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**DEPARTAMENTO DE CIÊNCIAS FISIOLÓGICAS**
**LABORATÓRIO DE FISIOLOGIA DO EXERCÍCIO**

# *EFEITOS DA DELEÇÃO DE REST DO MÚSCULO ESQUELÉTICO SOBRE A COMPOSIÇÃO CORPORAL E FUNÇÃO MUSCULAR DE CAMUNDONGOS C57BL/6.*

**Tese de doutorado**

**Gustavo Henrique Rigo Canevazzi**

**Orientador: Prof. Dr. Sérgio E. A. Perez**

**Coorientador: Raynald Bergeron**

**São Carlos**

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**UNIVERSDIDADE FEDERAL DE SÃO CARLOS**
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Tese apresentada ao Programa Interinstitucional de Pós-Graduação em Ciências Fisiológicas, do Centro de Ciências Biológicas e da Saúde da Universidade Federal de São Carlos, como parte dos requisitos para a obtenção do título de Doutor em Ciências Fisiológicas. Área de concentração: Fisiologia.

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Centro de Ciências Biológicas e da Saúde
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## Folha de Aprovação

Assinaturas dos membros da comissão examinadora que avaliou e aprovou a Defesa de Tese de Doutorado do candidato Gustavo Henrique Rigo Canevazzi, realizada em 26/10/2018:

Prof. Dr. Sergio Eduardo de Andrade Perez
UFSCar

Profa. Dra. Doris Hissako Sumida
UNESP

Prof. Dr. Claudio Alexandre Gobatto
UNICAMP

Profa. Dra. Ana Claudia Garcia de Oliveira Duarte
UFSCar

Prof. Dr. Wladimir Rafael Beck
UFSCar

## Apresentação

História acadêmica

Sempre fui um apaixonado por esportes, e como qualquer menino, sonhava em ser jogador de futebol. Após anos de dedicação as lesões me afastaram da prática esportiva e, ao mesmo tempo, me aproximaram do que seria a minha profissão – da fisioterapia, é claro!

Foi na graduação em fisioterapia que descobri que o funcionamento do corpo humano me fascinava. A partir de então, comecei a me dedicar aos estudos para tentar compreender os mecanismos envolvidos no movimento, nas lesões, nas doenças, nas funções biológicas. Descobri um mundo cheio de informações e, pela primeira vez na vida, havia encontrado algo que realmente gostava de estudar. Lembro que as aulas de fisiologia humana e fisiologia do exercício com o Prof. Dr. Robson Quitério eram um show à parte na construção do conhecimento. Mal imaginava que a fisiologia iria mudar minha vida.

Com o fim da graduação, procurando cursos relacionados a performance, encontrei o curso de pós-graduação em fisiologia do exercício da UFSCar. Minha vida estava prestes a mudar de forma radical. Quando comecei a estudar a fisiologia de modo mais "profundo" tive a certeza de que aquele era um caminho sem volta. Não era fácil! Tinha que trabalhar em Bebedouro, viajar quinzenalmente para São Carlos para a pós, o dinheiro era curto, dormia de favor na casa de amigos, mas as aulas compensavam todo o esforço. Quando abriram as inscrições para monitoria no laboratório de fisiologia do exercício fiquei animado com a ideia e fui conversar com o Prof. Dr. Vilmar Baldissera. Ele me disse que poderia tentar, mas morar em outra cidade e ficar no laboratório somente nos finais de semana seria insuficiente para as demandas inerentes a um monitor. Eu teria que mudar para São Carlos. Essa foi uma decisão difícil, mas o apoio da minha família ajudou bastante. A ideia de vivenciar a realidade do laboratório de fisiologia do exercício e, talvez, fazer um mestrado ou até mesmo um doutorado na fisiologia foi tão forte que juntei dois meses de salário, pedi demissão em Bebedouro e mudei de "mala e cuia" para São Carlos.

A vivência no laboratório me trouxe a possibilidade de ajudar em experimentos, aprender técnicas, discutir ideias, aprender algo novo todos os dias. Isso me motivou a querer fazer mestrado. Conversei com o Prof. Dr. Sérgio Perez e ele me disse que abriria 2 vagas para o ano seguinte e que eu poderia prestar o processo seletivo. Foi isso que fiz. Terminei a especialização, continuei estudando e trabalhando para conseguir o tão almejado mestrado. Em 2011 passei na prova e comecei a pós.

Em março de 2012 comecei a viver a realidade de projetos, ler artigos em inglês, cursar disciplinas e fazer experimentos. Estava conhecendo novamente um outro mundo de informações. Comecei a me interessar por tudo o que a pós-graduação oferece. Participei de atividades de ensino, pesquisa e extensão. Comecei a fazer parte da Associação de Pós-Graduandos (APG) da UFSCar e, com isso, consegui compreender melhor o funcionamento da universidade. Fiquei na APG durante 3 gestões.

O mestrado foi um período de muito aprendizado e superação com erros, acertos, mudanças de projeto, verba curta para análises, parcerias e muitas outras coisas que me fizeram amadurecer bastante. A fisiologia muscular me encantou e em 2014 finalizei meu mestrado. No mesmo ano comecei o doutorado. Novas e maiores responsabilidades estariam por vir.

Durante o doutorado mantive a mesma vontade de participar de atividades de ensino, pesquisa e extensão. Fui membro da Associação Nacional de Pós-Graduandos e participei de grandes eventos sobre a pós-graduação no Brasil, ajudei a organizar eventos de divulgação científica, participei de conselhos universitários, fui representante discente, desenvolvi projetos, dei aula, escrevi e ajudei a escrever artigos. Muita coisa aconteceu durante este tempo! O projeto que almejava inicialmente não foi aprovado pela FAPESP, faltou dinheiro, muita água rolou, mas fizemos parcerias e tocamos em frente.

Em 2016 consegui uma bolsa para fazer doutorado sanduíche. Coincidentemente, o Prof. Dr. Jean-Marc Lavoie da Universidade de Montreal – Canadá - tinha vindo ao nosso laboratório para a defesa de doutorado da Luciane Magri. Em uma “conversa” com ele (meu inglês era de chorar), me disse que o Prof. Dr. Raynald Bergeron estudava fisiologia muscular e que eu deveria entrar em contato. Ele desenvolvia algumas técnicas que não tínhamos em nosso laboratório e seria uma ótima oportunidade de ter contato com outras formas de se fazer pesquisa. Depois de algumas conversas com o Prof. Dr.

Raynald Bergeron comecei a preparar as coisas para fazer a primeira viagem internacional da minha vida. Depois de muitas aulas de inglês, o frio na barriga e a barreira do idioma ainda eram grandes desafios.

Em julho de 2017 cheguei ao departamento de cinesiologia da Universidade de Montreal. Foram 6 meses de muito aprendizado com aprofundamento teórico e diversas experiências de vida. Contei com muita ajuda e paciência do Prof. Raynald que mostrou os caminhos para superar este desafio. Desenvolvemos dois projetos em parceria com o Prof. Dr. Vahab Soleimani da Universidade McGill, fizemos análises, amizades, sorri, chorei, vi as folhas caírem e a neve chegar.

De volta ao Brasil, trouxe na bagagem muitas histórias, lembranças e experiências. Trouxe muita vontade de colocar em prática tudo que me foi ofertado durante todo o meu processo de formação.

Agora estou finalizando mais uma etapa da minha vida. Entregando minha tese e trazendo no coração os ensinamentos que foram plantados e cultivados por cada um dos professores e professoras que passaram por minha vida. Após anos de estudos, tenho completa e total consciência da minha ignorância frente ao conhecimento. O conhecimento é amplo, as perguntas são diversas, as experiências são muitas e a vida é curta. Certa vez Sócrates, um dos fundadores da filosofia ocidental, disse a conhecida frase: “Só sei que nada sei”. Essa frase me trouxe muita reflexão e me fez perceber, mesmo que momentaneamente, o meu lugar no mundo.

Segundo Aristóteles, a maior virtude de nossa “alma racional” é o exercício do pensamento. A felicidade é um estilo de vida no qual o ser humano precisa exercitar constantemente o melhor que tem dentro dele. Nietzsche caracteriza a felicidade como força vital, espírito de luta contra todos os obstáculos, superação de dificuldades. Para Ortega e Gasset, a felicidade consiste em encontrar algo que nos satisfaça plenamente. Por outro lado, Bertrand Russel descreve que a felicidade é a eliminação do egocentrismo. No entanto, Slavoj Zizek postula que a felicidade é uma questão de opinião, e não de verdade. O ser humano é um eterno insatisfeito porque na realidade não sabe o que quer. Hoje, independente da definição de felicidade utilizada, me sinto feliz e estou ávido em dedicar a minha vida na disseminação do conhecimento, em fazer

perguntas e tentar responder algumas delas. Talvez este seja o caminho da minha felicidade!

**Agradecimentos**

Creio que sozinho nunca conseguimos construir nada. Somos reflexo de todas as experiências e contatos que desenvolvemos durante a vida. Assim, o ambiente que nos cerca contribui de forma efetiva na construção de nossas ações, sentimentos e realizações.

Nesse sentido, meus pais, Carlos e Eliete, foram alicerces centrais nessa caminhada. Se privaram de muita coisa para dar estudos aos filhos. Dedicaram cada minuto das suas vidas para nos transmitir conceitos elementares como honestidade, sinceridade, empatia, amor. Eles proporcionaram um ambiente fértil para que nosso crescimento fosse o melhor possível e, que frente as tempestades inerentes a vida, tivéssemos o controle necessário para superar a adversidade e a capacidade de sorrir novamente com o brilho do sol.

Meus irmãos, Lucas e Amanda, sempre foram motivo de muito orgulho e carinho. A Amanda sempre com seu jeito calmo (parte Canevazzi da família) e o Lucas mais direto e explosivo (parte Rigo da família), participaram em cada momento dando força e acalento.

Meus avós, Antônio e Janete, que mesmo distantes sempre se preocuparam querendo compreender o meu trabalho, querendo saber se eu estava bem. Sou muito grato por toda ajuda e apoio.

Minha companheira, namorada, "pau pra toda obra", Carol, sabe mais do que ninguém que os percalços foram muitos. Ela soube ouvir, falar, criticar, ter paciência, chorar, rir. Soube fazer companhia, ter empatia e compreender as dificuldades.

Meu orientador, Dr. Sérgio Perez, que abriu as portas do laboratório, confiou e me deu liberdade para as diferentes tomadas de decisão. Soube dar conforto e cobrar quando necessário. Fico muito feliz de ser o seu último aluno e desejo tudo de bom nessa nova caminhada.

