., and Pozo, M. J. (2010). Tempol protects the gallbladder against ischemia/reperfusion. *J. Physiol. Biochem*. 66, 161–172. doi: 10.1007/s13105-010-0021-y

Granchelli, J. A., Pollina, C., and Hudeck, M. S. (2000). Pre-clinical screening of drugs using the MDX mouse. *Neuromusc. Disord.* 10, 235–239. doi: 10.1016/S0960-8966(99)00126-1

Grounds, M. D., Radley, H. G., Lynch, G. S., Nagaraju, K., and De Luca, A. (2008). Towards developing standard operating procedures for pre-clinical testing in the MDX mouse model of Duchenne muscular dystrophy. *Neurobiol. Dis*. 31, 1–19. doi: 10.1016/j.nbd.2008.03.008

Guiraud, S., and Davies, K. E. (2017). Pharmacological advances for treatment in Duchenne muscular dystrophy. *Curr. Opin. Pharmacol.* 34, 36–48. doi: 10.1016/j.coph.2017.04.002

Hermes, T. A., Macedo, A. B., Fogaça, A. R., Moraes, L. H. R., Faria, F. M., Kido, L. A., et al. (2016). Beneficial cilostazol therapeutic effects in MDX dystrophic skeletal muscle. *Clin. Exp. Pharmacol. Physiol.* 43, 259–267. doi: 10.1111/1440-1681.12521

Hermes, T. A., Mâncio, R. D., Macedo, A. B., Mizobuti, D. S., Rocha, G. L., Cagnon, V. H. A., et al. (2019). Tempol treatment shows phenotype improvement in MDX mice. *PLoS One* 14:e0215590. doi: 10.1371/journal.pone.0215590

Hermes, T. A., Mizobuti, D. S., Da Rocha, G. L., Da Silva, H. N. M., Covatti, C., Pereira, E. C. L., et al. (2020). Tempol improves redox status in MDX dystrophic diaphragm muscle. *Int. J. Exp. Pathol.* 101, 289–297. doi: 10.1111/iep.12376

Hyzewicz, J., Ruegg, U. T., and Takeda, S. (2015). Comparison of experimental protocols of physical exercise for MDX mice and duchenne muscular dystrophy patients. *J. Neuromusc. Dis.* 2, 325–342. doi: 10.3233/JND-150106

Kiriakidis, S., Andreakos, E., Monaco, C., Foxwell, B., Feldmann, M., and Paleolog, E. (2003). VEGF expression in human macrophages is NF-κB-dependent: studies using adenoviruses expressing the endogenous NF-κB inhibitor IκBα and a kinase-defective form of the IκB kinase 2. *J. Cell Sci.* 116, 665–674. doi: 10.1242/jcs.00286

Kobayashi, Y. M., Rader, E. P., Crawford, R. W., and Campbell, K. P. (2012). Endpoint measures in the MDX mouse relevant for muscular dystrophy pre-clinical studies. *Neuromusc. Disord.* 22, 34–42. doi: 10.1016/j.nmd.2011.08.001

Kumar, A., and Boriek, A. M. (2003). Mechanical stress activates the nuclear factor-kappa b pathway in skeletal muscle fibers: a possible role in Duchenne muscular dystrophy. *FASEB J.* 17, 386–396. doi: 10.1096/fj.02-0542com

Liu, J., Liang, X., Zhou, D., Lai, L., Xiao, L., Liu, L., et al. (2016). Coupling of mitochondrial function and skeletal muscle fiber type by a miR−499/Fnip1/AMPK circuit. *EMBO Mol. Med.* 8, 1212–1228. doi: 10.15252/emmm.201606372

Livak, K. J., and Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 25, 402–408. doi: 10.1006/meth.2001.1262

Mâncio, R. D., Hermes, T. A., Macedo, A. B., Mizoboti, D. S., Rupic, I. F., and Minatel, E. (2017b). Dystrophic phenotype improvement in the diaphragm muscle of MDX mice by diacerhein. *PLoS One* 12:e0182449. doi: 10.1371/journal.pone.0182449

Mâncio, R. D., Hermes, T. A., Macedo, A. B., Mizobuti, D. S., Valduga, A. H., Rupcic, I. F., et al. (2017a). Vitamin E treatment decreases muscle injury in MDX mice. *Nutrition* 43, 39–46. doi: 10.1016/j.nut.2017.07.003

Mastropasqua, F., Girolimetti, G., and Shoshan, M. (2018). PGC1α: friend or foe in cancer? *Genes* 9:48. doi: 10.3390/genes9010048

Neufeld, G., Cohen, T., Gengrinovitch, S., and Poltorak, Z. (1999). Vascular endothelial growth factor (VEGF) and its receptors. *FASEB J.* 13, 9–22.

Phua, W. W. T., Wong, M. X. Y., Liao, Z., and Tan, N. S. (2018). An aPPARent functional consequence in skeletal muscle physiology via peroxisome proliferator-activated receptors. *Int. J. Mol. Sci.* 19:1425. doi: 10.3390/ijms19051425

Podkalicka, P., Mucha, O., Dulak, J., and Loboda, A. (2019). Targeting angiogenesis in Duchenne muscular dystrophy. *Cell. Mol. Life Sci*. 76, 1507–1528. doi: 10.1007/s00018-019-03006-7

Radley-Crabb, H., Terrill, J., Shavlakadze, T., Tonkin, J., Arthur, P., and Grounds, M. (2012). A single 30min treadmill exercise session is suitable for 'proof-of concept studies' in adult MDX mice: a comparison of the early consequences of two different treadmill protocols. *Neuromuscul. Disord*. 22, 170–182. doi: 10.1016/j.nmd.2011.07.008

Rius-Pérez, S., Torres-Cuevas, I., Millán, I., Ortega, ÁL., and Pérez, S. (2020). PGC-1α, inflammation, and oxidative Stress: an integrative view in metabolism. *Oxid. Med. Cell. Longev.* 2020:1452696. doi: 10.1155/2020/1452696

Tonon, E., Ferretti, R., Shiratori, J. H., Neto, H. S., Marques, M. J., and Minatel, E. (2012). Ascorbic acid protects the diaphragm muscle against myonecrosis in MDX mice. *Nutrition* 28, 686–690. doi: 10.1016/j.nut.2011.09.013

Treat-NMD Neuromuscular Network UK (2018). *Use of Treadmill and Wheel Exercise for Impact on MDX Mice Phenotype*. Available online at: https://treat-nmd.org/wp-content/uploads/2016/08/MDX-DMD_M.2.1.001.pdf (accessed March 15, 2018).

Van Ruiten, H. J., Straub, V., Bushby, K., and Guglieri, M. (2014). Improving recognition of Duchenne muscular dystrophy: a retrospective case note review. *Arch. Dis. Child.* 99, 1074–1077. doi: 10.1136/archdischild-2014-306366

Verma, M., Shimizu-Motohashi, Y., Asakura, Y., Ennen, J., Bosco, J., Zou, Z., et al. (2019). Amelioration of muscular dystrophy phenotype in MDX mice by inhibition of Flt1. *bioRxiv* [Preprint]. doi: 10.1101/609735

Whitehead, N. P., Yeung, E. W., and Allen, D. G. (2006). Muscle damage in MDX (dystrophic) mice: role of calcium and reactive oxygen species. *Clin. Exp. Pharmacol. Physiol.* 33, 657–662. doi: 10.1111/j.1440-1681.2006.04394

Wilcox, C. S. (2010). Effects of tempol and redox-cycling nitroxides in models of oxidative stress. *Pharmacol. Ther.* 126, 119–145. doi: 10.1016/j.pharmthera.2010.01.003

Yucel, N., Chang, A. C., Day, J. W., Rosenthal, N., and Blau, H. M. (2018). Humanizing the MDX mouse model of DMD: the long and the short of it. *NPJ Regen. Med.* 3:4. doi: 10.1038/s41536-018-0045-4

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Physiology

REVIEW
published: 20 May 2021
doi: 10.3389/fphys.2021.668600

The Role of Embryonic Chick Muscle Cell Culture in the Study of Skeletal Myogenesis

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The mechanisms involved in the development of skeletal muscle fibers have been studied in the last 70 years and yet many aspects of this process are still not completely understood. A myriad of *in vivo* and *in vitro* invertebrate and vertebrate animal models has been used for dissecting the molecular and cellular events involved in muscle formation. Among the most used animal models for the study of myogenesis are the rodents rat and mouse, the fruit fly Drosophila, and the birds chicken and quail. Here, we describe the robustness and advantages of the chick primary muscle culture model for the study of skeletal myogenesis. In the myoblast culture obtained from embryonic chick pectoralis muscle it is possible to analyze all the steps involved in skeletal myogenesis, such as myoblast proliferation, withdrawal from cell cycle, cell elongation and migration, myoblast alignment and fusion, the assembly of striated myofibrils, and the formation of multinucleated myotubes. The fact that *in vitro* chick myotubes can harbor hundreds of nuclei, whereas myotubes from cell lines have only a dozen nuclei demonstrates the high level of differentiation of the autonomous chick myogenic program. This striking differentiation is independent of serum withdrawal, which points to the power of the model. We also review the major pro-myogenic and anti-myogenic molecules and signaling pathways involved in chick myogenesis, in addition to providing a detailed protocol for the preparation of embryonic chick myogenic cultures. Moreover, we performed a bibliometric analysis of the articles that used this model to evaluate which were the main explored topics of interest and their contributors. We expect that by describing the major findings, and their advantages, of the studies using the embryonic chick myogenic model we will foster new studies on the molecular and cellular process involved in muscle proliferation and differentiation that are more similar to the actual *in vivo* condition than the muscle cell lines.

Keywords: myogenesis, chick embryo, skeletal muscle, muscle differentiation, myoblast, myotube

OPEN ACCESS

Edited by:
Anselmo Sigari Moriscot, University of São Paulo, Brazil

Reviewed by:
Elisabeth Ehler, King's College London, United Kingdom
Maeli Dal-Pai-Silva, São Paulo State University, Brazil

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Specialty section:
This article was submitted to Striated Muscle Physiology, a section of the journal Frontiers in Physiology

Received: *16 February 2021*
Accepted: *21 April 2021*
Published: *20 May 2021*

Citation:
Costa ML, Jurberg AD and Mermelstein C (2021) The Role of Embryonic Chick Muscle Cell Culture in the Study of Skeletal Myogenesis. Front. Physiol. 12:668600. doi: 10.3389/fphys.2021.668600

A BRIEF VIEW OF SKELETAL MYOGENESIS

Among the various processes involved in the organogenesis of vertebrates, the formation of skeletal muscle (i.e., skeletal myogenesis) initiates in the paraxial mesoderm of the developing embryo, which gradually segments into somites (Christ and Ordahl, 1995; Chal and Pourquié, 2017). Epithelial-derived mesenchymal cells give rise to round proliferative presumptive mononucleated

myoblasts. Signaling molecules, mainly from the Wnt/beta-catenin, Sonic hedgehog (Shh), Bone morphogenetic protein (BMP), and Notch signaling pathways, participate in the initial steps of embryonic myogenesis (Cossu et al., 1996; Ikeya and Takada, 1998; Goichberg et al., 2001; Elia et al., 2007; Wang et al., 2010; Bentzinger et al., 2012; Maltzahn et al., 2012). Presumptive mononucleated myoblasts, which do not express myofibrillar proteins, will proliferate a few rounds of replication, and exit cell cycle. The activation of the muscle specific-master switch genes MyoD and Myf5 leads to the differentiation of presumptive myoblasts into elongated post-mitotic myoblasts (Davis et al., 1987; Choi et al., 1990). MyoD and Myf5, in conjunction with Myogenin and MRF4, are highly conserved genes known as myogenic regulatory factors (MRFs). They are organized into hierarchical gene expression networks that control skeletal muscle differentiation (Bentzinger et al., 2012). For instance, their activation drives the expression of muscle specific and myofibrillar proteins (Holtzer et al., 1991; Costa et al., 2004). Elongated post-mitotic myoblasts (or bipolar myoblasts) adhere to each other through a cell membrane-dependent recognition event, which is subsequently followed by cell fusion (that is, "primary fusion"). Myoblast fusion relies on membrane proteins, mainly cadherins, myomaker, and myomerger, to form multinucleated myotubes (Zeschnigk et al., 1995; Costa, 2014; Sampath et al., 2018; **Figures 1–3** and **Supplementary Figure 1**). Initially, the nuclei of myotubes are centrally placed, but they migrate to the periphery (Fear, 1977) while striated actomyosin-based myofibrils organize within the sarcoplasm of the multinucleated syncytium (**Figures 1–3** and **Table 1**). The growth of myotubes results from a secondary phase of cell fusion, which involves the fusion of mononucleated myoblasts into the multinucleated myotubes. During the formation of mature multinucleated myotubes, myofibrillar proteins start to organize into long contractile structures encompassing several muscle-specific proteins, such as actin, myosin, troponins C/T/I, tropomyosin, alpha-actinin, titin, nebulin, desmin, myosin binding protein-C (MyBP-C), myomesin, and tropomodulin (**Figures 1–3** and **Table 1**).

Different animal models have been used for dissecting the above-described steps of skeletal muscle differentiation, including rodents (mouse and rat), fishes, fruit-flies, frogs, and birds (chicken and quail), as well as human muscle cells. Among these models, the chick embryo is one of the major contributors of our current knowledge of muscle development (Buckingham et al., 2003). The chick embryonic skeletal muscle primary culture is a robust *in vitro* model for the study of skeletal muscle differentiation because of its autonomous myogenic program (Holtzer et al., 1991; Mermelstein et al., 1996; Oliveira et al., 2015; Ojima et al., 2016; Rosa de Andrade et al., 2018). All the before mentioned steps of skeletal myogenesis can be observed in the chick muscle cultures. Skeletal myogenesis proceeds in chick myoblast cultures through the following main sequential and temporal stages (some of which can be seen in **Figures 1–3** and **Table 1**): myoblast proliferation, cell cycle withdrawal, myoblast elongation and migration, myoblast alignment and fusion, and the formation of multinucleated myotubes, as well as the formation of contractile myofibrils.

Studies using chick myogenic cultures led to the discovery of many soluble factors that either stimulate or inhibit myoblast fusion and/or myotube formation, and which can be categorized into pro- or anti-myogenic. **Table 2** shows activators and inhibitors of embryonic chick skeletal myogenesis. Interestingly, there is a greater number of known molecules (˜3 fold) that inhibit myogenesis as compared to the ones that produced stimulatory outcomes. This difference can be explained by (i) the incompleteness of available knowledge, since it is usually easier to identify an inhibition or blockage of myofiber formation than a positive, sometimes subtle effect, and/or (ii) researchers in the field may be more interested in inhibitory molecules rather than stimulatory ones. Together, the information collected in **Table 2** could be useful to build a more complete picture of the molecular machinery controlling skeletal myogenesis.

The use of chick cells as a model of *in vitro* myogenesis has largely contributed for the description and molecular characterization of the aforementioned steps during myotube formation. In the section below, we will detail its main advantages, disadvantages, and particularities as an experimental model for skeletal myogenesis.

DISSECTING SKELETAL MYOGENESIS USING CHICK MYOBLAST CULTURES

The characterization and quantification of different aspects of skeletal myogenesis is relatively simple to achieve in culture of chick myoblasts (Abrunhosa et al., 2014; Bastos et al., 2019; Bagri et al., 2020). Among them, information regarding the number of mononucleated myoblasts and multinucleated myotubes, as well as fibroblasts, the number of nuclei within each myotube, area of muscle cells, and size of myotubes can be acquired from image processing tools, such as ImageJ (Schindelin et al., 2012). From these data, it is also possible to compute both the fusion index, which refers to the number of nuclei in myotubes divided by the total number of nuclei in a microscopy field of view, and the differentiation index, which is obtained from the number of nuclei in myosin heavy chain (MyHC)-positive cells divided by the total number of nuclei in a field. In this regard, the staining of MyHC can be replaced by other myofibrillar protein, such as desmin, alpha-actinin, troponin, tropomyosin, or titin.

One important aspect of chick skeletal myoblasts grown *in vitro* is their autonomous muscle differentiation program. Whereas the exposition of the mouse BC3H1 cell line to mitogens caused the downregulation of sarcomeric protein synthesis and cell cycle reentry (Taubman et al., 1989), chick muscle cells appear not to be subject to the exact same molecular mechanisms. This is reflected in the non-reliance of serum starvation to trigger chick myoblast differentiation into myotubes, unlike immortalized mice, rats, and human cells (Lawson and Purslow, 2000). For this reason, assays using chick myoblasts are the most suitable for tracking morphological and protein/gene expression changes over time during skeletal myogenesis. An example of this kind of studies is the analysis of the sequence of expression of muscle-specific proteins in

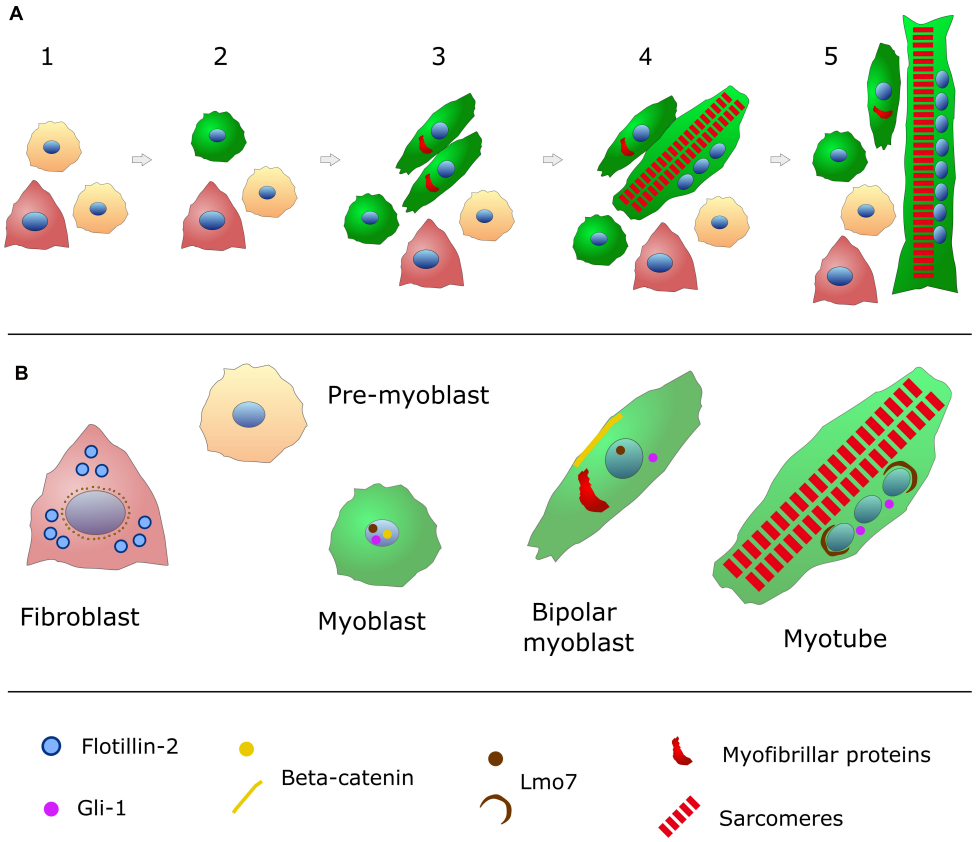

FIGURE 1 | Schematic representation of *in vitro* chick embryonic skeletal myogenesis. **(A)** Specific cell phenotypes can be characterized during skeletal muscle development (for more details, see **Table 1**). Presumptive myoblasts (or pre-myoblasts, shown in beige) are mononucleated (nuclei in blue), round shaped and proliferative cells that do not express myofibrillar proteins. When these cells withdraw cell cycle, they begin to express desmin (green in the cytoplasm) and they change to a bipolar shape. These bipolar myoblasts begin to express myofibrillar proteins (red) and they will align with other myoblasts. Cell adhesion and fusion happens between steps 3 and 5. Muscle differentiation culminates with the formation of long and multinucleated myotubes filled (steps 4 and 5) with contractile myofibrils (sarcomeres in red). Pre-myoblasts (beige cells), post-mitotic myoblasts (round green cells), bipolar myoblasts (elongated green cells), and fibroblasts (triangular-shaped and carmine-colored cells) are present in all steps of chick myogenic cultures (steps 1–5). **(B)** The intracellular distribution of the structural and signaling proteins flotillin-2 (light blue), beta-catenin (yellow), Lmo7 (brown), and myofibrillar proteins (red) are shown for each specific cell phenotype (myoblasts, fibroblasts and myotubes) found in chick myogenic cell cultures.

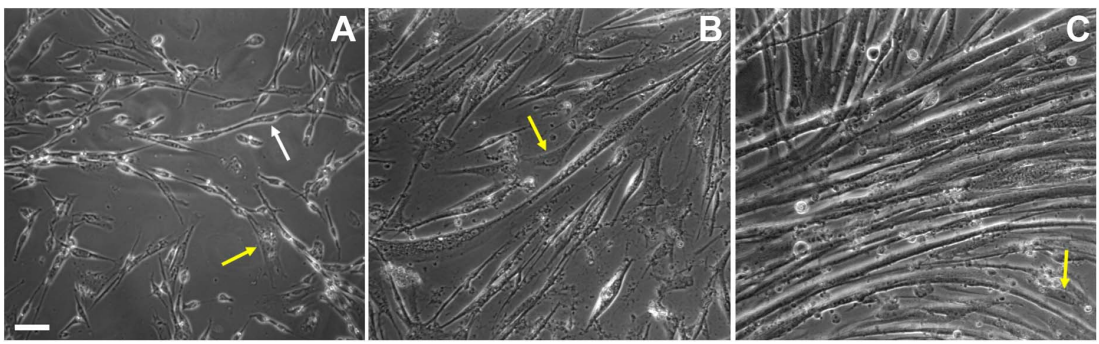

FIGURE 2 | Cell shape changes during *in vitro* chick myogenesis. Primary cultures of 11-day old chick myoblasts were grown for 24, 48, and 72 h and images were acquired under phase contrast microscopy. Bipolar myoblasts aligned in a pre-fusion chain-like structure (white arrow) are seen in a 24-h culture **(A)**, while thin myotubes are already present in a 48-h culture **(B)** and multinucleated myotubes are present in a 72-h culture **(C)**. Note the presence of fibroblasts in all the images (**A–C**, yellow arrows). Scale bar = 10 μm.

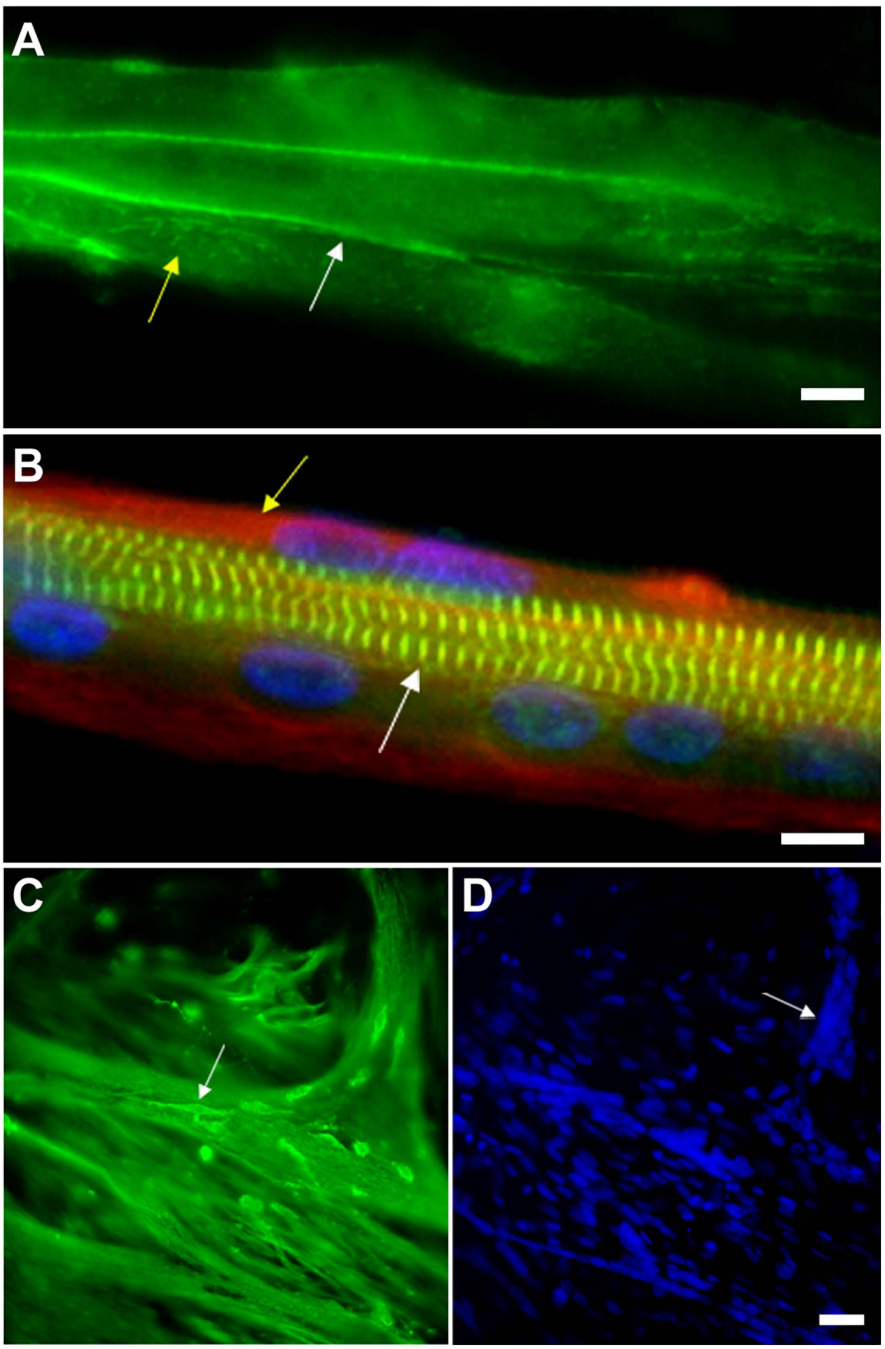

FIGURE 3 | Myoblast adhesion and myotube formation are hallmarks of skeletal myogenesis. **(A)** Cadherin and beta-catenin accumulate at cell-cell adhesion contacts in pre-fusion chick myoblasts. In chick myogenic cultures 24 h after cell plating, primary fusion (myoblast-myoblast fusion) can be observed as an intense immunolabeling of beta-catenin (green, white arrow in **A**) in continuous lines at cell-cell contacts. Note that beta-catenin is also present in a dot-like pattern in the whole sarcolemma (green, yellow arrow in **A**). Scale bar in **(A)** = 10 μm. **(B)** Myotubes from chick primary skeletal muscle cultures are multinucleated cells that contain highly organized myofibrils. Chick skeletal muscle cells were grown in culture for 72 h and stained with antibodies anti-sarcomeric alpha-actinin (green), anti-desmin (red, yellow arrow in **B**) and with the nuclear dye DAPI (blue). Note in the merged image the periodical labeling of alpha-actinin in the Z-bands of myofibrils (white arrow in **B**), the presence of desmin filaments at the periphery of the myotube (yellow arrow in **B**) and well-aligned nuclei. Scale bar in *B* = 10 μm. **(C,D)** Myoblasts adhere to myotubes in a secondary fusion process. Chick skeletal muscle cells were grown in culture for 72 h and stained with antibodies anti-alpha-tubulin (green, **C**) and with the nuclear dye DAPI (blue, **D**). Small mononucleated myoblasts (white arrow in **C**) can be seen at the top of a multinucleated myotube, which is evidenced by the microtubule staining. DAPI shows the presence of a high number of nuclei in each chick myotube cell (blue, **D**). Image **(A)** shows a 24-h primary myogenic culture, where most of the myoblasts are undergoing primary myoblast fusion (myoblast-myoblast fusion), while image **(C)** shows a 72-h primary myogenic culture, where secondary myoblast fusion (myoblast-myotube fusion) is frequently observed. Scale bar in **(D)** = 20 μm.

TABLE 1 | Characteristics of specific cell phenotypes during chick skeletal myogenesis.

	Presumptive myoblast	Round myoblast	Bipolar myoblast	Striated myoblast	Myotube
Proliferation status	Proliferative	Non-proliferative	Non-proliferative	Non-proliferative	Non-proliferative
Desmin expression	Desmin-negative	Desmin-positive	Desmin-positive	Desmin-positive	Desmin-positive
Myofibrillar proteins expression	Myofibrillar proteins-negative	Myofibrillar proteins-negative	Myofibrillar proteins-positive	Myofibrillar proteins-positive	Myofibrillar proteins-positive
Number of nuclei	mononucleated	mononucleated	mononucleated	Mononucleated	multinucleated
Presence of striations	Non-striated	Non-striated	Non-striated	Striated	Striated
Cell shape	Round shaped	Round shaped	Bipolar	Bipolar	Elongated

*The specific cell phenotypes that appear during an in vitro primary culture of embryonic chick skeletal muscle cells are characterized by specific events shown in this Table (for more details, see **Figure 1**). The differences between cell phenotypes depicted here are the replication capacity, expression of the muscle-specific intermediate filament desmin, expression of myofibrillar proteins, the number of nuclei per cell, presence of striations, and cell shape.*

TABLE 2 | Pro- or anti-myogenic effects of different molecules/substances during *in vitro* chick skeletal myogenesis.

Substance name	Substance activity	Substance concentration	References
Enhance myoblast fusion and/or muscle differentiation			
Alpha-cyclodextrin	Membrane phospholipid depleting agent	2 mM	Possidonio et al., 2011
6-Bromoindirubin-30-oxime (BIO)	GSK3b inhibitor	5 μM	Portilho et al., 2012
Interleukine 4 (IL-4)	Binds to IL-4 receptor	10 ng/mL	Possidonio et al., 2011
Isoproterenol (ISO)	Beta-adrenergic receptor agonist	100 nM	Bastos et al., 2019
Methyl-beta-cyclodextrin (MbCD)	Membrane cholesterol depleting agent	2 mM	Mermelstein et al., 2005b, 2007; Portilho et al., 2007, 2012
Wnt-3a	Canonical Wnt/beta-catenin pathway activator	L-Wnt3a-cells conditioned media	Portilho et al., 2007
Inhibits myoblast fusion and/or muscle differentiation			
Chloroquine Lys05	Lysosomal function inhibitor	1 μM	Bagri et al., 2020
4-{[2-[(2-Cyanobenzyl) thio]-4-oxothieno[3,2-d]pyrimidin-3(4H)-yl]methyl}benzoic acid (C3)	S-nitrosoglutathione reductase (GSNOR) inhibitor	10 μM	Yamashita et al., 2017
Cytochalasin B	Actin polymerization inhibitor	5 μM	Ojima et al., 2016
Dickkopf-related protein 1 (Dkk-1)	canonical Wnt/beta-catenin inhibitor	0.1 mg/mL	Portilho et al., 2012
Ethylene glycol tetraacetic acid (EGTA)	Calcium chelator	1.8 mM	Paterson and Strohman, 1972; Mermelstein et al., 2005a
Forskolin	Adenylate cyclase activator	100 μM	Baek et al., 1994
Ouabain	Na^+/K^+-ATPase inhibitor	10 μM	Oliveira et al., 2015
PD150606	Calpain inhibitor	20 μM	Buffolo et al., 2015
Rapamycin	mTOR signaling pathway inhibitor	3 μM	Bastos et al., 2019
Simvastatin	Cholesterol biosynthetic pathway inhibitor	0.5 μM	Portilho et al., 2012
Soluble frizzled receptor (Frzb-1)	Wnt pathway inhibitor	L-Fzrb1-cells conditioned media	Mermelstein et al., 2007
Sonic hedhehog (Shh)	Shh pathway activator	10 ng	Teixeira et al., 2018
12-O-Tetradecanoylphorbol-13-acetate (TPA)	protein kinase C activator	10^{-7} M	Cohen et al., 1977
Trifluoperazine (TFP)	Calmodulin antagonist	10 μM	Bar-Sagi and Prives, 1983
U0126	MEK-ERK pathway inhibitor	10 μM	Oliveira et al., 2015
Wnt-5a	Non-canonical Wnt pathway activator	L-Wnt5a-cells conditioned media	Portilho et al., 2007; Bastos et al., 2019

Only studies using embryonic chick muscle cultures obtained from pectoral (breast) skeletal muscle were included in this table. Studies using embryonic chick leg muscles were not included. Nearly all the studies described in the table used the indicated concentrations of substances over a 24-h period of incubation in vitro.

muscle cells during differentiation in culture (Lin et al., 1994). Upon terminal cell division, chick myoblasts start to express genes of myofibrillar and structural proteins in a specific order before cell fusion. Desmin is the first structural muscle-specific protein to appear after cell cycle withdrawal and it is followed by alpha-actin, troponin-I, alpha-actinin, MyHC, titin, and nebulin in an apparently stochastic order (Lin et al., 1994). In the period between 15 and 24 h after cell cycle exit, these contractile proteins first organize into nonstriated myofibrils and them into striated myofibrils, concurrently with the expression of myomesin and MyBP-C in the developing A-bands (Lin et al., 1994).

One of the most studied and yet not completely understood step of skeletal muscle differentiation is myoblast fusion, which is

a complex process regulated by several molecules and pathways (**Figures 1–3** and **Supplementary Figure 1**). Myoblast fusion in chick cells occurs after 50–65 h of culture, but it takes about 6 h after the first cell-to-cell contact begin. Cell fusion usually follows an end-to-end rather than side-to-side alignment of myoblasts (Fear, 1977). Lipids, proteins, ions, and cell organelles have been implicated in the fusion of chick myoblasts. Together, they are part of an extensive and growing list, which includes cholesterol, sphingosine, inositol phospholipid, cadherins, calpains, monensin, myomaker, protein kinase C, acetylcholine receptors, ascorbic acid, okadaic acid, nitric oxide synthase, Na^+-K^+-ATPase, prostaglandin, concanavalin A, small GTPases, cyclic-AMP, cyclic-GMP, interleukin 4 (IL-4), fibroblast growth factors (FGF), phorbol 12-myristate 13-acetate (TPA), hyaluronic acid, transglutaminase, calcium, zinc, cesium, oxygen, vesicles, and the Golgi apparatus (Shimada, 1971; Knudsen and Horwitz, 1977; Neff et al., 1984; Mermelstein et al., 2005b; Possidonio et al., 2011; Buffolo et al., 2015; Sieiro et al., 2017). The above list is extensive, at least in part, because it contains isolated members (membranar, cytoplasmic, effector and/or nuclear molecules) of the same intracellular signaling pathways.

Among all these aforementioned players, the membrane lipid cholesterol is perhaps the one we know best about its role in myoblast fusion (van der Bosch et al., 1973; Cornell et al., 1980; Sekiya et al., 1984; Hirayama et al., 2001; Nakanishi et al., 2001). Cholesterol is a multifunctional molecule involved in plasma membrane fluidity and permeability, in addition to take part of the synthesis of steroid hormones and others biological processes. It also plays a role in the formation and maintenance of membrane microdomains, which act as signaling centers (Simons and Sampaio, 2011). Our group have studied the role of cholesterol and cholesterol-enriched lipid domains in the sarcolemma of chick myoblast cells by using the cholesterol depletion drug methyl-beta-cyclodextrin (MbCD). This drug shows high affinity for membrane cholesterol, being widely used to extract this molecule from the cell plasma membrane (Ilangumaran and Hoessli, 1998). Upon MbCD treatment, the disorganization of lipid rafts induced an increase in myoblast proliferation and fusion, which led to the formation of larger spontaneously contracting multinucleated myotubes (Mermelstein et al., 2005b, 2007; Portilho et al., 2012). Interestingly, the depletion of cholesterol induced the translocation of beta-catenin into the nuclei of myoblasts and the activation of the canonical Wnt/beta-catenin pathway (Mermelstein et al., 2007; Portilho et al., 2007). A transcriptome analysis of MbCD-treated chick muscle cells revealed multiple changes, involving cell proliferation (cell cycle and p53 signaling), cell adhesion and cytoskeleton (focal adhesion, tight junctions, adherens junctions, gap junctions, and actin cytoskeleton), membrane trafficking-related processes (phagosome, lysosome, and endocytosis), and cell death (apoptosis and autophagy). Among all transcripts that were affected by cholesterol depletion, the levels of Lim domain only protein 7 (Lmo7) mRNA were the most upregulated (Possidonio et al., 2014a, 2016). Lmo7 is a multifunctional protein that can be found in the nucleus, cytoplasm and/or at adhesion junctions in many tissues (**Figure 1**), with high levels of expression in skeletal muscle, where it has a role as a transcription factor regulating the expression of many skeletal muscle genes, including Pax3, Pax7, MyoD, and Myf5 (Holaska et al., 2006; Possidonio et al., 2016).

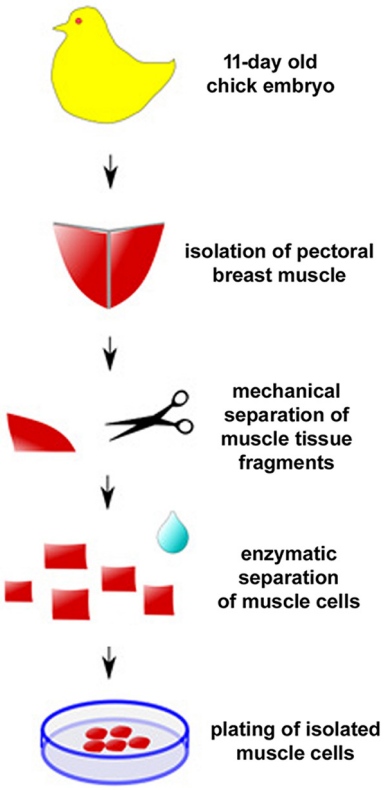

FIGURE 4 | Protocol for primary culture of embryonic chick myogenic cells. Schematic representation of the main steps involved in the culture of embryonic chick pectoral breast muscle cells.

Recently, two muscle-specific membrane proteins, myomaker and myomixer, have been identified as fusogenic regulators in vertebrates (Millay et al., 2013; Luo et al., 2015; Bi et al., 2017; Chen et al., 2020). Both myomaker and myomixer have the capacity to directly control the myogenic fusion process. While the experimental deletion of myomaker blocks myoblast fusion in cultured muscle cells and in zebrafish, it does not seem to be involved with other myogenic processes. Mutant myomaker gene causes the human myopathy Carey-Fineman-Ziter syndrome (Di Gioia et al., 2017). Myomaker is regulated by MyoD and myogenin, and its inhibition by miR-140-3p blocks chick myoblast fusion (Luo et al., 2015). Myomixer (also called myomerger or minion) is a small transmembrane peptide that when disrupted prevents myoblast fusion in knockout mice but its forced expression in non-muscle cells, together with myomaker, promotes cell fusion (Bi et al., 2017). Myomixer is also regulated by MyoD and myogenin and its expression pattern is similar to myomaker. It is likely that myomaker and myomixer work together, myomaker initiating myoblast fusion and myomixer expanding the membrane pore (Chen et al., 2020).

One aspect of the transition between myoblast fusion and myotube formation that is of particular interest is the movement and positioning of nuclei derived from mononucleated myoblasts into newly formed multinucleated fibers (Elhanany-Tamir et al., 2012; Folker and Baylies, 2013; Volk, 2013; Liu et al., 2020). Interestingly, Elhanany-Tamir et al. (2012) demonstrated a direct link between myonuclei positioning and proper muscle function in Drosophila, which relies on a network of polarized astral microtubules and KASH proteins that enables the dynamic movement and uniform spacing between the nuclei in each muscle fiber. Earlier studies revealed that nuclei of myotubes can undergo two different movements: (i) translocation along the long axis of the cell, and (ii) three-dimensional rotation in either clockwise or counterclockwise directions (Capers, 1960; Cooper and Konigsberg, 1961; Englander and Rubin, 1987). The meaning of these nuclear movements is still elusive during skeletal myogenesis, but the perinuclear region is becoming a focus of studies because of its highly dynamic and specialized concentration of molecules. The juxtanuclear area of muscle cells preferentially harbor specific organelles, such as centrosomes and lysosomes, as well as a number of structural and signaling proteins, such as desmin, Gli-1, and Lmo7 (**Figure 1**; Mermelstein et al., 2006; Possidonio et al., 2016; Teixeira et al., 2018; Bagri et al., 2020).

Interestingly, at the end of skeletal muscle differentiation in primary chick muscle cell cultures, a single myotube may exhibit hundreds of nuclei within its cytoplasm (**Figure 3**), whereas myotubes formed in cultures of widely used skeletal muscle cell lines, such as mouse C2C12, rat L6 and L8 cells, and human CL25, are usually composed by only a few (2–20) nuclei. As mentioned above, this is probably related to the autonomous nature of the chick muscle differentiation program. In addition, the formation of branched myotubes is also a common observation in chick muscle cell cultures (Konigsberg, 1963), but they have not been reported *in vivo*. Their branching appears to occur when myotubes are not aligned parallel to each other *in vitro*, which produces a contact between the end of one myotube and the lateral surface of another one (Fear, 1977). Furthermore, *in vitro* myotubes differ from *in vivo* myotubes in that the former are relatively hypernucleated (Okazaki and Holtzer, 1966).

After myoblast fusion, sarcomeres assemble to form contractile myofibrils in multinucleated myotubes, in a process called myofibrillogenesis. The formation of a myofibril occurs by the stepwise addition of several different myofibrillar proteins. This complex and dynamic process is based on the conversion of actin microfilament scaffolds decorated with alpha-actinin and other actin-associated proteins into stress fiber-like structures (also called pre-myofibrils; Almenar-Queralt et al., 1999). While these alpha-actinin deposits coalesce into periodically distributed nascent Z lines, they bind to the Z-line epitope of titin. Concomitant with the progressive alignment of Z-line molecules, thick filaments, formed by the muscle isoform of myosin II, are linked to the nascent myofibrils. Lastly, M-lines are assembled with the addition of myomesin and other proteins, including the M-line part of titin (Sanger et al., 2010; Wang et al., 2020). In summary, the assembly of sarcomeres involves interaction between two organizing centers, (i) one involves the assembly of thick filaments and their associated proteins, including titin, and (ii) the other involves the assembly of thin filaments and their associated proteins, forming the I-Z-I complex (Hill et al., 1986). Myofibril assembly is based on force transduction through the attachment of myofibrils to the sarcolemma and, through cell adhesion complexes, to the extracellular matrix.

A great advantage of the primary culture of chick muscle cells, besides its reliability and independence of differentiation stimuli, is the possibility of observation of molecules (proteins, lipids and RNAs) in cells at single cell- and/or at subcellular-levels. In opposition to biochemical analysis, where a whole cell culture extract (a mixture of different cells) is analyzed, in chick muscle cultures, unlabeled or fluorescently labeled-live cells can be observed at high resolution microscopy and provide valuable information of the behavior and structural characteristics of different myogenic cell phenotypes (proliferative myoblasts, post-mitotic myoblasts, fibroblasts, young myotubes, and mature myotubes). Importantly, chick skeletal muscle cell cultures have some disadvantages, such as the lack of widespread genetic manipulations, which are cutting edge approaches in other animal models of myogenesis, such as the fruit fly Drosophila (Bryantsev et al., 2019). Nevertheless, the genetic approach weakness of the chick muscle culture model has been, in part, surpassed by some recent advances in the transfection of chick cultured cells with siRNA targeted to specific proteins and fluorescent/luminescent protein reporter systems (Mermelstein et al., 2007; Possidonio et al., 2016; Yamashita et al., 2017; Bagri et al., 2020; de Andrade Rosa et al., 2020).

It is important to mention that chick primary muscle cell cultures have been used in the last 70 years as a two-dimensional (2D) layer of cells, and a recent study reported a promising work using three-dimensional (3D) chick skeletal muscle cell cultures to promote muscle differentiation (Gupta et al., 2021). These authors harvested thigh muscle cells from 10-day-old chick embryos, seeded them onto gelatin hydrogels and observed that myoblasts fused into aligned, multinucleated myotubes with robust sarcomere formation in long-term cultures. These results open new venues for engineered *in vitro* models of skeletal muscle which could be useful as platforms for muscle tissue development, function, disease, injury, and drug responses in a controlled setting.

A DETAILED PROTOCOL FOR CULTURING CHICK MUSCLE CELLS

One of the first protocols described for the culture of primary chick embryonic muscle cells is quite simple and therefore widely used (**Figure 4**; Haba et al., 1966). Briefly, pectoral muscle tissue is removed from 11-day old chicken embryos (usually obtained from a certified poultry farm) with the use of fine watchmaker forceps (N° 5). It is crucial to use all sterile materials and reagents, being extremely careful during the entire cell culture procedure to avoid unwanted contaminants (mostly, bacteria, fungi, or mycoplasma). After separation of the connective tissue under a dissecting scope, muscle tissue is minced with surgery knives and digested with 0.025% trypsin at 37°C for 20–30 min

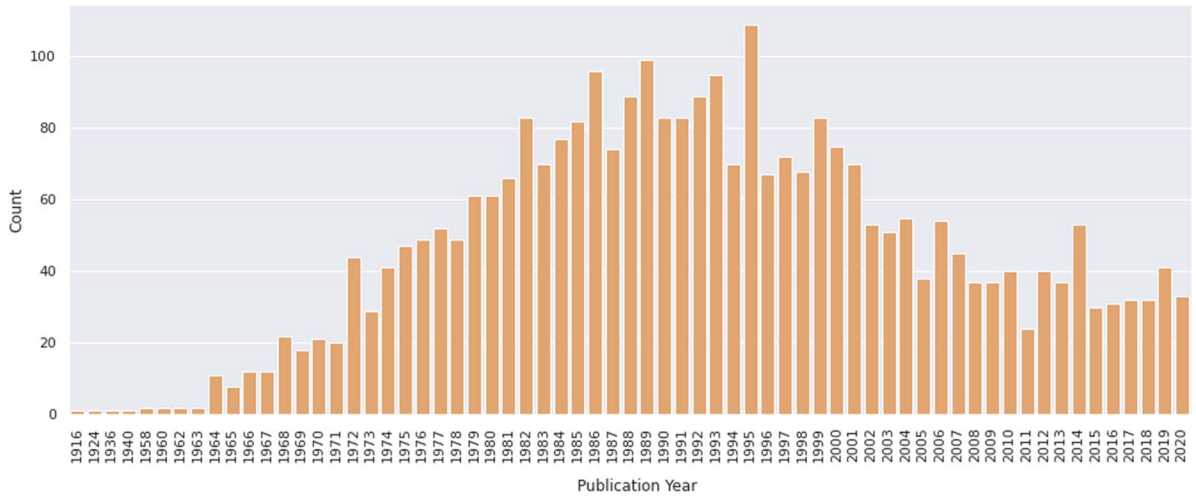

FIGURE 5 | Number of papers on primary culture of embryonic chick myogenic cells per year. Distribution of publications retrieved from PubMed. The retrieved dataset only included papers until the year of 2020.

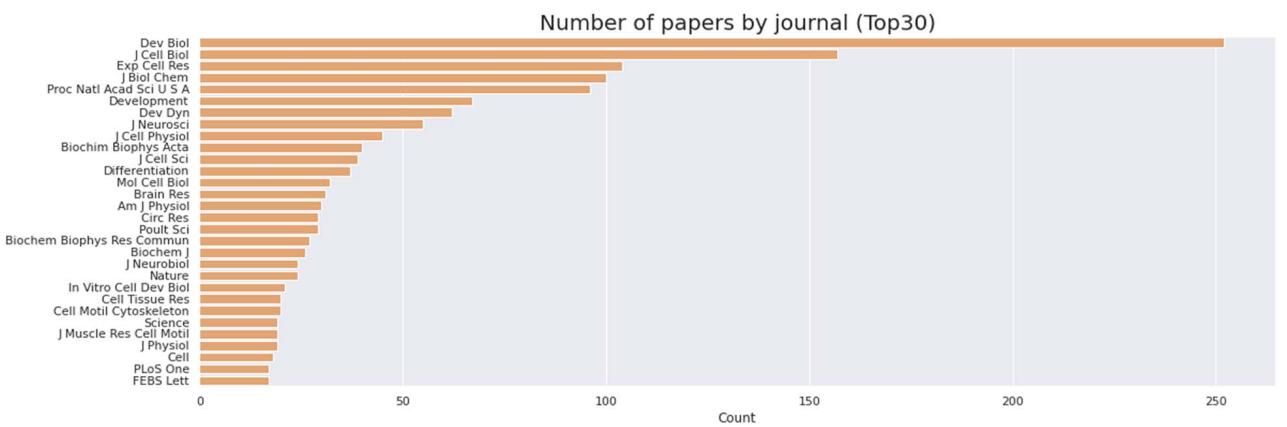

FIGURE 6 | Journals that published most papers on primary culture of embryonic chick myogenic cells. Number of papers by journal, as indicated. The retrieved PubMed dataset only included papers until the year of 2020.

in a 5% CO_2 incubator. To stop the digestion, a small volume of 8-1-0.5 medium (Minimum Essential Medium with 10% horse serum, 0.5% chick embryo extract, 1% L-glutamine, and 1% penicillin-streptomycin) is added to the sample, which is then centrifuged at 300 × g for 5 min. After removing the supernatant, the pellet is suspended in 8-1-0.5 medium and filtered through a 70 μm nylon cell strainer to produce a mononucleated cell suspension. Isolated mononucleated cells are counted using a hemocytometer chamber and then plated onto collagen-coated dishes, flasks, multi-well, or coverslips for optimal adhesion. In our experience, an initial density of 7.5 × 10^5 cells/35 mm culture dish in 2 mL of 8-1-0.5 medium is ideal for muscle differentiation. After the first 24 h after plating, cultures are fed daily with fresh 8-1-0.5 medium and kept in a humidified 5% CO_2 atmosphere at 37°C. It is important to observe muscle cultures every day under a phase contrast microscope to evaluate cell proliferation, death and density, as well as cell adhesion, elongation, myoblast fusion and the formation of multinucleated myotubes.

Initially, primary cultures of chick myogenic cells are composed by two major mononucleated cell types, myoblasts, and fibroblasts. Under phase contrast microscopy, mononucleated myoblasts are bipolar, with a central cytoplasmic expansion containing a phase dark nucleus, and two cytoplasmic processes. Differently, mononucleated fibroblasts are triangular or pleomorphic, with a less dense nucleus and a granular cytoplasm (Fear, 1977). In addition, chick myoblasts and fibroblasts can be also distinguished by their nuclear morphology and DNA content as revealed by a fluorescent nuclear probe, such as DAPI. In DAPI-stained chick myogenic cultures, muscle fibroblasts show large flattened pale nuclei, whereas myoblasts exhibit small round bright nuclei (Yamashita et al., 2017). The use of specific protein markers can also aid to identify each cell type. Myoblasts are positive for the intermediate filament

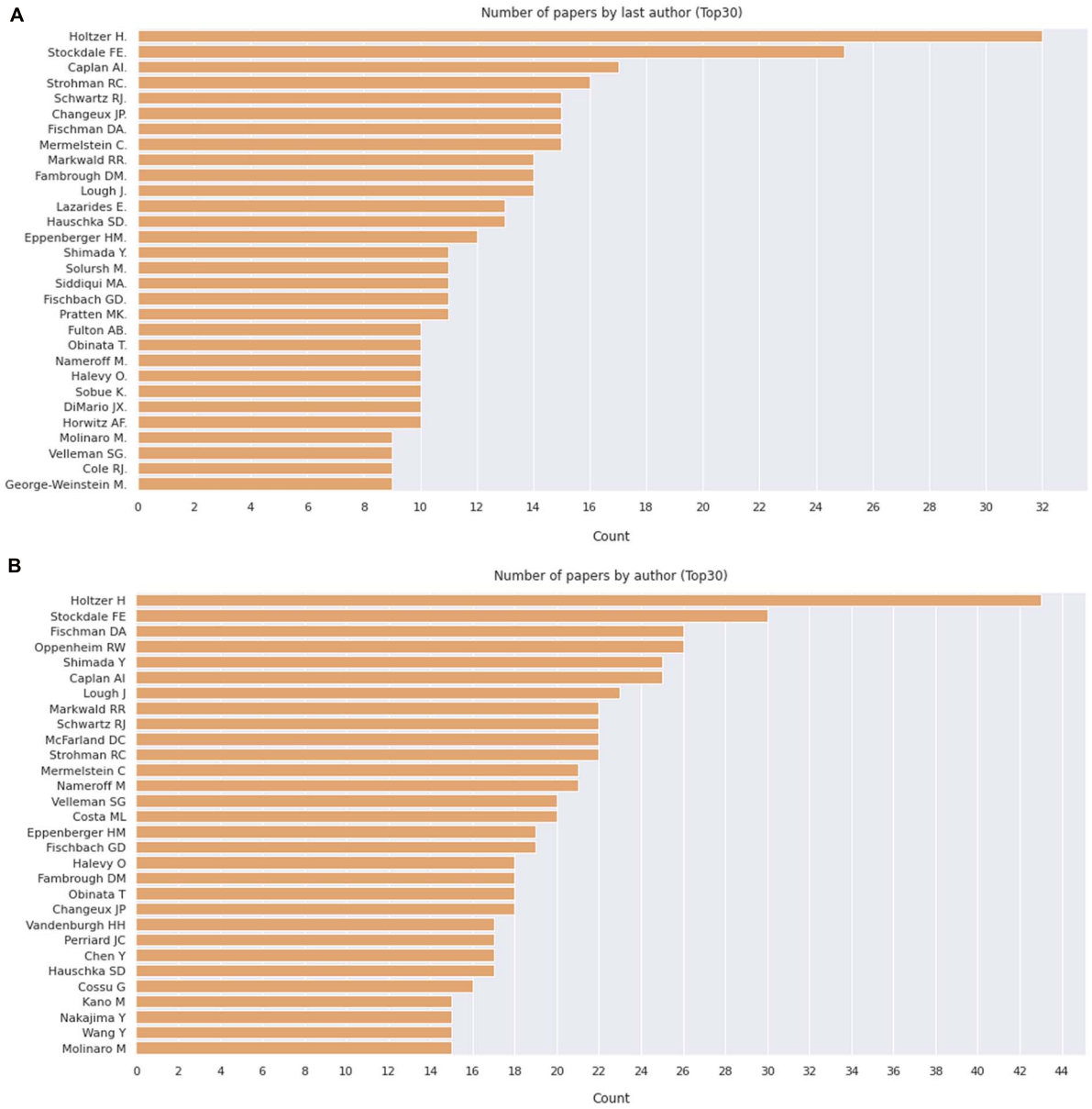

FIGURE 7 | Number of apers by last author. **(A)** Number of papers by journal, as indicated. **(B)** Pie chart depicting the relative proportion of journals grouped by the number of published papers on the subject. The retrieved PubMed dataset only included papers until the year of 2020.

desmin (Costa et al., 2004), whereas fibroblasts are negative for desmin and positive for the vesicle-associated protein flotillin-2 (Possidonio et al., 2014b). Thus, the percentage of myoblasts can be calculated by the double-staining of 24-h cultures with a nuclear dye, such as DAPI, and a muscle-specific protein marker, such as an anti-desmin antibody. Subsequently, it is only necessary to count the number of desmin-positive mononucleated cells out of the total number of cells in the field. On average, myoblasts make up 80% of each culture and non-myogenic cells comprise 20%. Additional immunolabeling with an antibody against flotillin-2 can confirm that all non-myogenic cells in these cultures are fibroblasts (Possidonio et al., 2014b). If necessary, it is possible to treat 24-h chick muscle cultures with anti-mitogenic drugs such as 1 μg/mL of arabinoside cytosine (ara-c) to reduce the number of fibroblasts. As an alternative to further decrease the number of fibroblasts in these cultures, is to use the differential adhesion characteristics of myoblasts and fibroblasts to the culture dish surface. More specifically, myoblasts are more loosely attached to the plastic surface than fibroblasts (Kaighn et al., 1966; Richler and Yaffe, 1970). Nevertheless, fibroblasts are important to muscle cell differentiation in culture, since they secrete many components of the extracellular matrix, such as collagen, laminin, and fibronectin, as well

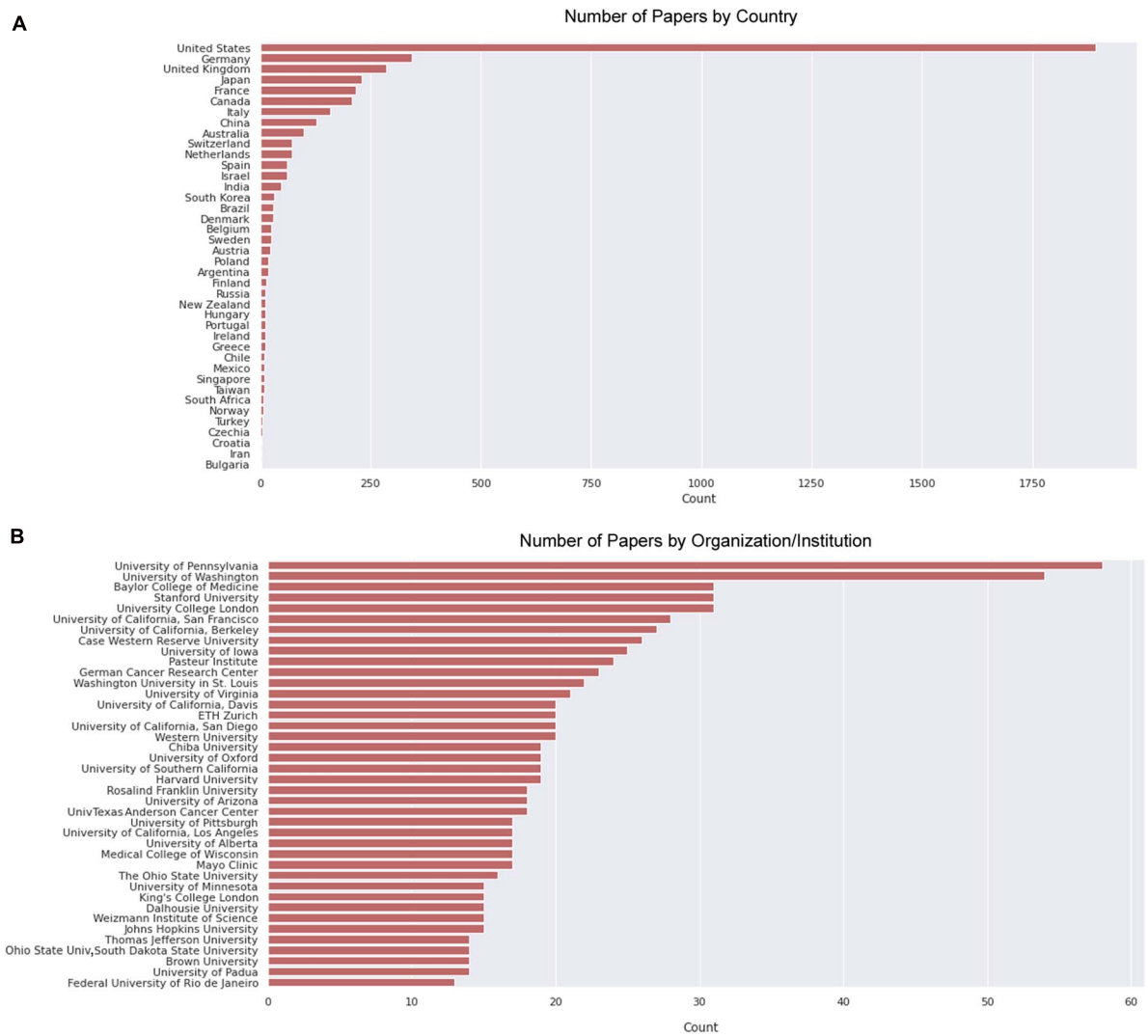

FIGURE 8 | Countries and institutions that harbored most papers on primary culture of embryonic chick myogenic cells. **(A)** Number of papers by country of the leading institution, as indicated. **(B)** Number of papers by leading institution. We defined the leading institution as the first affiliation depicted in the retrieved Dimensions dataset, which included publications until the year of 2020.

as numerous growth factors (Chapman et al., 2016). Their use in chick muscle cultures mimic the *in vivo* environment, where bundles of muscle fibers are in close association with fibroblast enriched-connective tissue (in the endomysium, perimysium, and epimysium). Chick muscle cells can grow up to 15 days in culture and after that, myotubes begin to detach from the culture dish because of their size and intense contraction.

Chick myogenic cells are platted on rat tail collagen-coated dishes. Collagen I is the main component of collagen fibers found in rat tails and it is also one of the main components found in the connective tissue layers (endomysium, perimysium, and epimysium) that envelops muscle fibers. It has been shown that collagen provides an ideal extracellular matrix substratum for the adhesion, survival, growth, and differentiation of chick muscle cells grown *in vitro* (Haba et al., 1975). Collagen I enriched solutions can be easily prepared from rat tails (either frozen or freshly isolated from rats). Briefly, rat tails (between 3 and 10) are soaked in a Balanced Salt Solution (BSS, Gibco, United States) for 24 h at 4°C. Then, they are placed in 95% ethanol solution and collagen fibers are isolated by pulling them out of the tails with the use of scissors and forceps. Finally, the isolated collagen fibers are trimmed into small pieces and immersed in 1% acetic acid solution (60–100 mL per rat tail) for 48 h under at 4°C, for the degradation of collagen fibers. This solution is then centrifuged at 5,000 rpm for 15 min and the supernatant is collected and stored in 50 mL tubes at −20°C for up to 2 years. The viscosity of the final collagen solution needs to be verified and further dilution with acetic acid are usually needed prior to use in cell dishes.

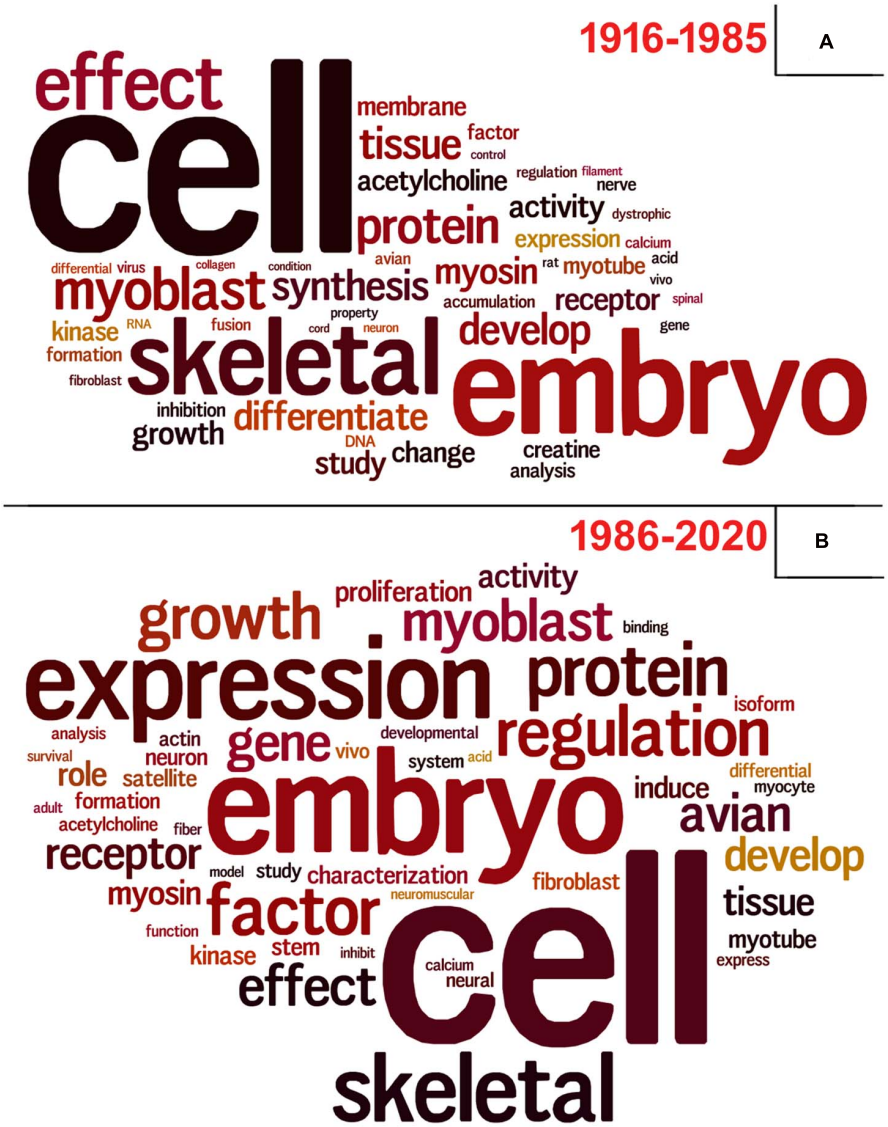

FIGURE 9 | Vocabulary analysis of the articles using chick muscle cultures. **(A)** A word cloud was generated using the titles of articles on primary culture of embryonic chick myogenic cells. This method gives greater prominence to frequent words. **(B)** Word frequency list. The retrieved PubMed dataset only included papers until the year of 2020.

A BIBLIOMETRIC GLIMPSE OF THE CONTRIBUTIONS OF CHICK CELL CULTURE TO THE STUDY OF SKELETAL MYOGENESIS

To evaluate the contribution of chicken cell culture to the field of skeletal myogenesis, we performed exploratory analyses of data retrieved from the PubMed[1] database. The query was performed on January 31, 2021 by using the following descriptors: (development OR differentiation OR myogenesis) AND (muscle OR myogenic OR muscular) AND (chick OR chicken) AND (vitro OR culture). We found a total of 3032 articles in a period that spanned the years 1916–2020 (**Figure 5**). The early years (1916–1963) exhibited only one or two articles published per year, whereas years 1964–1995 showed a continuous and significant increase in the number of publications, which reached a peak in 1995 with more than 100 papers in a single year (**Figure 5**). In turn, the years 1996–2011 revealed a decline in publications per year, until reaching a fairly regular plateau of 30–40 papers per year in between 2012 and 2020. The higher number of publications observed from 1972 to 1989 may be associated with the extensive use of chick cell culture in seminal studies of muscle development. Despite a relative drop in numbers of papers in recent years, it is relevant to note that the culture of chick muscle cells continues to be used nowadays. This trend is probably

[1]https://pubmed.ncbi.nlm.nih.gov/

related to the tweaking of new molecular and cellular biology techniques to be used in chick cell cultures. Among them, we can cite tools to study gene function through cell transfection, such as plasmids for reporter-based assays (such as TOP-Flash assay for the Wnt/beta-catenin pathway), gene overexpression, siRNA-dependent knockdown, or CRISPR-based gene editing (Mermelstein et al., 2007; Possidonio et al., 2016; Yamashita et al., 2017; Bagri et al., 2020; de Andrade Rosa et al., 2020).

We then delve into the main players of the chick muscle cell field. The scientific journals that published most of the studies using culture of chick muscle cells in the evaluated period were Developmental Biology, Journal of Cell Biology, Experimental Cell Research, Journal of Biological Chemistry, Proceedings of the National Academy of Sciences (PNAS), Development, and Developmental Dynamics. The top 30 journals that published papers on *in vitro* chick myogenesis are on the fields of developmental biology, cell biology, and biochemistry, in addition to a few multidisciplinary journals (**Figure 6**). However, more than half of the journals that communicated papers which used chick cell culture published only one paper, whereas about 9% of the identified journals were responsible for reporting more than 10 papers in the period (**Figure 6**). In addition, we examined the researchers involved in these publications. The largest contributor in number of articles using culture of chick muscle cells was Professor Howard Holtzer, from the University of Pennsylvania (United States), with 43 publications (of these, 32 as the last author; **Figure 7**). Then, Professor Frank E. Stockdale, from Stanford University (United States) also used this model in 30 studies (of these 25, as the last author). A list of the top 30 researchers that used culture of chick cells and their respective numbers can be found in **Figure 7**. The list of the top 30 researchers is in accordance with the lists of organizations/institutions and countries that have harbored the largest number of studies using chick muscle cells (**Figure 8**). Many of the researchers found in the list of major contributors of the chick muscle research field were members of the Pennsylvania Muscle Institute[2] (PMI, United States), which is an interdisciplinary group of research investigators whose goal is to discover the mechanisms of muscle function, muscle disease, muscle contraction and development.

To perform these analyzes, we used data retrieved from the Dimensions database[3] using the same descriptors as query, since PubMed did not provide this information. Lastly, we questioned ourselves what subjects/themes were most used in articles using chick muscle cell cultures. To answer this question, we gathered all the titles from the retrieved papers and examined the word frequency upon lemmatization (Peng et al., 2020; Yuhao et al., 2020). We separated our analysis in two groups: articles published between 1916–1985 and 1986–2020, to be able to compare possible word differences over the years. As expected, the used descriptors ("cell," "embryo," and "skeletal") were found among the most common words (**Figure 9**). Should we disregard them for subsequent analysis, we observed some interesting differences over the years in the use of specific words in papers in the chick muscle cell culture field. A higher frequency of cellular process-related words (e.g., "expression," "regulation," "growth," "gene," and "proliferation") was observed in the later years (1916–2020), whereas a higher frequency of words related to molecules, structures or organelles ("acetylcholine," "membrane," "creatine," "DNA," and "RNA") was observed in the early years (1986–1985) (**Figure 9**). These data points to the evolution of the muscle biology field from mostly descriptive studies toward a mechanistic understanding of muscle cell structure and function, including how different intracellular signaling pathways and networks regulate gene expression and cell physiology in muscle.

[2]https://www.med.upenn.edu/pmi/

[3]https://app.dimensions.ai/discover/publication

SUMMARY

The collection of data presented here point to the robustness of chick myoblast culture as a tool for the understanding of the role of different molecules and signaling pathways during the skeletal muscle differentiation program. We expect that by describing the major findings, and their advantages, of the studies using the embryonic chick myogenic model we will foster new studies on the molecular and cellular processes involved in muscle proliferation and differentiation that are more similar to the actual *in vivo* condition than the muscle cell lines.

AUTHOR CONTRIBUTIONS

CM wrote the manuscript. AJ and MC revised the manuscript critically. AJ, CM, and MC performed the bibliometric analysis and prepared the figures. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by Brazilian grants from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Fundação Carlos Chagas Filho de Apoio à Pesquisa do Estado do Rio de Janeiro (FAPERJ). AJ was a fellow of the Estácio de Sá University (UNESA) Research Productivity Program and Institutos Nacionais de Ciência e Tecnologia (INCT/CAPES, 88887.568853/2020-00).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2021.668600/full#supplementary-material

Supplementary Figure 1 | Myoblast fusion dynamics. Movie showing several myoblasts in a row during fusion in a 24-h chick myogenic culture. Note that fusion tended to follow end-to-end rather than side-to-side alignment of myoblasts.

REFERENCES

Abrunhosa, V. M., Soares, C. P., Batista Possidonio, A. C., Alvarenga, A. V., Costa-Felix, R. P., Costa, M. L., et al. (2014). Induction of skeletal muscle differentiation in vitro by therapeutic ultrasound. *Ultrasound. Med. Biol.* 40, 504–512. doi: 10.1016/j.ultrasmedbio.2013.10.013

Almenar-Queralt, A., Gregorio, C. C., and Fowler, V. M. (1999). Tropomodulin assembles early in myofibrillogenesis in chick skeletal muscle: evidence that thin filaments rearrange to form striated myofibrils. *J. Cell Sci.* 112, 1111–1123.

Baek, H. J., Jeon, Y. J., Kim, H. S., Kang, M. S., Chung, C. H., and Há, D. B. (1994). Cyclic AMP negatively modulates both Ca2+/calmodulin-dependent phosphorylation of the 100-kDa protein and membrane fusion of chick embryonic myoblasts. *Dev. Biol.* 165, 178–184. doi: 10.1006/dbio.1994.1244

Bagri, K. M., Rosa, I. A., Corrêa, S., Yamashita, A., Brito, J., Bloise, F., et al. (2020). Acidic compartment size, positioning, and function during myogenesis and their modulation by the Wnt/beta-catenin pathway. *Biomed. Res. Int.* 2020:6404230. doi: 10.1155/2020/6404230

Bar-Sagi, D., and Prives, J. (1983). Trifluoperazine, a calmodulin antagonist, inhibits muscle cell fusion. *J. Cell Biol.* 97, 1375–1380. doi: 10.1083/jcb.97.5.1375

Bastos, U. M. C., de Andrade Rosa, I., Teixeira, J. D., Gonçalves, G., Costa, M. L., Quintas, L. E. M., et al. (2019). Isoproterenol induces an increase in muscle fiber size by the proliferation of Pax7-positive cells and in a mTOR-independent mechanism. *Cell Biol. Int.* 43, 1425–1434. doi: 10.1002/cbin.11190

Bentzinger, C. F., Wang, Y. X., and Rudnicki, M. A. (2012). Building muscle: molecular regulation of myogenesis. *Cold Spring Harb. Perspect. Biol.* 4:2. doi: 10.1101/cshperspect.a008342

Bi, P., Ramirez-Martinez, A., Li, H., Cannavino, J., McAnally, J. R., Shelton, J. M., et al. (2017). Control of muscle formation by the fusogenic micropeptide myomixer. *Science* 356, 323–327. doi: 10.1126/science.aam9361

Bryantsev, A. L., Castillo, L., Oas, S. T., Chechenova, M. B., Dohn, T. E., and Lovato, T. L. (2019). Myogenesis in Drosophila melanogaster: dissection of distinct muscle types for molecular analysis. *Methods Mol. Biol.* 1889, 267–281. doi: 10.1007/978-1-4939-8897-6_16

Buckingham, M., Bajard, L., Chang, T., Daubas, P., Hadchouel, J., Meilhac, S., et al. (2003). The formation of skeletal muscle: from somite to limb. *J. Anat.* 202, 59–68. doi: 10.1046/j.1469-7580.2003.00139.x

Buffolo, M., Batista Possidonio, A. C., Mermelstein, C., and Araujo, H. (2015). A conserved role for calpains during myoblast fusion. *Genesis* 53, 417–430. doi: 10.1002/dvg.22870

Capers, C. R. (1960). Multinucleation of skeletal muscle in vitro. *J. Biophys. Biochem. Cytol.* 7, 559–566. doi: 10.1083/jcb.7.3.559

Chal, J., and Pourquié, O. (2017). Making muscle: skeletal myogenesis in vivo and in vitro. *Development* 144, 2104–2122. doi: 10.1242/dev.151035

Chapman, M. A., Meza, R., and Lieber, R. L. (2016). Skeletal muscle fibroblasts in health and disease. *Differentiation* 92, 108–115. doi: 10.1242/dmm.042192

Chen, B., You, W., Wang, Y., and Shan, T. (2020). The regulatory role of myomaker and myomixer-myomerger-minion in muscle development and regeneration. *Cell Mol. Life Sci.* 77, 1551–1569. doi: 10.1007/s00018-019-03341-9

Choi, J., Costa, M. L., Mermelstein, C. S., Chagas, C., Holtzer, S., and Holtzer, H. (1990). MyoD converts primary dermal fibroblasts, chondroblasts, smooth muscle, and retinal pigmented epithelial cells into striated mononucleated myoblasts and multinucleated myotubes. *Proc. Natl. Acad. Sci. U.S.A.* 87, 7988–7992. doi: 10.1073/pnas.87.20.7988

Christ, B., and Ordahl, C. P. (1995). Early stages of chick somite development. *Anat. Embryol. (Berl)* 191, 381–396. doi: 10.1007/BF00304424

Cohen, R., Pacifici, M., Rubinstein, N., Biehl, J., and Holtzer, H. (1977). Effect of a tumour promoter on myogenesis. *Nature* 266, 538–540. doi: 10.1038/266538a0

Cooper, W. G., and Konigsberg, I. R. (1961). Dynamics of myogenesis in vitro. *Anat. Rec.* 140, 195–205. doi: 10.1002/ar.1091400305

Cornell, R. B., Nissley, S. M., and Horwitz, A. F. (1980). Cholesterol availability modulates myoblast fusion. *J. Cell Biol.* 86, 820–824. doi: 10.1083/jcb.86.3.820

Cossu, G., Tajbakhsh, S., and Buckingham, M. (1996). How is myogenesis initiated in the embryo? *Trends Genet.* 12, 218–223. doi: 10.1016/0168-9525(96)10025-1

Costa, M. L. (2014). Cytoskeleton and adhesion in myogenesis. *ISRN Dev. Biol.* 2014, 1–15.

Costa, M. L., Escaleira, R. C., Cataldo, A., Oliveira, F., and Mermelstein, C. S. (2004). Desmin: molecular interactions and putative functions of the muscle intermediate filament protein. *Braz. J. Med. Biol. Res.* 37, 1819–1830. doi: 10.1590/s0100-879x2004001200007

Davis, R. L., Weintraub, H., and Lassar, A. B. (1987). Expression of a single transfected cDNA converts fibroblasts to myoblasts. *Cell* 51, 987–1000. doi: 10.1016/0092-8674(87)90585-x

de Andrade Rosa, I., Corrêa, S., Costa, M. L., and Mermelstein, C. (2020). The scaffolding protein calpain-3 has multiple distributions in embryonic chick muscle cells and it is essential for the formation of muscle fibers. *Tissue Cell* 67:101436. doi: 10.1016/j.tice.2020.101436

Di Gioia, S. A., Connors, S., Matsunami, N., Cannavino, J., Rose, M. F., Gilette, N. M., et al. (2017). A defect in myoblast fusion underlies Carey-Fineman-Ziter syndrome. *Nat. Commun.* 8:16077.

Elhanany-Tamir, H., Yu, Y. V., Shnayder, M., Jain, A., Welte, M., and Volk, T. (2012). Organelle positioning in muscles requires cooperation between two KASH proteins and microtubules. *J. Cell Biol.* 198, 833–846. doi: 10.1083/jcb.201204102

Elia, D., Madhala, D., Ardon, E., Reshef, R., and Halevy, O. (2007). Sonic hedgehog promotes proliferation and differentiation of adult muscle cells: Involvement of MAPK/ERK and PI3K/Akt pathways. *Biochim. Biophys. Acta.* 1773, 1438–1446. doi: 10.1016/j.bbamcr.2007.06.006

Englander, L. L., and Rubin, L. L. (1987). Acetylcholine receptor clustering and nuclear movement in muscle fibers in culture. *J. Cell Biol.* 104, 87–95. doi: 10.1083/jcb.104.1.87

Fear, J. (1977). Observations on the fusion of chick embryo myoblasts in culture. *J. Anat.* 124, 437–444.

Folker, E. S., and Baylies, M. K. (2013). Nuclear positioning in muscle development and disease. *Front. Physiol.* 4:363. doi: 10.3389/fphys.2013.00363

Goichberg, P., Shtutman, M., Ben-Ze'ev, A., and Geiger, B. (2001). Recruitment of beta-catenin to cadherin-mediated intercellular adhesions is involved in myogenic induction. *J. Cell Sci.* 114, 1309–1319.

Gupta, D., Santoso, J. W., and McCain, M. L. (2021). Characterization of gelatin hydrogels cross-linked with microbial transglutaminase as engineered skeletal muscle substrates. *Bioengineering (Basel)* 8, 6. doi: 10.3390/bioengineering8010006

Haba, G. L., Cooper, G. W., and Elting, V. (1966). Hormonal requirements for myogenesis of striated muscle in vitro: insulin and somatotropin. *Proc. Nat. Acad. Sci. U.S.A.* 56, 1719–1723. doi: 10.1073/pnas.56.6.1719

Haba, G. L., Kamali, H. M., and Tiede, D. M. (1975). Myogenesis of avian striated muscle in vitro: role of collagen in myofiber formation. *Proc. Natl. Acad. Sci. U.S.A.* 72, 2729–2732. doi: 10.1073/pnas.72.7.2729

Hill, C. S., Duran, S., Lin, Z. X., Weber, K., and Holtzer, H. (1986). Titin and myosin, but not desmin, are linked during myofibrillogenesis in postmitotic mononucleated myoblasts. *J. Cell Biol.* 103, 2185–2196. doi: 10.1083/jcb.103.6.2185

Hirayama, E., Sasao, N., Yoshimasu, S., and Kim, J. (2001). K252a, an indrocarbazole derivative, causes the membrane of myoblasts to enter a fusion-capable state. *Biochem. Biophys. Res. Commun.* 285, 1237–1243. doi: 10.1006/bbrc.2001.5327

Holaska, J. M., Rais-Bahrami, S., and Wilson, K. L. (2006). Lmo7 is an emerin-binding protein that regulates the transcription of emerin and many other muscle-relevant genes. *Hum. Mol. Genet.* 15, 3459–3472.

Holtzer, H., Dilullo, C., Costa, M. L., Lu, M., Choi, J., Mermelstein, C. S., et al. (1991). "Striated myoblasts and multinucleated myotubes induced in non-muscle cells by MyoD are similar to normal in vivo and in vitro counterparts," in *Frontiers in Muscle Research*, eds E. Ozawa, T. Masaki, and Y. Nabeshima (Amsterdam: Elsevier), 187–207.

Ikeya, M., and Takada, S. (1998). Wnt signaling from the dorsal neural tube is required for the formation of the medial dermomyotome. *Development* 125, 4969–4976.

Ilangumaran, S., and Hoessli, D. C. (1998). Effects of cholesterol depletion by cyclodextrin on the sphingolipid microdomains of the plasma membrane. *Biochem. J.* 335, 433–440. doi: 10.1042/bj3350433

Kaighn, M. E., Ebert, J. D., and Stott, P. M. (1966). The susceptibility of differentiating muscle clones to *Rous sarcoma* virus. *Anatomy* 56, 133–139. doi: 10.1073/pnas.56.1.133

Knudsen, K. A., and Horwitz, A. F. (1977). Tandem events in myoblast fusion. *Dev. Biol.* 58, 328–338. doi: 10.1016/0012-1606(77)90095-1

Konigsberg, I. R. (1963). Clonal analysis of myogenesis. *Science* 140, 1273–1284. doi: 10.1126/science.140.3573.1273

Lawson, M. A., and Purslow, P. P. (2000). Differentiation of myoblasts in serum-free media: effects of modified media are cell line-specific. *Cells Tissues Organs* 167, 130–137. doi: 10.1159/000016776

Lin, Z., Lu, M. H., Schultheiss, T., Choi, J., Holtzer, S., DiLullo, C., et al. (1994). Sequential appearance of muscle-specific proteins in myoblasts as a function of time after cell division: evidence for a conserved myoblast differentiation program in skeletal muscle. *Cell Motil. Cytoskeleton* 29, 1–19. doi: 10.1002/cm.970290102

Liu, J., Huang, Z. P., Nie, M., Wang, G., Silva, W. J., Yang, Q., et al. (2020). Regulation of myonuclear positioning and muscle function by the skeletal muscle-specific CIP protein. *Proc. Natl. Acad. Sci. U.S.A.* 117, 19254–19265. doi: 10.1073/pnas.1922911117

Luo, W., Li, E. X., Nie, Q. H., and Zhang, X. Q. (2015). Myomaker, regulated by MYOD, MYOG and miR-140-3p, promotes chicken myoblast fusion. *Int. J. Mol. Sci.* 16, 26186–26201.