Meu coorientador, Dr. Raynald Bergeron (Universidade de Montreal), que me recebeu de braços abertos em seu laboratório. Soube iluminar o caminho para que eu não tropeçasse, soube ensinar e cobrar, teve paciência, foi companheiro. Me mostrou um mundo novo e ilustrou didaticamente para que eu compreendesse sempre com seu jeito tranquilo, atencioso e honesto.

Dr. Yasuhiro Yamada, da Universidade de Tóquio, que gentilmente cedeu os animais $REST^{f/f}$ e o Dr. Vahab Soleimani, líder do projeto REST (Instituto Lady Davis da Universidade McGill), que nos deu todo apoio técnico e forneceu os animais para a realização do projeto.

Amigos e companheiros no Canadá, Luciane e João Paulo, que dividiram não só um lar, mas também os anseios, dificuldades, os sorrisos e até mesmo o frio. Ajudaram na adaptação, mostraram os atalhos e contribuíram para que tudo desse certo.

Jean-Marc que abriu as portas da sua casa para nos receber, fez questão de nos apresentar diversos lugares incríveis e proporcionar experiências de vida singulares. Sempre com um sorriso cativante e uma vitalidade motivante.

Não posso me esquecer de agradecer todos os professores e professoras que passaram em minha vida. Cada um deixou uma semente que carrego sempre comigo. Nunca esquecerei o quanto cada um contribuiu para formar minha história, personalidade e senso crítico.

Os amigos de república, em especial o Giz e o Djava, por compartilhar vivências, experiências, debates, amizades verdadeiras. Conversas sempre regadas de conhecimento e carinho.

Os amigos de São Carlos, Tite, Léo, Mari, Hérissom, Poly, Giovanni, Daniel, Dani, Zé, Anderson, Noele, Markão, Rodrigo, Jefinho, Lu, JP, dentre muitos outros que esta cidade me trouxe. Foi um período maravilhoso cheio de lembranças e sentimentos.

Os amigos de Bebedouro, Preto, Ju, Zelão, Baiano, Daniel, Ennio e tantos outros que cresceram e conviveram durante muito tempo comigo. Vocês ajudaram a construir momentos bonitos que sempre carreguei em meu coração nas dificuldades, nos sorrisos e nas comemorações.

Aos amigos e colegas de departamento e de pós-graduação, Keico, Márcia, Heloísa, Anabelle, Thaty, Vilmar, Gilberto, Gerson, Uliana, Gra, Ana Claudia, Victor, Cleiton e Léo que sempre tentaram ajudar de alguma forma nos projetos, nos momentos difíceis, dando conselhos, fazendo críticas e dando sugestões para desenvolver um trabalho melhor.

Agradeço também o apoio financeiro da CAPES que viabilizou o desenvolvimento da minha pós-graduação e proporcionou uma fantástica experiência de trabalhar em um importante laboratório no Canadá. This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) – Finance Code 001.

Muito obrigado a todos!!!

# *EFEITOS DA DELEÇÃO DE REST DO MÚSCULO ESQUELÉTICO SOBRE A COMPOSIÇÃO CORPORAL E FUNÇÃO MUSCULAR DE CAMUNDONGOS C57BL/6.*

**RESUMO:** Para elucidar o papel da proteína REST (*RE1 Silencing Transcription Factor*) do músculo esquelético sobre a composição corporal e funcionalidade muscular de camundongos adultos, objetivamos avaliar os efeitos da deleção específica de REST do músculo esquelético (REST MKO) sobre a morfologia de camundongos, força muscular in vivo e atividade locomotora e propriedades mecânicas in vitro de músculos esqueléticos de contração rápida e lenta isolados. Vinte e cinco camundongos foram utilizados em nosso estudo: 12 do tipo selvagem (WT; 6 machos e 6 fêmeas) e 13 REST MKO (8 machos e 5 fêmeas). Após 2 semanas de aclimatação, os animais foram alojados em gaiolas montadas com uma roda de corrida (Dia 0) para avaliar o nível de atividade física voluntária. Subsequentemente (Dia 4), os animais retornaram às gaiolas padrão e 3 dias depois (Dia 7) foram avaliadas a força de preensão e a composição corporal. A eutanásia foi realizada de dois a quatro dias depois da última intervenção (dias 9, 10 e 11; n = 2-4 camundongos/dia) durante os quais foram coletados os músculos de contração lenta soleus (SOL) e o de contração rápida extensor longo dos dedos (EDL) para avaliar suas propriedades mecânicas durante protocolos de estimulação elétrica. Não foram encontradas diferenças estatísticas entre camundongos REST MKO e WT em camundongos fêmeas. Com base nessa observação, apenas os dados obtidos em camundongos machos foram utilizados. REST MKO não apresentou efeito significativo no peso corporal, força de preensão relativa e atividade física voluntária; no entanto, REST MKO aumentou significativamente a massa gorda (+ 9%) e diminuiu a massa magra (-9%). O peso relativo do músculo foi 31% maior no SOL e 19% menor no EDL do REST MKO em relação ao grupo WT (ambos $p < 0,05$). A avaliação das propriedades mecânicas dos músculos esqueléticos isolados durante os protocolos de eletroestimulação indicou que REST MKO não apresentou efeito sobre a força relativa máxima no SOL e no EDL. Em relação aos efeitos musculares, o REST MKO promoveu maior desenvolvimento de força e reduziu fadiga no EDL durante o protocolo de fadiga muscular. Em contraste, nos músculos SOL a deleção de REST levou a uma diminuição na produção de força nas baixas frequências de estimulação e maior fadiga. Nós mostramos que o fator de transcrição REST do músculo esquelético parece ser importante para a manutenção da massa magra e sua perda leva a implicações funcionais em diferentes tecidos musculares esqueléticos.

**Palavras chaves:** REST, músculo esquelético, força-frequência e fadiga.

**INTERINSTITUTIONAL PROGRAM OF POST GRADUATION IN PHYSIOLOGICAL SCIENCES**
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**DEPARTMENT OF PHYSIOLOGICAL SCIENCES**
**EXERCISE PHYSIOLOGY LABORATORY**

***Doctoral Thesis of Gustavo Henrique Rigo Canevazzi***

***Supervisor: Prof. Dr. Sérgio E. A. Perez***

***Co-supervisor: Prof. Dr. Raynald Bergeron***

# Effects of skeletal muscle REST deletion on body composition and muscle function in C57BL/6 mice.

**ABSTRACT:** In order to elucidate the role of skeletal muscle REST (*RE1 Silencing Transcription Factor*) protein on body composition and muscle functionality, we aimed at evaluating the effects of the specific deletion of REST from skeletal muscle (REST MKO) on mouse morphology, *in vivo* muscle strength and locomotor activity, and *in vitro* mechanical properties of isolated fast and slow skeletal muscles. Twenty-five mice were used in our study: 12 wild type (WT; 6 males and 6 females) and 13 REST MKO (8 males and 5 females). After 2 weeks of acclimation, the animals were housed in cages mounted with a running wheel (Day 0) so as to assess the level of voluntary physical activity. Subsequently (Day 4), the animals returned to standard cages and 3 days later (Day 7) grip strength and body composition were assessed. Euthanasia was carried out in the following two to four days (Days 9, 10 and 11; n = 2-4 mice/day) during which slow-twitch soleus (SOL) and fast-twitch extensor digitorum longus (EDL) muscles were collected to assess their mechanical properties during electrical-stimulation protocols. No statistical differences between REST MKO and WT mice were found in female mice. Based on this observation, only the data obtained in male mice were used. REST MKO had no significant effect on body weight, relative grip strength and voluntary physical activity; however, it increased fat (+9%) and decreased lean (-9%) masses significantly. Muscle relative weight was 31% higher in SOL and 19% smaller in the EDL of the REST MKO as compared to WT group (both $p<0,05$). Evaluation of isolated skeletal muscles mechanical properties during electrostimulation protocols indicated that REST MKO had no effect on maximal relative force in both the SOL and EDL. Regarding the muscle effects, REST MKO promoted greater force development and reduced fatigability in the EDL during the fatigue protocol. In contrast, in SOL muscles the deletion of REST led to a decrease in force production at low frequencies of stimulation and greater fatigue. We have shown that skeletal muscle REST protein seems to be important for the maintenance of lean mass and its loss leads to functional implications in different skeletal muscle tissues.

**Key Words:** REST, skeletal muscle, force-frequency and fatigue.

## FIGURE LIST

## TABLE LIST

## ABBREVIATION LIST

**½Tmax -** fifty percent of the maximal force

**BW -** body weight

**CIHR -** Canadian Institute for Health Research

**EDL** - extensor digitorum longus

**MTJ -** muscle tendon junction

**NRSF** - *Neuron-Restrictive Silencer Factor*

**REST** - *RE1 Silencing Transcription Factor*

**REST MKO** - specific deletion of REST from skeletal muscle

**SD -** standard deviation

**SOL** – soleus

**TA -** tibialis anterior

**Tmax -** maximal force production

**WT -** wild type

**SUMMARY**

# 1 Introduction

## 1.1 REST functions

*RE1 Silencing Transcription Factor* (REST), also named as *Neuron-Restrictive Silencer Factor* (NRSF) is a key transcriptional regulator involved in vertebrates development (Kuwahara 2013; Xie et al. 2012; Yu et al. 2013; Wang et al. 2012; Kreisler et al. 2010). In addition, REST was also described to have different and important roles in vertebrates physiology.

One of the most important function of REST is related to neuronal cells physiology. REST expression causes repression of neuronal genes in non-neuronal tissues. It is widely expressed during embryogenesis and can play a strategic role in end-stage neuronal differentiation (Chen et al. 1998). In addition, in pluripotent stem cells and neural progenitors REST acts via epigenetic remodelling to actively repress a vast number of coding and noncoding neuronspecific genes. These genes are involved in synaptogenesis, axonal pathfinding, synaptic plasticity and structural remodelling, including synaptic vesicle proteins, ion channels, receptors, transporters, and neuron-specific microRNAs that regulate networks of non-neuronal genes (Hwang & Zukin 2018).

REST is downregulated after neuronal differentiation and can be upregulated by many factors, such as cytokines, stress, and brain injuries (Lu et al. 2014; Zhao et al. 2017). It also mediates the coupling of neurogenesis and gliogenesis, which might contribute to the neuronal-glial interactions that are associated with synaptic, neuronal network plasticity and homeostasis in the brain (Aoki et al. 2012).