Maltzahn, J. V., Chang, N. C., Bentzinger, C. F., and Rudnicki, M. A. (2012). Wnt Signaling in Myogenesis. *Trends Cell Biol.* 22, 602–609. doi: 10.1016/j.tcb.2012.07.008

Mermelstein, C. S., Andrade, L. R., Portilho, D. M., and Costa, M. L. (2006). Desmin filaments are stably associated with the outer nuclear surface in chick myoblasts. *Cell Tissue Res.* 323, 351–357. doi: 10.1007/s00441-005-0063-6

Mermelstein, C. S., Costa, M. L., Chagas Filho, C., and Moura Neto, V. (1996). Intermediate filament proteins in TPA-treated skeletal muscle cells in culture. *J. Muscle Res. Cell Motil.* 17, 199–206. doi: 10.1007/BF00124242

Mermelstein, C. S., Amaral, L. M., Rebello, M. I. L., Reis, J. S. N., Borojevic, R., and Costa, M. L. (2005a). Changes in cell shape and desmin intermediate filament distribution are associated with down-regulation of desmin expression in C2C12 myoblasts grown in the absence of extracellular Ca^{2+}. *Braz. J. Med. Biol. Res.* 38, 1025–1032. doi: 10.1590/s0100-879x2005000700005

Mermelstein, C. S., Portilho, D. M., Medeiros, R. B., Matos, A. R., Einicker-Lamas, M., Tortelote, G. G., et al. (2005b). Cholesterol depletion by methyl-β-cyclodextrin enhances myoblast fusion and induces the formation of myotubes with disorganized nuclei. *Cell Tissue Res.* 319, 289–297. doi: 10.1007/s00441-004-1004-5

Mermelstein, C. S., Portilho, D. M., Mendes, F. A., Costa, M. L., and Abreu, J. G. (2007). Wnt/beta-catenin pathway activation and myogenic differentiation are induced by cholesterol depletion. *Differentiation* 75, 184–192. doi: 10.1111/j.1432-0436.2006.00129.x

Millay, D. P., O'Rourke, J. R., Sutherland, L. B., Bezprozvannaya, S., Shelton, J. M., Bassel-Duby, R., et al. (2013). Myomaker is a membrane activator of myoblast fusion and muscle formation. *Nature* 499, 301–305. doi: 10.1038/nature12343

Nakanishi, M., Hirayama, E., and Kim, J. (2001). Characterization of myogenic cell membrane: II. Dynamic changes in membrane lipids during the differentiation of mouse C2 myoblast cells. *Cell Biol. Int.* 25, 971–979. doi: 10.1006/cbir.2001.0750

Neff, N., Decker, C., and Horwitz, A. (1984). The kinetics of myoblast fusion. *Exp. Cell Res.* 153, 25–31. doi: 10.1016/0014-4827(84)90444-0

Ojima, K., Lin, Z. X., Andrade, I. R., Costa, M. L., and Mermelstein, C. (2016). Distinctive effects of cytochalasin b in chick primary myoblasts and fibroblasts. *PLoS One* 11:e0154109. doi: 10.1371/journal.pone.0154109

Okazaki, K., and Holtzer, H. (1966). Myogenesis: fusion, myosin synthesis, and the mitotic cycle Proc. *Natl. Acad. Sci. U.S.A.* 56, 1484–1490. doi: 10.1073/pnas.56.5.1484

Oliveira, T. N., Possidonio, A. C., Soares, C. P., Ayres, R., Costa, M. L., Quintas, L. E., et al. (2015). The role of Na+/K+-ATPase during chick skeletal myogenesis. *PLoS One* 10:e0120940. doi: 10.1371/journal.pone.0120940

Paterson, B., and Strohman, R. C. (1972). Myosin synthesis in cultures of differentiating chicken embryo skeletal muscle. *Dev. Biol.* 29, 113–138. doi: 10.1016/0012-1606(72)90050-4

Peng, Q., Yuhao, Z., Yuhui, Z., Jason, B., and Christopher, D. M. (2020). "Stanza: a python natural language processing toolkit for many human languages," in *Proceedings of the 58th Annual Meeting of the Association for Computational Linguistics: System Demonstrations* (Stroudsburg, PA: Association for Computational Linguistics), 101–108.

Portilho, D. M., Martins, E. R., Costa, M. L., and Mermelstein, C. S. (2007). A soluble and active form of Wnt3a protein is involved in myogenic differentiation after cholesterol depletion. *FEBS Lett.* 581, 5787–5795. doi: 10.1016/j.febslet.2007.11.047

Portilho, D. M., Soares, C. P., Morrot, A., Thiago, L. S., Butler-Browne, G., Savino, W., et al. (2012). Cholesterol depletion by methyl-β-cyclodextrin enhances cell proliferation and increases the number of desmin-positive cells in myoblast cultures. *Eur. J. Pharmacol.* 694, 1–12. doi: 10.1016/j.ejphar.2012.07.035

Possidonio, A. C., Miranda, M., Gregoracci, G. B., Thompson, F. L., Costa, M. L., and Mermelstein, C. (2014a). Cholesterol depletion induces transcriptional changes during skeletal muscle differentiation. *BMC Genomics* 15:544. doi: 10.1186/1471-2164-15-544

Possidonio, A. C., Senna, M. L., Portilho, D. M., Pontes Soares, C., da Silva Sampaio, L., Einicker-Lamas, M., et al. (2011). α-Cyclodextrin enhances myoblast fusion and muscle differentiation by the release of IL-4. *Cytokine* 55, 280–287. doi: 10.1016/j.cyto.2011.04.018

Possidonio, A. C., Soares, C. P., Fontenele, M., Morris, E. R., Mouly, V., Costa, M. L., et al. (2016). Knockdown of Lmo7 inhibits chick myogenesis. *FEBS Lett.* 590, 317–329. doi: 10.1002/1873-3468.12067

Possidonio, A. C., Soares, C. P., Portilho, D. M., Midlej, V., Benchimol, M., Butler-Browne, G., et al. (2014b). Differences in the expression and distribution of flotillin-2 in chick, mice and human muscle cells. *PLoS One* 9:e103990. doi: 10.1371/journal.pone.0103990

Richler, C., and Yaffe, D. (1970). The in vitro cultivation and differentiation capacities of myogenic cell lines. *Dev. Biol.* 23, 1–22. doi: 10.1016/s0012-1606(70)80004-5

Rosa de Andrade, I., Corrêa, S., Fontenele, M., de Oliveira Teixeira, J. D., Abdelhay, E., Costa, M. L., et al. (2018). γ-Secretase inhibition induces muscle hypertrophy in a notch-independent mechanism. *Proteomics* 18:1700423. doi: 10.1002/pmic.201700423

Sampath, S. C., Sampath, S. C., and Millay, D. P. (2018). Myoblast fusion confusion: the resolution begins. *Skeletal. Muscle* 8:3. doi: 10.1186/s13395-017-0149-3

Sanger, J. W., Wang, J., Fan, Y., White, J., and Sanger, J. M. (2010). Assembly and dynamics of myofibrils. *J. Biomed. Biotechnol.* 2010:858606. doi: 10.1155/2010/858606

Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., et al. (2012). Fiji: an open-source platform for biological-image analysis. *Nat. Methods* 9, 676–682. doi: 10.1038/nmeth.2019

Sekiya, T., Takenawa, T., and Nozawa, Y. (1984). Reorganization of membrane cholesterol during membrane fusion in myogenesis in vitro: a study using the filipin-cholesterol complex. *Cell Struct. Funct.* 9, 143–155. doi: 10.1247/csf.9.143

Shimada, Y. (1971). Electron microscope observations on the fusion of chick myoblasts in vitro. *J. Cell Biol.* 48, 128–142. doi: 10.1083/jcb.48.1.128

Sieiro, D., Véron, N., and Marcelle, C. (2017). The chicken embryo as an efficient model to test the function of muscle fusion genes in amniotes. *PLoS One* 12:e0177681. doi: 10.1371/journal.pone.0177681

Simons, K., and Sampaio, J. L. (2011). Membrane organization and lipid rafts. *Cold Spring Harb. Perspect. Biol.* 3:a004697. doi: 10.1101/cshperspect.a004697

Taubman, M. B., Smith, C. W., Izumo, S., Grant, J. W., Endo, T., Andreadis, A., et al. (1989). The expression of sarcomeric muscle-specific contractile protein genes in BC3H1 cells: BC3H1 cells resemble skeletal myoblasts that are defective for commitment to terminal differentiation. *J. Cell Biol.* 108, 1799–1806. doi: 10.1083/jcb.108.5.1799

Teixeira, J. D., de Andrade Rosa, I., Brito, J., Maia de Souza, Y. R., Paulo, de Abreu Manso, P., et al. (2018). Sonic Hedgehog signaling and Gli-1 during embryonic chick myogenesis. *Biochem. Biophys. Res. Commun.* 507, 496–502. doi: 10.1016/j.bbrc.2018.11.071

van der Bosch, J., Schudt, C., and Pette, D. (1973). Influence of temperature, cholesterol, dipalmitoyllecithin and Ca2+ on the rate of muscle cell fusion. *Exp. Cell Res.* 82, 433–438. doi: 10.1016/0014-4827(73)90362-5

Volk, T. (2013). Positioning nuclei within the cytoplasm of striated muscle fiber: cooperation between microtubules and KASH proteins. *Nucleus Austin* 4, 18–22.

Wang, H., Noulet, F., Edom-Vovard, F., Le Grand, F., and Duprez, D. (2010). Bmp signaling at the tips of skeletal muscles regulates the number of fetal muscle progenitors and satellite cells during development. *Dev. Cell.* 18, 643–654. doi: 10.1016/j.devcel.2010.02.008

Wang, J., Fan, Y., Dube, S., Agassy, N. W., Dube, D. K., Sanger, J. M., et al. (2020). Myofibril assembly and the roles of the ubiquitin proteasome system. *Cytoskeleton* 77, 456–479. doi: 10.1002/cm .21641

Yamashita, A. M. S., Ancillotti, M. T. C., Rangel, L. P., Fontenele, M., Figueiredo-Freitas, C., Possidonio, A. C., et al. (2017). Balance between S-nitrosylation and denitrosylation modulates myoblast proliferation independently of soluble guanylyl cyclase activation. *Am. J. Physiol. Cell Physiol.* 313, C11–C26. doi: 10.1152/ajpcell.00140. 2016

Yuhao, Z., Yuhui, Z., Peng, Q., Christopher, D. M., and Curtis, P. L. (2020). Biomedical and clinical english model packages in the stanza python nlp library. *arXiv* [Preprint]. arXiv:2004.14530.

Zeschnigk, M., Kozian, D., Kuch, C., Schmoll, M., and Starzinski-Powitz, A. (1995). Involvement of M-cadherin in terminal differentiation of skeletal muscle cells. *J. Cell Sci.* 108, 2973–2981.

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Physiology

ORIGINAL RESEARCH
published: 07 June 2021
doi: 10.3389/fphys.2021.678610

Increasing Fracture Risk Associates With Plasma Circulating MicroRNAs in Aging People's Sarcopenia

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Aging generally coincides with a gradual decline in mass and strength of muscles and bone mineral density (BMD). Sarcopenia is closely linked to osteoporosis in the elderly, which can lead to abnormal gait, balance disorders, and dysfunctions, as well as increase in the risks of falls, fractures, weakness, and death. MicroRNAs (miRNAs, miRs) are a kind of short and non-coding RNA molecules but can regulate posttranscriptional protein expression. However, we have known little about their participation in age-associated osteoporosis and sarcopenia. The current study aims to confirm those miRNAs as biomarkers for age-related reduction in muscular atrophy associated with human blood fractures. In our study, 10 fracture-risk-related miRNAs (miR-637, miR-148a-3p, miR-125b-5p, miR-124-3p, miR-122-5p, miR-100-5p, miR-93-5p, miR-21-5p, miR-23a-3p, and miR-24-3p) were analyzed. For the initial screening, we determined the abundance of fracture-risk-associated miRNAs by RT-PCR most frequently detected in enrolled 93 elderly with sarcopenia and non-sarcopenia, respectively. Statistically, the relative expression levels of plasma miR-23a-3p, miR-93-5p, and miR-637 in the sarcopenia group were significantly lower than that in the non-sarcopenia group, while the levels of other miRNAs did not change significantly. Moreover, we showed that the levels of ASM/height^2, handgrip strength, and 4-m velocity in the sarcopenia group were significantly lower than in the non-sarcopenia group. Whereafter, we expanded the sample for further detection and analysis and revealed that the levels of plasma miR-23a-3p, miR-93-5p, and miR-637 in the sarcopenia group were significantly lower than that in the non-sarcopenia group, which is consistent with the initial screening experiment. From our analysis, changes in levels of plasma miR-93-5p and miR-637 were dramatically related to ASM/height^2. Furthermore, changes in miR-23a and miR-93-5p were significantly affected by ASM/height^2 in female individuals, with no significant correlations between miRNAs changes and these diagnostic indexes in male individuals after adjusting sex. The study showed that plasma miRNAs changed in an aging-related sarcopenia manner and were associated with increased fracture risk. In aging patients, plasma miR-23a-3p, miR-93-5p, and miR-637 have the potential as biomarkers of sarcopenia, which can affect the development of physiological dysfunction and may be also used in the fracture risk assessment of these patients.

Keywords: sarcopenia, circulating microRNAs, fracture, aging, plasma

OPEN ACCESS

Edited by:
Anselmo Sigari Moriscot, University of São Paulo, Brazil

Reviewed by:
Flavia Fonseca Bloise, Federal University of Rio de Janeiro, Brazil
Morteza Taheri, Imam Khomeini International University, Iran

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Specialty section:
This article was submitted to Exercise Physiology, a section of the journal Frontiers in Physiology

Received: *10 March 2021*
Accepted: *26 April 2021*
Published: *07 June 2021*

Citation:
He N, Zhang Y, Zhang Y, Feng B, Zheng Z, Wang D, Zhang S and Ye H (2021) Increasing Fracture Risk Associates With Plasma Circulating MicroRNAs in Aging People's Sarcopenia. Front. Physiol. 12:678610. doi: 10.3389/fphys.2021.678610

INTRODUCTION

Sarcopenia is a common geriatric syndrome (Marzetti et al., 2017; Larsson et al., 2019; Tournadre et al., 2019). In normal aging, a gradual decline occurs after a maximum of about 30 years of age and a decline of 20–40% annually after 40 years of age, leading to corresponding muscle dysfunction, which also can be considered as one of the indicators of frailty in the elderly. Currently, sarcopenia is defined as a clinical syndrome related to aging-related loss such as skeletal muscle mass, strength, and function, which will cause an increase in the risk of occurring physical disability, poor quality of life, and mortality (Landi et al., 2018). Sarcopenia has widespread seriousness of harm, which will lead to abnormal gait, balance disorders, and incapacitation, with an increased risk of falls, fractures, weakness, and death in the elderly (Yang et al., 2019). It also can be regarded as a factor of risk for poor prognosis of some chronic diseases such as cirrhosis, type 2 diabetes, and tumor (Angulo et al., 2016). Therefore, effective measures should be taken to slow down or even reverse the progression of sarcopenia in the elderly to the occurrence of adverse clinical outcomes, with the improvement in patients' quality of life. Exercise is positive to the muscle strength and body function in the elderly. Nutritional supplementation can improve the exercise effect of healthy people and can improve the effect of an exercise intervention on sarcopenia. Given the positive role of resistance exercise on human muscle mass, more and more studies have added resistance exercise to the treatment of sarcopenia. A study showed that one cycle (12–16 weeks) of resistance training increased the subjects' thigh circumference by 11.4% and muscle volume by 3.8% (Van Roie et al., 2013). What is more exciting, plenty of studies have proved that muscle growth caused by resistance exercise can occur at any age, even elderly people in their 1990s (Hunter et al., 2000; Westcott, 2009). According to Khadijeh et al., exercise in water can improve muscular balance and muscle strength and provide appropriate postural mobility without the fear of falling in older adults with inappropriate body shapes (Irandoust et al., 2018). Studies have shown that regular physical activity can reduce abdominal fat intake, improve musculoskeletal control, reduce lower back pain, and improve the quality of balance and walking speed in the elderly (Irandoust et al., 2018, 2019). Similarly, pilates exercise can decrease body fat mass and improve muscle atrophy, balance, and walking speed in inactive middle-aged women (Seghatoleslami et al., 2018). It was shown that skeletal muscle atrophy and skeletal muscle strength were significantly improved in elderly patients with sarcopenia after adequate vitamin D and amino acid treatment (Bauer et al., 2015). It is now thought that nutrition combined with exercise can better improve muscle strength and function. Studies have found that combining resistance exercise with protein and vitamin D supplements is the most effective way to improve sarcopenia or myasthenia in the elderly (Anton et al., 2018; Yamada et al., 2019; Eshaghi et al., 2020). However, the complex pathogenesis of sarcopenia and numerous influencing factors have not been fully understood.

As the society gets aging, the prevalence of musculoskeletal diseases is growing rapidly, showing that bone is closely related to muscle tissue: both of them are not only adjacent to the anatomical location but also have common paracrine and endocrine regulation, similar molecular signal regulation pathways, and common therapeutic targets and drugs (Bonewald et al., 2013; Girgis, 2015), which are biologically and functionally in line with increasing the risks of fracture in the elderly (Wong et al., 2019). Aging is involved in the loss of bone and muscle functions (Laurent et al., 2019). In particular, losing mass, strength, and function of muscles allied to aging significantly increases the risks in causing osteoporosis and fractures; furthermore, age-related decline in bone strength will also significantly increase the incidence of sarcopenia (Edwards et al., 2015; Oliveira and Vaz, 2015). The increased risk in causing fracture in patients with sarcopenia and osteoporosis is due to decreased muscle mass and strength, decreased bone density, and limited movement (Tarantino et al., 2016; Steihaug et al., 2017). Elderly people with sarcopenia are three times more likely to fall, and there is growing evidence that sarcopenia is closely linked to fractures (Marques and Queirós, 2018; Yeung et al., 2019). On top of that, there have been recent reports of a high prevalence of sarcopenia in patients with fractures, which is alarming for clinicians. However, there are currently still few clinical data to suggest that there is a causal relation between osteoporosis and sarcopenia.

MicroRNAs (miRNAs) are endogenous, non-coding RNAs that are 19–25 nucleotides long, which are able to achieve negative regulation of gene expressing at the posttranscription level through degradation or translational inhibition of messenger RNA (mRNA) (Saliminejad et al., 2019). A single miRNA, as a central regulator of gene networks, can directly or indirectly regulate the expression of hundreds of gene targets, which may be regulated by a suite of miRNAs as well. miRNAs are critical in various physiological and pathological processes including sarcopenia with its deregulation (Soriano-Arroquia et al., 2016; Yin et al., 2020). Interestingly, miRNAs are very stable and can be easily detected in blood circulation (e.g., plasma), where the profile of miRNAs can reflect health status, suggesting that circulating miRNAs (c-miRNAs) are considered as new, potential, and even more sensitive biomarkers for disease diagnosis and related treatment (Kosaka et al., 2010; Zendjabil et al., 2017; Gareev et al., 2020), whose signature for patients with osteoporotic fractures has been reported. The level of one of these osteoporosis-associated miR-21-5p and other nine related ones has been predicted to be fracture risk in patients with osteoporosis more recently (Seeliger et al., 2014; Panach et al., 2015; Kocijan et al., 2016; Kelch et al., 2017). It has recently been reported that muscles and bones are regulated by many common genes, endocrine regulatory networks, and signaling pathways. These common molecular regulations can significantly increase the incidence of sarcopenia and osteoporosis in the elderly, which often coexist (Chen et al., 2019; Reiss et al., 2019). Considering that bone and muscle are closely related to mechanotransduction and metabolic signaling, we can reasonably assume that sarcopenia can be assessed by measuring bone-specific c-miRNAs which can reflect resorption and formation of bones. Nevertheless, the regulation of c-miRNA in osteoporosis, fractures, tendonopenia, and a variety of other human conditions has not been totally understood yet.

Finally, considering the link between bone and muscle in mechanotransduction and metabolic signaling, osteoporosis and sarcopenia are both key factors in fractures, which may be useful to contain sarcopenia status in fracture risk model. Thus, this study is mainly to explore the relationship between well-functioning specific circulating miRNAs and reduced muscular atrophy and fracture risk in the elderly, and second, it is to determine the associations between these specific c-miRNAs and musculoskeletal variables. Specifically, we identified changes in circulating miRNA levels in the elderly with and without nodular reduction. More longitudinal studies are needed to assess whether circulating miR-23a-3p, miR-93-5p, and miR-637 have the potential as biomarkers of sarcopenia in the elderly.

MATERIALS AND METHODS

Study Population

In the present study, participants were chosen from Ximen Community of Ningbo, China. There were 1,047 elderly people aged 65 years old or more who were involved in our examination and completed a comprehensive geriatric assessment from November 2016 to March 2017. Inclusion criteria were the following: people aged 65 years old or more who were eligible to participate and people who can independently finish a comprehensive geriatric assessment, including tests of walking speed, grip strength and muscle mass. Exclusion criteria were the following: people refusing to take part in this study, people failing to complete the items that they were required to be inspected independently, and people who were aged <65 years old. Based on the above criteria, participants with the record of hand strength, 4-m speed or body composition (n = 28), or measurement of baPWV (n = 13) or the filled questionnaire (n = 4) were excluded. Thus, a total of 1,002 participants were analyzed. According to the diagnostic criteria of the Asian Working Group for Sarcopenia (AWGs) for sarcopenia, the participants were further divided into sarcopenia group (n = 93) and non-sarcopenia group (n = 93), as the matching factors of gender and age.

The study was carried out in accordance with the principles of the Declaration of Helsinki. All participants were informed in advance of the procedures, benefits, and probable adverse events associated with the protocol. Study subjects provided written informed consent, including permission to use the collected data for research purposes only. The study protocol has been approved by the Ethics Committee of Ningbo No. 2 Hospital.

Definition of Sarcopenia

Sarcopenia was diagnosed as low muscle mass, low muscle strength, and/or low physical performance by the Asian Working Group for Sarcopenia (AWGS) criteria. For low muscle mass, ASM/Ht^2 was <7.0 kg/m^2 for male and 5.7 kg/m^2 for female individuals, respectively. For low muscle strength, male handgrip strength was <2 kg, and female handgrip strength was <18 kg, respectively. Four-meter walking velocity <0.8 m/s could low physical performance.

Assessment of Muscle Strength and Physical Performance

The direct segmental multifrequency bioelectrical impedance analysis was used to analyze body composition features. Appendicular skeletal muscle mass (ASM) was summed as the total skeletal muscle in arms and legs. Relative skeletal muscle mass index (ASM/Ht^2) was defined as ASM divided by height squared in meters. Muscle strength was collected to the nearest 0.1 kg with an accurate handgrip dynamometer. Four-meter walking speed was tested on a straight corridor with a 6-m mark on the ground.

Other Measurements

Each participant was asked about age, sex, occupation, medical history, drug intake, smoking, and drinking habits via standardized questionnaires by experienced staff. Height and waist circumference were measured to the nearest 0.5 cm. Body mass index was weight in kilograms divided by the height squared in meters. Office blood pressure was measured with the Omron HEM-1300 monitor (Omron Healthcare, Inc., Kyoto, Japan). After resting in the sitting position for at least 5 min, the subjects took three blood pressure readings in a row, as recommended by the European Society of Hypertension. In the analysis, the mean office hypertension of three readings was blood pressure (BP) of at least 140 mmHg systolic or 90 mmHg diastolic.

In order to evaluate bone mineral density (BMD), all the participants underwent calcaneal ultrasound osteometry, with the analysis of BMD according to the manufacturer's recommendations. Results were expressed as T-scores. T-value was obtained by subtracting the measured value from the peak value of normal young people's bones. The smaller the difference, the better the bone quality. This value is used to diagnose osteoporosis and predict fracture risk.

Each participant was assessed for physical activity function through the International Physical Activity Questionnaire (IPAQ), which was designed specifically to assess physical activity in epidemiological studies of people over 65 years old.

Venous blood samples were obtained after overnight fasting, for the automatic enzymatic analysis on serum total cholesterol, high density lipoprotein cholesterol, serum creatinine, uric acid, and plasma glucose. Hypertension was caused by a previous diagnosis or the use of antihypertensive drugs. Diabetes mellitus was defined as having a glycosylated hemoglobin level of 7.0% or higher, taking antidiabetic medications, or having a history of diabetes. Dyslipidemia was defined as having a total cholesterol concentration higher than 5.0 mmol/L, with high-density lipoprotein (HDL) below 1.2 mmol/L in women or 1.0 mmol/L in men, or having been on lipid-lowering medications.

International Physical Activity Questionnaire-Short Forms

The short IPAQ administered by the interviewers determined the frequency and duration of moderate and vigorous leisure, transportation, and occupational physical activity, walking physical activity, and inactivity during the past week. IPAQ

has created three physical activity categories, namely, walking, moderate intensity, and high intensity.

Participants are asked about the frequency of different intensity activities in 1 week and the accumulated time of each day. Each question was set up with examples of moderate, vigorous, and walking activity for the participants to choose from, along with physiological cues for breathing and heart rate to help them recall activity at the appropriate intensity. One metabolic equivalent task (MET)-minute is defined as the intensity of a MET multiplied by the minutes of activity weekly. A MET roughly equals to active metabolic rate divided by resting metabolic rate, which represents the energy expended while sitting quietly at rest (21). MET intensity levels used for the iPAQ score are vigorous (8.0 METs), moderate (4.0 METs), and walking (3.3 METs). The principle of data outlier elimination is as follows: First, the daily cumulative time of each activity needs to be converted into minutes. Any missing data on activity frequency or time will not be included in the analysis. Assuming that each person had at least 8 h of sleep per day, if the cumulative time of three kinds of physical activity reported by the individual exceeded 960 min (16 h) per day, the individual was not included in the analysis.

Plasma Sampling and RNA Isolation

To obtain plasma, in silicone-coated serum tubes with increased silica act clot activator, venous blood was collected and then treated within 1 h after collection. The blood samples were separated by centrifuge at 845 × *g*, at 4°C for 15 min, with plasma and erythrocytes separated. Plasma were collected in aliquots into RNase/DNase-free tubes and stored at −80°C for further analysis.

Total RNA was isolated from the plasma with a mirVana PARIS isolation kit (Ambion, Austin, Texas) under the manufacturer's instructions. To avoid discrepancies in results, all samples were extracted and analyzed in a single batch to minimize repeated freeze–thaw cycles of plasma samples. Briefly, total RNA was extracted from 400 μl of plasma. After adding the same volume of denaturing solution, the plasma miRNA level was normalized by adding 50 pmol/L of *Caenorhabditis elegans* miR-39 (cel-miR-39) as the peak control. All the samples were eluted with 100 μl of RNAse-free water.

Analysis of Circulating miRNAs

The iScript complementary DNA (cDNA) reverse transcription kit (Bio-Rad) was used to reverse transcribe into cDNA from RNA. miRNA expressions were detected by Bulge-Loop TM miRNA qPCR Primer Sets (RiboBio) and quantitative reverse transcription polymerase chain reactions (qRT-PCRs) with iTaq^{TM} Universal SYBR Green Supermix (BIO-RAD) to quantify circulating miRNA levels. An Applied Biosystems 7900HT Fast Real-Time PCR device was used to perform all qRT-PCR reactions in triplicate. The patients with Ct values <35 were accepted for the analysis. The amplification efficiency of reference and studied miRNAs is near 100%, and the difference between the reference and studied miRNAs are <5%. Each sample was normalized to a reference sample across the plates. ΔCt values equals $\Delta Ct = \text{mean } Ct_{miR-X} - \text{mean } Ct_{miR(reference)}$. The formula $2^{(-\Delta\ \Delta\ Ct)}$ was used to calculate the fold-change of RNA species with Cel-miR-39 as spike-in control.

Statistical Analysis

GraphPad Prism 6 software (La Jolla, CA, United States) was used to performed statistical analysis. Means ± standard deviation (SD) represents subject characteristics, biochemical measurements, and general echocardiographic indexes. Through the analysis on qRT-PCR data, the $2^{-\Delta\ \Delta\ Ct}$ method was used to calculate the relative expression level for each miRNA, and data were as mean ± SD. An appropriate *t*-test was performed on the unpaired samples to analyze the miRNA changes. The Pearson's method was used to conducted the correlation analysis between changes in circulating miRNAs and diagnostic indexes of sarcopenia (ASM/height^2, handgrip strength and 4-m velocity) as appropriate for data distribution. $P < 0.05$ showed the statistical significance.

RESULTS

Participant Characteristics

The cohort used in this study was in accordance with previously reported (Zhang et al., 2019). The characteristics of the participants in the different groups are shown in **Table 1**. The height was higher in the no-sarcopenia group than in the sarcopenia group (159.73 ± 0.78 vs. 156.03 ± 0.74, $p < 0.05$). Body mass was greater in non-sarcopenia group than that in sarcopenia group (64.72 ± 1.19 vs. 52.97 ± 0.80, $p < 0.05$). The mean BMI was statistically significantly higher in the non-sarcopenia group than that in the sarcopenia group (25.32 ± 0.41 vs. 21.75 ± 0.29, $p < 0.05$). These data confirmed that the underweight condition of sarcopenia is frequent. Both groups had similar levels of diabetes mellitus. The incidence of hypertension was significantly higher in the non-sarcopenia group than that in the sarcopenia group. The detailed anthropometric indexes of these participants are indicated in **Table 2**. ASM/height^2, handgrip strength, knee extension, and 4-m velocity were significantly lower in the sarcopenia group than those in the non-sarcopenia group ($p < 0.05$). BMD, osteoarthritis, and physical activity scale were used for participants' fracture evaluation. As expected, patients with sarcopenia had a higher incidence of reduced BMD, reduced daily

TABLE 1 | Clinical characteristic of participants.

Clinical parameters	Non-sarcopenia (*n* = 93)	Sarcopenia (*n* = 93)	*P*
Age (years)	76.19 ± 0.58	76.15 ± 0.58	>0.05
Gender (male/female)			
Male	34 (36.6%)	34 (36.6%)	
Female	59 (63.4%)	59 (63.4%)	
Height (cm)	159.73 ± 0.78	156.03 ± 0.74	<0.05
Body mass (kg)	64.72 ± 1.19	52.97 ± 0.80	<0.05
BMI (kg/m^2)	25.32 ± 0.41	21.75 ± 0.29	<0.05
Hypertension	83 (89.2%)	72 (77.4%)	<0.05
Diabetes mellitus	38 (40.8%)	26 (27.9%)	>0.05

TABLE 2 | Anthropometric indexes of participants by sarcopenia status.

Anthropometric indexes	Non-sarcopenia group	Sarcopenia group	*p*
ASM/Height^2 (kg/m^2)	6.71 ± 0.10	5.63 ± 0.07	<0.05
Handgrip strength (kg)	24.18 ± 0.94	17.33 ± 0.55	<0.05
4-m velocit y (m/s)	1.12 ± 0.02	1.00 ± 0.03	<0.05
Knee extension (kg)	19.93 ± 0.81	14.76 ± 0.59	<0.05

TABLE 3 | Evaluated parameters in participants with fracture.

Physiological functions	Non-sarcopenia (*n* = 93)	Sarcopenia (*n* = 93)	*P*
Bone mineral density (*T*-score)	−1.37 ± 0.11	−1.60 ± 0.12	>0.05
Osteoarthritis	9 (9.7%)	10 (10.8%)	>0.05
Physical activity (IPAQ)	1,376.29 ± 172.45	1,188.61 ± 85.73	>0.05

physical activity index, and osteoarthritis, indicating a risk of fracture, compared with those without osteopenia (**Table 3**).

Decrease in Fracture Risk-Associated Circulating MiR-23a-3p, MiR-93-5p, and MiR-637 in Response to Sarcopenia in the Elderly

The expression of fracture risk-associated miRNAs [miR-21-5p (Seeliger et al., 2014), miR-23a-3p (Seeliger et al., 2014; Perksanusak et al., 2018), miR-24-3p (Kelch et al., 2017; Verdelli et al., 2020), miR-93-5p (Seeliger et al., 2014; Kelch et al., 2017), miR-100-5p (Ding et al., 2019), miR-122-5p, miR-124-3p (Qadir et al., 2015; Tang et al., 2017; Zou et al., 2017), miR-125b-5p (Lisse et al., 2013), miR-148a-3p (Hong et al., 2013; Verdelli et al., 2020), and miR-637 (Zhang et al., 2011; Gámez et al., 2014)] were determined. For the initial screening, we determined the expression level of miR-21-5p, miR-23a-3p, miR-24-3p, miR-93-5p, miR-100-5p, miR-122-5p, miR-124-3p, miR-125b-5p, miR-148a-3p, and miR-637 by RT-PCR most frequently detected in enrolled 93 sarcopenia elderly and 93 non-sarcopenia elderly plasma. The findings indicated that the levels of plasma miR-23a-3p, miR-93-5p, and miR-637 significantly decreased in the sarcopenia group compared to those in the non-sarcopenia group. On the contrary, miR-21-5p, miR-24-3p, miR-100-5p, miR-122-5p, miR-124-3p, miR-125b-5p, and miR-148a-3p did not change dramatically (**Figure 1**). According to continuous analyses, the experimental sample size was further expanded to verify the expression trend of these miRNAs that were differentially expressed, showing that the levels of miR-23a-3p, miR-93-5p, and miR-637 significantly decreased in the sarcopenia group compared to that in the non-sarcopenia group (**Figure 2**). The insulin signal decreases with age and is a special problem in patients with diabetes mellitus, which may be related to the c-miRNA response. We have excluded patients with diabetes mellitus from the analyzed data, suggesting that the levels of plasma miR-23a-3p, miR-93-5p, and miR-637 significantly decreased in the sarcopenia group compared to that in the non-sarcopenia group, with the same profile as the ones with diabetes mellitus (**Supplementary Figure 1**).

Changes in Fracture Risk-Associated MiR-23a-3p, MiR-93-5p, and MiR-637 Correlate With ASM/height^2, Handgrip Strength, or 4-M Velocity

The AWGS criteria used to diagnose muscular dystrophy are described below: low muscle mass, low muscle strength, and/or low physical strength. The detection was performed with ASM/height^2, handgrip strength, and 4-m velocity, respectively, whose levels significantly decreased in the elderly with sarcopenia (**Table 2**). The expression levels of circulating miR-23a-3p, miR-93-5p, and miR-637 significantly decreased in elderly patients with sarcopenia. Then, we investigated whether there was any correlation between these miRNAs and the diagnostic indicators of sarcopenia (ASM/height^2, handgrip strength, and 4-m speed). Our data indicated that the changes in miR-93-5p and miR-637 were remarkably related to ASM/height^2 ($r = 0.185$, $p < 0.05$; $r = 0.157$, $p < 0.05$), while the changes in other miRNAs were not strongly correlated with these diagnostic indexes (**Figure 3**). To explore more the data presenting as cluster separation regarding the miRNA levels and the different sarcopenia predictors, we have analyzed the correlation between the lowest and highest quartiles of miRNA levels and the corresponding predictors of sarcopenia in each group, showing that the miR-93-5p expression was markedly allied to ASM/height^2 (kg/m^2), which is consistent with the results of previous analysis (**Supplementary Figure 2**). In further analyses with adjustments applied for sex, we found that the changes in miR-23a and miR-93-5p were significantly allied to ASM/height^2 ($r = 0.192$, $p < 0.05$; $r = 0.246$, $p < 0.05$) in women (**Figure 4**). No significant correlations were found between miRNAs changes and these diagnostic indexes in men (**Figure 5**).

DISCUSSION

As a progressive and generalized skeletal muscle disorder involving the accelerated loss of muscle mass and function, sarcopenia is allied to aging, limiting the physical capabilities of the elderly (Marzetti et al., 2017; Larsson et al., 2019; Tournadre et al., 2019). Although the molecular mechanisms in sarcopenia remains entirely unclear, it is well documented that changes in miRNA during aging are major causes of sarcopenia (Siracusa et al., 2018; Jung et al., 2019; Yin et al., 2020). This study aims to investigate changes in specific circulating miRNAs associated with fracture risk in elderly patients with sarcopenia. From our data, it is noteworthy that the levels of plasma miR-23a-3p, miR-93-5p, and miR-637 significantly decreased in the sarcopenia group compared to those in the non-sarcopenia group, showing the association between the decrease in miR-23a-3p, miR-93-5p, and miR-637 with the predictor of sarcopenia, namely, the changes in miR-93-5p and miR-637 were significantly allied to ASM/height^2. After adjusting sex, the changes in miR-23a and miR-93-5p were significantly allied to ASM/height^2 in women, with no significant correlations between miRNAs changes and

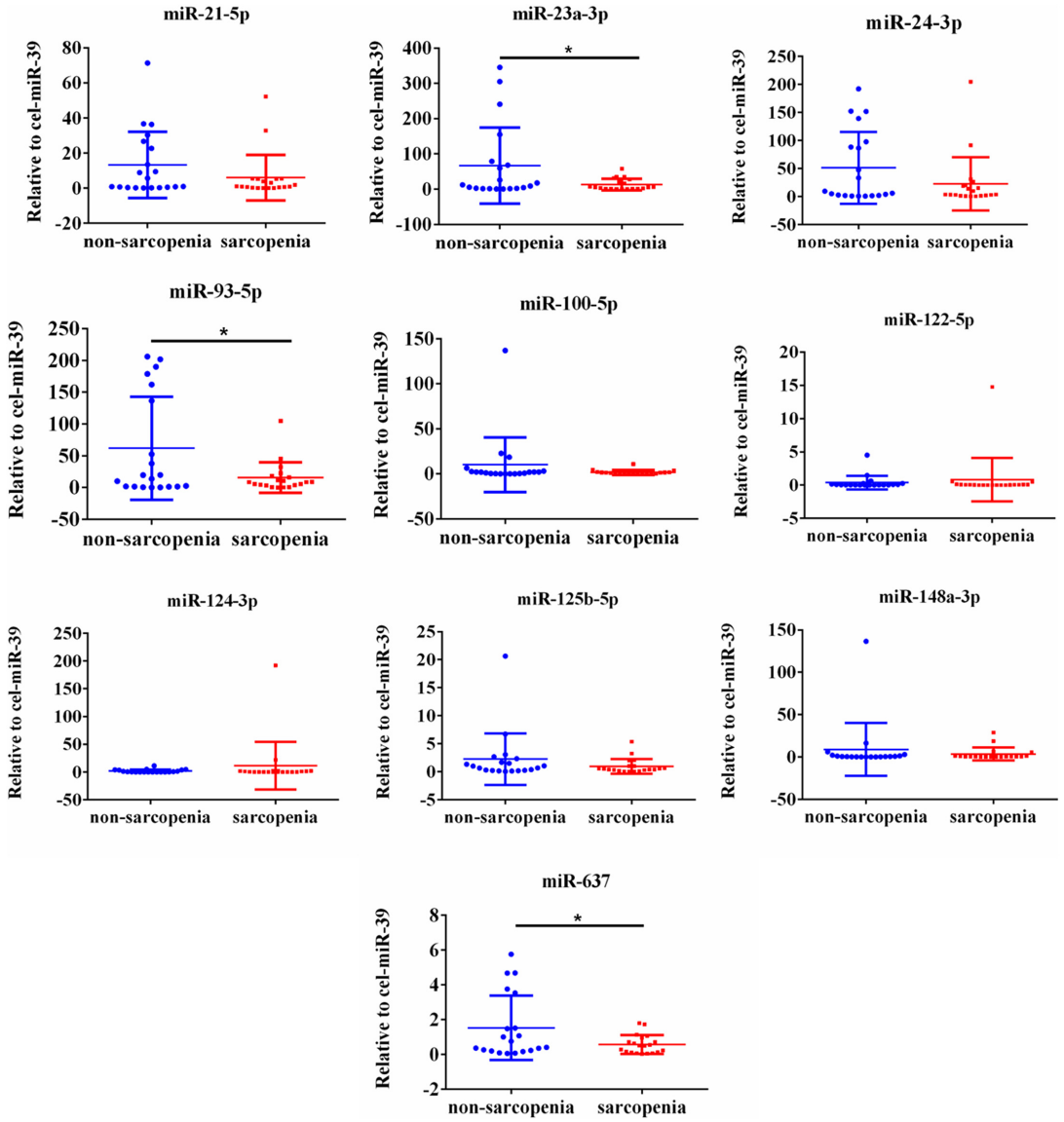

FIGURE 1 | Changes in circulating microRNAs in response to sarcopenia in the elderly. The expression level of microRNAs (miRNAs) were normalized using spike-in cel-miR-39. Unpaired *t*-test was used for these data. *Compared to non-sarcopenia of the elderly; $^{*}P < 0.05$; $n = 20$.

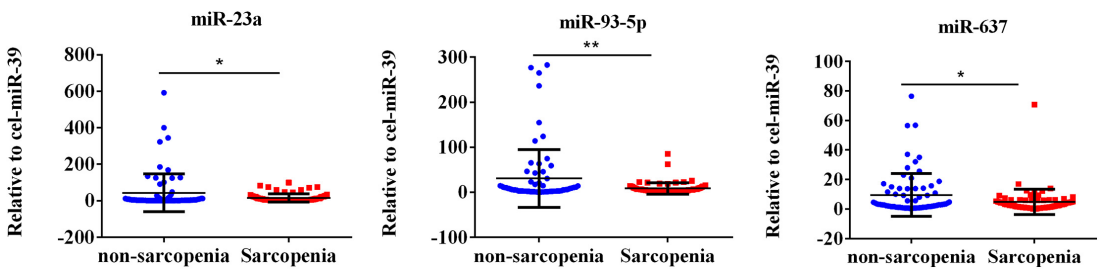

FIGURE 2 | Fracture risk-associated circulating miR-23a-3p, miR-93-5p, and miR-637 decrease in response to sarcopenia in the elderly. The expression level of microRNAs (miRNAs) were normalized using spike-in cel-miR-39. Unpaired *t*-test was used for these data. *Compared to non-sarcopenia of the elderly; $^{*}P < 0.05$, $^{**}P < 0.01$; $n = 73$.

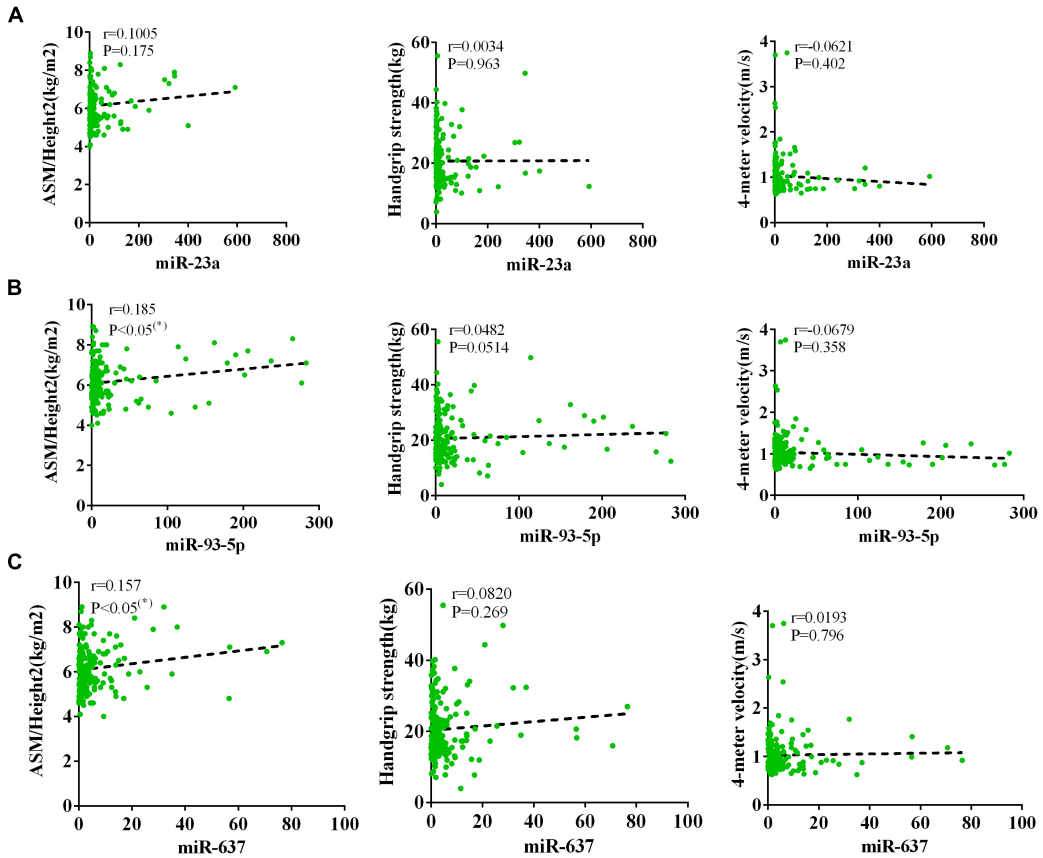

FIGURE 3 | Correlation analysis between the changes in **(A)** miR-23a-3p, **(B)** miR-93-5p, and **(C)** miR-637 and ASM/height^2 (kg/m^2), handgrip strength (kg), and 4-m velocity (m/s). The correlation between the changes in miR-23a-3p, miR-93-5p, and miR-637 and the diagnostic indicators of sarcopenia (ASM/height^2, handgrip strength, and 4-m velocity) in sarcopenic and non-sarcopenic subjects. Pearson's method was used for correlation analyses;*$P < 0.05$; $n = 186$.

these diagnostic indexes in men. These results suggest the practicality and feasibility of detecting changes in miRNAs in the blood to detect early signs of sarcopenia and fractures during aging. Nevertheless, an accurate diagnosis of sarcopenia remains challenging. Thus, we urgently need to explore new biomarkers to predict sarcopenia in the elderly.

The circulating miRNA profile reflects the expression of miRNA in cells as a marker of biological processes occurring in cells. Because of their position in the hierarchy of gene expression before translating the genetic code into proteins, information obtained from their circulating levels may ultimately predict early biological responses to specific conditions, as fine tuners (Hamam et al., 2017; Valihrach et al., 2020). The discovery of relatively abundant levels of miRNAs in serum/plasma has opened the door to their exploitation as biomarkers for numerous diseases, including sarcopenia and aging-related diseases (Andersen and Tost, 2020; Müller et al., 2020; Sabre et al., 2020). Several diagnostic indicators of sarcopenia measured have been reported to effectively provide preventive measures in the elderly, including ASM/height^2, handgrip strength, and 4-m velocity. It has been found that miRNAs exist in circulation in a consistent and reproducible manner, making them attractive for biomarker in patients with sarcopenia.

Selected miRNAs are involved in osteoblast formation and/or osteoclast formation and osteocyte function and/or clinical relevance, as they are allied to BMD and fracture risk. A growing evidence has suggested that several miRNAs expressed differentially in patients with osteoporotic and non-osteoporotic fractures (Mandourah et al., 2018; Zarecki et al., 2020). Specifically, miR-21, miR-23a, miR-24, miR-93, miR-100, miR-122a, miR-124a, miR-125b, and miR-148a were upregulated in osteoporotic serum, while miR-21, miR-23a, miR-24, miR-25, miR-100, and miR-125b were upregulated in osteoporotic bone tissues (Seeliger et al., 2014). Furthermore, miR-122a-5p, mir-125b-5p, and miR-21-5p were taken as osteoporotic markers for their fractures (Panach et al., 2015). Several circulating miRNAs have also been identified as closely related to sarcopenia in the elderly (Fan et al., 2016; Rusanova et al., 2018; Siracusa et al., 2018). Sarcopenia is closely related to osteoporosis, which together increases the risk of fractures (Atik, 2019; Greco et al., 2019). Thus, sarcopenia and osteoporosis are important risk factors of the frailty that leads to fracture. However, there is still no research to explore the common molecules leading to sarcopenia and osteoporosis in the elderly. Recently, our study has, for the first time, shown that miR-23a-3p, miR-93-5p, and miR-637, which are associated with fracture risk, are

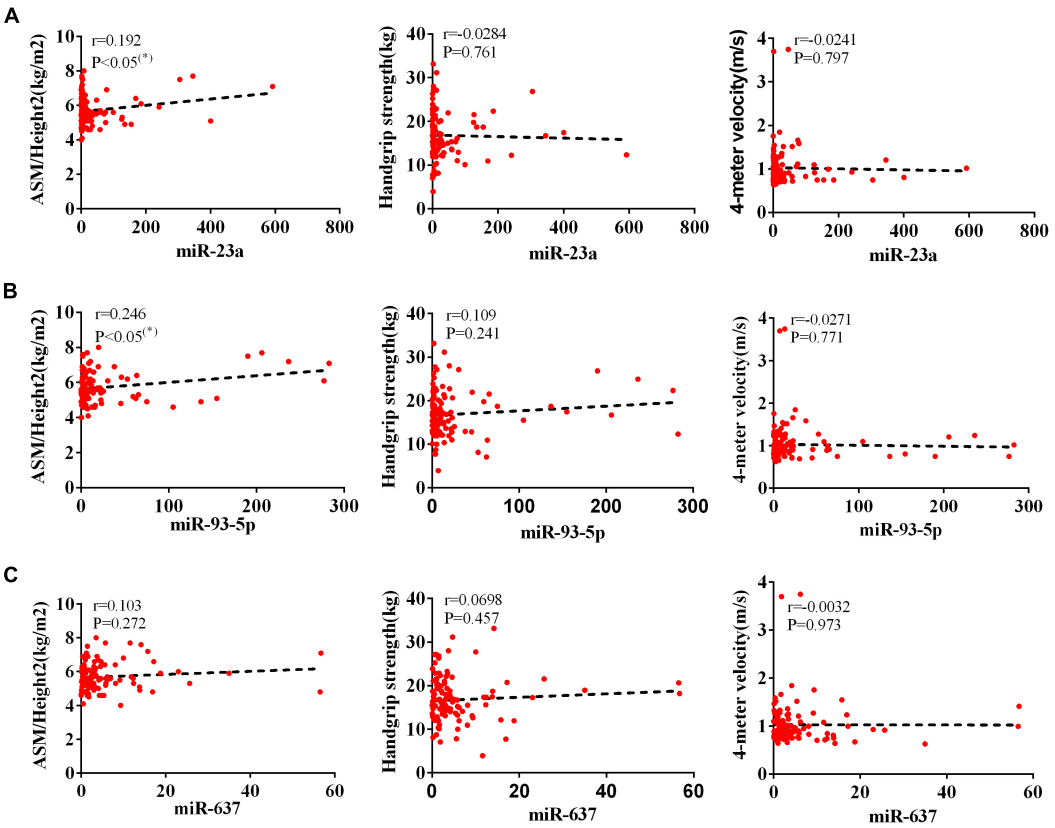

FIGURE 4 | Correlation analysis between the changes in **(A)** miR-23a-3p, **(B)** miR-93-5p, and **(C)** miR-637 and ASM/height^2 (kg/m^2), handgrip strength (kg), and 4-m velocity (m/s) in women. The correlation analysis of miR-23a-3p and miR-93-5p and miR-637 with these indicators in female subjects. Pearson's method was used for correlation analyses; *$P < 0.05$; $n = 119$.

decreased in sarcopenia response in the elderly, while other miRNAs identified in this study were not altered, suggesting that sarcopenia and osteoporosis may be regulated by these miRNAs. Moreover, considering that most osteoblastogenesis and/or osteoclastogenesis-related miRNAs did not change in the current study, future studies are still needed to investigate the functions of miRNAs in patients with sarcopenia.

We did find that there were statistically significant differences in c-miRNA levels of sarcopenia and non-sarcopenia groups. Circulating miR-23a-3p, miR-93-5p, and miR-637 achieved significantly decreased values in the sarcopenia group. Thus, circulating miR-23a-3p, miR-93-5p, and miR-637 may act as a common signature for sarcopenia. Most of the previous studies indicated that sarcopenia is significantly associated with fractures (Edwards et al., 2015; Oliveira and Vaz, 2015; Yeung et al., 2019). Here, we report a significant decrease in plasma levels of fracture-related miR-23a-3p, miR-93-5p, and miR-637 in the elderly with sarcopenia, supporting its relation with sarcopenia and fractures. However, the mechanism of changes in the circulating levels of miRNA associated with fracture risk in sarcopenia remains unclear. Considering the critical function of miRNAs in regulating bone metabolism, we can reasonably assume that bone turnover could be evaluated by measuring changes in the level of bone-specific c-miRNAs that reflect bone resorption and bone formation. Besides, miRNAs can regulate many common signaling pathways in the metabolism of muscle and skeletal muscle cell (Horak et al., 2016; Zheng et al., 2018). Wnt/β-catenin signaling pathway regulates osteoblast activity, which is also associated with regeneration of muscles. Osteocytes can secrete osteosclerosis, Wnt3a, and prostaglandins, regulate Wnt/β-catenin pathway, and affect the metabolism and functions of bones and muscles, respectively. Muscle contraction is an important mechanical stimulus for bones, which can activate the Wnt/β-catenin pathway of bone cells by enhancing the sheer force of fluid flow, thereby regulating osteoblast activity (Wannenes et al., 2014; Huang et al., 2017). Another signaling pathway closely related to muscle and bone is the PI3K/Akt pathway, through which insulin-like growth factor-1 exerts a beneficial effect on muscle and bone to promote anabolic metabolism (Yang et al., 2010; Cui et al., 2020). Finally, given that muscles and bones are regulated by many common genes, endocrine regulatory networks, and signaling pathways, both osteoporosis and sarcopenia are key factors to fractures. Including sarcopenia status may be useful in the fracture risk model. In the present study, our results on plasma circulating miRNA expression provide new insights in diagnosing and treating sarcopenia.

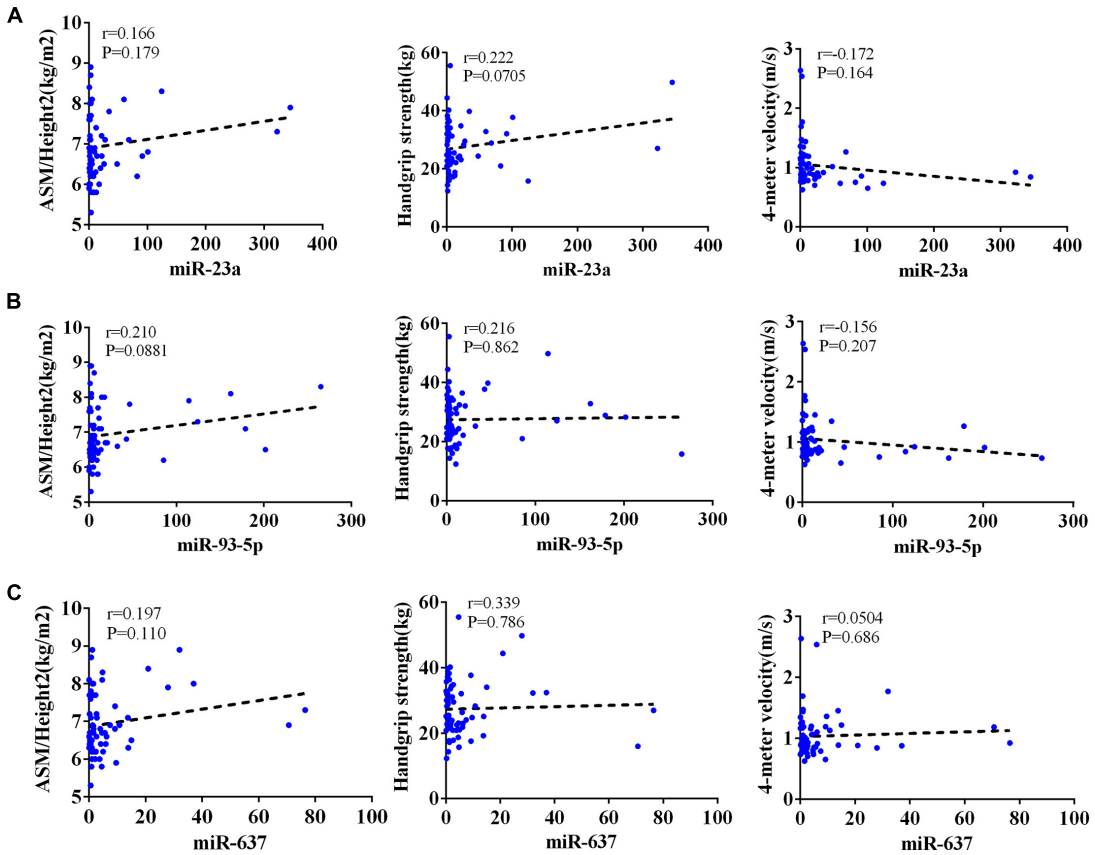

FIGURE 5 | Correlation analysis between the changes in **(A)** miR-23a-3p, **(B)** miR-93-5p, and **(C)** miR-637 and ASM/height^2 (kg/m^2), handgrip strength (kg), and 4-m velocity (m/s) in men. The correlation analysis of miR-23a-3p and miR-93-5p and miR-637 with these indicators in male subjects. Pearson's method was used for correlation analyses; *$P < 0.05$; $n = 67$.

Sarcopenia can be diagnosed as low muscle mass, low muscle strength, and/or low physical performance by AWGS criteria (Chen et al., 2014). Currently, the diagnostic criteria for sarcopenia are ASM/height^2, handgrip strength, and 4-m velocity, which can indicate the severity of sarcopenia. Interestingly, we found that the relative expressions of miR-93-5p and miR-637 were significantly positively associated with ASM/height^2, respectively, with a significant relation between the changes in miR-23a and miR-93-5p and ASM/height^2 in women while no significant correlations in men after adjusting sex. For one thing, researchers have consistently found that circulating miR-23a, miR-93-5p, and miR-637 are associated with fractures in patients with osteoporosis (Sansoni et al., 2018). Frequently, sarcopenia and osteoporosis are major syndromes in elderly patients, which are associated with weakness, including weight loss, low muscle strength, slow walking speed and instability, and more importantly, often leading to falls and fractures. Most studies have suggested that the incidence of sarcopenia differs among older men and women after a fragility fracture (Anderson et al., 2017; Laurent et al., 2019; Wong et al., 2019). Sarcopenia has some risk factors, and the decline in sex hormones may be one reason for sex differences, which may explain the difference in the correlation between changes in miRNAs levels and the diagnostic indicators of sarcopenia in different genders in our study. In addition, 63.4% of the subjects were female, and only 36.6% were male in our study. The possibility of the lack of this correlation in the male may be due to fewer participants.

There are still some limitations in our research. First, it is a single-center clinical trial with a small sample, the results of which need to be verified in a large sample, coverage area, and multicenter. If more patients of different races could be included, the results would be more reliable. Second, only eight targeted miRNAs that were associated with a fracture in the literature were examined because of cost; thus, there may be other unstudied miRNAs that may be better indicators of osteoporosis and sarcopenia status. Moreover, while the people in the study were relatively healthy, there may still be some patients with certain diseases. miRNA expression may alter with disease conditions, such as degenerative diseases, malignancies, and autoimmune diseases.

In summary, it has been proven that c-miRNAs may be considered as possible biomarkers for sarcopenia as a new diagnostic tool to monitor response to treatment. Further research into sarcopenia and osteoporosis-related fractures is also urgently needed to understand their relationships and mechanisms. All these mentioned above can provide more

evidence to develop potential interventions and improve clinical outcomes.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The Ethics Committee of Ningbo No. 2 Hospital. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

HY was in charge of conceiving and designing and revising the manuscript. NH was in charge of performing the experiments and writing the manuscript. NH, YLZ, and ZZ were in charge of collecting and analyzing the data. NH, BF, DW, and SZ were in charge of contributing the reagents, materials, and analysis tools used in this study. All authors contributed to the article and approved the submitted version.

FUNDING

This study was supported by the Ningbo Health Branding Subject Fund (PPXK2018-01), the Ningbo Medical Science and Technology Project (2016Z01), Zhejiang Provincial Public Service and Application Research Foundation, China (LGF20H250001), and the Ningbo HwaMei Research Fund (2019HMZD09) for funding.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2021.678610/full#supplementary-material

Supplementary Figure 1 | Fracture risk-associated circulating miR-23a-3p, miR-93-5p and miR-637 decrease in response to sarcopenia without diabetes in the elderly. The expression level of miRNAs were normalized using spike-in cel-miR-39; unpaired *T*-test was used for these data; *compared to non-sarcopenia of the elderly; $^*P < 0.05$; $n = 40$.

Supplementary Figure 2 | Correlation analysis between the lowest and highest quartile of the miR-23a-3p **(A)**, miR-93-5p **(B)**, miR-637 **(C)**, and ASM/Height^2 (kg/m^2), Handgrip strength (kg) and 4-m velocity (m/s). Pearson's method was used for Correlation analyses; $^*P < 0.05$; $n = 92$.

REFERENCES

Andersen, G. B., and Tost, J. (2020). Circulating miRNAs as biomarker in cancer. *Recent Results Cancer Res.* 215, 277–298. doi: 10.1007/978-3-030-26439-0_15

Anderson, L. J., Liu, H., and Garcia, J. M. (2017). Sex differences in muscle wasting. *Adv. Exp. Med. Biol.* 1043, 153–197. doi: 10.1007/978-3-319-70178-3_9

Angulo, J. C., Assar, M. E., and Rodriguezmanas, L. (2016). Frailty and sarcopenia as the basis for the phenotypic manifestation of chronic diseases in older adults. *Mol. Aspects Med.* 50, 1–32.

Anton, S. D., Hida, A., Robert, M., Andrew, L., Solberg, L. M., Mainous, A. G., et al. (2018). Nutrition and Exercise in Sarcopenia. *Curr. Protein Peptide Sci.* 19, 649–667.

Atik, O. (2019). There is an association between Sarcopenia, osteoporosis, and the risk of hip fracture. *Eklem Hastalik Cerrahisi* 30:1. doi: 10.5606/ehc.2019.001

Bauer, J. M., Verlaan, S., Bautmans, I., Brandt, K., Donini, L. M., Maggio, M., et al. (2015). Effects of a vitamin D and leucine-enriched whey protein nutritional supplement on measures of sarcopenia in older adults, the PROVIDE study: a randomized, double-blind, placebo-controlled trial. *J. Am. Med. Dir. Assoc.* 16, 740–747. doi: 10.1016/j.jamda.2015.05.021

Bonewald, L. F., Kiel, D. P., Clemens, T. L., Esser, K., Orwoll, E. S., O'Keefe, R. J., et al. (2013). Forum on bone and skeletal muscle interactions: summary of the proceedings of an ASBMR workshop. *J. Bone Miner. Res.* 28, 1857–1865. doi: 10.1002/jbmr.1980

Chen, L. K., Liu, L. K., Woo, J., Assantachai, P., Auyeung, T. W., Bahyah, K. S., et al. (2014). Sarcopenia in Asia: consensus report of the Asian Working Group for Sarcopenia. *J. Am. Med. Dir. Assoc.* 15, 95–101. doi: 10.1016/j.jamda.2013.11.025

Chen, Z., Bemben, M. G., and Bemben, D. A. (2019). Bone and muscle specific circulating microRNAs in postmenopausal women based on osteoporosis and sarcopenia status. *Bone* 120, 271–278. doi: 10.1016/j.bone.2018.11.001

Cui, C., Han, S., Shen, X., He, H., Chen, Y., Zhao, J., et al. (2020). ISLR regulates skeletal muscle atrophy via IGF1-PI3K/Akt-Foxo signaling pathway. *Cell Tissue Res.* 381, 479–492. doi: 10.1007/s00441-020-03251-4

Ding, W., Ding, S., Li, J., Peng, Z., Hu, P., Zhang, T., et al. (2019). Aberrant expression of miR-100 in plasma of patients with osteoporosis and its potential diagnostic value. *Clin. Lab.* 65, 9. doi: 10.7754/Clin.Lab.2019.190327

Edwards, M. H., Dennison, E. M., Aihie Sayer, A., Fielding, R., and Cooper, C. (2015). Osteoporosis and sarcopenia in older age. *Bone* 80, 126–130. doi: 10.1016/j.bone.2015.04.016

Eshaghi, S., Morteza, T., Khadijeh, I., Knechtle, B., Nikolaidis, P. T., and Chtourou, H. (2020). The effect of aerobic training and vitamin D supplements on the neurocognitive functions of elderly women with sleep disorders. *Biol. Rhythm Res.* 51, 1–8.

Fan, J., Kou, X., Yang, Y., and Chen, N. (2016). MicroRNA-regulated proinflammatory cytokines in Sarcopenia. *Mediators Inflamm.* 2016:1438686. doi: 10.1155/2016/1438686

Gámez, B., Rodriguez-Carballo, E., and Ventura, F. (2014). MicroRNAs and post-transcriptional regulation of skeletal development. *J. Mol. Endocrinol.* 52, R179–R197. doi: 10.1530/jme-13-0294

Gareev, I., Beylerli, O., Yang, G., Sun, J., Pavlov, V., Izmailov, A., et al. (2020). The current state of MiRNAs as biomarkers and therapeutic tools. *Clin. Exp. Med.* 20, 349–359. doi: 10.1007/s10238-020-00627-2

Girgis, C. M. (2015). Integrated therapies for osteoporosis and sarcopenia: from signaling pathways to clinical trials. *Calcif. Tissue Int.* 96, 243–255. doi: 10.1007/s00223-015-9956-x

Greco, E. A., Pietschmann, P., and Migliaccio, S. (2019). Osteoporosis and Sarcopenia increase frailty syndrome in the elderly. *Front. Endocrinol. (Lausanne)* 10:255. doi: 10.3389/fendo.2019.00255

Hamam, R., Hamam, D., Alsaleh, K. A., Kassem, M., Zaher, W., Alfayez, M., et al. (2017). Circulating microRNAs in breast cancer: novel diagnostic and prognostic biomarkers. *Cell Death Dis.* 8:e3045. doi: 10.1038/cddis.2017.440

Hong, I. S., Lee, H. Y., Choi, S. W., Kim, H. S., Yu, K. R., Seo, Y., et al. (2013). The effects of hedgehog on RNA binding protein Msi1 during the osteogenic differentiation of human cord blood-derived mesenchymal stem cells. *Bone* 56, 416–425. doi: 10.1016/j.bone.2013.07.016

Horak, M., Novak, J., and Bienertova-Vasku, J. (2016). Muscle-specific microRNAs in skeletal muscle development. *Dev. Biol.* 410, 1–13. doi: 10.1016/j.ydbio.2015.12.013

Huang, J., Romero-Suarez, S., Lara, N., Mo, C., Kaja, S., Brotto, L., et al. (2017). Crosstalk between MLO-Y4 osteocytes and C2C12 muscle cells is mediated by the Wnt/β-catenin pathway. *JBMR Plus* 1, 86–100. doi: 10.1002/jbm4.10015

Hunter, G. R., Wetzstein, C. J., Fields, D. A., Brown, A., and Bamman, M. M. (2000). Resistance training increases total energy expenditure and free-living physical activity in older adults. *J. Appl. Physiol. (1985)* 89, 977–984. doi: 10.1152/jappl.2000.89.3.977

Irandoust, K., Taheri, M., and Shavikloo, J. (2018). The effect of water-based aerobic training on the dynamic balance and walking speed of obese elderly men with low back pain. *Sleep Hypnosis* 20, 233–240.

Irandoust, K., Taheri, M., Mirmoezzi, M., H'mida, C., Chtourou, H., Trabelsi, K., et al. (2019). The effect of aquatic exercise on postural mobility of healthy older adults with endomorphic somatotype. *Int. J. Environ. Res. Public Health* 16:4387.

Jung, H. J., Lee, K. P., Kwon, K. S., and Suh, Y. (2019). MicroRNAs in skeletal muscle aging: current issues and perspectives. *J. Gerontol. A Biol. Sci. Med. Sci.* 74, 1008–1014. doi: 10.1093/gerona/gly207

Kelch, S., Balmayor, E. R., Seeliger, C., Vester, H., Kirschke, J. S., and van Griensven, M. (2017). miRNAs in bone tissue correlate to bone mineral density and circulating miRNAs are gender independent in osteoporotic patients. *Sci. Rep.* 7:15861. doi: 10.1038/s41598-017-16113-x

Kocijan, R., Muschitz, C., Geiger, E., Skalicky, S., Baierl, A., Dormann, R., et al. (2016). Circulating microRNA signatures in patients with idiopathic and postmenopausal osteoporosis and fragility fractures. *J. Clin. Endocrinol. Metab.* 101, 4125–4134. doi: 10.1210/jc.2016-2365

Kosaka, N., Iguchi, H., and Ochiya, T. (2010). Circulating microRNA in body fluid: a new potential biomarker for cancer diagnosis and prognosis. *Cancer Sci.* 101, 2087–2092. doi: 10.1111/j.1349-7006.2010.01650.x

Landi, F., Calvani, R., Cesari, M., Tosato, M., Martone, A. M., Ortolani, E., et al. (2018). Sarcopenia: an overview on current definitions, diagnosis and treatment. *Curr. Protein Pept. Sci.* 19, 633–638. doi: 10.2174/1389203718666170607113459

Larsson, L., Degens, H., Li, M., Salviati, L., Lee, Y., Thompson, W. J., et al. (2019). Sarcopenia: aging-related loss of muscle mass and function. *Physiol. Rev.* 99, 427–511.

Laurent, M. R., Dedeyne, L., Dupont, J., Mellaerts, B., Dejaeger, M., and Gielen, E. (2019). Age-related bone loss and sarcopenia in men. *Maturitas* 122, 51–56. doi: 10.1016/j.maturitas.2019.01.006

Lisse, T. S., Chun, R. F., Rieger, S., Adams, J. S., and Hewison, M. (2013). Vitamin D activation of functionally distinct regulatory miRNAs in primary human osteoblasts. *J. Bone Miner. Res.* 28, 1478–1488. doi: 10.1002/jbmr.1882

Mandourah, A. Y., Ranganath, L., Barraclough, R., Vinjamuri, S., Hof, R. V., Hamill, S., et al. (2018). Circulating microRNAs as potential diagnostic biomarkers for osteoporosis. *Sci. Rep.* 8:8421. doi: 10.1038/s41598-018-26525-y

Marques, A., and Queirós, C. (2018). "Frailty, Sarcopenia and falls," in *Fragility Fracture Nursing: Holistic Care and Management of the Orthogeriatric Patient*, eds K. Hertz, and J. Santy-Tomlinson (Cham: Springer), 15–26.

Marzetti, E., Calvani, R., Tosato, M., Cesari, M., Bari, M. D., Cherubini, A., et al. (2017). Sarcopenia: an overview. *Aging Clin. Exp. Res.* 29, 11–17.

Müller, S., Janke, F., Dietz, S., and Sültmann, H. (2020). Circulating MicroRNAs as potential biomarkers for lung cancer. *Recent Results Cancer Res.* 215, 299–318. doi: 10.1007/978-3-030-26439-0_16

Oliveira, A., and Vaz, C. (2015). The role of sarcopenia in the risk of osteoporotic hip fracture. *Clin. Rheumatol.* 34, 1673–1680. doi: 10.1007/s10067-015-2943-9

Panach, L., Mifsut, D., Tarín, J. J., Cano, A., and García-Pérez, M. (2015). Serum circulating MicroRNAs as biomarkers of osteoporotic fracture. *Calcif. Tissue Int.* 97, 495–505. doi: 10.1007/s00223-015-0036-z

Perksanusak, T., Panyakhamlerd, K., Hirankarn, N., Suwan, A., Vasuratna, A., and Taechakraichana, N. (2018). Correlation of plasma microRNA-21 expression and bone turnover markers in postmenopausal women. *Climacteric* 21, 581–585. doi: 10.1080/13697137.2018.1507020

Qadir, A. S., Um, S., Lee, H., Baek, K., Seo, B. M., Lee, G., et al. (2015). miR-124 negatively regulates osteogenic differentiation and in vivo bone formation of mesenchymal stem cells. *J. Cell. Biochem.* 116, 730–742. doi: 10.1002/jcb.25026

Reiss, J., Iglseder, B., Alzner, R., Mayr-Pirker, B., Pirich, C., Kässmann, H., et al. (2019). Sarcopenia and osteoporosis are interrelated in geriatric inpatients. *Z. Gerontol. Geriatr.* 52, 688–693. doi: 10.1007/s00391-019-01553-z

Rusanova, I., Diaz-Casado, M. E., Fernández-Ortiz, M., Aranda-Martínez, P., Guerra-Librero, A., García-García, F. J., et al. (2018). Analysis of plasma MicroRNAs as predictors and biomarkers of aging and frailty in humans. *Oxid. Med. Cell. Longev.* 2018:7671850. doi: 10.1155/2018/7671850

Sabre, L., Punga, T., and Punga, A. R. (2020). Circulating miRNAs as potential biomarkers in myasthenia gravis: tools for personalized medicine. *Front. Immunol.* 11:213. doi: 10.3389/fimmu.2020.00213

Saliminejad, K., Khorram Khorshid, H. R., Soleymani Fard, S., and Ghaffari, S. H. (2019). An overview of microRNAs: biology, functions, therapeutics, and analysis methods. *J. Cell. Physiol.* 234, 5451–5465. doi: 10.1002/jcp.27486

Sansoni, V., Perego, S., Vernillo, G., Barbuti, A., Merati, G., La Torre, A., et al. (2018). Effects of repeated sprints training on fracture risk-associated miRNA. *Oncotarget* 9, 18029–18040. doi: 10.18632/oncotarget.24707

Seeliger, C., Karpinski, K., Haug, A. T., Vester, H., Schmitt, A., Bauer, J. S., et al. (2014). Five freely circulating miRNAs and bone tissue miRNAs are associated with osteoporotic fractures. *J. Bone Miner. Res.* 29, 1718–1728. doi: 10.1002/jbmr.2175

Seghatoleslami, A., Afif, A. H., Irandoust, K., and Taheri, M. (2018). The impact of pilates exercises on motor control of inactive middle-aged women. *Sleep Hypnosis* 20, 262–266.

Siracusa, J., Koulmann, N., and Banzet, S. (2018). Circulating myomiRs: a new class of biomarkers to monitor skeletal muscle in physiology and medicine. *J. Cachexia Sarcopenia Muscle* 9, 20–27. doi: 10.1002/jcsm.12227

Soriano-Arroquia, A., House, L., Tregilgas, L., Canty-Laird, E., and Goljanek-Whysall, K. (2016). The functional consequences of age-related changes in microRNA expression in skeletal muscle. *Biogerontology* 17, 641–654. doi: 10.1007/s10522-016-9638-8

Steihaug, O. M., Gjesdal, C. G., Bogen, B., Kristoffersen, M. H., Lien, G., and Ranhoff, A. H. (2017). Sarcopenia in patients with hip fracture: a multicenter cross-sectional study. *PLoS One* 12:e0184780. doi: 10.1371/journal.pone.0184780

Tang, L., Yin, Y., Liu, J., Li, Z., and Lu, X. (2017). MiR-124 attenuates osteoclastogenic differentiation of bone marrow monocytes via targeting Rab27a. *Cell. Physiol. Biochem.* 43, 1663–1672. doi: 10.1159/000484027

Tarantino, U., Baldi, J., Scimeca, M., Piccirilli, E., Piccioli, A., Bonanno, E., et al. (2016). The role of sarcopenia with and without fracture. *Injury* 47(Suppl. 4), S3–S10. doi: 10.1016/j.injury.2016.07.057

Tournadre, A., Vial, G., Capel, F., Soubrier, M., and Boirie, Y. (2019). Sarcopenia. *Joint Bone Spine* 86, 309–314. doi: 10.1016/j.jbspin.2018.08.001

Valihrach, L., Androvic, P., and Kubista, M. (2020). Circulating miRNA analysis for cancer diagnostics and therapy. *Mol. Aspects Med.* 72:100825. doi: 10.1016/j.mam.2019.10.002

Van Roie, E., Delecluse, C., Coudyzer, W., Boonen, S., and Bautmans, I. (2013). Strength training at high versus low external resistance in older adults: effects on muscle volume, muscle strength, and force-velocity characteristics. *Exp. Gerontol.* 48, 1351–1361. doi: 10.1016/j.exger.2013.08.010

Verdelli, C., Sansoni, V., Perego, S., Favero, V., Vitale, J., Terrasi, A., et al. (2020). Circulating fractures-related microRNAs distinguish primary hyperparathyroidism-related from estrogen withdrawal-related osteoporosis in postmenopausal osteoporotic women: a pilot study. *Bone* 137:115350. doi: 10.1016/j.bone.2020.115350

Wannenes, F., Papa, V., Greco, E. A., Fornari, R., Marocco, C., Baldari, C., et al. (2014). Abdominal fat and Sarcopenia in women significantly alter osteoblasts homeostasis in vitro by a WNT/ β -catenin dependent mechanism. *Int. J. Endocrinol.* 2014:278316. doi: 10.1155/2014/278316

Westcott, W. L. (2009). Strength training for frail older adults. *J. Active Aging* 8, 52–59.

Wong, R. M. Y., Wong, H., Zhang, N., Chow, S. K. H., Chau, W. W., Wang, J., et al. (2019). The relationship between sarcopenia and fragility fracture-a systematic review. *Osteoporos. Int.* 30, 541–553. doi: 10.1007/s00198-018-04828-0

Yamada, M., Kimura, Y., Ishiyama, D., Nishio, N., Otobe, Y., Tanaka, T., et al. (2019). Synergistic effect of bodyweight resistance exercise and protein supplementation on skeletal muscle in sarcopenic or dynapenic older adults. *Geriatr. Gerontol. Int.* 19, 429–437.

Yang, M., Liu, Y., Zuo, Y., and Tang, H. (2019). Sarcopenia for predicting falls and hospitalization in community-dwelling older adults: EWGSOP versus EWGSOP2. *Sci. Rep.* 9:17636. doi: 10.1038/s41598-019-53522-6

Yang, S. Y., Hoy, M., Fuller, B., Sales, K. M., Seifalian, A. M., and Winslet, M. C. (2010). Pretreatment with insulin-like growth factor I protects skeletal muscle cells against oxidative damage via PI3K/Akt and ERK1/2 MAPK pathways. *Lab. Invest.* 90, 391–401. doi: 10.1038/labinvest.2009.139

Yeung, S. S. Y., Reijnierse, E. M., Pham, V. K., Trappenburg, M. C., Lim, W. K., Meskers, C. G. M., et al. (2019). Sarcopenia and its association with falls and fractures in older adults: a systematic review and meta-analysis. *J. Cachexia Sarcopenia Muscle* 10, 485–500. doi: 10.1002/jcsm.12411

Yin, J., Qian, Z., Chen, Y., Li, Y., and Zhou, X. (2020). MicroRNA regulatory networks in the pathogenesis of sarcopenia. *J. Cell. Mol. Med.* 24, 4900–4912. doi: 10.1111/jcmm.15197

Zarecki, P., Hackl, M., Grillari, J., Debono, M., and Eastell, R. (2020). Serum microRNAs as novel biomarkers for osteoporotic vertebral fractures. *Bone* 130:115105. doi: 10.1016/j.bone.2019.115105

Zendjabil, M., Favard, S., Tse, C., Abbou, O., and Hainque, B. (2017). [The microRNAs as biomarkers: what prospects?]. *C. R. Biol.* 340, 114–131. doi: 10.1016/j.crvi.2016.12.001

Zhang, J. F., Fu, W. M., He, M. L., Wang, H., Wang, W. M., Yu, S. C., et al. (2011). MiR-637 maintains the balance between adipocytes and osteoblasts by directly targeting Osterix. *Mol. Biol. Cell* 22, 3955–3961. doi: 10.1091/mbc.E11-04-0356

Zhang, L., Guo, Q., Feng, B.-L., Wang, C.-Y., Han, P.-P., Hu, J., et al. (2019). A cross-sectional study of the association between arterial stiffness and Sarcopenia in Chinese community-dwelling elderly using the Asian working group for sarcopenia criteria. *J. Nutr. Health Aging* 23, 195–201.

Zheng, Y., Kong, J., Li, Q., Wang, Y., and Li, J. (2018). Role of miRNAs in skeletal muscle aging. *Clin. Interv Aging* 13, 2407–2419. doi: 10.2147/cia.s169202

Zou, L., Zhang, G., Liu, L., Chen, C., Cao, X., and Cai, J. (2017). A MicroRNA-124 polymorphism is associated with fracture healing via modulating BMP6 expression. *Cell. Physiol. Biochem.* 41, 2161–2170. doi: 10.1159/000475570

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Physiology

REVIEW
published: 05 August 2021
doi: 10.3389/fphys.2021.704306

Autoregulation in Resistance Training for Lower Limb Tendinopathy: A Potential Method for Addressing Individual Factors, Intervention Issues, and Inadequate Outcomes

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Musculoskeletal disorders, such as tendinopathy, are placing an increasing burden on society and health systems. Tendinopathy accounts for up to 30% of musculoskeletal disorders, with a high incidence in athletes and the general population. Although resistance training has shown short-term effectiveness in the treatment of lower limb tendinopathy, more comprehensive exercise protocols and progression methods are required due to poor long-term outcomes. The most common resistance training protocols are predetermined and standardized, which presents significant limitations. Current standardized protocols do not adhere to scientific resistance training principles, consider individual factors, or take the importance of individualized training into account. Resistance training programs in case of tendinopathy are currently not achieving the required intensity and dosage, leading to high recurrence rates. Therefore, better methods for individualizing and progressing resistance training are required to improve outcomes. One potential method is autoregulation, which allows individuals to progress training at their own rate, taking individual factors into account. Despite the finding of their effectiveness in increasing the strength of healthy athletes, autoregulation methods have not been investigated in case of tendinopathy. The purpose of this narrative review was 3-fold: firstly, to give an overview and a critical analysis of the individual factors involved in tendinopathy and current resistance training protocols and their limitations. Secondly, to give an overview of the history, methods, and application of autoregulation strategies both in sports performance and physiotherapy. Finally, a theoretical adaptation of a current tendinopathy resistance training protocol using autoregulation methods is presented, providing an example of how the method could be implemented in clinical practice or future research.

Keywords: tendinopathy, resistance training, autoregulation, exercise, musculoskeletal-disorders

OPEN ACCESS

Edited by:
Anselmo Sigari Moriscot, University of São Paulo, Brazil

Reviewed by:
Stefano Masiero, University of Padua, Italy
Leandro Campos De Brito, University of São Paulo, Brazil

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Specialty section:
This article was submitted to Exercise Physiology, a section of the journal Frontiers in Physiology

Received: *02 May 2021*
Accepted: *06 July 2021*
Published: *05 August 2021*

Citation:
Burton I (2021) Autoregulation in Resistance Training for Lower Limb Tendinopathy: A Potential Method for Addressing Individual Factors, Intervention Issues, and Inadequate Outcomes. Front. Physiol. 12:704306. doi: 10.3389/fphys.2021.704306

KEY POINTS

- Specific resistance training protocols for tendinopathy rehabilitation have been widely adopted in clinical practice and research for more than 20 years. However, the protocols remain largely underdeveloped and have evolved little since their initial development. Current protocols lacking adherence to the scientific principles of progressive resistance training perhaps contribute to their poor long-term outcomes. There is an increasing attention being paid to the application of the scientific principles that underlie an adaptation to manipulating the exercise stimulus in a progressive manner, which potentially improve training prescription and outcomes in case of tendinopathy.
- A significant range of physical, genetic, and psychological individual factors has been identified across patients with tendinopathy, resulting in a large heterogenous population. Tendinopathies are also present in various stages of a continuum, with each stage potentially responding differently to the training stimulus. These factors can impact on individual performance, adaptation, adherence, and response to resistance training interventions. Therefore, protocols should be personalized and individually modified as opposed to the current predetermined and standardized protocols.
- Autoregulation represents a potential method of personalizing tendinopathy resistance training protocols, allowing for a modification based on the individual training response across the tendinopathy continuum. Future research should examine the efficacy of such an approach, like the example presented here, as a potential method of improving the outcomes of common tendinopathy resistance training interventions.

INTRODUCTION

Recently published studies have highlighted how musculoskeletal disorders are collectively a leading global cause of disability and pain, impacting on a broad demographic spectrum of society (Vavken and Dorotka, 2011; Moraes et al., 2014; Smith et al., 2014; Hoy et al., 2015; Onuora, 2019). Tendinopathy accounts for up to 50% of all musculoskeletal disorders and is often associated with chronic pain, decreased physical function, and reduced participation in activities (Maffulli et al., 2003; Dos Santos Franco et al., 2019). Although the etiology of tendinopathy is still not fully understood, it is known to be caused by repetitive tendon microtrauma and failed tendon healing (Magnussen et al., 2009; Fu et al., 2010; Lui et al., 2011; Simpson et al., 2016; Magnusson and Kjaer, 2019). This disrupted healing process manifests as increased tendon thickness, neovascularization, and the incidence of calcium deposits or calcification (Andres and Murrell, 2008; Rui et al., 2011). The previously used term "tendinitis" is referred to an inflammatory process, and, in case of the presence of inflammatory cells, the term tendinopathy has been adopted to reflect the often degenerative and non-inflammatory nature of the disorder (Jarvinen et al., 2005; Sharma and Maffulli, 2005; Rees et al., 2014). Athletes exposed to frequent loading through training and competition are particularly susceptible to developing tendinopathy due to repeated tendon stress and overloading (Ganestam et al., 2016). Patellar tendinopathy is reported to have a prevalence of 45% in elite volleyball players and 32% in elite basketball players (Abat et al., 2017). The lifetime risk of developing Achilles tendinopathy in elite runners amounts to 52%, reporting an incidence of 24% among competitive athletes, and 18% among young athletes compared to that in sedentary populations amounting to 5.9% (Ahmad et al., 2013).