In normal aging, REST has a neuroprotective role suppressing genes involved in neuronal death, thereby providing neuroprotection (Lu et al. 2014). However, in age-related degenerative brain disorders, such as Alzheimer's disease, loss of REST in hippocampal neurons leads to neuronal susceptibility to oxidative stress and exacerbates the disease characteristics (Lu et al. 2014). In addition, the disruption of the interaction of REST with its target genes was suggested to be one of the causes of aberrant changes in neuronal gene expression reported in epileptic seizures (Bassuk et al. 2008), in Huntington’s disease (Zuccato et al. 2007) and in Down’s syndrome (Canzonetta et al. 2008; Lepagnol-Bestel et al. 2009).

Besides the functions already mentioned, REST has influence in different conditions. Knockout of REST alone did not have an effect in colon carcinogenesis, therefore, this known effect of REST in this type of cancer can be associated with additional genetic/epigenetic abnormalities that occur during colon cancer development (Hatano et al. 2011). However, downregulation of REST in spinal cord contributes to the development of bone cancer pain in mice (Wang & Yu 2016). Additionally, REST was also described to have an important function in lens fiber cells formation, which is necessary for maintaining an intact lens structure (Aoki et al. 2016) and, interestingly, REST knockout was associated with changes in the behaviour of zebrafish swim, which leads to erratical swimming patterns and displays atypical spatial preferences in adults (Moravec et al. 2015).

Regarding the different effects of REST on muscle tissue, studies reveal that the process of development in mammalian hearts is also regulated by REST (Zhang et al. 2016). Furthermore, REST seems to be important for maintaining normal cardiac integrity once REST inhibition in the heart leads to cardiac dysfunction and sudden arrhythmic death accompanied by re-expression of various fetal genes, including those encoding fetal ion channels (Kuwahara et al. 2003). REST deletion inhibits the cardiomyocyte cell cycle and proliferation in embryonic or regenerating hearts, therefore REST appears to be required for mouse cardiac development and regeneration (Zhang et al. 2017).

The abovementioned studies suggest that REST has an important role in the differentiation, proliferation, and maintenance of cells pool in different types of tissues. However, there are no studies describing the different effects of REST on skeletal muscle tissue.

## 1.2 Muscle aging, satellite cells and REST

The progressive loss of skeletal muscle mass associated with decreases in muscle strength during aging is known as sarcopenia (Rolland et al. 2008). This process of muscle atrophy results in a lower muscular power and muscle quality of older individuals (Brady et al. 2014). The mechanisms of sarcopenia are complex and still unclear; however, it is well known that muscle atrophy is associated with a decline in number and/or efficiency of satellite cells (Relaix & Zammit 2012; Cisterna et al. 2016).

A remarkable property of satellite cells is their unique ability to maintain lineage specific heterochromatin. Therefore, alterations in the heterochromatin state adversely affect their lineage and compromise their potency in regeneration (Zeng et al. 2013; Mendelsohn & Larrick 2015). Reduced regenerative capacity of satellite cells may be related to the convergence of different pathways that ultimately lead to changes in the chromatin structure and, consequently, modifies gene expression resulting in mass and muscle strength losses. In this sense, recent studies suggest that heterochromatin loss is an important factor in aging (Djeghloul et al. 2016; Pegoraro & Misteli 2009; Larson et al. 2012; Zhang & Tang 2015). Comparative data of gene expression between cells of elderly and young rats showed differences in gene activation (Chambers et al. 2007; Cruickshanks & Adams 2011), supporting the rationale that the loss of heterochromatin during aging is regional and probably caused by the reduced activity of transcriptional regulators (Smeal et al. 1996; Suh et al. 2004; Berardi et al. 2003).

The landscape of REST-mediated chromatin remodelling is a dynamic and complex process that involves several histone modifying enzymes (Zheng et al. 2009). In addition, REST also regulates proliferation of neuronal stem cells by repressing pathways of neurogenic differentiation (Gao et al. 2011). Importantly, targeting the altered chromatin for disease intervention and understanding the aging muscle process requires knowledge of factors that regulate critical aspects of stem cell function such as self-renewal and differentiation. However, the role of REST in the skeletal myogenesis remains unexplored.

### 1.3 Aging effects and muscle quality

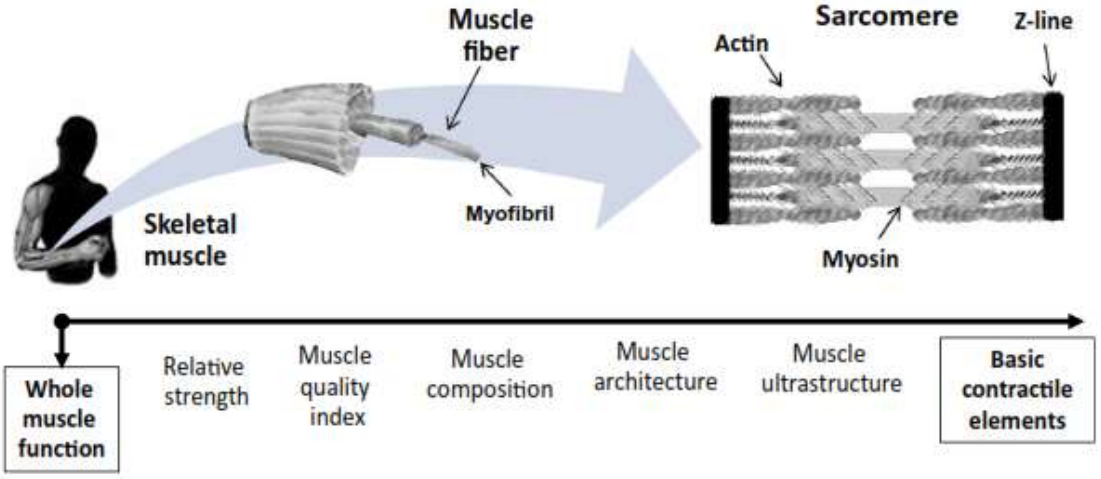
Progressive muscle degeneration during aging has been considered a risk factor for mortality in elderly (Edmunds et al. 2018). Aging of the skeletal muscle system has been related to a reduction in both number and size of muscle fibers (mainly of type II) (Larsson & et al. 1978; Lexell 1995), compromising intracellular $Ca^{2+}$ homeostasis (Zhao et al. 2008; Payne et al. 2004), impairing contractility, increasing deposition of non-contractible tissues such as fat and fibrotic tissues (Janssen et al. 2004), reducing muscle strength (Chan & Stewart I Head 2010), increasing fatigability (J. A. Faulkner 1995) and leading to a reduction of physical activity (Newman et al. 2003). Additionally, the aging process is accompanied by changes in body composition, specifically an increase in adiposity and a decrease in muscle mass

(Goodpaster et al. 2006). Taken together, these factors affect the function and quality of muscle tissue and, consequently, contribute to increase frailty, reduce mobility, increase the risk of falls and impair the quality of life of elderly people (Tinetti et al. 1986).

Decreased muscle mass with aging has been associated to the loss of slow and fast motor units and its reduction is more pronounced in fast motor units. This affects differently slow and fast twitch muscles, appearing to be related to more atrophy of type II fibers (Lexell et al. 1995). The muscle fibers of the lost motor units can be recruited and converted to the remaining motor units types. Thus, there is a general conversion of type II fibers (fast twitch) into type I fibers (slow twitch). Clinically, the loss of rapid motor units and consequently of the type II fibers, results in decreased strength and muscular energy which is required for physical movements (Lang et al. 2010). Another important morphological aspect in the reduction of muscle strength is the infiltration of lipids in this tissue, either by an increase in the number of adipocytes (Shefer et al. 2006; Shefer & Yablonka-Reuveni 2007) or by an increase in lipid deposition in muscle fibers (Dubé & Goodpaster 2006; Kraegen & Cooney 2007).

While declines in physical function such as reduced mobility and functional impairments have traditionally been attributed to the age-related decreases in muscle mass, recent evidence indicates that the quality of the muscle tissue may have greater functional relevance. Muscle quality refers to the capacity of the tissue to perform its different functions, including muscle contraction, metabolism and electrical conduction, spanning from the broad aspect of whole muscle force production to muscle composition and morphology to the level of the sarcomere. Additionally, there has also been a growing interest in defining the structural, physiological, and biological determinants of muscle quality, such as in individual muscle cells, cellular components and metabolic pathways (Fragala et al. 2015). However, muscle quality indices are ultimately dependent on the qualitative features of the muscle tissue, including the composition, architecture/morphology and ultrastructure of the contractile apparatus (Figure 1).

Figure 1 - Skeletal muscle qualitative features can span from the level of whole muscle functioning to features of the basic contractile elements.




From Fragala et al. 2015.

Muscle quality can be quantified through muscle's contractile function and is often assessed as the muscle's ability to generate tension from which mechanical properties such force (strength), velocity of contraction, power and resistance to fatigue can be assessed. Measuring the muscle's ability to generate tension represents an index of muscle quality. Such indices of muscle quality include measures of relative strength (force normalized to muscle mass) and fatigability (Fragala et al. 2015).

On the other hand, using changes in strength as a means of measuring age-related contractile alterations may mask other age-related muscle changes such as those related to the sensitivity of calcium release from the sarcoplasmic reticulum (Brotto et al. 2001). *Ex vivo* preparations of whole isolated muscle provides an enclosed environment to manipulate the concentrations of ions ($Ca^{2+}$, $Na^{+}$ and $K^{+}$) and chemicals (ATP and glucose) that are necessary for optimal force generation and this offers a great opportunity to study the effect of these variables on force production (Hakim et al. 2013). Measurements of contractile force and fatigability are important for the overall evaluation of skeletal muscle function and may help to understand how certain muscle changes, such as during aging, can affect muscle contractile properties. Hence, force can be assessed force in response to varying frequencies of electrical stimulation in order to generate force-frequency curve for each individual muscle. Typically,

soleus (SOL) muscle depicts a force-frequency curve which is shifted leftward in comparison to extensor digitorum longus (EDL) muscle. This is interpreted to reflect a more sensitive release of Ca2+ from the sarcoplasmic reticulum in response to low frequencies of stimulation. Following such force-frequency experiments, frequencies causing maximal force production (Tmax) and fifty percent of the maximal force (½Tmax) can be identified. Those frequencies can then be used to induce muscle fatigue. It was reported that fatigue experiments conducted at ½ Tmax emphasize $Ca^{2+}$ regulation and excitation-contraction coupling events while fatigue experiments conducted at Tmax should provide information on the contractile machinery events/modulation (Park et al. 2012).