A diverse range of common treatments, such as anti-inflammatory medications, corticosteroid injections, low-level laser therapy, ultrasound, platelet-rich plasma injections, prolotherapy, glycerol trinitrate patches, manual therapy, shockwave therapy, and exercise, are used in tendinopathy rehabilitation (Cardoso et al., 2019; Girgis and Duarte, 2020). Exercise is the most common and recommended treatment option, including heavy slow resistance training (HSRT) and eccentric strengthening interventions having positive outcomes for various lower limb tendinopathies such as plantar heel pain (PHP), Achilles, and patellar tendinopathy (Dimitrios, 2015; Riel et al., 2019b). Despite positive short-term outcomes, long-term outcomes remain poor, with up to 40% of patients having symptoms 2 years after resistance training interventions (Rathleff and Thorborg, 2015). Recent evidence suggests that current resistance training interventions have not achieved the required loading dosages to stimulate mechano-biological changes, such as increases in strength and changes in tendon (Riel et al., 2019a). Current resistance training interventions may not follow scientific training principles and may require more accurate modification of training variables, such as frequency, intensity, and volume (Abat et al., 2017). A shift to a more scientifically supported training model, such as periodization models used in training athletes, may improve outcomes (Fleck, 2011). Autoregulation is a concept of training derived from periodization models, which are related to manipulating the training variables based on the circumstances of an individual, such as response to training and daily readiness to train (Mann et al., 2010).

Recently, several individual factors have been identified in case of tendinopathy, highlighting the heterogeneity of this patient population (Steinmann et al., 2020). Therefore, it is likely not appropriate that all patients receive the same rigid training protocol in the standardized resistance training. The implementation of autoregulation methods during tendinopathy resistance training may achieve the individualization of training loads and dosage, which may maximize clinical outcomes compared to the following standardized protocols not being tailored to the individual (Helms et al., 2018). However, to the authors' knowledge, autoregulation methods during tendinopathy resistance training interventions have not previously been investigated. Therefore, the purpose of this review is to synthesize the current research on resistance training interventions for lower limb tendinopathies, their limitations, and potential autoregulation strategies. The review begins with an overview of individual factors and resistance training protocols in tendinopathy before providing an overview of the theory and implementation of autoregulation methods. Finally, an overview of the potential application of

autoregulation methods will be applied to a current tendinopathy protocol, allowing for a potential clinical application to improve clinical outcomes.

INDIVIDUAL FACTORS

Tendinopathies are challenging to treat and are considered to have a multifactorial pathogenesis resulting from extrinsic and intrinsic factors (Seitz et al., 2011; Deans et al., 2012). Abnormal or repeated tendon loading factors combined with genetic, biological, and cellular variations may lead to tendon changes and damage (Wu et al., 2017). Despite a condition that is considered to be common in athletes and physically active, tendinopathies are also commonly seen in sedentary patients and the elderly (Moosmayer et al., 2009). Gender differences may also present in case of Achilles tendon injuries, that is, being more common in men than women (Andrew and Jonathan, 2014). Tendinopathies in different locations, i.e., upper and lower limbs, present different pathophysiological and etiological circumstances, which will require individual treatment approaches despite the treatment often being standardized across all tendinopathies (Clayton and Court-Brown, 2008).

Individual variability in biomechanics, such as anatomical differences, malalignment, neuromuscular capacity, weakness or strength, foot structure, flexibility, repeated training errors, and movement kinetics and kinematics may lead to repetitive, excessive, or compressive loading on tendons (Benjamin et al., 2008; Sharma and Maffulli, 2008; Dean et al., 2017; Ferreira et al., 2020). In patellar tendinopathy, almost 37 different individual biomechanical risk variables have been identified in relation to jump-landing strategy alone (Harris et al., 2020). The identified external risk factors may have a complex interplay with individual intrinsic and biological factors, namely age, gender, bodyweight, hormones, genetics, height, prior tendon injury, and health conditions such as obesity, diabetes, hyperlipidemia, rheumatological conditions, and osteoarthritis (Kjaer et al., 2006, 2009; Leblanc et al., 2017). Environmental factors, such as daily activities, smoking, alcohol consumption, poor nutrition or sleep habits, physically demanding workplace, stress, inappropriate footwear, and medications such as corticosteroids, statins, aromatase inhibitors, and fluoroquinolone antibiotics, may also contribute to the individual tendinopathy risk (van der Linden et al., 2002; Knobloch, 2016). Individual differences and factors, such as loading capacity and tolerance, motor control, education, beliefs, exercise types and preferences, and psychosocial and contextual factors, have also been shown to be important factors related to the individual response to rehabilitation and treatment outcomes (Silbernagel et al., 2007a; Kongsgaard et al., 2010; Littlewood et al., 2012; Cook and Docking, 2015; Hanratty et al., 2016; O'Keeffe et al., 2016; Thiese et al., 2016; Dejaco et al., 2017; Gillespie et al., 2017; Ingwersen et al., 2017; Mallows et al., 2017a,b; Mc Auliffe et al., 2017; Raschhofer et al., 2017; Plinsinga et al., 2020).

Andia and Maffulli (2019) highlighted that the response to tendinopathy treatment interventions is strictly individualistic and can be inherent to the genetic background, demography, immune status, microbiota function, associated metabolic diseases, systemic drugs or local anesthetics, the characteristics and stages of recipient tissue, including inflammation, angiogenic state, and cellularity. Positive responses to resistance training are influenced by a multitude of individual factors, including fitness level, training history, nutritional intake, psychological and social states, sleep and recovery, age, weight, and overall training workload (Pickering and Kiely, 2019). However, some of these factors may prove difficult to evaluate in clinical practice (Abate et al., 2020), with a thorough clinical assessment of each individual and the requirement of their personal factors prior to prescribing resistance training interventions (Maestroni et al., 2020). A wide variety of potential factors involved in the development, progression, and rehabilitation of tendinopathy leads to a highly diverse and broad range of potential cellular responses and ultimately a broad pathological spectrum of disease, leading to a vastly heterogenous tendinopathic patient population.

Due to a plethora of potential individual patient characteristics and risk factors involved in any type of tendinopathy, their presentation is highly heterogenous in nature, with a multitude of potential factors contributing to triggering the onset of tendinopathy (Steinmann et al., 2020). Individual patient characteristics (age, gender, and genetics) can interplay with lifestyle habits (smoking, alcohol consumption, and physical activity levels) leading to a diverse spectrum of potential tendinopathy risk and onset factors. All cases of tendinopathy do not clinically present uniformly, with each individual case of tendinopathy having a varying degree of symptomatic presentation of pain, swelling, weakness, and functional ability. Pain experienced from tendinopathy is not always associated with tissue damage, suggesting that other neurophysiological mechanisms may be involved (Rio et al., 2015a, 2016; Hainline et al., 2017). Central nervous system hypersensitivity and the role of pain central sensitization have recently emerged as a potential contributing factor, which can vary in the presentation among individuals (Littlewood et al., 2013a; Plinsinga et al., 2015, 2018; Sanchis et al., 2015).

Although the importance of an individualized approach to a heterogenous tendinopathy population has been repeatedly stressed in the tendinopathy literature, it is clearly a limitation while developing studies due to the effort and the need to homogenize samples. However, there is a special need to make progress in the knowledge of treating patients with tendinopathy even in the case of no possibility of perfect homogeneity (Abat et al., 2017). The heterogeneous presentation of tendinopathies means that a "one-size-fits-all" treatment approach may be inappropriate and related to poor long-term treatment outcomes (Silbernagel, 2014). The Achilles tendon, for example, can experience a positive adaptation when exposed to the strains of a specific range or "sweet spot," which depends on several factors known to differ between the individuals, requiring treatment interventions that account for these interindividual differences (Pizzolato et al., 2019). Physiotherapists have reported to use complex clinical reasoning to adapt eccentric training research protocols for individual patients. For example, the need to change eccentric loading protocols in patients experiencing pain

prevented them from being adherent to the published protocols, and therefore substituting with isotonic or isometric loading initially. To make recommendations clearer, future research should focus on variables of the eccentric training program that could impact on efficacy, such as exercise speed, duration, progression and loading rate, and chronicity and severity of the tendinopathy (Rowe et al., 2012).

As the etiology of tendinopathy is multifactorial, rehabilitation should be focused on identifying the multiple risk factors that affect a specific individual (Peters et al., 2016). It could be considered as a disservice to the patient if the factors contributing to the development of their tendinopathy are not addressed (Kulig et al., 2015). It is important to consider that each individual will respond differently to rehabilitation, and each program must be tailored to the individual with "Recipe" approaches to tendinopathy rehabilitation failing to capitalize on the potential for recovery (Cook, 2011; Cook and Purdam, 2014). The needs of an individual must be addressed in the rehabilitation program, which was highlighted in a recent e-Delphi survey to establish a patellar tendinopathy rehabilitation framework. The results quantified three central aspects, including functional abilities, individualized rehabilitation, and load tolerance, which formed the foundation of the rehabilitation framework (Morgan et al., 2018a). Factors relevant, when accessing, adapting, and applying training protocols, to the patient include the mapping information gathered from a subjective assessment to pathophysiological knowledge and likely treatment responses to enable clinical decisions that utilize interventions in an individualized way (Morrissey, 2015). Comorbidities, symptom development, patient beliefs, psychosocial and cultural factors, possible tissue demands, and previous intervention outcomes may influence treatment outcomes. Different tendinopathies and pathological tendon states combined with individual circumstances require an individualized treatment approach, which must be applied to progressive resistance training interventions (Morrissey, 2015).

Despite the necessity of addressing individual factors in tendinopathy, they are often not considered in rehabilitation, with the treatment offered to a young elite male athlete often the same as that for a postmenopausal woman (Cook and Purdam, 2009). Cook and Purdam (2009) proposed that tendinopathy pathology is not absolute and presents on a continuum with three distinct stages, with each having implications for the treatment. The staging model acknowledges the heterogeneity of tendinopathy presentation, with the three phases being (1) early reactive tendinopathy due to acute overload, (2) tendon disrepair after failed healing responses, and (3) degenerative tendinopathy with permanent structural and cellular damage (Cook et al., 2016). The individual tendinopathy risk factors discussed earlier may play a role in altering the progression throughout this continuum and will possibly contribute to individual treatment response (September et al., 2008). The tendinopathy continuum model recommends that rehabilitation is optimized by individually tailored treatment to the tendinopathy stage and targeting the primary driver and interrelated alterations. While exercise is considered essential in tendinopathy rehabilitation, a plethora of other treatment options for tendinopathy exists, which can increase the complexity of the clinical decision-making process. Tendinopathies have a heterogeneous clinical presentation due to the variable changes that exist between the individuals in a cellular matrix structure, pain, function, and disability (Malliaras et al., 2010; Malliaras and Cook, 2011). Phenotyping of patients based on multifactorial components of pain, disability, and load capacity and tolerance, may allow clinicians to direct appropriate interventions at such critical limiting factors (Cook et al., 2016). A recent model proposed by Steinmann et al. (2020) has demonstrated the complex interplay of individual factors in tendinopathy pathogenesis and how they relate to the tendon continuum model. A further step would be the integration of individualized treatment strategies into this model in each pathology stage.

RESISTANCE TRAINING IN TENDINOPATHY

Resistance Training Protocols

Stanish et al. (1986) were the first to publish on the concept of a predominately eccentric-based resistance training protocol for the treatment of lower limb tendinopathy. However, the publication of the Alfredson eccentric heel-drop protocol for Achilles tendinopathy in 1998 had helped to popularize the concept of using an eccentric-based training protocol for treating Achilles tendinopathy (Alfredson et al., 1998). Following the publication of the Alfredson protocol, eccentric-focused resistance training has become a dominant exercise-based strategy for lower limb tendinopathies for the last 20 years (Malliaras et al., 2013). There are, however, some important differences between these protocols, with Stanish recommending that patients perform the eccentric exercises with no pain, and, on the other hand, Alfredson recommended pushing through pain (Sayana and Maffulli, 2007). The parameters of the Alfredson protocol have also been adapted in several studies in the form of an eccentric decline squat protocol to treat patellar tendinopathy (Young et al., 2005; Visnes and Bahr, 2007). Although eccentric training has positive effects in treating tendinopathies, there are several issues related to eccentric-based training protocols. Despite widespread clinical adoption, there is a dearth of high-quality research confirming that removal of the concentric action from isotonic contractions is appropriate or more useful than full isotonic contractions when treating chronic tendinopathies (Couppe et al., 2015). However, for Achilles (Mafi et al., 2001), patellar (Jonsson and Alfredson, 2005), and tennis elbow tendinopathies (Peterson et al., 2014), eccentric-only training has shown better results when directly compared with concentric-only.

Despite the results from individual studies, comprehensive systematic reviews have consistently concluded that high quality pooling evidence of positive clinical outcomes for isolated eccentric resistance training in lower limb tendinopathies is lacking (Lim and Wong, 2018; Murphy et al., 2019; Mendonca et al., 2020b). The original study on the Alfredson eccentric protocol was conducted with a cohort of athletes, which may be related to the high success rate of the protocol. However, these

outcomes may not be generalizable to the general population or sedentary demographics, and it is clear from previous studies that different patient characteristics (activity level and age) potentially have an impact on the response and outcomes from the eccentric-based training (Sayana and Maffulli, 2007). For example, Sayana and Maffulli (2007) found that 45% of the patients in a general population had a failed treatment outcome with eccentric training based on pain and function outcome measures, which suggests that eccentric-only training may not be suitable for all types of patients. However, other single and combined contraction types of resistance training have been investigated, namely concentric-eccentric, isolated concentric, and combined plyometric training (Silbernagel et al., 2020).

Heavy slow resistance training with heavy-load isotonic contractions is a more recent conception in tendinopathy rehabilitation, which has been widely adopted in both clinical research and practice (Malliaras et al., 2013). The HSRT approach was first investigated in case of patellar tendinopathy and was found to have significant positive outcomes for pain and function improvement (Kongsgaard et al., 2009). Similar HSRT interventions based on the same loading and progression parameters were recently investigated with the positive outcomes in Achilles tendinopathy (Beyer et al., 2015) and PHP (Rathleff et al., 2015a). The Alfredson eccentric protocol involves a strict fixed-loading protocol of 180 repetitions and 2 training sessions daily. However, the HSRT protocols can be considered to adhere more to scientific resistance training principles, including progressive increases in volume and load, with increased rest periods between the sessions (**Table 1**). Individual RCTs have found that HSRT protocols have superior clinical outcomes for pain and function and greater patient satisfactory outcomes compared to eccentric-only training for the treatment of lower limb tendinopathies (Kongsgaard et al., 2009; Beyer et al., 2015; Rathleff et al., 2015a). Despite of possessing positive outcomes, it has been suggested that even HSRT protocols do not achieve the intended dosage levels for tendon adaptation and may be enhanced by improved loading and progression methods, including adopting an individualized intervention rather than a standardized protocol (Riel et al., 2019a). Slow performing isotonic contractions during HSRT interventions are thought to stimulate tendon adaptations by providing high loads and forces across tendons, which may improve tendon compliance and activate remodeling processes when applied progressively, including reducing neovascularization and increasing collagen production (Murtaugh and Ihm, 2013). It is well-known that exercise can influence human cell homeostasis and activate tissue changes and healing in both muscles and tendons, through the process known as mechano-transduction (Khan and Scott, 2009). Despite this capability of exercise and particularly HSRT, it has been suggested that the symptom improvement seen in HSRT protocols may not be due to the changes in tendon architecture but rather improved mechanical properties of the tendon (Farnqvist et al., 2020). Resistance training has also been shown to be more effective than stretching for improving flexibility, reducing the injury risk inducing length changes in short muscles *via* sarcomerogenesis (Weppler and Magnusson, 2010; O'Sullivan et al., 2012; Askling et al., 2013; Konrad and Tilp, 2014; Freitas and Mil-Homens, 2015; Timmins et al., 2016).

Loading Issues

There are more questions still to be answered concerning the appropriate dosage of exercise for tendinopathy, which requires further investigation on the optimal dosage and methods to better individualize resistance training interventions (Silbernagel, 2014). It appears to be a tendency during the lower limb tendinopathy rehabilitation to dismiss exercise as a sufficient treatment option if patients do not respond to the one-size-fits-all exercise program and instead pursue other more costly and invasive treatment options (Silbernagel, 2014). To suitably address individual needs, resistance training prescription and dosage need to be individualized to ensure that it is adequate and appropriate. The staging of tendinopathy and subsequent implications on exercise selection are the other areas that require the consideration of an individual (Barratt et al., 2017). At present, there is insufficient evidence to define the exact speed, intensity, and duration of resistance training needed or to predict, certainly, the long-term effects of resistance training for lower limb tendinopathy. It is relatively common for patients with Achilles tendinopathy to fail to respond to the conservative treatment. For example, in an 8-year study of 83 patients with chronic Achilles tendinopathy, 29% failed conservative management and required surgical intervention (Paavola et al., 2000). The individualization of training progression is also required with the selection of appropriate loads for the individual patient required to attenuate for their sport and performance level. Individual progression of resistance training to plyometric exercises is needed to effectively progress athletes to return-to-sport training volume and intensity (Malliaras et al., 2015).

The overload principle states that it is necessary to stress biological tissues beyond their current thresholds to increase their tolerance to subsequent stresses and avoid future injuries. Training with heavier loads may produce greater load tolerance of tendons, contributing to the positive effects of tissue remodeling and improved structural properties of the degenerated tendons (Kulig et al., 2009). However, it is unlikely that the sufficiently high training loads required for these adaptations are being achieved with the Alfredson protocol due to the parameter of two times daily training with no rest days for 12 weeks. It is clear from the literature on tendon responses to loading that no more than three high-intensity resistance training sessions should be undertaken within 1 week in tendinopathy, to allow adequate collagen synthesis, remodeling, adaptation, and tendon recovery (Malliaras et al., 2015; Magnusson and Kjaer, 2019). It is essential that healing tendons receive adequate rest and recovery time during rehabilitation to allow the anabolic and catabolic processes involved in tendon remodeling and adaptation to occur successfully (Waugh et al., 2018). Sufficient tendon adaptations and improved clinical outcomes from HSRT protocols may also require longer than the current gold-standard intervention time of 12 weeks in lower limb tendinopathy rehabilitation (Sussmilch-Leitch et al., 2012). It is believed that the exercise load has to be relatively high to stimulate tendon remodeling, which can explain why forced eccentric toe-raises,

TABLE 1 | Evidence-based resistance training protocols in lower limb tendinopathy.

Protocol	Tendinopathy	Exercise type	Sets, repetitions	Frequency	Duration	Progression	Pain
Characteristics of resistance training protocols in lower limb tendinopathy							
Stanish et al. (1986)	Achilles	Eccentric-concentric, power	3, 10–20	Daily	12 weeks	Speed then load	Enough load to be painful in 3rd set
Alfredson et al. (1998)	Achilles	Eccentric	3, 15	2 × daily	12 weeks	Increase load as able (backpack)	Enough load to achieve moderate pain
Silbernagel et al. (2007b)	Achilles	Eccentric-concentric, balance, plyometric	Various	Daily	12 weeks	Volume, type of exercise	Acceptable within defined limits
Beyer et al. (2015)	Achilles	Eccentric-concentric (HSRT)	3–4, 15–6	3 × week	12 weeks	15–6RM, increase load as able (external weight machine)	Acceptable if not worse after exercise
Rathleff et al. (2015a)	Plantar heel	Eccentric-concentric (HSRT)	3–5, 12–8	3 × week	12 weeks	12–8RM, Increase load as able (backpack)	Acceptable if not worse after exercise
Kongsgaard et al. (2009)	Patellar	Eccentric-concentric (HSRT)	4, 15–6	3 × week	12 weeks	15–6RM, Increase load as able (external weight machine)	Acceptable if not worse after exercise

RM, repetition maximum; HSRT, Heavy slow resistance training.

giving high loads, also give better results than using regular concentric/eccentric toe-raises. There is, however, no direct research showing that high eccentric loading is the best option for lower limb tendinopathies (Silbernagel et al., 2001). The dosage parameters (e.g., training frequency, the number of repetitions and sets, rest, and the duration of a contraction) and the types of exercise are the important characteristics of an exercise program, with loads as high as 80% of 1 repetition maximum (RM) being used for isometric (80% 1RM) and 80% of 8RM for isotonic exercises in some studies (van Ark et al., 2016). Tendons require high load per repetition to derive more optimal tendon healing effects with a high percentage of RM in leg extension exercises, which are also shown to improve neural activation and increase muscle strength (van Ark et al., 2016). However, the optimal dosage and loading strategy for positive outcomes, along with a precise mechanism of the effect of heavy loading, remain to be elucidated and require further investigation (van Ark et al., 2016).

Several studies have advocated the need for higher tendon loading in lower limb tendinopathy rehabilitation programs with the type of load even being considered inconsequential (Cuddeford et al., 2018). In most studies, the Alfredson protocol is the most commonly used protocol and consists of up to 180 repetitions daily, with the addition of additional weight *via* a backpack. However, performing an eccentric exercise with a backpack in excess of 20 kg requires significant therapist involvement and may be one of the reasons of only using bodyweight with high repetitions in most studies (Cuddeford et al., 2018). Adequate adherence to training programs may also be falsely reported in studies as it has been found that accurate recording of dosage in participant training diaries is often not performed or misinterpreted. The assumption that active individuals or athletes have a better compliance for the prescribed exercises than non-active individuals, which may also play a role as the studies involving athletes have previously had better outcomes from resistance training interventions in tendinopathy (Alfredson et al., 1998; Sayana and Maffulli, 2007; de Jonge et al., 2010). Despite this, in one study conducted with an athletic cohort, over one-quarter of patients reported performing the exercises at an intensity that was less than half of what was initially prescribed (Abate et al., 2020). Regardless of the intervention population, individualized exercise programs should be adequately supervised, reviewed, and progressed to ensure the adherence and resolution of the tendinopathy (Vicenzino, 2015). The effectiveness of resistance training interventions may be enhanced by implementing intensive care taking procedures, such as having patients to perform exercises with supervision four times per week for 8 weeks as opposed to at home and unsupervised (Stergioulas et al., 2008).

There is a lack of consensus in the literature regarding which training variables may influence the training outcomes, such as whether exercise should be painful, exercise speed and intensity, training duration, progression methods, and unsupervised home vs. supervised clinic-based training (Rompe et al., 2009). Large randomized controlled trials with blinded assessors and long-term follow-up periods investigating differences in these training parameters are warranted. Resistance training at high-intensity levels alongside gradual increases in loading is considered essential to create positive effects and adaptation in human muscles and tendons. Although the importance of loading in tendinopathy rehabilitation through heavy resistance training is well-established, the optimal intervention parameters and dosages require further research investigation and may include the consideration of motor control, biomechanics, kinetic chain, and reactive strength (Silbernagel et al., 2007b). The outcomes of different resistance training approaches in tendinopathy need to be investigated before accurate clinical practice recommendations can be made, with the outcomes for one specific tendinopathy likely not being generalizable to

other tendinopathies (Riel et al., 2018). As a previous study on lower limb tendinopathies has not found the superiority of one specific resistance training approach, the long-term effects of different resistance training protocols in tendinopathy remain to be investigated (Riel et al., 2018). Current interventions may also not be prescribing true resistance training, with low-load eccentric protocols perhaps being better described as active stretching due to their high frequency and low intensity (Purdam et al., 2004; Abat et al., 2017). It appears from the literature that only HSRT can be identified as true resistance training as it produces the minimum level of load and tension based on scientific resistance training principles. To cause tendon adaptations, including mechanical, material, and morphological tendon changes, research has suggested that an intensity threshold of >70% of the maximum is required to derive adaptations (Bohm et al., 2015; Arampatzis et al., 2020). High repetitions with inadequate loading in current protocols may be related to their poor long-term outcomes. Alternative strategies of performing more sets with fewer repetitions may allow for sufficient loading, volume, and intensity throughout the training to stimulate adaptations (Davies et al., 2020).

Kinetic Chain Strength Issues

Strengthening of the entire kinetic chain, particularly, the gluteal muscles in tendinopathy rehabilitation has been advocated considering its successful application to a plethora of musculoskeletal disorders with similar etiologies (Nakagawa et al., 2008; Cibulka et al., 2009; Meira and Brumitt, 2011; Delitto et al., 2012; Khayambashi et al., 2012; Reiman et al., 2012; Barton et al., 2013, 2019; Rathleff et al., 2014; Baldon Rde et al., 2015; Lack et al., 2015; Rio et al., 2015b; Thomson et al., 2016; Mucha et al., 2017). Gluteal strengthening for the loss of lumbopelvic control impairing motor control and the kinetic chain has been recommended in patellar and Achilles tendinopathies (Kountouris and Cook, 2007). Recently, several studies have shown the improvements in pain and function from the targeted hip strengthening exercises in PHP (Kamonseki et al., 2016; Santos et al., 2016; Lee et al., 2019). Hip muscle weakness can cause an excessive adduction and medial rotation of the hip causing dynamic knee valgus and over pronation (Powers, 2003; Khamis and Yizhar, 2007; Barton et al., 2012). Reduction in the strength of the hip abductors and rotators is identified as a risk factor for lower limb tendinopathy due to altered biomechanics (Harty et al., 2005; McPoil et al., 2008; Labovitz et al., 2011; Bolivar et al., 2013; Beeson, 2014; Martin et al., 2014). Therefore, strengthening these muscles could improve dynamic lower limb alignment and could reduce the excessive strain on lower limb tissues (Snyder et al., 2009; Fukuda et al., 2010; Dolak et al., 2011). Hip muscle activation can also impact the force production at an ankle joint impacting gait, which may contribute to the lower limb pain (Komura et al., 2004; Wainner et al., 2007).

Some authors have recommended that isotonic resistance training interventions are superior to eccentric-biased training in lower limb tendinopathy, particularly for those with concentric muscle weakness that would not be addressed with primarily eccentric training. Individual differences and heterogeneity in tendinopathy presentation necessitate assessing and targeting the kinetic chain weaknesses of individuals, which would not be targeted with eccentric-only training (Goom et al., 2016). For example, athletes with patellar tendinopathy have been shown to respond better to comprehensive resistance training interventions, which include addressing the muscle-tendon strength and function of the kinetic chain (Zwerver et al., 2011). To optimally manage tendinopathies, overall functional capacity should be assessed to facilitate progressive overload of the tendon itself and the kinetic chain (Thompson, 2017). Failure to adequately address isolated muscle deficits, kinetic chain deficits or biomechanics may lead to a suboptimal rehabilitation in tendinopathy (Malliaras et al., 2015). Therefore, it could be considered as essential to address any intrinsic or extrinsic risk factor individually during rehabilitation and consider making environmental adaptations if warranted (Morgan et al., 2018b).

Lower limb tendinopathy rehabilitation may be enhanced by the presence of progressive loading of an muscle-tendon unit alongside targeting the whole lower extremity and kinetic chain. However, an optimal loading protocol to improve outcomes is unclear at present (Cook, 2018). For athletes, individual needs can be addressed through a comprehensive evaluation by physiotherapists to identify particular areas of weakness in the biomechanical kinetic chain (Rudavsky and Cook, 2014). Developing an individualized rehabilitation protocol must consider that elite athletes have increased training and competition demands and will require more comprehensive rehabilitation than amateur athletes for a successful return to sport and avoiding a relapse of the tendinopathy (Rudavsky and Cook, 2014). It may be considered as essential to address any whole body strength deficits in athletes with tendinopathy due to their increased physical loading demands (Kulig et al., 2015). Patellar tendinopathy rehabilitation based on individualizing the rehabilitation intervention according to the participant's specific biomechanical deficiencies and load tolerance has demonstrated an improvement in pain and functionality in elite rugby union players with patellar tendinopathy (Morgan et al., 2018a).

Hip extensor muscles such as the gluteus maximus has been considered as an important target for increasing strength and function during lower limb tendinopathy rehabilitation (Scattone Silva et al., 2015). Previous research has found an association with weak hip extensor muscles and poor lumbopelvic control and the risk of developing lower limb tendinopathy, which may be due to an altered load distribution on the lower limb kinetic chain (Stasinopoulos and Malliaras, 2016). A recent Delphi study of clinical practice found that there was a strong predisposition (87%) toward hip strengthening as a component of patellar tendinopathy rehabilitation programs (Morgan et al., 2018a). Similarly, a recent survey of Brazilian physical therapist practice for patellar tendinopathy reported that hip strengthening exercises were secondary only to quadriceps eccentric training in terms of the frequency of prescription (Mendonca et al., 2020a). Rehabilitation interventions focusing on improving the hip extensor muscle strength in patellar tendinopathy have shown positive outcomes. An 8-week treatment protocol of jump-landing modification and hip-specific muscle strengthening improved pain, function, and biomechanics in an athlete with patellar tendinopathy (Scattone Silva et al., 2015). Biomechanical

studies have found a variety of risk factors, both local and distal to the knee joint, which may be associated with the development of lower limb tendinopathy (Powers, 2010). Physiotherapists must consider these components when tailoring a comprehensive intervention for treating athletes with patellar tendinopathy. The clinical effectiveness of existing rehabilitation strategies in lower limb tendinopathy management options, including eccentric resistance training, do not have satisfactory long-term clinical outcomes. Evidence suggests that not all patient types respond adequately to eccentric-only loading, highlighting the necessity of future research investigating alternative or additional resistance training strategies. Future research on resistance training interventions in tendinopathy should focus on addressing proximal, local, and distal factors including strengthening of the kinetic chain as opposed to isolated strengthening of the pathological tendon (Scattone Silva et al., 2015).

Reactive Strength Issues

Athletic activities such as running and jumping involve energy storage and release from tendons, supported by strong muscular contractions, known as the stretch-shortening cycle (SSC) (Taube et al., 2012). Plyometric exercise, which utilizes the SSC, is a common training practice among athletes. It has been shown to improve the maximal rate of torque development, maximal voluntary contraction, jump performance, running economy, and leg stiffness in active people (Spurrs et al., 2003; Kubo et al., 2007; Foure et al., 2010). In athletes, lower limb tendinopathy occurs most commonly in those participating in activities involving the SSC, such as running and jumping (Malliaras et al., 2013). During athletic activities, the Achilles tendon experiences loads 6–12 times of bodyweight. These high loads require a considerable strength and power to repeatedly generate force and enable the storage and release of energy during athletic activity (Cook et al., 2016). Therefore, rehabilitation programs focusing solely on strength *via* the use of various heel-raise programs may lead to suboptimal functional capabilities, persistent strength deficits, inadequate rehabilitation, and ultimately high recurrence rates of tendinopathy (Child et al., 2010; Gajhede-Knudsen et al., 2013).

Lack of consensus in physiotherapy of what constitutes strength may be a factor in the predominance of unidimensional rehabilitation programs. Little consensus exists on the presence of strength deficits in tendinopathy, leading to the implementation of ineffective rehabilitation programs focusing on one type of exercise only in clinical practice (McAuliffe et al., 2019). To clarify this uncertainty, several categories of strength have been suggested. Maximal strength involves a maximal force development through high-load and low-velocity movements. In contrast, explosive strength is considered as an ability to rapidly produce muscle force through medium to high-load and high-velocity movements such as the rate of force development. On the other hand, reactive strength is considered as the ability of the gastric-soleus muscle complex to store and release energy through a sufficient function of the SSC through low-load, high-velocity plyometric exercises such as hopping and jumping (Beattie et al., 2014). Deficits in SSC power and efficiency have been shown in Achilles tendinopathy as measured by leg stiffness during submaximal hopping and a reduction in maximal hopping distance (Maquirriain, 2012; Wang et al., 2012). After 6 months of recommended exercise rehabilitation in Achilles tendinopathy without plyometric exercise, persistent SSC impairments have been found after 1 year (Silbernagel et al., 2007a).

Heavy slow resistance training in healthy individuals has been found to increase tendon stiffness, whereas plyometric training increases joint stiffness and jumping performance (Kubo et al., 2007). This suggests that the plyometric exercise likely has complimentary effects to HSRT in tendinopathy rehabilitation and may reduce persistent SSC deficits, which can decrease rehabilitation effectiveness. A consistent barrier to implementing the plyometric exercise for lower limb tendinopathy in rehabilitation is that it is not implemented progressively and therefore aggravates symptoms (Chmielewski et al., 2006; Kountouris and Cook, 2007). The common belief that plyometric activities will have a negative impact on tendinopathy rehabilitation is not supported by evidence and it may be beneficial for improving outcomes (McAuliffe et al., 2019). In those with Achilles tendinopathy who continued plyometric exercise such as sprinting, jumping, and hopping, similar pain and function were reported to those who stopped all plyometric activities (Silbernagel et al., 2007b). This study identified that SSC loads are tolerable during tendinopathy rehabilitation in active people, provided they are guided by a pain- and symptom-monitoring model (Silbernagel et al., 2007b). Another study in Achilles tendinopathy used a loading intervention integrating plyometric exercise for 12 weeks to address the entire strength spectrum, which resulted in improvements in pain and disability at 12 months (Silbernagel et al., 2007a). Further studies integrating plyometric training in tendinopathy with traditional HSRT are required, particularly in PHP as it has yet to be investigated (Huffer et al., 2017).

A recent systematic review identified that individuals with Achilles tendinopathy demonstrate strength deficits across the strength spectrum of maximal, reactive, and explosive compared to the uninjured side or asymptomatic controls (McAuliffe et al., 2019). This study concluded that the current focus on maximal strength in tendinopathy rehabilitation and the lack of emphasis on reactive or explosive strength may not optimally match the entire strength spectrum. The authors argued that this could explain why Achilles tendinopathy rehabilitation is only moderately effective with recurrence as high as 27% and why SSC deficits persist post rehabilitation (van der Plas et al., 2012). Similarly, the lack of emphasis on plyometric training in PHP rehabilitation may also help to explain why the rehabilitation focused only on strengthening HSRT is only moderately effective (Riel et al., 2019a). Approximately 40% of the patients with PHP have pain and symptoms 2 years after the diagnosis and treatment, suggesting that new and effective treatments are warranted (Rathleff et al., 2015a). Training only one aspect of the strength profile of an individual, which is common in the lower limb tendinopathy rehabilitation with concentric-eccentric HSRT, will likely not optimally improve the performance across the entire strength spectrum (Allison and

Purdam, 2009). However, only Silbernagel et al. (2007a) have addressed the entire strength spectrum in Achilles tendinopathy rehabilitation, with no studies currently addressing it with PHP (Huffer et al., 2017). Plyometric loading could potentially be a more potent stimulus for muscles compared to tendon adaptation, thus it may compliment HSRT programs, which more potently stimulate tendon adaptation (Mersmann et al., 2017). Increased tendon stress and strain due to a non-uniform musculotendinous development have been observed recently in adolescent volleyball athletes, a high-risk group for tendinopathy (Mersmann et al., 2019). Previous findings highlight the importance of improving the current understanding of the interaction of physical maturation and loading, indicating that SSC training *via* plyometric exercise may act as a tendinopathy prevention strategy (McAuliffe et al., 2019).

Recently, a pain-guided progressive hopping intervention was found to be safe and feasible while adding to the currently recommended HSRT exercise and education for Achilles tendinopathy in recreational runners (Sancho et al., 2019b). This study also included kinetic chain isotonic strength exercises for the gluteus medius and maximus as recent studies have shown that weakness and delayed onset of the gluteal muscles are common while running in those with lower limb tendinopathy (Sancho et al., 2019a). Despite providing evidence of plyometric exercise possessing complimentary effects to HSRT and improving rehabilitation outcomes, there is a dearth of studies investigating the effects of plyometric exercise in tendinopathy rehabilitation (McAuliffe et al., 2019). This is perhaps due to the perception by patients and clinicians that plyometric exercise is potentially damaging and counterproductive in tendinopathy rehabilitation (Sancho et al., 2019b). However, providing education on the benefits of exercise for pain can reduce self-reported pain following an acute exercise compared to those not receiving pain education (Jones et al., 2017). The following sections provide an overview of the application of autoregulation limited to strength and conditioning (S&C), before finally considering the potential application of autoregulation in physiotherapy and its integration into current HSRT protocols in tendinopathy.

PERIODIZATION IN RESISTANCE TRAINING

In the profession of S&C, the use of periodized programming for training is generally accepted as a more effective way of ensuring strength gains for athletes compared to non-periodized programming (Harries et al., 2015). Periodization can be defined as the planned distribution of training to increase the potential for achieving optimal sports performance at a predetermined time point (Conlon et al., 2016; Kiely, 2018). Block periodization has been shown to effectively improve strength and power in athletes and is based on several distinct training mesocycles; hypertrophic, basic strength, and maximal strength (Issurin, 2008, 2010). The mesocycles are performed in an order with an increase in intensity and a decrease in the overall training volume throughout the model (Bartolomei et al., 2014). Maximal strength is considered one of the most important factors in maximizing athletic performance and has been shown to improve the performance in many running sports and reduce injury risk (Stone et al., 2004; Wisloff et al., 2004; Storen et al., 2008). In physiotherapy, strength exercise is one of the most common treatment prescriptions for musculoskeletal disorders such as tendinopathy (Babatunde et al., 2017; Booth et al., 2017). Although there has been research showing the applicability of using structured periodized training in the rehabilitation of athletes, there is a lack of studies on its use on athletes with tendinopathy or the general population in physiotherapy (Lorenz et al., 2010; Lorenz and Reiman, 2011; Reiman and Lorenz, 2011; Hoover et al., 2016; Ladlow et al., 2020).

Periodized strength training typically manipulates the intensity as a percentage of 1RM, which is obtained prior to beginning the training. This approach does not account for daily fluctuations in the training performance due to fatigue and strength levels, which may affect its accuracy and outcome. There can be significant variations in daily strength as high as 20%, resulting in a variable performance when using a fixed method (Hoover et al., 2016). Individual variability in factors, such as genetics, sleep, nutrition, psychological stress, lifestyle factors, total training load, and heart rate variability, may also affect the training performance (Mann et al., 2014; Costa et al., 2019). Fixed periodization also does not account for the individual training response and as such the potential for strength gains may be greater than the potential this model facilitates (Timmons, 2011; Fisher et al., 2017). Personalized exercise prescription and progression may be a more appropriate method, which better accounts for a large interindividual and biological variability that exists in response to training interventions (Mann et al., 2010). Therefore, the use of a method known as autoregulation during training programs would allow individuals to increase strength at their own capability and can allow a constant adjustment of repetitions, which may prevent plateaus in training (Mann et al., 2010).

AUTOREGULATION

Autoregulation is a form of periodization, which involves manipulating or adjusting the variables of strength training based on an individual's daily or weekly readiness to train (Mann et al., 2010). Due to the heterogeneous nature and the multifactorial complexities involved in individual responses to training, the implementation of autoregulated training derives improved training adaptations and outcomes compared to traditional fixed training models (Zourdos et al., 2016a; Rauch et al., 2020). Although autoregulation originated in physiotherapy research and has become widespread in sports performance training, there is a dearth of scientific enquiries evaluating its effectiveness. The principle of individualization is an essential consideration when designing a resistance training program to optimize the adaptations to training (Borresen and Lambert, 2009; Kiely, 2012). A few studies have shown that training adaptations are enhanced when training is tailored to an individual (Jones et al., 2016). Despite a paucity of interventional studies on

autoregulation, several studies have utilized autoregulation training *via* the progressive resistance exercise (PRE) model. The PRE model originated in rehabilitation research and is reported to involve progressively heavier sets of 10 repetitions: one set at 50% of 10RM, one set at 75% 10RM, and one set at 100% 10RM (Delorme et al., 1950).

A daily adjustable progressive resistance exercise (DAPRE) program was implemented with an autoregulated set, which accounted for daily responses in the rehabilitation of a patient (Knight, 1979). This approach allowed for an interactive protocol, which objectively determined the optimal time to increase resistance and the optimal amount of weight during exercise. This provided a more efficient rehabilitation accounting for the individualized reacquisition of strength (Knight, 1990). A specific autoregulatory program derived from the DAPRE method expanded in this concept to meet different training goals of hypertrophy and strength/power and allowed for continual body adaptation through a specific adaptation to imposed demand (SAID) principle (Siff, 2000; Cunanan et al., 2018). This method, termed as autoregulatory progressive resistance exercise (APRE), enhances the previous DAPRE method by introducing training cycles aimed at improving hypertrophy, strength, and power regimes of conditioning. This allows for a continual neuromuscular adaptation by systematically changing the program variables, thus promoting efficient performance gains (Horschig et al., 2014). The autoregulation approach helps to facilitate rapid strength gains without the risk of excessively overloading the joints and tissues, potentially causing injury (Wilson, 2008; Brumitt and Cuddeford, 2015). Without monitoring and adaptation, even the most well-considered protocol potentially becomes irrelevant. Furthermore, physiotherapists face the challenge of dealing with the complexities and heterogenous nature of an individual's recovery and healing process. Meanwhile, adherence and training consistency can enhance adaptation, planned training variation is also essential to ensure relevance on any given day. Therefore, modifiable programming based on relevant feedback is important, which can be achieved using autoregulation (Lorenz and Morrison, 2015).

Mann et al. (2010) adopted an APRE model of periodization and applied it with collegiate athletes. There was a set number of repetitions performed at the percentage of 10RM, 6RM, and 3RM, with athletes self-adjusting the weight after the third set. Athletes performed a maximum number of repetitions to failure during the third set with 100% of their 6RM, determining the load for the fourth set using the number of repetitions completed. This autoregulation method was not only daily adjusted but also decided the weekly changes in load; however, volume and intensity were not controlled due to the inclusion of sets with as many repetitions as possible. These individual differences in volume and intensity could be a reason for the effectiveness of autoregulation training (Siff, 2000). This autoregulation method proved to be effective in increasing maximal bench press and squat strength over 6 weeks in athletes compared to traditional linear periodization (Mann et al., 2010). This autoregulation approach was a modification of the DAPRE system that allows for a more flexible application than more traditional approaches (Rhea et al., 2006). **Tables 2**, **3** highlight the APRE training routines and the amount of load adjustment following the third set of repetitions to failure.

TABLE 2 | Adjusted progressive resistance exercise training routines.

Set	3RM routine	6RM routine	10RM routine
0	Warm-up	Warm-up	Warm-up
1	6 repetitions (50% of 3RM)	10 repetitions (50% of 6RM)	12 repetitions (50% of 10RM)
2	3 repetitions (75% of 3RM)	6 repetitions (75% of 6RM)	10 repetitions (75% of 10RM)
3	Repetitions to failure (3RM)	Repetitions to failure (6RM)	Repetitions to failure (10RM)
4	Adjusted repetitions to failure*	Adjusted repetitions to failure*	Adjusted repetitions to failure*

**Denotes training load must be adjusted according to this table.*
RM, repetition maximum.

Another study found that autoregulation training significantly increased leg press strength compared to non-linear periodization in novice weightlifters for 12 weeks (McNamara and Stearne, 2010). The autoregulation group was able to choose among the three training sessions of various intensities depending on their energy and motivation levels (10RM, 15RM, or 20RM, repetitions of free weight exercises). Unlike the previous study (Mann et al., 2010), volume and intensity were matched between groups, therefore the autoregulation group had a limited number of exercise selection. Although the autoregulation group performed better in leg press strength, both groups significantly improved, which have been attributed to all participants being new to strength training. Therefore, a rapid improvement in strength due to improved neuromuscular efficiency and motor unit recruitment would be expected (Folland and Williams, 2007). However, improvements in chest press strength were not significantly different between the two groups.

In a physiotherapy case report by Ardali (2014), a DAPRE protocol was implemented alongside standard rehabilitation to maximize the quadriceps muscle strength in a 61-year-old female following the total knee replacement. In phase 1 of the intervention, the emphasis was on edema management, improving the left knee range of motion, and reducing pain over the three sessions. The second phase consisted of two components: (1) a DAPRE protocol aimed at increasing quadriceps strength and (2) functional training aimed at normalizing the altered gait patterns, transfers, and dynamic balance. Patients significantly improved their functional performance and quadriceps strength in the 7 weeks following knee surgery and were able to return to daily living activities without knee pain. The early postsurgical implementation of a DAPRE protocol had no adverse effects and was successful in enhancing the functional performance and muscle strength in this case (Ardali, 2014).

In a physiotherapy case report by Horschig et al. (2014), an APRE model of periodization was investigated using the back squat during the rehabilitation of a 17-year-old male athlete recovering from ACL reconstruction. A secondary aim was to

TABLE 3 | Example adjustments for APRE protocols.

Adjustments for 3RM		Adjustments for 6RM		Adjustments for 10RM	
Repetitions	**Set 4**	**Repetitions**	**Set 4**	**Repetitions**	**Set 4**
1–2	Decrease 5–10 pounds	0–2	Decrease 5–10 pounds	4–6	Decrease 5–10 pounds
3–4	Same	3–4	Decrease 0–5 pounds	7–8	Decrease 0–5 pounds
5–6	Increase 5–10 pounds	5–7	Same	9–11	Same
7+	Increase 10–15 pounds	8–12	Increase 5–10 pounds	12–16	Increase 5–10 pounds
		13+	Increase 10–15 pounds	17+	Increase 10–15 pounds

RM, repetition maximum.

examine the applicability of this method in the transitional period from rehabilitation to S&C for which a current disconnect exists. Starting at 20 weeks postoperatively, the athlete was able to show a 10 pounds daily average increase with the 10RM protocol, a 6 pounds daily average increase during the 6RM protocol, and a 6.3 pounds average increase with the 3RM protocol. A 2RM of 390 lbs was performed in the back squat in the conclusion of the program at 39 weeks postoperatively. The results strengthen the current limited knowledge regarding periodization during the later phases of rehabilitation and the transition back to sport participation time period while at the same time providing new insights for future protocol considerations in rehabilitating athletes. The APRE method of periodization provides an individualized progressive resistive protocol that can be used to safely and effectively increase the strength in both healthy populations and individuals recovering from injury during short-term training cycles (Horschig et al., 2014). Although autoregulation research originated in rehabilitation and these two case reports provide encouraging results, there is a clear lack of longitudinal and interventional research of its effectiveness in physiotherapy.

Autoregulation training should not be considered as a stand-alone periodization model, and it can be applied individually during the periodization in three ways: to adjust load during training, to progress weekly load, or to select daily load prior to training (Klemp et al., 2016; Zourdos et al., 2016a). The modification of training volume with autoregulation has typically been the training variable successfully adopted in studies; however, other training variables have also been examined. The effectiveness of autoregulation of rest intervals in trained men compared to fixed rest intervals has been successfully demonstrated, resulting in greater performance outcomes (Goessler and Polito, 2013). A recent systematic review also concluded that autoregulated rest periods were as good as/better than the structured ones for strength and muscle gain (Henselmans and Schoenfeld, 2014).

AUTOREGULATION PROGRESSION METHODS

Rating of Perceived Exertion

The rating of perceived exertion (RPE) scale has been found to be a reliable measure of not only training session intensity but also specific exercise intensity during a session (Lins-Filho Ode et al., 2012; Kraft et al., 2014). Although most studies have found that RPE is an accurate measure of fatigue during strength training, some have found it is not (Day et al., 2004; Duncan et al., 2006; Gearhart et al., 2009; Tiggemann et al., 2010). However, the more experienced in training someone is, the more accurate an RPE scale becomes, with it being less accurate for younger or novice athletes (McGuigan et al., 2008; Testa et al., 2012). In physiotherapy, an RPE scale offers advantages as it allows intensity monitoring without establishing 1RM, which is contraindicated due to injury (Lorenz and Morrison, 2015). However, the Oddvar Holten curve and other models that exist estimate 1RM without lifting a true 1RM, which are used to establish an estimated 1RM based on submaximal loads taken to failure (Lorenz et al., 2010; Haff and Triplett, 2016). Recommendations have been published for using the Borg RPE scale with resistance band strength training in physiotherapy (Day et al., 2004; American College of Sports Medicine, 2009). A recent study on patients with patellofemoral pain adopted this model with an RPE >6 considered to be desirable (Drew et al., 2017). Although this model may be useful in general population patients only using resistance bands, exercise rehabilitation programs in athletes with tendinopathy typically require the use of much heavier external loads to derive the required adaptations. This suggests that more specific progression models for heavier resistance training in rehabilitation are necessary to achieve optimal progression.

Repetitions in Reserve

The Reactive Training Manual by Tuchscherer (2008) details an autoregulation training method common in powerlifting based on an RPE scale. The scale allows an individual to evaluate his/her performance based on the number of repetitions they perceive that they could have completed prior to muscular failure. The scale goes from 1 to 10, with 10 being the maximum number of repetitions. This model is like a repetitions in reserve (RIR) model and is considered by some authors to be more appropriate for resistance training than the common RPE methods used in endurance training (Zourdos et al., 2016b). An RPE of 8 indicated that two extra repetitions could be completed, for example, guiding the adjustments in training load. This type of RIR-based RPE scale may have more specificity than the traditional Borg scale RPE, which has shown submaximal scores even when individuals perform exercises to muscular failure (Shimano et al.,

2006; Pritchett et al., 2009; Hackett et al., 2012). Therefore, in S&C, it has been suggested this specific RPE is more accurate and superior for assessing the intensity compared to traditional RPE during resistance training (Helms et al., 2016; Hackett et al., 2017; Mansfield et al., 2020).

Recently, a resistance training-specific RPE scale has been demonstrated to be effective for measuring the effort during a training session and subsequently adjusting load (Zourdos et al., 2016b). Like the previous model (Tuchscherer, 2008), it measures RIR, where the individual records an RPE during or after completing a set, with 10 being the maximum, 10RPE = 0RIR. The model allows an individual to estimate the number of additional repetitions remaining at a given load. Consequently, if an achieved RPE is too low or high, training load can be altered accordingly and objectively (Zourdos et al., 2016b). For example, an RPE of 9 or 10 could require a load reduction of 2.5–5 kg. This method is preferable for the load assignment to APRE models as they involve training to failure at predetermined loads, meaning a lack of flexibility in training loads and exertion, whereas the RIR RPE model avoids training to failure (Zourdos et al., 2016b). For example, in APRE studies, athletes perform a maximum number of repetitions to failure during a third set with 100% of their 6RM, with the number of repetitions completed determining the load for the fourth set (Mann et al., 2010). Although this resistance training-specific scale has been validated in experienced and novice weightlifters, its application in rehabilitation such as musculoskeletal physiotherapy has not been evaluated (Zourdos et al., 2016b). Nevertheless, this training-specific RIR-based RPE scale is a potential method to regulate training load in clinical patients prescribed resistance training, such as those undertaking traditional resistance loading programs in tendinopathy (Fairman et al., 2017). Recently, the specific RIR-based RPE model was compared against a traditional 1RM load assignment in resistance trained men. Although both loading types were effective, the RPE strategy provided a small 1RM strength advantage in most participants (Helms et al., 2018). Another study compared the RIR autoregulation approach with a fixed-loading intervention based on 1RM for squat strength in trained men over 12 weeks. Although both groups improved squat performance, the RIR autoregulation group showed significantly greater improvements and was trained at an overall higher intensity (Graham and Cleather, 2019). The results from these studies suggest that RIR as a method of autoregulation training can lead to superior strength gains in athletes compared to traditional fixed approaches. However, as a subjective method, it is less effective compared to the objective methods of autoregulation (Shattock and Tee, 2020).

Velocity-Based Training

An objective monitoring method during training sessions is velocity-based training (VBT), which has become increasingly popular due to new technology for monitoring repetition velocity (Nevin, 2019). Despite the lack of research on VBT, several models have been developed that determine the intensity based on barbell velocity during lifting and the end of the set based on a predetermined velocity decrease (Gonzalez-Badillo et al., 2011; Dorrell et al., 2019). Technological devices are available to allow quantitative visualization of the effort of an individual, by recording repetition velocity (Jovanovic and Jukic, 2020). Research and practical application of VBT are becoming increasingly popular in S&C and, when VBT is applied systematically, it allows for an immediate feedback, a fatigue control, and the monitoring of biomechanical changes, and can guide the training process (Banyard et al., 2020; Lahti et al., 2020; Martinez-Cava et al., 2020; Perez-Castilla et al., 2020; Sindiani et al., 2020; Suarez-Arrones et al., 2020; Tsoukos et al., 2020). However, for obtaining accurate measurements, individuals must perform fast concentric actions with a good technique, and training adherence and motivation, which may be a barrier for use in rehabilitation (Kawamori et al., 2006). Recently, a VBT intervention was found to increase maximal strength and jump height in the trained men compared with a traditional 1RM percentage-based training approach for 6 weeks (Dorrell et al., 2020). The VBT group achieved these better outcomes and also having a reduction in total training volume compared with the traditional group, indicating a potentially more efficient training method. Despite a dearth of VBT research in physiotherapy, VBT may have a role on musculoskeletal rehabilitation, particularly on athletes who are involved in S&C or transitioning from rehabilitation into S&C (Lorenz and Morrison, 2015).

AUTOREGULATION IN PHYSIOTHERAPY

Self-Management in Physiotherapy

Recent UK government policy has emphasized the improvement of outcomes in the National Health Service (NHS) by placing the patient at the center of all decisions taken about them. However, the NHS is also facing financial challenges and the need to deliver gains in efficiency (Littlewood et al., 2013b). Self-management in physiotherapy has been suggested as a solution to these issues by reducing the demand for a regular contact with physiotherapists. Self-management also offers opportunities to individualize care, and there is evidence to suggest that an approach by which patients are encouraged to take responsibility for their own care is no less effective than the treatment requiring regular clinic attendance (Littlewood et al., 2013b). Self-management involves individuals to actively manage their own symptoms, treatments, consequences, and changes to lifestyle, which may be associated with their condition (Barlow et al., 2002). This process is facilitated through a partnership referred to as a therapeutic alliance between the physiotherapist and the patient. The self-management framework consists of components, which are considered as essential for enhancing self-efficacy and facilitating self-management such as skill acquisition, problem solving, self-monitoring, and goal setting (Holman and Lorig, 2004).

Real-Time Feedback

Evidence suggests that an adherence to the prescribed self-management exercise in physiotherapy patients can be improved by using interventions such as activity monitoring and feedback systems (Peek et al., 2016). Meanwhile, in S&C, feedback methods such as VBT aimed at helping individuals to maintain high exercise velocity in physiotherapy, exercise was often performed too fast in musculoskeletal rehabilitation (Rathleff et al., 2015b).

This results in contraction time being too short and too few repetitions, which can impede the recovery due to a low exercise dose (Osteras et al., 2013). As well as the overall load, factors such as adequate contraction time or "time under tension" can influence adaptations such as increasing muscle protein synthesis (Burd et al., 2012). Studies have found that both patients and healthy individuals often do not perform exercises in the predefined contraction time, which affects exercise compliance and outcomes (Faber et al., 2015). This could be particularly important during tendinopathy rehabilitation, where HSRT exercises are performed very slowly to maximize contraction time (Riel et al., 2019a). For example, a common HSRT program requires 8 s (3 s concentric, 2 s isometric, and 3 s eccentric) to complete one repetition (Rathleff et al., 2015a).

A new technology that can provide a real-time feedback on exercises performed with an elastic band is a sensor called BandCizer (BandCizer Aps, Odense, Denmark). It has been validated to quantify contraction time, the number of repetitions performed, and the force used to stretch the band by measuring its thickness (Rathleff et al., 2015b). Data from the sensor can be transmitted to an iPad (Apple Inc., Cupertino, CA, USA), with the BandCizer app providing a real-time exercise feedback (McGirr et al., 2015). It has been found feasible for measuring contraction time and exercise dose during musculoskeletal physiotherapy (Rathleff et al., 2017). Recently, the first RCT investigating BandCizer in musculoskeletal physiotherapy found that the real-time feedback on contraction time resulted in exercises being performed closer to the prescribed dose and induced larger strength gains than the control group (Riel et al., 2016). Although this study was performed in those with patellofemoral pain, the prescribed contraction time was 8 s, the same time as repetitions in the PHP HSRT program. This suggests that providing a similar feedback may be applicable if applied in tendinopathy rehabilitation to improve compliance and adequate loading, which has been identified as a barrier in achieving the desired outcomes (Riel et al., 2019a).

Pain-Monitoring Models

The common-sense model of self-regulation of health and illness suggests that patients interpret their individual pain response in a way to facilitate the use of exercise training as a management strategy being pivotal (Hale et al., 2007). Persistent beliefs that the pain is associated with tissue damage, and that the rest is required to enable tissue recovery can prevent the successful implementation of rehabilitation (McAndrew et al., 2008). Such an appraisal could lead to activity avoidance behavior, the prevention of necessary rehabilitation engagement, and adherence (Phillips et al., 2012). In this case, reliance is dependent on the adoption of a therapeutic alliance where concerns and worries can be expressed by the patient, with the reassurance offered by the physiotherapist, including an appropriate explanation of the cause of the problem (Littlewood et al., 2012). The appropriate interpretation of physiological symptoms and responses alongside adequate self-monitoring is considered an essential component of self-management (Newman, 2008).

Several studies on tendinopathy have encouraged patients to monitor their pain response during resistance training, *via* the recording of their pain scores in a self-report diary, with an instruction that the pain produced during training is acceptable but should be no worse after training (Jonsson and Alfredson, 2005; Bernhardsson et al., 2011; Littlewood et al., 2016). When the pain response during training decreases, this is regarded as an indication to progress the loaded exercise by increasing the resistance. In contrast to others who have used a numeric pain rating scale, which does not allow the pain to increase >5/10, to guide training progression, the self-monitoring intervention described allows patients to determine what is manageable in terms of symptom response (Holmgren et al., 2012). This decision reflects the individual perceptions of what constitutes acceptability in terms of pain and which differs between the individuals (Littlewood et al., 2012). Littlewood et al. (2016) proposed the SELF study, an RCT, aimed to evaluate the clinical effectiveness of a self-managed single exercise program, which was self-progressed using pain-monitoring vs. usual physiotherapy treatment for rotator cuff tendinopathy. In the 6- and 12-month follow-up, there was no significant difference between the groups. This study suggested that there is no superiority of one intervention over the other in the short-, mid-, or long-term and that a self-management program based around a single self-progressed exercise *via* pain-monitoring appears to be comparable to a usual physiotherapy treatment (Littlewood et al., 2016).

Progression *via* pain-monitoring in Achilles tendinopathy has also been found feasible. Patients with Achilles tendinopathy who are continuing plyometric exercises, such as sprinting, jumping, and hopping, reported pain and function similar to those who stopped all plyometric activities (Silbernagel et al., 2007b). This study identified that plyometric loads are tolerable during tendinopathy rehabilitation in active people, provided they are guided by a pain- and symptom-monitoring model (Silbernagel et al., 2007b). Recently, this model was expanded further in a feasibility cohort study. A pain-guided progressive plyometric hopping intervention was found to be safe and feasible when added to the currently recommended HSRT exercise and education for Achilles tendinopathy in recreational runners (Sancho et al., 2019b). Recreational and elite athletes with tendinopathy will need to adapt their training and rehabilitation parameters such as frequency, intensity, and duration due to persistent pain. This could have significant consequences for athletes as it can restrain them from training for long periods, decreasing fitness levels, and performance (Petraglia et al., 2017). However, there has been a lack of studies on the investigation of solutions to this problem despite the high prevalence of tendinopathy in athletes (Tahririan et al., 2012). The use of pain-monitoring models during rehabilitation may be a possible solution, to ensure that adequate training levels are maintained.

Historically, the rest from pain-provoking activities, namely running and jumping have been recommended as an initial treatment in tendinopathy (Paavola et al., 2000). However, recommendations in the literature are vague when recommending some types of modified rest in which the

pain provoking activity should be limited or avoided (Kader et al., 2002; Paavola et al., 2002). As athletes are generally very physically active, an over-long period of rest or a decrease in physical activity due to tendinopathy may have a negative effect on their quality of life and sporting performance (Petraglia et al., 2017). In tendinopathy research, it is now generally accepted that a complete rest period is not helpful, with the loaded exercise required for the healing process and continuing a moderate pain-provoking activity may be beneficial when adjusted appropriately (Silbernagel et al., 2007b, 2011).

Autoregulation in Tendinopathy

Since the initial research of autoregulation conducted on physiotherapy patients, there has been a lack of further research, particularly longitudinal research, with only case reports appearing in the physiotherapy literature (Ardali, 2014; Horschig et al., 2014). Despite the preponderance of research showing the effectiveness of resistance training for musculoskeletal disorders, it is usually prescribed or investigated using the predetermined protocols (Ciolac and Rodrigues-da-Silva, 2016). Although the importance of identifying methods for enabling patient self-management with the exercise is becoming increasingly emphasized in physiotherapy, the use of autoregulation methods for progressing a self-managed exercise has been given little attention (Peek et al., 2016). A common issue in the use of predetermined exercise programs in physiotherapy is that the prescription and progression of loaded sets and repetitions are often poorly defined and underdosed (Riel et al., 2019a). If the dosage of training loads is not enough, then the mechano-biological stimulus may not be adequate to stimulate healing, resulting in poor outcomes (Khan and Scott, 2009). Previous studies have shown an association between increased exercise dose and the recovery for musculoskeletal disorders (Osteras et al., 2013). An option for increasing training dosage would be to prescribe a larger exercise dose; however, an compliance with exercise can be compromised by a low self-efficacy (McLean et al., 2010). An alternative approach to increase the exercise dosage is to increase the self-efficacy of patients by empowering patients to be in control of their own rehabilitation (Jack et al., 2010). To the authors' knowledge, only two RCTs have investigated autoregulatory resistance training protocols vs. standardized protocols in tendinopathy. A self-dosed protocol in one study on Achilles tendinopathy (Stevens and Tan, 2014) and one study of PHP (Riel et al., 2019a) is compared with the conventional standardized heel-raise protocols according to the work of Alfredson and Rathleff, respectively. Although these studies have not used any true autoregulation methods or distinct load progression, they are still innovative as they allowed the experimental group to self-select the amount of exercise completed as opposed to following a non-flexible protocol.

Riel et al. (2019a) recently investigated this approach in patients with PHP by comparing a 12-week self-dosed HSRT program with a predetermined program. The HSRT program was based on the protocol, which was previously shown more effective than stretching for PHP (Rathleff et al., 2015a). Both groups performed standing heel raises; however, the experimental group was instructed to perform the exercise with the load as heavy as possible but no heavier than 8RM and for as many sets as possible. Participants in the control group were instructed to perform the exercise according to a rigid protocol progressing from 12RM to 8RM. Both groups performed exercises every 2nd day in a 12-week intervention. If participants felt they could perform more repetitions than their load corresponded to (e.g., 10 repetitions when the load was supposed to be 8RM), a backpack with books to add weight was used (Riel et al., 2019a). There was no significant between-group difference in the improvement of Foot Health Status Questionnaire pain after 12 weeks (adjusted MD 26.9 points, 95% CI 215.5–1.7). According to the Global Rating of Change, 24 of 33 in the experimental group and 20 of 32 in the control group were improved (RR = 1.16, 95% CI 0.83–1.64). However, only the four participants achieved the patient's acceptable symptom state, suggesting that more comprehensive interventions are warranted. Although tendinopathy loading programs are typically predetermined, this study indicates that standardized programs are unnecessary if the self-dosed programs aiming to maximize the load are equally effective (Riel et al., 2019a).

Similarly, Stevens and Tan (2014) found that performing a 6-week do-as-tolerated program of the Alfredson eccentric heel-drop exercises led to similar outcomes in Achilles tendinopathy pain and function compared to the standardized 180 repetitions per day protocol. About 28 patients were randomized to either the standard ($n = 15$) or the do-as-tolerated ($n = 13$) intervention for only 6 weeks. The group performing standard training completed 180 repetitions daily, by completing three sets of 15 repetitions, with either the knee fully extended or slightly flexed, two times per day. The experimental group completed the Alfredson protocol, with a guidance that they could choose to complete a repetition volume that was tolerable. All aspects of management were standardized between the groups other than the repetition volume completed. There was a statistically significant within-group improvement in the function for both the groups; however, only the do-as-tolerated group significantly improved the pain at the end of the intervention based on the VISA outcome scores. There were no statistically significant differences between the groups in the completion of the intervention. The mean numbers of repetitions completed per day by the participants were 112 and 166 for the do-as-tolerated and standard groups, respectively (Stevens and Tan, 2014).

Contrary to expectation, the self-dosed HSRT groups in both the studies did not substantially increase the achieved exercise dose compared with a predetermined regimen. Riel et al. (2019a) concluded that these HSRT regimens are not enough to achieve an acceptable symptom state in most people with PHP. Perhaps, this is because heel-raise HSRT alone does not cater for the deficits in a patient's entire strength spectrum, with the addition of other exercise types such as kinetic chain and plyometric exercise being needed to address strength deficits. The use of a "as many sets or repetitions as possible" method did not increase the number of sets performed in the self-dosed groups compared to the predetermined groups. Perhaps, the use of a

RIR-based RPE method of autoregulation could be a potential strategy to ensure that an adequate and optimal amount of load is achieved and progressed for each individual patient undertaking HSRT interventions. The use of RPE to dictate the number of repetitions and sets performed as a volume autoregulation method has been found effective to increase the total volume and strength gains in powerlifting athletes but has not been investigated for increasing the strength in tendinopathy rehabilitation (Helms et al., 2018).

PRACTICAL APPLICATIONS

Although the previously described autoregulated HSRT protocols (Stevens and Tan, 2014; Riel et al., 2019a) were innovative approaches attempting at improving the earlier protocols of Alfredson and Rathleff by allowing an individualized modification, patients did not perform neither an increased number of sets or repetitions nor increased load. Perhaps, the set progression by a RIR approach would be preferable to perform as many sets or repetitions as possible. This could also allow for within-session increases in load, accounting for an individual's daily readiness to train and their own individual factors. The intra-session changes and load achieved due to RIR monitoring could then be used to determine future daily load selection and weekly adjustment. For example, in the self-dosed Riel protocol, the target was to perform 8RM as heavy as possible for as many sets as possible at a specific load. However, if the patient was to record a 10RIR on the first set, then the load could be lowered to ensure the completion of the intended training volume. Intra-session external load adjustments could also be made using the RIR scale based upon the predetermined RIR goals. For example, if a target RIR is set at 5–6 and a RIR of 7 is recorded, a 2.5 kg load decrease could occur, and for a RIR of 9, a 5 kg load decrease occurs. Similarly, a RIR of 3–5 would require an adjustment of 2.5 kg increase in load, and an RIR of 1–2, a 5 kg increase (**Table 4**).

TABLE 4 | Theoretical load adjustment during autoregulated tendinopathy heel-raise resistance training based on the representation of rating of perceived exertion and corresponding repetitions in reserve vales.

Warm-up if required

Set 1: Based on load lifted during last set of last session

Set 2–4: Load adjusted per set based on RPE/RIR values during previous set

RPE	RIR	Description of perceived effort	Example load adjustment
10	0	Maximum effort, cannot increase load or repetitions	−5 kg
9.5	0.5	Cannot complete more repetitions but could increase load	−2.5 kg
9	1	1 repetition remaining	Maintain load
8.5	1.5	1–2 repetitions remaining	Maintain load
8	2	2 repetitions remaining	Maintain load
7.5	2.5	2–3 repetitions remaining	+2.5 kg
7	3	3 repetitions remaining	+2.5 kg
5–6	4–6	4–6 repetitions remaining	+5 kg
3–4	6+	Light effort	+7.5 kg
1–2	10+	Little to no effort	+10 kg

Following an initial set at a load determined by the previous session, sets 2–5 can be adapted in load based on RIR, with the load in the fifth set determining the starting load of the next session, as in the previous APRE protocols. Many tendinopathy resistance training protocols stipulate an increase of load increments of 2.5–5 kg between the sessions if there is no increase in pain. Adopting this intra-session autoregulated approach based on RIR would allow for individualized loading in response to an individual's daily readiness to train and their individual factors. This individualized approach to load adjustment may allow for a more specific load progression than the standardized protocols, which do not adjust the load during the sessions, potentially improving the outcomes of individuals to the intervention. The rest could also be autoregulated as this has been shown no less effective than the predetermined rest protocols for increasing strength and hypertrophy (Henselmans and Schoenfeld, 2014). A comparison of how this theoretical autoregulated protocol would be compared to the Rathleff and Riel protocols for PHP is presented in **Table 5**. The example provided is specific to HSRT for PHP, with autoregulation methods requiring feasibility and effectiveness investigations across all lower limb tendinopathies.

Despite the potential efficacy of such an autoregulation approach in tendinopathy, several potential limitations to its practical application exist. Although this theoretically autoregulated protocol only includes a progressive HSRT heel-raise exercise, future interventional studies should also consider adding plyometric exercises and exercises with high kinetic chain activation such as squats and deadlifts, progressed in the same autoregulated manner, to address potential strength spectrum deficits (Boren et al., 2011; Reiman et al., 2012). Accurate use of RPE scales has been shown to be higher in those with more training experience, and practice is required to become competent in using this method (McNamara and Stearne, 2010). The application may, therefore, be more suitable for athletes with tendinopathy who have significant training experience. The use of the previous sessions loading parameters to dictate the starting load for the following sessions does not consider daily readiness, which needs to be monitored at an individual level by each patient. Due to higher fatigue levels on any given day, the starting load may need to be subjectively reduced, which again will require an experience to become proficient in making such judgments. Guidance and intervention instruction by a physiotherapist with training in the clinical application of resistance training principles and methods could improve the efficacy and safety efficacy of applying the intervention methods, especially with participants who lack training experience. The use of an RPE RIR progression method will naturally have an element of unreliability due to its subjectivity, and objective autoregulation methods such as VBT have been found slightly more effective when compared

TABLE 5 | Descriptors of Rathleff, Riel and autoregulated interventions.

Descriptor	Exercise program		
	Rathleff	**Riel**	**Autoregulated**
Load magnitude	Weeks 1 + 2: 12RM Weeks 3 + 4: 10RM Weeks 5–12: 8RM	As heavy as possible, no heavier than 8RM	8RM
Monitoring per set	Not reported	Not reported	RPE RIR scale—adjust load based on response
Load progression and volume	Increase weight in backpack—not recorded	Increase weight in backpack—not recorded	Adjust weight per set based on RPE RIR scale—record weight
Number of repetitions per set	Weeks 1 + 2: 12 Weeks 3 + 4: 10 Weeks 5–12: 8	8, depending on load	8
Number of sets	Weeks 1 + 2: 3 Weeks 3 + 4: 4 Weeks 5–12: 5	As many as possible	5
Rest between sets	2 min	2 min	As much as needed
Session frequency	3/week		
Program duration	12 weeks		
Contraction modes per repetition	3 s concentric, 2 s isometric, 3 s eccentric		
Rest between repetitions	Nil		
Time under tension	Weeks 1–2: 8 s/repetition, 96 s/set, 288 s/training session Weeks 3–4: 8 s/repetition, 80 s/set, 320 s/training session Weeks 5–12: 8 s/repetition, 64 s/set, 320 s/training session Total over 12 weeks: 13 216 s	8 s/repetition, 64 s/set, Total over 12 weeks: varies between participants depending on number of sets performed	8 s/repetition, 64 s/set, 320 s/training session total over 12 weeks: 13,440 s
Muscle failure	Yes	Yes	Not mandatory
Range of motion	Full range of motion		
Between session recovery	48 h		
Exercise description	Standing heel raise, with forefoot on a step and a towel placed underneath the toes to dorsiflex them throughout the exercise. With a fully extended knee, the participant performed a heel raise		

against each other (Shattock and Tee, 2020). However, its advantages include an easier implementation while also being a potential method for increasing patient self-efficacy and allowing the self-management in the home setting without the need for expensive equipment, making it a more practical and cost-effective choice.

CONCLUSION

This review has provided an overview of individual factors in tendinopathy, the current resistance training protocols in lower limb tendinopathy and their limitations, and autoregulation methods culminating in a proposed example of the implementation of autoregulation under the current tendinopathy HSRT protocols. While current resistance training protocols are considered as the gold standard treatment intervention for a range of tendinopathies, they are not without limitations as they fail to address an individual's entire strength spectrum and the principle of individualization, resulting in inadequate long-term outcomes. This review provides an example of how these factors may be addressed through autoregulation methods, which may improve the outcomes for tendinopathy, which is a leading global cause of musculoskeletal pain and disability. Previous literature studies have demonstrated how the adoption of autoregulation methods in resistance training can improve strength gains and sports performance in healthy athletes. However, there has been a dearth of research applying these autoregulation principles to musculoskeletal rehabilitation despite the methodology originating in physiotherapy research. This review demonstrates how applying these autoregulation methods to widely adopted resistance training protocols in tendinopathy allows for tailoring the exercise dosage and considering the myriad of potential patient individual factors, which are not accounted for in the standardized and predetermined protocols. However, as a narrative review based on the authors' opinion with no interventions investigated, this review has limitations in terms of clinical applicability. Therefore, further interventional research is required to investigate the safety, feasibility, and efficacy of these methods in improving the outcomes of resistance training interventions in tendinopathy rehabilitation.

A key point from this review is noted that autoregulation should be used as a replacement for current interventions, but that it is a context-specific approach and may be used as a modification strategy to the current resistance training protocols in tendinopathy. Eccentric-only and HSRT protocols have been successfully implemented in lower limb tendinopathy rehabilitation around the world for two decades. However, despite their moderate success, a modification through improved prescription, monitoring, and progression based on the principle of individualization may lead to more optimal, individually tailored, and efficacious interventions. For several years, leading tendinopathy clinicians and researchers have been advocating that tendinopathy rehabilitation must be individualized to improve long-term outcomes. However, this has not been addressed, with predetermined and standardized protocols remaining the mainstay of tendinopathy rehabilitation, both clinically and in interventional research methods. It is clear from the recent literature studies that a paradigm shift in the resistance training application in lower limb tendinopathy is required, attempting to improve clinical outcomes for patients, which remain inadequate in the long term. Perhaps, a slight adaptation of these long-standing resistance training interventions through autoregulation methods may pave the way forward for ascertaining the most optimal and efficacious approach for tendinopathy rehabilitation, which has yet to be elucidated.

AUTHOR CONTRIBUTIONS

IB conceptualized the work, wrote the first draft of the manuscript, revised the manuscript, and approved the final manuscript.

REFERENCES

Abat, F., Alfredson, H., Cucchiarini, M., Madry, H., Marmotti, A., Mouton, C., et al. (2017). Current trends in tendinopathy: consensus of the ESSKA basic science committee. Part I: biology, biomechanics, anatomy and an exercise-based approach. *J. Exp. Orthop*. 4:18. doi: 10.1186/s40634-017-0092-6

Abate, M., Di Carlo, L., Belluati, A., and Salini, V. (2020). Factors associated with positive outcomes of platelet-rich plasma therapy in achilles tendinopathy. *Eur. J. Orthop. Surg. Traumatol*. 30, 859–867. doi: 10.1007/s00590-020-02642-1

Ahmad, J., Repka, M., and Raikin, S. M. (2013). Treatment of myotendinous achilles ruptures. *Foot Ankle Int*. 34, 1074–1078. doi: 10.1177/1071100713483115

Alfredson, H., Pietila, T., Jonsson, P., and Lorentzon, R. (1998). Heavy-load eccentric calf muscle training for the treatment of chronic achilles tendinosis. *Am. J. Sports Med*. 26, 360–366. doi: 10.1177/03635465980260030301

Allison, G. T., and Purdam, C. (2009). Eccentric loading for achilles tendinopathy–strengthening or stretching? *Br. J. Sports Med*. 43, 276–279. doi: 10.1136/bjsm.2008.053546

American College of Sports Medicine (2009). American college of sports medicine position stand. progression models in resistance training for healthy adults. *Med. Sci. Sports Exerc*. 41, 687–708. doi: 10.1249/MSS.0b013e3181915670

Andia, I., and Maffulli, N. (2019). Blood-derived products for tissue repair/regeneration. *Int. J. Mol. Sci*. 20. doi: 10.3390/ijms20184581

Andres, B. M., and Murrell, G. A. (2008). Treatment of tendinopathy: what works, what does not, and what is on the horizon. *Clin. Orthop. Relat. Res*. 466, 1539–1554. doi: 10.1007/s11999-008-0260-1

Andrew, G., and Jonathan, S. (2014). Comparison of achilles tendon loading between male and female recreational runners. *J. Hum. Kinet*. 44, 155–159. doi: 10.2478/hukin-2014-0121

Arampatzis, A., Mersmann, F., and Bohm, S. (2020). Individualized muscle-tendon assessment and training. *Front. Physiol*. 11:723. doi: 10.3389/fphys.2020.00723

Ardali, G. (2014). A daily adjustable progressive resistance exercise protocol and functional training to increase quadriceps muscle strength and functional performance in an elderly homebound patient following a total knee arthroplasty. *Physiother. Theory Pract*. 30, 287–297. doi: 10.3109/09593985.2013.868064

Askling, C. M., Tengvar, M., and Thorstensson, A. (2013). Acute hamstring injuries in swedish elite football: a prospective randomised controlled clinical trial comparing two rehabilitation protocols. *Br. J. Sports Med*. 47, 953–959. doi: 10.1136/bjsports-2013-092165

Babatunde, O. O., Jordan, J. L., Van der Windt, D. A., Hill, J. C., Foster, N. E., and Protheroe, J. (2017). Effective treatment options for musculoskeletal pain in primary care: a systematic overview of current evidence. *PLoS ONE* 12:e0178621. doi: 10.1371/journal.pone.0178621

Baldon Rde, M., Piva, S. R., Scattone Silva, R., and Serrao, F. V. (2015). Evaluating eccentric hip torque and trunk endurance as mediators of changes in lower limb and trunk kinematics in response to functional stabilization training in women with patellofemoral pain. *Am. J. Sports Med*. 43, 1485–1493. doi: 10.1177/0363546515574690

Banyard, H. G., Tufano, J. J., Weakley, J. J. S., Wu, S., Jukic, I., and Nosaka, K. (2020). Superior changes in jump, sprint, and change-of-direction performance but not maximal strength following 6 weeks of velocity-based training compared with 1-repetition-maximum percentage-based training. *Int. J. Sports Physiol. Perform*. 1, 1–11. doi: 10.1123/ijspp.2019-0999

Barlow, J., Wright, C., Sheasby, J., Turner, A., and Hainsworth, J. (2002). Self-management approaches for people with chronic conditions: a review. *Patient Educ. Couns*. 48, 177–187. doi: 10.1016/S0738-3991(02)00032-0

Barratt, P. A., Brookes, N., and Newson, A. (2017). Conservative treatments for greater trochanteric pain syndrome: a systematic review. *Br. J. Sports Med*. 51, 97–104. doi: 10.1136/bjsports-2015-095858

Bartolomei, S., Hoffman, J. R., Merni, F., and Stout, J. R. (2014). A comparison of traditional and block periodized strength training programs in trained athletes. *J. Strength Cond. Res*. 28, 990–997. doi: 10.1519/JSC.0000000000000366

Barton, C. J., de Oliveira Silva, D., Patterson, B. E., Crossley, K. M., Pizzari, T., and Nunes, G. S. (2019). A proximal progressive resistance training program targeting strength and power is feasible in people with patellofemoral pain. *Phys. Ther. Sport*. 38, 59–65. doi: 10.1016/j.ptsp.2019.04.010

Barton, C. J., Lack, S., Malliaras, P., and Morrissey, D. (2013). Gluteal muscle activity and patellofemoral pain syndrome: a systematic review. *Br. J. Sports Med*. 47, 207–214. doi: 10.1136/bjsports-2012-090953

Barton, C. J., Levinger, P., Crossley, K. M., Webster, K. E., and Menz, H. B. (2012). The relationship between rearfoot, tibial and hip kinematics in individuals with patellofemoral pain syndrome. *Clin. Biomech.* 27, 702–705. doi: 10.1016/j.clinbiomech.2012.02.007

Beattie, K., Kenny, I. C., Lyons, M., and Carson, B. P. (2014). The effect of strength training on performance in endurance athletes. *Sports Med*. 44, 845–865. doi: 10.1007/s40279-014-0157-y

Beeson, P. (2014). Plantar fasciopathy: revisiting the risk factors. *Foot Ankle Surg*. 20, 160–165. doi: 10.1016/j.fas.2014.03.003

Benjamin, M., Kaiser, E., and Milz, S. (2008). Structure-function relationships in tendons: a review. *J. Anat*. 212, 211–228. doi: 10.1111/j.1469-7580.2008.00864.x

Bernhardsson, S., Klintberg, I. H., and Wendt, G. K. (2011). Evaluation of an exercise concept focusing on eccentric strength training of the rotator cuff for patients with subacromial impingement syndrome. *Clin. Rehabil*. 25, 69–78. doi: 10.1177/0269215510376005

Beyer, R., Kongsgaard, M., Hougs Kjaer, B., Ohlenschlaeger, T., Kjaer, M., and Magnusson, S. P. (2015). Heavy slow resistance versus eccentric training as treatment for achilles tendinopathy: a randomized controlled trial. *Am. J. Sports Med*. 43, 1704–1711. doi: 10.1177/0363546515584760

Bohm, S., Mersmann, F., and Arampatzis, A. (2015). Human tendon adaptation in response to mechanical loading: a systematic review and meta-analysis of exercise intervention studies on healthy adults. *Sports Med. Open* 1, 1–18. doi: 10.1186/s40798-015-0009-9

Bolivar, Y. A., Munuera, P. V., and Padillo, J. P. (2013). Relationship between tightness of the posterior muscles of the lower limb and plantar fasciitis. *Foot Ankle Int*. 34, 42–48. doi: 10.1177/1071100712459173

Booth, J., Moseley, G. L., Schiltenwolf, M., Cashin, A., Davies, M., and Hubscher, M. (2017). Exercise for chronic musculoskeletal pain: a biopsychosocial approach. *Musculoskeletal Care* 15, 413–421. doi: 10.1002/msc.1191

Boren, K., Conrey, C., Le Coguic, J., Paprocki, L., Voight, M., and Robinson, T. K. (2011). Electromyographic analysis of gluteus medius and gluteus maximus during rehabilitation exercises. *Int. J. Sports Phys. Ther*. 6, 206–223.

Borresen, J., and Lambert, M. I. (2009). The quantification of training load, the training response and the effect on performance. *Sports Med*. 39, 779–795. doi: 10.2165/11317780-000000000-00000

Brumitt, J., and Cuddeford, T. (2015). Current concepts of muscle and tendon adaptation to strength and conditioning. *Int. J. Sports Phys. Ther*. 10, 748–759.

Burd, N. A., Andrews, R. J., West, D. W., Little, J. P., Cochran, A. J., Hector, A. J., et al. (2012). Muscle time under tension during resistance exercise stimulates differential muscle protein sub-fractional synthetic responses in men. *J. Physiol*. 590, 351–362. doi: 10.1113/jphysiol.2011.221200

Cardoso, T. B., Pizzari, T., Kinsella, R., Hope, D., and Cook, J. L. (2019). Current trends in tendinopathy management. *Best Pract. Res. Clin. Rheumatol*. 33, 122–140. doi: 10.1016/j.berh.2019.02.001

Child, S., Bryant, A. L., Clark, R. A., and Crossley, K. M. (2010). Mechanical properties of the achilles tendon aponeurosis are altered in athletes with achilles tendinopathy. *Am. J. Sports Med*. 38, 1885–1893. doi: 10.1177/0363546510366234

Chmielewski, T. L., Myer, G. D., Kauffman, D., and Tillman, S. M. (2006). Plyometric exercise in the rehabilitation of athletes: physiological responses and clinical application. *J. Orthop. Sports Phys. Ther*. 36, 308–319. doi: 10.2519/jospt.2006.2013

Cibulka, M. T., White, D. M., Woehrle, J., Harris-Hayes, M., Enseki, K., Fagerson, T. L., et al. (2009). Hip pain and mobility deficits–hip osteoarthritis: clinical practice guidelines linked to the international classification of functioning, disability, and health from the orthopaedic section of the american physical therapy association. *J. Orthop. Sports Phys. Ther*. 39, A1–A25. doi: 10.2519/jospt.2009.0301

Ciolac, E. G., and Rodrigues-da-Silva, J. M. (2016). Resistance training as a tool for preventing and treating musculoskeletal disorders. *Sports Med*. 46, 1239–1248. doi: 10.1007/s40279-016-0507-z

Clayton, R. A., and Court-Brown, C. M. (2008). The epidemiology of musculoskeletal tendinous and ligamentous injuries. *Injury* 39, 1338–1344. doi: 10.1016/j.injury.2008.06.021

Conlon, J. A., Newton, R. U., Tufano, J. J., Banyard, H. G., Hopper, A. J., Ridge, A. J., et al. (2016). Periodization strategies in older adults: impact on physical function and health. *Med. Sci. Sports Exerc*. 48, 2426–2436. doi: 10.1249/MSS.0000000000001053

Cook, J. (2011). Tendinopathy: no longer a 'one size fits all' diagnosis. *Br. J. Sports Med*. 45, 385. doi: 10.1136/bjsm.2011.084111

Cook, J. L. (2018). Ten treatments to avoid in patients with lower limb tendon pain. *Br. J. Sports Med*. 52:882. doi: 10.1136/bjsports-2018-099045

Cook, J. L., and Docking, S. I. (2015). "Rehabilitation will increase the 'capacity' of your...insert musculoskeletal tissue here...." defining 'tissue capacity': a core concept for clinicians. *Br. J. Sports Med*. 49, 1484–1485. doi: 10.1136/bjsports-2015-094849

Cook, J. L., and Purdam, C. R. (2009). Is tendon pathology a continuum? A pathology model to explain the clinical presentation of load-induced tendinopathy. *Br. J. Sports Med*. 43, 409–416. doi: 10.1136/bjsm.2008.051193

Cook, J. L., and Purdam, C. R. (2014). The challenge of managing tendinopathy in competing athletes. *Br. J. Sports Med*. 48, 506–509. doi: 10.1136/bjsports-2012-092078

Cook, J. L., Rio, E., Purdam, C. R., and Docking, S. I. (2016). Revisiting the continuum model of tendon pathology: what is its merit in clinical practice and research? *Br. J. Sports Med*. 50, 1187–1191. doi: 10.1136/bjsports-2015-095422

Costa, J., Figueiredo, P., Nakamura, F., Rago, V., Rebelo, A., and Brito, J. (2019). Intra-individual variability of sleep and nocturnal cardiac autonomic activity in elite female soccer players during an international tournament. *PLoS ONE* 14:e0218635. doi: 10.1371/journal.pone.0218635

Couppe, C., Svensson, R. B., Silbernagel, K. G., Langberg, H., and Magnusson, S. P. (2015). Eccentric or concentric exercises for the treatment of tendinopathies? *J. Orthop. Sports Phys. Ther*. 45, 853–863. doi: 10.2519/jospt.2015.5910

Cuddeford, T., Houck, J., Palmer, D., Beilstein, J., and Visser, J. (2018). What maximum ankle torque is appropriate for training patients with non-insertional achilles tendinopathy. *Orthopaedic Pract*. 30, 543–547.

Cunanan, A. J., DeWeese, B. H., Wagle, J. P., Carroll, K. M., Sausaman, R., Hornsby, W. G., et al. (2018). The general adaptation syndrome: a foundation for the concept of periodization. *Sports Med*. 48, 787–797. doi: 10.1007/s40279-017-0855-3

Davies, T. B., Halaki, M., Orr, R., Helms, E. R., and Hackett, D. A. (2020). Changes in bench press velocity and power after 8 weeks of high-load cluster- or traditional-set structures. *J. Strength Cond. Res*. 34, 2734–2742. doi: 10.1519/JSC.0000000000003166

Day, M. L., McGuigan, M. R., Brice, G., and Foster, C. (2004). Monitoring exercise intensity during resistance training using the session RPE scale. *J. Strength Cond. Res*. 18, 353–358. doi: 10.1519/00124278-200405000-00027

de Jonge, S., de Vos, R. J., Van Schie, H. T., Verhaar, J. A., Weir, A., and Tol, J. L. (2010). One-year follow-up of a randomised controlled trial on added splinting to eccentric exercises in chronic midportion achilles tendinopathy. *Br. J. Sports Med*. 44, 673–677. doi: 10.1136/bjsm.2008.052142

Dean, B. J. F., Dakin, S. G., Millar, N. L., and Carr, A. J. (2017). Review: emerging concepts in the pathogenesis of tendinopathy. *Surgeon* 15, 349–354. doi: 10.1016/j.surge.2017.05.005

Deans, V. M., Miller, A., and Ramos, J. (2012). A prospective series of patients with chronic achilles tendinopathy treated with autologous-conditioned plasma injections combined with exercise and therapeutic ultrasonography. *J. Foot Ankle Surg*. 51, 706–710. doi: 10.1053/j.jfas.2012.06.009

Dejaco, B., Habets, B., van Loon, C., van Grinsven, S., and van Cingel, R. (2017). Eccentric versus conventional exercise therapy in patients with rotator cuff tendinopathy: a randomized, single blinded, clinical trial. *Knee Surg. Sports Traumatol. Arthrosc*. 25, 2051–2059. doi: 10.1007/s00167-016-4223-x

Delitto, A., George, S. Z., Van Dillen, L. R., Whitman, J. M., Sowa, G., Shekelle, P., et al. (2012). Low back pain. *J. Orthop. Sports Phys. Ther*. 42, A1–A57. doi: 10.2519/jospt.2012.42.4.A1

Delorme, T. L., West, F. E., and Shriber, W. J. (1950). Influence of progressive resistance exercises on knee function following femoral fractures. *J. Bone Joint Surg. Am*. 32A, 910–924. doi: 10.2106/00004623-195032040-00022

Dimitrios, S. (2015). Exercise for tendinopathy. *World J. Methodol*. 5, 51–54. doi: 10.5662/wjm.v5.i2.51

Dolak, K. L., Silkman, C., Medina McKeon, J., Hosey, R. G., Lattermann, C., and Uhl, T. L. (2011). Hip strengthening prior to functional exercises reduces pain sooner than quadriceps strengthening in females with patellofemoral pain syndrome: a randomized clinical trial. *J. Orthop. Sports Phys. Ther*. 41, 560–570. doi: 10.2519/jospt.2011.3499

Dorrell, H. F., Moore, J. M., Smith, M. F., and Gee, T. I. (2019). Validity and reliability of a linear positional transducer across commonly practised resistance training exercises. *J. Sports Sci*. 37, 67–73. doi: 10.1080/02640414.2018.1482588

Dorrell, H. F., Smith, M. F., and Gee, T. I. (2020). Comparison of velocity-based and traditional percentage-based loading methods on maximal strength and power adaptations. *J. Strength Cond. Res*. 34, 46–53. doi: 10.1519/JSC.0000000000003089

Dos Santos Franco, Y. R., Miyamoto, G. C., Franco, K. F. M., de Oliveira, R. R., and Cabral, C. M. N. (2019). Exercise therapy in the treatment of tendinopathies of the lower limbs: a protocol of a systematic review. *Syst Rev*. 8, 1–6. doi: 10.1186/s13643-019-1058-9

Drew, B. T., Conaghan, P. G., Smith, T. O., Selfe, J., and Redmond, A. C. (2017). The effect of targeted treatment on people with patellofemoral pain: a pragmatic, randomised controlled feasibility study. *BMC Musculoskelet Disord*. 18, 1–11. doi: 10.1186/s12891-017-1698-7

Duncan, M. J., Al-Nakeeb, Y., and Scurr, J. (2006). Perceived exertion is related to muscle activity during leg extension exercise. *Res. Sports Med*. 14, 179–189. doi: 10.1080/15438620600854728

Faber, M., Andersen, M. H., Sevel, C., Thorborg, K., Bandholm, T., and Rathleff, M. (2015). The majority are not performing home-exercises correctly two weeks after their initial instruction-an assessor-blinded study. *PeerJ*. 3:e1102. doi: 10.7717/peerj.1102

Fairman, C. M., Zourdos, M. C., Helms, E. R., and Focht, B. C. (2017). A scientific rationale to improve resistance training prescription in exercise oncology. *Sports Med*. 47, 1457–1465. doi: 10.1007/s40279-017-0673-7

Farnqvist, K., Pearson, S., and Malliaras, P. (2020). Adaptation of tendon structure and function in tendinopathy with exercise and its relationship to clinical outcome. *J. Sport Rehab*. 29, 107–115. doi: 10.1123/jsr.2018-0353

Ferreira, V. M. L. M., Oliveira, R. R., Nazareno, T. S., Freitas, L. V., and Mendonca, L. D. (2020). Interaction of foot and hip factors identifies achilles tendinopathy occurrence in recreational runners. *Phys. Ther. Sport*. 45, 111–119. doi: 10.1016/j.ptsp.2020.06.006

Fisher, J., Steele, J., and Smith, D. (2017). High- and low-load resistance training: interpretation and practical application of current research findings. *Sports Med*. 47, 393–400. doi: 10.1007/s40279-016-0602-1

Fleck, S. J. (2011). Non-linear periodization for general fitness & athletes. *J. Hum. Kinet*. 29A, 41–45. doi: 10.2478/v10078-011-0057-2

Folland, J. P., and Williams, A. G. (2007). The adaptations to strength training: morphological and neurological contributions to increased strength. *Sports Med*. 37, 145–168. doi: 10.2165/00007256-200737020-00004

Foure, A., Nordez, A., and Cornu, C. (2010). Plyometric training effects on achilles tendon stiffness and dissipative properties. *J. Appl. Physiol.* 109, 849–854. doi: 10.1152/japplphysiol.01150.2009

Freitas, S. R., and Mil-Homens, P. (2015). Effect of 8-week high-intensity stretching training on biceps femoris architecture. *J. Strength Cond. Res*. 29, 1737–1740. doi: 10.1519/JSC.0000000000000800

Fu, S. C., Rolf, C., Cheuk, Y. C., Lui, P. P., and Chan, K. M. (2010). Deciphering the pathogenesis of tendinopathy: a three-stages process. Sports Med Arthrosc Rehabil Ther Technol. 2. doi: 10.1186/1758-2555-2-30

Fukuda, T. Y., Rossetto, F. M., Magalhaes, E., Bryk, F. F., Lucareli, P. R., and de Almeida Aparecida Carvalho, N. (2010). Short-term effects of hip abductors and lateral rotators strengthening in females with patellofemoral pain syndrome: a randomized controlled clinical trial. *J. Orthop. Sports Phys. Ther*. 40, 736–742. doi: 10.2519/jospt.2010.3246

Gajhede-Knudsen, M., Ekstrand, J., Magnusson, H., and Maffulli, N. (2013). Recurrence of achilles tendon injuries in elite male football players is more common after early return to play: an 11-year follow-up of the UEFA champions league injury study. *Br. J. Sports Med*. 47, 763–768. doi: 10.1136/bjsports-2013-092271

Ganestam, A., Kallemose, T., Troelsen, A., and Barfod, K. W. (2016). Increasing incidence of acute achilles tendon rupture and a noticeable decline in surgical treatment from 1994 to 2013. A nationwide registry study of 33,160 patients. *Knee Surg. Sports Traumatol. Arthrosc*. 24, 3730–3737. doi: 10.1007/s00167-015-3544-5

Gearhart, R. F. Jr., Lagally, K. M., Riechman, S. E., Andrews, R. D., and Robertson, R. J. (2009). Strength tracking using the OMNI resistance exercise scale in older men and women. *J. Strength Cond. Res*. 23, 1011–1015. doi: 10.1519/JSC.0b013e3181a2ec41

Gillespie, M. A. M., Cznik, A., Wassinger, C. A., and Sole, G. (2017). Rotator cuff-related pain: patients' understanding and experiences. *Musculoskelet Sci. Pract*. 30, 64–71. doi: 10.1016/j.msksp.2017.05.009

Girgis, B., and Duarte, J. A. (2020). Physical therapy for tendinopathy: an umbrella review of systematic reviews and meta-analyses. *Phys. Ther. Sport*. 46, 30–46. doi: 10.1016/j.ptsp.2020.08.002

Goessler, K. F., and Polito, M. D. (2013). Effect of fixed and self-suggested rest intervals between sets of resistance exercise on post- exercise cardiovascular behavior. *Rev. Bras Cineantropom Desempenho Hum*. 15, 467–475. doi: 10.5007/1980-0037.2013v15n4p467

Gonzalez-Badillo, J. J., Marques, M. C., and Sanchez-Medina, L. (2011). The importance of movement velocity as a measure to control resistance training intensity. *J. Jum Kinet*. 29A, 15–19. doi: 10.2478/v10078-011-0053-6

Goom, T. S., Malliaras, P., Reiman, M. P., and Purdam, C. R. (2016). Proximal hamstring tendinopathy: clinical aspects of assessment and management. *J. Orthop. Sports Phys. Ther*. 46, 483–493. doi: 10.2519/jospt.2016.5986

Graham, T., and Cleather, D. J. (2019). Autoregulation by "repetitions in reserve" leads to greater improvements in strength over a 12-week training program than fixed loading. *J. Strength Cond. Res*. doi: 10.1519/JSC.0000000000003164

Hackett, D. A., Cobley, S. P., Davies, T. B., Michael, S. W., and Halaki, M. (2017). Accuracy in estimating repetitions to failure during resistance exercise. *J. Strength Cond. Res*. 31, 2162–2168. doi: 10.1519/JSC.0000000000001683

Hackett, D. A., Johnson, N. A., Halaki, M., and Chow, C. M. (2012). A novel scale to assess resistance-exercise effort. *J. Sports Sci*. 30, 1405–1413. doi: 10.1080/02640414.2012.710757

Haff, G., and Triplett, N. T. (2016). *Essentials of Strength Training and Conditioning, 4th Edn*. Champaign, IL: Human Kinetics.

Hainline, B., Turner, J. A., Caneiro, J. P., Stewart, M., and Lorimer Moseley, G. (2017). Pain in elite athletes-neurophysiological, biomechanical and psychosocial considerations: a narrative review. *Br. J. Sports Med*. 51, 1259–1264. doi: 10.1136/bjsports-2017-097890

Hale, E. D., Treharne, G. J., and Kitas, G. D. (2007). The common-sense model of self-regulation of health and illness: how can we use it to understand and respond to our patients' needs? *Rheumatology* 46, 904–906. doi: 10.1093/rheumatology/kem060

Hanratty, C. E., Kerr, D. P., Wilson, I. M., McCracken, M., Sim, J., Basford, J. R., et al. (2016). Physical therapists' perceptions and use of exercise in the management of subacromial shoulder impingement syndrome: focus group study. *Phys. Ther*. 96, 1354–1363. doi: 10.2522/ptj.20150427

Harries, S. K., Lubans, D. R., and Callister, R. (2015). Systematic review and meta-analysis of linear and undulating periodized resistance training programs on muscular strength. *J. Strength Cond. Res*. 29, 1113–1125. doi: 10.1519/JSC.0000000000000712

Harris, M., Schultz, A., Drew, M. K., Rio, E., Adams, S., and Edwards, S. (2020). Thirty-seven jump-landing biomechanical variables are associated with asymptomatic patellar tendon abnormality and patellar tendinopathy: a systematic review. *Phys. Ther. Sport*. 45, 38–55. doi: 10.1016/j.ptsp.2020.03.011

Harty, J., Soffe, K., O'Toole, G., and Stephens, M. M. (2005). The role of hamstring tightness in plantar fasciitis. *Foot Ankle Int*. 26, 1089–1092. doi: 10.1177/107110070502601215

Helms, E. R., Cronin, J., Storey, A., and Zourdos, M. C. (2016). Application of the repetitions in reserve-based rating of perceived exertion scale for resistance training. *Strength Cond. J*. 38, 42–49. doi: 10.1519/SSC.0000000000000218

Helms, E. R., Cross, M. R., Brown, S. R., Storey, A., Cronin, J., and Zourdos, M. C. (2018). Rating of perceived exertion as a method of volume autoregulation within a periodized program. *J. Strength Cond. Res*. 32, 1627–1636. doi: 10.1519/JSC.0000000000002032

Henselmans, M., and Schoenfeld, B. J. (2014). The effect of inter-set rest intervals on resistance exercise-induced muscle hypertrophy. *Sports Med*. 44, 1635–1643. doi: 10.1007/s40279-014-0228-0

Holman, H., and Lorig, K. (2004). Patient self-management: a key to effectiveness and efficiency in care of chronic disease. *Public Health Rep*. 119, 239–243. doi: 10.1016/j.phr.2004.04.002

Holmgren, T., Bjornsson Hallgren, H., Oberg, B., Adolfsson, L., and Johansson, K. (2012). Effect of specific exercise strategy on need for surgery in patients with subacromial impingement syndrome: randomised controlled study. *BMJ* 344:e787. doi: 10.1136/bmj.e787

Hoover, D. L., VanWye, W. R., and Judge, L. W. (2016). Periodization and physical therapy: bridging the gap between training and rehabilitation. *Phys. Ther. Sport*. 18, 1–20. doi: 10.1016/j.ptsp.2015.08.003

Horschig, A. D., Neff, T. E., and Serrano, A. J. (2014). Utilization of autoregulatory progressive resistance exercise in transitional rehabilitation periodization of a high school football-player following anterior cruciate ligament reconstruction: a case report. *Int. J. Sports Phys. Ther*. 9, 691–698.

Hoy, D. G., Smith, E., Cross, M., Sanchez-Riera, L., Blyth, F. M., Buchbinder, R., et al. (2015). Reflecting on the global burden of musculoskeletal conditions: lessons learnt from the global burden of disease 2010 study and the next steps forward. *Ann. Rheum. Dis*. 74, 4–7. doi: 10.1136/annrheumdis-2014-205393

Huffer, D., Hing, W., Newton, R., and Clair, M. (2017). Strength training for plantar fasciitis and the intrinsic foot musculature: a systematic review. *Phys. Ther. Sport*. 24, 44–52. doi: 10.1016/j.ptsp.2016.08.008

Ingwersen, K. G., Jensen, S. L., Sorensen, L., Jorgensen, H. R., Christensen, R., Sogaard, K., et al. (2017). Three months of progressive high-load versus traditional low-load strength training among patients with rotator cuff tendinopathy: primary results from the double-blind randomized controlled RoCTEx trial. *Orthop. J. Sports Med*. 5. doi: 10.1177/2325967117723292

Issurin, V. (2008). Block periodization versus traditional training theory: a review. *J. Sports Med. Phys. Fitness* 48, 65–75.

Issurin, V. B. (2010). New horizons for the methodology and physiology of training periodization. *Sports Med*. 40, 189–206. doi: 10.2165/11319770-000000000-00000

Jack, K., McLean, S. M., Moffett, J. K., and Gardiner, E. (2010). Barriers to treatment adherence in physiotherapy outpatient clinics: a systematic review. *Man Ther*. 15, 220–228. doi: 10.1016/j.math.2009.12.004

Jarvinen, T. A., Kannus, P., Maffulli, N., and Khan, K. M. (2005). Achilles tendon disorders: etiology and epidemiology. *Foot Ankle Clin*. 10, 255–266. doi: 10.1016/j.fcl.2005.01.013

Jones, M. D., Valenzuela, T., Booth, J., Taylor, J. L., and Barry, B. K. (2017). Explicit education about exercise-induced hypoalgesia influences pain responses to acute exercise in healthy adults: a randomized controlled trial. *J. Pain*. 18, 1409–1416. doi: 10.1016/j.jpain.2017.07.006

Jones, N., Kiely, J., Suraci, B., Collins, D. J., de Lorenzo, D., Pickering, C., et al. (2016). A genetic-based algorithm for personalized resistance training. *Biol. Sport*. 33, 117–26. doi: 10.5604/20831862.1198210

Jonsson, P., and Alfredson, H. (2005). Superior results with eccentric compared to concentric quadriceps training in patients with jumper's knee: a prospective randomised study. *Br. J. Sports Med*. 39, 847–850. doi: 10.1136/bjsm.2005.018630

Jovanovic, M., and Jukic, I. (2020). Within-unit reliability and between-units agreement of the commercially available linear position transducer and barbell-mounted inertial sensor to measure movement velocity. *J. Strength Cond. Res. Oct* 9. doi: 10.1519/JSC.0000000000003776

Kader, D., Saxena, A., Movin, T., and Maffulli, N. (2002). Achilles tendinopathy: some aspects of basic science and clinical management. *Br. J. Sports Med*. 36, 239–249. doi: 10.1136/bjsm.36.4.239

Kamonseki, D. H., Goncalves, G. A., Yi, L. C., and Junior, I. L. (2016). Effect of stretching with and without muscle strengthening exercises for the foot and hip in patients with plantar fasciitis: a randomized controlled single-blind clinical trial. *Man Ther*. 23, 76–82. doi: 10.1016/j.math.2015.10.006

Kawamori, N., Rossi, S. J., Justice, B. D., Haff, E. E., Pistilli, E. E., O'Bryant, H. S., et al. (2006). Peak force and rate of force development during isometric and dynamic mid-thigh clean pulls performed at various intensities. *J. Strength Cond. Res*. 20, 483–491. doi: 10.1519/00124278-200608000-00005

Khamis, S., and Yizhar, Z. (2007). Effect of feet hyperpronation on pelvic alignment in a standing position. *Gait Posture* 25, 127–134. doi: 10.1016/j.gaitpost.2006.02.005

Khan, K. M., and Scott, A. (2009). Mechanotherapy: how physical therapists' prescription of exercise promotes tissue repair. *Br. J. Sports Med*. 43, 247–252. doi: 10.1136/bjsm.2008.054239

Khayambashi, K., Mohammadkhani, Z., Ghaznavi, K., Lyle, M. A., and Powers, C. M. (2012). The effects of isolated hip abductor and external rotator muscle strengthening on pain, health status, and hip strength in females with patellofemoral pain: a randomized controlled trial. *J. Orthop. Sports Phys. Ther*. 42, 22–29. doi: 10.2519/jospt.2012.3704

Kiely, J. (2012). Periodization paradigms in the 21st century: evidence-led or tradition-driven? *Int. J. Sports Physiol. Perform*. 7, 242–250. doi: 10.1123/ijspp.7.3.242

Kiely, J. (2018). Periodization theory: confronting an inconvenient truth. *Sports Med*. 48, 753–764. doi: 10.1007/s40279-017-0823-y

Kjaer, M., Langberg, H., Heinemeier, K., Bayer, M. L., Hansen, M., Holm, L., et al. (2009). From mechanical loading to collagen synthesis, structural changes and function in human tendon. *Scand. J. Med. Sci. Sports* 19, 500–510. doi: 10.1111/j.1600-0838.2009.00986.x

Kjaer, M., Magnusson, P., Krogsgaard, M., Boysen Moller, J., Olesen, J., Heinemeier, K., et al. (2006). Extracellular matrix adaptation of tendon and skeletal muscle to exercise. *J. Anat*. 208, 445–450. doi: 10.1111/j.1469-7580.2006.00549.x

Klemp, A., Dolan, C., Quiles, J. M., Blanco, R., Zoeller, R. F., Graves, B. S., et al. (2016). Volume-equated high- and low-repetition daily undulating programming strategies produce similar hypertrophy and strength adaptations. *Appl. Physiol. Nutr. Metab*. 41, 699–705. doi: 10.1139/apnm-2015-0707

Knight, K. L. (1979). Knee rehabilitation by the daily adjustable progressive resistive exercise technique. *Am. J. Sports Med*. 7, 336–337. doi: 10.1177/036354657900700605

Knight, K. L. (1990). Quadriceps strengthening with the DAPRE technique: case studies with neurological implications. *J. Orthop. Sports Phys. Ther*. 12, 66–71. doi: 10.2519/jospt.1990.12.2.66

Knobloch, K. (2016). Drug-induced tendon disorders. *Adv. Exp. Med. Biol*. 920, 229–238. doi: 10.1007/978-3-319-33943-6_22

Komura, T., Prokopow, P., and Nagano, A. (2004). Evaluation of the influence of muscle deactivation on other muscles and joints during gait motion. *J. Biomech*. 37, 425–436. doi: 10.1016/j.jbiomech.2003.09.022

Kongsgaard, M., Kovanen, V., Aagaard, P., Doessing, S., Hansen, P., Laursen, A. H., et al. (2009). Corticosteroid injections, eccentric decline squat training and heavy slow resistance training in patellar tendinopathy. *Scand. J. Med. Sci. Sports* 19, 790–802. doi: 10.1111/j.1600-0838.2009.00949.x

Kongsgaard, M., Qvortrup, K., Larsen, J., Aagaard, P., Doessing, S., Hansen, P., et al. (2010). Fibril morphology and tendon mechanical properties in patellar tendinopathy: effects of heavy slow resistance training. *Am. J. Sports Med*. 38, 749–756. doi: 10.1177/0363546509350915

Konrad, A., and Tilp, M. (2014). Increased range of motion after static stretching is not due to changes in muscle and tendon structures. *Clin. Biomech*. 29, 636–642. doi: 10.1016/j.clinbiomech.2014.04.013

Kountouris, A., and Cook, J. (2007). Rehabilitation of achilles and patellar tendinopathies. *Best Pract. Res. Clin. Rheumatol*. 21, 295–316. doi: 10.1016/j.berh.2006.12.003

Kraft, J. A., Green, J. M., and Gast, T. M. (2014). Work distribution influences session ratings of perceived exertion response during resistance exercise matched for total volume. *J. Strength Cond. Res*. 28, 2042–2046. doi: 10.1519/JSC.0000000000000342

Kubo, K., Morimoto, M., Komuro, T., Yata, H., Tsunoda, N., Kanehisa, H., et al. (2007). Effects of plyometric and weight training on muscle-tendon complex and jump performance. *Med. Sci. Sports Exerc*. 39, 1801–1810. doi: 10.1249/mss.0b013e31813e630a

Kulig, K., Lederhaus, E. S., Reischl, S., Arya, S., and Bashford, G. (2009). Effect of eccentric exercise program for early tibialis posterior tendinopathy. *Foot Ankle Int*. 30, 877–885. doi: 10.3113/FAI.2009.0877

Kulig, K., Noceti-DeWit, L. M., Reischl, S. F., and Landel, R. F. (2015). Physical therapists' role in prevention and management of patellar tendinopathy injuries in youth, collegiate, and middle-aged indoor volleyball athletes. *Braz. J. Phys. Ther*. 19, 410–420. doi: 10.1590/bjpt-rbf.2014.0126

Labovitz, J. M., Yu, J., and Kim, C. (2011). The role of hamstring tightness in plantar fasciitis. *Foot Ankle Spec*. 4, 141–144. doi: 10.1177/1938640010397341

Lack, S., Barton, C., Sohan, O., Crossley, K., and Morrissey, D. (2015). Proximal muscle rehabilitation is effective for patellofemoral pain: a systematic review with meta-analysis. *Br. J. Sports Med*. 49, 1365–1376. doi: 10.1136/bjsports-2015-094723

Ladlow, P., Conway, D., Hayhurst, D., Suffield, C., Cassidy, R. P., and Coppack, R. J. (2020). Integration of strength training into UK defence rehabilitation practice: current trends and future challenges. *BMJ Mil Health*. doi: 10.1136/bmjmilitary-2020-001590

Lahti, J., Jimenez-Reyes, P., Cross, M. R., Samozino, P., Chassaing, P., Simond-Cote, B., et al. (2020). Individual sprint force-velocity profile adaptations to in-season assisted and resisted velocity-based training in professional rugby. *Sports* 8:74. doi: 10.3390/sports8050074

Leblanc, D. R., Schneider, M., Angele, P., Vollmer, G., and Docheva, D. (2017). The effect of estrogen on tendon and ligament metabolism and function. *J. Steroid Biochem. Mol. Biol*. 172, 106–116. doi: 10.1016/j.jsbmb.2017.06.008

Lee, J. H., Park, J. H., and Jang, W. Y. (2019). The effects of hip strengthening exercises in a patient with plantar fasciitis: a case report. *Medicine* 98:e16258. doi: 10.1097/MD.0000000000016258

Lim, H. Y., and Wong, S. H. (2018). Effects of isometric, eccentric, or heavy slow resistance exercises on pain and function in individuals with patellar tendinopathy: a systematic review. *Physiother. Res. Int*. 23:e1721. doi: 10.1002/pri.1721

Lins-Filho Ode, L., Robertson, R. J., Farah, B. Q., Rodrigues, S. L., Cyrino, E. S., and Ritti-Dias, R. M. (2012). Effects of exercise intensity on rating of perceived

exertion during a multiple-set resistance exercise session. *J. Strength Cond. Res.* 26, 466–472. doi: 10.1519/JSC.0b013e31822602fa

Littlewood, C., Ashton, J., Mawson, S., May, S., and Walters, S. (2012). A mixed methods study to evaluate the clinical and cost-effectiveness of a self-managed exercise programme versus usual physiotherapy for chronic rotator cuff disorders: protocol for the SELF study. *BMC Musculoskelet Disord.* 13, 1–7. doi: 10.1186/1471-2474-13-62

Littlewood, C., Bateman, M., Brown, K., Bury, J., Mawson, S., May, S., et al. (2016). A self-managed single exercise programme versus usual physiotherapy treatment for rotator cuff tendinopathy: a randomised controlled trial (the SELF study). *Clin. Rehabil.* 30, 686–696. doi: 10.1177/0269215515593784

Littlewood, C., Malliaras, P., Bateman, M., Stace, R., May, S., and Walters, S. (2013a). The central nervous system–an additional consideration in 'rotator cuff tendinopathy' and a potential basis for understanding response to loaded therapeutic exercise. *Man Ther.* 18, 468–472. doi: 10.1016/j.math.2013.07.005

Littlewood, C., Malliaras, P., Mawson, S., May, S., and Walters, S. (2013b). Development of a self-managed loaded exercise programme for rotator cuff tendinopathy. *Physiotherapy* 99, 358–362. doi: 10.1016/j.physio.2012.12.002

Lorenz, D., and Morrison, S. (2015). Current concepts in periodization of strength and conditioning for the sports physical therapist. *Int. J. Sports Phys. Ther.* 10, 734–747.

Lorenz, D. S., and Reiman, M. P. (2011). Performance enhancement in the terminal phases of rehabilitation. *Sports Health* 3, 470–480. doi: 10.1177/1941738111415039

Lorenz, D. S., Reiman, M. P., and Walker, J. C. (2010). Periodization: current review and suggested implementation for athletic rehabilitation. *Sports Health* 2, 509–518. doi: 10.1177/1941738110375910

Lui, P. P., Maffulli, N., Rolf, C., and Smith, R. K. (2011). What are the validated animal models for tendinopathy? *Scand. J. Med. Sci. Sports* 21, 3–17. doi: 10.1111/j.1600-0838.2010.01164.x

Maestroni, L., Read, P., Bishop, C., Papadopoulos, K., Suchomel, T. J., Comfort, P., et al. (2020). The benefits of strength training on musculoskeletal system health: practical applications for interdisciplinary care. *Sports Med.* 50, 1431–1450. doi: 10.1007/s40279-020-01309-5

Maffulli, N., Wong, J., and Almekinders, L. C. (2003). Types and epidemiology of tendinopathy. *Clin. Sports Med.* 22, 675–692. doi: 10.1016/S0278-5919(03)00004-8

Mafi, N., Lorentzon, R., and Alfredson, H. (2001). Superior short-term results with eccentric calf muscle training compared to concentric training in a randomized prospective multicenter study on patients with chronic achilles tendinosis. *Knee Surg. Sports Traumatol. Arthrosc.* 9, 42–47. doi: 10.1007/s001670000148

Magnussen, R. A., Dunn, W. R., and Thomson, A. B. (2009). Nonoperative treatment of midportion achilles tendinopathy: a systematic review. *Clin. J. Sport Med.* 19, 54–64. doi: 10.1097/JSM.0b013e31818ef090

Magnusson, S. P., and Kjaer, M. (2019). The impact of loading, unloading, ageing and injury on the human tendon. *J. Physiol.* 597, 1283–1298. doi: 10.1113/JP275450

Malliaras, P., Barton, C. J., Reeves, N. D., and Langberg, H. (2013). Achilles and patellar tendinopathy loading programmes: a systematic review comparing clinical outcomes and identifying potential mechanisms for effectiveness. *Sports Med.* 43, 267–286. doi: 10.1007/s40279-013-0019-z

Malliaras, P., and Cook, J. (2011). Changes in anteroposterior patellar tendon diameter support a continuum of pathological changes. *Br. J. Sports Med.* 45, 1048–1051. doi: 10.1136/bjsm.2010.082180

Malliaras, P., Cook, J., Purdam, C., and Rio, E. (2015). Patellar tendinopathy: clinical diagnosis, load management, and advice for challenging case presentations. *J. Orthop. Sports Phys. Ther.* 45, 887–898. doi: 10.2519/jospt.2015.5987

Malliaras, P., Purdam, C., Maffulli, N., and Cook, J. (2010). Temporal sequence of greyscale ultrasound changes and their relationship with neovascularity and pain in the patellar tendon. *Br. J. Sports Med.* 44, 944–947. doi: 10.1136/bjsm.2008.054916

Mallows, A., Debenham, J., Walker, T., and Littlewood, C. (2017b). Association of psychological variables and outcome in tendinopathy: a systematic review. *Br. J. Sports Med.* 51, 743–748. doi: 10.1136/bjsports-2016-096154

Mallows, A. J., Debenham, J. R., Malliaras, P., Stace, R., and Littlewood, C. (2017a). Cognitive and contextual factors to optimise clinical outcomes in tendinopathy. *Br. J. Sports Med.* 51, 743–748 doi: 10.1136/bjsports-2017-098064

Mann, J. B., Thyfault, J. P., Ivey, P. A., and Sayers, S. P. (2010). The effect of autoregulatory progressive resistance exercise vs. linear periodization on strength improvement in college athletes. *J. Strength Cond. Res.* 24, 1718–1723. doi: 10.1519/JSC.0b013e3181def4a6

Mann, T. N., Lamberts, R. P., and Lambert, M. I. (2014). High responders and low responders: factors associated with individual variation in response to standardized training. *Sports Med.* 44, 1113–1124. doi: 10.1007/s40279-014-0197-3

Mansfield, S. K., Peiffer, J. J., Hughes, L. J., and Scott, B. R. (2020). Estimating repetitions in reserve for resistance exercise: an analysis of factors which impact on prediction accuracy. *J. Strength Cond. Res.* doi: 10.1519/JSC.0000000000003779

Maquirriain, J. (2012). Leg stiffness changes in athletes with achilles tendinopathy. *Int. J. Sports Med.* 33, 567–571. doi: 10.1055/s-0032-1304644

Martin, R. L., Davenport, T. E., Reischl, S. F., McPoil, T. G., Matheson, J. W., Wukich, D. K., et al. (2014). Heel pain-plantar fasciitis: revision 2014. *J. Orthop. Sports Phys. Ther.* 44, A1–A33. doi: 10.2519/jospt.2014.0303

Martinez-Cava, A., Hernandez-Belmonte, A., Courel-Ibanez, J., Moran-Navarro, R., Gonzalez-Badillo, J. J., and Pallares, J. G. (2020). Reliability of technologies to measure the barbell velocity: implications for monitoring resistance training. *PLoS ONE* 15:e0232465. doi: 10.1371/journal.pone.0232465

Mc Auliffe, S., Synott, A., Casey, H., Mc Creesh, K., Purtill, H., and O'Sullivan, K. (2017). Beyond the tendon: experiences and perceptions of people with persistent achilles tendinopathy. *Musculoskelet Sci. Pract.* 29, 108–114. doi: 10.1016/j.msksp.2017.03.009

McAndrew, L. M., Musumeci-Szabo, T. J., Mora, P. A., Vileikyte, L., Burns, E., Halm, E. A., et al. (2008). Using the common sense model to design interventions for the prevention and management of chronic illness threats: from description to process. *Br. J. Health Psychol.* 13(Pt 2), 195–204. doi: 10.1348/135910708X295604

McAuliffe, S., Tabuena, A., McCreesh, K., O'Keeffe, M., Hurley, J., Comyns, T., et al. (2019). Altered strength profile in achilles tendinopathy: a systematic review and meta-analysis. *J. Athl. Train* 54, 889–900. doi: 10.4085/1062-6050-43-18

McGirr, K., Harring, S. I., Kennedy, T. S., Pedersen, M. F., Hirata, R. P., Thorborg, K., et al. (2015). An elastic exercise band mounted with a bandcizer can differentiate between commonly prescribed home exercises for the shoulder. *Int. J. Sports Phys. Ther.* 10, 332–340.

McGuigan, M. R., Al Dayel, A., Tod, D., Foster, C., Newton, R. U., and Pettigrew, S. (2008). Use of session rating of perceived exertion for monitoring resistance exercise in children who are overweight or obese. *Pediatr. Exerc. Sci.* 20, 333–341. doi: 10.1123/pes.20.3.333

McLean, S. M., Burton, M., Bradley, L., and Littlewood, C. (2010). Interventions for enhancing adherence with physiotherapy: a systematic review. *Man Ther.* 15, 514–521. doi: 10.1016/j.math.2010.05.012

McNamara, J. M., and Stearne, D. J. (2010). Flexible nonlinear periodization in a beginner college weight training class. *J. Strength Cond. Res.* 24, 17–22. doi: 10.1519/JSC.0b013e3181bc177b

McPoil, T. G., Martin, R. L., Cornwall, M. W., Wukich, D. K., Irrgang, J. J., and Godges, J. J. (2008). Heel pain–plantar fasciitis: clinical practice guildelines linked to the international classification of function, disability, and health from the orthopaedic section of the american physical therapy association. *J. Orthop. Sports Phys. Ther.* 38, A1–A18. doi: 10.2519/jospt.2008.0302

Meira, E. P., and Brumitt, J. (2011). Influence of the hip on patients with patellofemoral pain syndrome: a systematic review. *Sports Health* 3, 455–465. doi: 10.1177/1941738111415006

Mendonca, L. M., Bittencourt, N. F. N., Alves, L. E. M., Resende, R. A., and Serrao, F. V. (2020a). Interventions used for rehabilitation and prevention of patellar tendinopathy in athletes: a survey of brazilian sports physical therapists. *Braz. J. Phys. Ther.* 24, 46–53. doi: 10.1016/j.bjpt.2018.12.001

Mendonca, L. M., Leite, H. R., Zwerver, J., Henschke, N., Branco, G., and Oliveira, V. C. (2020b). How strong is the evidence that conservative treatment reduces pain and improves function in individuals with patellar tendinopathy? A systematic review of randomised controlled trials including GRADE recommendations. *Br. J. Sports Med.* 54, 87–93. doi: 10.1136/bjsports-2018-099747

Mersmann, F., Bohm, S., and Arampatzis, A. (2017). Imbalances in the development of muscle and tendon as risk factor for tendinopathies in youth

athletes: a review of current evidence and concepts of prevention. *Front. Physiol*. 8:987. doi: 10.3389/fphys.2017.00987

Mersmann, F., Pentidis, N., Tsai, M. S., Schroll, A., and Arampatzis, A. (2019). Patellar tendon strain associates to tendon structural abnormalities in adolescent athletes. *Front. Physiol*. 10:963. doi: 10.3389/fphys.2019.00963

Moosmayer, S., Smith, H. J., Tariq, R., and Larmo, A. (2009). Prevalence and characteristics of asymptomatic tears of the rotator cuff: an ultrasonographic and clinical study. *J. Bone Joint Surg. Br*. 91, 196–200. doi: 10.1302/0301-620X.91B2.21069

Moraes, V. Y., Lenza, M., Tamaoki, M. J., Faloppa, F., and Belloti, J. C. (2014). Platelet-rich therapies for musculoskeletal soft tissue injuries. *Cochrane Database Syst Rev*. 12:CD010071. doi: 10.1002/14651858.CD010071.pub3

Morgan, S., Janse van Vuuren, E. C., and Coetzee, F. (2018a). Patellar tendinopathy: an international e-Delphi perspective. *SAJR SPER* 40, 115–128.

Morgan, S., Janse van Vuuren, E. C., and Coetzee, F. (2018b). Patellar tendinopathy: a rehabilitation intervention in elite rugby union players. *SAJR SPER* 40, 129–142.

Morrissey, D. (2015). Guidelines and pathways for clinical practice in tendinopathy: their role and development. *J. Orthop. Sports Phys. Ther*. 45, 819–822. doi: 10.2519/jospt.2015.0111

Mucha, M. D., Caldwell, W., Schlueter, E. L., Walters, C., and Hassen, A. (2017). Hip abductor strength and lower extremity running related injury in distance runners: a systematic review. *J. Sci. Med. Sport*. 20, 349–355. doi: 10.1016/j.jsams.2016.09.002

Murphy, M. C., Travers, M. J., Chivers, P., Debenham, J. R., Docking, S. I., Rio, E. K., et al. (2019). Efficacy of heavy eccentric calf training for treating mid-portion achilles tendinopathy: a systematic review and meta-analysis. *Br. J. Sports Med*. 53, 1070–1077. doi: 10.1136/bjsports-2018-099934

Murtaugh, B., and Ihm, J. M. (2013). Eccentric training for the treatment of tendinopathies. *Curr. Sports Med. Rep*. 12, 175–182. doi: 10.1249/JSR.0b013e3182933761

Nakagawa, T. H., Muniz, T. B., Baldon Rde, M., Dias Maciel, C., de Menezes Reiff, R. B., and Serrao, F. V. (2008). The effect of additional strengthening of hip abductor and lateral rotator muscles in patellofemoral pain syndrome: a randomized controlled pilot study. *Clin. Rehabil*. 22, 1051–1060. doi: 10.1177/0269215508095357

Nevin, J. (2019). Autoregulated resistance training: does velocity-based training represent the future? *Strength Cond. J*. 41, 34–39. doi: 10.1519/SSC.0000000000000471

Newman, S. P. (2008). Chronic disease self-management approaches within the complex organisational structure of a health care system. *Med. J. Aust*. 189(10 Suppl.), S7–S8. doi: 10.5694/j.1326-5377.2008.tb02201.x

O'Keeffe, M., Cullinane, P., Hurley, J., Leahy, I., Bunzli, S., O'Sullivan, P. B., et al. (2016). What influences patient-therapist interactions in musculoskeletal physical therapy? Qualitative systematic review and meta-synthesis. *Phys. Ther*. 96, 609–622. doi: 10.2522/ptj.20150240

Onuora, S. (2019). Burden of musculoskeletal diseases increasing. *Nat. Rev. Rheumatol*. 15:318. doi: 10.1038/s41584-019-0232-8

Osteras, B., Osteras, H., Torstensen, T. A., and Vasseljen, O. (2013). Dose-response effects of medical exercise therapy in patients with patellofemoral pain syndrome: a randomised controlled clinical trial. *Physiotherapy* 99, 126–131. doi: 10.1016/j.physio.2012.05.009

O'Sullivan, K., McAuliffe, S., and Deburca, N. (2012). The effects of eccentric training on lower limb flexibility: a systematic review. *Br. J. Sports Med*. 46, 838–845. doi: 10.1136/bjsports-2011-090835

Paavola, M., Kannus, P., Jarvinen, T. A., Khan, K., Jozsa, L., and Jarvinen, M. (2002). Achilles tendinopathy. *J. Bone Joint Surg. Am*. 84, 2062–2076. doi: 10.2106/00004623-200211000-00024

Paavola, M., Kannus, P., Paakkala, T., Pasanen, M., and Jarvinen, M. (2000). Long-term prognosis of patients with achilles tendinopathy. an observational 8-year follow-up study. *Am. J. Sports Med*. 28, 634–642. doi: 10.1177/03635465000280050301

Peek, K., Sanson-Fisher, R., Mackenzie, L., and Carey, M. (2016). Interventions to aid patient adherence to physiotherapist prescribed self-management strategies: a systematic review. *Physiotherapy* 102, 127–135. doi: 10.1016/j.physio.2015.10.003

Perez-Castilla, A., Jimenez-Alonso, A., Cepero, M., Miras-Moreno, S., Rojas, F. J., and Garcia-Ramos, A. (2020). Velocity performance feedback during ballistic training: which is the optimal frequency of feedback administration? *Motor Control*. 1, 1–14. doi: 10.1123/mc.2020-0039

Peters, J. A., Zwerver, J., Diercks, R. L., Elferink-Gemser, M. T., and van den Akker-Scheek, I. (2016). Preventive interventions for tendinopathy: a systematic review. *J. Sci. Med. Sport*. 19, 205–211. doi: 10.1016/j.jsams.2015.03.008

Peterson, M., Butler, S., Eriksson, M., and Svardsudd, K. (2014). A randomized controlled trial of eccentric vs. concentric graded exercise in chronic tennis elbow (lateral elbow tendinopathy). *Clin. Rehabil*. 28, 862–872. doi: 10.1177/0269215514527595

Petraglia, F., Ramazzina, I., and Costantino, C. (2017). Plantar fasciitis in athletes: diagnostic and treatment strategies. A systematic review. *Muscles Ligaments Tendons J*. 7, 107–118. doi: 10.11138/mltj/2017.7.1.107

Phillips, L. A., Leventhal, H., and Leventhal, E. A. (2012). Physicians' communication of the common-sense self-regulation model results in greater reported adherence than physicians' use of interpersonal skills. *Br. J. Health Psychol*. 17, 244–257. doi: 10.1111/j.2044-8287.2011.02035.x

Pickering, C., and Kiely, J. (2019). Do non-responders to exercise exist-and if so, what should we do about them? *Sports Med*. 49, 1–7. doi: 10.1007/s40279-018-01041-1

Pizzolato, C., Lloyd, D. G., Zheng, M. H., Besier, T. F., Shim, V. B., Obst, S. J., et al. (2019). Finding the sweet spot via personalised achilles tendon training: the future is within reach. *Br. J. Sports Med*. 53, 11–12. doi: 10.1136/bjsports-2018-099020

Plinsinga, M. L., Brink, M. S., Vicenzino, B., and van Wilgen, C. P. (2015). Evidence of nervous system sensitization in commonly presenting and persistent painful tendinopathies: a systematic review. *J. Orthop. Sports Phys. Ther*. 45, 864–875. doi: 10.2519/jospt.2015.5895

Plinsinga, M. L., Coombes, B. K., Mellor, R., and Vicenzino, B. (2020). Individuals with persistent greater trochanteric pain syndrome exhibit impaired pain modulation, as well as poorer physical and psychological health, compared with pain-free individuals: a cross-sectional study. *Pain Med*. 31. doi: 10.1093/pm/pnaa047

Plinsinga, M. L., van Wilgen, C. P., Brink, M. S., Vuvan, V., Stephenson, A., Heales, L. J., et al. (2018). Patellar and achilles tendinopathies are predominantly peripheral pain states: a blinded case control study of somatosensory and psychological profiles. *Br. J. Sports Med*. 52, 284–291. doi: 10.1136/bjsports-2016-097163

Powers, C. M. (2003). The influence of altered lower-extremity kinematics on patellofemoral joint dysfunction: a theoretical perspective. *J. Orthop. Sports Phys. Ther*. 33, 639–646. doi: 10.2519/jospt.2003.33.11.639

Powers, C. M. (2010). The influence of abnormal hip mechanics on knee injury: a biomechanical perspective. *J. Orthop. Sports Phys. Ther*. 40, 42–51. doi: 10.2519/jospt.2010.3337

Pritchett, R. C., Green, J. M., Wickwire, P. J., and Kovacs, M. S. (2009). Acute and session RPE responses during resistance training: bouts to failure at 60% and 90% of 1RM. *South Afr. J. Sports Med*. 21. doi: 10.17159/2078-516X/2009/v21i1a304

Purdam, C. R., Jonsson, P., Alfredson, H., Lorentzon, R., Cook, J. L., and Khan, K. M. (2004). A pilot study of the eccentric decline squat in the management of painful chronic patellar tendinopathy. *Br. J. Sports Med*. 38, 395–397. doi: 10.1136/bjsm.2003.000053

Raschhofer, R., Poulios, N., Schimetta, W., Kisling, R., and Mittermaier, C. (2017). Early active rehabilitation after arthroscopic rotator cuff repair: a prospective randomized pilot study. *Clin. Rehabil*. 31, 1332–1339. doi: 10.1177/0269215517694931

Rathleff, C. R., Bandholm, T., Spaich, E. G., Jorgensen, M., and Andreasen, J. (2017). Unsupervised progressive elastic band exercises for frail geriatric inpatients objectively monitored by new exercise-integrated technology-a feasibility trial with an embedded qualitative study. *Pilot Feasibility Stud*. 3, 1–16. doi: 10.1186/s40814-017-0202-3

Rathleff, M. S., Molgaard, C. M., Fredberg, U., Kaalund, S., Andersen, K. B., Jensen, T. T., et al. (2015a). High-load strength training improves outcome in patients with plantar fasciitis: a randomized controlled trial with 12-month follow-up. *Scand. J. Med. Sci. Sports* 25, e292–e300. doi: 10.1111/sms.12313

Rathleff, M. S., Rathleff, C. R., Crossley, K. M., and Barton, C. J. (2014). Is hip strength a risk factor for patellofemoral pain? A systematic review and meta-analysis. *Br. J. Sports Med*. 48:1088. doi: 10.1136/bjsports-2013-093305

Rathleff, M. S., and Thorborg, K. (2015). 'Load me up, scotty': mechanotherapy for plantar fasciopathy (formerly known as plantar fasciitis). *Br. J. Sports Med*. 49, 638–639. doi: 10.1136/bjsports-2014-094562

Rathleff, M. S., Thorborg, K., Rode, L. A., McGirr, K. A., Sorensen, A. S., Bogild, A., et al. (2015b). Adherence to commonly prescribed, home-based strength training exercises for the lower extremity can be objectively monitored using the bandcizer. *J. Strength Cond. Res*. 29, 627–636. doi: 10.1519/JSC.0000000000000675

Rauch, J. T., Ugrinowitsch, C., Barakat, C. I., Alvarez, M. R., Brummert, D. L., Aube, D. W., et al. (2020). Auto-regulated exercise selection training regimen produces small increases in lean body mass and maximal strength adaptations in strength-trained individuals. *J. Strength Cond. Res*. 34, 1133–1140. doi: 10.1519/JSC.0000000000002272

Rees, J. D., Stride, M., and Scott, A. (2014). Tendons–time to revisit inflammation. *Br. J. Sports Med*. 48, 1553–1557. doi: 10.1136/bjsports-2012-091957

Reiman, M. P., Bolgla, L. A., and Loudon, J. K. (2012). A literature review of studies evaluating gluteus maximus and gluteus medius activation during rehabilitation exercises. *Physiother. Theory Pract*. 28, 257–268. doi: 10.3109/09593985.2011.604981

Reiman, M. P., and Lorenz, D. S. (2011). Integration of strength and conditioning principles into a rehabilitation program. *Int. J. Sports Phys. Ther*. 6, 241–253.

Rhea, M. R., Hunter, R. L., and Hunter, T. J. (2006). Competition modeling of american football: observational data and implications for high school, collegiate, and professional player conditioning. *J. Strength Cond. Res*. 20, 58–61. doi: 10.1519/00124278-200602000-00010

Riel, H., Jensen, M. B., Olesen, J. L., Vicenzino, B., and Rathleff, M. S. (2019a). Self-dosed and pre-determined progressive heavy-slow resistance training have similar effects in people with plantar fasciopathy: a randomised trial. *J. Physiother*. 65, 144–151. doi: 10.1016/j.jphys.2019.05.011

Riel, H., Matthews, M., Vicenzino, B., Bandholm, T., Thorborg, K., and Rathleff, M. S. (2016). Efficacy of live feedback to improve objectively monitored compliance to prescribed, home-based, exercise therapy-dosage in 15 to 19 year old adolescents with patellofemoral pain- a study protocol of a randomized controlled superiority trial (the XRCISE-AS-INSTRUcted-1 trial). *BMC Musculoskelet Disord*. 17, 1–12. doi: 10.1186/s12891-016-1103-y

Riel, H., Olesen, J. L., Jensen, M. B., Vicenzino, B., and Rathleff, M. S. (2019b). Heavy-slow resistance training in addition to an ultrasound-guided corticosteroid injection for individuals with plantar fasciopathy: a feasibility study. *Pilot Feasibility Stud*. 5, 1–9. doi: 10.1186/s40814-019-0489-3

Riel, H., Vicenzino, B., Jensen, M. B., Olesen, J. L., Holden, S., and Rathleff, M. S. (2018). The effect of isometric exercise on pain in individuals with plantar fasciopathy: a randomized crossover trial. *Scand. J. Med. Sci. Sports*. doi: 10.1111/sms.13296

Rio, E., Kidgell, D., Moseley, G. L., and Cook, J. (2016). Elevated corticospinal excitability in patellar tendinopathy compared with other anterior knee pain or no pain. *Scand. J. Med. Sci. Sports* 26, 1072–1079. doi: 10.1111/sms.12538

Rio, E., Kidgell, D., Purdam, C., Gaida, J., Moseley, G. L., Pearce, A. J., et al. (2015a). Isometric exercise induces analgesia and reduces inhibition in patellar tendinopathy. *Br. J. Sports Med*. 49, 1277–1283. doi: 10.1136/bjsports-2014-094386

Rio, E., Mayes, S., and Cook, J. (2015b). Heel pain: a practical approach. *Aust. Fam. Phys*. 44, 96–101.

Rompe, J. D., Furia, J., and Maffulli, N. (2009). Eccentric loading versus eccentric loading plus shock-wave treatment for midportion achilles tendinopathy: a randomized controlled trial. *Am. J. Sports Med*. 37, 463–470. doi: 10.1177/0363546508326983

Rowe, V., Hemmings, S., Barton, C., Malliaras, P., Maffulli, N., and Morrissey, D. (2012). Conservative management of midportion achilles tendinopathy: a mixed methods study, integrating systematic review and clinical reasoning. *Sports Med*. 42, 941–967. doi: 10.1007/BF03262305

Rudavsky, A., and Cook, J. (2014). Physiotherapy management of patellar tendinopathy (jumper's knee). *J. Physiother*. 60, 122–129. doi: 10.1016/j.jphys.2014.06.022

Rui, Y. F., Lui, P. P., Chan, L. S., Chan, K. M., Fu, S. C., and Li, G. (2011). Does erroneous differentiation of tendon-derived stem cells contribute to the pathogenesis of calcifying tendinopathy? *Chin. Med. J.* 124, 606–610.

Sanchis, M. N., Lluch, E., Nijs, J., Struyf, F., and Kangasperko, M. (2015). The role of central sensitization in shoulder pain: a systematic literature review. *Semin. Arthritis Rheum*. 44, 710–716. doi: 10.1016/j.semarthrit.2014.11.002

Sancho, I., Malliaras, P., Barton, C., Willy, R. W., and Morrissey, D. (2019a). Biomechanical alterations in individuals with achilles tendinopathy during running and hopping: a systematic review with meta-analysis. *Gait Posture* 73, 189–201. doi: 10.1016/j.gaitpost.2019.07.121

Sancho, I., Morrissey, D., Willy, R. W., Barton, C., and Malliaras, P. (2019b). Education and exercise supplemented by a pain-guided hopping intervention for male recreational runners with midportion achilles tendinopathy: a single cohort feasibility study. *Phys. Ther. Sport*. 40, 107–116. doi: 10.1016/j.ptsp.2019.08.007

Santos, B. D., Correa, L. A., Teixeira Santos, L., Filho, N. A., Lemos, T., and Nogueira, L. A. (2016). Combination of hip strengthening and manipulative therapy for the treatment of plantar fasciitis: a case report. *J. Chiropr. Med*. 15, 310–313. doi: 10.1016/j.jcm.2016.08.001

Sayana, M. K., and Maffulli, N. (2007). Eccentric calf muscle training in non-athletic patients with achilles tendinopathy. *J. Sci. Med. Sport*. 10, 52–58. doi: 10.1016/j.jsams.2006.05.008

Scattone Silva, R., Ferreira, A. L., Nakagawa, T. H., Santos, J. E., and Serrao, F. V. (2015). Rehabilitation of patellar tendinopathy using hip extensor strengthening and landing-strategy modification: case report with 6-month follow-up. *J. Orthop. Sports Phys. Ther*. 45, 899–909. doi: 10.2519/jospt.2015.6242

Seitz, A. L., McClure, P. W., Finucane, S., Boardman, N. D. 3rd, and Michener, L. A. (2011). Mechanisms of rotator cuff tendinopathy: intrinsic, extrinsic, or both? *Clin. Biomech*. 26, 1–12. doi: 10.1016/j.clinbiomech.2010.08.001

September, A. V., Posthumus, M., van der Merwe, L., Schwellnus, M., Noakes, T. D., and Collins, M. (2008). The COL12A1 and COL14A1 genes and achilles tendon injuries. *Int. J. Sports Med*. 29, 257–263. doi: 10.1055/s-2007-965127

Sharma, P., and Maffulli, N. (2005). Tendon injury and tendinopathy: healing and repair. *J. Bone Joint Surg. Am*. 87, 187–202. doi: 10.2106/00004623-200501000-00030

Sharma, P., and Maffulli, N. (2008). Tendinopathy and tendon injury: the future. *Disabil. Rehabil*. 30, 1733–1745. doi: 10.1080/09638280701788274

Shattock, K., and Tee, J. C. (2020). Autoregulation in resistance training: a comparison of subjective versus objective methods. *J. Strength Cond. Res*. doi: 10.1519/JSC.0000000000003530

Shimano, T., Kraemer, W. J., Spiering, B. A., Volek, J. S., Hatfield, D. L., Silvestre, R., et al. (2006). Relationship between the number of repetitions and selected percentages of one repetition maximum in free weight exercises in trained and untrained men. *J. Strength Cond. Res*. 20, 819–823. doi: 10.1519/00124278-200611000-00015

Siff, M. C. (2000). *Supertraining*. Denver, CO: Supertraining Institute.

Silbernagel, K. G. (2014). Does one size fit all when it comes to exercise treatment for achilles tendinopathy? *J. Orthop. Sports Phys. Ther*. 44, 42–44. doi: 10.2519/jospt.2014.0103

Silbernagel, K. G., Brorsson, A., and Lundberg, M. (2011). The majority of patients with achilles tendinopathy recover fully when treated with exercise alone: a 5-year follow-up. *Am. J. Sports Med*. 39, 607–613. doi: 10.1177/0363546510384789

Silbernagel, K. G., Hanlon, S., and Sprague, A. (2020). Current clinical concepts: conservative management of achilles tendinopathy. *J. Athl. Train* 55, 438–447. doi: 10.4085/1062-6050-356-19

Silbernagel, K. G., Thomee, R., Eriksson, B. I., and Karlsson, J. (2007a). Full symptomatic recovery does not ensure full recovery of muscle-tendon function in patients with achilles tendinopathy. *Br. J. Sports Med*. 41:276; discussion: 280. doi: 10.1136/bjsm.2006.033464

Silbernagel, K. G., Thomee, R., Eriksson, B. I., and Karlsson, J. (2007b). Continued sports activity, using a pain-monitoring model, during rehabilitation in patients with achilles tendinopathy: a randomized controlled study. *Am. J. Sports Med*. 35, 897–906. doi: 10.1177/0363546506298279

Silbernagel, K. G., Thomee, R., Thomee, P., and Karlsson, J. (2001). Eccentric overload training for patients with chronic achilles tendon pain–a randomised controlled study with reliability testing of the evaluation methods. *Scand. J. Med. Sci. Sports* 11, 197–206. doi: 10.1034/j.1600-0838.2001.110402.x

Simpson, M., Rio, E., and Cook, J. (2016). At what age do children and adolescents develop lower limb tendon pathology or tendinopathy? A systematic review and meta-analysis. *Sports Med*. 46, 545–557. doi: 10.1007/s40279-015-0438-0

Sindiani, M., Lazarus, A., Iacono, A. D., and Halperin, I. (2020). Perception of changes in bar velocity in resistance training: accuracy levels within and between exercises. *Physiol. Behav*. 224:113025. doi: 10.1016/j.physbeh.2020.113025

Smith, E., Hoy, D. G., Cross, M., Vos, T., Naghavi, M., Buchbinder, R., et al. (2014). The global burden of other musculoskeletal disorders: estimates from the global burden of disease 2010 study. *Ann. Rheum. Dis*. 73, 1462–1469. doi: 10.1136/annrheumdis-2013-204680

Snyder, K. R., Earl, J. E., O'Connor, K. M., and Ebersole, K. T. (2009). Resistance training is accompanied by increases in hip strength and changes in lower extremity biomechanics during running. *Clin. Biomech.* 24, 26–34. doi: 10.1016/j.clinbiomech.2008.09.009

Spurrs, R. W., Murphy, A. J., and Watsford, M. L. (2003). The effect of plyometric training on distance running performance. *Eur. J. Appl. Physiol*. 89, 1–7. doi: 10.1007/s00421-002-0741-y

Stanish, W. D., Rubinovich, R. M., and Curwin, S. (1986). Eccentric exercise in chronic tendinitis. *Clin. Orthop. Relat. Res*. 65–68. doi: 10.1097/00003086-198607000-00014

Stasinopoulos, D., and Malliaras, P. (2016). Is the heavy slow resistance program effective for all patients with tendinopathy and effective for all its sites? *J. Sports Med. Phys. Fitness* 56, 1430–1431.

Steinmann, S., Pfeifer, C. G., Brochhausen, C., and Docheva, D. (2020). Spectrum of tendon pathologies: triggers, trails and end-state. *Int. J. Mol. Sci*. 21:844. doi: 10.3390/ijms21030844

Stergioulas, A., Stergioula, M., Aarskog, R., Lopes-Martins, R. A., and Bjordal, J. M. (2008). Effects of low-level laser therapy and eccentric exercises in the treatment of recreational athletes with chronic achilles tendinopathy. *Am. J. Sports Med*. 36, 881–887. doi: 10.1177/0363546507312165

Stevens, M., and Tan, C. W. (2014). Effectiveness of the Alfredson protocol compared with a lower repetition-volume protocol for midportion achilles tendinopathy: a randomized controlled trial. *J. Orthop. Sports Phys. Ther*. 44, 59–67. doi: 10.2519/jospt.2014.4720

Stone, M. H., Sands, W. A., Carlock, J., Callan, S., Dickie, D., Daigle, K., et al. (2004). The importance of isometric maximum strength and peak rate-of-force development in sprint cycling. *J. Strength Cond. Res*. 18, 878–884. doi: 10.1519/00124278-200411000-00034

Storen, O., Helgerud, J., Stoa, E. M., and Hoff, J. (2008). Maximal strength training improves running economy in distance runners. *Med. Sci. Sports Exerc*. 40, 1087–1092. doi: 10.1249/MSS.0b013e318168da2f

Suarez-Arrones, L., Nunez, F. J., Lara-Lopez, P., Di Salvo, V., and Mendez-Villanueva, A. (2020). Inertial flywheel knee- and hip-dominant hamstring strength exercises in professional soccer players: muscle use and velocity-based (mechanical) eccentric overload. *PLoS ONE* 15:e0239977. doi: 10.1371/journal.pone.0239977

Sussmilch-Leitch, S. P., Collins, N. J., Bialocerkowski, A. E., Warden, S. J., and Crossley, K. M. (2012). Physical therapies for achilles tendinopathy: systematic review and meta-analysis. *J. Foot Ankle Res*. 5, 1–16. doi: 10.1186/1757-1146-5-15

Tahririan, M. A., Motififard, M., Tahmasebi, M. N., and Siavashi, B. (2012). Plantar fasciitis. *J. Res. Med. Sci*. 17, 799–804.

Taube, W., Leukel, C., Lauber, B., and Gollhofer, A. (2012). The drop height determines neuromuscular adaptations and changes in jump performance in stretch-shortening cycle training. *Scand. J. Med. Sci. Sports* 22, 671–683. doi: 10.1111/j.1600-0838.2011.01293.x

Testa, M., Noakes, T. D., and Desgorces, F. D. (2012). Training state improves the relationship between rating of perceived exertion and relative exercise volume during resistance exercises. *J. Strength Cond. Res*. 26, 2990–2996. doi: 10.1519/JSC.0b013e31824301d1

Thiese, M. S., Hegmann, K. T., Kapellusch, J., Merryweather, A., Bao, S., Silverstein, B., et al. (2016). Psychosocial factors related to lateral and medial epicondylitis: results from pooled study analyses. *J. Occup. Environ. Med*. 58, 588–593. doi: 10.1097/JOM.0000000000000701

Thompson, R. C. (2017). Managing plantar fasciopathy and tendinopathy through conditioning and load monitoring within elite sport. *J. Aust. Strength Cond*. 25, 85–103.

Thomson, C., Krouwel, O., Kuisma, R., and Hebron, C. (2016). The outcome of hip exercise in patellofemoral pain: a systematic review. *Man Ther*. 26, 1–30. doi: 10.1016/j.math.2016.06.003

Tiggemann, C. L., Korzenowski, A. L., Brentano, M. A., Tartaruga, M. P., Alberton, C. L., and Kruel, L. F. (2010). Perceived exertion in different strength exercise loads in sedentary, active, and trained adults. *J. Strength Cond. Res*. 24, 2032–2041. doi: 10.1519/JSC.0b013e3181d32e29

Timmins, R. G., Ruddy, J. D., Presland, J., Maniar, N., Shield, A. J., Williams, M. D., et al. (2016). Architectural changes of the biceps femoris long head after concentric or eccentric training. *Med. Sci. Sports Exerc*. 48, 499–508. doi: 10.1249/MSS.0000000000000795

Timmons, J. A. (2011). Variability in training-induced skeletal muscle adaptation. *J. Appl. Physiol*. 110, 846–853. doi: 10.1152/japplphysiol.00934.2010

Tsoukos, A., Brown, L. E., Terzis, G., Veligekas, P., and Bogdanis, G. C. (2020). Potentiation of bench press throw performance using a heavy load and velocity-based repetition control. *J. Strength Cond. Res*. 8. doi: 10.1519/JSC.0000000000003633

Tuchscherer, M. (2008). *The reactive training manual: developing your own custom training program for powerlifting: reactive training systems*; Self-published.

van Ark, M., Cook, J. L., Docking, S. I., Zwerver, J., Gaida, J. E., van den Akker-Scheek, I., et al. (2016). Do isometric and isotonic exercise programs reduce pain in athletes with patellar tendinopathy in-season? A randomised clinical trial. *J. Sci. Med. Sport*. 19, 702–706. doi: 10.1016/j.jsams.2015.11.006

van der Linden, P. D., Sturkenboom, M. C., Herings, R. M., Leufkens, H. G., and Stricker, B. H. (2002). Fluoroquinolones and risk of achilles tendon disorders: case-control study. *BMJ* 324, 1306–1307. doi: 10.1136/bmj.324.7349.1306

van der Plas, A., de Jonge, S., de Vos, R. J., van der Heide, H. J., Verhaar, J. A., Weir, A., et al. (2012). A 5-year follow-up study of Alfredson's heel-drop exercise programme in chronic midportion achilles tendinopathy. *Br. J. Sports Med*. 46, 214–218. doi: 10.1136/bjsports-2011-090035

Vavken, P., and Dorotka, R. (2011). Burden of musculoskeletal disease and its determination by urbanicity, socioeconomic status, age, and sex: results from 14,507 subjects. *Arthritis Care Res*. 63, 1558–1564. doi: 10.1002/acr.20558

Vicenzino, B. (2015). Tendinopathy: evidence-informed physical therapy clinical reasoning. *J. Orthop. Sports Phys. Ther*. 45, 816–818. doi: 10.2519/jospt.2015.0110

Visnes, H., and Bahr, R. (2007). The evolution of eccentric training as treatment for patellar tendinopathy (jumper's knee): a critical review of exercise programmes. *Br. J. Sports Med*. 41, 217–223. doi: 10.1136/bjsm.2006.032417

Wainner, R. S., Whitman, J. M., Cleland, J. A., and Flynn, T. W. (2007). Regional interdependence: a musculoskeletal examination model whose time has come. *J. Orthop. Sports Phys. Ther*. 37, 658–660. doi: 10.2519/jospt.2007.0110

Wang, H. K., Lin, K. H., Su, S. C., Shih, T. T., and Huang, Y. C. (2012). Effects of tendon viscoelasticity in achilles tendinosis on explosive performance and clinical severity in athletes. *Scand. J. Med. Sci. Sports* 22, e147–e155. doi: 10.1111/j.1600-0838.2012.01511.x

Waugh, C. M., Alktebi, T., de Sa, A., and Scott, A. (2018). Impact of rest duration on achilles tendon structure and function following isometric training. *Scand. J. Med. Sci. Sports* 28, 436–445. doi: 10.1111/sms.12930

Weppler, C. H., and Magnusson, S. P. (2010). Increasing muscle extensibility: a matter of increasing length or modifying sensation? *Phys. Ther*. 90, 438–449. doi: 10.2522/ptj.20090012

Wilson, M. E. (2008). The daily adjustable progressive resistance exercise system: getting reacquainted with an old friend. *Strength Cond. J*. 30, 76–78. doi: 10.1519/SSC.0b013e31816a87d8

Wisloff, U., Castagna, C., Helgerud, J., Jones, R., and Hoff, J. (2004). Strong correlation of maximal squat strength with sprint performance and vertical jump height in elite soccer players. *Br. J. Sports Med*. 38, 285–288. doi: 10.1136/bjsm.2002.002071

Wu, F., Nerlich, M., and Docheva, D. (2017). Tendon injuries: basic science and new repair proposals. *EFORT Open Rev*. 2, 332–342. doi: 10.1302/2058-5241.2.160075

Young, M. A., Cook, J. L., Purdam, C. R., Kiss, Z. S., and Alfredson, H. (2005). Eccentric decline squat protocol offers superior results at 12 months compared with traditional eccentric protocol for patellar tendinopathy in volleyball players. *Br. J. Sports Med*. 39, 102–105. doi: 10.1136/bjsm.2003.010587

Zourdos, M. C., Jo, E., Khamoui, A. V., Lee, S. R., Park, B. S., Ormsbee, M. J., et al. (2016a). Modified daily undulating periodization model produces greater

performance than a traditional configuration in powerlifters. *J. Strength Cond. Res.* 30, 784–791. doi: 10.1519/JSC.0000000000001165

Zourdos, M. C., Klemp, A., Dolan, C., Quiles, J. M., Schau, K. A., Jo, E., et al. (2016b). Novel resistance training-specific rating of perceived exertion scale measuring repetitions in reserve. *J. Strength Cond. Res.* 30, 267–275. doi: 10.1519/JSC.0000000000001049

Zwerver, J., Hartgens, F., Verhagen, E., van der Worp, H., van den Akker-Scheek, I., and Diercks, R. L. (2011). No effect of extracorporeal shockwave therapy on patellar tendinopathy in jumping athletes during the competitive season: a randomized clinical trial. *Am. J. Sports Med.* 39, 1191–1199. doi: 10.1177/0363546510395492

Conflict of Interest: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Physiology

ORIGINAL RESEARCH
published: 06 August 2021
doi: 10.3389/fphys.2021.707116

Transcriptional Time Course After Rotator Cuff Tear

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OPEN ACCESS

Edited by:
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Reviewed by:
Steve Lancel, Université de Lille, France
Frank Suhr, KU Leuven, Belgium

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Specialty section:
This article was submitted to Striated Muscle Physiology, a section of the journal Frontiers in Physiology

Received: *09 May 2021*
Accepted: *06 July 2021*
Published: *06 August 2021*

Citation:
Vasquez-Bolanos LS, Gibbons MC, Ruoss S, Wu IT, Vargas-Vila M, Hyman SA, Esparza MC, Fithian DC, Lane JG, Singh A, Nasamran CA, Fisch KM and Ward SR (2021) Transcriptional Time Course After Rotator Cuff Tear. Front. Physiol. 12:707116. doi: 10.3389/fphys.2021.707116

Rotator cuff (RC) tears are prevalent in the population above the age of 60. The disease progression leads to muscle atrophy, fibrosis, and fatty infiltration in the chronic state, which is not improved with intervention or surgical repair. This highlights the need to better understand the underlying dysfunction in muscle after RC tendon tear. Contemporary studies aimed at understanding muscle pathobiology after RC tear have considered transcriptional data in mice, rats and sheep models at 2–3 time points (1 to 16 weeks post injury). However, none of these studies observed a transition or resurgence of gene expression after the initial acute time points. In this study, we collected rabbit supraspinatus muscle tissue with high temporal resolution (1, 2, 4, 8, and 16 weeks) post-tenotomy (n = 6/group), to determine if unique, time-dependent transcriptional changes occur. RNA sequencing and analyses were performed to identify a transcriptional timeline of RC muscle changes and related morphological sequelae. At 1-week post-tenotomy, the greatest number of differentially expressed genes was observed (1,069 up/873 down) which decreases through 2 (170/133), 4 (86/41), and 8 weeks (16/18), followed by a resurgence and transition of expression at 16 weeks (1,421/293), a behavior which previously has not been captured or reported. Broadly, 1-week post-tenotomy is an acute time point with expected immune system responses, catabolism, and changes in energy metabolism, which continues into 2 weeks with less intensity and greater contribution from mitochondrial effects. Expression shifts at 4 weeks post-tenotomy to fatty acid oxidation, lipolysis, and general upregulation of adipogenesis related genes. The effects of previous weeks' transcriptional dysfunction present themselves at 8 weeks post-tenotomy with enriched DNA damage binding, aggresome activity, extracellular matrix-receptor changes, and significant expression of genes known to induce apoptosis. At 16 weeks post-tenotomy, there is a range of enriched pathways including extracellular matrix constituent binding, mitophagy,

neuronal activity, immune response, and more, highlighting the chaotic nature of this time point and possibility of a chronic classification. Transcriptional activity correlated significantly with histological changes and were enriched for biologically relevant pathways such as lipid metabolism. These data provide platform for understanding the biological mechanisms of chronic muscle degeneration after RC tears.

Keywords: rotator cuff, rotator cuff muscle dysfunction, transcriptome (RNA-seq), time series data analysis, muscle biology, tenotomy, muscle atrophy

INTRODUCTION

Rotator cuff (RC) tears are prevalent in the general population with an incident rate of 20% after the age of 60 (Sayampanathan and Andrew, 2017; Collin et al., 2019). Although RC repairs are used to treat this condition, time between tear and repair can influence the success of surgery (Gladstone et al., 2018). However, clinical studies have proven that repair does not improve or reverse the muscle atrophy and fatty infiltration observed at chronic states of disease (Gerber et al., 2000; Gladstone et al., 2018). This highlights the need to better understand what dysfunction in RC tears is leading to persistent muscle atrophy and fatty infiltration to determine potential targets for reversibility.

The rabbit model is an advantageous system to use to study this question due to similar morphological changes such as increased fat, fibrosis, and degeneration (Rubino et al., 2007; Farshad et al., 2012; Valencia et al., 2018; Hyman et al., 2020, 2021, in revision; Vargas-Vila et al., 2021) to what is observed in the supraspinatus (SSP) in humans (Gibbons et al., 2017, 2018b) without the need of a neurectomy as in smaller animal models (Rowshan et al., 2010). The major physiological changes noted in this model include significant decrease in muscle fiber cross-sectional area (CSA) at 4 and 16 weeks, degeneration of muscle fibers with a ∼25% muscle mass reduction after 16 weeks, and an increase in collagen and fat between 4 to 16 weeks (Rubino et al., 2007; Farshad et al., 2012; Valencia et al., 2018; Hyman et al., 2020, 2021, in revision; Vargas-Vila et al., 2021). Understanding the progression of RC disease before repair may help determine the potential effectiveness of a surgical intervention with or without adjuvant therapeutics.

Currently, few human studies have characterized gene expression in RC muscle after tendon tear and how it relates to the severity of muscle changes (Reardon et al., 2001; Steinbacher et al., 2010; Choo et al., 2014; Chaudhury et al., 2016; Shah et al., 2017; Gibbons et al., 2018a; Ren et al., 2018; Ge et al., 2020; Tashjian et al., 2020), but these data could not be related to a time course neither compared to a healthy control. Gene expression data with two or more time points have been collected in a mouse RC tear model and a rat tenotomy and neurectomy model (Lee et al., 2018; Gumucio et al., 2019; Hu et al., 2019), typically focusing on specific programs and gene overlap over the time series. However, even fewer studies have considered the mechanistic effects of muscle unloading in an animal RC tear model which properly recapitulates the human pathophysiology of fatty infiltration. One example includes the sheep RC tear model, which recapitulates human pathophysiology (Gerber et al., 2004) and has been used to consider the role of mitochondrial dysfunction with transcriptomics at two different time points (2 and 16 weeks post-tenotomy; Flück et al., 2017, 2020), Given the lack of a broad understanding of the progression of RC disease there is an unmet need to investigate the development in a time dependent manner with greater time and transcriptional resolution.

This study aims to establish transcriptional responses to RC tendon tear as a function of time in the rabbit tenotomy injury model. Sequencing for all genes, rather than select ones, allows for an unbiased analysis of transcriptional changes over time. These data may provide a greater understanding of how RC tears progress to a chronic state of decreased muscle quality and function. We hypothesize, based on morphological changes previously observed in this model, that transcription of certain genes is time dependent, where early changes would favor atrophy and inflammation and late changes would favor fatty infiltration, degeneration, and fibrosis.

MATERIALS AND METHODS

Animals

In this study 30 female New Zealand White rabbits (∼6 months, Western Oregon Rabbit Company, Philomath, OR, United States) were used to evaluate post-tenotomy transcriptional changes over time (Vargas-Vila et al., 2021). Females were used due to housing safety concerns regarding mixing gender and the ease of sourcing older female animals. All protocols were approved by the University of California, San Diego Institutional Animal Care and Use Committee (protocol #S11246). All animals were assigned a number ID and cage location upon arrival and then at time of harvest were randomized to one of the study groups. Animals were single housed with food and water ad lib, environmental and food enrichment, and visual access to other animals. There were no adverse events in this study and no animals met the criteria for humane early endpoints.

Surgical Procedures

Rabbits were anesthetized with a subcutaneous injection of ketamine and xylazine (35 mg/kg ketamine/5 mg/kg xylazine, MWI Veterinary Supply, Boise, ID, United States). Following intubation, 2–4% isoflurane (VetOne, Boise, ID, United States) was utilized to keep the animals under anesthesia for the duration of the surgery. The surgical site was disinfected, and an incision

was made through the skin and deltoid muscle overlying the RC. After exposing the SSP tendon, a unilateral tenotomy was performed by transecting the tendon at its footprint on the greater tubercle of the humerus. Surrounding soft tissues were bluntly dissected to permit unobstructed retraction of the tendon. To avoid the formation of tissue adhesions, a Penrose drain (Medline, Northfield, IL, United States) was sutured to the tendon stump. The muscle and skin layers were subsequently sutured and stapled closed, and the animals were allowed to recover. A sham surgery was performed on the contralateral limb of the tenotomy to serve as a control for the procedure where only the skin was cut, tendon isolated, and then stitched up as normal. An E-collar was placed on the animals to prevent suture ripping, and after 10 days, the collar and any remaining staples were removed (Vargas-Vila et al., 2021).

Muscle Harvesting

After the study, animals were euthanized at 5 time points; 1, 2, 4, 8, and 16 weeks post-tenotomy. At the specified time points, animals were euthanized with an intravenous overdose of pentobarbital (Beuthanasia, 120 mg/kg, MWI Veterinary Supply, Boise, ID, United States). The SSP muscles from both shoulders were harvested and divided into four regions with the central tendon serving as the muscle midline between the anterior and posterior sides of the muscle. These four regions included anterior lateral (A1), posterior lateral (P1), anterior medial (A2), and posterior medial (P2), and one full-muscle thickness fragment was harvested from each location. The harvested muscle regions were pinned to *in vivo* length and flash frozen in liquid nitrogen-chilled isopentane for storage at −80°C (Vargas-Vila et al., 2021).

RNA Extraction

The muscle samples from the P1 region were removed from −80°C and brought to a cryostat where they were allowed to come up to −20°C. The P1 region was chosen due to consistently presenting the most affected region of muscle in this RC injury model compared to the anterior and medial regions (Vargas-Vila et al., 2021). One notable difference is his region experiences the greatest fiber type changes with increased type II and decreased type I influencing the metabolic activity (Vargas-Vila et al., 2021). A 50–75 mg piece was removed from the center of each pinned region and placed in a pyrogen-free tube. RNA extraction was performed using the QIAGEN Fibrous Tissue mini kit on a QIAGEN Qiacube robot (QIAGEN, Germantown, MD, United States). In brief, the tissue was immersed in buffer RLT and disrupted by bead in the QIAGEN TissueLyser II (QIAGEN, Germantown, MD, United States), before being transferred to the Qiacube for RNA extraction. Samples were digested with Proteinase K, (QIAGEN, Germantown, MD, United States) prior to extraction. A DNase digestion step was included in the protocol. RNA was stored at −80°C.

RNA Sequencing

Total RNA was assessed for quality using an Agilent Tapestation 4200, and samples with an RNA Integrity Number (RIN) greater than 8.0 were used to generate RNA sequencing libraries using the TruSeq Stranded mRNA Sample Prep Kit (Illumina, San Diego, CA, United States). Samples were processed following manufacturer's instructions, modifying RNA shear time to 5 min. Resulting libraries were multiplexed and sequenced with 75 basepair (bp) single reads (SR75) to a depth of approximately 25 million reads per sample on an Illumina HiSeq400. Samples were demultiplexed using bcl2fastq Conversion Software (Illumina, San Diego, CA, United States).

RNAseq Analysis

Quality control of the raw fastq files was performed using the software tool FastQC (Andrews, 2010). Sequencing reads were aligned to the rabbit genome (Ensembl OryCun2.0) using the STAR v2.5.1a aligner (Dobin et al., 2013). Read quantification was performed with RSEM (Li and Dewey, 2011; v1.3.0) and Ensembl annotation (Oryctolagus_cuniculus.OryCun2.0.91.gtf). The R BioConductor packages edgeR (Robinson et al., 2010) and limma (Ritchie et al., 2015) were used to implement limma-voom (Law et al., 2014) followed by empirical Bayes technique for differential expression analysis. Lowly expressed genes were filtered out (cpm $>$ 1 in at least one sample). Trimmed mean of *M*-values (TMM) normalization was applied (Robinson and Oshlack, 2010). The experimental design was modeled upon time point and treatment ($\sim$0 +time_treatment) with contrasts (Tenotomy – Sham) for each time point and all samples. All results will be presented as the tenotomy time point compared to sham unless specified otherwise. From the empirical Bayes result, differentially expressed (DE) genes were defined by an adjusted P-value $<$ 0.05 [based on the moderated t-statistic using the Benjamini-Hochberg (BH) method to control the false discovery rate (Benjamini et al., 2001)] and a $|\log2FC| > 1$ (**Supplementary Data 1**). G:Profiler was used to map rabbit Ensemble IDs to human Ensemble IDs, Entrez IDs, and symbols (Raudvere et al., 2019), **Supplementary Data 2**. Of the 11,535 total genes, 1,210 were not mapped to human and 296 were duplicates and were removed for the analysis. The resulting genes with Entrez IDs correspond to the set of "background or detected genes" consisting of 10,029 genes. We removed one 8 week sample that we identified as an outlier using PCA plots and unsupervised hierarchical clustering. With $n = 6$ animals per group, we were sufficiently powered to identify significantly DE genes with a power of 72%. Volcano plots were created using Enhanced Volcano package (v.1.60). Heatmaps were created using clustermap in the seasborn package (Waskom, 2021). The Venn Diagram was produced using Van de Peer lab tools (Van de Peer Lab, 2021). RT-qPCR validation was not used in this study due to the robust nature of RNAseq methods and data analysis and supporting literature (Feng et al., 2010; Coenye, 2021).