### 1.4 Research study rationale

In light of the literature reported in the previous sections, we wanted to know if REST protein knock out from skeletal muscle could affect skeletal muscle mass and function. Thus, using a murine model featuring a specific deletion of REST from skeletal muscle (REST MKO), we aimed at evaluating mouse body composition, in vivo muscle strength and locomotor activity, and in vitro mechanical properties of isolated both fast and slow skeletal muscles.

## 2 Objective

The aim of this study was to analyze the effects of the specific deletion of REST from skeletal muscle on body composition and muscle function in C57BL/6 mice.

### 2.1 Specific objective

Analyze the effects REST deletion from skeletal muscle on mice:

- Grip strength;
- Voluntary physical activity;
- Body composition;
- Muscle force on soleus and extensor digitorum longus muscles;
- Muscle fatigue on soleus and extensor digitorum longus muscles.

## 3 Hypothesis

We postulated that skeletal muscle REST deletion in mice decreases the grip strength, reduces force and increases fatigue in different muscles. In addition, this deletion can reduce the voluntary physical activity and affect the body composition, reducing lean mass.

# 4 Materials and methods

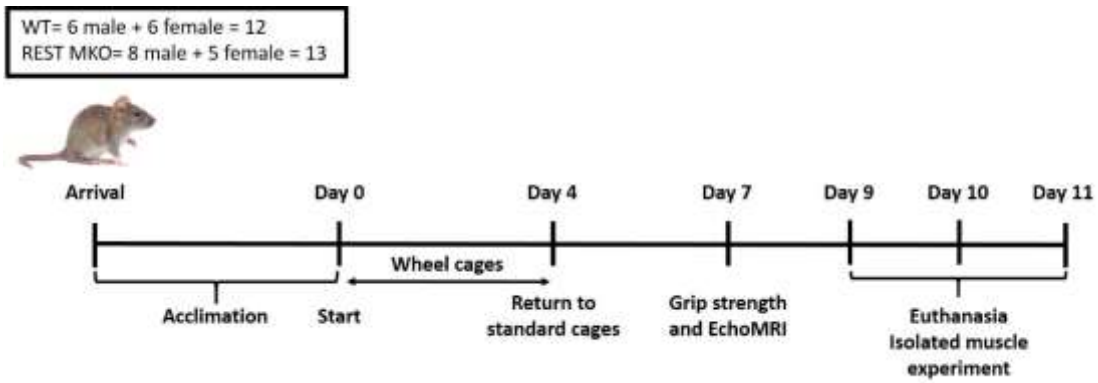
## 4.1 Animals

All animal experiments were approved by Montreal University Institutional Animal Care Committee (Protocol Number: 16-117) and were conducted accordingly to the directives of the Canadian Council on Animal Care. In total, twenty-five mice *C57BL/6* wild type (WT) and REST MKO nineteen weeks old were used in this study. REST MKO group harboring a deletion of REST were obtained by breeding $REST^{f/f}$ mice with $Pax7^{Cre/Cre}$ mice. $REST^{f/f}$ mice were kindly provided by Dr. Yasuhiro Yamada, University of Tokyo and $Pax7^{Cre/Cre}$ mice were obtained from the Jackson Laboratory (Bar Harbour, ME). This project was made in collaboration with Dr. Vahab Soleimani from the Lady Davis Institute for Medical Research at McGill University who is the principal investigator of this research program on the role of REST on muscle physiology and regeneration funded by the Canadian Institute for Health Research (CIHR). Before any animal experiment in this project all animals were genotyped to verify REST deletion. Animals had unrestricted access to food and water, were kept under constant temperature (22 ± 2$^{o}$C) and on a 12-hour alternating light and dark cycle. All tissue harvesting was performed under isoflurane anesthesia. Animals were euthanized by exsanguination and pneumothorax.

## 4.2 Groups and experimental design

Mice were divided into 2 groups: 12 WT (6 males and 6 females) and 13 REST MKO (8 males and 5 females) mice. After 2 weeks of acclimation, the animals were transferred to cages mounted with a running wheel (Day 0) in order to assess voluntary physical activity. Subsequently (Day 4), mice were returned to standard cages and three days later (Day 7) the grip strength test and body composition assessment were performed. Body weight was measured at days 0, 4, 7 and at euthanasia which was carried out on either days 9, 10 or 11 (n = 2-4 mice/day) when soleus (SOL) and extensor digitorum longus (EDL) were collected in order to perform the isolated muscle experiment (Figure 2).

Figure 2 - Procedures performed during the experimental protocol.




## 4.3 Grip strength test

This experiment, through a traction plate attached to a digital force gauge, aims to measure the maximum force exerted by the animal's forelimbs (Force Gauge Model DFG55-10, Omega®). The traction plate comprises a rectangular frame (7.5 x 5.0 cm) having at its center a wire weave net onto which mouse will naturally grab. Keeping the animal stabilized by the tail and swinging it over the tension plate, the mouse grabs it with his forelimbs and then by dragging the mouse over the plate in a straight horizontal line (2-3 cm/s), until the mouse releases the traction plate (Figure 3). After this procedure, the mice returned to their respective cages. Each mouse was evaluated five times with five minutes rest between each test. The average of the best three grip tests was used to report the maximal grip strength. Values acquired in this analysis are reported in grams of force.

Figure 3 - Grip strength test.

### 4.4 Whole body composition

Whole body composition was evaluated using nuclear magnetic resonance (EchoMRI$^{TM}$, Houston) as described earlier (Fontés et al. 2015). Awake animals were placed in a restraining tube inserted into the instrument and analysis were conducted according to the manufacturer's instructions (Figure 4). The body composition analysis of fat and lean mass in mice lasts approximately 3 min per animal. Those were returned to their respective cages after this assessment. Values are reported in grams.

Figure 4 - Nuclear magnetic resonance (NMR) used to analyze whole body composition

### 4.5 Voluntary physical activity

Voluntary physical activity was analyzed using wheel cages (Figure 5) with the following dimensions: 12.7 cm, 5.72 cm, 0.40 m/revolution (diameter : width : distance, respectively - Lafayette Instruments; IN). Each wheel was equipped with a sensor, which continuously recorded data relating to the number of revolutions. The average of the distance traveled (kilometers/day) was used to evaluate the voluntary physical activity of each mouse.

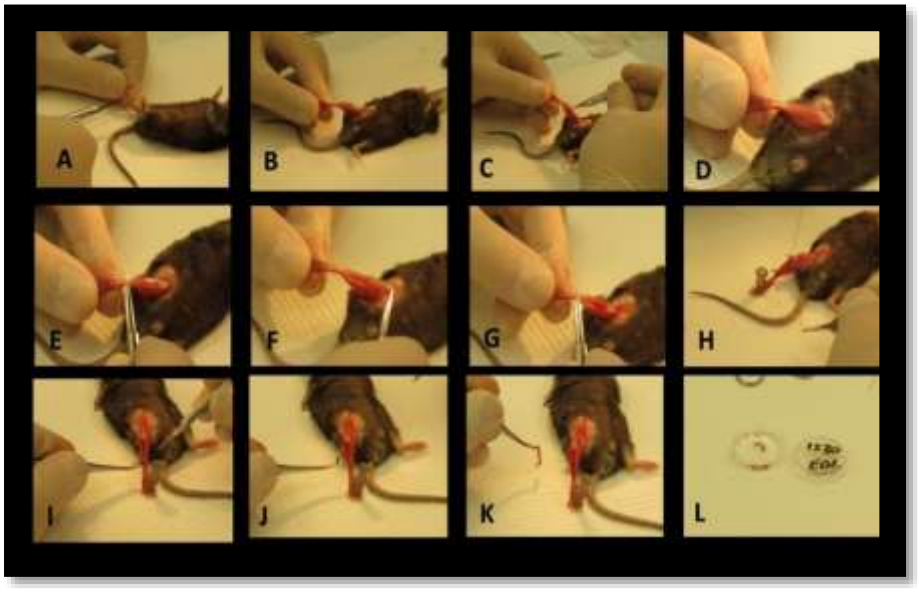
Figure 5 - Cages mounted with a running wheel to analyze the voluntary physical activity.

### 4.6 Muscle removal procedure

In order to perform isolated muscle experiment, SOL and EDL, respectively slow and fast-twitch muscles (Brotto et al. 2001), were collected following an adapted version of a previously published procedure (Hakim et al. 2013; Park et al. 2012).

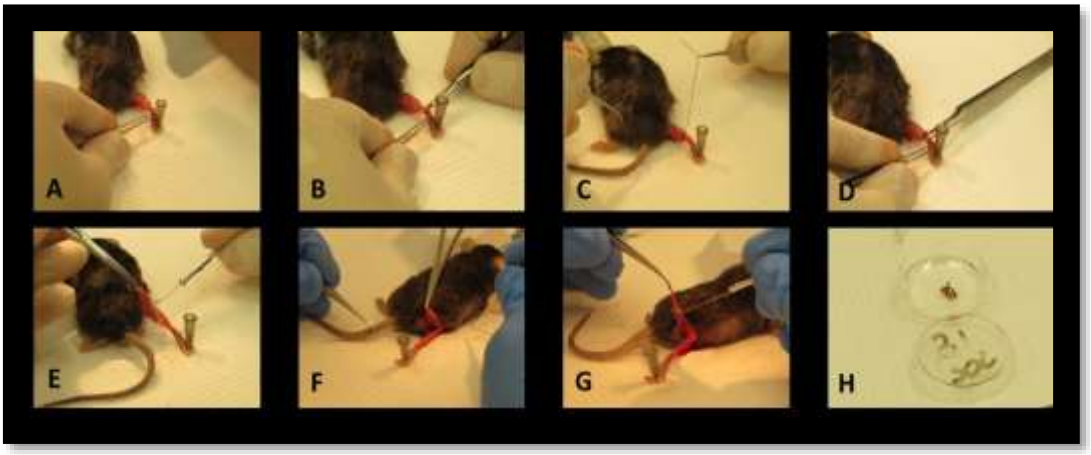
For the EDL removal, we exposed the distal tibialis anterior (TA) tendon and the extensor ligament by dissecting the skin toward the foot, we gently remove the fascia covering the TA muscle and cut the extensor ligament to release the distal TA tendon. In addition, we cut the distal TA tendon and use it to peel off the TA muscle. Carefully, the TA muscle was removed at its proximal attachment. Subsequently, we tie a double loop knot using a suture silk at the muscle tendon junction (MTJ) of the distal EDL muscle and an incision was made in the distal portion of the biceps femoris muscle to expose the proximal EDL muscle. Then the same set of knots at the MTJ of the proximal EDL tendon were prepared. We cut the proximal EDL tendon superior to the proximal suture knot, lift up the EDL muscle, and cut the vasculature beneath the muscle. In addition, we cut the distal EDL tendon inferior to the distal suture knot to remove the EDL muscle from the hind limb (Figure 6).