Enrichment Analysis

Assignment of functional categories was based on the Gene Ontology (GO) categories "Biological process," "Molecular function," and "Cellular component." Enrichment analysis of GO categories was performed in R (version 4.0.2; http://www.r-project.org) using the "weight01" method from the Bioconductor topGO (v. 2.40.0) package with the org.Hs.eg.db_3.11.4 human database (Carlson, 2019; Alexa and Rahnenfuhrer, 2020). Node

size was set to 10, and Fisher's exact test was used for assessing GO term significance. Overrepresentation of functional categories was calculated for DE genes as compared with the 10,030 "background" genes, and significant GO terms were identified as those having P-value < 0.05 (**Supplementary Data 3**). KEGG analysis was also done in R using KEGGREST package (v. 1.28.0) with list of pathways and genes. A Wilcox rank-sum test was performed for each pathway, the Entrez ID along with the adjusted p-values of the gene expression as input. Testing whether p-values of genes included in that pathway are smaller than outside p-values. Overrepresentation of KEGG pathways was calculated for DE genes as compared with the 10,030 "background" genes, and significant KEGG pathways were identified as those having a p-value < 0.05 (**Supplementary Data 4**).

Gene Pathways of Interest

To highlight the changes in common genetic programs of interest, the RC muscle literature was searched to build a list of essential genes (**Supplementary Data 5**) found in programs such as myogenesis, inflammation, adipogenesis, and fibrosis (Gibbons et al., 2018a). Additional genes relating to adipogenesis identified as correlated with RC radiographic assessments (Shah et al., 2017) were also included. The final lists were compared to other RC transcriptome analyses in other animal models (Lee et al., 2018; Gumucio et al., 2019; Flück et al., 2020) for confirmation of overlap although in more broadly described categories.

Correlation Analysis

Weighted correlation network analysis (WGCNA) was performed using WGCNA R package (v. 1.70-3; Langfelder and Horvath, 2008) with transcriptional and phenotypic data (**Supplementary Data 6**) with the tenotomy only samples as there was no significance found in the combined analysis. This analysis works by building an unbiased network of modules which represents a cluster of genes, and then correlations (**Supplementary Data 7**) can be investigated with phenotype traits through gene membership. The phenotypic data included in this study is fiber area, central nucleation, fat quantification, collagen content, and degeneration, all of which are reported in detail elsewhere (Vargas-Vila et al., 2021) but are from the same animals used in this study. However, Hematoxylin and Eosin-stained (H&E) stained sections of representative muscles in each group, at each time point, can be seen in **Figure 1**.

RESULTS

Transcriptome Profiles Changed From Acute to Chronic State

Visually there were obvious structural muscle changes over time between post-tenotomy and sham samples (**Figure 1**). Changes included decreased muscle CSA, increased muscle degeneration, and increased fat and collagen deposition (Vargas-Vila et al., 2021).

At 1 week post-tenotomy, the initial acute time point, there was substantial number of significantly up- and down-regulated DE genes (**Figure 2A**), followed by a sharp decrease in differentially regulated transcripts at 2 (**Figure 2B**), 4 (**Figure 2C**), and 8 weeks post-tenotomy (**Figure 2D**). However, at 16 weeks post-tenotomy (**Figure 2E**) there was a resurgence in the number of DE genes, which considers this time point at a chronic state because although the expression is similar in intensity to 1 week post-tenotomy (**Figure 2A**), it had more up-regulated genes than down-regulated genes. The difference between the labeled genes in **Figures 2A–E** include that the genes at 1 week post-tenotomy do not appear in the described top 10 genes at any other time point, meanwhile there was overlap with the other time points, although with no clear commonality. Each time point's corresponding principal component analysis (**Figures 2F–J**) illustrated that standard analytical methods easily separate tenotomy from sham.

The Venn diagram (**Figure 3A**) shows the distribution of DE genes between each time point post-tenotomy, where 1 week had 1,111 unique genes, 2 weeks with 27, 4 weeks with 9, 8 weeks with 2, and 16 weeks post-tenotomy with 876. There are 6 DE genes [KLHL34, LSMEM2 (Leucine Rich Single-Pass Membrane Protein 2), UCP2 (Uncoupling Protein 2), TP63 (Tumor Protein P63), MYT1L (Myelin Transcription Factor 1 Like), ADPRHL1 (ADP-Ribosylhydrolase Like 1)] in common at all the time points which generally have to do with energy or transcription factors. The 1 week and 16 weeks post-tenotomy time points uniquely shared 581 DE genes (only 25% of their respective total DE genes). The total amount of DE genes by week was 1,942 with 1,069 up and 873 down for 1 week, 303 with 170 up and 133 down for 2 weeks, 127 with 86 up and 41 down for 4 weeks, 34 with 16 up and 18 down for 8 weeks and 1,714 with 1,421 up and 293 down for 16 weeks post-tenotomy (**Figure 3B**). Despite 1 and 16 weeks post-tenotomy shared an approximately equivalent number of DE genes, instead of the DE genes being split by up- and down-regulated as seen at the 1 week post-tenotomy, the 16 weeks post-tenotomy demonstrated 83% of up-regulated DE genes (**Figure 3B**). This contrast between 1 and 16 weeks post-tenotomy can also be demonstrated by the clustering in the heatmap. The clustering grouped 5 out of 6 of 1 week post-tenotomy samples separately from 16 weeks post-tenotomy (**Figure 3C**). The sham samples generally clustered together with some tenotomy sample exceptions (**Figure 3C**).

Enrichment Analysis

To determine the larger scale function the DE genes, overrepresentation analyses were performed using GO and KEGG at each time point (**Figure 4**). For 1 week post-tenotomy, the GO cellular component (yellow bars – **Figure 4A**) was enriched for transcripts relating to mitochondria. The molecular function (green bars – **Figure 4A**) was enriched for transcripts related to energy, and transporter and transferase activity. Biological processes (blue bars – **Figure 4A**) were enriched regarding catabolic, metabolic, and signaling specific pathways.

Gene ontology analysis at 2 weeks post-tenotomy continued to be enriched for components relating to mitochondria, molecular function relating to enzymes of the electron transport chain for

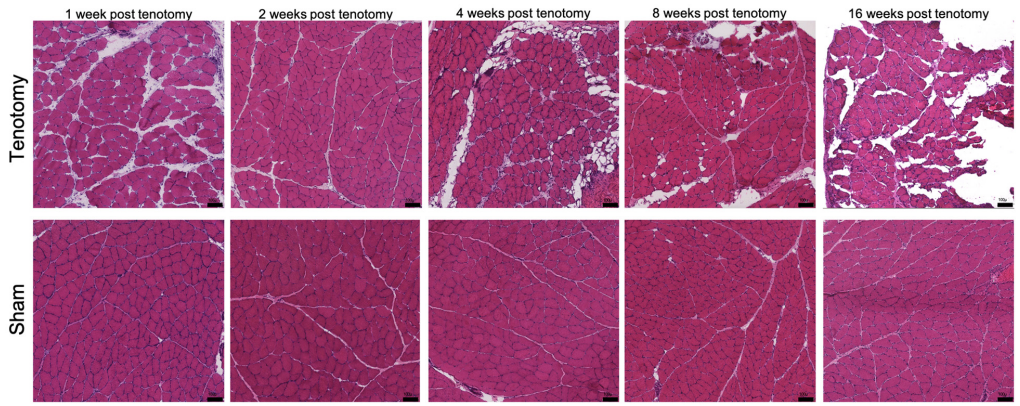

FIGURE 1 | Representative histological H&E sections of muscle at each time point for post-tenotomy and sham. Demonstrating over time a decrease in muscle CSA, muscle mass, increase in collagen content and fat reported in Vargas-Vila et al. (2021). Scale bar 100 μm.

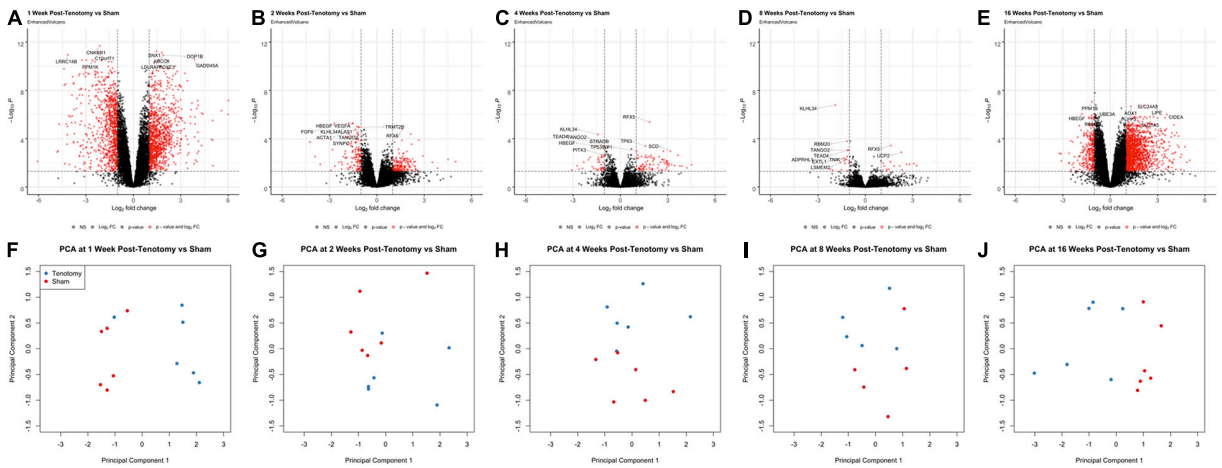

FIGURE 2 | Volcano plots **(A–E)** highlight differentially expressed (DE) genes (red dots) at each time point post-tenotomy defined as a logFC > 1 and an adjusted *p*-value < 0.05. The genes labeled in each volcano plot **(A–E)** consist of the top 10 genes with the smallest *p*-value. Corresponding PCA plots **(F–J)** at each time point demonstrate that the analytical methods separate tenotomy and sham.

ATP, NADH, cytochrome-c, and iron-sulfur binding (**Figure 4B**). The electron transport chain activity continued to be in enriched in the biological process along with positive regulation of the calcineurin-NFAT signaling cascade (**Figure 4B**).

At 4 weeks post-tenotomy, the cellular components were enriched for complexes related to signaling and neurotransmission (**Figure 4C**). Molecular function at 4 weeks was enriched for binding (activin, IGF1, and I-SMAD), catalytic activity on DNA, and proton transport (**Figure 4C**). There was continued enrichment of positive regulation of the calcineurin-NFAT signaling cascade at 4 weeks in biological process along with response of vitamin A and leucine (**Figure 4C**). This was the first time point where positive regulation of fatty acid oxidation appeared along with negative regulation of protein import into the nucleus.

The percent of genes in a given GO term decreases over time and was at its lowest at 8 weeks post-tenotomy, with most having 25% or less genes in the term (**Figure 4D**). The cellular components had broad enrichment at 8 weeks, ranging from aggresome, *Z*-disk, synapse, mitochondrial envelope to nucleus (**Figure 4D**). Interestingly, damaged DNA binding and transcription repressor activity appeared enriched in molecular function at 8 weeks, along with ubiquitin protease activity, virus receptor activity, and antioxidant activity (**Figure 4D**). Positive regulation of calcineurin-related signaling again was enriched in biological process at 8 weeks with more regulation related to IL-8, translation in response to stress, and skeletal muscle cell differentiation.

An increase of ∼50% in significant genes within a GO term from 8 weeks post-tenotomy to 16 weeks post-tenotomy was seen with a range of cellular components enriched for the synapse, collagen trimer, mitochondrial respiratory chain complex I, and pseudopodium (**Figure 4E**). Molecular function at 16 weeks was enriched for binding (PDGF, collagen, proteoglycan, and phosphatidylinositol-4-phosphate) and extracellular matrix constituents (**Figure 4E**). Positive

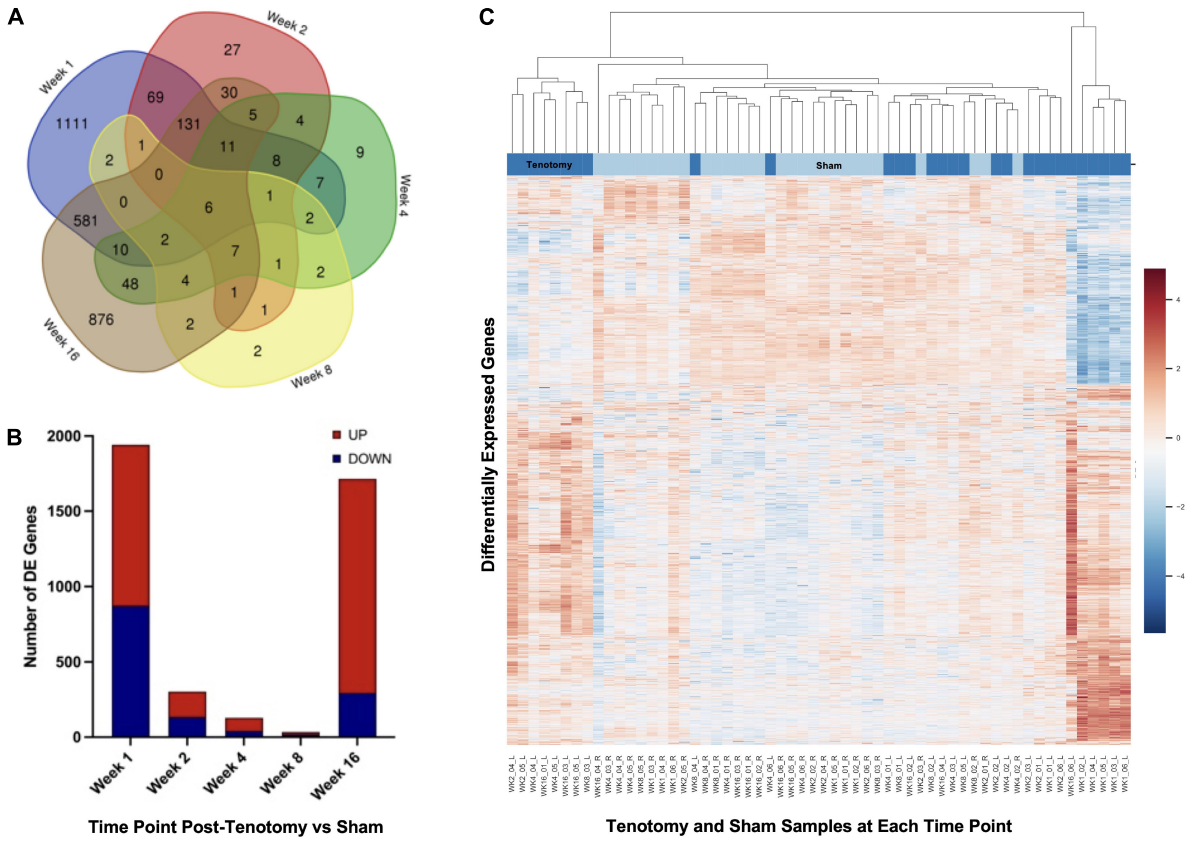

FIGURE 3 | Distribution of samples by tenotomy vs sham and DE genes over each time point post-tenotomy. Venn diagram **(A)** highlights the DE genes at each time point and the overlap with other time points. The bar chart **(B)** displays the number of DE genes which are up or down regulated at each timepoint. Data in the heatmap **(C)** is presented as normalized expression for each tenotomy and sham sample at each time point post-tenotomy with a *z*-score scale by rows and an average hierarchical clustering by columns.

regulation of reactive oxygen species (ROS) generation was enriched in biological process at 16 weeks, along with synapse maturation, axonal transport of mitochondrion, leukocytes rolling, and membrane fission (**Figure 4E**).

KEGG analyses highlighted changes of broader pathways over time (**Figure 5**). At 1 week post-tenotomy, all metabolism terms were significantly enriched and generally continued into 2 weeks post-tenotomy (**Figure 5** – metabolism). Metabolism at 4 weeks post-tenotomy identified new enriched metabolism terms such as tryptophan metabolism (**Figure 5** – amino acid metabolism), retinol metabolism (**Figure 5** – vitamins and cofactors metabolism), sugar metabolism (**Figure 5** – carbohydrate metabolism), cholesterol metabolism, and steroid biosynthesis (**Figure 5** – lipid metabolism). Only glycine, serine, threonine metabolism (**Figure 5** – amino acid metabolism), and biosynthesis of unsaturated fatty acid (**Figure 5** – lipid metabolism) were enriched at 8 weeks post-tenotomy. By 16 weeks post-tenotomy there were only a few pathways in each metabolism category that were enriched significantly. In the signaling transduction cluster, MAPK, FoxO, Rap1 signaling pathways were the only terms enriched at all time points. Most terms in this cluster were enriched at 16 weeks post-tenotomy with 5 pathways (cGMP-PKG, Apelin, Wnt, Hedgehog, and Sphingolipid signaling pathway) that became enriched only at this time point. Focusing on the immune system cluster, there was a clear activation of all terms at 1 week which decreased slightly by 2 weeks, was not present at 4 weeks and only antigen processing and presentation was enriched at 8 weeks, but by 16 weeks post-tenotomy there was a reactivation of many of the 1 week terms in addition to B and T cell receptor signaling pathway (**Figure 5** – immune system). There were a few enriched terms in the endocrine system cluster at 1, 2, and 4 weeks with double the number at 8 weeks and over 9 at 16 weeks post-tenotomy (**Figure 5** – endocrine system). At 4 weeks post-tenotomy, regulation of lipolysis in adipocytes, insulin resistance and signaling pathway were enriched and continued to be at 16 weeks post-tenotomy along with many more endocrine system terms. 16 weeks post-tenotomy also had many significantly enriched terms in the nervous system cluster which contrasted with the behavior at 1 week post-tenotomy. The cell processes cluster highlighted terms with the cell environment where 1 week post-tenotomy was enriched for apoptosis, ABC transporters, cytokine-cytokine receptor interaction, 2 weeks with ribosome, 4 weeks with p53 signaling pathway, 8 weeks with neuroactive

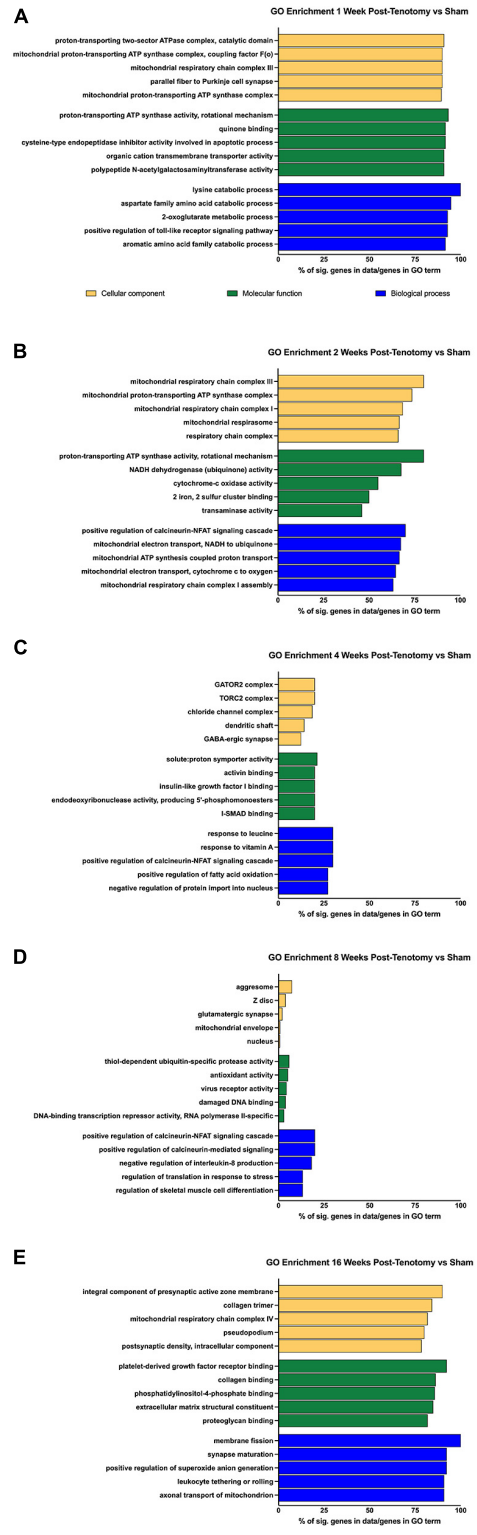

FIGURE 4 | GO enrichment analysis for **(A)** 1 week post-tenotomy, **(B)** 2 weeks post-tenotomy, **(C)** 4 weeks post-tenotomy, **(D)** 8 weeks post-tenotomy, and **(E)** 16 weeks post-tenotomy. Data presented as top 5 greatest gene coverage (# of significant genes/total genes in GO term) in each category: cellular component (yellow), molecular function (green), and biological process (blue).

ligand-receptor interaction, adherens junction, and 16 weeks with mitophagy and ubiquitin mediated proteolysis.

Gene Programs of Interest Changed Over Time After Tear

Using a literature-driven approach, specific genes from the literature relating to myogenic, anti-myogenic (suppressing muscle formation, cell death, and degradation), adipogenic, inflammation, and fibrotic programs were highlighted across the five time points (**Figure 6**). The myogenic program (**Figure 6** – green bars) was activated the strongest after 1 week post-tenotomy (**Figure 6A**) and was faint or not significant at all other time points (**Figures 6B–E**). The anti-myogenic program (**Figure 6** – red bars) was also expressed strongly at 1 week post-tenotomy with continued expression at all the time points, although with fewer genes at the 2–8 week time points, and then presented a second robust response at 16 weeks. Although some genes within the adipogenic program (**Figure 6** – yellow bars) were significantly expressed in 1 and 2 weeks, the entire program was fully expressed initially at 4 weeks and again at 16 weeks post-tenotomy. Inflammation (**Figure 6** – orange bars) was observed with a large expression at 1 week post-tenotomy and decreasing through 8 weeks, with a resurgence at 16 weeks. The fibrotic program (**Figure 6** – pink bars) was also initially expressed at 1 week with fewer expressed genes at 2 weeks followed by no significant genes at 4 and 8 weeks (**Figures 6C,D** – dashed bars) and another robust response at 16 weeks post-tenotomy (**Figure 6E**).

Transcriptional Data Correlations With Phenotypic Traits

Weighted correlation network analysis revealed gene modules significantly related to phenotype characteristics quantified by histology (**Supplementary Figure 1**). Modules 10 and 4 encompass the greatest number of significant correlations with phenotype traits, and genes in these modules were significantly enriched for interesting pathways related to lipid metabolism, and general RNA activity (**Supplementary Data 8**). The phenotype traits degeneration (p = 0.07) and collagen content (p = 0.03) were only correlated with module 10, which is enriched for lipid metabolism. The strongest correlation coefficient (-0.71, p = 2e-05) is associated with the centralized nuclei trait (a marker of muscle degeneration-regeneration) and module 13 which is enriched for pathways related to DNA binding and regulation. Centralized nuclei trait is also correlated significantly to modules 9, 11, and 14 and these genes are enriched for skeletal muscle adaptation/fast-slow fiber transition, endoplasmic reticulum related protein binding, and ubiquitin-like protease activity, respectively, (**Supplementary Figure 1** and **Supplementary Data 8**). Module 7 is significantly associated with interfascicular fat and fat and degeneration phenotype traits whose genes are enriched for interesting pathways as Ragulator complex which is anchored to lipid rafts in late endosomes and protein import related to peroxisomes membrane (**Supplementary Figure 1** and **Supplementary Data 8**).

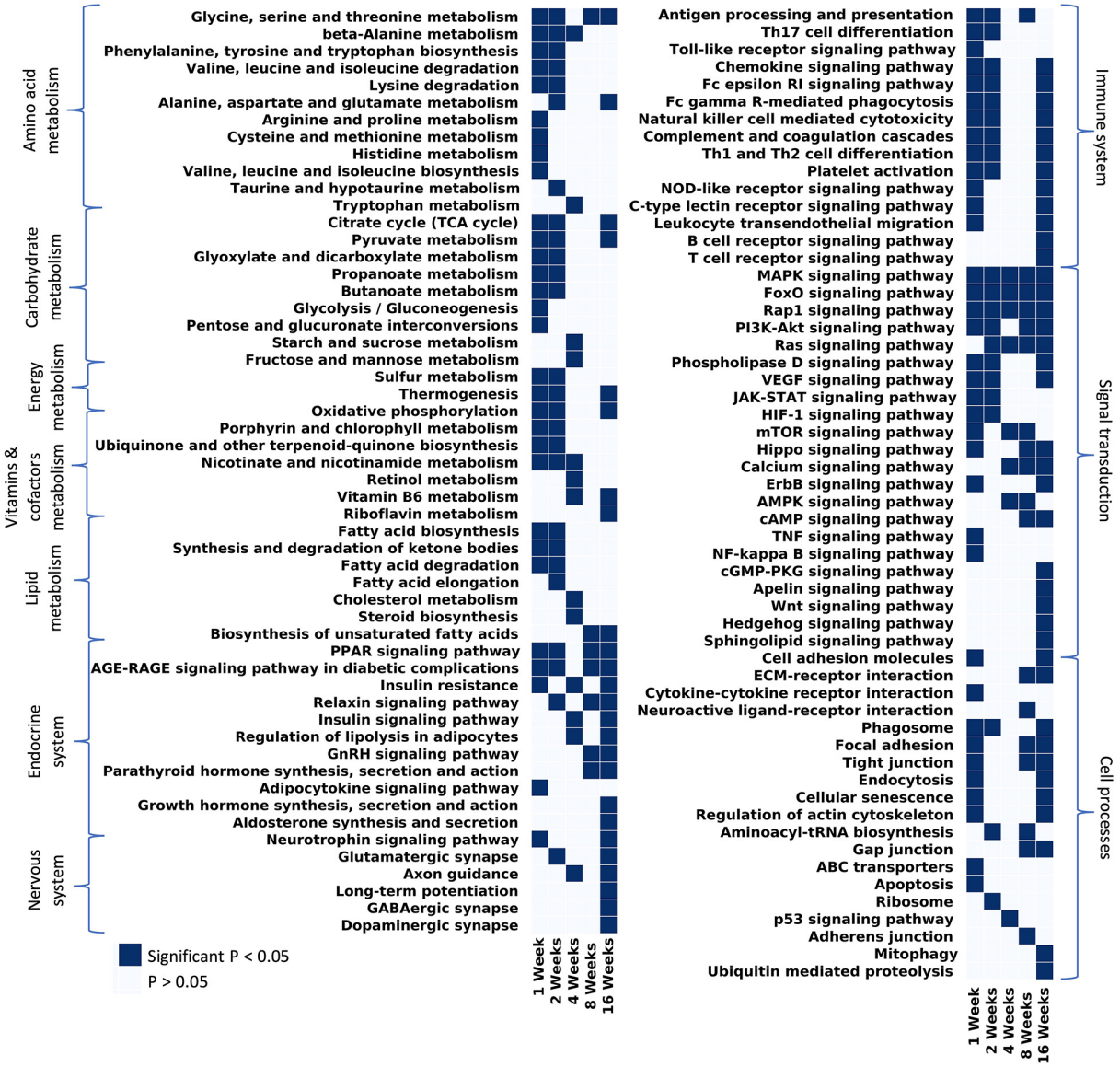

FIGURE 5 | Considering all pathways that have at least 1 significant *p*-value and filtering out disease/tissue specific pathways, the remaining pathways were grouped by KEGG hierarchy into amino acid metabolism, carbohydrate metabolism, vitamins and cofactors metabolism, energy metabolism, lipid metabolism, endocrine system, nervous system, immune system, signal transduction, and cell processes.

DISCUSSION

The purpose of this study was to establish transcriptional changes as a function of time in a rabbit RC tear model. We hypothesize, based on morphological changes previously observed in this model, that transcription of certain genes is time dependent, where early changes would favor atrophy and inflammation and late changes would favor fatty infiltration, degeneration, and fibrosis. There were clear transcriptional differences between each time point, which support the concept of differential timing of inflammation, adipogenesis, fibrosis, and cell death programs that lead to muscle atrophy, degeneration, and fatty infiltration. Likewise, phenotypic traits correlated significantly with gene groupings in unbiasedly defined modules which were enriched for biological relevant pathways such as lipid metabolism.

At 1 week post-tenotomy, as expected, there was a large transcriptional response in both up- and down regulation (**Figures 2A**, **3B**). Functional enrichment elucidates that these genes are related to the immune system, energy metabolism, catabolism and a wide range of signaling pathways we would expect to see at an acute response to injury (**Figures 4A**, **5A**). 2 weeks post-tenotomy the response trends in areas of metabolism, particularly with the mitochondria (**Figure 4B**), signaling pathways, and immune system with fewer enriched

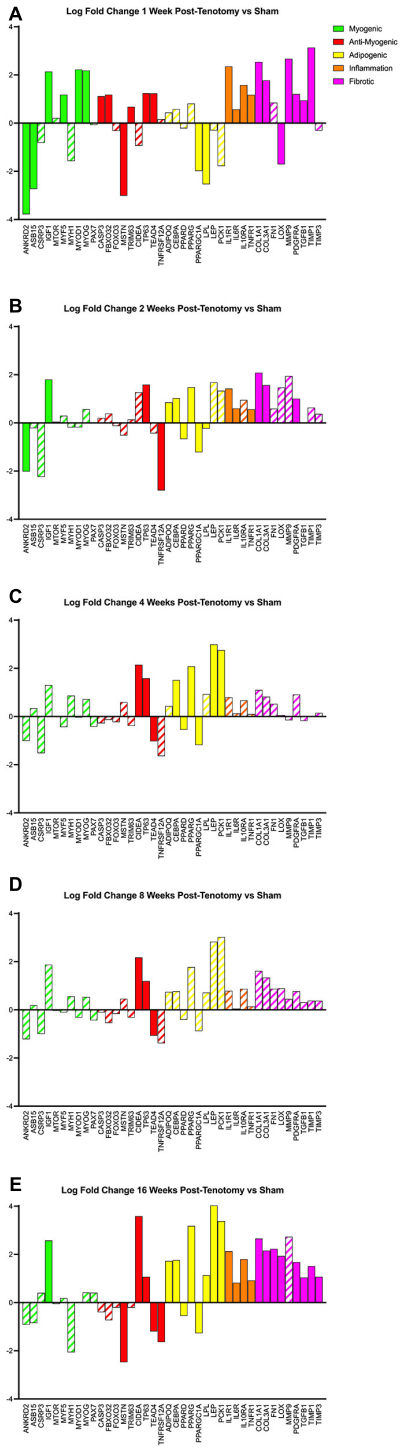

FIGURE 6 | Changes in transcriptome of genetic programs of interest at **(A)** 1 week post-tenotomy, **(B)** 2 weeks post-tenotomy, **(C)** 4 weeks post-tenotomy, **(D)** 8 weeks post-tenotomy, and **(E)** 16 weeks post-tenotomy. Log fold change (logFC) is the difference of tenotomy and sham. Solid bars represent a significant adjusted *p*-value ($p < 0.05$) and partially filled in bars are not significant. Green bars represent myogenic related genes, red represents anti-myogenic, yellow represents adiopogenic, orange represents inflammation, and pink represents fibrotic genes. Data are presented as average logFC.

terms (**Figure 5**). Overall, 2 weeks post-tenotomy had a more muted response than the previous week with greater emphasis on ubiquinone, a metabolite involved in the electron transport chain in the mitochondria and free-radical scavenger antioxidant, related build up (**Figure 4B**).

A turning point appeared to occur at 4 weeks post-tenotomy where the expression, despite decreasing from 2 weeks (**Figure 2C**), shares a greater overlap of DE genes with the 16 weeks time point as opposed to 1 and 2 weeks (**Figure 3A**). The enrichment analyses supported the gene shift toward lipids and adipogenesis, with enriched pathways of positive regulation of fatty acid oxidation (**Figure 4C**) and regulation of lipolysis in adipocytes (**Figure 6**). Likewise, when literature-specific adipogenesis genes were considered, this time point demonstrated significant upregulation of these genes (**Figure 6C**).

The 8 weeks post-tenotomy time point, with only 34 DE genes, was the time point with the lowest levels of DE expression demonstrating a possible trend toward steady state of transcription expression (**Figures 2D**, **3**). These genes are related to GO terms associated with DNA damage binding, transcription repression, regulation of translation in response to stress, and interestingly aggresome, which serves as a location for misfolded proteins and is used when there is too much protein degradation for the cell to handle (**Figure 4D**). These changes are all associated with low transcriptional activity and degradation. ECM-receptor interaction, including neuroactive ligand-receptor interaction, GAP junctions first appeared enriched at this time point highlighting a change in the cell environment (**Figure 6**). Given how few significant genes there were at 8 weeks post-tenotomy, only CIDEA, TP63, and TEAD4 from the literature specific genes (**Figure 6D**) were significantly expressed in the anti-myogenic category, which interestingly, are related to apoptosis.

At 16 weeks post-tenotomy, a resurgence in expression, well after the acute stage response, suggests the possibility of a unique, chronic transcriptional signature. Based on morphology from previous studies (Vargas-Vila et al., 2021), this was when fatty infiltration was most present. Transcriptionally, there are 876 unique DE genes at 16 weeks post-tenotomy not present at any other time point highlighting the difference in response from 1 week post-tenotomy (**Figure 2A**). Positive regulation of superoxide (a type of ROS) anion generation, ubiquitin mediated proteolysis, general immune system response was enriched at this time point (**Figure 4E**). Similarly, extracellular matrix (ECM) binding such as collagen, proteoglycans, neuronal activity relating to synapse components, maturation and axonal transport of mitochondrion are enriched (**Figure 4E**). Mitophagy, in particular, was enriched at this time point suggesting there was sufficient mitochondrial damage/stress that needed to be degraded by autophagy. Not including the myogenic specific genes, all literature-based programs are significantly expressed and more so than at any other time point (**Figure 6E**).

The distribution of DE genes with the first acute time point having the most and the decrease of expression (**Figure 2**) was similar to other transcriptional time-series studies (Lee et al., 2018; Gumucio et al., 2019) in a mouse RC tear model (1 and 4 weeks) and a rat (10, 30, and 60 days) RC tear and denervation

model. However, neither of these studies captured what was observed at the 16 weeks post-tenotomy time point (**Figure 2E**), which was a resurgence of expression to a similar level of the most acute time point at 1 week post-tenotomy, but with 876 new genes (**Figure 3A**) and a greater ratio of up-regulated genes instead of down-regulated (**Figure 3B**) where autophagy and ECM binding dominate. These studies also demonstrated a large overlap of DE genes at all recorded time points (Lee et al., 2018; Gumucio et al., 2019; Hu et al., 2019), which does not encompass the RC tear progression over time since this study only recorded 6 genes in common at all time points (**Figure 3**). This is an advantage of this study due to the time and sequence resolution used in order to capture unique time points during RC tear progression. In regard to enrichment there were similar trends in expression of ECM related genes increasing over-time in a rat tenotomy and neurectomy model (Gumucio et al., 2019) which was similar to the genes in the literature-defined fibrotic program that increased at 16 weeks post-tenotomy in this study (**Figure 6**).

In a more clinically relevant sheep RC tear model (Flück et al., 2017, 2020), with two time points (2 and 16 weeks) highlighted that there were 350 transcripts reported different at either time point. Enrichment analyses highlighted similarities with pathways related to focal adhesions, calcium binding, and extracellular space, which were also enriched at 16 weeks post-tenotomy in our study (**Figure 5**). Comparing to a human qPCR study of torn RC muscle (Gibbons et al., 2018a), the gene expression across the cell programs in our study at 16 weeks post-tenotomy (**Figure 6E**) closely resembled biopsies taken from high-fat characterized muscle compared to no-muscle or intact biopsies (Gibbons et al., 2018a). Highlighting essential genes from the literature, we observed the adipogenic pathway expressed starting at 4 and 16 weeks post-tenotomy (**Figure 6**). Likewise, the difference between 1- and 16 weeks post-tenotomy emphasizes how expression shifts toward more adipogenic and fibrotic genes.

Given a unique, chronic transcriptional profile which emphasized ROS build up, protein degradation, change in ECM/cell environment, the cause(s) leading to these changes merit further investigation to better understand the mechanisms of chronic fatty, fibrotic muscle atrophy observed in RC disease. Some intriguing possibilities based on these data are; changes in metabolism with the mitochondria and oxidative stress, late stage dysregulation of the inflammatory system, or a combination of metabolic and inflammatory dysregulation encouraging autophagy. These potential hypotheses, and other based on these unique data, should be tested mechanistically by deliberately manipulating this now better-defined system.

We are unaware of any other correlation between RNAseq data and muscle structural changes over time in RC muscle. Specifically, unbiased gene sets were correlated with histological measurements in our tenotomy samples, where certain gene sets correlated positively with most histological measurements (**Supplementary Figure 1**). The fact that there was an obvious statistical correlation between transcriptional activity and structure reinforces our confidence in the physiological significance of the RNAseq data presented in this study. Importantly, although sets of genes, as opposed to individual genes or even pathways, are likely most relevant to the broad degenerative changes seen in tenotomized muscle, we are not implying that direct cause-effect relationships between specific gene set dysregulation and structural changes as observed in this analysis. That being said, the connection between altered lipid metabolism, DNA regulation, and ubiquitination are all logically appealing pathways to probe contributing to future studies in the muscle physiology field along with better understanding RC tear injury dysfunction. Future studies will need to manipulate gene sets, or pathways, positively and negatively to determine if they are mechanistically related to degeneration. Additionally, given the heterogeneity of muscle degeneration across individual muscles, relating transcriptional activity to specific regions of muscle will be a valuable technological advance.

CONCLUSION

Defining transcriptional changes in a RC tear model such as rabbit, which is more similar to human RC disease progression, allows for the possibility of further mechanistic studies to understand the muscle dysfunction leading to muscle atrophy and fatty infiltration observed. The field needs to better understand the progression of tear in order to make more informed decisions regarding RC repair and therapeutics. This study outlines the timeline of transcriptional changes in muscle after RC tear such as the immune response at 1 week post-tenotomy, mitochondrial activity at 2 weeks post tenotomy, adipogenesis active at 4 weeks post-tenotomy, transcriptional steady state at 8 weeks post-tenotomy, and not previously reported resurgence of transcription at 16 weeks post-tenotomy, which may represent a chronic transcriptional signature, and correlates gene sets to structural muscle changes over time.

DATA AVAILABILITY STATEMENT

The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (Edgar et al., 2002) and are accessible through GEO Series accession number GSE173234 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE173234).

ETHICS STATEMENT

The animal study was reviewed and approved by University of California, San Diego Institutional Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

MG, MV-V, JL, AS, and SW were responsible for the conception and design of the study. LV-B, MG, SR, MV-V, IW, SH, ME,

DF, JL, AS, CN, and KF contributed to collection and analysis of data. LV-B was responsible for the design and drafting of the manuscript. All authors revised the manuscript and gave final approval.

FUNDING

The project described was partially supported by the National Institutes of Health, Grant UL1TR001442 of CTSA. The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH.

ACKNOWLEDGMENTS

We acknowledge the contribution of UCSD IGM for sequencing services. Padmini Rangamani and Pradipta Ghosh for helpful discussion of the data.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2021.707116/full#supplementary-material

REFERENCES

Alexa, A., and Rahnenfuhrer, J. (2020). *topGO: Enrichment Analysis for Gene Ontology. R package version 2.40.0*.

Andrews, S. (2010). FastQC: A Quality Control Tool For High Throughput Sequence Data. Available online at: http://www.bioinformatics.babraham.ac.uk/projects/fastqc/

Benjamini, Y., Drai, D., Elmer, G., Kafkafi, N., and Golani, I. (2001). Controlling the false discovery rate in behavior genetics research. *Behav. Brain Res.* 125, 279–284. doi: 10.1016/S0166-4328(01)00297-2

Carlson, M. (2019). *Genome Wide Annotation For Human. R Package Version 3.8.2*.

Chaudhury, S., Xia, Z., Thakkar, D., Hakimi, O., and Carr, A. J. (2016). Gene expression profiles of changes underlying different-sized human rotator cuff tendon tears. *J. Shoulder. Elb. Surg.* 25, 1561–1570. doi: 10.1016/j.jse.2016.02.037

Choo, A., McCarthy, M., Pichika, R., Sato, E. J., Lieber, R. L., Schenk, S., et al. (2014). Muscle gene expression patterns in human rotator cuff pathology. *J. Bone Jt. Surg Am.* 96, 1558–1565. doi: 10.2106/JBJS.M.01585

Coenye, T. (2021). Do results obtained with RNA-sequencing require independent verification? *Biofilm* 3:100043. doi: 10.1016/j.bioflm.2021.100043

Collin, P., Thomazeau, H., Walch, G., Gerber, C., Mansat, P., Favard, L., et al. (2019). Clinical and structural outcome twenty years after repair of isolated supraspinatus tendon tears. *J. Shoulder Elbow Surg.* 28, 1–196. doi: 10.1016/j.jse.2018.07.023

Dobin, A., Davis, C. A., Schlesinger, F., Drenkow, J., Zaleski, C., Jha, S., et al. (2013). STAR: ultrafast universal RNA-seq aligner. *Bioinformatics* 29, 15–21. doi: 10.1093/bioinformatics/bts635

Edgar, R., Domrachev, M., and Lash, A. E. (2002). Gene Expression Omnibus: NCBI gene expression and hybridization array data repository. *Nucleic Acids Res.* 30, 207–210. doi: 10.1093/nar/30.1.207

Farshad, M., Meyer, D. C., Nuss, K. M., and Gerber, C. (2012). A modified rabbit model for rotator cuff tendon tears: functional, histological and radiological characteristics of the supraspinatus muscle. *Shoulder Elbow* 4, 90–94. doi: 10.1111/j.1758-5740.2011.00170.x

Feng, L., Liu, H., Liu, Y., Lu, Z., Guo, G., Guo, S., et al. (2010). Power of deep sequencing and agilent microarray for gene expression profiling study. *Mol. Biotechnol.* 45, 101–110. doi: 10.1007/s12033-010-9249-6

Flück, M., Fitze, D., Ruoss, S., and Valdivieso, P. (2020). Down-regulation of mitochondrial metabolism after tendon release primes lipid accumulation in rotator cuff muscle. *Am. J. Pathol.* 190, 1513–1529. doi: 10.1016/j.ajpath.2020.03.019

Flück, M., Ruoss, S., Möhl, C. B., Valdivieso, P., Benn, M. C., von Rechenberg, B., et al. (2017). Genomic and lipidomic actions of nandrolone on detached rotator cuff muscle in sheep. *J. Steroid Biochem. Mol. Biol.* 165, 382–395. doi: 10.1016/j.jsbmb.2016.08.005

Ge, Z., Tang, H., Lyu, J., Zhou, B., Yang, M., Tang, K., et al. (2020). Conjoint analysis of lncRNA and mRNA expression in rotator cuff tendinopathy. *Ann. Transl. Med.* 8, 335–335. doi: 10.21037/atm.2020.02.149

Gerber, C., Fuchs, B., and Hodler, J. (2000). The results of repair of massive tears of the rotator cuff. *J. Bone Joint Surg.* 82:505.

Gerber, C., Meyer, D. C., Schneeberger, A. G., Hoppeler, H., and von Rechenberg, B. (2004). Effect of tendon release and delayed repair on the structure of the muscles of the rotator cuff: an experimental study in sheep. *J. Bone Joint Surg. Am.* 86, 1973–1982. doi: 10.2106/00004623-200409000-00016

Gibbons, M. C., Fisch, K. M., Pichika, R., Cheng, T., Engler, A. J., Schenk, S., et al. (2018a). Heterogeneous muscle gene expression patterns in patients with massive rotator cuff tears. *PLoS One* 13:1–17. doi: 10.1371/journal.pone.0190439

Gibbons, M. C., Singh, A., Anakwenze, O., Cheng, T., Pomerantz, M., Schenk, S., et al. (2017). Histological evidence of muscle degeneration in advanced human rotator cuff disease. *J. Bone Joint Surg.* 99, 190–199. doi: 10.2106/JBJS.16.00335

Gibbons, M. C., Singh, A., Engler, A. J., and Ward, S. R. (2018b). The role of mechanobiology in progression of rotator cuff muscle atrophy and degeneration. *J. Orthop. Res.* 36, 546–556. doi: 10.1002/jor.23662

Gladstone, J. N., Bishop, J. Y., Lo, I. K. Y., and Flatow, E. L. (2018). Fatty infiltration and atrophy of the rotator cuff do not improve after rotator cuff repair and correlate with poor functional outcome. *Am. J. Sports Med.* 35, 719–728. doi: 10.1177/0363546506297539

Gumucio, J. P, Qasawa, A. H, Ferrara, P. J, Malik, A. N, Funai, K., Mcdonagh, B., et al. (2019). Reduced mitochondrial lipid oxidation leads to fat accumulation in myosteatosis. *FASEB J* 33, 7857–7863. doi: 10.1096/fj.201802457RR

Hu, P., Jiang, L., and Wu, L. (2019). Identify differential gene expressions in fatty infiltration process in rotator cuff. *J. Orthop. Surg. Res.* 14, 1–10. doi: 10.1186/s13018-019-1182-1

Hyman, S., Norman, M. B., Dorn, S. N., Bremner, S. N., Esparza, M. C., Lieber, R. L., et al. (2020). In vivo supraspinatus muscle contractility and architecture in rabbit. *J. Appl. Physiol. (1985)* 129, 1405–1412. doi: 10.1152/japplphysiol.00609.2020

Langfelder, P., and Horvath, S. (2008). WGCNA: an r package for weighted correlation network analysis. *BMC Bioinform.* 9:559. doi: 10.1186/1471-2105-9-559

Law, C. W., Chen, Y., Shi, W., and Smyth, G. K. (2014). Voom: precision weights unlock linear model analysis tools for RNA-seq read counts. *Geno. Biol.* 15, 1–17. doi: 10.1186/gb-2014-15-2-r29

Lee, Y. S., Kim, J. Y., Kim, H. N., Lee, D. W., and Chung, S. W. (2018). Gene expression patterns analysis in the supraspinatus muscle after a rotator cuff tear in a mouse model. *Biomed. Res. Int.* 2018, 21–25. doi: 10.1155/2018/5859013

Li, B., and Dewey, C. N. (2011). RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. *BMC Bioinform.* 12:323. doi: 10.1186/1471-2105-12-323

Raudvere, U., Kolberg, L., Kuzmin, I., Arak, T., Adler, P., Peterson, H., et al. (2019). G:Profiler: a web server for functional enrichment analysis and conversions of gene lists (2019 update). *Nucleic Acids Res.* 47, W191–W198. doi: 10.1093/nar/gkz369

Reardon, K. A., Davis, J., Kapsa, R. M. I., Choong, P., and Byrne, E. (2001). Myostatin, insulin-like growth factor-1, and leukemia inhibitory factor mRNAs are upregulated in chronic human disuse muscle atrophy. *Muscle Nerve* 24, 893–899. doi: 10.1002/mus.1086.abs

Ren, Y. M., Duan, Y. H., Sun, Y. B., Yang, T., and Tian, M. Q. (2018). Bioinformatics analysis of differentially expressed genes in rotator cuff tear patients using microarray data. *J. Orthop. Surg. Res.* 13, 1–9. doi: 10.1186/s13018-018-0989-5

Ritchie, M. E., Phipson, B., Wu, D., Hu, Y., Law, C. W., Shi, W., et al. (2015). Limma powers differential expression analyses for RNA-sequencing and microarray studies. *Nucleic Acids Res.* 43:e47. doi: 10.1093/nar/gkv007

Robinson, M. D., and Oshlack, A. A. (2010). Scaling normalization method for differential expression analysis of RNA-seq data. *Genome Biol.* 11:R25.

Robinson, M. D., McCarthy, D. J., and Smyth, G. K. (2010). edgeR: a bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics* 26, 139–140. doi: 10.1093/bioinformatics/btp616

Rubino, L. J., Stills, H. F., Jr., Sprott, D. C., and Crosby, L. A. (2007). Fatty infiltration of the torn rotator cuff worsens over time in a rabbit model. *Arthroscopy* 23, 717–722. doi: 10.1016/j.arthro.2007.01.023

Rowshan, K., Hadley, S., Pham, K., Caiozzo, V., Lee, T. Q., and Gupta, R. (2010). Development of fatty atrophy after neurologic and rotator cuff injuries in an animal model of rotator cuff pathology. *J. Bone Joint Surg. Am.* 92, 2270–2278. doi: 10.2106/jbjs.i.00812

Sayampanathan, A. A., and Andrew, T. H. C. (2017). Systematic review on risk factors of rotator cuff tears. *J. Orthop. Surg. (Hong Kong).* 25, 1–9. doi: 10.1177/2309499016684318

Shah, S. A., Kormpakis, I., Cavinatto, L., Killian, M. L., Thomopoulos, S., and Galatz, L. M. (2017). Rotator cuff muscle degeneration and tear severity related to myogenic, adipogenic, and atrophy genes in human muscle. *J. Orthop. Res.* 35, 2808–2814. doi: 10.1002/jor.23593

Steinbacher, P., Tauber, M., Kogler, S., Stoiber, W., Resch, H., and Sänger, A. M. (2010). Effects of rotator cuff ruptures on the cellular and intracellular composition of the human supraspinatus muscle. *Tissue Cell.* 42, 37–41. doi: 10.1016/j.tice.2009.07.001

Tashjian, R. Z., Lock, I., Granger, E. K., Wang, Y., Lee, Y., Chalmers, P. N., et al. (2020). Gene expression in torn rotator cuff tendons determined by RNA sequencing. *Orthop. J. Sport. Med.* 8, 1–8. doi: 10.1177/2325967120927480

Valencia, A. P., Lai, J. K., Iyer, S. R., Mistretta, K. L., Spangenburg, E. E., Davis, D. L., et al. (2018). Fatty infiltration is a prognostic marker of muscle function after rotator cuff tear. *Am. J. Sports Med.* 46, 2161–2169. doi: 10.1177/0363546518769267

Vargas-Vila, M. A., Gibbons, M. C., Wu, I. T., Esparza, M. C., Kato, K., Johnson, S. D., et al. (2021). *The "second hit" of rotator cuff repair in a chronic tear rabbit model. J. Orthop. Res.* (in press).

Van de Peer Lab (2021). Available online at: http://bioinformatics.psb.ugent.be/webtools/Venn/ (accessed January 15, 2021).

Waskom, M. L. (2021). seaborn: statistical data visualization. *J. Open Source Softw.* 6:3021. doi: 10.21105/joss.03021

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frontiers in Physiology

CORRECTION
published: 29 October 2021
doi: 10.3389/fphys.2021.775297

Corrigendum: Transcriptional Time Course After Rotator Cuff Tear

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Keywords: rotator cuff, rotator cuff muscle dysfunction, transcriptome (RNA-seq), time series data analysis, muscle biology, tenotomy, muscle atrophy

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Edited and reviewed by:
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Specialty section:
This article was submitted to Striated Muscle Physiology, a section of the journal Frontiers in Physiology

Received: *13 September 2021*
Accepted: *07 October 2021*
Published: *29 October 2021*

Citation:
Vasquez-Bolanos LS, Gibbons MC, Ruoss S, Wu IT, Vargas-Vila M, Hyman SA, Esparza MC, Fithian DC, Lane JG, Singh A, Nasamran CA, Fisch KM and Ward SR (2021) Corrigendum: Transcriptional Time Course After Rotator Cuff Tear. Front. Physiol. 12:775297. doi: 10.3389/fphys.2021.775297

A Corrigendum on

Transcriptional Time Course After Rotator Cuff Tear

by Vasquez-Bolanos, L. S., Gibbons, M. C., Ruoss, S., Wu, I. T., Vargas-Vila, M., Hyman, S. A., Esparza, M. C., Fithian, D. C., Lane, J. G., Singh, A., Nasamran, C. A., Fisch, K. M., and Ward, S. R. (2021). Front. Physiol. 12:707116. doi: 10.3389/fphys.2021.707116

In the original article, there was a mistake in **Figure 3** as published. **An older version of the figure was published by mistake.** The corrected **Figure 3** appears below.

The authors apologize for this error and state that this does not change the scientific conclusions of the article in any way. The original article has been updated.

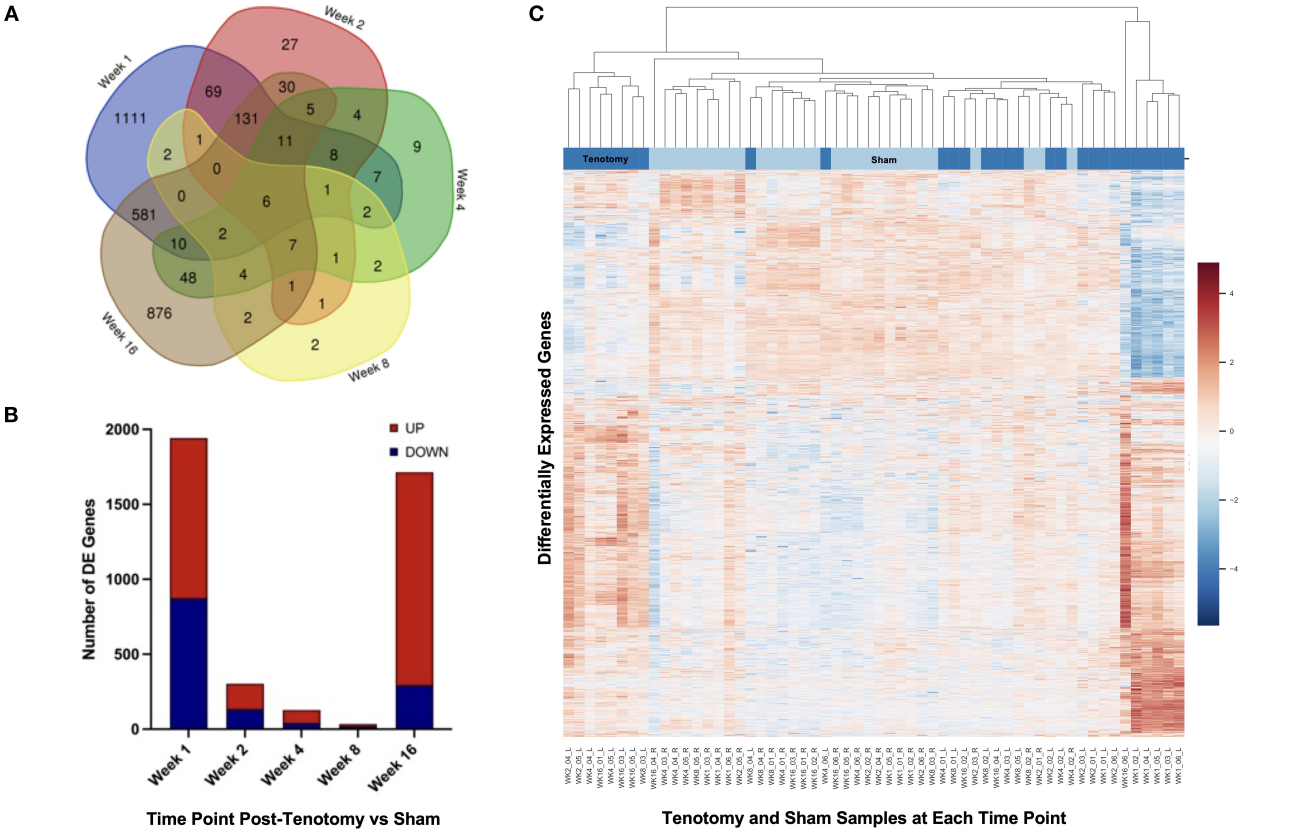

FIGURE 3 | Distribution of samples by tenotomy vs sham and DE genes over each time point post-tenotomy. Venn diagram **(A)** highlights the DE genes at each time point and the overlap with other time points. The bar chart **(B)** displays the number of DE genes which are up or down regulated at each timepoint. Data in the heatmap **(C)** is presented as normalized expression for each tenotomy and sham sample at each time point post-tenotomy with a z-score scale by rows and an average hierarchical clustering by columns.

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REVIEW
published: 24 August 2021
doi: 10.3389/fphys.2021.691954

Engineering Musculoskeletal Grafts for Multi-Tissue Unit Repair: Lessons From Developmental Biology and Wound Healing

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Edited by:
Rita de Cassia Marqueti, University of Brasilia, Brazil

Reviewed by:
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Specialty section:
This article was submitted to Striated Muscle Physiology, a section of the journal Frontiers in Physiology

Received: 07 April 2021
Accepted: 22 July 2021
Published: 24 August 2021

Citation:
Zhang X, Wang D, Mak K-LK, Tuan RS and Ker DFE (2021) Engineering Musculoskeletal Grafts for Multi-Tissue Unit Repair: Lessons From Developmental Biology and Wound Healing. Front. Physiol. 12:691954. doi: 10.3389/fphys.2021.691954

In the musculoskeletal system, bone, tendon, and skeletal muscle integrate and act coordinately as a single multi-tissue unit to facilitate body movement. The development, integration, and maturation of these essential components and their response to injury are vital for conferring efficient locomotion. The highly integrated nature of these components is evident under disease conditions, where rotator cuff tears at the bone-tendon interface have been reported to be associated with distal pathological alterations such as skeletal muscle degeneration and bone loss. To successfully treat musculoskeletal injuries and diseases, it is important to gain deep understanding of the development, integration and maturation of these musculoskeletal tissues along with their interfaces as well as the impact of inflammation on musculoskeletal healing and graft integration. This review highlights the current knowledge of developmental biology and wound healing in the bone-tendon-muscle multi-tissue unit and perspectives of what can be learnt from these biological and pathological processes within the context of musculoskeletal tissue engineering and regenerative medicine. Integrating these knowledge and perspectives can serve as guiding principles to inform the development and engineering of musculoskeletal grafts and other tissue engineering strategies to address challenging musculoskeletal injuries and diseases.

Keywords: bone, tendon, muscle, multi-tissue units, musculoskeletal developmental biology, wound healing, musculoskeletal tissue engineering, inflammation

INTRODUCTION

Bone, tendon, and skeletal muscle are essential components of the musculoskeletal system whose development, integration, and response to injury are vital for conferring efficient body movement. Specifically, joint motion occurs as a result of bone, tendon, and skeletal muscle coordinately acting as a single multi-tissue unit, muscle-generated contractile force is transmitted to compliant tendons, which efficiently stores and subsequently releases elastic strain energy

to result in bone movement. During development, these tissues are specified in a concerted manner, resulting in a highly integrated unit that further matures due to post-natal mechanical loading. This highly integrated nature is evident when injury is sustained. For example, in rotator cuff tears, although the bone-tendon interface or enthesis is often the site of injury, distal pathological alterations such as skeletal muscle degeneration in the form of fibrosis and fatty degeneration (Gilbert et al., 2018) as well as bone loss (Galatz et al., 2005) have been reported. Inflammation is a crucial factor that determines the outcome of tissue healing as well as the response to medical devices such as grafts. As such, knowledge gleaned from developmental biology and wound healing can serve as guiding principles to inform the development and engineering of musculoskeletal grafts.

Musculoskeletal tissue engineering aims to apply combinations of cells, signaling molecules including growth factors and inflammation-modulating factors, and biomaterials to generate mechanically-robust and/or bioactive grafts/scaffolds for treatment of injured or diseased tissues. Within the context of this review, we define a biomaterial as the base material or substance from which a scaffold or graft is fabricated. This means that a scaffold or graft is the engineered form of a biomaterial with the term "scaffold" being used in pre-clinical studies and "graft" being used in clinical settings. To appropriately utilize musculoskeletal development and wound healing knowledge and concepts, it is vital to recognize that the bone-tendon-muscle unit is a single functioning entity for locomotion (Ker et al., 2011a,b; Wang et al., 2021b). Therefore, understanding how these musculoskeletal tissues along with their interfaces develop, integrate, and mature (section "Brief Overview of Bone, Tendon, Muscle Development, Integration, and Maturation") as well as the impact of inflammation on musculoskeletal healing and graft integration (section "Musculoskeletal Tissue Healing and Graft Integration") is crucial. In addition, we present our perspective of what can be learnt from these biological processes within the context of musculoskeletal tissue engineering and regenerative medicine (section "Perspective: What Can We Learn From Developmental Biology and Wound Healing for Musculoskeletal Tissue Engineering") as well as potential treatment strategies (section "Outlook"). Integrating such knowledge will facilitate the development of rational and informed tissue engineering strategies for addressing challenging musculoskeletal injuries and diseases.

BRIEF OVERVIEW OF BONE, TENDON, MUSCLE DEVELOPMENT, INTEGRATION, AND MATURATION

Musculoskeletal Tissue Development and Integration

During embryonic development, the mesoderm is the prime contributor to musculoskeletal formation. The paraxial and lateral plate mesoderm, contribute toward somite and limb bud formation, respectively, which in turn are responsible for musculoskeletal formation in the trunk (primarily somitic in origin) and appendicular skeleton (primarily limb bud in origin; **Figure 1**; Cserjesi et al., 1995; Schweitzer et al., 2001; Wolpert et al., 2002; Mitchell and Sharma, 2005; Thomopoulos et al., 2010; Berendsen and Olsen, 2015; Endo, 2015; Jensen et al., 2018). The details for embryonic development of musculoskeletal tissue are comprehensively described in other excellent book chapters and reviews (Walker, 1991; Wolpert et al., 2002; Mitchell and Sharma, 2005; Schweitzer et al., 2010; Huang, 2017).

During trunk musculoskeletal development, somites are initially comprised of sclerotome and dermomyotome regions only but subsequently subdivide into the sclerotome, syndetome, myotome, and dermatome, which contribute toward bone, tendon, muscle, and skin elements, respectively (Cserjesi et al., 1995; Schweitzer et al., 2001; Wolpert et al., 2002; Mitchell and Sharma, 2005; Thomopoulos et al., 2010; Berendsen and Olsen, 2015; Endo, 2015; Jensen et al., 2018). Patterning signals including Sonic hedgehog (SHH; Fan and Tessier-Lavigne, 1994; Bumcrot and McMahon, 1995; Musumeci et al., 2015), Noggin (Berendsen and Olsen, 2015; Musumeci et al., 2015), Wingless/Integrated (Wnt), Neutrophin, and Fibroblast growth factor (FGF) as well as transcription factors including paraxis, paired box protein (PAX)-1, and PAX-9 are crucial to somite development (Love and Tuan, 1993; Smith and Tuan, 1995; Barnes et al., 1996a,b, 1997; LeClair et al., 1999; Alexander and Tuan, 2010). Within the sclerotome, cells form mesenchymal condensations that contribute to skeletal formation indirectly (*via* cartilaginous endochondral bone formation) or directly (*via* intramembranous bone formation; Berendsen and Olsen, 2015). Disrupting *Pax1* expression using antisense oligonucleotides or valproic acid disrupts sclerotomal differentiation to cause skeletal defects (Love and Tuan, 1993; Smith and Tuan, 1995; Barnes et al., 1996a,b, 1997; LeClair et al., 1999; Alexander and Tuan, 2010). As somite development progresses, a group of cells migrate away from the dorso-lateral edges of the dermomyotome toward the original boundary of the sclerotome and dermomyotome (Musumeci et al., 2015). This action segregates the dermomyotome into myotome and dermatome regions, and cells within this region undergo rapid muscle differentiation (Musumeci et al., 2015). Concurrently, muscle progenitor cells also undergo long-range migration to the limb buds, where they contribute toward appendicular muscle formation (Cossu et al., 1996; Musumeci et al., 2015). As the somite matures, a new domain termed the syndetome forms at the interface of sclerotome and myotome. Within the syndetome, tendon specification is initiated with the expression of scleraxis (*Scx*), a basic helix–loop–helix transcription factor that marks a tendon/ligament progenitor population and is subsequently present in terminally-differentiated tendon/ligament cells (Cserjesi et al., 1995; Brent et al., 2003). SCX is also known to upregulate tenomodulin (TNMD), a transmembrane protein found in tenocytes (Shukunami et al., 2006) as well as collagen type I, a main component of the tendon extracellular matrix (ECM; Lejard et al., 2007). Interactions among the abutting musculoskeletal compartments are crucial for further tissue development with transforming growth factor (TGF) and FGF signals from these regions

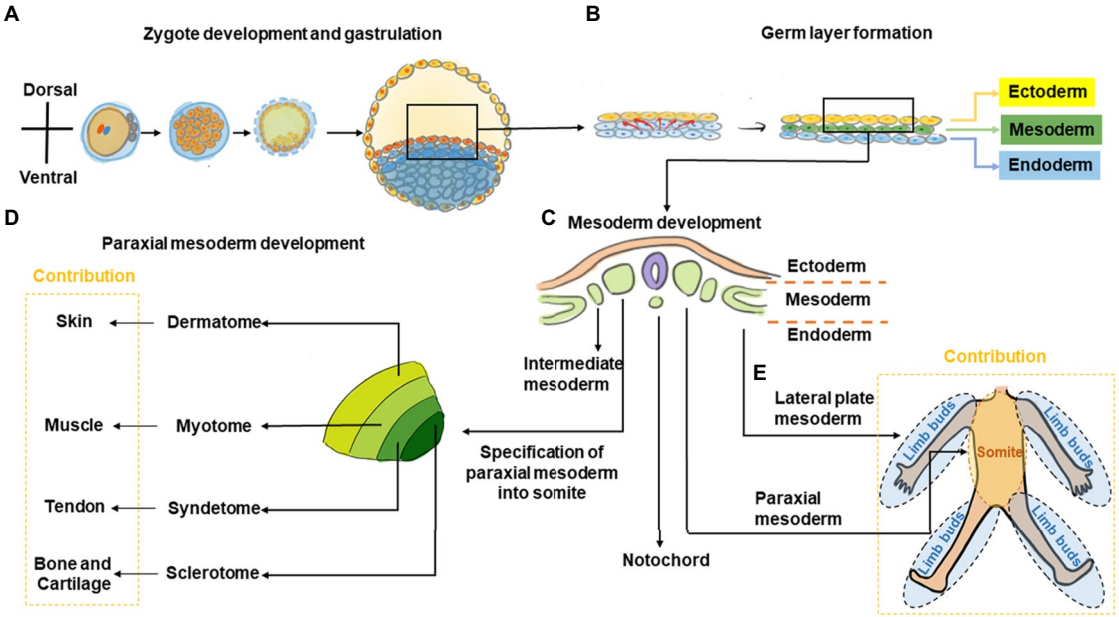

FIGURE 1 | Graphic overview of musculoskeletal development in the trunk and limbs. **(A)** The fertilized zygote transitions through several embryonic structures before forming the blastula, which contains the two-layered embryonic or germ disk consisting of epiblast and hypoblast. **(B)** As development proceeds, a tri-laminar gastrula structure consisting of ectoderm, mesoderm, and endoderm layers is formed. **(C)** Further during gastrulation, the mesoderm subdivides into the paraxial, intermediate, and lateral plate mesoderm. **(D)** Subsequently, somites are formed from paraxial mesoderm. The somite further subdivides into the sclerotome, syndetome, myotome, and dermatome regions, which contribute toward cartilage and bone, tendon, muscle, and skin elements, respectively. **(E)** The lateral plate mesoderm gives rise to the limb bud, which forms the cartilage/bone and tendon elements of the appendicular skeleton, while migrating myoblasts from the myotome contribute toward muscle elements.

regulating *Scx* expression and musculoskeletal integration (Brent and Tabin, 2004; Brent et al., 2005; Pryce et al., 2009). For example, mouse embryos with null mutations in Myogenic factor-5 (*Myf5*) and Myogenic differentiation 1 (*MyoD*) are devoid of skeletal muscle and their absence results in disruption of tendon development (Brent et al., 2005).

During development of the appendicular musculature and skeleton, the lateral plate mesoderm forms the limb bud, which contributes to bone and tendon elements, while migrating muscle cells originating from the myotome contribute to muscle elements (Cserjesi et al., 1995; Schweitzer et al., 2001; Wolpert et al., 2002; Mitchell and Sharma, 2005; Thomopoulos et al., 2010; Berendsen and Olsen, 2015; Endo, 2015; Jensen et al., 2018). Homeobox (*Hox*) transcription factor genes are important to this process and contribute toward patterning in the developing limb to define the stylopod (*Hox10*), zeugopod (*Hox11*), and autopod (*Hox13*) structures, which eventually give rise to the humerus/femur, radius and ulna/tibia and fibula, and hand/foot bones, respectively (Pineault and Wellik, 2014). Similar to its role in trunk skeletogenesis, *Pax1* is also involved in formation of limb skeletal elements where it is initially expressed in the anterior proximal margin of limb buds (LeClair et al., 1999). Skeletal formation of the limb is subsequently orchestrated Sex determining region Y (SRY)-box 9/Sox9-positive mesenchymal cartilage progenitors that form condensations within the limb bud and undergo sequential endochondral ossification to form bone (Pineault and Wellik, 2014). As previously mentioned, limb skeletal muscle originates from cells within the somites, which then migrate to the developing limb bud (Cossu et al., 1996; Musumeci et al., 2015). These progenitor cells delaminate from the dorso-lateral region of the developing dermomyotome, become transiently inhibited from differentiating by expression of *Pax3* (Relaix et al., 2005), and subsequently express Tyrosine protein kinase met (*C-Met*; Cossu et al., 1996), a Hepatocyte growth factor (HGF) receptor. When *HGF* is expressed by mesenchymal cells of the limb bud, these muscle progenitors migrate toward these regions (Musumeci et al., 2015) and subsequently differentiate into muscle (Chevallier et al., 1977). Tendon progenitors arise from the lateral plate mesoderm and its specification is independent of muscle (Kieny and Chevallier, 1979). These tendon progenitors express *Scx*, and its protein levels are negatively- and positively-regulated by bone morphogenetic protein (BMP) and Noggin signaling, respectively (Schweitzer et al., 2001). Further musculoskeletal development of these tissues is interdependent. For example, removal of tendon primordia disrupts limb muscle formation (Kardon, 1998), whereas loss of limb muscle only results in transient tendon formation (Kieny and Chevallier, 1979). Other studies have also shown that the tendon/ligament marker *Scx* is crucial for musculoskeletal integration (Yoshimoto et al., 2017). Indeed, a population of cells positive for both *Scx* and *Sox9* are found at the interface between tendon and as-yet unmineralized bone, which contributes to eventual formation of the bone-tendon interface (Blitz et al., 2013; Sugimoto et al., 2013). For example,

Scx has been shown to regulate *Bmp4* expression in tendon cells and this in turn subsequently directs formation of bone ridges at the deltoid tuberosity of humeral bone, which provides a stable anchoring point and stress dissipation for musculoskeletal tissue attachment (Blitz et al., 2009).

Thus, bone and tendon formation in the limb is derived from lateral plate mesoderm, whereas muscle formation originates from migrating precursors of paraxial mesoderm-derived somite.

Role of Mechanical Forces in Musculoskeletal Tissue Maturation

In addition to the specification, development, and integration of bone, tendon, and muscle, maturation of these musculoskeletal tissues occurs both *in utero* and postnatally. For obvious reasons, mechanical forces play a substantial role in muscle, tendon, and bone maturation. While this topic is broad, excellent reviews of this subject include those by Felsenthal and Zelzer (Felsenthal and Zelzer, 2017; mechanical forces during musculoskeletal development), Mammoto et al. (Mammoto et al., 2012), Jansen et al. (Jansen et al., 2015; mechanosensitive mechanisms), Geoghegan et al. (Geoghegan et al., 2019; bone mechanobiology), and Lavagnino et al. (Lavagnino et al., 2015; tendon mechanobiology), as well as Fischer et al. (Fischer et al., 2016) and Schiaffino et al. (Schiaffino et al., 2013; muscle mechanobiology; **Figure 2**).

One area of rapid advancement is the Hippo network, a highly conserved pathway associated with organ size regulation, mediated in part, by two transcriptional co-activators – Yes-associated protein (YAP) and Transcriptional co-activator with PDZ-binding motif (TAZ). YAP/TAZ activity is defined by its intracellular locations – nuclear YAP/TAZ is active as exemplified by culturing cells on a stiff substrate, which often results in a spread cell morphology; cytoplasmic YAP/TAZ is inactive as exemplified by culturing cells on a soft substrate, which often results in a rounded cell morphology. At the cellular level, musculoskeletal actors including osteoblasts, osteocytes, tenocytes, myocytes, and various progenitors are highly sensitive to mechanical forces, contributing toward the overall and final properties of the tissue. However, details of the relevant intracellular signaling mechanisms that transduce mechanical cues remain largely unclear. Recent studies have shown that YAP and TAZ are regulated by ECM elasticity and cell geometry (Dupont et al., 2011; Wada et al., 2011). Crucially, the control of YAP/TAZ subcellular localization by mechanical cues is highly conserved among different cell types such as adult tissue-derived mesenchymal stem cells (MSCs), which contribute toward musculoskeletal differentiation and tissue homeostasis. Indeed, the shifting of MSC differentiation from osteogenic (rigid substrate) to adipogenic (soft substrate) lineage is governed by YAP/TAZ activity (Dupont et al., 2011; Seo et al., 2013). Also, there is increasing evidence that YAP/TAZ signaling is implicated in multiple events of the musculoskeletal system, including the regulation of endochondral bone ossification and fracture healing (Deng et al., 2016; Li et al., 2018), articular cartilage maintenance (Deng et al., 2018b), collagen secretion from tendon fibroblasts (Yeung et al., 2015), and promotion of muscle growth (Fischer et al., 2016; Huraskin et al., 2016; Stearns-Reider et al., 2017). Future investigations are expected to elucidate how YAP/TAZ regulates and transduces various mechanical stimuli into intracellular signaling in these musculoskeletal cell types.

Like their tissue counterparts, the interfaces of musculoskeletal tissues are mechanosensitive and remain highly dynamic throughout life. For example, bone-tendon/bone-ligament interfaces, such as the medial collateral ligament and periodontal ligament, migrate with skeletal growth (Wang et al., 2013, 2014) and biomechanical loading (Lin et al., 2017), respectively, while the architecture and composition of muscle-tendon interfaces are highly responsive to exercise-induced loading (Jakobsen et al., 2017).

At the bone-tendon/bone-ligament interface or enthesis, four classically-distinct regions are defined – bone, mineralized fibrocartilage, unmineralized fibrocartilage, and tendon regions (Yang and Temenoff, 2009). Recent work has shown a greater degree of nuance, with a gradual transition in both mineral accumulation (Moffat et al., 2008; Genin et al., 2009; Schwartz et al., 2012) and organization of collagen fibers (Genin et al., 2009; Rossetti et al., 2017) observed across this interface. Together, these interfacial features not only mediate attachment of flexible tendon to rigid bone but also act to minimize the risk of tissue rupture and detachment by reducing stress concentrations. Growth factors and ECM components associated with the development and maturation of the humeral bone-supraspinatus tendon interface include TGF-β s, such as TGF-β1 and TGF-β3, as well as various fibrous collagen types, such as collagen types I, II, and X (Galatz et al., 2007).

Postnatal development of this interface is initiated at the tendon-epiphyseal cartilage/unmineralized bone interface by Hedgehog-responsive cells expressing the transcription factor Gli1 (Schwartz et al., 2015). For some entheses, these cells are synonymous with a *Scx*- and *Sox9*-positive embryonic cell population identified in prior studies (Blitz et al., 2013; Sugimoto et al., 2013; Felsenthal et al., 2018), and eventually divide into two subpopulations – one of which contributes toward formation of mineralized fibrocartilage and eventually becomes non-responsive to Hedgehog signaling, while the other contributes toward formation of unmineralized fibrocartilage and retains its responsiveness to Hedgehog signaling (Schwartz et al., 2015).

Muscle loading plays a crucial role in enthesis development, as botulinum toxin-induced muscle paralysis can alter Hedgehog signaling and disrupt enthesis maturation as well as biomechanical properties (Derwin et al., 2006; Deymier et al., 2019). Muscle unloading for 21 days affected both structure and biomechanical properties of musculoskeletal tissue units at multiple hierarchical levels (Deymier et al., 2019). At the macro scale, supraspinatus tendon tensile strength and bone-tendon interfacial toughness were decreased by 36.6–66.7% and 70.0–74.3%, respectively, whereas ultimate load and stiffness remained unaffected in muscle-unloaded samples (Deymier et al., 2019). At the nanometer scale, the level of bioapatite carbonate across the unloaded bone-tendon interface was decreased by 6.25%, which when coupled with changes in bioapatite crystal orientation led to decreased total energy dissipation (Deymier et al., 2019). As such, the resulting loss of organization in unloaded musculoskeletal tissue units across multiple length scales

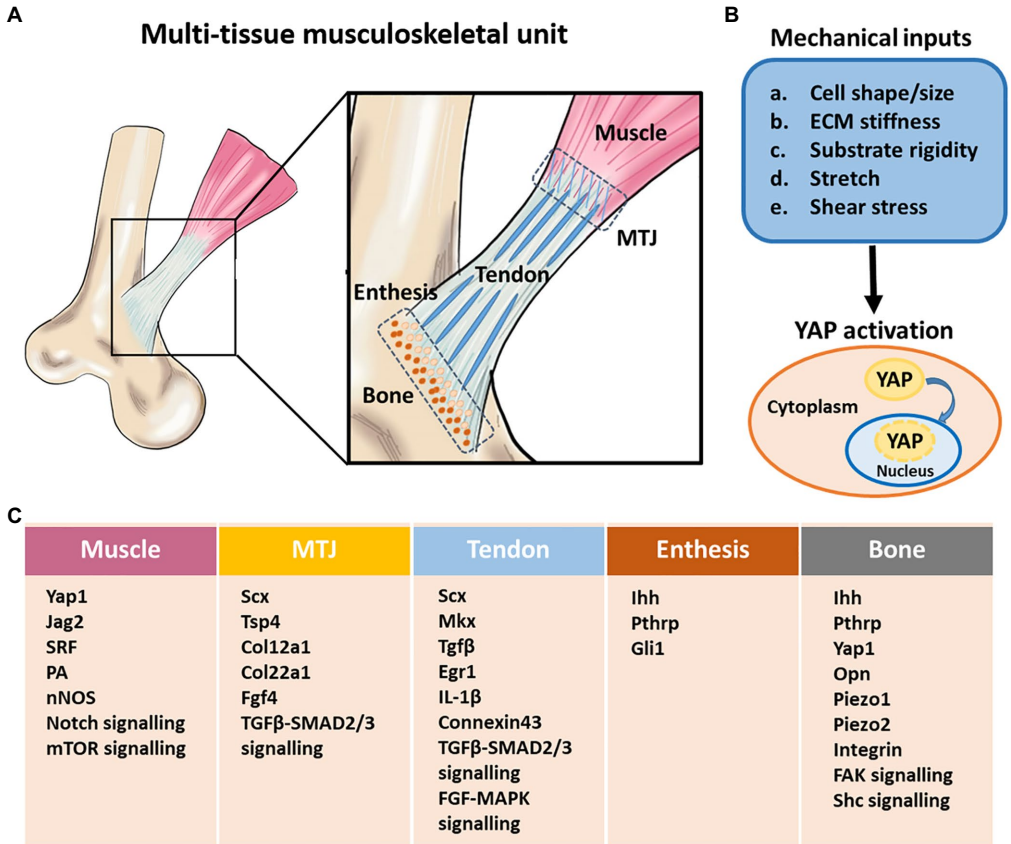

FIGURE 2 | Structural components, and mechanobiological response and gene regulation in the multi-tissue musculoskeletal unit. **(A)** A schematic illustration of multi-tissue musculoskeletal unit containing bone, tendon, muscle, bone-tendon enthesis, and myotendinous junction (MTJ). The proper formation of tendon, muscle, and the attachment between them requires mechanical load. **(B)** Different mechanical inputs regulate Yes-associated protein (YAP) activity. YAP is localized to the nucleus and active under mechanical conditions that lead to high intracellular tension. **(C)** Mechanically regulated key signaling pathways, factors and genes involved in musculoskeletal system development. MTJ, myotendinous junction. Adapted with permission from Felsenthal and Zelzer (2017).

increased injury risk *via* a decreased ability to absorb energy prior to material failure (Deymier et al., 2019).

When fully-formed, bone-tendon/bone-ligament interfaces may be "stationary," whereas others such as those found in the medial collateral ligament migrate with skeletal growth (Wang et al., 2013, 2014). During this process, load-induced Parathyroid hormone-related protein (PTHRP) causes bone resorption above the interface to form an unmineralized tract (Wang et al., 2013, 2014). This unmineralized region specifies the future destination of the migrating interface, and migration to this site occurs as a result of coupled osteoclast- and osteoblast-driven bone resorption and formation, respectively (Wang et al., 2013, 2014). Together, these studies highlight the dynamic nature of bone-tendon/bone-ligament interface.

At the muscle-tendon interface, three distinct regions, tendon, myotendinous junction (MTJ), and muscle, are formed (Yang and Temenoff, 2009). Together, these interfacial features not only mediate attachment of flexible tendon to the contractile force generation apparatus in skeletal muscle but also act to minimize the risk of tissue rupture and detachment by reducing stress concentrations.

Unlike the bone-tendon interface, these distinct regions are already formed during embryonic development and have already undergone *in utero* maturation to some extent. For example, the myotendinous interface of zebrafish embryos remodels from an ECM milieu that is originally rich in fibronectin to one that is laminin-rich (Jenkins et al., 2016). Laminins are crucial in this respect as they play important roles in both skeletal muscle integrity and force transmission (Holmberg and Durbeej, 2013). This is important as *in utero* muscle loading, such as kicking movements, are vital to regulation of musculoskeletal tissue mechanical properties, including tendon (Pan et al., 2018).

During postnatal maturation of the muscle-tendon interface, an increase in tissue complexity is observed (Nagano et al., 1998). For example, the ends of skeletal muscle fibers in Chinese hamsters undergo morphogenesis from relatively simple cone-shaped structures into complex structures with an increased number of finger-like projections and clefts. Such dynamic changes are likely a response to increased muscle loading and are echoed in studies, where different exercise regimens result in the remodeling of muscle-tendon organization and structure (Curzi et al., 2016; Rissatto Sierra et al., 2018).

In summary, both embryonic and post-natal development contribute toward the specification, maturation, and integration of bone-tendon-muscle units. Embryonic processes are primarily responsible for initiating cell differentiation and early multi-tissue patterning, whereas post-natal processes, under the influence of biomechanical loading, guide and regulate the maturation and remodeling of musculoskeletal interfaces.

MUSCULOSKELETAL TISSUE HEALING AND GRAFT INTEGRATION

General Overview of Wound Healing

To effectively integrate engineered musculoskeletal grafts, it is important to understand the role of wound healing. In general, wound healing is comprised of four sequential but overlapping stages – hemostasis, inflammation, proliferation, and maturation (Marieb, 1999).

Briefly, wound healing first commences with hemostasis, which initiates inflammation, recruits cells, and mediates wound closure. During this phase, platelet activation and degranulation results in: (1) the initiation of coagulation *via* increased expression of cell surface protein αIIb/β3 (which exhibits high affinity for fibrinogen to form the provisional ECM) as well as the secretion of adenosine diphosphate (ADP) and von Willebrand factor, which aid in platelet adhesion and aggregation; (2) the release of a multitude of growth factors that orchestrate inflammation and recruit cells including but not limited to fibroblasts, immune cells such as macrophages, and stem cells, which participate in wound healing and regeneration (Marieb, 1999; Aurora and Olson, 2014; Eming et al., 2014; Manning et al., 2014). Second, inflammation establishes the initial wound microenvironment by removing both pathogens and damaged cells (Marieb, 1999; Aurora and Olson, 2014; Eming et al., 2014; Manning et al., 2014), as well as *via* the secretion of inflammatory mediators that attract stem and progenitor cells for subsequent tissue repair (Rustad and Gurtner, 2012). Third, proliferation regenerates tissue-resident cells *via* granulation tissue formation and myofibroblast-mediated wound contraction (Marieb, 1999; Aurora and Olson, 2014; Eming et al., 2014; Manning et al., 2014). Fourth, tissue remodeling removes transient cells and ECM, reorganizing and closing the wound to re-establish native tissue (Marieb, 1999; Aurora and Olson, 2014; Eming et al., 2014; Manning et al., 2014). However, if the injury is severe or chronic, such as in the case of tendinopathy, healing may be incomplete with the formation of mechanically-weaker scar tissues as well as the persistence of aberrant inflammatory conditions (Marieb, 1999; Aurora and Olson, 2014; Eming et al., 2014; Manning et al., 2014). Thus, wound healing is a crucial consideration for simultaneous engineering and integration of multiple musculoskeletal tissue grafts.

Importance of Inflammation in Tendon, Skeletal Muscle, and Bone Healing

Given its aforementioned role in establishing the initial wound microenvironment, inflammation has emerged as a crucial consideration for musculoskeletal regeneration. For example, inflammatory cells such as macrophages were increased at the muscle-tendon interface following exercise (Jakobsen et al., 2017). Presumably, these macrophages are responsible for orchestrating tissue repair and adaptation as part of the body's homeostatic program. Indeed, macrophages have been reported to organize the wound microenvironment for regenerating entire limbs in model organisms such as the adult salamander (Godwin et al., 2013).

With respect to tendon injury, many inflammation-associated mediators and cell types have been identified. These include Interleukins (ILs; IL-1β, IL-6, IL-10, IL-17A, IL-21, and IL-33), Tumor necrosis factor-alpha (TNF-α), Substance P, and alarmin molecules, as well as neutrophils, macrophages, mast cells, lymphocytes, fibroblasts, tenocytes, and stem/progenitor cells (Jensen et al., 2018; Tang et al., 2018; Vinhas et al., 2018). While the precise roles and contributions of these signaling molecules and immunocytes remain unclear and are actively being studied (Tang et al., 2018), it is evident that an unbalanced inflammatory response is detrimental, and is associated with rotator cuff overuse injuries (Perry et al., 2005) and tears (Yadav et al., 2009). It is also worth noting that the majority of rotator cuff tears involve tendinopathic changes. For example, pro-inflammatory-associated IL-1β treatment has been shown to increase expression of ECM-destructive enzymes including matrix metalloproteinases (MMPs) such as MMP-1, MMP-3, MMP-13 as well as aggrecanase-1 in human tenocytes (Tsuzaki et al., 2003), which positively correlate with increased rotator cuff tear size and severity (Shindle et al., 2011). However, this does not imply that direct inhibition of pro-inflammatory responses will thus lead to improved tendon healing. For example, non-steroidal anti-inflammatory drugs, including indomethacin and celecoxib, are often prescribed after orthopaedic procedures to alleviate pain. In an acute rat rotator cuff injury study, depressing the inflammatory response using indomethacin or celecoxib also retarded tendon healing with decreased collagen organization and inferior biomechanical properties (Cohen et al., 2006).

Both classically pro- and anti-inflammatory responses are necessary for tendon healing. Following tendon tissue damage, alarmin molecules such as High-mobility group box 1 (HMGB1) along with other pro-inflammatory molecules, including caspase-1, IL-1β, receptor for advanced glycation end products, Toll-like receptors (TLRs) such as TLR-2 and TLR-4, and triggering receptor expressed on myeloid cells were upregulated at early time points (1–2 weeks) and subsequently declined (3–4 weeks), which correlated well with the healing response (Thankam et al., 2018). Although, the secretion of pro-inflammatory molecules such as IL-1β is associated with ECM-degradation, such effects are presumed to be beneficial for tendon remodeling and repair when induced at moderate levels (Yang et al., 2005). Stromal cell-derived factor-1, an associated downstream mediator of pro-inflammatory IL-1β (Akbar et al., 2017), then mediates the next phase of wound healing by facilitating infiltration of pro-regenerative M2 macrophages and bone marrow-derived stem cells into supraspinatus muscle (Tellier et al., 2018). At later stages of

tendon healing, anti-inflammatory-associated IL-10 is expressed (Ricchetti et al., 2008; Sugg et al., 2014) and promotes proliferation and migration of tendon-derived stem cells concurrently with inhibition of tenocyte differentiation (Deng et al., 2018a). Taken together, these studies demonstrate that a delicate balance between tissue-destructive pro-inflammatory and tissue-reconstructive anti-inflammatory responses is crucial for chemotaxis and expansion of musculoskeletal stem/progenitor cells into physiologically relevant numbers sufficient for effecting tendon modeling and repair (**Figure 3**). When this delicate balance is disrupted, such as in the case of persistent inflammation, chronic ECM degradation leads to disorganized tendon ECM organization and results in mechanically weak scar tissue formation.

Similar to tendon, inflammation also has catabolic and anabolic effects on skeletal muscle and bone. An over-exuberant pro-inflammatory response induces bone resorption (Goldring, 2015) and muscle wasting (Londhe and Guttridge, 2015), which are linked to the pathophysiology of joint diseases, including rheumatoid arthritis and seronegative spondyloarthropathies (Goldring, 2015; Londhe and Guttridge, 2015). Similar inflammatory mediators, including TNF-α, IL-1β, IL-4, and IL-10, as well as cell types, including macrophages, T cells, B cells, fibroblasts, osteoblasts, osteoclasts, myocytes, and musculoskeletal stem/progenitor cells, are involved (Goldring, 2015; Londhe and Guttridge, 2015; Turner et al., 2018; **Figure 3**).

As such, inflammation-associated catabolic and anabolic events can affect patients afflicted by musculoskeletal disease or injury. In rheumatoid arthritis, joint-resident synovial fibroblasts, by virtue of their immune-associated cell receptors and chemokine secretion, can both respond to and promote inflammation (Turner et al., 2018). This response allows them to modulate leukocyte infiltration as well as the activity of bone-building osteoblasts and bone-resorbing osteoclasts, respectively, to perturb bone remodeling (Turner et al., 2018). This is important in the context of rotator cuff repair, since the number of shoulder arthroplasty patients with rheumatoid arthritis is increasing (Leroux et al., 2018), and increased rotator cuff tear severity is positively correlated with increased synovium inflammation (Shindle et al., 2011). Similarly, in rotator cuff muscles, increased macrophage density (10–100-fold relative to healthy tissue) has been observed (Gibbons et al., 2017). Presumably, these increased macrophage numbers contribute toward fatty atrophy and fibrosis of skeletal muscle tissue *via*

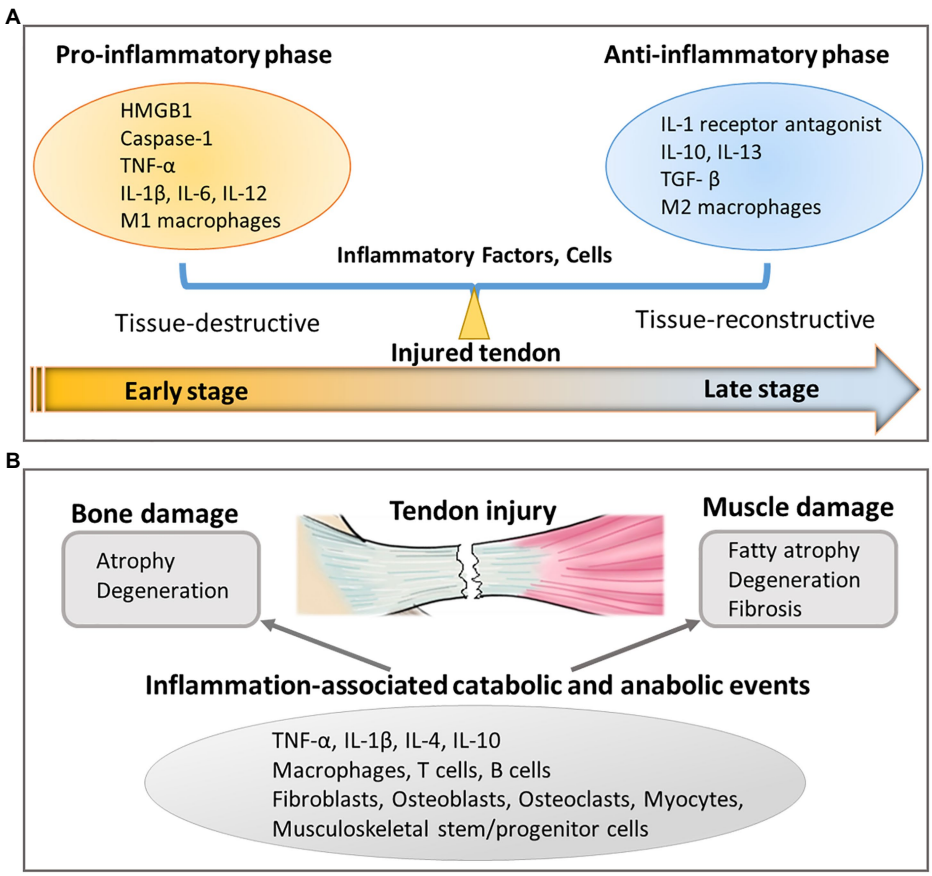

FIGURE 3 | Inflammation-related responses resulting from tendon injury. **(A)** Unbalanced pro-inflammatory and anti-inflammatory response in injured tendon affects tendon healing by secreting inflammatory factors and cells. **(B)** Tendon injury also leads to bone and muscle damage through inflammation-associated catabolic and anabolic events.

Ras homolog gene family member A (RhoA) signaling (Davies et al., 2017). Rebalancing pro- and anti-inflammatory responses *via* application of anti-inflammatory drugs such as Licofelone (Oak et al., 2014) and Simvastatin (Davis et al., 2015) decreased muscle fibrosis. Thus, a crucial balance between pro- and anti-inflammatory is also necessary to prevent atrophy/degeneration/retraction of skeletal muscle and bone components of a musculoskeletal unit.

Importance of Inflammation in Integration of Clinical Grafts and Devices

In addition to their anabolic and catabolic effects on musculoskeletal tissues, inflammation also plays a pivotal role in considering the efficacy of medical devices such as sutures, suture anchors, and grafts. Such inflammation-associated responses share similarities with but are ultimately distinct from those involved in disease progression or normal wound healing. For example, in an acute rabbit supraspinatus tendon resection model, non-absorbable sutures induced higher levels of inflammation relative to absorbable and partially-absorbable sutures (Su et al., 2018). This increased pro-inflammatory response was associated with poorer biomechanical properties and incomplete bone-tendon healing (Su et al., 2018). Similarly, severe pro-inflammatory responses associated with either tendon grafts (Walton et al., 2007) or suture anchors (Barber, 2007; Cobaleda Aristizabal et al., 2014; Joo Han et al., 2015) have also led to failed rotator cuff repairs, requiring patients to undergo revision surgeries.

For these circumstances, it is challenging to ascertain a single underlying cause of failure. However, an important consideration is the non-autogenic nature of these clinical devices (Barber, 2007; Walton et al., 2007; Cobaleda Aristizabal et al., 2014; Joo Han et al., 2015; Su et al., 2018), which can induce different levels of inflammation. Indeed, xenogenic grafts induced an adverse pro-inflammatory response (Walton et al., 2007), whereas allogenic grafts had minimal levels of inflammation (Snyder et al., 2009). In addition, scaffolds and clinical grafts can also be broadly divided into biological, synthetic, or biosynthetic (hybrid of biological and synthetic) in origin. Typically, synthetic scaffolds are favored for their ease of production and reproducible attributes such as mechanical properties, which is important for facilitating joint locomotion, but have traditionally been considered as strong inducers of chronic inflammation and the foreign body response relative to their biological counterparts (Crupi et al., 2015; Saleh and Bryant, 2018; Zhou and Groth, 2018; Sadtler et al., 2019). Indeed, in a muscle wound environment, synthetic materials (polyethylene and polyethylene glycol) elicited a type-1-like immune response with recruitment of a high proportion of neutrophils and CD86-positive pro-inflammatory macrophages, which was attributed to biomaterial stiffness, whereas a biological material (porcine urinary bladder ECM), elicited a type-2-like immune response with upregulation of *Il4*, *Cd163*, *Mrc1*, and *Chil3* genes, which was attributed to damage-associated molecular patterns signaling (Sadtler et al., 2019). Similarly, in a bone wound environment, inflammatory mediators such as TLR-4 have been shown to play vital roles in mediating calvarial bone healing as well as bone graft-mediated calvarial repair (Wang et al., 2015, 2017).

The magnitude of inflammation and the ensuing foreign body reaction are highly dependent upon immune cell-biomaterial interactions which occur at the tissue/material interface (Anderson et al., 2008). Following implantation of a clinical device, plasma proteins are adsorbed, or opsonized onto the biomaterial surface followed by complement activation, macrophage adhesion, and macrophage fusion into foreign body giant cells that mediate degradation (Anderson et al., 2008). If this opsonization process creates a detrimental or chronic wound microenvironment, device failure and impaired shoulder function will result (Walton et al., 2007), whereas if a microenvironment conducive to regeneration is presented, graft remodeling and successful integration with host tissue will ensue (Snyder et al., 2009). Contributing factors related to this include biomaterial stiffness, biomaterial surface chemistry, scaffold/graft topography, scaffold/graft degradation rate, and physico-chemical effect of degradation products, scaffold/graft porosity, and presence of inflammatory antigens such as α-galactosidase (Crupi et al., 2015; Zhou and Groth, 2018; Sadtler et al., 2019). This has led to the development of diverse anti-inflammatory strategies, which include use of scaffold topography such as grooves to reduce immune cell pro-inflammatory response, modification of biomaterial surfaces to present weakly inflammatory functional chemical groups such as hydroxyls and carboxylic acids, use of anti-fouling coatings, attachment of anti-inflammatory or immunomodulatory reagents, and removal of inflammatory antigens such as α-galactosidase *via* enzymatic treatment (Crupi et al., 2015; Zhou and Groth, 2018). Thus, these studies highlight the importance of biomaterial or scaffold/graft characteristics and their impact on inflammation, which ultimately determines successful engraftment of devices used in clinical repair.

Importance of Vascularization for Clinical Grafts and Devices

In tissue engineering, one major challenge for the long-term survival and function of clinical implants and devices is ensuring sufficient vascularization after implantation. There are two principal strategies are used – angiogenesis and inosculation. Angiogenesis focuses on stimulating the ingrowth of newly formed blood vessels into implanted constructs from the surrounding host tissue(s), whereas the inosculation requires a preformed microvascular networks generated within tissue constructs that subsequently develop interconnections with the host microvasculature (Laschke and Menger, 2012). Strategies to address this include modification of scaffolds/grafts to induce a mild proinflammatory response that may be beneficial to vascularization, incorporation of signaling molecules that promote angiogenesis or blood vessel maturation such as FGF-2, PDGF, VEGF, TGF-β, Angiopoietin-1, and Angiopoietin-2, and incorporation of vascular or proangiogenic cells. The details of the vascularization process as well as the principles and strategies are extensively described by Laschke and Menger (2012) and Herrmann et al. (2014).

In particular, angiogenesis can also be impacted by the magnitude of inflammation. In a recent study, mice were intraperitoneally administered with arthritic serum repeatedly, resulting in clinical manifestations of rheumatoid arthritis including prolonged systemic inflammation (Wang et al., 2021a). Following bone fracture injury, such mice exhibited fracture nonunion with reduced cartilaginous and bony callus formation, fibrotic scarring, and diminished angiogenesis (Wang et al., 2021a). Subsequent mechanistic studies showed that this reduced angiogenesis was attributed to downregulation of SPP1 and CXCL12 in chondrocytes (Wang et al., 2021a). By employing a biodegradable polycaprolactone scaffold loaded with pro-angiogenic SPP1 and CXCL12, improved angiogenesis and osteogenesis, as evidenced by increased blood vessel count, increased bone area and higher biomechanical properties (torque) were achieved (Wang et al., 2021a). In a similar vein, different scaffolds and grafts can present varying levels of angiogenesis and vascular ingrowth, which can be affected by the magnitude of inflammation. In a mouse subcutaneous implantation study, 3D-printed poly(L-lactide-co-glycolide) and 3D-printed collagen-chitosan-hydroxyapatite hydrogel scaffolds were directly compared (Rücker et al., 2006). Intravital fluorescence microscopy analyses, histological as well as immunohistochemical staining, and cytotoxicity assays showed that 3D-printed poly(L-lactide-co-glycolide) scaffolds induced a mild pro-inflammatory response (slight increase in leukocyte recruitment), which was associated with increased angiogenesis and good microvascular ingrowth 14 days post-implantation, whereas 3D-printed collagen-chitosan-hydroxyapatite hydrogel scaffolds induced a severe pro-inflammatory response (around 15-fold increase of leukocyte-endothelial cell interactions), which was associated with poor microvascular ingrowth to the scaffolds (Rücker et al., 2006). Thus, scaffold or graft neovascularization, which is crucial to the long-term survival and function of an implant is dependent on a moderate pro-inflammatory response.

In summary, both pro- and anti-inflammatory responses are essential for healing of musculoskeletal tissue units and contribute toward integration of medical devices including vascularization. Pro- and anti-inflammatory responses are primarily, although not exclusively, responsible for catabolic and anabolic tissue responses, respectively, and a coordinated as well as balanced inflammatory response is essential to successful biomaterial integration and tissue repair.

PERSPECTIVE: WHAT CAN WE LEARN FROM DEVELOPMENTAL BIOLOGY AND WOUND HEALING FOR MUSCULOSKELETAL TISSUE ENGINEERING

Applicability

Concepts and knowledge generated from developmental biology and wound healing studies can aid the development and assessment of musculoskeletal tissue engineering strategies. In other words, information gained from developmental biology can offer a "cheat sheet" for assessing post-natal musculoskeletal differentiation as well as inspire novel therapeutic strategies to regenerate and repair injured musculoskeletal tissues (Tuan, 2004; Huang et al., 2015). For bone, growth factors such as BMPs, Wnts, and Indian Hedgehog have been identified as bone-promoting cues, while collagen type I, Runt-related transcription factor 2 (RUNX2), BMP-2, and BMP-6 serve as useful ECM and transcription factor markers to assess osteoblast differentiation (Wang et al., 2020). For tendon, growth factors such as TGF-βs, FGF, BMPs, Wnts, and Connective tissue growth factor (CTGF) have been identified as tendon-promoting cues, while collagen types I and III, tenascin-C, TNMD, decorin, biglycan, SCX, mohawk (MKX), and Early growth response 1 (EGR1) serve as useful ECM and transcription factor markers to assess tenocyte differentiation (Huang, 2017; Liu et al., 2017). For skeletal muscle, growth factors such as insulin-like growth factor-1, HGF, FGF-2, and PDGF have been identified as skeletal muscle-promoting cues (Syverud et al., 2016), while collagen type I, laminins, myogenin, MYOD, MYF5 serve as useful ECM and transcription factor markers to assess myocyte differentiation (Holmberg and Durbeej, 2013; Syverud et al., 2016; Huang, 2017). In addition to identification of appropriate cues and markers, signaling pathways governing musculoskeletal tissue formation can be manipulated to develop novel therapies. For example, stable cartilage formation has been achieved using targeted chemical inhibition of the BMP pathway without affecting TGF-β signaling (Occhetta et al., 2018). Similarly, stable cartilage formation has also been observed *in vivo via* application of the Wnt/β-catenin inhibitor XAV939 to manipulate Wnt signaling, which is crucial in the development, growth, and maintenance of both cartilage and bone (Deng et al., 2019). These studies demonstrate a remarkable achievement as prior attempts at stable chondrocyte differentiation typically result in a transient chondrocyte phenotype that rapidly proceeds toward a hypertrophic and mineralizing fate, eventually forming unwanted bone (Correa et al., 2015). Thus, integrating knowledge gleaned from developmental biology studies into tissue engineering approaches can prove valuable.

Wound healing studies can model injury-relevant settings for understanding the pathophysiological process as well as evaluating novel therapeutic strategies. For understanding pathophysiological processes, various models have been utilized. In rats, the supraspinatus tendon passes under an enclosed arch similar to humans (Soslowsky et al., 1996). This similarity in shoulder anatomy makes the rat ideal for modeling rotator cuff diseases such as subacromial impingement, whereby friction between the rotator cuff tendons and the coracoacromial arch during muscular movement leads to pain and reduced range of joint motion. In rabbits, detachment of the rotator cuff tendon(s) resulted in prominent fatty degeneration of skeletal muscle (Abdou et al., 2019), and has been used to simulate chronic rotator cuff tears to study the effect of hypercholesterolemia on skeletal muscle fatty degeneration and bone-to-tendon healing. Indeed, use of such models identified αSMA-positive cells as primary contributors to scar formation (Moser et al., 2020), which may pave the way for identification of new therapeutic targets. For evaluating novel therapeutic strategies, similar animal

models have been used. For example, both rats and rabbits have been used to evaluate the effect of growth factors, such as FGF-2, Platelet-derived growth factor-BB (PDGF), and TGF-β1, on musculoskeletal tissue healing for rotator cuff repair (Tokunaga et al., 2015a,b, 2017; Arimura et al., 2017). Also, when coupled with appropriate outcome measurements, animal models can be used to understand why therapeutics may result in unfavorable outcomes. For example, biomechanical, biochemical, and proteomics studies showed that application of adipose-derived stromal cells with tenogenic BMP-12 in a fibrin-based scaffold for intrasynovial tendon repair amplified unfavorable responses including inflammation, stress response, and matrix degradation, leading to poor healing (Gelberman et al., 2016). Such studies highlight the need for avoidance of negative local reactions in cell- and growth factor-based therapies.

Thus, knowledge derived from developmental biology and wound healing studies can guide both the development and assessment of therapies for musculoskeletal tissue engineering and regenerative medicine by identifying musculoskeletal regenerative cues, differentiation markers, and potential therapeutic targets as well as facilitate evaluation of such therapies in injury-relevant models.

Limitations

Despite the tremendous advances and insights gleaned from developmental biology and wound healing studies, there are noteworthy limitations. Thus, great care must be taken in interpreting their results and integrating this information in translational medicine efforts for two reasons.

First, within the context of developmental studies, there are intrinsic differences between non-adult and adult musculoskeletal cells as well as their tissue environments. For example, it is widely known that adult tendons have incomplete healing, which entails scar tissue formation (Longo et al., 2008, 2011), whereas fetal or neonatal tendons exhibit scarless wound healing (Andarawis-Puri et al., 2015; Galatz et al., 2015; Howell et al., 2017). In a mouse Achilles tendon resection study, improved neonatal healing was attributed to recruitment of Scx-positive cells, which are absent in adults (Howell et al., 2017). In lieu of Scx-positive cells, smooth muscle α-actin-positive cells mediated wound healing *via* fibrovascular scar tissue formation, leading to impaired musculoskeletal function (Howell et al., 2017). Transplantation of wounded adult and fetal sheep tendons into immunocompromised adult mice demonstrated that fetal cells exhibited superior intrinsic healing capacities (Favata et al., 2006). Wounded fetal tendons demonstrated both wound closure and recovery of tendon biomechanical properties including peak stress, peak load, modulus, and stiffness relative to unwounded controls, while wounded adult tendons did not (Favata et al., 2006). Also, fetal or postnatal tissue-specific microenvironments differ from that in adult tissue and can alter the wound healing response. These differences include high cellularity and low ECM content in developing tendons, which stand in stark contrast to the hypocellular and predominantly ECM-based nature of adult tendons (Dahners, 2005). For example, subtle compositional differences in the ECM of developing tendons such as lysyl oxidase-mediated collagen crosslinking increased mechanical properties such as stiffness (Marturano et al., 2013), which can affect tenogenic differentiation (Schiele et al., 2013). Another major difference in tissue environment includes differences in the inflammatory microenvironment between non-adult and adult tendon (Galatz et al., 2015). To-date, direct comparisons between adult and fetal tendon inflammation have not been reported, but in related musculoskeletal systems such as articular cartilage, improved wound healing is typically associated with lower inflammatory response (Ribitsch et al., 2018). However, more studies are needed to identify potential inflammatory factors associated with improved wound healing (Galatz et al., 2015). This is because the relationship between cells and their tissue environments is highly dynamic and interdependent. For example, dermally-derived fetal fibroblasts exhibited greater regenerative potential relative to their adult counterparts (Tang et al., 2014). Using a 2 mm mouse Achilles tendon defect model, tendon cell sheets engineered from fetal cells demonstrated improved biomechanical properties, such as higher stiffness and tensile modulus, as well as tissue organization, such as increased tissue birefringence and collagen fibril size (Tang et al., 2014). Improved tendon healing in this scenario was attributed to increased fetal fibroblast proliferation and decreased immunocyte presence at the injury site concomitant with decreased expression of pro-inflammatory cytokines including IL-1β, IL-6, and CD44 (Tang et al., 2014). Such studies highlight the need for careful interpretation of findings from developmental studies for therapeutic development.

Second, knowledge gleaned from the study of animal models has limitations. Developmental and wound healing studies have used a vast array of model organisms to advance our understanding of tendon biology. These include invertebrates such as drosophila (Schweitzer et al., 2010), vertebrates such as fish (Chen and Galloway, 2014, 2017), chick (Schweitzer et al., 2001; Edom-Vovard et al., 2002; Edom-Vovard and Duprez, 2004; Havis et al., 2016), and mice (Thomopoulos et al., 2007; Schweitzer et al., 2010; Schwartz et al., 2012, 2013, 2015, 2017; Huang et al., 2015; Havis et al., 2016; Huang, 2017). However, species-specific differences may limit the applicability of growth factors identified for musculoskeletal tissue engineering. For example, it has been suggested that FGF signaling is important for tendon development in chick but less so for mouse (Havis et al., 2016). Also, TGF-β3 is implicated in tendon development (Liu et al., 2017) but its application resulted in more disorganized scar tissue relative to repair only and carrier only controls (Manning et al., 2011). In addition, there is growing recognition that the hierarchical nature of tendon organization in smaller animal models such as rats, while useful for studying musculoskeletal biology, do not mimic those seen in larger organisms, such as horse or humans, owing to their lack of high load-bearing fascicles (Lee and Elliott, 2018). Also, it is imperative to both devise an appropriately relevant injury model for evaluation. For example, severe rotator cuff tears are typically chronic in nature (Gartsman, 2009). As such, chronic injury models, such as those which entail skeletal muscle fatty degeneration (Gupta and Lee, 2007), are more ideal for evaluating a therapy meant to address large-to-massive injuries. At the same time, it is

important to carefully interpret animal model studies that employ acute injuries for an intervention meant to address a chronic wound. Such studies highlight the need for careful design of studies and interpretation of their results.

Thus, developmental biology and wound healing studies can offer tremendous insights that aid musculoskeletal tissue engineering and regenerative medicine, but factors such as intrinsic differences between non-adult and adult cells and microenvironment, species-specific differences, and relevance of wound model must be carefully considered.

OUTLOOK

In summary, developmental and wound healing studies can serve as useful inspirations to engineer multi-tissue, musculoskeletal units. Most importantly, it is evident that musculoskeletal development and integration of bone, tendon, and muscle tissues are highly interdependent, while common inflammatory and anti-inflammatory signals mediate healing of these tissues as well as integration of clinical grafts. Collectively, such inquiries have advanced our understanding of musculoskeletal biology by identifying useful markers of musculoskeletal differentiation and promising cues for therapeutic development. However, integrating this knowledge for translational applications requires careful consideration of differences in embryonic or postnatal development with adult wound healing as well as the limitations of animal models used for studying developmental biology and simulating injury. Overcoming such differences will greatly advance efforts to engineer or regenerate multi-tissue musculoskeletal units.

AUTHOR CONTRIBUTIONS

XZ wrote the manuscript and prepared the figures. DW and K-LM wrote the manuscript. DK and RT conceived and wrote the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

DK was supported by The Chinese University of Hong Kong (Faculty Innovation Award, FIA2018/A/01), Innovation and Technology Commission (Tier 3 Award, ITS/090/18), and Research Grants Council of Hong Kong (Early Career Scheme Award, 24201720). DW was supported by The Chinese University of Hong Kong (Direct Grant Award, 2018.092), Innovation and Technology Commission (Tier 3 Award, ITS/333/18), Food and Health Bureau (Health Medical and Research Fund, 07180686), and Research Grants Council of Hong Kong (General Research Fund Award, 14118620). RT was supported by Lee Quo Wei and Lee Yick Hoi Lun Professorship in Tissue Engineering and Regenerative Medicine.

ACKNOWLEDGMENTS

The authors thank Amanda Shuting Huang for her illustrations in **Figure 1**. The support of the Lee Quo Wei and Lee Yick Hoi Lun Professorship in Tissue Engineering and Regenerative Medicine (RST) is also gratefully acknowledged.

REFERENCES

Abdou, M. A., Kim, G. E., Kim, J., Kim, B. H., Kim, Y. K., Jeong, S. E., et al. (2019). How long should we wait to create the goutallier stage 2 fatty infiltrations in the rabbit shoulder for repairable rotator cuff tear model? *Biomed. Res. Int.* 2019:7387131. doi: 10.1155/2019/7387131

Akbar, M., Gilchrist, D. S., Kitson, S. M., Nelis, B., Crowe, L. A. N., Garcia-Melchor, E., et al. (2017). Targeting danger molecules in tendinopathy: the hmgb1/tlr4 axis. *RMD Open* 3:e000456. doi: 10.1136/rmdopen-2017-000456

Alexander, P. G., and Tuan, R. S. (2010). Role of environmental factors in axial skeletal dysmorphogenesis. *Birth Defects Res. C Embryo Today* 90, 118–132. doi: 10.1002/bdrc.20179

Andarawis-Puri, N., Flatow, E. L., and Soslowsky, L. J. (2015). Tendon basic science: development, repair, regeneration, and healing. *J. Orthop. Res.* 33, 780–784. doi: 10.1002/jor.22869

Anderson, J. M., Rodriguez, A., and Chang, D. T. (2008). Foreign body reaction to biomaterials. *Semin. Immunol.* 20, 86–100. doi: 10.1016/j.smim.2007.11.004

Arimura, H., Shukunami, C., Tokunaga, T., Karasugi, T., Okamoto, N., Taniwaki, T., et al. (2017). Tgf-beta1 improves biomechanical strength by extracellular matrix accumulation without increasing the number of tenogenic lineage cells in a rat rotator cuff repair model. *Am. J. Sports Med.* 45, 2394–2404. doi: 10.1177/0363546517707940

Aurora, A. B., and Olson, E. N. (2014). Immune modulation of stem cells and regeneration. *Cell Stem Cell* 15, 14–25. doi: 10.1016/j.stem.2014.06.009

Barber, F. A. (2007). Biodegradable shoulder anchors have unique modes of failure. *Arthroscopy* 23, 316–320. doi: 10.1016/j.arthro.2007.01.014

Barnes, G. L., Alexander, P. G., Hsu, C. W., Mariani, B. D., and Tuan, R. S. (1997). Cloning and characterization of chicken paraxis: a regulator of paraxial mesoderm development and somite formation. *Dev. Biol.* 189, 95–111. doi: 10.1006/dbio.1997.8663

Barnes, G. L., Hsu, C. W., Mariani, B. D., and Tuan, R. S. (1996a). Chicken pax-1 gene: structure and expression during embryonic somite development. *Differentiation* 61, 13–23. doi: 10.1046/j.1432-0436.1996.6110013.x

Barnes, G. L., Mariani, B. D., and Tuan, R. S. (1996b). Valproic acid-induced somite teratogenesis in the chick embryo: relationship with pax-1 gene expression. *Teratology* 54, 93–102. doi: 10.1002/(SICI)1096-9926(199606)54:2<93::AID-TERA5>3.0.CO;2-5

Berendsen, A. D., and Olsen, B. R. (2015). Bone development. *Bone* 80, 14–18. doi: 10.1016/j.bone.2015.04.035

Blitz, E., Sharir, A., Akiyama, H., and Zelzer, E. (2013). Tendon-bone attachment unit is formed modularly by a distinct pool of scx- and sox9-positive progenitors. *Development* 140, 2680–2690. doi: 10.1242/dev.093906

Blitz, E., Viukov, S., Sharir, A., Shwartz, Y., Galloway, J. L., Pryce, B. A., et al. (2009). Bone ridge patterning during musculoskeletal assembly is mediated through scx regulation of bmp4 at the tendon-skeleton junction. *Dev. Cell* 17, 861–873. doi: 10.1016/j.devcel.2009.10.010

Brent, A. E., Braun, T., and Tabin, C. J. (2005). Genetic analysis of interactions between the somitic muscle, cartilage and tendon cell lineages during mouse development. *Development* 132, 515–528. doi: 10.1242/dev.01605

Brent, A. E., Schweitzer, R., and Tabin, C. J. (2003). A somitic compartment of tendon progenitors. *Cell* 113, 235–248. doi: 10.1016/S0092-8674(03)00268-X

Brent, A. E., and Tabin, C. J. (2004). Fgf acts directly on the somitic tendon progenitors through the ets transcription factors pea3 and erm to regulate scleraxis expression. *Development* 131, 3885–3896. doi: 10.1242/dev.01275

Bumcrot, D. A., and Mcmahon, A. P. (1995). Somite differentiation: sonic signals somites. *Curr. Biol.* 5, 612–614. doi: 10.1016/S0960-9822(95)00123-0

Chen, J. W., and Galloway, J. L. (2014). The development of zebrafish tendon and ligament progenitors. *Development* 141, 2035–2045. doi: 10.1242/dev.104067

Chen, J. W., and Galloway, J. L. (2017). Using the zebrafish to understand tendon development and repair. *Methods Cell Biol.* 138, 299–320. doi: 10.1016/bs.mcb.2016.10.003

Chevallier, A., Kieny, M., and Mauger, A. (1977). Limb-somite relationship: origin of the limb musculature. *J. Embryol. Exp. Morpholog.* 41, 245–258.

Cobaleda Aristizabal, A. F., Sanders, E. J., and Barber, F. A. (2014). Adverse events associated with biodegradable lactide-containing suture anchors. *Arthroscopy* 30, 555–560. doi: 10.1016/j.arthro.2014.02.011

Cohen, D. B., Kawamura, S., Ehteshami, J. R., and Rodeo, S. A. (2006). Indomethacin and celecoxib impair rotator cuff tendon-to-bone healing. *Am. J. Sports Med.* 34, 362–369. doi: 10.1177/0363546505280428

Correa, D., Somoza, R. A., Lin, P., Greenberg, S., Rom, E., Duesler, L., et al. (2015). Sequential exposure to fibroblast growth factors (fgf) 2, 9 and 18 enhances hmsc chondrogenic differentiation. *Osteoarthr. Cartil.* 23, 443–453. doi: 10.1016/j.joca.2014.11.013

Cossu, G., Kelly, R., Tajbakhsh, S., Di Donna, S., Vivarelli, E., and Buckingham, M. (1996). Activation of different myogenic pathways: Myf-5 is induced by the neural tube and myod by the dorsal ectoderm in mouse paraxial mesoderm. *Development* 122, 429–437. doi: 10.1242/dev.122.2.429

Crupi, A., Costa, A., Tarnok, A., Melzer, S., and Teodori, L. (2015). Inflammation in tissue engineering: the janus between engraftment and rejection. *Eur. J. Immunol.* 45, 3222–3236. doi: 10.1002/eji.201545818

Cserjesi, P., Brown, D., Ligon, K. L., Lyons, G. E., Copeland, N. G., and Gilbert, D. J. (1995). Scleraxis: a basic helix-loop-helix protein that prefigures skeletal formation during mouse embryogenesis. *Development* 121, 1099–1110. doi: 10.1242/dev.121.4.1099

Curzi, D., Sartini, S., Guescini, M., Lattanzi, D., Di Palma, M., Ambrogini, P., et al. (2016). Effect of different exercise intensities on the myotendinous junction plasticity. *PLoS One* 11:e0158059. doi: 10.1371/journal.pone.0158059

Dahners, L. E. (2005). "Growth and development of tendons," in *Tendon Injuries Basic Science and Clinical Medicine*. eds. N. Maffulli, P. Renstrom and W. B. Leadbetter (London: Springer-Verlag).

Davies, M. R., Lee, L., Feeley, B. T., Kim, H. T., and Liu, X. (2017). Lysophosphatidic acid-induced rhoa signaling and prolonged macrophage infiltration worsens fibrosis and fatty infiltration following rotator cuff tears. *J. Orthop. Res.* 35, 1539–1547. doi: 10.1002/jor.23384

Davis, M. E., Korn, M. A., Gumucio, J. P., Harning, J. A., Saripalli, A. L., Bedi, A., et al. (2015). Simvastatin reduces fibrosis and protects against muscle weakness after massive rotator cuff tear. *J. Shoulder Elb. Surg.* 24, 280–287. doi: 10.1016/j.jse.2014.06.048

Deng, Y., Lei, G., Lin, Z., Yang, Y., Lin, H., and Tuan, R. S. (2019). Engineering hyaline cartilage from mesenchymal stem cells with low hypertrophy potential via modulation of culture conditions and wnt/beta-catenin pathway. *Biomaterials* 192, 569–578. doi: 10.1016/j.biomaterials.2018.11.036

Deng, G., Li, K., Chen, S., Chen, P., Zheng, H., Yu, B., et al. (2018a). Interleukin-10 promotes proliferation and migration, and inhibits tendon differentiation via the jak/stat3 pathway in tendon derived stem cells in vitro. *Mol. Med. Rep.* 18, 5044–5052. doi: 10.3892/mmr.2018.9547

Deng, Y., Lu, J., Li, W., Wu, A., Zhang, X., Tong, W., et al. (2018b). Reciprocal inhibition of yap/taz and nf-κb regulates osteoarthritic cartilage degradation. *Nat. Commun.* 9:4564. doi: 10.1038/s41467-018-07022-2

Deng, Y., Wu, A., Li, P., Li, G., Qin, L., Song, H., et al. (2016). Yap1 regulates multiple steps of chondrocyte differentiation during skeletal development and bone repair. *Cell Rep.* 14, 2224–2237. doi: 10.1016/j.celrep.2016.02.021

Derwin, K. A., Baker, A. R., Spragg, R. K., Leigh, D. R., and Iannotti, J. P. (2006). Commercial extracellular matrix scaffolds for rotator cuff tendon repair. Biomechanical, biochemical, and cellular properties. *J. Bone Joint Surg. Am.* 88, 2665–2672. doi: 10.2106/JBJS.E.01307

Deymier, A. C., Schwartz, A. G., Cai, Z., Daulton, T. L., Pasteris, J. D., Genin, G. M., et al. (2019). The multiscale structural and mechanical effects of mouse supraspinatus muscle unloading on the mature enthesis. *Acta Biomater.* 83, 302–313. doi: 10.1016/j.actbio.2018.10.024

Dupont, S., Morsut, L., Aragona, M., Enzo, E., Giulitti, S., Cordenonsi, M., et al. (2011). Role of yap/taz in mechanotransduction. *Nature* 474, 179–183. doi: 10.1038/nature10137

Edom-Vovard, F., and Duprez, D. (2004). Signals regulating tendon formation during chick embryonic development. *Dev. Dyn.* 229, 449–457. doi: 10.1002/dvdy.10481

Edom-Vovard, F., Schuler, B., Bonnin, M. A., Teillet, M. A., and Duprez, D. (2002). Fgf4 positively regulates scleraxis and tenascin expression in chick limb tendons. *Dev. Biol.* 247, 351–366. doi: 10.1006/dbio.2002.0707

Eming, S. A., Martin, P., and Tomic-Canic, M. (2014). Wound repair and regeneration: mechanisms, signaling, and translation. *Sci. Transl. Med.* 6:265sr266. doi: 10.1126/scitranslmed.3009337

Endo, T. (2015). Molecular mechanisms of skeletal muscle development, regeneration, and osteogenic conversion. *Bone* 80, 2–13. doi: 10.1016/j.bone.2015.02.028

Fan, C.-M., and Tessier-Lavigne, M. (1994). Patterning of mammalian somites by surface ectoderm and notochord: evidence for sclerotome induction by a hedgehog homolog. *Cell* 79, 1175–1186. doi: 10.1016/0092-8674(94)90009-4

Favata, M., Beredjiklian, P. K., Zgonis, M. H., Beason, D. P., Crombleholme, T. M., Jawad, A. F., et al. (2006). Regenerative properties of fetal sheep tendon are not adversely affected by transplantation into an adult environment. *J. Orthop. Res.* 24, 2124–2132. doi: 10.1002/jor.20271

Felsenthal, N., Rubin, S., Stern, T., Krief, S., Pal, D., Pryce, B. A., et al. (2018). Development of migrating tendon-bone attachments involves replacement of progenitor populations. *Development* 145:dev.165381. doi: 10.1242/dev.165381

Felsenthal, N., and Zelzer, E. (2017). Mechanical regulation of musculoskeletal system development. *Development* 144, 4271–4283. doi: 10.1242/dev.151266

Fischer, M., Rikeit, P., Knaus, P., and Coirault, C. (2016). Yap-mediated mechanotransduction in skeletal muscle. *Front. Physiol.* 7:41. doi: 10.3389/fphys.2016.00041

Galatz, L. M., Gerstenfeld, L., Heber-Katz, E., and Rodeo, S. A. (2015). Tendon regeneration and scar formation: the concept of scarless healing. *J. Orthop. Res.* 33, 823–831. doi: 10.1002/jor.22853

Galatz, L., Rothermich, S., Vanderploeg, K., Petersen, B., Sandell, L., and Thomopoulos, S. (2007). Development of the supraspinatus tendon-to-bone insertion: localized expression of extracellular matrix and growth factor genes. *J. Orthop. Res.* 25, 1621–1628. doi: 10.1002/jor.20441

Galatz, L. M., Rothermich, S. Y., Zaegel, M., Silva, M. J., Havlioglu, N., and Thomopoulos, S. (2005). Delayed repair of tendon to bone injuries leads to decreased biomechanical properties and bone loss. *J. Orthop. Res.* 23, 1441–1447. doi: 10.1016/j.orthres.2005.05.005.1100230629

Gartsman, G. M. (2009). *Shoulder Arthroscopy. 2nd Edn.* Philadelphia: W. B. Saunders.

Gelberman, R. H., Shen, H., Kormpakis, I., Rothrauff, B., Yang, G., and Tuan, R. S. (2016). The effect of adipose-derived stromal cells and bmp12 on intrasynovial tendon repair: a biomechanical, biochemical, and proteomics study. *J. Orthop. Res.* 34, 630–640. doi: 10.1002/jor.23064

Genin, G. M., Kent, A., Birman, V., Wopenka, B., Pasteris, J. D., Marquez, P. J., et al. (2009). Functional grading of mineral and collagen in the attachment of tendon to bone. *Biophys. J.* 97, 976–985. doi: 10.1016/j.bpj.2009.05.043

Geoghegan, I. P., Hoey, D. A., and Mcnamara, L. M. (2019). Integrins in osteocyte biology and mechanotransduction. *Curr. Osteoporos. Rep.* 17, 195–206. doi: 10.1007/s11914-019-00520-2

Gibbons, M. C., Singh, A., Anakwenze, O., Cheng, T., Pomerantz, M., Schenk, S., et al. (2017). Histological evidence of muscle degeneration in advanced human rotator cuff disease. *J. Bone Joint Surg. Am.* 99, 190–199. doi: 10.2106/JBJS.16.00335

Gilbert, F., Meffert, R. H., Schmalzl, J., Weng, A. M., Kostler, H., and Eden, L. (2018). Grade of retraction and tendon thickness correlates with mr-spectroscopically measured amount of fatty degeneration in full thickness supraspinatus tears. *BMC Musculoskelet. Disord.* 19:197. doi: 10.1186/s12891-018-2096-5

Godwin, J. W., Pinto, A. R., and Rosenthal, N. A. (2013). Macrophages are required for adult salamander limb regeneration. *Proc. Natl. Acad. Sci.* 110, 9415–9420. doi: 10.1073/pnas.1300290110

Goldring, S. R. (2015). Inflammatory signaling induced bone loss. *Bone* 80, 143–149. doi: 10.1016/j.bone.2015.05.024

Gupta, R., and Lee, T. Q. (2007). Contributions of the different rabbit models to our understanding of rotator cuff pathology. *J. Shoulder Elb. Surg.* 16, S149–S157. doi: 10.1016/j.jse.2007.05.002

Havis, E., Bonnin, M. A., Esteves de Lima, J., Charvet, B., Milet, C., and Duprez, D. (2016). Tgfbeta and fgf promote tendon progenitor fate and act downstream of muscle contraction to regulate tendon differentiation during chick limb development. *Development* 143, 3839–3851. doi: 10.1242/dev.136242

Herrmann, M., Laschke, M.W., Alini, M., Scherberich, A., and Verrier, S. (2014). "Chapter 14: Vascularization, survival, and functionality of tissue-engineered constructs," in *Tissue Engineering*. 2*nd Edn*. eds. C. A. V. Blitterswijk and J. De Boer (Oxford: Academic Press), 471–496.

Holmberg, J., and Durbeej, M. (2013). Laminin-211 in skeletal muscle function. *Cell Adhes. Migr.* 7, 111–121. doi: 10.4161/cam.22618

Howell, K., Chien, C., Bell, R., Laudier, D., Tufa, S. F., Keene, D. R., et al. (2017). Novel model of tendon regeneration reveals distinct cell mechanisms underlying regenerative and fibrotic tendon healing. *Sci. Rep.* 7:45238. doi: 10.1038/srep45238

Huang, A. H. (2017). Coordinated development of the limb musculoskeletal system: tendon and muscle patterning and integration with the skeleton. *Dev. Biol.* 429, 420–428. doi: 10.1016/j.ydbio.2017.03.028

Huang, A. H., Lu, H. H., and Schweitzer, R. (2015). Molecular regulation of tendon cell fate during development. *J. Orthop. Res.* 33, 800–812. doi: 10.1002/jor.22834

Huraskin, D., Eiber, N., Reichel, M., Zidek, L. M., Kravic, B., Bernkopf, D., et al. (2016). Wnt/β-catenin signaling via axin2 is required for myogenesis and, together with yap/taz and tead1, active in iia/iix muscle fibers. *Development* 143, 3128–3142. doi: 10.1242/dev.139907

Jakobsen, J. R., Mackey, A. L., Knudsen, A. B., Koch, M., Kjær, M., and Krogsgaard, M. R. (2017). Composition and adaptation of human myotendinous junction and neighboring muscle fibers to heavy resistance training. *Scand. J. Med. Sci. Sports* 27, 1547–1559. doi: 10.1111/sms.12794

Jansen, K. A., Donato, D. M., Balcioglu, H. E., Schmidt, T., Danen, E. H. J., and Koenderink, G. H. (2015). A guide to mechanobiology: where biology and physics meet. *Biochim. Biophys. Acta* 1853, 3043–3052. doi: 10.1016/j.bbamcr.2015.05.007

Jenkins, M. H., Alrowaished, S. S., Goody, M. F., Crawford, B. D., and Henry, C. A. (2016). Laminin and matrix metalloproteinase 11 regulate fibronectin levels in the zebrafish myotendinous junction. *Skelet. Muscle* 6:18. doi: 10.1186/s13395-016-0089-3

Jensen, P. T., Lambertsen, K. L., and Frich, L. H. (2018). Assembly, maturation, and degradation of the supraspinatus enthesis. *J. Shoulder Elb. Surg.* 27, 739–750. doi: 10.1016/j.jse.2017.10.030

Joo Han, O., Byung Wook, S., and Tae-Yon, R. (2015). Two cases of biodegradable suture anchor displacement diagnosed with ultrasonography following arthroscopic rotator cuff repair. *Clin. Shoulder Elbow* 18, 254–260. doi: 10.5397/cise.2015.18.4.254

Kardon, G. (1998). Muscle and tendon morphogenesis in the avian hind limb. *Development* 125, 4019–4032. doi: 10.1242/dev.125.20.4019

Ker, E. D., Chu, B., Phillippi, J. A., Gharaibeh, B., Huard, J., Weiss, L. E., et al. (2011a). Engineering spatial control of multiple differentiation fates within a stem cell population. *Biomaterials* 32, 3413–3422. doi: 10.1016/j.biomaterials.2011.01.036

Ker, E. D., Nain, A. S., Weiss, L. E., Wang, J., Suhan, J., Amon, C. H., et al. (2011b). Bioprinting of growth factors onto aligned sub-micron fibrous scaffolds for simultaneous control of cell differentiation and alignment. *Biomaterials* 32, 8097–8107. doi: 10.1016/j.biomaterials.2011.07.025

Kieny, M., and Chevallier, A. (1979). Autonomy of tendon development in the embryonic chick wing. *J. Embryol. Exp. Morpholog.* 49, 153–165.

Laschke, M. W., and Menger, M. D. (2012). Vascularization in tissue engineering: angiogenesis versus inosculation. *Eur. Surg. Res.* 48, 85–92. doi: 10.1159/000336876

Lavagnino, M., Wall, M. E., Little, D., Banes, A. J., Guilak, F., and Arnoczky, S. P. (2015). Tendon mechanobiology: current knowledge and future research opportunities. *J. Orthop. Res.* 33, 813–822. doi: 10.1002/jor.22871

LeClair, E. E., Bonfiglio, L., and Tuan, R. S. (1999). Expression of the paired-box genes pax-1 and pax-9 in limb skeleton development. *Dev. Dyn.* 214, 101–115. doi: 10.1002/(SICI)1097-0177(199902)214:2<101::AID-AJA1>3.0.CO;2-4

Lee, A. H., and Elliott, D. M. (2018). Comparative multi-scale hierarchical structure of the tail, plantaris, and achilles tendons in the rat. *J. Anat.* 234, 252–262. doi: 10.1111/joa.12913

Lejard, V., Brideau, G., Blais, F., Salingcarnboriboon, R., Wagner, G., and Roehrl, M. H. (2007). Scleraxis and nfatc regulate the expression of the pro-alpha1(i) collagen gene in tendon fibroblasts. *J. Biol. Chem.* 282, 17665–17675. doi: 10.1074/jbc.M610113200

Leroux, T. S., Basques, B. A., Saltzman, B. M., Nicholson, G. P., Romeo, A. A., and Verma, N. N. (2018). Shoulder arthroplasty in patients with rheumatoid arthritis: a population-based study examining utilization, adverse events, length of stay, and cost. *Am. J. Orthop.* 47, 1–13. doi: 10.12788/ajo.2018.0046

Li, W., Deng, Y., Feng, B., and Mak, K. K.-L. (2018). Mst1/2 kinases modulate glucose uptake for osteoblast differentiation and bone formation. *J. Bone Miner. Res.* 33, 1183–1195. doi: 10.1002/jbmr.3413

Lin, J. D., Jang, A. T., Kurylo, M. P., Hurng, J., Yang, F., Yang, L., et al. (2017). Periodontal ligament entheses and their adaptive role in the context of dentoalveolar joint function. *Dent. Mater* 33, 650–666. doi: 10.1016/j.dental.2017.03.007

Liu, Y., Suen, C. W., Zhang, J. F., and Li, G. (2017). Current concepts on tenogenic differentiation and clinical applications. *J. Orthop. Translat.* 9, 28–42. doi: 10.1016/j.jot.2017.02.005

Londhe, P., and Guttridge, D. C. (2015). Inflammation induced loss of skeletal muscle. *Bone* 80, 131–142. doi: 10.1016/j.bone.2015.03.015

Longo, U. G., Berton, A., Khan, W. S., Maffulli, N., and Denaro, V. (2011). Histopathology of rotator cuff tears. *Sports Med. Arthrosc.* 19, 227–236. doi: 10.1097/JSA.0b013e318213bccb

Longo, U. G., Franceschi, F., Ruzzini, L., Rabitti, C., Morini, S., Maffulli, N., et al. (2008). Histopathology of the supraspinatus tendon in rotator cuff tears. *Am. J. Sports Med.* 36, 533–538. doi: 10.1177/0363546507308549

Love, J. M., and Tuan, R. S. (1993). Pair-rule gene expression in the somitic stage chick embryo: association with somite segmentation and border formation. *Differentiation* 54, 73–83. doi: 10.1111/j.1432-0436.1993.tb01590.x

Mammoto, A., Mammoto, T., and Ingber, D. E. (2012). Mechanosensitive mechanisms in transcriptional regulation. *J. Cell Sci.* 125, 3061–3073. doi: 10.1242/jcs.093005

Manning, C. N., Havlioglu, N., Knutsen, E., Sakiyama-Elbert, S. E., Silva, M. J., and Thomopoulos, S. (2014). The early inflammatory response after flexor tendon healing: a gene expression and histological analysis. *J. Orthop. Res.* 32, 645–652. doi: 10.1002/jor.22575

Manning, C. N., Kim, H. M., Sakiyama-Elbert, S., Galatz, L. M., Havlioglu, N., and Thomopoulos, S. (2011). Sustained delivery of transforming growth factor beta three enhances tendon-to-bone healing in a rat model. *J. Orthop. Res.* 29, 1099–1105. doi: 10.1002/jor.21301

Marieb, E. N. (1999). *Human Anatomy and Physiology*. San Francisco: Benjamin Cummings.

Marturano, J. E., Arena, J. D., Schiller, Z. A., Georgakoudi, I., and Kuo, C. K. (2013). Characterization of mechanical and biochemical properties of developing embryonic tendon. *Proc. Natl. Acad. Sci.* 110, 6370–6375. doi: 10.1073/pnas.1300135110

Mitchell, B., and Sharma, R. (2005). *Embryology: An Illustrated Colour Text*. Amsterdam: Elsevier.

Moffat, K. L., Sun, W. H., Pena, P. E., Chahine, N. O., Doty, S. B., Ateshian, G. A., et al. (2008). Characterization of the structure-function relationship at the ligament-to-bone interface. *Proc. Natl. Acad. Sci.* 105, 7947–7952. doi: 10.1073/pnas.0712150105

Moser, H. L., Abraham, A. C., Howell, K., Laudier, D., Zumstein, M. A., Galatz, L. M., et al. (2020). Cell lineage tracing and functional assessment of supraspinatus tendon healing in an acute repair murine model. *J. Orthop. Res.* doi: 10.1002/jor.24769 [Epub ahead of print]

Musumeci, G., Castrogiovanni, P., Coleman, R., Szychlinska, M. A., Salvatorelli, L., Parenti, R., et al. (2015). Somitogenesis: from somite to skeletal muscle. *Acta Histochem.* 117, 313–328. doi: 10.1016/j.acthis.2015.02.011

Nagano, Y., Matsuda, Y., Desaki, J., Oki, S., Kitaoka, K., Okumura, H., et al. (1998). Morphodifferentiation of skeletal muscle fiber ends at the myotendinous junction in the postnatal chinese hamster: a scanning electron microscopic study. *Arch. Histol. Cytol.* 61, 89–92. doi: 10.1679/aohc.61.89

Oak, N. R., Gumucio, J. P., Flood, M. D., Saripalli, A. L., Davis, M. E., Harning, J. A., et al. (2014). Inhibition of 5-lox, cox-1, and cox-2 increases tendon healing and reduces muscle fibrosis and lipid accumulation after rotator cuff repair. *Am. J. Sports Med.* 42, 2860–2868. doi: 10.1177/0363546514549943

Occhetta, P., Pigeot, S., Rasponi, M., Dasen, B., Mehrkens, A., Ullrich, T., et al. (2018). Developmentally inspired programming of adult human mesenchymal stromal cells toward stable chondrogenesis. *Proc. Natl. Acad. Sci.* 115, 4625–4630. doi: 10.1073/pnas.1720658115

Pan, X. S., Li, J., Brown, E. B., and Kuo, C. K. (2018). Embryo movements regulate tendon mechanical property development. *Philos. Trans. R. Soc. Lond. Ser. B Biol. Sci.* 373:20170325. doi: 10.1098/rstb.2017.0325

Perry, S. M., Mcilhenny, S. E., Hoffman, M. C., and Soslowsky, L. J. (2005). Inflammatory and angiogenic mrna levels are altered in a supraspinatus tendon overuse animal model. *J. Shoulder Elb. Surg.* 14, 79S–83S. doi: 10.1016/j.jse.2004.09.020

Pineault, K. M., and Wellik, D. M. (2014). Hox genes and limb musculoskeletal development. *Curr. Osteoporos. Rep.* 12, 420–427. doi: 10.1007/s11914-014-0241-0

Pryce, B. A., Watson, S. S., Murchison, N. D., Staverosky, J. A., Dünker, N., and Schweitzer, R. (2009). Recruitment and maintenance of tendon progenitors by tgfβ signaling are essential for tendon formation. *Development* 136, 1351–1361. doi: 10.1242/dev.027342

Relaix, F., Rocancourt, D., Mansouri, A., and Buckingham, M. (2005). A pax3/pax7-dependent population of skeletal muscle progenitor cells. *Nature* 435, 948–953. doi: 10.1038/nature03594

Ribitsch, I., Mayer, R. L., Egerbacher, M., Gabner, S., Kandula, M. M., Rosser, J., et al. (2018). Fetal articular cartilage regeneration versus adult fibrocartilaginous repair: secretome proteomics unravels molecular mechanisms in an ovine model. *Dis. Model. Mech.* 11:dmm033092. doi: 10.1242/dmm.033092

Ricchetti, E. T., Reddy, S. C., Ansorge, H. L., Zgonis, M. H., Van Kleunen, J. P., Liechty, K. W., et al. (2008). Effect of interleukin-10 overexpression on the properties of healing tendon in a murine patellar tendon model. *J. Hand. Surg. Am.* 33, 1843–1852. doi: 10.1016/j.jhsa.2008.07.020

Rissatto Sierra, L., Favaro, G., Cerri, B. R., Rocha, L. C., de Yokomizo de Almeida, S. R., Watanabe, I. S., et al. (2018). Myotendinous junction plasticity in aged ovariectomized rats submitted to aquatic training. *Microsc. Res. Tech.* 81, 816–822. doi: 10.1002/jemt.23040

Rossetti, L., Kuntz, L. A., Kunold, E., Schock, J., Muller, K. W., Grabmayr, H., et al. (2017). The microstructure and micromechanics of the tendon-bone insertion. *Nat. Mater.* 16, 664–670. doi: 10.1038/nmat4863

Rücker, M., Laschke, M. W., Junker, D., Carvalho, C., Schramm, A., Mülhaupt, R., et al. (2006). Angiogenic and inflammatory response to biodegradable scaffolds in dorsal skinfold chambers of mice. *Biomaterials* 27, 5027–5038. doi: 10.1016/j.biomaterials.2006.05.033

Rustad, K. C., and Gurtner, G. C. (2012). Mesenchymal stem cells home to sites of injury and inflammation. *Adv. Wound Care* 1, 147–152. doi: 10.1089/wound.2011.0314

Sadtler, K., Wolf, M. T., Ganguly, S., Moad, C. A., Chung, L., Majumdar, S., et al. (2019). Divergent immune responses to synthetic and biological scaffolds. *Biomaterials* 192, 405–415. doi: 10.1016/j.biomaterials.2018.11.002

Saleh, L. S., and Bryant, S. J. (2018). The host response in tissue engineering: crosstalk between immune cells and cell-laden scaffolds. *Curr. Opin. Biomed. Eng.* 6, 58–65. doi: 10.1016/j.cobme.2018.03.006

Schiaffino, S., Dyar, K. A., Ciciliot, S., Blaauw, B., and Sandri, M. (2013). Mechanisms regulating skeletal muscle growth and atrophy. *FEBS J.* 280, 4294–4314. doi: 10.1111/febs.12253

Schiele, N. R., Marturano, J. E., and Kuo, C. K. (2013). Mechanical factors in embryonic tendon development: potential cues for stem cell tenogenesis. *Curr. Opin. Biotechnol.* 24, 834–840. doi: 10.1016/j.copbio.2013.07.003

Schwartz, A. G., Galatz, L. M., and Thomopoulos, S. (2017). Enthesis regeneration: a role for gli1+ progenitor cells. *Development* 144, 1159–1164. doi: 10.1242/dev.139303

Schwartz, A. G., Lipner, J. H., Pasteris, J. D., Genin, G. M., and Thomopoulos, S. (2013). Muscle loading is necessary for the formation of a functional tendon enthesis. *Bone* 55, 44–51. doi: 10.1016/j.bone.2013.03.010

Schwartz, A. G., Long, F., and Thomopoulos, S. (2015). Enthesis fibrocartilage cells originate from a population of hedgehog-responsive cells modulated by the loading environment. *Development* 142, 196–206. doi: 10.1242/dev.112714

Schwartz, A. G., Pasteris, J. D., Genin, G. M., Daulton, T. L., and Thomopoulos, S. (2012). Mineral distributions at the developing tendon enthesis. *PLoS One* 7:e48630. doi: 10.1371/journal.pone.0048630

Schweitzer, R., Chyung, J. H., Murtaugh, L. C., Brent, A. E., Rosen, V., Olson, E. N., et al. (2001). Analysis of the tendon cell fate using scleraxis, a specific marker for tendons and ligaments. *Development* 128, 3855–3866. doi: 10.1242/dev.128.19.3855

Schweitzer, R., Zelzer, E., and Volk, T. (2010). Connecting muscles to tendons: tendons and musculoskeletal development in flies and vertebrates. *Development* 137, 2807–2817. doi: 10.1242/dev.047498

Seo, E., Basu-Roy, U., Gunaratne, P. H., Coarfa, C., Lim, D.-S., Basilico, C., et al. (2013). Sox2 regulates yap1 to maintain stemness and determine cell fate in the osteo-adipo lineage. *Cell Rep.* 3, 2075–2087. doi: 10.1016/j.celrep.2013.05.029

Shindle, M. K., Chen, C. C. T., Robertson, C., Ditullio, A. E., Paulus, M. C., Clinton, C. M., et al. (2011). Full-thickness supraspinatus tears are associated with more synovial inflammation and tissue degeneration than partial-thickness tears. *J. Shoulder Elb. Surg.* 20, 917–927. doi: 10.1016/j.jse.2011.02.015

Shukunami, C., Takimoto, A., Oro, M., and Hiraki, Y. (2006). Scleraxis positively regulates the expression of tenomodulin, a differentiation marker of tenocytes. *Dev. Biol.* 298, 234–247. doi: 10.1016/j.ydbio.2006.06.036

Smith, C. A., and Tuan, R. S. (1995). Functional involvement of pax-1 in somite development: somite dysmorphogenesis in chick embryos treated with pax-1 paired-box antisense oligodeoxynucleotide. *Teratology* 52, 333–345. doi: 10.1002/tera.1420520604

Snyder, S. J., Arnoczky, S. P., Bond, J. L., and Dopirak, R. (2009). Histologic evaluation of a biopsy specimen obtained 3 months after rotator cuff augmentation with graftjacket matrix. *Arthroscopy* 25, 329–333. doi: 10.1016/j.arthro.2008.05.023

Soslowsky, L. J., Carpenter, J. E., Debano, C. M., Banerji, I., and Moalli, M. R. (1996). Development and use of an animal model for investigations on rotator cuff disease. *J. Shoulder Elb. Surg.* 5, 383–392. doi: 10.1016/S1058-2746(96)80070-X

Stearns-Reider, K. M., D'Amore, A., Beezhold, K., Rothrauff, B., Cavalli, L., Wagner, W. R., et al. (2017). Aging of the skeletal muscle extracellular matrix drives a stem cell fibrogenic conversion. *Aging Cell* 16, 518–528. doi: 10.1111/acel.12578

Su, W., Qi, W., Li, X., Zhao, S., Jiang, J., and Zhao, J. (2018). Effect of suture absorbability on rotator cuff healing in a rabbit rotator cuff repair model. *Am. J. Sports Med.* 46, 2743–2754. doi: 10.1177/0363546518787181

Sugg, K. B., Lubardic, J., Gumucio, J. P., and Mendias, C. L. (2014). Changes in macrophage phenotype and induction of epithelial-to-mesenchymal transition genes following acute achilles tenotomy and repair. *J. Orthop. Res.* 32, 944–951. doi: 10.1002/jor.22624

Sugimoto, Y., Takimoto, A., Akiyama, H., Kist, R., Scherer, G., Nakamura, T., et al. (2013). Scx+/sox9$^+$ progenitors contribute to the establishment of the junction between cartilage and tendon/ligament. *Development* 140, 2280–2288. doi: 10.1242/dev.096354

Syverud, B. C., Vandusen, K. W., and Larkin, L. M. (2016). Growth factors for skeletal muscle tissue engineering. *Cells Tissues Organs* 202, 169–179. doi: 10.1159/000444671

Tang, C., Chen, Y., Huang, J., Zhao, K., Chen, X., Yin, Z., et al. (2018). The roles of inflammatory mediators and immunocytes in tendinopathy. *J. Orthop. Translat.* 14, 23–33. doi: 10.1016/j.jot.2018.03.003

Tang, Q. M., Chen, J. L., Shen, W. L., Yin, Z., Liu, H. H., Fang, Z., et al. (2014). Fetal and adult fibroblasts display intrinsic differences in tendon tissue engineering and regeneration. *Sci. Rep.* 4:5515. doi: 10.1038/srep07483

Tellier, L. E., Krieger, J. R., Brimeyer, A. L., Coogan, A. C., Falis, A. A., Rinker, T. E., et al. (2018). Localized sdf-1α delivery increases pro-healing bone marrow-derived cells in the supraspinatus muscle following severe rotator cuff injury. *Regen. Eng. Transl. Med.* 4, 92–103. doi: 10.1007/s40883-018-0052-4

Thankam, F. G., Roesch, Z. K., Dilisio, M. F., Radwan, M. M., Kovilam, A., Gross, R. M., et al. (2018). Association of inflammatory responses and ecm disorganization with hmgb1 upregulation and nlrp3 inflammasome activation in the injured rotator cuff tendon. *Sci. Rep.* 8:8918. doi: 10.1038/s41598-018-27250-2

Thomopoulos, S., Genin, G. M., and Galatz, L. M. (2010). The development and morphogenesis of the tendon-to-bone insertion—what development can teach us about healing. *J. Musculoskelet. Neuronal Interact.* 10, 35–45.

Thomopoulos, S., Kim, H. M., Rothermich, S. Y., Biederstadt, C., Das, R., and Galatz, L. M. (2007). Decreased muscle loading delays maturation of the tendon enthesis during postnatal development. *J. Orthop. Res.* 25, 1154–1163. doi: 10.1002/jor.20418

Tokunaga, T., Ide, J., Arimura, H., Nakamura, T., Uehara, Y., Sakamoto, H., et al. (2015a). Local application of gelatin hydrogel sheets impregnated with platelet-derived growth factor bb promotes tendon-to-bone healing after rotator cuff repair in rats. *Arthroscopy* 31, 1482–1491. doi: 10.1016/j.arthro.2015.03.008

Tokunaga, T., Karasugi, T., Arimura, H., Yonemitsu, R., Sakamoto, H., Ide, J., et al. (2017). Enhancement of rotator cuff tendon-bone healing with fibroblast

growth factor 2 impregnated in gelatin hydrogel sheets in a rabbit model. *J. Shoulder Elb. Surg.* 26, 1708–1717. doi: 10.1016/j.jse.2017.03.020

Tokunaga, T., Shukunami, C., Okamoto, N., Taniwaki, T., Oka, K., Sakamoto, H., et al. (2015b). Fgf-2 stimulates the growth of tenogenic progenitor cells to facilitate the generation of tenomodulin-positive tenocytes in a rat rotator cuff healing model. *Am. J. Sports Med.* 43, 2411–2422. doi: 10.1177/0363546515597488

Tsuzaki, M., Guyton, G., Garrett, W., Archambault, J. M., Herzog, W., Almekinders, L., et al. (2003). Il-1 beta induces cox2, mmp-1, −3 and −13, adamts-4, il-1 beta and il-6 in human tendon cells. *J. Orthop. Res.* 21, 256–264. doi: 10.1016/S0736-0266(02)00141-9

Tuan, R. S. (2004). Biology of developmental and regenerative skeletogenesis. *Clin. Orthop. Relat. Res.* 427, S105–S117. doi: 10.1097/01.blo.0000143560.41767.ee

Turner, J. D., Naylor, A. J., Buckley, C., Filer, A., and Tak, P.-P. (2018). "Fibroblasts and osteoblasts in inflammation and bone damage," in *Stromal Immunology*. eds. B. M. J. Owens and M. A. Lakins (Basel: Springer International Publishing), 37–54.

Vinhas, A., Rodrigues, M. T., and Gomes, M. E. (2018). Exploring stem cells and inflammation in tendon repair and regeneration. *Adv. Exp. Med. Biol.* 1089, 37–46. doi: 10.1007/5584_2018_258

Wada, K.-I., Itoga, K., Okano, T., Yonemura, S., and Sasaki, H. (2011). Hippo pathway regulation by cell morphology and stress fibers. *Development* 138, 3907–3914. doi: 10.1242/dev.070987

Walker, J. M. (1991). Musculoskeletal development: a review. *Phys. Ther.* 71, 878–889. doi: 10.1093/ptj/71.12.878

Walton, J. R., Bowman, N. K., Khatib, Y., Linklater, J., and Murrell, G. A. (2007). Restore orthobiologic implant: not recommended for augmentation of rotator cuff repairs. *J. Bone Joint Surg. Am.* 89, 786–791. doi: 10.2106/JBJS.F.00315

Wang, D., Gilbert, J. R., Shaw, M. A., Shakir, S., Losee, J. E., Billiar, T. R., et al. (2015). Toll-like receptor 4 mediates the regenerative effects of bone grafts for calvarial bone repair. *Tissue Eng. Part A* 21, 1299–1308. doi: 10.1089/ten.tea.2014.0215

Wang, D., Gilbert, J. R., Zhang, X., Zhao, B., Ker, D. F. E., and Cooper, G. M. (2020). Calvarial versus long bone: implications for tailoring skeletal tissue engineering. *Tissue Eng. Part B Rev.* 26, 46–63. doi: 10.1089/ten.teb.2018.0353

Wang, M., Nasiri, A., Vanhouten, J. N., Tommasini, S. M., and Broadus, A. E. (2014). The remarkable migration of the medial collateral ligament. *J. Anat.* 224, 490–498. doi: 10.1111/joa.12145

Wang, D., Taylor, G. M., Gilbert, J. R., Losee, J. E., Sodhi, C. P., Hackam, D. J., et al. (2017). Enhanced calvarial bone healing in cd11c-tlr4$^{-/-}$ and myd88$^{-/-}$ mice. *Plast. Reconstr. Surg.* 139, 933e–940e. doi: 10.1097/PRS.0000000000003206

Wang, M., Vanhouten, J. N., Nasiri, A. R., Johnson, R. L., and Broadus, A. E. (2013). Pthrp regulates the modeling of cortical bone surfaces at fibrous insertion sites during growth. *J. Bone Miner. Res.* 28, 598–607. doi: 10.1002/jbmr.1801

Wang, C., Ying, J., Nie, X., Zhou, T., Xiao, D., Swarnkar, G., et al. (2021a). Targeting angiogenesis for fracture nonunion treatment in inflammatory disease. *Bone Res.* 9:29. doi: 10.1038/s41413-021-00150-4

Wang, D., Zhang, X., Huang, S., Liu, Y., Fu, B. S., Mak, K. K., et al. (2021b). Engineering multi-tissue units for regenerative medicine: bone-tendon-muscle units of the rotator cuff. *Biomaterials* 272:120789. doi: 10.1016/j.biomaterials.2021.120789

Wolpert, L., Jessell, T., Lawrence, P., Meyerowitz, E., and Smith, J. (2002). *Principles of Development*. Oxford: Oxford University Press.

Yadav, H., Nho, S., Romeo, A., and Macgillivray, J. D. (2009). Rotator cuff tears: pathology and repair. *Knee Surg. Sports Traumatol. Arthrosc.* 17, 409–421. doi: 10.1007/s00167-008-0686-8

Yang, G., Im, H. J., and Wang, J. H. (2005). Repetitive mechanical stretching modulates il-1beta induced cox-2, mmp-1 expression, and pge2 production in human patellar tendon fibroblasts. *Gene* 363, 166–172. doi: 10.1016/j.gene.2005.08.006

Yang, P. J., and Temenoff, J. S. (2009). Engineering orthopedic tissue interfaces. *Tissue Eng. Part B Rev.* 15, 127–141. doi: 10.1089/ten.teb.2008.0371

Yeung, C. Y. C., Zeef, L. A. H., Lallyett, C., Lu, Y., Canty-Laird, E. G., and Kadler, K. E. (2015). Chick tendon fibroblast transcriptome and shape depend on whether the cell has made its own collagen matrix. *Sci. Rep.* 5:13555. doi: 10.1038/srep13555

Yoshimoto, Y., Takimoto, A., Watanabe, H., Hiraki, Y., Kondoh, G., and Shukunami, C. (2017). Scleraxis is required for maturation of tissue domains for proper integration of the musculoskeletal system. *Sci. Rep.* 7:45010. doi: 10.1038/srep45010

Zhou, G., and Groth, T. (2018). Host responses to biomaterials and anti-inflammatory design—a brief review. *Macromol. Biosci.* 18:1800112. doi: 10.1002/mabi.201800112

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GLOSSARY

Terms	Definitions
ADP	Adenosine diphosphate
BMP	Bone morphogenetic protein
CTGF	Connective tissue growth factor
EGR1	Early growth response 1
ECM	Extracellular matrix
FGF	Fibroblast growth factor
HGF	Hepatocyte growth factor
HMGB1	High-mobility group box 1
HOX	Homeobox
IL	Interleukin
MMP	Matrix metalloproteinase
MKX	Mohawk
MYOD	Myogenic differentiation 1
MYF5	Myogenic factor-5
PTHRP	Parathyroid hormone-related protein
PAX	Paired box protein
PDGF	Platelet-derived growth factor
RHOA	Ras homolog gene family member A
RUNX2	Runt-related transcription factor 2
SCX	Scleraxis
SHH	Sonic hedgehog
SOX9	SRY (Sex determining region Y)-box 9
TNMD	Tenomodulin
TGF	Transforming growth factor
TAZ	Transcriptional co-activator with PDZ-binding motif
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor-alpha
C-Met	Tyrosine protein kinase met
VEGF	Vascular endothelial growth factor
WNT	Wingless/integrated
YAP	Yes-associated protein

frontiers in Physiology

ORIGINAL RESEARCH
published: 07 September 2021
doi: 10.3389/fphys.2021.704044

Sepsis Disrupts Mitochondrial Function and Diaphragm Morphology

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OPEN ACCESS

Edited by:
Anselmo Sigari Moriscot, University of São Paulo, Brazil

Reviewed by:
Jia-feng Wang, Second Military Medical University, China
Steve Lancel, Université de Lille, France

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Specialty section:
This article was submitted to Striated Muscle Physiology, a section of the journal Frontiers in Physiology

Received: *01 May 2021*
Accepted: *02 August 2021*
Published: *07 September 2021*

Citation:
Oliveira TS, Santos AT, Andrade CBV, Silva JD, Blanco N, Rocha NN, Woyames J, Silva PL, Rocco PRM, da-Silva WS, Ortiga-Carvalho TM and Bloise FF (2021) Sepsis Disrupts Mitochondrial Function and Diaphragm Morphology. Front. Physiol. 12:704044. doi: 10.3389/fphys.2021.704044

Background: The diaphragm is the primary muscle of inspiration, and its dysfunction is frequent during sepsis. However, the mechanisms associated with sepsis and diaphragm dysfunction are not well understood. In this study, we evaluated the morphophysiological changes of the mitochondrial diaphragm 5 days after sepsis induction.

Methods: Male C57Bl/6 mice were divided into two groups, namely, cecal ligation and puncture (CLP, *n* = 26) and sham-operated (*n* = 19). Mice received antibiotic treatment 8 h after surgery and then every 24 h until 5 days after surgery when mice were euthanized and the diaphragms were collected. Also, diaphragm function was evaluated *in vivo* by ultrasound 120 h after CLP. The tissue fiber profile was evaluated by the expression of myosin heavy chain and SERCA gene by qPCR and myosin protein by using Western blot. The *Myod1* and *Myog* expressions were evaluated by using qPCR. Diaphragm ultrastructure was assessed by electron microscopy, and mitochondrial physiology was investigated by high-resolution respirometry, Western blot, and qPCR.

Results: Cecal ligation and puncture mice developed moderated sepsis, with a 74% survivor rate at 120 h. The diaphragm mass did not change in CLP mice compared with control, but we observed sarcomeric disorganization and increased muscle thickness (38%) during inspiration and expiration (21%). Septic diaphragm showed a reduction in fiber myosin type I and IIb mRNA expression by 50% but an increase in MyHC I and IIb protein levels compared with the sham mice. Total and healthy mitochondria were reduced by 30% in septic mice, which may be associated with a 50% decrease in *Ppargc1a* (encoding PGC1a) and *Opa1* (mitochondria fusion marker) expressions in the septic diaphragm. The small and non-functional OPA1 isoform also increased 70% in the septic diaphragm. These data suggest an imbalance in mitochondrial function. In fact, we observed downregulation of all respiratory chain complexes mRNA expression, decreased complex III and IV protein levels, and reduced oxygen

consumption associated with ADP phosphorylation (36%) in CLP mice. Additionally, the septic diaphragm increased proton leak and downregulated *Sod2* by 70%.

Conclusion: The current model of sepsis induced diaphragm morphological changes, increased mitochondrial damage, and induced functional impairment. Thus, diaphragm damage during sepsis seems to be associated with mitochondrial dysfunction.

Keywords: diaphragm, sepsis, mitochondria, CLP, oxidative phosphorylation, muscle

INTRODUCTION

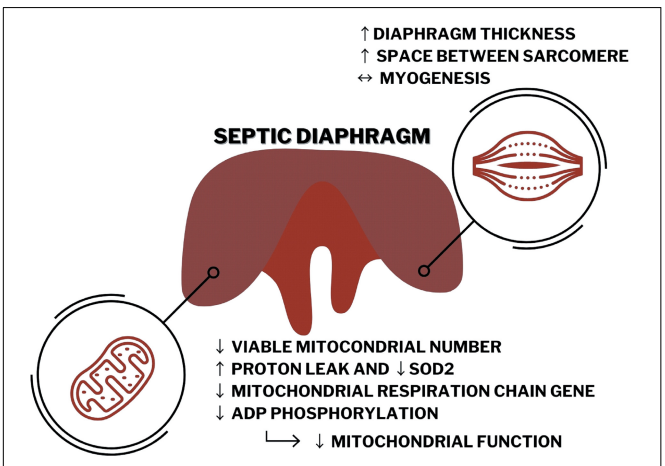
Sepsis is a life-threatening organ dysfunction associated with an imbalance host response to infection (Singer et al., 2016). Sepsis accounts for approximately half of hospital deaths in the United States, and patients usually need intensive care treatment (Thompson et al., 2017). The global sepsis incidence was estimated to be 48 million persons, and 11 million had septic-related deaths in 2017 (Rudd et al., 2020). These data suggest that 20% of worldwide death was related to sepsis (Rudd et al., 2020). Thus, sepsis is a significant public health concern in both high- and low-income countries.

Respiratory failure is a common cause for the admission of septic patients in intensive care units. Additionally, sepsis is a risk factor for developing acute respiratory distress syndrome (ARDS) (Thompson et al., 2017). ARDS accounts for approximately 10% of patients under intensive care treatment and more than 20% of patients under mechanical ventilation, and presents a high mortality rate (Bellani et al., 2016; Thompson et al., 2017). ARDS survivors have higher risks of developing skeletal-muscle weakness and exercise limitations up to 5 years after hospital discharge (Herridge et al., 2011; Thompson et al., 2017; Rudd et al., 2020).

Recent study suggests that respiratory muscles have an essential role in developing ventilatory failure, especially the diaphragm (Bloise et al., 2016; Penuelas et al., 2019). Mechanical ventilation itself can cause diaphragm weakness (Penuelas et al., 2019). However, recent findings demonstrated that diaphragm failure occurs before the mechanical ventilation intervention (Demoule et al., 2013; Supinski and Callahan, 2013; Supinski et al., 2018). Surprisingly, very few groups evaluated the diaphragm physiology during sepsis (Supinski and Callahan, 2013). Patients with the most severe diaphragm weakness presented the worst prognostic (Supinski and Callahan, 2013). Post-sepsis muscle weakness affects patients with no previous comorbidities and can impact life quality after hospital discharge for more than 6 months (Yende et al., 2016; Inayat et al., 2020).

Endotoxemia animal models demonstrated that bacterial lipopolysaccharide (LPS) signaling cascade could increase muscle proteolysis (Ono et al., 2020). Sepsis myopathy is also associated with increased proteasomal degradation, sarcomeric disorganization, and decreased insulin signaling in the peripheral muscle (Callahan and Supinski, 2009). Additionally, skeletal muscle response to sepsis differs according to the muscle fiber type (Owen et al., 2019). The slow-oxidative type I fibers are less affected by sepsis than the fast-glycolytic type II fibers (Owen et al., 2019). The murine septic model by cecal ligation and puncture (CLP) demonstrated decreased skeletal muscle cross-sectional area in the diaphragm and tibial anterior 24 h after surgery (Moarbes et al., 2019). However, the diaphragm returns to the control condition 96 h after CLP, while the tibial anterior cross-sectional area is still reduced compared with the control (Moarbes et al., 2019).

It is crucial to notice that the diaphragm is a continuous active skeletal muscle formed by almost equal amounts of type I and II fibers (Polla et al., 2004). Thus, the diaphragm physiology during sepsis can differ from peripheral muscle (Stana et al., 2017; Talarmin et al., 2017). Understanding these changes could help to treat the septic patients with respiratory failure better. Diaphragm weakness can be correlated with the mortality rate in intensive care units (Supinski et al., 2018). Our group recently demonstrated that severe untreated acute sepsis could reduce the mitochondrial number in the diaphragm and show unbalance in thyroid hormone signaling related to mitochondrial impairment in the diaphragm (Bloise et al., 2020). The mechanisms associated with diaphragm dysfunction during treated and more prolonged sepsis are still not well understood. In this study, we explored molecular pathways and morphological changes in the diaphragm and mitochondria during moderate sepsis to suggest the physiological routes that could disrupt diaphragm physiology.



GRAPHICAL ABSTRACT | Septic diaphragm has impaired morphology and increased thickness that seems to be associated, at least in part, with decreased mitochondrial function related to reducing in *Pgc1α* expression, ATP production, mitochondrial number, and quality in the CLP mice compared with the control group.

MATERIALS AND METHODS

Ethics Statement and Animal Experiment

All animals aged 4–5 weeks used were purchased from the Multidisciplinary Center for Biological Research Chronic – UNICAMP. Animals were acclimated and maintained at the Multiuser Biological Model Laboratory from the Carlos Chagas Filho Institute of Biophysics – UFRJ. The experiments were performed using 12–14 weeks old C57BL/6 male mice weighing 20–26 g. The conventional animals were housed in ventilated cages, 3–4 animals per cage in a 12-h light/dark cycle at a constant temperature (22°C). Water and chow (standard diet AIN93) were available *ad libitum*. CLP is the gold standard murine sepsis model (Laudanski, 2021). Sepsis was induced in the experimental group by CLP; control animals were sham-operated as described previously (Rittirsch et al., 2009; Bloise et al., 2020). In brief, after anesthetized with ketamine (100 mg/kg; Cristália, Itapira, Brazil) and xylazine (10 mg/kg; Syntec, Barueri, Brazil) intraperitoneally, the cecum was located, and below the ileocecal valve the cecal ligation was performed. Then, using an 18G needle, the cecum was perforated once between the ligation and its end. Sepsis severity was scored before any animal manipulation at 8 h after surgery and then every 24 h until 5 days after surgery (the score for measure sepsis severity description is given in Supplementary Material). Animals were treated with Meropenem (20 mg/kg, Aspen Pharmacare, Durban, South Africa) intraperitoneally 8, 24, 48, 72, and 120 h after surgery. Animals were euthanized under anesthesia with 1.5–2.0% of isoflurane (Isoforine, Cristália, SP, Brazil) 120 h after surgery. Diaphragms were collected, immediately frozen at liquid nitrogen, and stored at −70°C for the molecular biology analysis. The freshly dissected diaphragms were also collected for mitochondrial physiology measurement or immerse in 4% of paraformaldehyde for further microscopy analysis. The animal handling and euthanasia procedures were approved by the Federal University of Rio de Janeiro Animal Care Committee (CEUA-088/15).

mRNA Isolation and qPCR

The total RNA from diaphragm samples (15 mg) was extracted using the TRIzol Reagent (Invitrogen, Carlsbad, CA, United States) and Macherey Nagel Kit (Macherey Nagel, Düren, DE) as described previously (Herridge et al., 2011). The cDNA synthesis was performed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, CA, United States) with 800 ng of total RNA according to the protocols of the manufacturer. After the cDNA synthesis, mRNA expressions were evaluated by qPCR using the HOT FIREPol® Evagreen® qPCR Supermix (Solis Biodyne, Denmark) and the Mastercycler Realplex system (Eppendorf, Germany). **Table 1** contains the sequences of the primer pair. In summary, the following fiber profiles were evaluated: *Myhc7*, *Myhc4*, *Atp2a1*, and *Atp2a2*; the following myogenesis are investigated: *Myod1* and Myog; the following mitochondrial physiology are analyzed: *Ppargc1a*, *Opa1*, and *Dnm1l*; the following respiratory chains were investigated: *Ndufb8*, *Sdhb*, *Uqcrc2*, and *Cox4i1*; and the following reactive oxygen defenses are investigated: mitochondrial superoxide dismutase 2 (SOD 2; *Sod2*). Relative mRNA expression quantification was calculated using the standard curve method, and the expression level is related to the mean of the reference gene values (*Phctr* and *Pib* for diaphragm and *Vdac* for genes located in the mitochondrion). The best reference genes were chosen according to their *Cq* values and variances between the groups. PCR programs were as follows: denaturation 12 min 95°C, 40 cycles of 15 s 95°C, 30 s 60°C, and 30 s 72°C, following melting program. qPCR quality and genomic DNA contaminations were checked using Intron-spanning primers, reverse transcriptase-negative samples from cDNA synthesis, and melting curve analysis obtained from each reaction.