Figure 6 - A series of digital images showing the dissection of the EDL muscle from anesthetized mice.




To dissect the SOL muscle, we accessed the posterior lateral side of the leg, then pushed aside the gastrocnemius muscle that covers the soleus and localized the muscle. At its origin (proximal), we tie a double loop knot using a suture silk at the MTJ and cut the ligaments connecting to the proximal half of posterior tibia along the soleal line. Next, at the insertion (distal), we tie a double loop as described above and cut the calcaneal tendon that inserts into the posterior calcaneus (Figure 7).

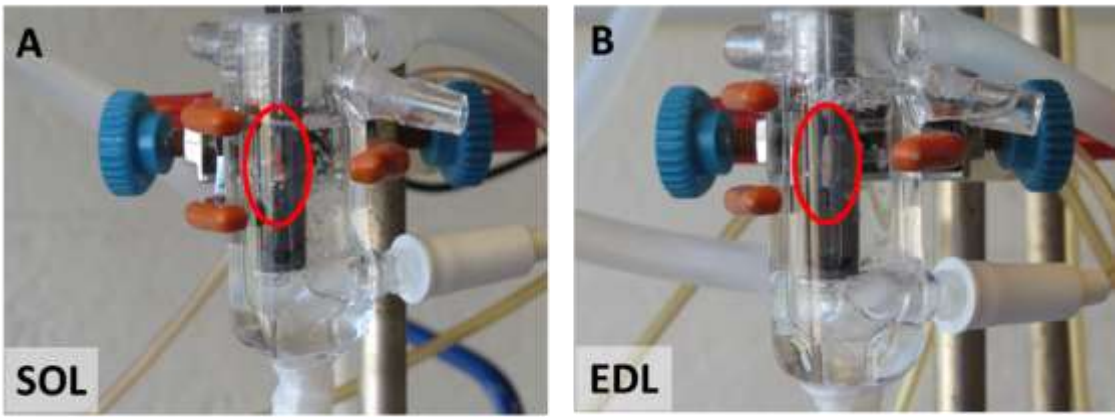
Figure 7 - A series of digital images showing the dissection of the SOL muscle from anesthetized mice.



### 4.7 Isolated muscle experiment

Immediately after dissection, EDL and soleus muscles were placed into a small petri dish containing enough Tyrode solution to fully immerge the muscles. Rapidly, each muscle was then transferred to one of the four tissue bath unit (Multi-channel Myobath II tissue bath system, World Precision Instruments, Sarasota, FL), filled with Tyrode solution (121mM NaCl, 5mM KCl, 24mM $NaHCO_3$, 0,4mM $NaH_2PO_4$, 1.8mM $CaCl_2.2H_2O$, 0.5mM $MgCl_2$, 0.1mM EDTA and 5.5mM glucose) (Sandström et al. 2006). The solution was continuously bubbled with 95% $O_2$–5% $CO_2$, resulting in pH of 7.4 and maintained at 24ºC. One end of the muscle was fixed while the other end was attached to a force transducer (Figure 8). Changes in muscle tension were digitally recorded by using a customized software. Muscles were stimulated with electrical trains of 500 ms duration at a rate of 0.033 tps (3 ms and 5 ms pulse duration respectively for SOL and EDL, 100 Hz and 150 Hz pulse rate respectively for SOL and EDL and 60 Volts of stimulus intensity for both muscles) to identify the optimal length that gave the maximal force response upon stimulation. This muscle length was maintained throughout the experiments. Muscles were then allowed to rest for 30 min in the oxygenated Tyrode solution before measurements were performed.

Figure 8 - Isolated muscle experiment.




Muscles in a stimulation chamber. A: soleus muscle; B: extensor digitorum longus.

A force-frequency test was performed by stimulating muscles once every 30 sec (train rate: 0.033 train per sec). Muscle tension developed at the following frequencies (SOL: 1, 5, 10, 15, 20, 25, 30, 40, 50, 60, 70, 80, 90 and 100 Hz; and EDL: 20, 30, 40, 50, 60, 70, 80, 90,

100 and 120Hz) were recorded and plotted to generate a force-frequency curve from which the following contractile properties were obtained: maximal force (absolute force), maximal relative force (force normalized to muscle mass), frequency at 100% of the maximum force (Tmax) and frequency at 50% of the maximum force (½Tmax) which represents the sensitivity of contractile machinery to calcium release. After 30 minutes of recovery, a fatigue test was performed. Muscles received stimuli at the individually identified frequency generating ½Tmax, at a rate of 0.5 tps (equivalent to one 500 ms long electrical train every 2 seconds) for 5 minutes. The decline in the recorded force during over this time is an indication of muscle fatigability. After 2 minutes of recovery following the fatigue test, a second force-frequency curve test was performed to evaluate the fatigue recovery responses. All data generated by muscles were analyzed and subsequently statistical analysis were performed as described in the specific section.

### 4.8 Tibia length

These measurements were performed using a caliper (0.05 mm accuracy) following the referential standardization: from the tibial plateau (proximal region) to the medial malleolus (distal region). Values were obtained in millimeters.

### 4.9 Statistical analysis

Data were expressed as mean +/- standard deviation (SD). For all samples the Student's unpaired t-tests were used, except for body weight, voluntary physical activity and force-frequency curves unfatigued/fatigued test, wherein a two-way ANOVA followed by the Bonferroni's post hoc test were used to compare the variables between groups. Values of $p < 0.05$ were considered significant. Statistical and graph analysis were performed with GraphPad Prism 6 version 6.01 for Windows (GraphPad Software, CA, USA).

## 5 Results

Both males and females have been studied in the present study. Of notice, for almost all of the variables that were studied, no statistical differences between REST MKO and WT mice were found in female mice. Based on this observation, only the data obtained in male mice are reported below.

At the end of the experiment, animals presented statistically the same age as shown in Table 1 (22.8±1.3 weeks WT and 23.4±0.5 weeks REST MKO) as well as tibia length.

Table 1 - Anthropometric data in male WT and REST MKO mice.

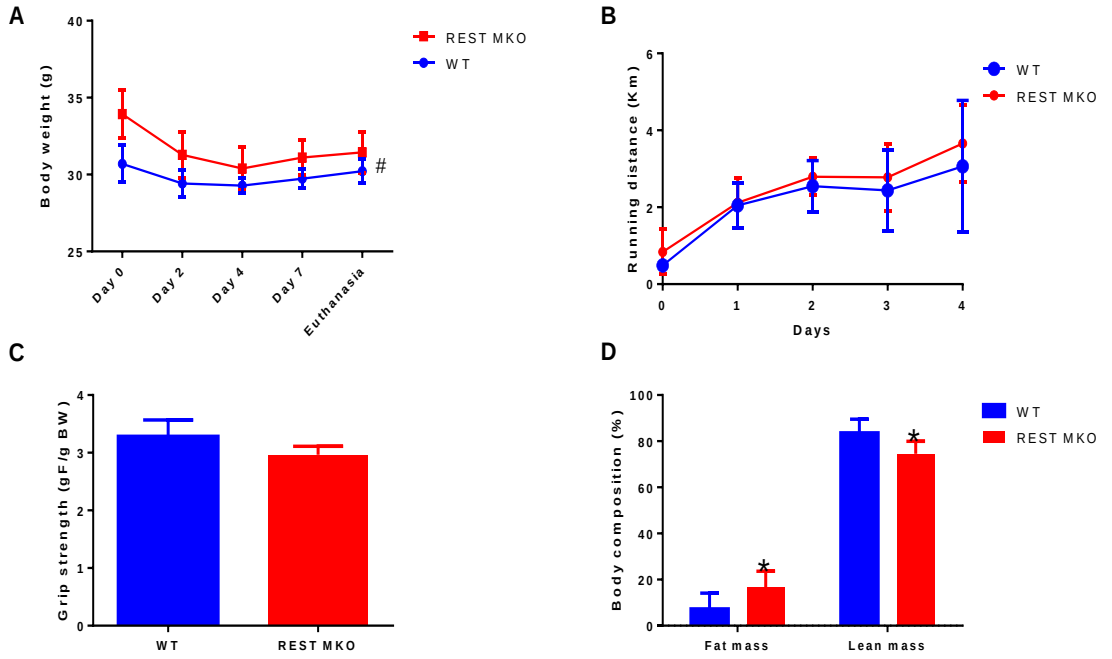
| Groups | Dissection age (weeks) | Tibia length (mm) | Relative gonadal fat mass (g/g BW) |
|---|---|---|---|
| WT | 22.8±1.3 | 7.4±0.1 | 8.6±2.1 |
| REST MKO | 23.4±0.5 | 7.6±0.1 | 16.0±2.1* |

Values are means ± SD. BW, body weight. Data only from male mice WT (n=6) and REST MKO (n=8). *p<0.05 compared to WT group.

Body weight showed interaction between groups during the intervention period, however there was no statistical difference in the body weight between groups on the day of euthanasia (Figure 9A). Additionally, the deletion of REST from skeletal muscle had no significant effect on voluntary physical activity (Figure 9B) and also on relative grip strength (Figure 9C).

Interestingly, male REST MKO mice showed significant increases in the percentage of whole body fat mass (+9%) as compared to WT mice (Figure 9D). Supporting this data on percent whole body composition, the relative gonadal fat mass was bigger in the REST MKO group as compared to WT (Table 1) (p<0,05).

Figure 9 - Body weight, voluntary physical activity, relative grip strength and body composition.




Knocking out from skeletal muscle (REST MKO) had no effect on body weight (A), voluntary physical activity (B) and relative grip strength (C). However, it modified the body composition (D) by increasing the percentage of whole body fat mass and decreasing the percentage of whole body lean mass. Data are means +/- SD. BW, body weight. Data only from male mice WT (n=6) and REST MKO (n=8). #p<0.05 interaction genotype x time; *p<0.05 compared to WT group.

In contrast, the percentage of whole body lean mass was decreased by approximately 9% in REST MKO mice as compared to WT mice (Figure 9D) ($p<0.05$). Interestingly, not all muscles were affected by the genetic deletion of REST from skeletal muscle in a similar manner. Specifically, relative SOL muscle weight was 31% heavier in SOL of REST MKO mice whereas their EDL was 19% lighter (and significantly shorter; see muscle length in Table 2) as compared to similar muscles from the WT group suggesting that skeletal muscle REST protein may affect muscle in a fiber type specific manner.

Table 2 - Muscle morphological characteristics and contractile properties in REST KO and WT mice.

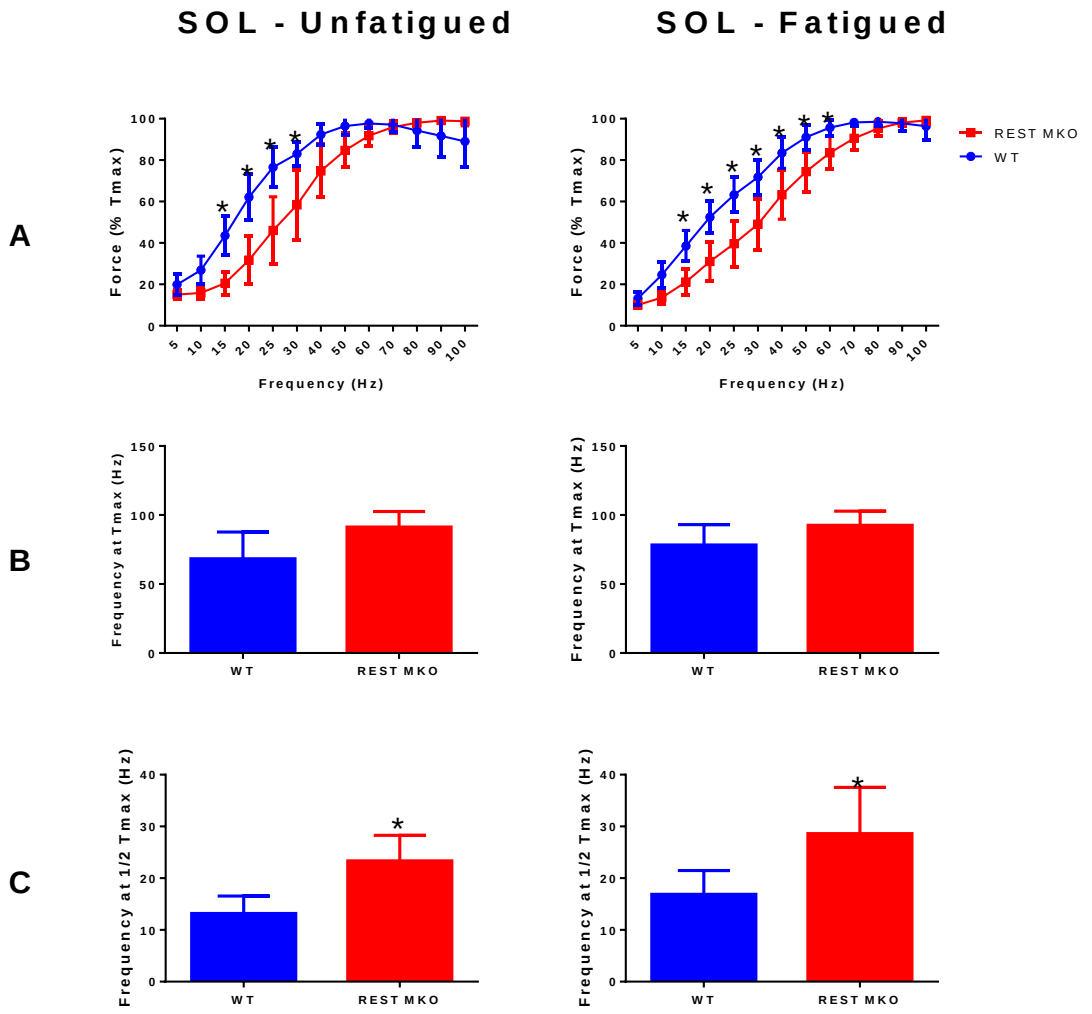
| | SOL | | EDL | |
|---|---|---|---|---|
| | WT | REST MKO | WT | REST MKO |
| Relative muscle weight (g/g BW) | 0.31±0.02 | 0.45±0.02* | 0.32±0.02 | 0.26±0.01* |
| Muscle length (mm) | 94.7±4.1 | 97.5±2.3 | 103.6±2.5 | 94.4±2.8* |
| Maximal force (g) | 10.9±3.5 | 19.2±4.7* | 16.0±5.8 | 11.8±4.5 |
| Maximal relative force (g/mg muscle) | 1176.2±376.8 | 1379.1±398.0 | 1514.1±404.1 | 1392.2±398.6 |

Values are mean ± SD. BW, body weight. Data only from male mice WT (n=6) and REST MKO (n=8). *$p<0.05$ compared to WT group.

Interestingly, maximal force was increased in SOL of REST MKO as compared to WT mice, however this was not observed in EDL (Table 2). On the other hand, no difference was identified in maximal relative force in both the SOL and EDL in REST MKO group (Table 2). This could indicate that the increase in maximal SOL force in REST MKO vs WT is due to the larger muscle mass in SOL of REST MKO mice (Table 2).

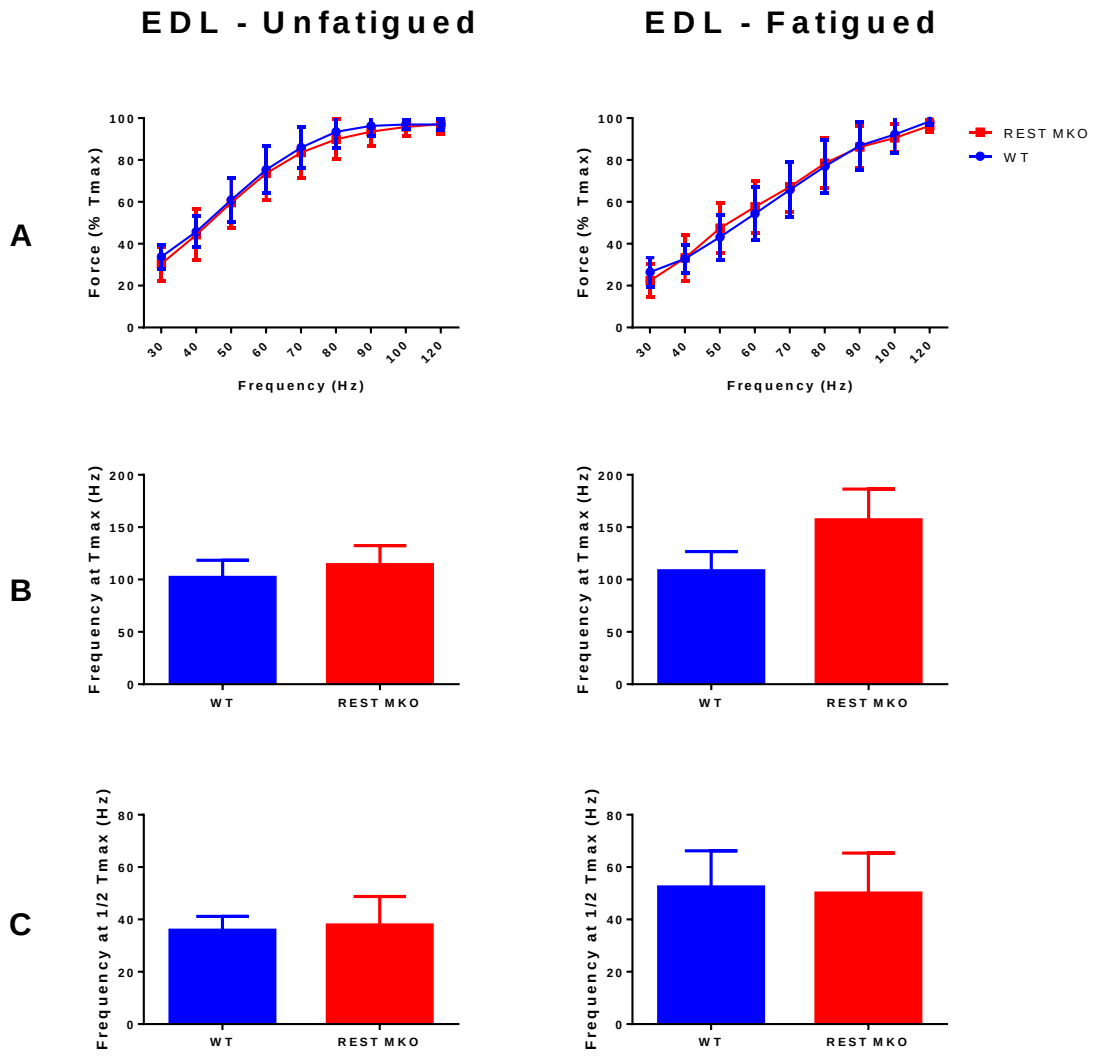
Although no differences in maximal strength between REST MKO and WT was observed in SOL and EDL when the data was normalized to the muscle mass, careful analyses of force production in response to a range of low to high stimulation frequencies revealed a different phenotype in SOL muscle of REST MKO as compared to WT mice (Figure 10A). More specifically, force-frequency curve in the SOL muscle from REST MKO mice was shifted rightward, indicated by less force before (15-30 Hz) and after fatigue (15-50 Hz) at lower frequencies (Figure 10A). In addition, although SOL muscle from REST MKO and WT mice achieved maximal force (Tmax) (Figure 10B) at stimulation frequencies that were not significantly different between both genotypes, a significantly higher frequency of stimulation was necessary in REST MKO to achieve 50% of muscle maximal force (½Tmax) both before and after the fatigue protocol as compared to SOL muscles from WT mice (Figure 10C). Nonetheless, REST MKO had no effect on force-frequency in an unfatigued (left panels) and fatigued (right panels) conditions in EDL muscle (Figure 11A). Additionally, REST MKO had no effect on frequencies at Tmax (Figure 11B) and ½ Tmax (Figure 11C) in EDL muscle.

Figure 10 - Force-frequency experiments under unfatigued and fatigued conditions in SOL muscles.




REST MKO curves were shifted to the right before and after fatigue (A). Similar frequencies of stimulation were required to achieve Tmax (B) while higher frequencies were necessary to achieve ½ Tmax both before and after fatigue protocols (C) in SOL of REST MKO vs WT mice. Data are means +/- STD. Tmax: 100% of maximal muscle force. ½Tmax: 50% of maximal force. Data only from male mice WT (n=6) and REST MKO (n=8). *p<0.05 compared to WT group.

Figure 11 - Force-frequency experiments under unfatigued and fatigued conditions in EDL muscles.