Skeletal Muscle High-Resolution Respirometry

For the high-resolution respirometry (HRR), 3 mg of the diaphragm was dissected from the right area of the costal muscle domain. The tissue was washed in chilled BIOPS buffer (2.77 mM CaK_2EGTA, 7.23 mM K_2EGTA, 20 mM imidazole, 20 mM taurine, 6.56 mM $MgCl_2$, 5.77 mM ATP, 15 mM phosphocreatine, 0.5 mM dithiothreitol, and 50 mM K-MES, pH 7.1). Then, it was permeabilized in cold BIOPS buffer supplemented with saponin (50 μg/ml) on ice for 40 min with gentle agitation. Permeabilized tissues were weighed, and samples were then transferred to chilled Mir05 buffer (110 mM sucrose, 60 mM K-MES, 0.5 mM EGTA, 3 mM $MgCl_2$, 20 mM taurine, 10 mM KH_2PO_4, 20 mM K-HEPES, 1 g/L de bovine serum albumin (BSA) fatty acid free, pH 7.1). Tissues were maintained in Mir05 buffer for at least 10 min under mild agitation to remove saponin and metabolites and then transferred to measure oxygen consumption with an O2K (Oroboros Instruments GmbH, Innsbruck, Austria). Each O2K chamber was calibrated with Mir05 for at least 20 min before data acquisition.

Two sequential runs were performed for each tissue using one chamber for the sham tissue and the other for the CLP. In the next run, freshly permeabilized tissue was positioned in the opposite chambers to avoid errors associated with the equipment. The data of each run were treated as duplicate data for each animal. Thus, during further data analysis, the experimenter did not know which sample was from the diaphragms of sham and CLP. The background oxygen level from the tissue in Mir05 alone was recorded for at least 5 min, followed by substrate or drug administration. All substrate and drug administrations were titrated previously during test standardization. The oxygen consumption was recorded until stabilization after the addition of drugs or substrates as mentioned in the following sequence: 5 mM pyruvate and 5 mM malate (for recording basal respiration after substrate administration and stimulating complex I), 3 mM ADP (saturated ADP concentration to record coupled respiration), 10 μM cytochrome *c* (for assessing mitochondrial damage during permeabilization, O_2 consumption increase above 5% was considered damage induced by the tissue process, and the data were excluded from the analysis), 10 mM succinate (coupled respiration associated with complex II), 2 μg/ml oligomycin

TABLE 1 | Primer list.

Gene	Primer sequences (forward top, reverse bottom)	GenBank accession no.	Amplicon size	Protein encoded (related function)
Phactr4	ACTTTACACGCTACCATCGCCCAT AAGGGGAGCACAAGGACACG	NM_175306	190 bp	Phosphatase and Actin Regulator 4 (reference gene)
Ppib	GAGACTTCACCAGGGG CTGTCTGTCTTGGTGCTCTCC	NM_011149	253 bp	Peptidylprolyl Isomerase B (reference gene)
Vdac	GGGCTGACGTTTACAGAGAAG CTCATAGCCAAGCACCAGAGC	NM_001362693	240 bp	Voltage-Dependent Anion Channel 1 (mitochondrial reference gene)
Ppargc1a	CAATGAATGCAGCGGTCTA GTGTGAGGAGGGTCATCGTT	NM_008904.2	112 bp	Peroxisome Proliferator-Activated Receptor Gamma Coactivator 1-Alpha (PGC1α – a key regulatory factor of mitochondria biogenesis and function)
Opa1	TGGAAAATGGTTCGAGAGTCAG CATTCCGTCTCTAGGTTAAAGCG	NM_001199177.1 NM_133752.3	77 bp	OPA1 Mitochondrial Dynamin Like GTPase (promotes mitochondria fusion)
Dnm1l	TAAGCCCTGAGCCAATCCATC CATTCCCGGTAAATCCACAAGT	NM_001360010.1	77 bp	Dynamin 1 Like (promotes mitochondria fission)
Ndufb8	TGTTGCCGGGGTCATATCCTA AGCATCGGGTAGTCGCCATA	NM_026061	127 bp	NADH:Ubiquinone Oxidoreductase Subunit B8 (a subunit of the respiratory complex I)
Sdhb	AATTTGCCATTTACCGATGGGA AGCATCCAACACCATAGGTCC	NM_001355515	104 bp	Succinate Dehydrogenase Complex Iron-Sulfur Subunit B (a subunit of the respiratory complex II)
Uqcrc2	TCTCTGGAAAACTATGCTCCTCT AAATGTGAGGTTCCCAAGTTGT	NM_025899.2	95 bp	Ubiquinol-Cytochrome C Reductase Core Protein 2 (a subunit of the respiratory complex III - gene in the mitochondrion)
Cox4i1	TCCCCACTTACGCTGATCG GATGCGGTACAACTGAACTTTCT	NM_009941	149 bp	Cytochrome C Oxidase Subunit 4I1 (a subunit of the respiratory complex IV)
Sod2	CCAAGGGAGATGTTACAACTCAG GGGCTCAGGTTTGTCCAGAA	NM_013671	100 bp	Superoxide Dismutase 2 (mitochondrial defense against oxygen reactive species, converts superoxide into hydrogen peroxide and diatomic oxygen)

(ATP synthase inhibitor – oxygen consumption related to proton leak or the uncoupled respiration, the dose was divided into two additions, but only the O_2 consumption value after the second pulse was used for the analysis), 600 nM carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (mitochondrial uncoupling agent, related to maximal respiration), and 10 mM potassium cyanide (KCN) (inhibitor of cytochrome *c* oxidase and non-mitochondrial oxygen consumptions). All O_2 consumption raw data were subtracted from KCN values to discard non-mitochondrial oxygen consumption. After succinate, the subtraction of oxygen consumption values from the values after oligomycin gives us the oxygen consumption related to ADP phosphorylation (coupled respiration). The pmol O_2 consumption was normalized by corresponding sample weight. The pyruvate, saponin, and KCN solutions were freshly made before each experiment.

Transmission Electron Microscopy

Transmission electron microscopy (TEM) and mitochondrial quantification were performed as described previously (Bloise et al., 2020). In brief, diaphragms were immersed in 4% of paraformaldehyde for at least 48 h. The samples were then processed and analyzed (qualitatively and quantitatively) using a JEM1011 microscope (JEOL, Akishima, Tokyo, Japan). The fragments were washed in phosphate-buffered saline, fixed in 2.5% of glutaraldehyde sodium cacodylate (100 mM) buffer (pH 7.2) for 24 h, and washed three times in the same buffer. The tissue was post-fixed in 1% osmium tetroxide and sodium cacodylate buffer, dehydrated in acetone (30, 50, 70, 90, and 100%), and embedded in Poly/Bed812 resin (Ted Pella Inc., Redding, CA, United States). Ultrathin sections were contrasted with uranyl acetate-lead citrate and were collected in three grids with a distance of 7 μm between them. A total of 10 images were acquired from random fields in each grid (AMT XR80 CCD digital camera – Advanced Microscopy Techniques, Woburn, MA, United States; at 12,000× magnification, 70 nm). The total amount of mitochondria and the number of healthy and injured mitochondria were quantified in each electron micrograph. At least three images per tissue were analyzed. The qualitative evaluation consisted of analyzing the integrity of mitochondrial internal and external membranes, the mitochondrial morphology, and the matrix electron density. Healthy mitochondria have intact external and internal (mitochondrial cristae) membranes, with no signs of membrane disruption, continuous folds called crista, and a very electron-dense matrix (**Supplementary Figure 1**).

In vivo Diaphragm Ultrasonography

Ultrasonography was performed and analyzed as described previously (Whitehead et al., 2016; Buras et al., 2019). The methodology was previously validated as a non-invasive methodology to access murine diaphragm function *in vivo* in a high-resolution ultrasonography system designed for mouse echocardiography, i.e., the Vevo 2100 (Fujifilm VisualSonics, Toronto, Canada) from the National Center for Structural Biology and Bioimaging (CENABIO-UFRJ, RJ, Brazil). The mice

were anesthetized in an isoflurane chamber (1.5% isoflurane and 1.5% O_2 L/min). Mice were fixated on the table by the four limbs taped onto the platform copper leads. Anesthesia was maintained using a nose-cone perfused with 1% isoflurane and 1.5% oxygen L/min. The chest and the abdomen of the mouse were shaved using depilatory cream to avoid any interference during the acquisition of the images. A small amount of ultrasound gel was applied between the animal skin and the probe (MS400) to guarantee proper image recording, as well as on the platform copper leads (**Supplementary Figure 2**). For diaphragm ultrasonography, each mouse was placed in a dorsal decubitus position, and the platform was tilted horizontally by 30° with the head of the mouse lower with respect to the feet. The probe was fixed transversally to the mid-sternal of the mouse at 180° with respect to the platform. Image acquisition was performed in mono-dimensional (M-Mode) and bi-dimensional (B-Mode). We obtained the following data: diaphragm thickness during inspiration and expiration, the thickness fraction, diaphragm excursion amplitude (excursion), inspiratory duration, peak-to-peak time, the excursion–time (E–T) index (product of diaphragm excursion and inspiratory period), and inspiratory duty cycle (quotient of inspiratory duration/peak-to-peak time). The thickness fraction is the measure of the thickness on inspiration (Ti) minus the thickness on expiration (Te) divided by the thickness on expiration; thus, the formula is (Ti - Te)/Te.

Western Blot Analysis

The total protein was extracted from diaphragm fragments (±30 mg) in pH 7.4 RIPA lysis buffer (50 mM Tris–HCl, 150 mM NaCl, 1.0% Triton X-100, 0.1% SDS, 5 mM EDTA, 50 mM NaF, 30 mM sodium pyrophosphate tetrabasic, and 1 mM sodium orthovanadate) with complete protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN, United States) using the TissueLyser LT (QIAGEN). The total protein was quantified using a Pierce BCA Protein Assay Kit (Thermo Scientific) and was separated (10 μg) by SDS–PAGE in 8, 10, or 12% polyacrylamide gels and transferred onto a polyvinylidene difluoride membrane (Hybond-P 0.45 mm PVDF; Amersham Biosciences, United Kingdom) for 90 min at 0.25 A in a Bio-Rad wet system (Bio-Rad Laboratories, United States). The membranes were incubated with 5% of BSA (Sigma Life Science, United States) for 90 min at room temperature to block non-specific binding sites and then incubated overnight at 4°C with primary antibodies. The antibodies used were as follows: anti-Total OXPHOS Rodent WB Antibody Cocktail (Gel 12%; 1:1,000 dilution; cat. no. ab110413, Abcam, United Kingdom), OPA1 (Gel 10%; cat. no. 80471; 1:1,000 dilution, Cell Signaling Technology, Inc.), MyHC I (Gel 8%; 300 ng/ml; BA-F8; Developmental Studies Hybridoma Bank, United States), MyHC II A (Gel 8%; 300 ng/ml; SC-71; Developmental Studies Hybridoma Bank, United States), MyHC II B (Gel 10%; 300 ng/ml; BF-F3; Developmental Studies Hybridoma Bank, United States), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 1:10,000 dilution; cat. no. 14C10, Cell Signaling Technology, United States) as the loading control. Alternatively, the membranes were then washed and incubated with peroxidase-labeled secondary anti-mouse antibody (1:10,000 dilution, cat. no. 62-6520, Invitrogen, United States) or anti-rabbit antibody (1:10,000 dilution, cat. no. 65-6120, Invitrogen, United States) for 150 min at room temperature. Immunoreacted proteins were detected by SuperSignal West Pico PLUS substrate or SuperSignal West Femto substrate (Thermo Fisher) in an ImageQuantLAS 4000 system, followed by densitometric analyses (GE Healthcare Life Sciences, United States). The Ponceau staining (Sigma Life Science, United States; cat. no. P7170) was used as the loading control in 8% gel. Data were corrected by the loading control and expressed relative to the Sham group.

Statistical Analysis

The Kolmogorov–Smirnov test was used to test data normality and Grubbs' test was used to remove outliers (alpha = 0.05). We used the Mann–Whitney *U* test (non-normally distributed data) or Student's *t*-test (normally distributed data) to analyze the differences between groups. The Kaplan–Meier analysis was performed to investigate the survivor rate using Log-rank (Mantel-Cox) test to compare the mortality rates between CLP and sham groups. The results are shown as mean values ±SD of at least three animals per group. Statistical analysis was performed using the GraphPad Prism 5 software (GraphPad Software, Inc., San Diego, CA, United States). Differences were considered to be significant at $p < 0.05$. Symbols were used to describe p values < 0.05.

RESULTS

Sepsis Clinical Score and Mortality

Sepsis was induced by CLP plus antibiotic treatment for 5 days. CLP mice presented a higher mortality rate compared with sham. Almost half of the septic mice did not survive (**Figure 1A**). Additionally, the survivor rate markedly decreases 72 h after the CLP. The majority of CLP animals developed moderate sepsis throughout the experiment (**Figure 1B**).

Prolonged Sepsis Disturbs Diaphragm Ultrastructure but Does Not Function

Cecal ligation and puncture-induced sepsis modified diaphragm ultrastructure presenting unallied and thinner sarcomeres, as can be observed by discontinuous Z lines and the low-electrodense spaces between sarcomeres (**Figures 2A,B**). These morphological changes could indicate a decrease in muscle mass. However, we did not observe a reduction in diaphragm weight (**Figures 2C,D**). Since the morphological change in the septic diaphragms could be related to an imbalance in tissue regeneration, the expressions of two myogenesis master regulators, namely, *Myod1* and *Myog*, were investigated, but no significant differences were observed between CLP and sham diaphragms (**Figures 2E,F**).

Then, we evaluated the expression fiber profile markers to better understand the physiological changes related to the diaphragm ultrastructural modification in CLP mice. The diaphragm is a mixed fiber and thus expresses both type I

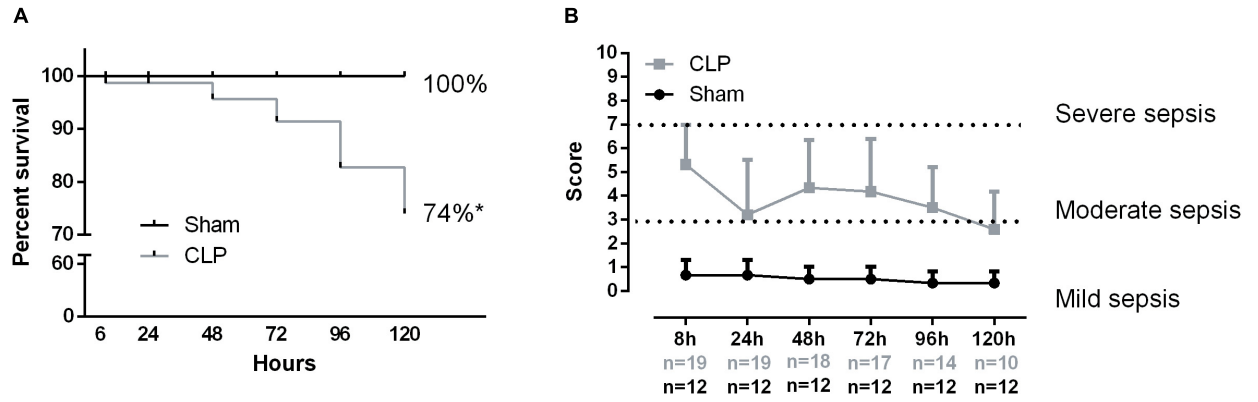


FIGURE 1 | Sepsis clinical score and mortality. Animal survival rate **(A)**. The percentage compares the total live animal numbers at the beginning (0 h) and end of the experiment (120 h). Sepsis score time evolution **(B)**. The following variables evaluated the severity of sepsis: piloerection, alterations in gait, lethargy, alterations in respiratory rate, lacrimation, loss of grip strength, decreased body tone, respiratory difficulty after manipulation, lack of exploratory behavior, and body temperature alterations if present animal received 1 point. The sum of the total score reflected the severity of sepsis. Score 2–3 denotes mild sepsis, 4–7 moderated sepsis, 8–10 severe sepsis. Values under the *x*-axis time point represent the live animal number in each group, gray numbers represent CLP total animals, and black numbers represent the sham group. Black dots represent sham animals caused by surgical stress (n = 12), and gray squares represent CLP – cecal ligation puncture technique (n = 19 at 0 h and n = 10 at 120 h). Values expressed as mean ± SD, *p = 0.0007 chi-square, and **p = 0.0001 unpaired t-test compared with sham mice.

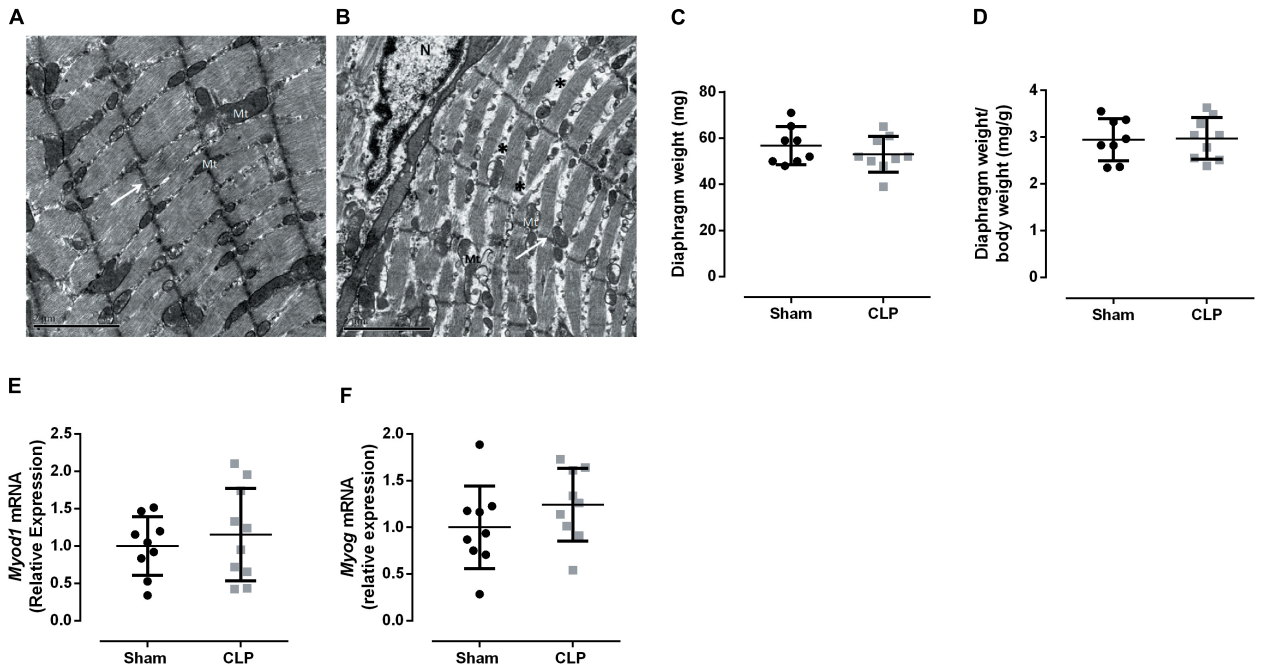


FIGURE 2 | Prolonged sepsis disturbs diaphragm morphology. Transmission electron micrograph of Sham **(A)** and CLP **(B)** diaphragm fragments, white arrows indicate Z lines, * exemplify low electrodense areas between sarcomeres, *N* indicates the nucleus and Mt mitochondria, scales bars represent 2 μm. Diaphragm total weight **(C)** and related to animal body weight **(D)**. Expression of *Myod1* (expressed by activated satellite cells and myoblast; **E**), *Myog* (expressed by myocytes and early myotubes **F**). Black dots represent sham animals caused by surgical stress, and gray squares represent CLP – cecal ligation puncture technique. Values are expressed as mean ± SD, *$p < 0.05$, and **$p < 0.005$ compared with the sham group.

fibers, characterized by myosin I and SERCA 2a expression, and type II fibers, represented by myosin II (a, x, and b) and SERCA 1 expression (Greising et al., 2015). We investigated myosin and expression of SERCA isoforms from type I and type II fibers, *Myhc7* (myosin I, expressed in slow oxidative fibers), Atp2a2 (SERCA 2a), and *Myh4* (myosin IIb, the faster myosin expressed in the more glycolytic fiber), *Atp2a1* (SERCA 1), respectively. Related to the fiber I profile, we observed a decrease in myosin I and SERCA 2a mRNA expressions by 40 and 30% in septic animals, respectively, compared with sham. The myosin IIb expression and SERCA 1 decreased by 60 and 40% in the CLP diaphragm compared with the control expression,

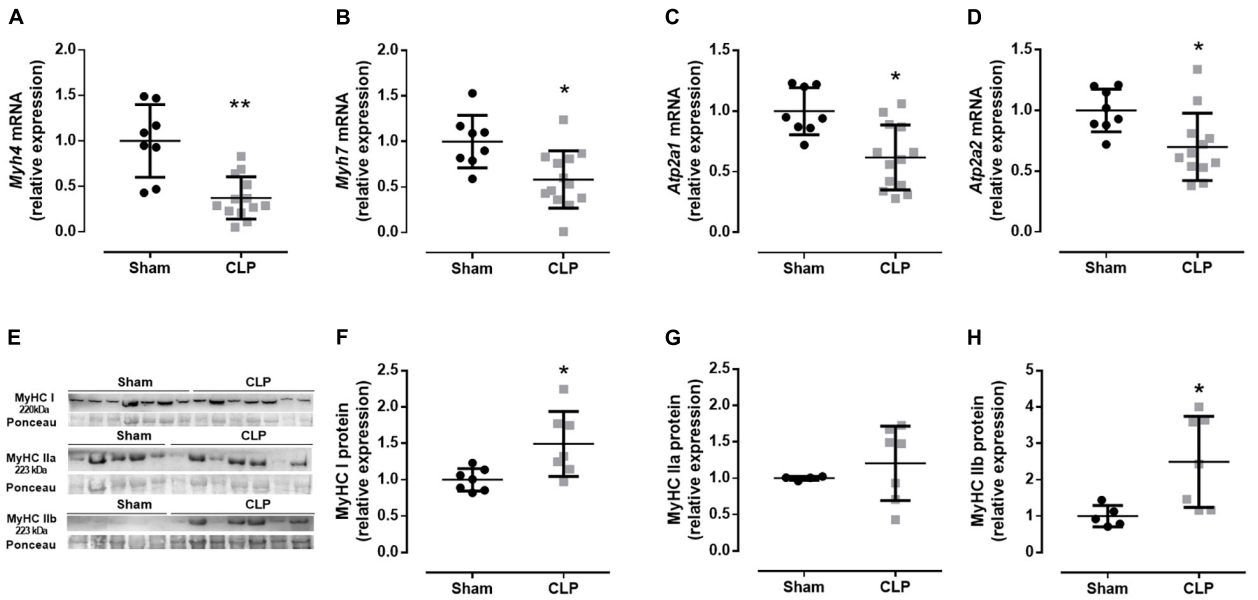


FIGURE 3 | Moderate sepsis modulates fiber profile. mRNA expression of *Myh4* (encodes MHC IIb expressed in fast-glycolytic type II fiber; **A**), *Myh7* (encodes MHC I expressed in slow-oxidative type I fiber; **B**), *Atp2a1* (encodes SERCA1 expressed in fast-glycolytic type II fiber; **C**), and *Atp2a2* (encodes SERCA2 expressed in slow-oxidative type I fiber; **D**) in the diaphragm. Western blot representative figure for measuring MyHC I (fiber type I marker), MyHC IIa (fiber type IIa marker), and MyHC IIb (fiber type IIb marker; **E**). Relative protein expression of MyHC I **(F)**, MyHC IIa **(G)**, and MyHC IIb **(H)**. Black dots represent sham animals caused by surgical stress, and gray squares represent CLP – cecal ligation puncture technique. Values are expressed as mean ± SD, $^{*}p < 0.05$, and $^{**}p < 0.005$ compared with the sham group.

respectively (**Figures 3A–D**). The mRNA data suggested a reduction in type I and II fibers. However, the myosin protein expression demonstrated an increase in type I and IIb fibers and no modulation in type IIa fiber in the CLP diaphragm (**Figures 3E–H**). We observed an increase in MyHC I (49%) and IIb (140%) in the CLP diaphragm compared with sham (**Figures 3E,F,H**).

Then, we investigated diaphragm function *in vivo* using ultrasonography (**Figure 4**). Although diaphragm mass did not change during sepsis (**Figures 2C,D**), we observed an increase in diaphragm thickness in inspiration (38%) and expiration (21%) in CLP compared with sham (**Figures 4A,B**). Thickness fraction, amplitude excursion, and excursion velocity did not change in CLP mice (**Figures 4C–E**). The other functional ultrasound data, i.e., duty cycle (the fraction of each breath spent during inspiration) and E–T index, did not change in CLP compared with control (**Figures 4F,G**).

Sepsis Disrupted Diaphragm Mitochondrial Function

The electron micrograph from **Figure 2** suggested a decrease in the mitochondrial amount in the CLP diaphragm. The total and healthy mitochondria decreased by 35% in septic mice than sham-operated (**Figures 5A,B**). Then, we investigated the PGC1α gene expression (encodes by Ppargc1a), a key mitochondriogenesis stimulator. We observed that *Ppargc1a* expression decreased by half in the septic diaphragm (**Figure 5C**), suggesting that mitochondriogenesis was not triggered by the decrease in healthy mitochondria available in the septic diaphragm.

Subsequently, we investigated the fusion and fission dynamics by *Opa1* (a mitochondrial fusion marker) and *Dmn1l* (a mitochondrial fission marker) mRNA expression. In fact, we observed a 50% reduction in the fusion marker *Opa1* in septic diaphragms (**Figure 5D**). The functional long OPA1 isoform (l-OPA1) did not change between the groups (**Figures 5E–G**), but the non-functional small OPA1 (s-OPA1) protein level increased by 70% in the septic diaphragm (**Figures 5E–G**). The fission marker *Dmn1l* mRNA expression did not change between groups (**Figure 5H**). Additionally, we then investigated the antioxidant mitochondrial defense expression, namely, the superoxide dismutase 2 (Sod2). The diaphragm *Sod2* expression decreased by 70% in the CLP group compared with the control group (**Figure 5I**).

The decrease in healthy mitochondrial amount (**Figure 5**) could suggest a reduction in oxidative phosphorylation function during sepsis. Therefore, we analyzed the respiratory chain complexes I–IV (*Ndufb8*, *Sdhb*, *Uqcrc2*, *Cox4i1*, respectively) mRNA expressions and protein levels. All gene expressions were reduced in the septic diaphragm group (**Figures 6A–D**). The protein levels of respiratory chain complexes III (UQCR2) and IV (MTCO-1) decreased by 36 and 42%, respectively, in the CLP group compared with the control (**Figures 6E–J**). These reductions could impair mitochondrial transmembrane electrochemical gradient and, thus, disrupt oxidative phosphorylation. Then, we investigated diaphragm oxygen consumption by HRR. After addition of pyruvate and

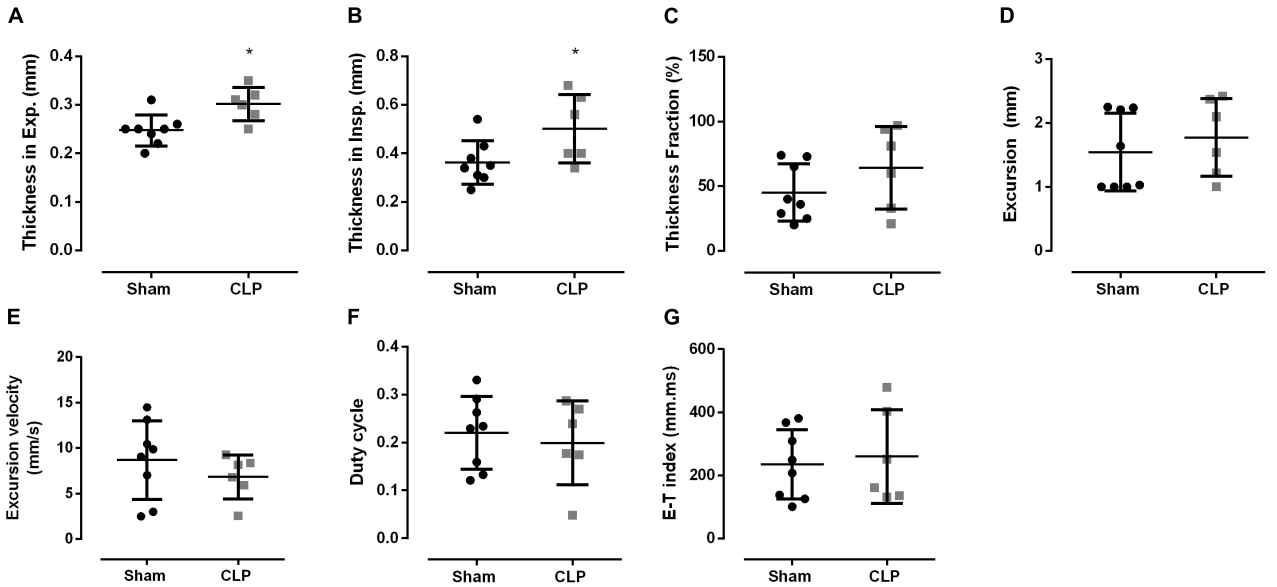


FIGURE 4 | Diaphragm function is maintained during sepsis. Diaphragm thickness during expiration **(A)** and inspiration **(B)**; thickness fraction, an indication of contractability work, is the measure of the thickness on inspiration minus the thickness on expiration divided by the thickness on expiration **(C)**; excursion (movement amplitude, **D**); excursion velocity **(E)**, duty cycle (inspiratory duration/peak to peak, **F**), E–T index **(G)**. Black dots represent sham animals caused by surgical stress, and gray squares represent CLP – cecal ligation puncture technique. Values are expressed as mean ± SD and $^{*}p < 0.05$ compared with the sham group.

malate, the basal respiration did not change between CLP and sham diaphragm (sham: 25.60 ± 2.34 pmol O_2/mg*s, $N = 10$; CLP: 21.35 ± 2.59 pmol O_2/mg*s, $N = 13$). Also, the maximal respiration measured after the addition of FCCP did not change (sham: 39.48 ± 3.85 pmol O_2/mg*s, $N = 10$; CLP: 34.38 ± 1.69 pmol O_2/mg*s, $N = 13$). However, we observed a 40% decrease in the oxygen consumption associated with ADP phosphorylation in septic mice (**Figure 6K**). Additionally, the oxygen consumption related to proton leak increased by 35% in septic diaphragms related to basal respiration (**Figure 6L**), but this absolute value did not change (sham: 26.40 ± 2.69 pmol O_2/mg*s, $N = 10$; CLP: 25.89 ± 2.22 pmol O_2/mg*s, $N = 13$).

DISCUSSION

In lung physiology, respiratory disruption caused by sepsis is well established (Bloise et al., 2016; Penuelas et al., 2019). However, recent findings suggest that sepsis also decreases peripheral skeletal muscle function (Owen et al., 2019). In fact, sarcomeric disorganization, increased protein degradation, and unbalancing energy production are present in peripheral muscle from critically ill patients (Callahan and Supinski, 2009). In contrast, the impact of sepsis on the diaphragm, the primary respiratory skeletal muscle, is not well understood. Herein, we demonstrated that antibiotic-treated moderate sepsis leads to decreased mitochondrial function and changes in diaphragm ultrastructure (Graphical Abstract).

Sepsis affects peripheral skeletal muscle mass differently depending on the fiber type (Bloise et al., 2016; Owen et al., 2019). The fast-glycolytic muscle mass decreases more than the slow-oxidative muscle mass during sepsis (Owen et al., 2019). Moreover, the gastrocnemius type II fiber showed sepsis-mediated atrophy, while the type I fiber demonstrated minor atrophy (Owen et al., 2019). Thus, the data from peripheral skeletal muscle may not be directly translated to the diaphragm, a mixed fiber muscle.

The Extensor digitorum longus (EDL), fast-twitch muscle, *ex vivo*-specific force decreases 1 week and 1 month in septic induced by cecal slurry compared with control mice (Owen et al., 2019). Moreover, the *ex vivo* diaphragm function was reduced 6 and 24 h after sepsis induction (Zolfaghari et al., 2015; Eyenga et al., 2021). This model resamples severe acute sepsis. Our sepsis animal model induces 5-day moderate sepsis (**Figure 1**). Additionally, our group demonstrated that acute and chronic systemic inflammation causes differential modulation in diaphragm physiology (Bloise et al., 2016). In this study, we showed that moderate sepsis reduces mitochondrial function and leads to diaphragm adaptation to maintain functional activity.

An increase in diaphragm thickness is associated with local inflammation or diaphragm work (Picard et al., 2012; Vivier et al., 2012). We did not observe inflammatory signs on the septic diaphragm on the electron micrographs. Thus, the augment in the diaphragm thickness in CLP mice might be related to an increase in diaphragmatic work. Therefore, our data suggest a compensatory adaptation during the septic progression.

Additionally, *ex vivo* data demonstrated that maximal isometric force generated was reduced in 24 h septic diaphragm strips compared with control fibers (Zolfaghari et al., 2015). The *ex vivo* force measure of the extensor digitorum longus (a muscle rich in type II fibers) demonstrated a decrease in specific

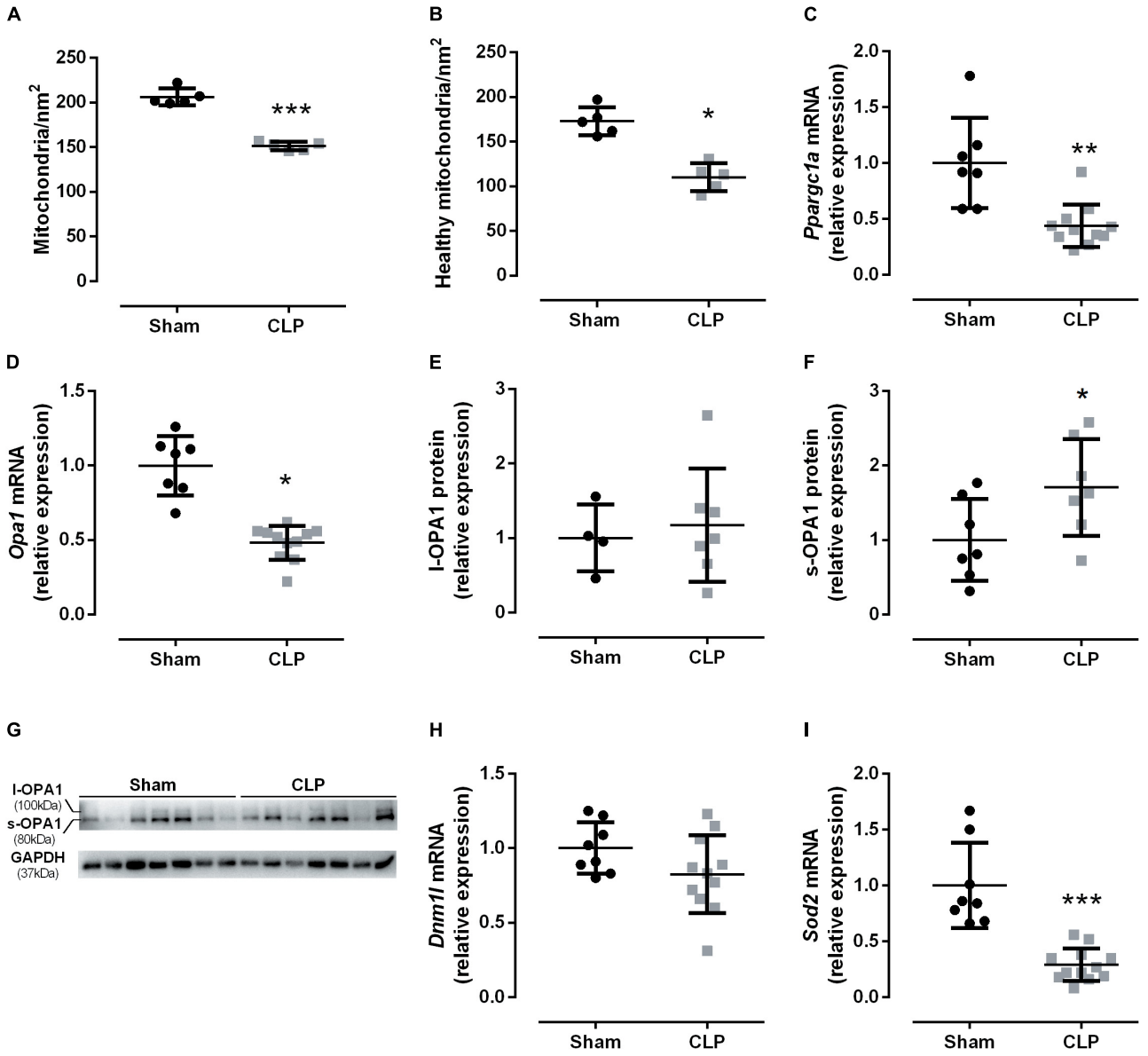


FIGURE 5 | Sepsis disrupted diaphragm mitochondrial dynamics. Quantification of mitochondrial total **(A)** and healthy **(B)** quantity. The mitochondrial quality was assessed by the following factors: disrupting the mitochondrial membranes (injured mitochondria), the mitochondrial morphology: the preservation of the mitochondrial cristae, and the matrix electron density (healthy mitochondria), intact external membranes (healthy mitochondria), intact cristae and continuous folds (healthy mitochondria), and matrix electron density (injure mitochondria present low electron-dense matrix). mRNA expression of *Ppargc1a* (encodes PGC1α; **C**); *Opa1* (fusion marker; **D**); relative protein expression of the functional l-OPA1, 100 kDa isoform **(E)**, cleaved isoform s-OPA1, 80 kDa **(F)**, OPA1 and GAPDH (loading control) representative Western blot gels **(G)**. mRNA expression of *Dnm1l* (fission marker; **H**) and *Sod2* (encodes mitochondrial superoxide dismutase – SOD2; **I**). Black dots represent sham animals caused by surgical stress, and gray squares represent CLP – cecal ligation puncture technique. Values are expressed as mean ± SD, $^{*}p < 0.05$, $^{**}p < 0.005$, and $^{***}p = 0.0002$ compared with sham group.

force generation in 2 weeks and 1-month sepsis survivor mice (Owen et al., 2019). This group did not observe a change in soleus, gastrocnemius, tibial anterior, and extensor digitorum longus weight corrected to body weight (Owen et al., 2019). In our sepsis model, the diaphragm weight did not change as well. Although, the ultrasound data demonstrated an increase in the tissue thickness and no alteration in thickness fraction, indicating contractability work. Thus, we cannot discard that the augment in the diaphragm thickness is a physiological adaptation of the survivor septic mice that helped maintain the muscle work 120 h after CLP.

It is essential to notice that moderated sepsis manifestation represents most human septic cases (Paoli et al., 2018). Thus, understanding the diaphragm adaptations during prolongated sepsis models could help improve the knowledge associated with the impaired respiratory capacity observed in septic patients.

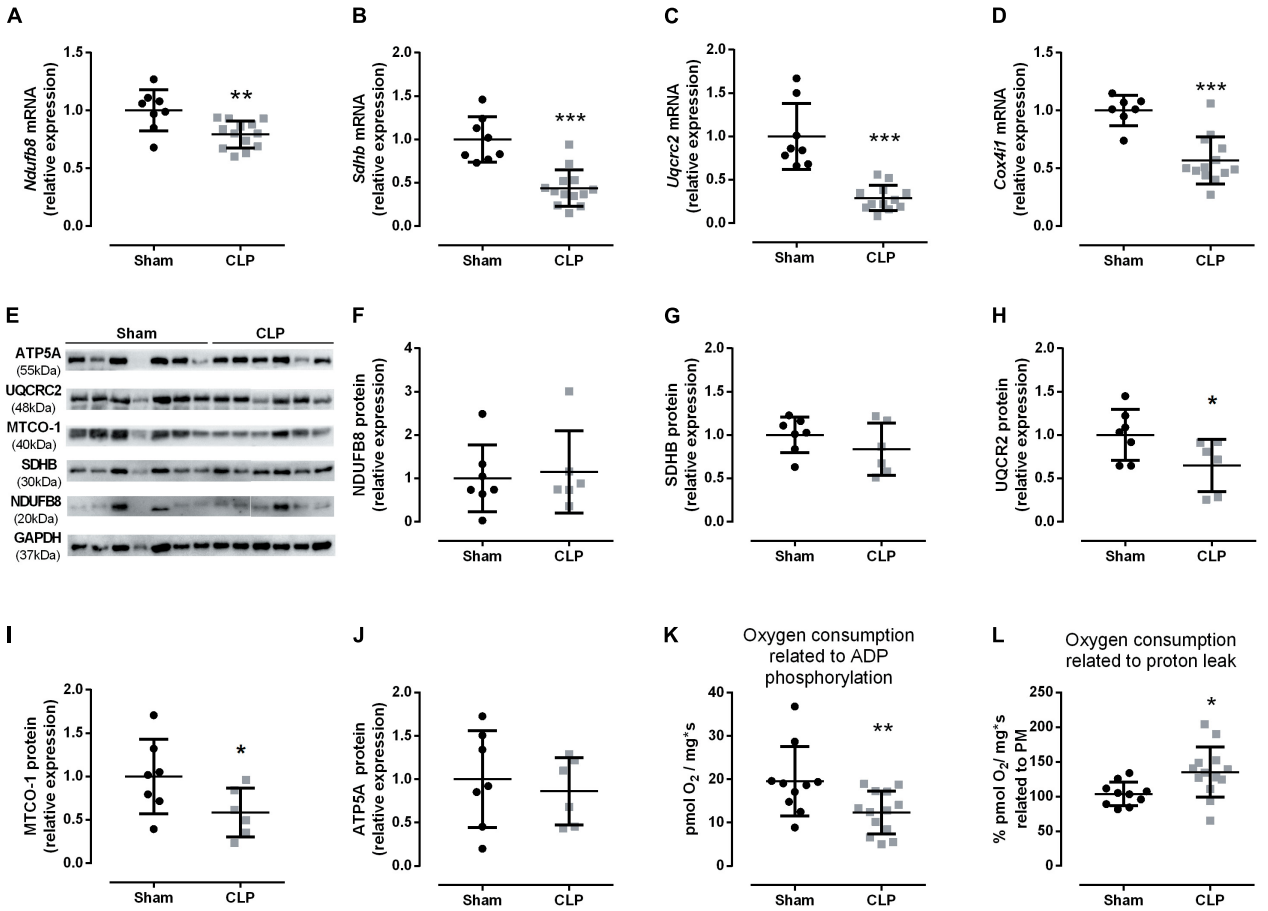


FIGURE 6 | Sepsis impairs diaphragm mitochondrial function. Expression of complex I (*Ndufb8* – **A**); II (*Sdhb* – **B**); III (*Uqcrc2* – **C**), and IV (*Cox4i1* – **D**) in the diaphragm. Representative protein expression blot from complexes I–V **(E)**; protein quantification of complex I (NDUFB8 – **F**); complex II (SDHB – **G**); complex III (UQCR2 – **H**); complex IV (MTCO1 – **I**); ATP synthase (complex V; ATP5A – **J**). Diaphragm HHR: oxygen consumption coupled to ADP phosphorylation (O_2 consumption after succinate administration values minus O_2 consumption after oligomycin administration values), the data values are an absolute measure of O_2 consumption **(K)**; oxygen consumption related to proton leak **(L)**, values represent the percentage of O_2 consumption after oligomycin administration related to pyruvate and malate O_2 consumption values. Black dots represent sham animals caused by surgical stress, and gray squares represent CLP – cecal ligation puncture technique. Values are expressed as mean ± SD, $^{*}p < 0.05$, $^{**}p < 0.005$, and $^{***}p < 0.001$ compared with sham group.

Herein, we observed sarcomeric disorganization associated with modulating the fiber profile in the septic diaphragm (**Figures 2**, **3**).

Endotoxemia induces muscle wasting *via* autophagy and proteolysis 48 h after LPS injection (Ono et al., 2020). Additionally, soleus (muscle rich in slow oxidative type I fibers) demonstrated a decrease in cross-sectional area of all type II fibers 4 days after sepsis (Owen et al., 2019). Autophagy and proteasome pathways were increased in the septic diaphragm (Stana et al., 2017). We observed an increase in the spaces between sarcomeres in the CLP diaphragm, suggesting tissue wasting or injury (**Figures 2A,B**). The expression of myogenic transcription regulators did not change in the septic diaphragm (**Figures 2E,F**). Suggesting there is not a regenerative process occurring at this time point in the septic diaphragm. However, the fiber profile was modulated in the CLP muscle. We observed a reduction in myosin I and IIb and SERCA1 and 2a mRNA expression (**Figures 3A–D**), while the myosin I and IIb protein expression increased (**Figures 3E–H**). During muscle plasticity, the mRNA and protein levels can be different (Andersen and Schiaffino, 1997). Septic diaphragm fiber size and cross-sectional area decrease, also protein degradation increase during the first 24 h after sepsis (Stana et al., 2017; Talarmin et al., 2017; Moarbes et al., 2019). However, these parameters are restored 96 h to 7 days after sepsis (Stana et al., 2017; Talarmin et al., 2017; Moarbes et al., 2019). Thus, our myosin mRNA and protein data suggest that the septic diaphragm is a transitional tissue. Probably, more prolonged sepsis could lead to a decrease in type I and IIb fibers.

During stress, the mitochondrial fusion process attenuates the damage caused by injured organelles (Gustafsson and Dorn, 2019). Thus, healthy and partially damaged mitochondria fuse to maintain overall function (Youle and van der Bliek, 2012; Gustafsson and Dorn, 2019). Herein, we demonstrated that septic diaphragms reduce the healthy mitochondrial amount. Additionally, the septic diaphragm decreased the *Opa1* mRNA and increased s-OPA1 protein isoform, which does not induce

mitochondrial fusion (Wai et al., 2015; **Figures 5D–G**). Thus, our HRR data suggest that damaged mitochondria were not restored. Therefore, it can lead to a decrease in energy production. In fact, we observed a reduction in ADP phosphorylation in the diaphragm from the CLP group (**Figure 6K**). Moreover, the data from intercostal respiratory muscle from septic patients also decreased citrate synthase and complex I activity (Fredriksson and Rooyackers, 2007).

Pgc1α stimulates mitochondrial genesis and functions in skeletal muscle (Eisele and Handschin, 2014; Bloise et al., 2018). The Pgc1α expression upregulates during an increase in energy demand and intensification in ROS production (Eisele and Handschin, 2014). However, systemic inflammatory reactions can deregulate Pgc1α action in the peripheral skeletal muscle *via* NFκb signaling (Eisele and Handschin, 2014). Therefore, we investigated *Ppargc1a* expression (encodes Pgc1α) in the diaphragm during sepsis. We observed a decrease in *Ppargc1a* expression in the CLP diaphragm (**Figure 5C**). Pgc1α is a rapidly degraded coactivator associated with genomic and mitochondrial DNA, which is a crucial regulator of mitochondrial function, as oxidative phosphorylation, number, and mass (Villena, 2015). Skeletal muscle highly expresses Pgc1α, especially the slow type I fibers, associated with the adaptation to high energy demand (Villena, 2015). Thus, the reduction in *Ppargc1a* expression could be related to decreased mitochondrial energy production observed in the septic diaphragm (**Figure 6K**). Additionally, Pgc1α induces the expression of detoxifying ROS enzymes such as Sod2 (Eisele and Handschin, 2014). Septic animals also decreased the expression of *Sod2* (**Figure 5I**).

Additionally, the diaphragm seems more resistant to oxidative damage than limb muscle with no increase in lipid peroxidation (Talarmin et al., 2017). However, it did not present a significant change in the SOD protein level (Talarmin et al., 2017). Another group demonstrated that acute sepsis increased H_2O_2 production in the diaphragm (Eyenga et al., 2021). Reduction in *Sod* expression observed herein can be a consequence of the decrease in *Pgc1α* observed (**Figures 5**, **6**). However, we cannot discard that other factors might be involved in the antioxidative enzyme control, since we did not evaluate the antioxidative defense enzymatic activity and oxidative damage (Shan et al., 2015; Ahmed et al., 2017).

The increased unhealthy mitochondrial amount (**Figure 5B**) could be one of the causes of tissue damage. CLP diaphragm presented an increase in proton leak (**Figure 6L**). The proton leak is a cellular defense from the increased ROS production (Zhao et al., 2019). Thus, it could be an adaptation process to handle septic mitochondrial damage. We previously demonstrated that acute sepsis (24 h CLP) decreases the critical cellular antioxidative protein expression in septic diaphragms (Bloise et al., 2020). Therefore, the disruption in mitochondrial physiology induced by CLP demonstrated here could be one factor that leads to diaphragm dysfunction during sepsis.

Whitehead et al. (2016) demonstrated a positive correlation between diaphragm excursion amplitude measurement by ultrasound and diaphragm-specific force measure by traditional *ex vivo* force measurements (Whitehead et al., 2016). The E–T index is associated with forcing generation (Palkar et al., 2018). In the sepsis diaphragm, the E–T index did not change (**Figure 4G**). Also, in the duty cycle, another functional data (Buras et al., 2019), the diaphragm contraction parameters seem not to be affected by sepsis (**Figure 4E**). Thus, besides the decrease in the mitochondrial function, the diaphragm appears to be adapted to maintain the contractile work in the prolonged septic group. It is crucial to notice that our data are related to the survivor group. Therefore, we cannot assume that the same modulation occurred in the non-survivor septic animals. We have demonstrated previously that 24 h after CLP, the mitochondrial function is already decreased. However, the diaphragm sarcomeric organization is preserved (Bloise et al., 2020). Herein, the diaphragm function did not change; however, the sarcomeric organization seems impaired (**Figure 2B**). Thus, in the survivor septic group, diaphragm ultrastructural disorganization and decreased mitochondrial function were not enough to impair the primary respiratory muscle contraction. Furthermore, the diaphragm E–T index is used in clinical particles to guide the extubation time of patients. Hence, it is important to notice that a normal diaphragm contraction function may not reflect restored diaphragm morphology.

Herein, we demonstrated that 5 days after moderate sepsis induction led to mitochondrial damage and diaphragm morphological changes. Additionally, we observed disruption in diaphragm fiber ultrastructure. These changes might be associated with diaphragm failure during sepsis conditions.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Federal University of Rio de Janeiro Animal Care Committee.

AUTHOR CONTRIBUTIONS

TO, AS, NR, and FB performed and designed the experiments and analyzed and interpreted the data. CA, JS, NB, and JW performed the experiments. PS and PR interpreted the data and wrote the manuscript. Wd-S designed the experiments, supervised the data acquisition, wrote the manuscript, and interpreted the data. TO-C and FB conceived and designed the experiments, supervised the data acquisition, interpreted the data, and wrote the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This study was supported by the Brazilian Society of Endocrine and Metabolism – Thyroid Department (SBEM), Carlos Chagas Filho Research Foundation of the State of Rio de Janeiro

(FAPERJ, CNE E26/292798/2018, E-26/010.002215/2019, and CNE E26-/202.930/2019), National Council for Scientific and Technological Development (CNPq, 304667/2016-1 and 314070/2018-4), and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES, Code001). FB was a recipient of the Domestic Post-Doctorate Program (PNPD, CAPES). AS was a recipient of the Ph.D. program (CNPq). TO was a recipient of the master program "FAPERJ nota 10" (FAPERJ).

ACKNOWLEDGMENTS

We thank Alan Moraes for the technical support and the Electron Microscopy Laboratory of the Biology Institute from the Fluminense Federal University for the transmission electron microscope (JEM 1011) use. We are also grateful to Juliana Pena Gonçalves and Hannaily Ribeiro for the research support during HRR experiments and animal handling, respectively. Additionally, we thank the support of Debora Bastos Mello during the ultrasonography data acquisition and analysis at the National Center for Structural Biology and Bioimaging (CENABIO-UFRJ, Rio de Janeiro, Brazil).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2021.704044/full#supplementary-material

REFERENCES

Ahmed, S. M., Luo, L., Namani, A., Wang, X. J., and Tang, X. (2017). Nrf2 signaling pathway: pivotal roles in inflammation. *Biochim. Biophys. Acta Mol. Basis Dis.* 1863, 585–597. doi: 10.1016/j.bbadis.2016.11.005

Andersen, J. L., and Schiaffino, S. (1997). Mismatch between myosin heavy chain mRNA and protein distribution in human skeletal muscle fibers. *Am. J. Physiol.* 272, C1881–C1889.

Bellani, G., Laffey, J. G., Pham, T., Fan, E., Brochard, L., Esteban, A., et al. (2016). Epidemiology, Patterns of Care, and Mortality for Patients With Acute Respiratory Distress Syndrome in Intensive Care Units in 50 Countries. *JAMA* 315, 788–800. doi: 10.1001/jama.2016.0291

Bloise, F. F., Cordeiro, A., and Ortiga-Carvalho, T. M. (2018). Role of thyroid hormone in skeletal muscle physiology. *J. Endocrinol.* 236, R57–R68.

Bloise, F. F., Santos, A. T., Brito, J. T., Andrade, C. B. V., Oliveira, T. S., Souza, A. F., et al. (2020). Sepsis Impairs Thyroid Hormone Signaling and Mitochondrial Function in the Mouse Diaphragm. *Thyroid* 30, 1079–1090. doi: 10.1089/thy.2019.0124

Bloise, F. F., van der Spek, A. H., Surovtseva, O. V., Ortiga-Carvalho, T. M., Fliers, E., and Boelen, A. (2016). Differential Effects of Sepsis and Chronic Inflammation on Diaphragm Muscle Fiber Type, Thyroid Hormone Metabolism, and Mitochondrial Function. *Thyroid* 26, 600–9. doi: 10.1089/thy.2015.0536

Buras, E. D., Converso-Baran, K., Davis, C. S., Akama, T., Hikage, F., Michele, D. E., et al. (2019). Fibro-Adipogenic Remodeling of the Diaphragm in Obesity-Associated Respiratory Dysfunction. *Diabetes.* 68, 45–56. doi: 10.2337/db18-0209

Callahan, L. A., and Supinski, G. S. (2009). Sepsis-induced myopathy. *Crit. Care Med.* 37, S354–S367.

Demoule, A., Jung, B., Prodanovic, H., Molinari, N., Chanques, G., Coirault, C., et al. (2013). Diaphragm dysfunction on admission to the intensive care unit. Prevalence, risk factors, and prognostic impact-a prospective study. *Am. J. Respir. Crit. Care Med.* 188, 213–219. doi: 10.1164/rccm.201209-1668oc

Eisele, P. S., and Handschin, C. (2014). Functional crosstalk of PGC-1 coactivators and inflammation in skeletal muscle pathophysiology. *Semin. Immunopathol.* 36, 27–53. doi: 10.1007/s00281-013-0406-4

Eyenga, P., Roussel, D., Rey, B., Ndille, P., Teulier, L., Eyenga, F., et al. (2021). Mechanical ventilation preserves diaphragm mitochondrial function in a rat sepsis model. *Intensive Care Med. Exp.* 9:19.

Fredriksson, K., and Rooyackers, O. (2007). Mitochondrial function in sepsis: respiratory versus leg muscle. *Crit. Care Med.* 35, S449–S453.

Greising, S. M., Medina-Martinez, J. S., Vasdev, A. K., Sieck, G. C., and Mantilla, C. B. (2015). Analysis of muscle fiber clustering in the diaphragm muscle of sarcopenic mice. *Muscle Nerve.* 52, 76–82. doi: 10.1002/mus.24641

Gustafsson, A. B., and Dorn, G. W. II (2019). Evolving and Expanding the Roles of Mitophagy as a Homeostatic and Pathogenic Process. *Physiol Rev.* 99, 853–892. doi: 10.1152/physrev.00005.2018

Herridge, M. S., Tansey, C. M., Matte, A., Tomlinson, G., Diaz-Granados, N., Cooper, A., et al. (2011). Functional disability 5 years after acute respiratory distress syndrome. *N Engl J Med.* 364, 1293–1304.

Inayat, S., Nazariahangarkolaee, M., Singh, S., McNaughton, B. L., Whishaw, I. Q., and Mohajerani, M. H. (2020). Low acetylcholine during early sleep is important for motor memory consolidation. *Sleep* 43:zsz297.

Laudanski, K. (2021). Humanized Mice as a Tool to Study Sepsis-More Than Meets the Eye. *Int J Mol Sci.* 22:2403. doi: 10.3390/ijms22052403

Moarbes, V., Mayaki, D., Huck, L., Leblanc, P., Vassilakopoulos, T., Petrof, B. J., et al. (2019). Differential regulation of myofibrillar proteins in skeletal muscles of septic mice. *Physiol Rep.* 7:e14248.

Ono, Y., Maejima, Y., Saito, M., Sakamoto, K., Horita, S., Shimomura, K., et al. (2020). TAK-242, a specific inhibitor of Toll-like receptor 4 signalling, prevents endotoxemia-induced skeletal muscle wasting in mice. *Sci Rep.* 10:694.

Owen, A. M., Patel, S. P., Smith, J. D., Balasuriya, B. K., Mori, S. F., Hawk, G. S., et al. (2019). Chronic muscle weakness and mitochondrial dysfunction in the absence of sustained atrophy in a preclinical sepsis model. *Elife* 8:e49920.

Palkar, A., Narasimhan, M., Greenberg, H., Singh, K., Koenig, S., Mayo, P., et al. (2018). Diaphragm Excursion-Time Index: a New Parameter Using Ultrasonography to Predict Extubation Outcome. *Chest* 153, 1213–1220. doi: 10.1016/j.chest.2018.01.007

Paoli, C. J., Reynolds, M. A., Sinha, M., Gitlin, M., and Crouser, E. (2018). Epidemiology and Costs of Sepsis in the United States-An Analysis Based on Timing of Diagnosis and Severity Level. *Crit. Care Med.* 46, 1889–1897. doi: 10.1097/ccm.0000000000003342

Penuelas, O., Keough, E., Lopez-Rodriguez, L., Carriedo, D., Goncalves, G., Barreiro, E., et al. (2019). Ventilator-induced diaphragm dysfunction: translational mechanisms lead to therapeutical alternatives in the critically ill. *Intensive Care Med. Exp.* 7:48.

Picard, M., Jung, B., Liang, F., Azuelos, I., Hussain, S., Goldberg, P., et al. (2012). Mitochondrial dysfunction and lipid accumulation in the human diaphragm during mechanical ventilation. *Am. J. Respir. Crit. Care Med.* 186, 1140–1149. doi: 10.1164/rccm.201206-0982oc

Polla, B., D'Antona, G., Bottinelli, R., and Reggiani, C. (2004). Respiratory muscle fibres: specialisation and plasticity. *Thorax* 59, 808–817. doi: 10.1136/thx.2003.009894

Rittirsch, D., Huber-Lang, M. S., Flierl, M. A., and Ward, P. A. (2009). Immunodesign of experimental sepsis by cecal ligation and puncture. *Nat. Protoc.* 4, 31–36. doi: 10.1038/nprot.2008.214

Rudd, K. E., Johnson, S. C., Agesa, K. M., Shackelford, K. A., Tsoi, D., Kievlan, D. R., et al. (2020). Global, regional, and national sepsis incidence and mortality, 1990-2017: analysis for the Global Burden of Disease Study. *Lancet* 395, 200–211.

Shan, Y., Akram, A., Amatullah, H., Zhou, D. Y., Gali, P. L., Maron-Gutierrez, T., et al. (2015). ATF3 protects pulmonary resident cells from acute and ventilator-induced lung injury by preventing Nrf2 degradation. *Antioxid. Redox. Signal.* 22, 651–668. doi: 10.1089/ars.2014.5987

Singer, M., Deutschman, C. S., Seymour, C. W., Shankar-Hari, M., Annane, D., Bauer, M., et al. (2016). The Third International Consensus Definitions for Sepsis and Septic Shock (Sepsis-3). *JAMA* 315, 801–810.

Stana, F., Vujovic, M., Mayaki, D., Leduc-Gaudet, J. P., Leblanc, P., Huck, L., et al. (2017). Differential Regulation of the Autophagy and Proteasome Pathways in Skeletal Muscles in Sepsis. *Crit. Care Med.* 45, e971–e979.

Supinski, G. S., and Callahan, L. A. (2013). Diaphragm weakness in mechanically ventilated critically ill patients. *Crit. Care* 17:R120.

Supinski, G. S., Morris, P. E., Dhar, S., and Callahan, L. A. (2018). Diaphragm Dysfunction in Critical Illness. *Chest* 153, 1040–1051. doi: 10.1016/j.chest.2017.08.1157

Talarmin, H., Derbre, F., Lefeuvre-Orfila, L., Leon, K., Droguet, M., Pennec, J. P., et al. (2017). The diaphragm is better protected from oxidative stress than hindlimb skeletal muscle during CLP-induced sepsis. *Redox. Rep.* 22, 218–226. doi: 10.1080/13510002.2016.1223793

Thompson, B. T., Chambers, R. C., and Liu, K. D. (2017). Acute Respiratory Distress Syndrome. *N. Engl. J. Med.* 377, 562–572.

Villena, J. A. (2015). New insights into PGC-1 coactivators: redefining their role in the regulation of mitochondrial function and beyond. *FEBS J.* 282, 647–672. doi: 10.1111/febs.13175

Vivier, E., Mekontso Dessap, A., Dimassi, S., Vargas, F., Lyazidi, A., Thille, A. W., et al. (2012). Diaphragm ultrasonography to estimate the work of breathing during non-invasive ventilation. *Intensive Care Med.* 38, 796–803. doi: 10.1007/s00134-012-2547-7

Wai, T., Garcia-Prieto, J., Baker, M. J., Merkwirth, C., Benit, P., Rustin, P., et al. (2015). Imbalanced OPA1 processing and mitochondrial fragmentation cause heart failure in mice. *Science* 350:aad0116. doi: 10.1126/science.aad0116

Whitehead, N. P., Bible, K. L., Kim, M. J., Odom, G. L., Adams, M. E., and Froehner, S. C. (2016). Validation of ultrasonography for non-invasive assessment of diaphragm function in muscular dystrophy. *J. Physiol.* 594, 7215–7227. doi: 10.1113/jp272707

Yende, S., Austin, S., Rhodes, A., Finfer, S., Opal, S., Thompson, T., et al. (2016). Long-Term Quality of Life Among Survivors of Severe Sepsis: analyses of Two International Trials. *Crit. Care Med.* 44, 1461–1467. doi: 10.1097/ccm.0000000000001658

Youle, R. J., and van der Bliek, A. M. (2012). Mitochondrial fission, fusion, and stress. *Science* 337, 1062–1065. doi: 10.1126/science.1219855

Zhao, R. Z., Jiang, S., Zhang, L., and Yu, Z. B. (2019). Mitochondrial electron transport chain. ROS generation and uncoupling (Review). *Int. J. Mol. Med.* 44, 3–15.

Zolfaghari, P. S., Carre, J. E., Parker, N., Curtin, N. A., Duchen, M. R., and Singer, M. (2015). Skeletal muscle dysfunction is associated with derangements in mitochondrial bioenergetics (but not UCP3) in a rodent model of sepsis. *Am. J. Physiol. Endocrinol. Metab.* 308, E713–E725.

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Physiology

ORIGINAL RESEARCH
published: 27 September 2021
doi: 10.3389/fphys.2021.706128

Paternal Resistance Exercise Modulates Skeletal Muscle Remodeling Pathways in Fathers and Male Offspring Submitted to a High-Fat Diet

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Although some studies have shown that a high-fat diet (HFD) adversely affects muscle extracellular matrix remodeling, the mechanisms involved in muscle trophism, inflammation, and adipogenesis have not been fully investigated. Thus, we investigated the effects of 8 weeks of paternal resistance training (RT) on gene and protein expression/activity of critical factors involved in muscle inflammation and remodeling of fathers and offspring (offspring exposed to standard chow or HFD). Animals were randomly distributed to constitute sedentary fathers (SF; $n = 7$; did not perform RT) or trained fathers (TF $n = 7$; performed RT), with offspring from mating with sedentary females. After birth, 28 male pups were divided into four groups ($n = 7$ per group): offspring from sedentary father submitted either to control diet (SFO-C) or high-fat diet (SFO-HF) and offspring from trained father submitted to control diet (TFO-C) or high-fat diet (TFO-HF). Our results show that an HFD downregulated collagen mRNA levels and upregulated inflammatory and atrophy pathways and adipogenic transcription factor mRNA levels in offspring gastrocnemius muscle. In contrast, paternal RT increased MMP-2 activity and decreased IL-6 levels in offspring exposed to a control diet. Paternal RT upregulated *P70s6k* and *Ppara* mRNA levels and downregulated *Atrogin1* mRNA

OPEN ACCESS

Edited by:
Raphael Ritti-Dias, Universidade Nove de Julho, Brazil

Reviewed by:
Mauricio Krause, Federal University of Rio Grande do Sul, Brazil
Richelle Mychasiuk, Monash University, Australia
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Specialty section:
This article was submitted to Exercise Physiology, a section of the journal Frontiers in Physiology

Received: *06 May 2021*
Accepted: *02 September 2021*
Published: *27 September 2021*

Citation:
Salomão R, Neto IVdS, Ramos GV, Tibana RA, Durigan JQ, Pereira GB, Franco OL, Royer C, Neves FdAR, Carvalho ACAd, Nóbrega OT, Haddad R, Prestes J and Marqueti RdC (2021) Paternal Resistance Exercise Modulates Skeletal Muscle Remodeling Pathways in Fathers and Male Offspring Submitted to a High-Fat Diet. Front. Physiol. 12:706128. doi: 10.3389/fphys.2021.706128

levels, while decreasing NFκ-B, IL-1β, and IL-8 protein levels in offspring exposed to an HFD. Paternal physical training influences key skeletal muscle remodeling pathways and inflammatory profiles relevant for muscle homeostasis maintenance in offspring submitted to different diets.

Keywords: exercise, intergenerational, gastrocnemius, atrophy/hypertrophy signaling, proinflammatory cytokines, protein turnover, adipogenic

INTRODUCTION

Obesity and its comorbidities represent a global health problem that has become a major social and economic burden worldwide. Overweight and obesity are often causally linked to marked alterations in lifestyle, such as increases in high-energy dietary intake, as well as a high prevalence of physical inactivity. In rodent models, a high-fat diet (HFD) promotes accumulation of intramyocellular lipids that induce muscular lipotoxicity, chronic inflammation, oxidative stress, and glucose metabolism abnormalities (Bollheimer et al., 2012; Trajcevski et al., 2013). Among the relevant signaling pathways, NF-κB has emerged as a critical transcription factor that induces the expression of many proinflammatory cytokines (TNF-α, IL-1β, IL-6, IL-8, and IL-12) involved in the skeletal muscle atrophy process (Carlsen et al., 2009). In addition, NF-κB upregulation becomes evident in many catabolic scenarios associated with an increased ubiquitin-proteasome system and autophagy (Demasi and Laurindo, 2012; Sandri, 2013; Abrigo et al., 2016), which in turn might lead to cell damage and muscle disorders.

The inflammatory condition is also closely related to dysfunctional extracellular matrix (ECM) remodeling in skeletal muscle, corroborating musculoskeletal disorders (Tam et al., 2015). Tam et al. (2015) found downregulation of mRNA levels in collagen I, III, and VI in the gastrocnemius muscle of C57BL/6 mice fed an HFD for 5 weeks, indicating imbalance in ECM homeostasis, whose remodeling is regulated by matrix metalloproteinases (MMPs) essential to maintain a favorable environment for structural support, mechanical stability, and transmission of signals from muscle cells. It has been shown that MMP-2 deficiency promotes abnormal changes in collagen (Martinez-Huenchullan et al., 2017), which is crucial to force transmission among fibers and fascicles in the muscle contraction process (de Sousa Neto et al., 2017). The decrease in MMP-2 activity may also impair the release of local growth factors (TGF-β, CTGF, and IGF-1) and proliferation, differentiation, and migration of satellite cells responsible for muscle hypertrophy (Martinez-Huenchullan et al., 2017). Although some studies have already shown that HFD adversely affects muscle ECM remodeling, the mechanisms involved in the muscle trophism, inflammation, and adipogenesis have not yet been fully investigated. Hence, further studies are required to determine the harmful effects caused by an HFD in skeletal muscle.

Resistance exercise training (RT) is a potent lipolysis mediator, preventing intramyocellular lipid expansion in skeletal muscle, linked to the PPARy-mediated mechanism (Ribeiro et al., 2017). The role of RT on skeletal muscle confers beneficial effects on adipogenic transcriptional factors (CCAAT/enhancer-binding proteins), suppressing proinflammatory factors (Guedes et al., 2020) and abnormal ECM remodeling (Guzzoni et al., 2018). Furthermore, RT protects against obesity-induced muscle atrophy, via activation of the anabolic signaling axis *(IGF1*, *AKT, P70sk6,* and *MyoD)* (Glover and Phillips, 2010). Currently, it is widely accepted that a future generation inherits protective exercise-mediated effects through epigenetics inheritance (Vieira de Sousa Neto et al., 2021). The RT performed by fathers represents a potential regulator of intergenerational inheritance and is a cost-effective strategy to improve the health of the first offspring exposed to an HFD (Vieira de Sousa Neto et al., 2021). Thus, the father's behavior may modulate key players responsible for skeletal muscle remodeling in the offspring.

Previous evidence demonstrated that paternal RT (8 weeks, three times per week) induced protective effects on the left ventricle and tendon proteome of offspring exposed to an HFD through the increase in abundance proteins involved in cellular survival pathways, tissue integrity maintenance, and overall homeostasis (ECM organization, heart contraction, metabolic processes, antioxidant activity, transport, and transcription regulation) (de Sousa Neto et al., 2020a,b). Likewise, Stanford et al. (2018) showed that voluntary paternal running on a wheel (3 weeks with running distances averaging 5.8 ± 0.4 km/day) suppresses the detrimental effects of paternal HFD on offspring, reducing fat mass and glucose uptake in the gastrocnemius, tibialis anterior, extensor digitorum longus, and soleus, which suggests that paternal programming may regulate developmental heritages of offspring diseases. However, the extent to which paternal RT leads to orchestrated molecular changes in offspring skeletal muscle exposed to different diets has not been investigated. The climbing a ladder provides an alternative means of inducing force overload with advantages when improving rat musculoskeletal properties and neuromuscular function (Hornberger and Farrar, 2004), while voluntary wheel running may have limited effects on muscle hypertrophy process (Legerlotz et al., 2008; Kurosaka et al., 2009).

Skeletal muscle adaptation is a complex process involving crosstalk of multiple regulatory mechanisms and intracellular pathways (Potthoff et al., 2007). Identifying essential genes and proteins involved in signaling inflammation, atrophy/hypertrophy molecules, and ECM remodeling could provide meaningful insights into the offspring muscle dysfunctions. A mechanistic framework of controlled paternal RT effects may yield clinically useful information and design interventions to effectively counterbalance harmful responses inherent to HFD. Thus, whether the father's lifestyle modulates the musculoskeletal system remains a valid question for muscle health and treatment guidance. The purpose of this study was

to investigate the effects of 8 weeks of paternal RT on gene and protein expression/activity of crucial factors involved in muscle inflammation and remodeling of fathers and offspring (offspring exposed to standard chow or HFD). We hypothesized that paternal RT could minimize maladaptive responses at the transcriptional level (inflammatory factors, atrophy-associated genes, and ECM markers), proinflammatory cytokines levels, and ECM protein turnover pathways impaired by HFD.

MATERIALS AND METHODS

Animals and Experimental Groups

All procedures were conducted following the Guide for the Care and Use of Laboratory Animals (National Research Council, 1996) in compliance with the ARRIVE guidelines. The research protocol received approval from the Ethics Committee on Animal Experimentation from the Universidade Católica de Brasília, Brasília (protocol No. 010/13). Initially, fourteen ($n = 14$) 4-month-old *Wistar* rats (*Rattus norvegicus albinus*, weighing 359 g $\pm$ 32.4) were obtained from the Central Vivarium of the Faculty of Physical Education of the Universidade Católica de Brasilia. The animals were housed in collective cages (maximum of four rats per cage) and received water and standard feed for rodents *ad libitum* during the experimental period. The animals were randomly distributed into two subgroups: sedentary fathers (SF; $n = 7$; did not perform RT) and trained fathers (TF; $n = 7$; performed RT).

Paternal Resistance Training

The RT protocol was adapted from Hornberger and Farrar (2004), according to the requirements of this study. In addition, the procedures have been described in previous intergenerational studies (de Sousa Neto et al., 2020a,b). The RT protocol was carried out for 8 weeks, with the climbs performed three times a week (Monday, Wednesday, and Friday) in the afternoon (between 2 and 4 p.m.). Initially, the rats were adapted to the resistance exercise protocol, which required the animals to climb a vertical ladder (1.1 $\times$ 0.18 m, 2 cm step, 80% climb angle) with the load apparatus fixed to their tail, via a self-adhesive foam strip wrapped around the proximal portion. The size of the steps meant the animals performed 8–12 movements per climb. In the familiarization phase, the rats were placed at the bottom of the stairs and were stimulated to climb with a weight attached to the tail. If necessary, a stimulus through finger tweezers was applied to the animal's tail to start the movement. At the top of the stairs, the rats reached a house chamber (20 $\times$ 20 $\times$ 20 cm) where they rested for 2 min. This procedure was repeated until the animals could voluntarily climb the subsequent set without stimulation (de Sousa Neto et al., 2018). The familiarization sessions were performed three times with an interval of 48 h.

Three days after familiarization, each animal was evaluated to determine its maximum load capacity, which consisted of 4–8 ladder climbs with gradually heavier loads. The initial climb consisted of carrying a load corresponding to 75% of the animal's body weight. After that, weights of 30 g were added progressively until the rat was unable to complete the climb. Failure to climb was determined when the animal was unable to complete the climb after three successive tail stimuli. The highest load carried to the top of the stairs was considered the maximum load capacity of the rat for that specific training session. After defining the maximum load capacity, the training sessions comprised eight climbs (TF), two sets with each load of 50, 75, 90, and 100% of the animal's maximum load capacity. Average feed intake was measured for each experimental group 24 and 48 h after the first RT session and 72 h after the second RT session.

In each week, respecting the progressive overload principle of the training, 30 g (approximately 13% of the animal's body weight) were added, referring to the weight of the previous week with 100% of the maximum loading capacity, in order to guarantee the desired stress resulting from the exercise.

Offspring Groups

The offspring came from mating with sedentary females. The estrous cycle of the females was checked daily after completing the RT. The male rats were mated with primigravidae females during the proestrus phase, housing together for two consecutive days with free access to a control diet (Purina®, Descalvado-SP, Brazil) water.

After birth, the experimental groups in this study consisted of 28 male pups. The litters were standardized to 7 pups each to avoid litters of different sizes and were left with their mothers for 21 days of breastfeeding. The litters belonging to the same experimental group were descended from different parents.

After weaning, male pups were divided into 4 groups ($n = 7$ per group): offspring from sedentary father submitted either to control diet (SFO-C) or high-fat diet (SFO-HF) and offspring from trained father submitted to control diet (TFO-C) or high-fat diet (TFO-HF). The animals were housed in polypropylene cages with a temperature of 23 $\pm$ 2°C and light:dark cycle of 12:12 h. The pups were weighed, in grams, on a digital scale, weekly for 6 months (Filizola®, São Paulo, Brazil).

Offspring Diet

The offspring submitted to the control diet (SFO-C and TFO-C) were fed with standard feed (66.00% carbohydrates, 24.00% protein, and 10.00% lipids, totaling 3.48 Kcal/g, Labina Presence®, Paulínia, São Paulo, Brazil) and water *ad libitum*. Females were also kept on the control diet during gestation and lactation periods. The SFO-HF and TFO-HF groups were submitted to the commercially acquired HF diet (20.27% carbohydrates, 19.89% protein, and 59.38% lipids, totaling 5.20 Kcal/g) (Prag® solutions, Biosciences, Jaú, Brazil) and an overload of 200 mL of soft drink (high-fructose corn syrup, caramel coloring, caffeine, and phosphoric acid) per week (100% carbohydrates—21 g, sodium: 10 mg totaling 0.85 Kcal/g), in addition to having free access to water from the 21st day after birth for 6 months. Detailed compositions of the experimental diets were reported previously (de Sousa Neto et al., 2020b).

Some studies have demonstrated the efficacy of this HFD model in body weight gain and adiposity in *Wistar* rats (Goncalves et al., 2018; de Sousa Neto et al., 2020b). Soft drinks for rats are used as a complement and characterization of the HF diet and are an effective strategy to promote the increase

in total energy intake and body weight gain in the long term (Messier et al., 2007; Lozano et al., 2016). The offspring were weighed weekly on a digital scale (Filizola, São Paulo, Brazil) until euthanasia. The experimental design of the study is shown in **Figure 1**.

Euthanasia

Fathers were euthanized with an intraperitoneal injection of xylazine solution (12 mg/kg body weight) and ketamine (95 mg/kg body weight), 48 h after the training period to avoid the acute effects of exercise. The offspring were euthanized with the same combination of solutions after 6 months of exposure to the HFD. Afterward, the medial gastrocnemius muscles were immediately dissected from each posterior hindlimb and one part was frozen in RNase-free tubes using liquid nitrogen (for qPCR, cytokines, zymography, and western blotting) and then stored at −80°C for further molecular analysis related to skeletal muscle remodeling. An experienced researcher dissected all muscles to prevent contamination of other tissues.

RNA Extraction

Tissue samples from 28 animals (7 per group) were homogenized in a tube containing five stainless steel balls (diameter, 2.3 mm) (BioSpec Products, Bartlesville, OK, United States), three sharp silicon carbide particles (1 mm) (BioSpec Products), and TRIzol, by shaking using a FastPrep-24 instrument (M.P. Biomedicals, Solon, OH, United States) (Marqueti et al., 2012). In order to thoroughly homogenize the tissue, the shaking process was repeated five times at speed 4. The tubes were kept in ice-cooling for 2 min to avoid RNA degradation between each shaking step. The total RNA was extracted according to the TRIzol method described by Chomczynski and Sacchi (1987). A NanoDrop® spectrophotometer (ND-1000; NanoDrop Technologies Inc., Wilmington, DE, United States) was used to quantify the RNA concentrations in each sample, determining the absorbance rate of 260–280 nm. Subsequently, samples were frozen at −80°C up to purification.

One Step RT-qPCR

To assess the gastrocnemius muscle gene expression, a total of 100–350 ng of RNA extracted from each sample was converted to cDNA (final volume 20 μL) using GoTaq® Probe 1-Step RT-qPCR (Promega-Cat. A6120X) based on the manufacturer's protocol. The standard cycling conditions to perform reverse transcription and amplification of samples were: (a) Reverse transcription (1 cycle): 45°C for 15 min; (b) reverse transcriptase

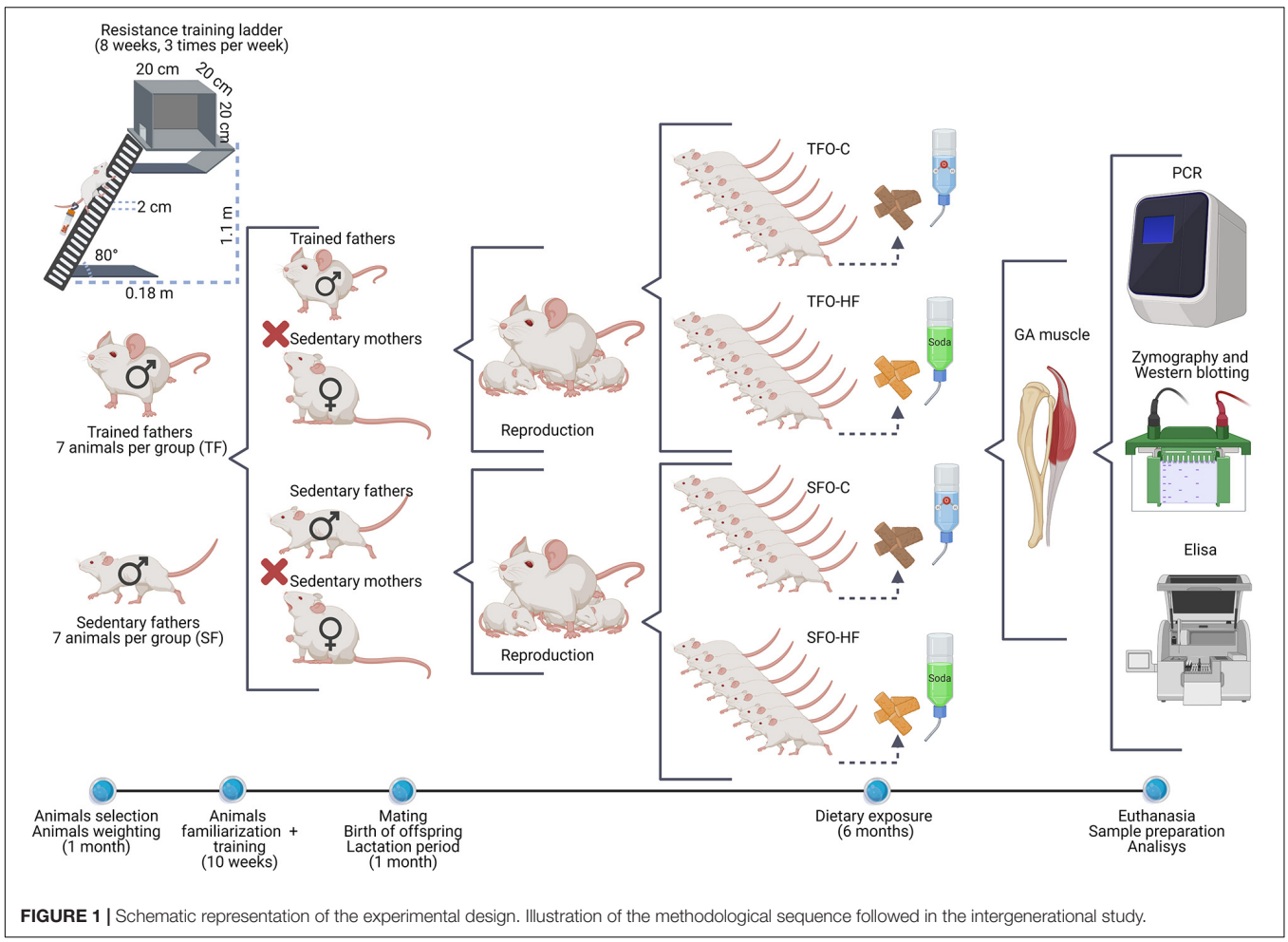

FIGURE 1 | Schematic representation of the experimental design. Illustration of the methodological sequence followed in the intergenerational study.

inactivation and GoTaq® DNA Polymerase activation (1 cycle): 95°C for 2 min; (c) Denaturation (40 cycles): 95°C for 15 s; and (d) Annealing and extension (40 cycles): 60°C for 1 min.