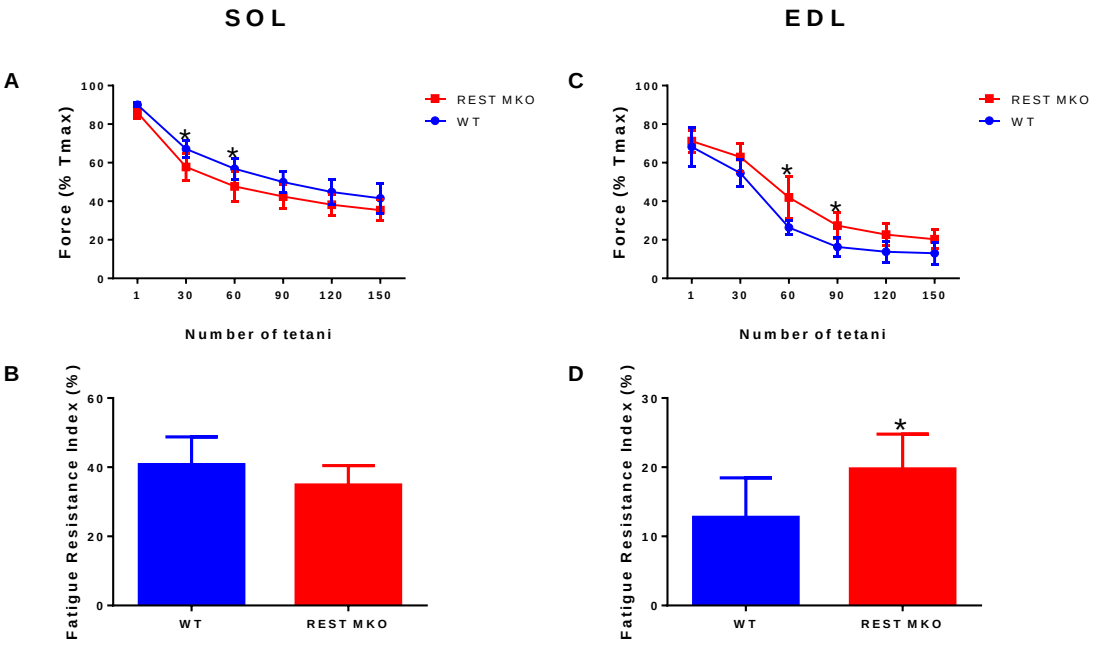



REST MKO had no effect on force-frequency in unfatigued and fatigued conditions (A). Similar frequencies of stimulation were required to achieve Tmax (B) and ½ Tmax (C) both before and after fatigue protocols in EDL of REST MKO vs WT mice. Data are means +/- STD. Tmax: 100% of maximal muscle force. ½Tmax: 50% of maximal force. Data only from male mice WT (n=6) and REST MKO (n=8). *p<0.05 compared to WT group.

Fatigue experiments had opposite effects on the different types of muscles in the REST MKO group. During the fatigue protocol, SOL, a slow twitch muscle, presented a greater force decline with increasing number of tetanus at 30 and 60 Hz, indicating greater fatigue development in the REST MKO group (Figure 12A). However, this effect was not evident in the resistance to fatigue index (Figure 12B). On the other hand, the same experimental fatiguing

protocol in EDL, which is a fast twitch muscle, produced opposite results. EDL from REST MKO mice demonstrated greater force development at 60 and 90 tetanus (Figure 12C) and 6.5% more resistance to fatigue (Figure 12D) as compared to EDL from WT mice.

Figure 12 - Fatigue and fatigue resistance index curves.




During the fatigue protocol, the SOL muscles presented a greater force decline with increasing number of tetanus, indicating greater fatigue in the REST MKO (A); and this effect was not observed in the resistance to fatigue index (B). EDL muscle presented a lesser force decline (C) and a larger fatigue resistance index in the REST MKO group (D). Data are means +/- SD. Tmax: 100% of maximal muscle force. Data only from male mice WT (n=6) and REST MKO (n=8). *p<0.05 compared to WT.

# 6 Discussion

In the present study, we demonstrated that the deletion of REST from skeletal muscle has an important role on muscle function and this specific deletion can also affect body composition in male mice. To our knowledge, this is the first time that a study reports data on the effects of the specific deletion of REST from skeletal muscle on the whole organism and skeletal muscle physiology.

Reductions in habitual physical activity, adverse changes in body composition and declines in muscle capacity are all important factors contributing to physical function impairment in older adults (Brady et al. 2014). Studies with old mice revealed that voluntary physical activity decreases over the weeks, this functional discrepancy is also evident in old mice when compared to younger counterparts (Soffe et al. 2016; Cheng et al. 2013; Fujimaki et al. 2014). Intriguingly, despite the REST knockout has been previously associated with changes in the behaviour of zebrafish swim that leads to reduction in the locomotor activity in larvae (Moravec et al. 2015), our results indicated that the specific deletion of REST from skeletal muscle did not modify voluntary physical activity in mice when housed in cages mounted with a running wheel.

Knockout out REST from skeletal muscle changed other parameters such as body composition and muscle function. Changes in body composition, specifically an increase in adiposity and a decrease in muscle mass are related to the aging process (Goodpaster et al. 2006; Brady et al. 2014). Furthermore, greater amounts of fat mass associated with loss of skeletal muscle mass, also known as sarcopenia, have been associated with reductions in muscle capacity, with implications for physical function, increasing risk for physical disability (Jankowski et al. 2008; Valentine et al. 2009).

The underlying mechanisms contributing to sarcopenia are multifactorial and include altered endocrine function, increased inflammation, mitochondrial dysfunction, inadequate nutrition, and apoptosis (Rolland et al. 2008). Our results demonstrated that the body weight of the animals did not present significant differences between WT and REST MKO groups. On the other hand, the increase in the relative content of fat mass and the reduction in the lean mass

may suggest that REST MKO could reduce the amount of contractile proteins and increase the deposition of non-contractile tissues in the skeletal muscle.

Studies have shown that besides the key role of REST in the neural control of stem cell proliferation and differentiation (Yang et al. 2012), REST maintains self-renewal and pluripotency of embryonic stem cells (Singh et al. 2008). Upon REST silencing, a study showed the suppression of cell proliferation and migration, while apoptosis was not affected in glioblastoma cells (D. Zhang et al. 2016). In addition, REST knockdown disrupted the mTOR signalling pathway in squamous cell carcinoma, indicating that REST plays an important role in the survival of oral cancer cells by regulating the mTOR signalling pathway (Cho et al. 2015), which is also a crucial regulator pathway of skeletal muscle hypertrophy and can prevent muscle atrophy *in vivo* (Bodine et al. 2001).

Impairment of muscle function, such as the loss force, may be related to an increase in non-contractile extracellular tissue in aged skeletal muscle (Payne et al. 2003). However, force may also decline with age (González et al. 2000; Jiménez-Moreno et al. 2008), suggesting that the reduction of muscle strength may be related to intrinsic factors of the muscle fibers themselves. These possible factors include structural changes of myosin (Lowe et al. 2001), changes in actin-myosin cross bridge kinetics (Lowe et al. 2002) and impairments in the excitation-contraction coupling process (Jiménez-Moreno et al. 2008; Payne et al. 2009). Nevertheless, in our study no significant differences were found in relative grip strength as well as relative maximal force in the muscles that were analyzed.

Interestingly, studies have reported a development of slow-twitch muscle characteristics with advancing age (Larsson & Edström 1986; Brown & Hasser 1996; Moran et al. 2005). Slow-twitch muscle characteristics are related to the reduction in the proportion of fast-twitch fibers, especially type IIb, with an increase in the proportion of slow-twitch fibers as reported previously in aging muscle (Luff 1998). This process seems to occur due to the denervation of fast fibers and their reinnervation by axonal sprouting from slow fibers (ARAKI 1988; Kadhiresan et al. 1996). In addition, a reduced rate of $Ca^{2+}$ uptake by the sarcoplasmic reticulum caused by the reduction in $Ca^{2+}$-ATPase activity impairs the function of the EDL excitation-contraction coupling system and leads to a slower twitch response (Chan & Stewart I. Head

2010). However, our results showed that the force-frequency curve for EDL was not affected by the specific deletion of REST from skeletal muscle.

Likewise, we found a reduced fatigability of EDL muscles in the REST MKO group. The decline in the percentage force being generated during the fatiguing protocol was significantly less as compared to the WT group. Additionally, REST MKO presented a higher resistance to fatigue index for EDL when compared to the WT group. Similar results with EDL were described in aged muscles by different research groups (Brown & Hasser 1996; Chan & Stewart I. Head 2010). This result might arise due to loss of fast-twitch muscle fibers and their replacement by slow-twitch muscle fibers, which are more resistant to fatigue (ARAKI 1988; Kadhiresan et al. 1996). Taken together, the above-mentioned findings indicate that the EDL muscles from the REST MKO group might present a possible interconversion of fast fibers to slow fibers. However, the muscle fibers typing analysis was not performed in the present study, which could confirm this hypothesis or not.

On the other hand, the force-frequency curve for SOL from REST MKO mice was shifted rightwards, producing less force within a range of low frequency stimulations before and after fatigue. This significantly lower contraction-tetanus ratio may be related to changes in the excitatory-contraction coupling process regulated by $Ca^{2+}$ (Park et al. 2012). In addition, SOL required a greater frequency to achieve 50% of muscle force before and after fatigue in REST MKO group. Similar results with SOL were described in aged muscles (Brotto et al. 2001). These responses could be explained by alterations in excitation-contraction coupling, accounting for the age-dependent decline in intracellular $Ca^{2+}$ mobilization (Jiménez-Moreno et al. 2008; Payne et al. 2009). In aged muscle, the activation of $Ca^{2+}$ sparks is reduced due to the reduction of MG29 expression, known as a synaptophysin-related membrane protein. This underexpression may lead to an increased threshold for $Ca^{2+}$-induced activation of ryanodine channel, which would require the development of stimuli at higher frequencies to achieve the same force production. Additionally, the fragmentation of sarcoplasmic reticulum enables the generation of a segregated sarcoplasmic reticulum $Ca^{2+}$ pool, that uncouples from the normal voltage-induced $Ca^{2+}$ release process, impairing the release of calcium by the sarcoplasmic reticulum and, consequently, altering the excitation-contraction coupling process (Weisleder et al. 2006).