Quantitative Real-Time Polymerase Chain Reaction

The quantitative real-time polymerase chain reaction (qRT-PCR) was performed with the aid of the GoTaq® Probe 1-Step RT-qPCR System (Promega—Cat. A6120X). Ten microliters of the GoTaq Probe qPCR Master Mix with dUTP were homogenized with 1 μL of the 20 × primer, an amount of RNA (100–350 ng), 0.4 μL GoScript RT Mix for 1-Step RT-qPCR 1X, and water for a final volume of 20 μL. The qRT-PCR was performed using a QuantStudio 3 Real-Time PCR system (Applied Biosystems) for the following genes (**Table 1**):

The normalization of the expression from each target gene occurred through the expression of the constitutive gene RPLP0, which was used as the endogenous control of RNA, due to the lower intra and intergroup variability compared with the other housekeeping genes tested (Beta-actin and Gapdh, also tested in this study). The ΔCt values of the samples were determined by subtracting the average Ct value of the target gene from the average Ct value of the maintenance gene. Next, the ΔΔCt values were calculated by subtracting the ΔCt value from the condition of interest from the ΔCt value from the control condition. Finally, $2^{-\Delta\Delta Ct}$ values were calculated to present data of relative expression. The sample quantity and the reaction efficiency of each gene analyzed in the present study were determined from a standardization curve, with slope reference parameters equal to −3.3, R^2 = 0.9–1.0, and efficiency above 90%. These genes were chosen because a HFD induces substantial muscle atrophy, inflammation, and dysfunctional ECM remodeling in the skeletal muscle (Tam et al., 2015; Abrigo et al., 2016).

TABLE 1 | List of tested genes associated with extracellular matrix, inflammation, metabolism, and myogenesis.

mRNA	Code (life technologies)	mRNA	Code (life technologies)
Actb	rn00667869	*Murf1*	rn00590197
Akt1	rn00583646	*Myod*	rn01511496
Atrogin1	rn00591730	*Nfkb*	rn00595794
Cebpa	rn00560963	*Ppara*	rn00566193
Col1a1	rn01463848	*P70s6k*	rn00583148
Col3a1	rn01437681	*Rplp0*	rn03302271
Ctgf	rn01537279	*Tgfb1*	rn00572010
Foxo1	rn01494868	*Tnfa*	dr03126850
Gapdh	rn01775763	*Tweak*	rn01461586
Igf1a	rn00710306	*Mmp2*	rn01538170
Il6	rn01410330		

Actb, Actin beta; Akt1, AKT serine/threonine kinase 1; F-box protein 32 (also known as Atrogin1); Cebpa, CCAAT/enhancer binding protein alpha; Col1a1 and Col3a1, respectively, collagen 1 alpha 1 and collagen 3 alpha 1; Ctgf, connective tissue growth factor;Foxo1, forkhead box O1; Igf1, insulin-like growth factor 1; Il6, interleukin 6; Mmp2, matrix metallopeptidase 2; Murf1, tripartite motif containing 63; Myod, myogenic differentiation; Nfkb, nuclear factor kappa beta; Ppara, peroxisome proliferator-activated receptor alpha; ribosomal protein S6 kinase beta-1 (also known as P70s6k), Rplp0, ribosomal protein lateral stalk subunit P0; Tgfb1, transforming growth factor, beta 1; Tnfa, tumor necrosis factor alpha; Tweak, TNF superfamily member 12.

Cytokine Levels

The quantification of total proteins was performed in a NanoDrop® 2000 spectrophotometer (Thermo Fisher Scientific) using a 260/280 nm relation. Cytokine concentrations were determined by a multiplexed flow cytometry method using bead-based immunoassays developed by the BD Biosciences® (CBA, San Diego, CA, United States), specific for the following mediators: Interleukin (IL)-1beta (IL-1β), IL-6, IL-8, IL-10, IL-12p70, and tumor necrosis factor (TNF-α). Assessments were performed according to the manufacturer's protocols. Reconstituted cytokine standards and the thawed serum samples were processed using the BD FACS Verse cytometer. Two hundred events or more were acquired for each cytokine bead. Data were analyzed using FCAP software, version 3.0 (BD Biosciences®, San Diego, CA, United States). Standard curves for each cytokine were generated using serial dilutions of the mediators supplied, with each sample titrated by linear interpolation. All samples were determined in duplicate to guarantee reliability.

Gelatin Zymography

The zymography technique was used to measure MMP-2 activity. The gastrocnemius muscle extracts were homogenized and incubated in extraction buffer (10 mM cacodylic acid, pH 5.0; 0,15 M NaCl; 1 μM $ZnCl_2$; 20 mM $CaCl_2$; 1.5 mM NaN_3, and 0.01% Triton X-100) with five stainless steel balls (diameter, 2.3 mm) (BioSpec Products, Bartlesville, OK, United States) and three silicon-carbide sharp particles (1 mm) (BioSpec Products) by being shaken in a FastPrep-24 instrument (MP Biomedicals, Solon, OH, United States). Next, the solution was centrifuged for 30 min (13,000 g at 4°C), and the supernatant was reserved. A NanoDrop spectrophotometer (ND-1000; NanoDrop Technologies Inc., Wilmington, DE, United States) was used to quantify protein concentrations. Thirty mg of total protein were loaded into each lane. The samples were concentrated in 30 μg of protein and 10 μL of sample buffer without β-mercaptoethanol (reducing agent) and were resolved by electrophoresis on polyacrylamide gel with SDS and gelatin, in the final concentration of 1 mg/mL. After the run, the gel was washed twice for 20 min in 2.5% Triton X-100 solution to remove the SDS. Next, the gel was incubated in the substrate buffer (Tris- HCl 50 mM pH 8.0, CaCl 2.5 mM; NaN_3 0.02%, and $ZnCl_2$ 10 mM), at 37°C, for 18 h. Subsequently, the gel was stained with Coomassie Brilliant Blue for 60 min. Afterward, the gel was washed in a solution of methanol 30% and acetic acid 10%. The averages of the band intensities were measured using Image Master 2D Platinum 7.0 software and conducted by a blinded researcher, attenuating possible bias related to this process. Pro and active isoform bands were identified via standard techniques using molecular weight criteria. The bands found in all groups were 72–64 kDa, as proposed by previous studies that evaluated MMP-2 in the skeletal muscle (de Sousa Neto et al., 2017, 2018). The assurance of the analysis

accuracy, the gels for zymography were prepared simultaneously using the same solutions. Gels electrophoresis was carried out simultaneously using fresh buffers at the same condition and temperature (inside the fridge) to minimize the variation between the gels. Furthermore, protein normalization, voltage and time during electrophoresis, as well gel staining background, have been carefully standardized.

Western Blot Assay

Total proteins were extracted from tissues using phosphate buffer saline (PBS 1%, pH 7.4) supplemented with protease inhibitor cocktail (Roche, Germany) with five stainless steel balls (diameter, 2.3 mm) (BioSpec Products, Bartlesville, OK, United States) and three silicon-carbide sharp particles (1 mm) (BioSpec Products) by being shaken in a FastPrep-24 instrument (MP Biomedicals, Solon, OH, United States). After this, the samples were centrifuged (14,000 g for 30 min at 4°C). The supernatant was transferred to a new tube and the protein concentration was determined using a NanoDrop® spectrophotometer (ND-1000; NanoDrop Technologies Inc., Wilmington, DE, United States). The protein (20 μg) was separated using 10% SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes (Merck Millipore). The membranes were blocked using 5% non-fat milk in TBST buffer [200 mM Tris-HCl (pH 7.5), 1.5 M NaCl, 0.1% Tween 20] for 2 h and then incubated with the primary monoclonal antibodies: COL1A1 (Sigma Aldrich, C2456), NF-Kβ p65 (Cell Signaling Technology, #4764), and PPAR-α (Santa Cruz Biotechnology, sc-9000) overnight at 4°C. Next, the membrane was incubated with secondary anti-mouse IgG antibody -HRP (Santa Cruz Biotechnology, Sc -2005) or anti-rabbit IgG antibody -HRP (Cell Signaling Technology, #7074) for 1 h at room temperature. Chemiluminescence was detected using an ECL western blotting detection kit (GE Healthcare, Chicago, IL, United States). Densitometry was performed using ImageJ (National Institutes of Health, Bethesda, MD). The intensity related to the analyzed protein was normalized to the GAPDH band (A1978, Sigma Aldrich). The gels for western blot assay were prepared simultaneously using the same solutions to assure the analysis accuracy. Gels electrophoresis was carried out simultaneously using fresh buffers at the same condition and temperature to minimize the variation between the gels. Furthermore, protein normalization, voltage and time during electrophoresis have been carefully standardized. We also choose the best normalization standard; it was confirmed the primary antibody's quality (monoclonal), prevented signal saturation and accurately quantifying the signal intensity of the target protein.

Statistical Analysis

The results are expressed as mean ± standard deviation (SD). The Shapiro-Wilk test was used to determine the normality of data, and the Levene test was used to analyze the homogeneity of the variance. Two-way ANOVA (diet x paternal training) was used to compare the dependent variables between the four groups of offspring. When a significant difference was detected, the Tukey *post hoc* test was applied to identify where the difference occurred. An alpha level of $p \leq 0.05$ was considered significant. The software GraphPad Prism 8.3 (San Diego, CA, United States) was used for statistical analysis and graphics design. The results from gene expression were presented according (Heinemeier et al., 2007, Marqueti et al., 2018; Barin et al., 2019). The mRNA gene expression was log-transformed for the statistical analyses to obtain normal distribution. Data are presented in graphs as geometric mean ± back-transformed standard error (SE) calculated as follows: Geometric mean = 2^ or 10^ logarithmic mean. The geometric mean at 2^ was used only when the logarithmic value was higher than 1,000. Back-transformed positive SE = 2^ or 10^ (logarithmic mean + logarithmic SE) – geometric mean. Back-transformed negative SE = geometric mean – 10^(logarithmic mean + logarithmic SE). The mRNA data are presented on a logarithmic scale as fold changes relative to the mean of the young sedentary group. For this reason, the values for SF and SFO-C groups are always "1." Biorender web-based software was used to create the study design and the final figure (License Number QH22U934HD).

RESULTS

Effects of High-Fat Diet and Paternal Resistance Training on Offspring Body Weight Gain and Adiposity Index

Offspring exposed to the HFD showed a higher body weight and adiposity index when compared with the offspring exposed to the control diet ($p = 0.001$) following 6 months of diet exposure, based on previously published works (de Sousa Neto et al., 2020a,b). Paternal RT attenuated the body weight gain (TFO-HF = 398.8 ± 8.1 vs. SFO-HF 433.9 ± 34.7; $p = 0.001$) and adiposity index (TFO-HF = 2.63 ± 0.68 vs. SFO-HF = 4.75 ± 0.95) in the offspring submitted to an HFD. No statistical changes in body weight were observed between the TFO-C and SFO-C groups.

Effects of High-Fat Diet and Paternal Resistance Training on Structural Matrix Proteins, Matrix Remodeling Enzymes, and Growth Factor mRNA Levels in the Gastrocnemius Muscle

There were no differences between sedentary and trained fathers in mRNA levels of *Col1a1, Col3a1, Tgfβ1, Ctgf* ($p > 0.05$; **Figures 2A,C,G,I**, respectively), while *Mmp2* and *Igf1a* mRNA levels were significantly upregulated in the trained fathers ($p = 0.03$ and $p = 0.001$, **Figures 2E,K**, respectively). Regarding offspring, C*ol1a1* mRNA levels were significantly downregulated with HFD (SFO-HF vs. SFO-C; $p = 0.03$; **Figure 2B**). Furthermore, HFD downregulated C*ol3a1* mRNA levels in the offspring from trained fathers (TFO-HF vs. TFO-C group; $p = 0.04$, **Figure 2D**). However, paternal RT did not modulate structural matrix proteins, matrix remodeling enzymes, and growth factor mRNA levels in the offspring ($p > 0.05$; **Figures 2F,H,J,L**, respectively).

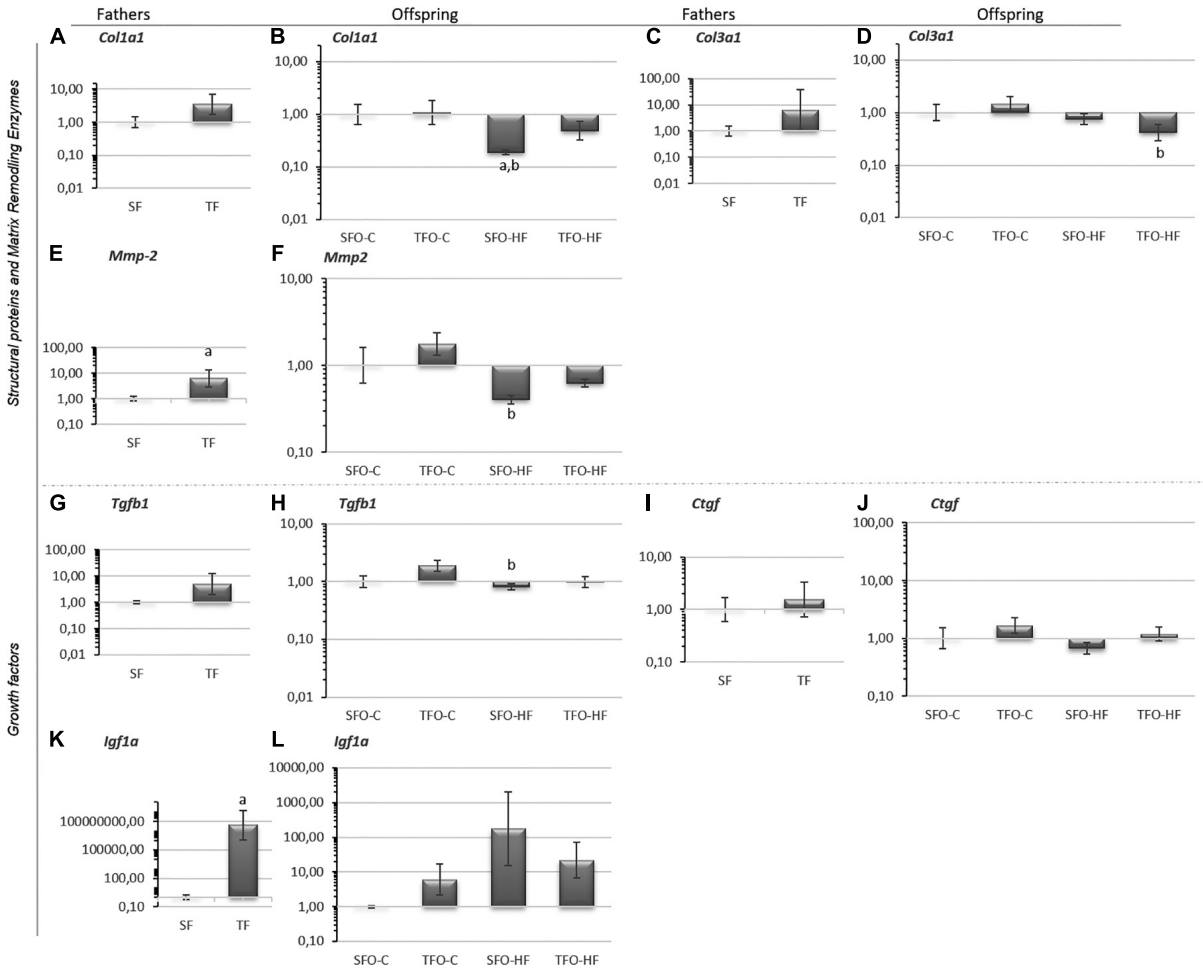

FIGURE 2 | Structural matrix proteins, matrix remodeling enzymes, and growth factor. mRNA levels in the gastrocnemius muscle of fathers and offspring exposed to control diet and HFD. The data are geometric mean ± standard error. The $2^{-\Delta\Delta CT}$ represents the expression level. **(A,B)** Collagen, type I, alpha 1 (*Col1a1*). **(C,D)** Collagen, type III, alpha 3 (*Col3a1*). **(E,F)** Matrix metalloproteinase-2 (*Mmp2*). **(G,H)** Transforming growth factor-beta 1 (TGF-β1). **(I,J)** Connective tissue growth factor (*Ctgf*). **(K,L)** Pro-insulin like growth factor IA (*Igf1a*). SF, sedentary fathers; TF, trained fathers; SFO-C, offspring from sedentary fathers, exposed to control diet; TFO-C, offspring from trained fathers exposed to control diet; SFO-HF, offspring from sedentary fathers exposed to high-fat diet; TFO-HF, offspring from trained fathers exposed to a high-fat diet. Statistically significant differences compared to: [a]SFO-C; [b]TFO-C; $p \leq 0.05$ ($n = 7$ per group).

Effects of High-Fat Diet and Paternal Resistance Training on Synthesis and Atrophy Pathway mRNA Levels in the Gastrocnemius Muscle

Akt1, MyoD, P70S6K, and *Murf1* mRNA levels were increased after RT in trained fathers ($p = 0.01$; **Figures 3A,C,E,K**, respectively). Despite the difference presented in the Akt graph (SFO-HF vs. TFO-C; $p = 0.01$; **Figure 3B**) it did not cause any significant comparison. No changes were observed in fathers for mRNA levels of *Foxo1* ($p > 0.05$; **Figure 3G**) and *Atrogin1* ($p > 0.05$; **Figure 3I**). Regarding offspring, *Atrogin1* and *Murf1* mRNA levels were significantly upregulated with HFD (SFO-HF vs. SFO-C; $p = 0.008$ and $p = 0.01$; **Figures 3J–L**). Paternal RT upregulated *P70s6k* and *Foxo1* mRNA levels in the TFO-HF group when compared to the SFO-HF group ($p = 0.03$ and $p = 0.001$; **Figures 3F,H**, respectively). Additionally, paternal RT mitigated the HFD-associated increase in *Atrogin1* mRNA levels of offspring (TFO-HF vs. SFO-HF; $p = 0.007$; **Figure 3J**). No changes were observed in mRNA levels of *MyoD* between offspring groups ($p > 0.05$; **Figure 3D**).

Effects of the High-Fat Diet and Paternal Resistance Training on Inflammatory Pathways, Adipogenic Transcription Factor, and Transcription Factor Regulator of Lipid Metabolism mRNA Levels in the Gastrocnemius Muscle

No changes were observed in inflammatory pathway mRNA levels in the fathers ($p > 0.05$; **Figures 4A,C,E,G**). *Cebpa* and *Ppara* mRNA levels were increased in the trained fathers when

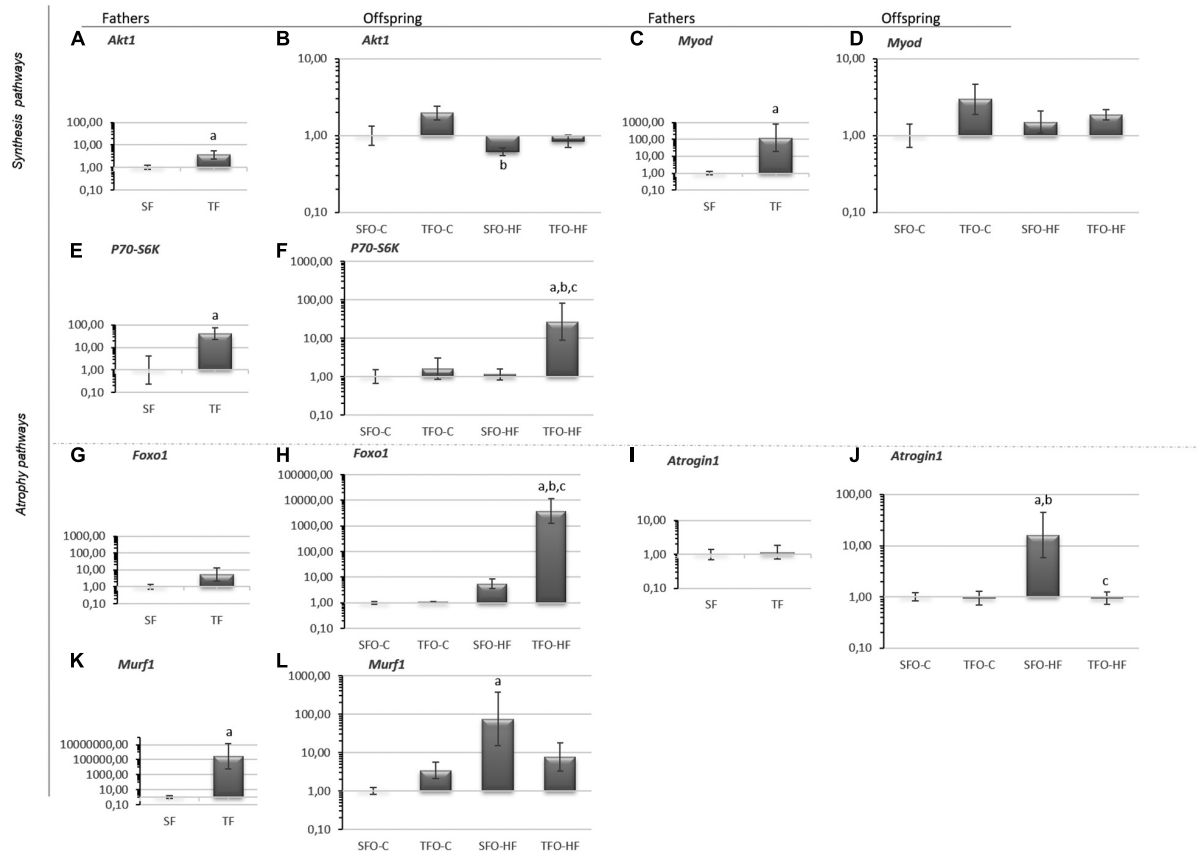

FIGURE 3 | Synthesis and atrophy pathway mRNA levels in the gastrocnemius muscle of fathers and offspring exposed to control diet and HFD. The data are geometric mean ± standard error. The expression level is represented by the $2^{-\Delta\Delta CT}$. **(A,B)** Protein kinase B (*Akt1*). **(C,D)** Myoblast determination protein 1 (*Myod*). **(E,F)** Ribosomal protein S6 kinase beta-1 (*P70s6k*). **(G,H)** Forkhead Box O1 (*Foxo11*). **(I,J)** Atrogin 1 (*Atrogin 1*). **(K,L)** Muscle RING-finger protein-1 (*Murf1*). SF, sedentary fathers; TF, trained fathers; SFO-C, offspring from sedentary fathers, exposed to control diet; TFO-C, offspring from trained fathers exposed to control diet; SFO-HF, offspring from sedentary fathers exposed to high-fat diet; TFO-HF, offspring from trained fathers exposed to a high-fat diet. Statistically significant differences compared to: [a]SFO-C; [b]TFO-C; [c]SFO-HF, $p \leq 0.05$ ($n = 7$ per group).

compared with sedentary fathers ($p = 0.04$ and $p = 0.01$, respectively; **Figures 4I,K**). Regarding offspring, no changes were observed in *Tnfa* mRNA levels ($p > 0.05$; **Figure 4B**). *Tweak*, *Cebpa*, and *Nfkb* mRNA levels were significantly upregulated with HFD (SFO-HF vs. SFO-C; $p = 0.01$; **Figures 4D,J,H**, respectively). Paternal RT upregulated *Ppara* mRNA levels in the TFO-HF group when compared to the SFO-HF group ($p = 0.0001$, **Figure 4L**). No changes were observed in mRNA levels of *Il6* between offspring groups ($p > 0.05$; **Figure 4F**).

Effects of High-Fat Diet and Paternal Resistance Training on Proinflammatory and Anti-Inflammatory Cytokines Levels in the Gastrocnemius Muscle

HFD did not modulate proinflammatory and anti-inflammatory cytokine levels in the offspring's gastrocnemius muscle ($p > 0.05$; **Figure 5**). However, paternal RT decreased IL-6 levels in the TFO-C group when compared to the SFO-C group ($p = 0.03$; **Figure 5C**). Moreover, paternal RT decreased the IL-1β levels in the offspring exposed to HFD. (TFO-HF vs. SFO-HF; $p = 0.03$; **Figure 5A**). Of note, IL-8 levels changed similarly in fathers and offspring, showing downregulation in the groups under the RT regimen (TF vs. SF; $p = 0.04$ and TFO-HF vs. SFO-HF; $p = 0.004$; **Figures 5E,F**, respectively). No changes were observed in IL-10, IL-12, and TNF-α levels between offspring groups ($p > 0.05$; **Figures 5H,J,L**, respectively).

Effects of High-Fat Diet and Paternal Resistance Training on MMP-2 Activity in the Gastrocnemius Muscle

The pro and active MMP-2 activity increased in the gastrocnemius muscle of fathers in response to RT (TF vs. SF; $p = 0.001$ and $p = 0.002$, respectively, **Figures 6A,C**). No changes were observed in the pro-MMP-2 activity of offspring ($p > 0.05$; **Figure 6B**). The active MMP-2 activity was significantly decreased with HFD in the offspring from trained fathers (TFO-C vs. TFO-HF; $p = 0.003$; **Figure 6D**). On the other hand, paternal RT increased active MMP-2 activity in the

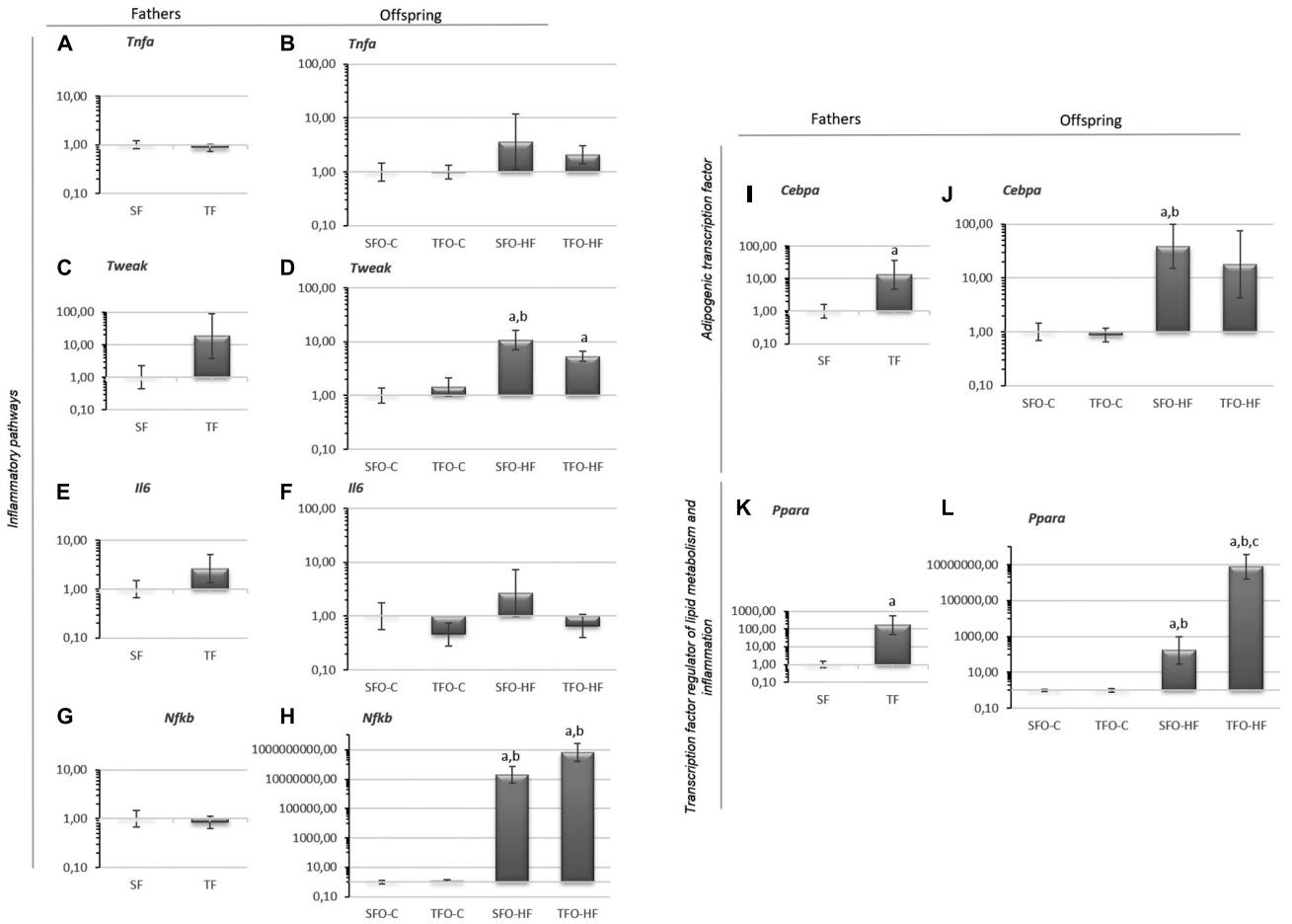

FIGURE 4 | Inflammatory pathways, adipogenic transcription factor and transcription factor regulator of lipid metabolism mRNA levels in the gastrocnemius muscle of fathers and offspring exposed to control diet and HFD. **(A,B)** Tumor necrosis factor alpha (*Tnfa*). **(C,D)** Tumor necrosis factor ligand superfamily member 12 (*Tweak*). **(E,F)** Interleukin-6 (*Il6*). **(G,H)** Factor nuclear kappa B (*Nfkb*). **(I,J)** CCAAT/enhancer binding protein (*Cebpa*). **(K,L)** Peroxisome proliferator-activated receptor (*Ppara*). The data are geometric mean ± standard error. The expression level is represented by the $2^{-\Delta\Delta CT}$. SF, sedentary fathers; TF, trained fathers; SFO-C, offspring from sedentary fathers, exposed to control diet; TFO-C, offspring from trained fathers exposed to control diet; SFO-HF, offspring from sedentary fathers exposed to high-fat diet; TFO-HF, offspring from trained fathers exposed to a high-fat diet. Statistically significant differences compared to: [a]SFO-C; [b]TFO-C; [c]SFO-HF, $p \leq 0.05$ ($n = 7$ per group).

TFO-C group when compared to the SFO-C group ($p = 0.01$; **Figure 6D**). MMP-9 activity was not detected by zymography.

High-Fat Diet and Paternal Resistance Training on Col1, NFκ-B, and PPAR-α Protein Levels in the Gastrocnemius Muscle

There were no differences between sedentary and trained fathers in Col1, NFκ-B, and PPAR-α protein levels in the gastrocnemius muscle ($p > 0.05$.; **Figures 7A,C,E**, respectively). HFD did not modulate Col1, NFκ-B, and PPAR-α protein levels in the offspring from sedentary fathers (SFO-HF vs. SFO-C $p > 0.05$; **Figures 7B,D,F**, respectively). Intriguingly, the TFO-C group displayed higher levels of NFκ-B when compared to the TFO-HF group ($p = 0.0001$; **Figure 7D**). However, paternal RT decreased the NFκ-B protein level in offspring exposed to HFD (TFO-HF vs. SFO-HF; $p = 0.01$; **Figure 7D**). **Supplementary Data 1** shows p-values of factors (diet and paternal training) and interaction.

DISCUSSION

The present study was designed to explore the role of pre-conceptional paternal RT on essential molecules involved in the skeletal muscle remodeling of offspring exposed to standard chow or HFD. Our results support the initial hypothesis, showing that HFD downregulated collagen mRNA levels, while upregulating inflammatory and atrophy pathways and adipogenic transcription factor mRNA levels in the offspring gastrocnemius muscle. It was hypothesized that these two findings combined might contribute to the impaired functional integrity of muscle. On the other hand, we demonstrated that paternal RT increased MMP-2 activity in offspring exposed

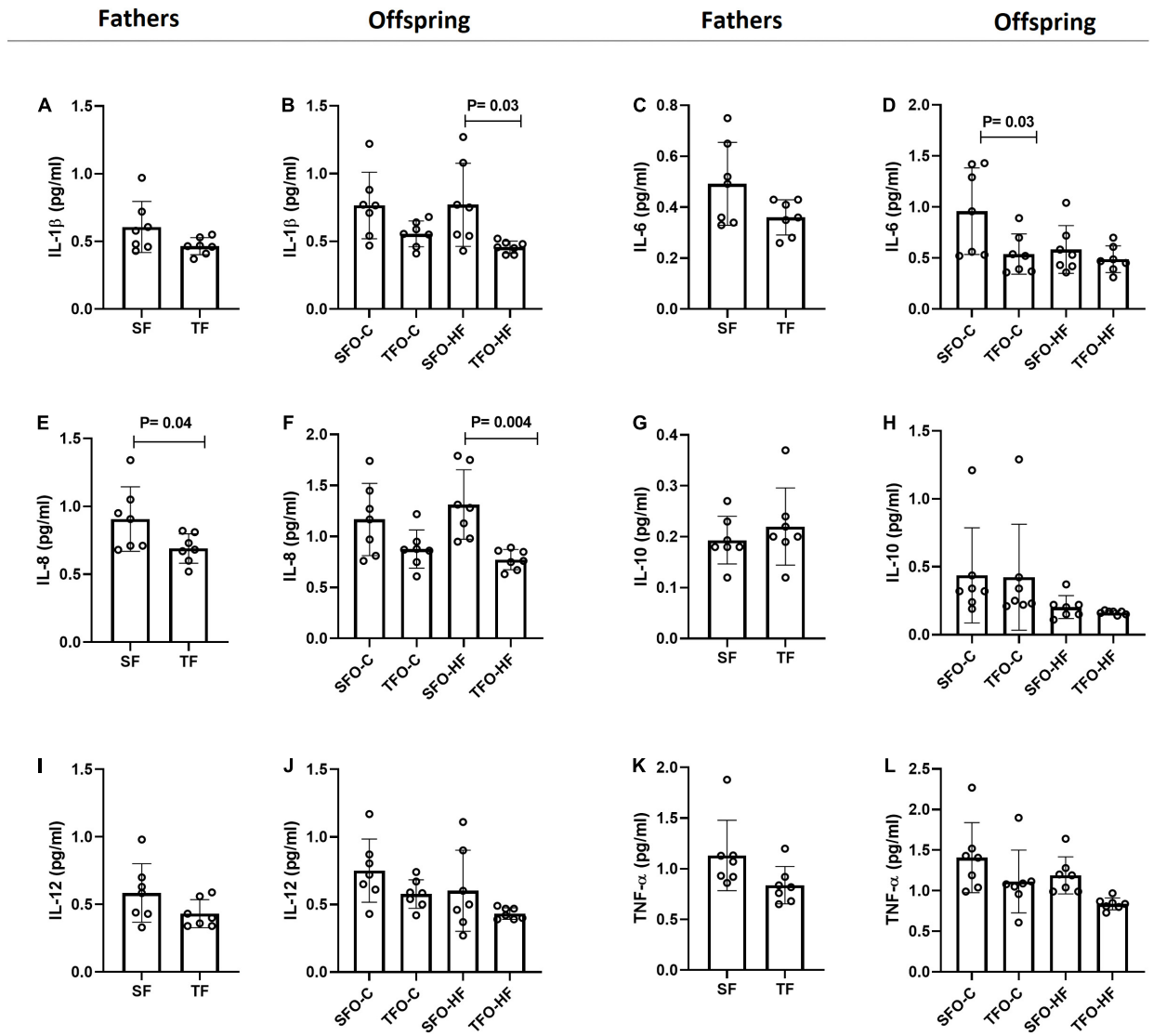

FIGURE 5 | Pro-inflammatory and anti-inflammatory cytokine levels in the gastrocnemius muscle of fathers and offspring exposed to control diet and HFD. The data are mean ± standard deviation. **(A,B)** Interleukin 1β (IL-1β). **(C,D)** Interleukin-6 (IL-6). **(E,F)** Interleukin 8 (IL-8). **(G,H)** Interleukin 10 (IL-10). **(I,J)** Interleukin 12 (IL-12). **(K,L)** Tumor necrosis factor alpha (Tnf-α). SF, sedentary fathers; TF, trained fathers; SFO-C, offspring from sedentary fathers, exposed to control diet; TFO-C, offspring from trained fathers exposed to control diet; SFO-HF, offspring from sedentary fathers exposed to high-fat diet; TFO-HF, offspring from trained fathers exposed to a high-fat diet ($n = 7$ per group).

to a control diet, accompanied by a decrease in IL-6 levels. More significantly, paternal RT upregulated *P70s6k* and *Ppara* mRNA levels, as well as downregulating *Atrogin1* mRNA levels and decreasing NFκ-B, IL-1β, and IL-8 protein levels in offspring exposed to HFD. These data support that paternal RT might modulate key skeletal muscle remodeling pathways and inflammatory profiles in offspring submitted to different diets, which may be relevant for cellular function, longevity, and muscle homeostasis maintenance. Our findings raise valuable insights into the molecular mechanisms involved in the intergenerational impacts of paternal exercise on the musculoskeletal system of offspring. This molecular approach progresses the current knowledge on muscle biology.

Effects of resistance training on gene and protein expression/activity involved in muscle inflammation and remodeling of fathers.

Skeletal muscle is a highly plastic organ that plays a pivotal role in physical maintenance and metabolic health (Blaauw et al., 2013). Strategies focused on increasing the quality of this tissue are relevant, and RT is an effective non-pharmacological tool to promote positive impacts on the remodeling process. However, the regulatory mechanisms that mediate muscle adaptations are complex (Blaauw et al., 2013). The present molecular analysis comparing the TF group vs. SF group (fathers) reinforces that RT might modulate multiple routes involved in muscle remodeling. We observed that RT upregulated anabolic markers (*Igf1, Akt,*

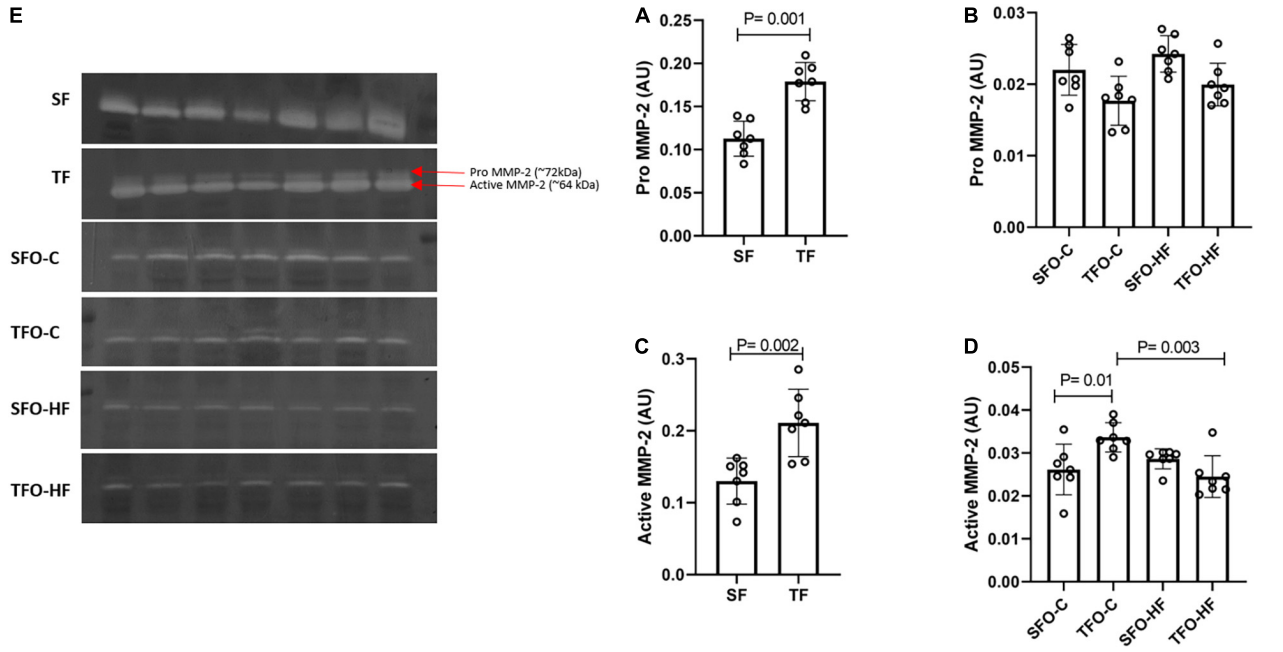

FIGURE 6 | MMP-2 activity in the gastrocnemius muscle of fathers and offspring exposed to control diet and HFD. Optical densitometry of zymographic bands of MMP-2 in arbitrary units (AU). The data are mean ± standard deviation. **(A)** Pro MMP-2 activity in fathers. **(B)** Pro MMP-2 activity in offspring. **(C)** Active MMP-2 activity in fathers. **(D)** Active MMP-2 activity in offspring. **(E)** Optical densitometry of zymographic bands. SF, sedentary fathers; TF, trained fathers; SFO-C, offspring from sedentary fathers, exposed to control diet; TFO-C, offspring from trained fathers exposed to control diet; SFO-HF, offspring from sedentary fathers exposed to high-fat diet; TFO-HF, offspring from trained fathers exposed to a high-fat diet ($n = 7$ per group).

P70sk6, and *Myod*), *Murf1*, *Cebp*a, *Ppara* mRNA levels, and MMP-2 activity in the gastrocnemius muscle in trained fathers, accompanied by a decrease in IL-8 levels, which may suggest that RT induces molecular adaptation through an integrated signs network. Muscle adaptability is mediated by a complex interplay between a myriad of signaling pathways, joined to several transcription and translation factors (Karalaki et al., 2009; Hoppeler, 2016). The pleiotropic effects of RT and the complexity of responses at a molecular level in our study suggest that there is no singular pathway mediating exercise adaptation. Thus, it is important to emphasize that cellular homeostasis is achieved by a delicate balance between multiple pathways required to carry out complex physiological processes.

Effects of high-fat diet on gene and protein expression/activity involved in muscle inflammation and remodeling of offspring.

The increase in high-energy dietary and unbalanced calorie intake are well-known risk factors that negatively affect skeletal muscle functionality (Lee et al., 2015; Herrenbruck and Bollinger, 2020). The harmful changes are linked to the increase in intramyocellular lipids, associated with lipotoxicity, ECM dysfunctional remodeling, and changes in muscle atrophy—related genes (Abrigo et al., 2016). Indeed, HFD downregulated collagen mRNA levels while upregulating inflammatory (*Tweak* and *Nfkb*) and atrophy pathways (*Atrogin1* and *Murf1*), and Cebpa mRNA levels in the offspring gastrocnemius muscle (SFO-HF vs. SFO-C). This result is in agreement with Kumar et al. (2012), who showed that the *Tweak-Nfkb* axis could be involved in protein breakdown and play an important role in regulating *Murf1* and *Atrogin1* genes. These changes might result in a more inflammatory and catabolic phenotype linked to the adipogenesis integrated process, leading to a panel of adverse remodeling. Nevertheless, we found that gene expression changes were not reflected at the level of proteins at the time-point analyzed, probably due to delayed synthesis between mRNA or the protein during state transition, as well as proteins disconnected from the transcripts (Liu et al., 2016). It is known that protein levels are regulated by transcriptional regulatory elements such as enhancers and post-transcriptional control mechanisms (Sadhale et al., 2007). Furthermore, protein turnover is often under the control of the ubiquitin-proteasome system. One possibility that should not be ruled out is that NF-kB might be associated with ubiquitin-proteasome system overexpression by HFD diet (Abrigo et al., 2016), which may have affected protein patterns and function in the present study.

Other results confirm that HFD has considerable adverse effects on skeletal muscle remodeling due to down-regulation of *Col3a1* mRNA levels and MMP-2 activity, accompanied by up-regulation of *Nfkb* and *Foxo1* mRNA levels in the offspring from trained fathers (TFO-HF vs. TFO-C). The present findings seem to point to progression toward a degenerative phenotype, typical features of muscle wasting and weakness. Abnormal muscle remodeling may impact cell activity, tissue polarity, and muscle repair, inducing chronic disease initiation or progression. Regarding plausible mechanisms, Abrigo et al. (2016) explained that HFD decreases multipotent mesenchymal stromal cells

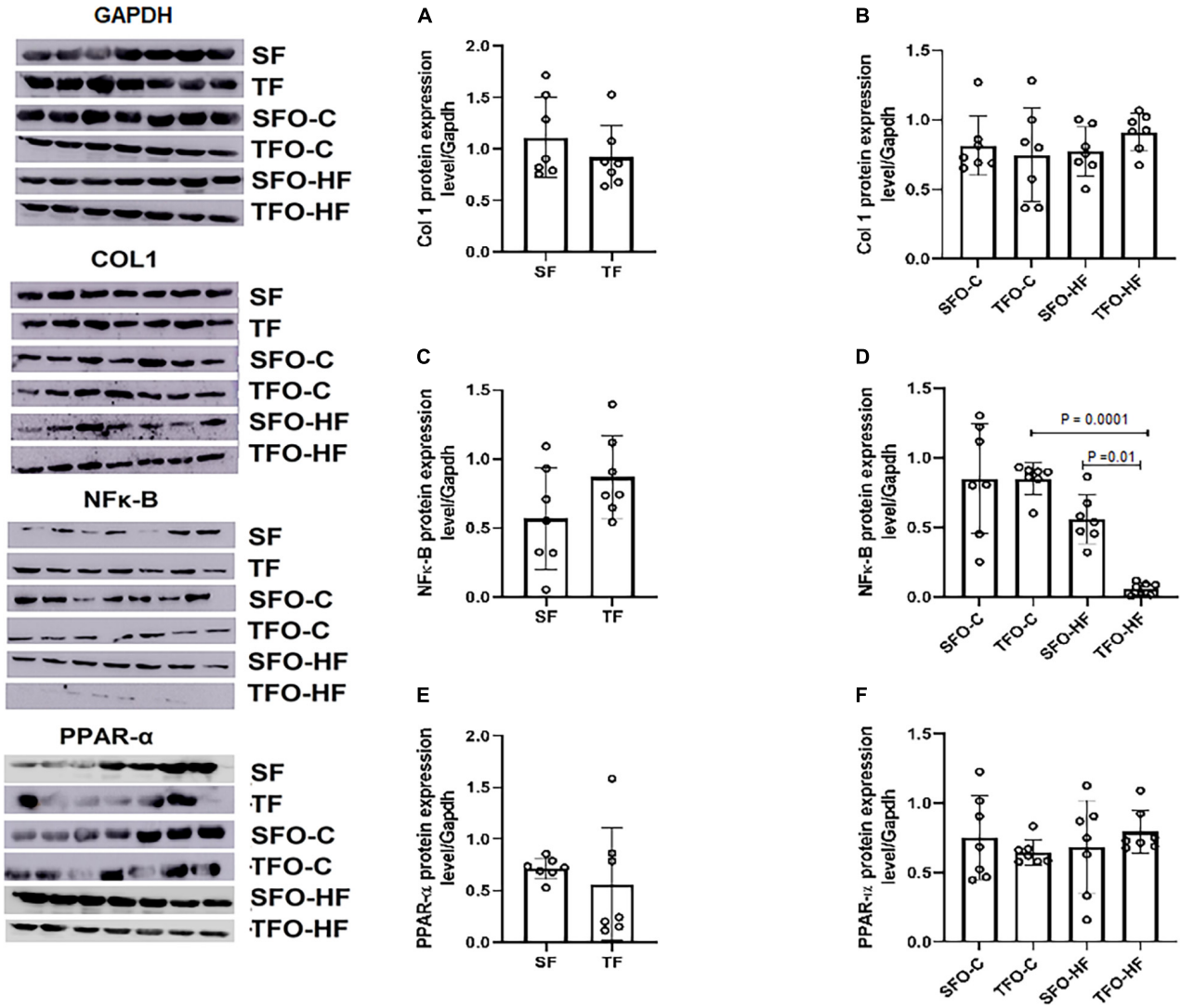

FIGURE 7 | Collagen type I, NF-κB, and PPAR-α protein expression in the gastrocnemius muscle of fathers and offspring exposed to control diet and HFD. The data are mean ± standard deviation. **(A)** Col 1 protein expression level/Gapdh in the fathers. **(B)** Col 1 protein expression level/Gapdh in the offspring. **(C)** NF-κB protein expression level/Gapdh in the fathers. **(D)** NF-κB protein expression level/Gapdh in the offspring. **(E)** PPAR-α protein expression level/Gapdh in the fathers. **(F)** PPAR-α protein expression level/Gapdh in the offspring. SF, sedentary fathers; TF, trained fathers; SFO-C, offspring from sedentary fathers, exposed to control diet; TFO-C, offspring from trained fathers exposed to control diet; SFO-HF, offspring from sedentary fathers exposed to high-fat diet; TFO-HF, offspring from trained fathers exposed to a high-fat diet ($n = 7$ per group).

through complex crosstalk of inflammation, oxidative stress, satellite cell dysfunction, and myonuclear apoptosis which, in turn, induces an atrophic effect on skeletal muscle. Furthermore, the adipogenesis process also leads to inflammation and atrophy in muscle cells during obesity (Pellegrinelli et al., 2015), which explains an increase in the *Cebpa*, *Tweak,* and *Nfkb*, *Atrogin1,* and *Murf1* mRNA levels simultaneously in the SFO-HF group.

Effects of paternal resistance training on gene and protein expression/activity involved in muscle inflammation and remodeling of offspring exposed to control diet.

An exciting result was that paternal RT upregulated MMP-2 activity while downregulating *Il6* levels in offspring exposed to the control diet. This suggests that the father's lifestyle can imply changes in ECM remodeling and the inflammatory profile when offspring are not exposed to a harmful diet. The increase in MMP-2 activity contributes to the remodeling of fibers and connective tissue during normal homeostasis (Chen and Li, 2009). MMP-2 is involved in the morphogenesis and angiogenesis process (de Sousa Neto et al., 2018), playing a critical role in differentiation, muscle repair, and regeneration (de Sousa Neto et al., 2017; Martinez-Huenchullan et al., 2017). Hojman et al. (2019) reported that any ECM alteration initiates the membrane conformational changes needed to release the IL-6-containing intrafibrillar vesicles. Therefore, it seems reasonable to speculate that optimal muscle remodeling modulates inflammation adjustment, considered substantial for

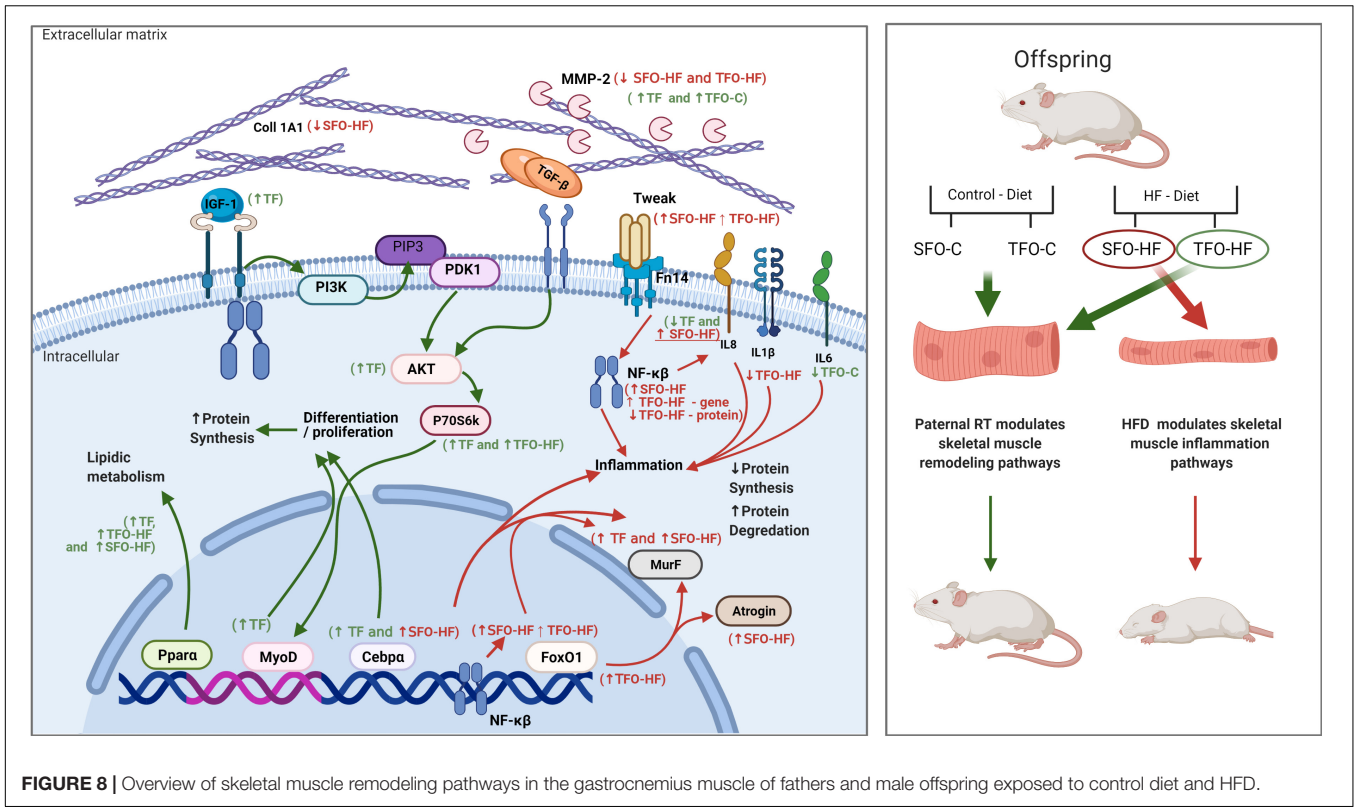

FIGURE 8 | Overview of skeletal muscle remodeling pathways in the gastrocnemius muscle of fathers and male offspring exposed to control diet and HFD.

muscle adaptation mechanisms. Additional studies should be carried out to test this hypothesis and clarify the causal link between these two biological phenomena. de Sousa Neto et al. (2020b) reported that paternal RT induces modifications at the protein level in the left ventricle of eutrophic offspring. However, this modulation was more prominent in the tendon when the offspring were subjected to HFD (de Sousa Neto et al., 2020a). Therefore, these results support the idea that molecular responses in the offspring might differ between tissue types.

The results of the present study are in agreement with Lira et al., 2009, who demonstrated the potential exercise effect on downregulation of IL-6 levels in healthy rat skeletal muscle. Regarding possible physiological significance, decreased muscle-derived IL-6 production may work to inhibit the effects of pro-inflammatory cytokines, as well as contributing to glucose homeostasis maintenance and lipid metabolism (Lira et al., 2009; Munoz-Canoves et al., 2013). It is therefore possible to speculate that, as well as the attenuation of HPD-associated chronic inflammation, paternal RT may also lead to a decreased risk of the incidence of various diseases associated with an offspring sedentary lifestyle, considering that even in a eutrophic condition (i.e., TFO-C vs. SFO-C) it shows a significant anti-inflammatory effect.

Effects of paternal resistance training on gene and protein expression/activity involved in muscle inflammation and remodeling of offspring exposed to a high-fat diet.

Another novelty of our study was that paternal RT upregulated *P70s6k,* effective in mitigating the HFD-associated increase in *Atrogin1* mRNA levels in offspring exposed to HFD, which can inhibit protein degradation and promote synthesis. Possibly, these adaptations are important to alleviate musculoskeletal damage, the inflammatory microenvironment, and nuclear apoptosis (Abrigo et al., 2016). Thus, paternal RT should be considered an important component to combat skeletal muscle atrophy with HFD.

Remarkably, short (3 weeks) and long-term voluntary wheel protocol (12 weeks) proved to be capable of suppressing the effects of a paternal high-fat diet on offspring, reversing impairment in glucose intolerance, fat mass, and glucose uptake in skeletal muscles (Krout et al., 2018; Stanford et al., 2018). This protection occurred through increased expression of insulin signaling pathways (GLUT4, IRS1, and PI3K) in the skeletal muscle of the offspring (Krout et al., 2018). However, voluntary wheel running possibly has a more considerable aerobic component when compared to weighted stair climbing, which can limit the effects on muscle hypertrophy as previously reported (Legerlotz et al., 2008; Kurosaka et al., 2009). Climbing a ladder provides an alternative means of inducing force overload with advantages when improving musculoskeletal properties and neuromuscular function (Hornberger and Farrar, 2004), and consequent signaling pathway activation that controls skeletal muscle hypertrophy. We consider that the current RT protocol (8 weeks, three times per week) should receive special attention since might open new avenues for preventing and treating muscle wasting in metabolic and neuromuscular diseases in offspring exposed to the risk factors.

Curiously, we found that paternal RT upregulated *Foxo1* mRNA levels in the offspring exposed to HFD. Although *Foxo1*

exacerbated activation associated with muscle atrophy, this protein can exhibit ambivalent and pleiotropic functions (Seiler et al., 2013; Sanchez et al., 2014). This suggestion corroborates other studies which showed that *Foxo1* modulates the subcellular localization of the membrane fatty acid translocase that permits fatty acid uptake into muscle cells (Kamei et al., 2003; Bastie et al., 2005). Evidence suggests that *Foxo1* can act as a switch for a shift toward lipids instead of glucose as a fuel substrate under HFD conditions (Constantin-Teodosiu et al., 2012; Sanchez et al., 2014). Additionally, *Ppara* may contribute to adaptive changes in fatty acid metabolism in response to overexpressed *Foxo1* (Constantin-Teodosiu et al., 2012), supporting our TFO-HF group findings. Together, our results suggest that paternal RT can orchestrate factors that might contribute to muscle fat oxidation in the offspring.

In this sense, paternal RT also upregulated *Ppara* mRNA levels, besides decreasing NFκ-B and IL-8 protein levels in offspring exposed to HFD, implying a protective factor against the undesired effects of HFD. Upregulated *Ppara* reduces triglyceride levels by increasing lipolysis, contributing to anti-inflammatory activity and metabolic improvements (Gross et al., 2017; Wang et al., 2020). PPAR-α activation interacts with a diverse group of molecules (Viswakarma et al., 2010); for example, PPAR-α inhibits the NF-κB pathway and reduces inflammatory markers, such as IL-1β and IL-8, which in turn suppress atrophy process at multiple levels (Rigamonti et al., 2008). Recently, several plausible studies have revealed that these potent proinflammatory cytokines are involved in calcium homeostasis, mitogenesis, inhibition of angiogenesis, chemotaxis, neutrophil degranulation, and leukocyte activation, which might be an essential contributor to the inflammatory mechanism (Pernhorst et al., 2013; Chuang and Khorram, 2014; Kofanova et al., 2019). Considering our findings, paternal RT may attenuate lipid storage in the skeletal muscle and modulate inflammatory status through IL-1β and IL-8 levels. This result is in agreement with the decrease in *Atrogin1* mRNA levels observed in the TFO-HF group, since it has been demonstrated that IL-1β and IL-8 also play a vital role in atrophy signaling (Callaway et al., 2019). A schematic representation of the intracellular and extracellular environment demonstrates the up and downregulation, and the role of the main genes and proteins identified in this study (**Figure 8**).

The canonical signaling pathways that induce NF-kB activation converge on the IkB kinase (IKK) complex, which is responsible for IkB phosphorylation and is essential for subsequent translocation of active NF-κB from the cytoplasm to nucleus (Lawrence, 2009). In addition, Toll like receptor (TLR) stimulation and reactive oxygen species (ROS) inherent to HFD provoke activation of multiple signaling pathways, including Ikb/NF-kB axis, JNK/AP-1 signaling, and MAP kinases (ERK1/2, JNK, and p38), promoting subsequent expression of proinflammatory cytokines (Zhang et al., 2016). Alternatively, exercise training decreases TLR expression, while stimulating the expression of antioxidant enzymes and sirtuin 1 (SIRT1), which inhibits NF-κB activity through deacetylation and induces PPARα signaling pathway activation (Gleeson et al., 2006; Liu and Chang, 2018). Collectedly, these adjacent molecular pathways and mutual crosstalk could clarify the possible protective paternal RT effects on skeletal muscle inflammation of offspring in the current study.

Paternal RT did not modulate structural matrix proteins or matrix remodeling enzyme mRNA levels, like Col1 protein level and MMP-2 activity in the gastrocnemius muscle of offspring exposed to HFD, which could be related to similar *Ctgf* and *Tgfb* mRNA levels between the TFO-HF and SFO-HF groups. Skeletal muscle ECM is perturbed under insulin-resistant conditions (Kang et al., 2011, 2013; Graae et al., 2019); however we demonstrated that paternal RT did not decrease the area under the curve (AUC) during the oral glucose tolerance test (de Sousa Neto et al., 2020b), which may have contributed to the limited effects of paternal RT on ECM adaptations in the HFD condition.

It has been shown that paternal exercise in advanced aged sires (>P100) suppresses the effects of paternal high-fat diet on offspring, reversing the observed impairment in glucose tolerance, percentage of fat mass, and glucose uptake in different skeletal muscles of the offspring (Stanford et al., 2018). In the current study, adult rats (4-month-old) affect gene and protein expression in the skeletal muscle of offspring, which suggests that different fathers' age might modulate the muscle physiology of offspring. Future studies should include the different species of animals and offspring sex and paternal exercise detraining. Furthermore, the effect of lifelong exercise training, not just for a predetermined time intervention or father's age remains a provocative hypothesis for further investigation. One of the essential aspects of the practical application of this study is to encourage fathers to perform regular exercise throughout life because this might delay the vicious cycle of increased unhealthy risks propagating across generations.

The implementation of paternal RT may become a primary means for combating the ever-growing burden of musculoskeletal system disorders that presently threaten the functional health and wellness of future generations. An epigenetic inheritance mechanistic view could provide crucial clues for public health and clinical practice. Therefore, the tendency or prevalence of muscle abnormalities in the offspring could be partially explained by the father's environment and lifestyle.

Limitations

Some limitations of the present study should be highlighted, such as the impossibility of analyzing morphology properties (cross-sectional area, intramyocellular lipids, and ECM markers) and muscle functional assessments (strength and isolated whole-muscle contractility). Our results are also limited to our time diet exposure (i.e., 24 weeks) and male offspring. Further research must include female and male offspring in order to identify specific molecular changes or overlaps between the sexes. The reason for this sex-specificity is uncertain and requires further research. The assessment of only a single point in time is also a considerable limitation. Different time-points from the beginning of dietary intake would be highly valuable in order to clarify the time-course effects of other diets on molecular responses. Non-coding RNA and DNA methylation in the sperm are major routes,

which are probably responsible for transmitting the epigenetic landscape from fathers to future generations (Kusuyama et al., 2020; Vieira de Sousa Neto et al., 2021). Additional studies are required to evaluate the link between the sperm epigenetic profile and offspring skeletal muscle remodeling pathways.

CONCLUSION

In summary, the father's lifestyle and the offspring's diet distinctly modified the key genes and protein expression involved in the muscle inflammation and remodeling pathways. HFD activates inflammatory factors, which in turn might induce dysregulation of signaling pathways associated with the ECM structure and balance between muscle synthesis/breakdown. Interestingly, paternal RT is a critical factor capable of reprogramming different remodeling pathways regardless of the offspring's diet. In particular, MMP-2 and IL-6 for offspring exposed to the control diet, and the synthesis/atrophy pathways, PPAR-α, and NFκ-B/cytokine axis for offspring submitted to HFD are relevant candidates modulated by paternal RT that may become potential therapeutic targets for treating skeletal muscle disorders. Of relevance, these critical molecules could be contributing toward a muscle-healthy phenotype in the offspring. We believe our findings provide a significant advance to map key paternal exercise-regulated pathways in offspring skeletal muscle.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The research protocol received approval from the Ethics Committee on Animal Experimentation from the Universidade Católica de Brasília, Brasília (protocol no. 010/13).

AUTHOR CONTRIBUTIONS

RS, IN, RT, JP, and RM conceived and planned the design of the experiments. RS, IN, GR, RT, and AC performed the experiments, designed the figures, and analyzed the data. RS, IN, GR, JP, and RM interpreted the results and worked on the writing of the manuscript. JD, GP, ON, OF, CR, FN, and RH involved in planning and supervising the work, also contributed to reagents, materials, and analysis tools, implementation of the research and provided critical feedback of the manuscript. All authors discussed the results and contributed to the final manuscript.

FUNDING

The authors are grateful for the financial support provided by the Fundação de Apoio a Pesquisa do Distrito Federal (FAPDF) (grant nos. 193.001.452/2016, 193.000.653/2015, 00193.00000641/2018-45, and 00193.0000168/2019- 87) and FAPESP (2013/00798-2 and 2011/11229-3), the Decanato de Pesquisa e Inovação e de Pós-Graduação (grant nos. DPI/DPG N.° 02/2020, DPI/DPG N.° 02/2021, and DPI/DPG N.° 04/2021), the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES Finance Code 001), and Conselho Nacional de Desenvolvimento Científco e Tecnológico (CNPq) (grant nos. 445069/2014-7 and 312136/2018-8) from Brazil. ON, JP, JD, and RM were recipients of fellowships for productivity (CNPq). OF was also funded by FAPDF and FUNDECT.

ACKNOWLEDGMENTS

We thank Robin Hambly for reviewing the English.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2021.706128/full#supplementary-material

REFERENCES

Abrigo, J., Rivera, J. C., Aravena, J., Cabrera, D., Simon, F., Ezquer, F., et al. (2016). High fat diet-induced skeletal muscle wasting is decreased by mesenchymal stem cells administration: implications on oxidative stress, ubiquitin proteasome pathway activation, and myonuclear apoptosis. *Oxid. Med. Cell Longev.* 2016:9047821. doi: 10.1155/2016/9047821

Barin, F. R., de Sousa Neto, I. V., Vieira Ramos, G., Szojka, A., Ruivo, A. L., Anflor, C. T. M., et al. (2019). Calcaneal tendon plasticity following gastrocnemius muscle injury in rat. *Front. Physiol.* 10:1098. doi: 10.3389/fphys.2019.01098

Bastie, C. C., Nahle, Z., McLoughlin, T., Esser, K., Zhang, W., Unterman, T., et al. (2005). FoxO1 stimulates fatty acid uptake and oxidation in muscle cells through CD36-dependent and -independent mechanisms. *J. Biol. Chem.* 280, 14222–14229. doi: 10.1074/jbc.M413625200

Blaauw, B., Schiaffino, S., and Reggiani, C. (2013). Mechanisms modulating skeletal muscle phenotype. *Compr. Physiol.* 3, 1645–1687. doi: 10.1002/cphy.c130009

Bollheimer, L. C., Buettner, R., Pongratz, G., Brunner-Ploss, R., Hechtl, C., Banas, M., et al. (2012). Sarcopenia in the aging high-fat fed rat: a pilot study for modeling sarcopenic obesity in rodents. *Biogerontology* 13, 609–620. doi: 10.1007/s10522-012-9405-4

Callaway, C. S., Delitto, A. E., Patel, R., Nosacka, R. L., D'Lugos, A. C., Delitto, D., et al. (2019). IL-8 released from human pancreatic cancer and tumor-associated stromal cells signals through a CXCR2-ERK1/2 axis to induce muscle atrophy. *Cancers (Basel)* 11:1863. doi: 10.3390/cancers11121863

Carlsen, H., Haugen, F., Zadelaar, S., Kleemann, R., Kooistra, T., Drevon, C. A., et al. (2009). Diet-induced obesity increases NF-kappaB signaling in reporter mice. *Genes Nutr.* 4, 215–222. doi: 10.1007/s12263-009-0133-6

Chen, X., and Li, Y. (2009). Role of matrix metalloproteinases in skeletal muscle: migration, differentiation, regeneration and fibrosis. *Cell Adh. Migr.* 3, 337–341. doi: 10.4161/cam.3.4.9338

Chomczynski, P., and Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem.* 162, 156–159. doi: 10.1006/abio.1987.9999

Chuang, T. D., and Khorram, O. (2014). miR-200c regulates IL8 expression by targeting IKBKB: a potential mediator of inflammation in leiomyoma pathogenesis. *PLoS One* 9:e95370. doi: 10.1371/journal.pone.0095370

Constantin-Teodosiu, D., Constantin, D., Stephens, F., Laithwaite, D., and Greenhaff, P. L. (2012). The role of FOXO and PPAR transcription factors in diet-mediated inhibition of PDC activation and carbohydrate oxidation during exercise in humans and the role of pharmacological activation of PDC in overriding these changes. *Diabetes* 61, 1017–1024. doi: 10.2337/db11-0799

de Sousa Neto, I. V., Durigan, J. L. Q., Guzzoni, V., Tibana, R. A., Prestes, J., de Araujo, H. S. S., et al. (2018). Effects of resistance training on matrix metalloproteinase activity in skeletal muscles and blood circulation during aging. *Front. Physiol.* 9:190. doi: 10.3389/fphys.2018.00190

de Sousa Neto, I. V., Tibana, R. A., da Cunha Nascimento, D., Vieira, D. C., Durigan, J. L., Pereira, G. B., et al. (2017). Effects of resistance training volume on MMPs in circulation, muscle and adipose tissue. *Int. J. Sports Med.* 38, 307–313. doi: 10.1055/s-0042-123192

de Sousa Neto, I. V., Tibana, R. A., da Silva, L. G. O., de Lira, E. M., do Prado, G. P. G., de Almeida, J. A., et al. (2020a). Paternal resistance training modulates calcaneal tendon proteome in the offspring exposed to high-fat diet. *Front. Cell Dev. Biol.* 8:380. doi: 10.3389/fcell.2020.00380

de Sousa Neto, I. V., Tibana, R. A., Prestes, J., de Oliveira da Silva, L. G., Almeida, J. A., Franco, O. L., et al. (2020b). Paternal resistance training induced modifications in the left ventricle proteome independent of offspring diet. *Oxid. Med. Cell Longev.* 2020:5603580. doi: 10.1155/2020/5603580

Demasi, M., and Laurindo, F. R. (2012). Physiological and pathological role of the ubiquitin-proteasome system in the vascular smooth muscle cell. *Cardiovasc. Res.* 95, 183–193. doi: 10.1093/cvr/cvs128

Gleeson, M., McFarlin, B., and Flynn, M. (2006). Exercise and Toll-like receptors. *Exerc. Immunol. Rev.* 12, 34–53.

Glover, E. I., and Phillips, S. M. (2010). Resistance exercise and appropriate nutrition to counteract muscle wasting and promote muscle hypertrophy. *Curr. Opin. Clin. Nutr. Metab. Care* 13, 630–634. doi: 10.1097/MCO.0b013e32833f1ae5

Goncalves, T. T., Lazaro, C. M., De Mateo, F. G., Campos, M. C., Mezencio, J. G., Claudino, M. A., et al. (2018). Effects of glucosyl-hesperidin and physical training on body weight, plasma lipids, oxidative status and vascular reactivity of rats fed with high-fat diet. *Diabetes Metab. Syndr. Obes.* 11, 321–332. doi: 10.2147/DMSO.S153661

Graae, A. S., Grarup, N., Ribel-Madsen, R., Lystbaek, S. H., Boesgaard, T., Staiger, H., et al. (2019). ADAMTS9 regulates skeletal muscle insulin sensitivity through extracellular matrix alterations. *Diabetes* 68, 502–514. doi: 10.2337/db18-0418

Gross, B., Pawlak, M., Lefebvre, P., and Staels, B. (2017). PPARs in obesity-induced T2DM, dyslipidaemia and NAFLD. *Nat. Rev. Endocrinol.* 13, 36–49. doi: 10.1038/nrendo.2016.135

Guedes, J. M., Pieri, B., Luciano, T. F., Marques, S. O., Guglielmo, L. G. A., and Souza, C. T. (2020). Muscular resistance, hypertrophy and strength training equally reduce adiposity, inflammation and insulin resistance in mice with diet-induced obesity. *Einstein (Sao Paulo)* 18:eAO4784. doi: 10.31744/einstein_journal/2020AO4784

Guzzoni, V., Ribeiro, M. B. T., Lopes, G. N., de Cassia Marqueti, R., de Andrade, R. V., Selistre-de-Araujo, H. S., et al. (2018). Effect of resistance training on extracellular matrix adaptations in skeletal muscle of older rats. *Front. Physiol.* 9:374. doi: 10.3389/fphys.2018.00374

Heinemeier, K. M., Olesen, J. L., Haddad, F., Langberg, H., Kjaer, M., Baldwin, K. M., et al. (2007). Expression of collagen and related growth factors in rat tendon and skeletal muscle in response to specific contraction types. *J. Physiol.* 582(Pt 3), 1303–1316. doi: 10.1113/jphysiol.2007.127639

Herrenbruck, A. R., and Bollinger, L. M. (2020). Role of skeletal muscle autophagy in high-fat-diet-induced obesity and exercise. *Nutr. Rev.* 78, 56–64. doi: 10.1093/nutrit/nuz044

Hojman, P., Brolin, C., Norgaard-Christensen, N., Dethlefsen, C., Lauenborg, B., Olsen, C. K., et al. (2019). IL-6 release from muscles during exercise is stimulated by lactate-dependent protease activity. *Am. J. Physiol. Endocrinol. Metab.* 316, E940–E947. doi: 10.1152/ajpendo.00414.2018

Hoppeler, H. (2016). Molecular networks in skeletal muscle plasticity. *J. Exp. Biol.* 219(Pt 2), 205–213. doi: 10.1242/jeb.128207

Hornberger, T. A. Jr., and Farrar, R. P. (2004). Physiological hypertrophy of the FHL muscle following 8 weeks of progressive resistance exercise in the rat. *Can. J. Appl. Physiol.* 29, 16–31. doi: 10.1139/h04-002

Kamei, Y., Mizukami, J., Miura, S., Suzuki, M., Takahashi, N., Kawada, T., et al. (2003). A forkhead transcription factor FKHR up-regulates lipoprotein lipase expression in skeletal muscle. *FEBS Lett.* 536, 232–236. doi: 10.1016/s0014-5793(03)00062-0

Kang, L., Ayala, J. E., Lee-Young, R. S., Zhang, Z., James, F. D., Neufer, P. D., et al. (2011). Diet-induced muscle insulin resistance is associated with extracellular matrix remodeling and interaction with integrin alpha2beta1 in mice. *Diabetes* 60, 416–426. doi: 10.2337/db10-1116

Kang, L., Lantier, L., Kennedy, A., Bonner, J. S., Mayes, W. H., Bracy, D. P., et al. (2013). Hyaluronan accumulates with high-fat feeding and contributes to insulin resistance. *Diabetes* 62, 1888–1896. doi: 10.2337/db12-1502

Karalaki, M., Fili, S., Philippou, A., and Koutsilieris, M. (2009). Muscle regeneration: cellular and molecular events. *In Vivo* 23, 779–796.

Kofanova, O., Bellora, C., Quesada, R. A., Bulla, A., Panadero-Fajardo, S., Keipes, M., et al. (2019). IL8 and EDEM3 gene expression ratio indicates peripheral blood mononuclear cell (PBMC) quality. *J. Immunol. Methods* 465, 13–19. doi: 10.1016/j.jim.2018.11.012

Krout, D., Roemmich, J. N., Bundy, A., Garcia, R. A., Yan, L., and Claycombe-Larson, K. J. (2018). Paternal exercise protects mouse offspring from high-fat-diet-induced type 2 diabetes risk by increasing skeletal muscle insulin signaling. *J. Nutr. Biochem.* 57, 35–44. doi: 10.1016/j.jnutbio.2018.03.013

Kumar, A., Bhatnagar, S., and Paul, P. K. (2012). TWEAK and TRAF6 regulate skeletal muscle atrophy. *Curr. Opin. Clin. Nutr. Metab. Care* 15, 233–239. doi: 10.1097/MCO.0b013e328351c3fc

Kurosaka, M., Naito, H., Ogura, Y., Kojima, A., Goto, K., and Katamoto, S. (2009). Effects of voluntary wheel running on satellite cells in the rat plantaris muscle. *J. Sports Sci. Med.* 8, 51–57.

Kusuyama, J., Alves-Wagner, A. B., Makarewicz, N. S., and Goodyear, L. J. (2020). Effects of maternal and paternal exercise on offspring metabolism. *Nat. Metab.* 2, 858–872. doi: 10.1038/s42255-020-00274-7

Lawrence, T. (2009). The nuclear factor NF-kappaB pathway in inflammation. *Cold Spring Harb. Perspect. Biol.* 1:a001651. doi: 10.1101/cshperspect.a001651

Lee, S. R., Khamoui, A. V., Jo, E., Park, B. S., Zourdos, M. C., Panton, L. B., et al. (2015). Effects of chronic high-fat feeding on skeletal muscle mass and function in middle-aged mice. *Aging Clin. Exp. Res.* 27, 403–411. doi: 10.1007/s40520-015-0316-5

Legerlotz, K., Elliott, B., Guillemin, B., and Smith, H. K. (2008). Voluntary resistance running wheel activity pattern and skeletal muscle growth in rats. *Exp. Physiol.* 93, 754–762. doi: 10.1113/expphysiol.2007.041244

Lira, F. S., Koyama, C. H., Yamashita, A. S., Rosa, J. C., Zanchi, N. E., Batista, M. L. Jr., et al. (2009). Chronic exercise decreases cytokine production in healthy rat skeletal muscle. *Cell Biochem. Funct.* 27, 458–461. doi: 10.1002/cbf.1594

Liu, H. W., and Chang, S. J. (2018). Moderate exercise suppresses NF-κB signaling and activates the SIRT1-AMPK-PGC1alpha axis to attenuate muscle loss in diabetic *db/db* mice. *Front. Physiol.* 9:636. doi: 10.3389/fphys.2018.00636

Liu, Y., Beyer, A., and Aebersold, R. (2016). On the dependency of cellular protein levels on mRNA abundance. *Cell* 165, 535–550. doi: 10.1016/j.cell.2016.03.014

Lozano, I., Van der Werf, R., Bietiger, W., Seyfritz, E., Peronet, C., Pinget, M., et al. (2016). High-fructose and high-fat diet-induced disorders in rats: impact on diabetes risk, hepatic and vascular complications. *Nutr. Metab. (Lond)* 13:15. doi: 10.1186/s12986-016-0074-1

Marqueti, R. C., Durigan, J. L. Q., Oliveira, A. J. S., Mekaro, M. S., Guzzoni, V., Aro, A. A., et al. (2018). Effects of aging and resistance training in rat tendon remodeling. *FASEB J.* 32, 353–368. doi: 10.1096/fj.201700543R

Marqueti, R. C., Heinemeier, K. M., Durigan, J. L., de Andrade Perez, S. E., Schjerling, P., Kjaer, M., et al. (2012). Gene expression in distinct regions of rat tendons in response to jump training combined with anabolic androgenic steroid administration. *Eur. J. Appl. Physiol.* 112, 1505–1515. doi: 10.1007/s00421-011-2114-x

Martinez-Huenchullan, S., McLennan, S. V., Verhoeven, A., Twigg, S. M., and Tam, C. S. (2017). The emerging role of skeletal muscle extracellular matrix remodelling in obesity and exercise. *Obes. Rev.* 18, 776–790. doi: 10.1111/obr.12548

Messier, C., Whately, K., Liang, J., Du, L., and Puissant, D. (2007). The effects of a high-fat, high-fructose, and combination diet on learning, weight, and glucose regulation in C57BL/6 mice. *Behav. Brain Res.* 178, 139–145. doi: 10.1016/j.bbr.2006.12.011

Munoz-Canoves, P., Scheele, C., Pedersen, B. K., and Serrano, A. L. (2013). Interleukin-6 myokine signaling in skeletal muscle: a double-edged sword? *FEBS J.* 280, 4131–4148. doi: 10.1111/febs.12338

National Research Council (1996). *Guide for the Care and Use of Laboratory Animals*. Washington, DC: National Academy Press.

Pellegrinelli, V., Rouault, C., Rodriguez-Cuenca, S., Albert, V., Edom-Vovard, F., Vidal-Puig, A., et al. (2015). Human adipocytes induce inflammation and atrophy in muscle cells during obesity. *Diabetes* 64, 3121–3134. doi: 10.2337/db14-0796

Pernhorst, K., Herms, S., Hoffmann, P., Cichon, S., Schulz, H., Sander, T., et al. (2013). TLR4, ATF-3 and IL8 inflammation mediator expression correlates with seizure frequency in human epileptic brain tissue. *Seizure* 22, 675–678. doi: 10.1016/j.seizure.2013.04.023

Potthoff, M. J., Olson, E. N., and Bassel-Duby, R. (2007). Skeletal muscle remodeling. *Curr. Opin. Rheumatol.* 19, 542–549. doi: 10.1097/BOR.0b013e3282efb761

Ribeiro, M. B. T., Guzzoni, V., Hord, J. M., Lopes, G. N., Marqueti, R. C., de Andrade, R. V., et al. (2017). Resistance training regulates gene expression of molecules associated with intramyocellular lipids, glucose signaling and fiber size in old rats. *Sci. Rep.* 7:8593. doi: 10.1038/s41598-017-09343-6

Rigamonti, E., Chinetti-Gbaguidi, G., and Staels, B. (2008). Regulation of macrophage functions by PPAR-alpha, PPAR-gamma, and LXRs in mice and men. *Arterioscler. Thromb. Vasc. Biol.* 28, 1050–1059. doi: 10.1161/ATVBAHA.107.158998

Sadhale, P., Verma, J., and Naorem, A. (2007). Basal transcription machinery: role in regulation of stress response in eukaryotes. *J. Biosci.* 32, 569–578. doi: 10.1007/s12038-007-0056-6

Sanchez, A. M., Candau, R. B., and Bernardi, H. (2014). FoxO transcription factors: their roles in the maintenance of skeletal muscle homeostasis. *Cell Mol. Life Sci.* 71, 1657–1671. doi: 10.1007/s00018-013-1513-z

Sandri, M. (2013). Protein breakdown in muscle wasting: role of autophagy-lysosome and ubiquitin-proteasome. *Int. J. Biochem. Cell Biol.* 45, 2121–2129. doi: 10.1016/j.biocel.2013.04.023

Seiler, F., Hellberg, J., Lepper, P. M., Kamyschnikow, A., Herr, C., Bischoff, M., et al. (2013). FOXO transcription factors regulate innate immune mechanisms in respiratory epithelial cells. *J. Immunol.* 190, 1603–1613. doi: 10.4049/jimmunol.1200596

Stanford, K. I., Rasmussen, M., Baer, L. A., Lehnig, A. C., Rowland, L. A., White, J. D., et al. (2018). Paternal exercise improves glucose metabolism in adult offspring. *Diabetes* 67, 2530–2540. doi: 10.2337/db18-0667

Tam, C. S., Power, J. E., Markovic, T. P., Yee, C., Morsch, M., McLennan, S. V., et al. (2015). The effects of high-fat feeding on physical function and skeletal muscle extracellular matrix. *Nutr. Diabetes* 5:e187. doi: 10.1038/nutd.2015.39

Trajcevski, K. E., O'Neill, H. M., Wang, D. C., Thomas, M. M., Al-Sajee, D., Steinberg, G. R., et al. (2013). Enhanced lipid oxidation and maintenance of muscle insulin sensitivity despite glucose intolerance in a diet-induced obesity mouse model. *PLoS One* 8:e71747. doi: 10.1371/journal.pone.0071747

Vieira de Sousa Neto, I., Fontes, W., Prestes, J., and de Cassia Marqueti, R. (2021). Impact of paternal exercise on physiological systems in the offspring. *Acta Physiol. (Oxf)* 231:e13620. doi: 10.1111/apha.13620

Viswakarma, N., Jia, Y., Bai, L., Vluggens, A., Borensztajn, J., Xu, J., et al. (2010). Coactivators in PPAR-Regulated gene expression. *PPAR Res.* 2010:250126. doi: 10.1155/2010/250126

Wang, Y., Nakajima, T., Gonzalez, F. J., and Tanaka, N. (2020). PPARs as metabolic regulators in the liver: lessons from liver-specific PPAR-Null mice. *Int. J. Mol. Sci.* 21:2061. doi: 10.3390/ijms21062061

Zhang, J., Wang, X., Vikash, V., Ye, Q., Wu, D., Liu, Y., et al. (2016). ROS and ROS-Mediated cellular signaling. *Oxid. Med. Cell Longev.* 2016:4350965. doi: 10.1155/2016/4350965

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