Likewise, we found greater fatigue in the SOL from REST MKO group. This supports the above-mentioned findings of force-frequency curves for the SOL in the REST MKO group, which reinforces the idea that possibly the specific deletion of REST from skeletal muscle negatively influenced the excitation-contraction coupling process regulated by $Ca^{2+}$. Events dependent on the $Ca^{2+}$ pool located just below the plasma membrane, which are controlled primarily by the influx, are seriously hampered by the reduction of REST expression and can affect several other processes involved in signalling, growth and other cell functions (Ariano et al. 2010). Interestingly, a study indicated that REST suppression contributed to increased cardiac expression of the T-type $Ca^{2+}$ channels observed under pathological conditions. In addition, inhibition of REST may lead to left ventricular enlargement, cardiac dysfunction, sudden arrhythmic death accompanied by re-expression of multiple fetal cardiac genes, including those that encode fetal ion channels (such as T-type $Ca^{2+}$ channels), which impairs normal cardiac systolic function and electrical stability (Kuwahara 2013). Thus, based on our results it is tempting to speculate that REST MKO can negatively influence the excitation-contraction coupling process by $Ca^{2+}$ alterations in the SOL muscle. In this sense, more studies will be needed to explore this possibility.

# 7 Conclusion

In this study, we showed that the specific deletion of REST from skeletal muscle does not affect relative force, grip strength and voluntary physical activity. On the other hand, it changed the body composition increasing fat mass and reducing lean mass.

Regarding the muscle effects, REST MKO promoted greater force development and reduced fatigability in the EDL during the fatigue protocol. In contrast, in SOL muscles the deletion of REST led to a decrease in force production at low frequencies of stimulation and greater fatigue.

In summary, we have shown that REST may have an important role in the lean mass maintenance and its loss leads to different functional implications in slow and fast twitch muscles.

## 8 Study limitations

We acknowledge that this study has some limitations, we did not analyze the changes in fiber-type composition, as well as $Ca^{2+}$ alterations. Likewise, we have worked to quantify muscle fiber types and their respective cross-sectional areas, however the data were not available in time to include in this study. In this sense, complementary studies should be performed in order to elucidate how the specific deletion of REST from skeletal muscle affects not only the function of the skeletal muscle system, but also the roles in the regeneration, muscle maintenance and possible functions in muscle aging process.

## 9 Future perspectives

In summary, we have shown that the specific deletion of REST from skeletal muscle has an important role in the maintenance of lean mass and its loss has contrasting functional implications in slow and fast twitch muscles in adult mice. A wide research project should be developed to discover if the specific deletion of REST can promote stronger effects in older mice (52 weeks old) when compared to young ones and if resistance training can attenuate or even reverse the muscle functional alterations and body composition changes found in adult mice.

## 10 Synthesis of the thesis in Portuguese

RE1 *Silencing Transcription Factor* (REST), é um regulador transcricional envolvido no desenvolvimento de vertebrados (Kuwahara 2013; Xie et al. 2012; Yu et al. 2013; Wang et al. 2012; Kreisler et al. 2010). Além disso, REST também foi relacionado a diferentes e importantes papéis na fisiologia de vertebrados.

No envelhecimento normal, REST possui um papel neuroprotetor suprimindo genes envolvidos na morte neuronal (Lu et al. 2014). No entanto, em desordens degenerativas do cérebro relacionadas a idade, como a doença de Alzheimer, a redução de REST em neurônios do hipocampo leva a uma maior susceptibilidade neural ao estresse oxidativo e maximiza as características da doença (Lu et al. 2014).

Com relação aos diferentes efeitos de REST sobre o tecido muscular, estudos demonstraram que o processo de desenvolvimento do coração de mamíferos é regulado pelo REST (Zhang et al. 2016). Nesse sentido, o REST parece ser importante para a manutenção da integridade cardíaca normal, uma vez que a inibição de REST no coração leva a uma disfunção cardíaca, acompanhada pela reexpressão de vários genes fetais, incluindo aqueles que codificam canais iônicos fetais (Kuwahara et al. 2003). A deleção de REST inibe a proliferação e o ciclo celular dos cardiomiócitos em corações embrionários. Assim, o REST parece estar envolvido na regeneração e desenvolvimento cardíaco de camundongos (Zhang et al. 2017).

Em resumo, este fator de transcrição, REST, possui um papel importante na diferenciação, proliferação e manutenção do *pool* de células em diferentes tipos de tecidos. No entanto, não existem estudos descrevendo os diferentes efeitos do REST no tecido muscular esquelético.

A degeneração muscular progressiva durante o envelhecimento tem sido considerada um fator de risco para mortalidade em idosos (Edmunds et al. 2018). O envelhecimento do sistema muscular esquelético está relacionado com a redução do número e tamanho das fibras musculares (principalmente das fibras do tipo II) (Larsson & et al. 1978; Lexell 1995), juntamente com o comprometimento da homeostase intracelular de $Ca^{2+}$ (Zhao et al. 2008; Payne et al. 2004), prejuízo da contratilidade

muscular, aumento da deposição de tecidos não-contráteis como gordura e tecidos fibróticos (Janssen et al. 2004), redução da força muscular (Chan & Stewart I Head 2010), aumento da fadigabilidade (J. A. Faulkner 1995) promovendo uma redução da atividade física (Newman et al. 2003). Além disso, o processo de envelhecimento é acompanhado por mudanças na composição corporal na qual ocorre um aumento na adiposidade e redução da massa muscular (Goodpaster et al. 2006). Em conjunto, estes fatores afetam a função e a qualidade do tecido muscular e, consequentemente, contribuem para aumentar a fragilidade, reduzir a mobilidade, aumentando o risco de quedas e prejudicando a qualidade de vida da população idosa (Tinetti et al. 1986).

À luz da literatura relatada nas seções anteriores, queríamos saber se a deleção da proteína REST do músculo esquelético poderia afetar a massa e a função do músculo esquelético. Dessa forma, para elucidar o papel de REST do músculo esquelético sobre a composição corporal e funcionalidade muscular de camundongos adultos, objetivamos avaliar os efeitos da deleção específica de REST do músculo esquelético (REST MKO) sobre a composição corporal de camundongos, força muscular *in vivo*, atividade locomotora e propriedades mecânicas *in vitro* de músculos esqueléticos de contração rápida e lenta isoladamente. Para isso, vinte e cinco camundongos foram utilizados em nosso estudo sendo eles: 12 animais controle (WT: *wild type*; 6 machos e 6 fêmeas) e 13 animais REST MKO (8 machos e 5 fêmeas). Após 2 semanas de aclimatação, os animais foram alojados em gaiolas montadas com uma roda de corrida para avaliar o nível de atividade física voluntária (Dia 0). Decorridos 4 dias, os animais retornaram às gaiolas padrão e 3 dias depois (Dia 7) foram avaliadas a força de preensão palmar e a composição corporal. A eutanásia foi realizada de dois a quatro dias depois da avaliação da composição corporal e da força de preensão palmar (dias 9, 10 e 11; n = 2-4 camundongos/dia). Neste dia, foram coletados os músculos de contração lenta sóleo (SOL) e o de contração rápida extensor longo dos dedos (EDL) para avaliar suas propriedades mecânicas durante os protocolos de estimulação elétrica.

Nas medidas avaliadas não foram encontradas diferenças entre camundongos REST MKO e WT em camundongos fêmeas. Com base nessa observação, apenas os dados obtidos em camundongos machos foram utilizados.

O grupo REST MKO não apresentou efeitos significativos no peso corporal, na força de preensão relativa e na atividade física voluntária. No entanto, REST MKO aumentou significativamente a massa gorda (+ 9%) e diminuiu a massa magra (-9%). O peso relativo do músculo foi 31% maior no SOL e 19% menor no EDL nos animais REST MKO em relação ao grupo WT (ambos $p < 0,05$). A avaliação das propriedades mecânicas dos músculos esqueléticos isolados durante os protocolos de eletroestimulação indicou que REST MKO não apresentou efeito sobre a força relativa máxima no SOL e no EDL. Em relação aos efeitos musculares, o REST MKO promoveu maior desenvolvimento de força e reduziu fadiga no EDL durante o protocolo de fadiga muscular. Em contraste, nos músculos SOL a deleção de REST levou a uma diminuição na produção de força nas baixas frequências de estimulação e maior fadiga.

Nesse sentido, o conjunto de nossos resultados demonstraram que o silenciamento do fator de transcrição REST do músculo esquelético, parece ser importante para a manutenção da massa magra corporal e sua perda leva a implicações funcionais em diferentes tecidos musculares esqueléticos.

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# 12 Attachments

## 12.1 Published articles during the PhD

Physiology & Biochemistry

# Resistance Training and Ovariectomy: Antagonic Effects in Mitochondrial Biogenesis Markers in Rat Skeletal Muscle

Authors

M. R. Barbosa[1], G. E. Shiguemoto[2], L. M. Tomaz[3], F. C. Ferreira[4], M. F. C. Rodrigues[3], M. M. Domingues[3], M. V. C. Souza Master[5], G. H. R. Canevazzi[3], N. S. Silva-Magosso[3], H. S. Selistre-de-Araujo[3], S. EA Perez[3]

Affiliations

Affiliation addresses are listed at the end of the article

Key words
- mitochondrial biogenesis
- ovariectomy
- resistance training
- skeletal muscle

## Abstract

Estrogen reduction is associated with a decline in skeletal muscle mitochondrial biogenesis. Molecular events associated with improvements in markers of mitochondrial biogenesis after resistance training and estradiol replacement are unknown. This study aimed to investigate the effects of ovariectomy, resistance training, and estradiol replacement on markers of mitochondrial biogenesis and protein expression related to oxidative capacity in the rat gastrocnemius pool. Estradiol replacement was performed using Silastic® capsules. During the 12-week resistance training, animals climbed a ladder with weights attached to their tails. Gene expression was analysed by RT-PCR, and protein content was determined by western blotting. Ovariectomy decreased the gene expression of the mitochondrial biogenesis markers PGC-1α (~73%), NRF-1 (~44%), and TFAM (~53%) ($p < 0.05$) and decreased the protein expression of phosphorylated AMPK, CREB and AKT, which are related to oxidative capacity. Resistance training increased PGC-1α (~59%) and TFAM (~48%) expression compared to the Ovariectomy-Sedentary group. The combination of resistance training and estradiol replacement was superior to the ovariectomy-sedentary and ovariectomy-resistance training treatments regarding the gastrocnemius muscle. Estrogen deficiency altered the expression of genes and proteins that favour the development of a mitochondrial dysfunction phenotype, which was improved with resistance training and was partially improved by estradiol replacement.

## Highlights

- Ovariectomy reduced the mitochondrial biogenesis markers;
- Estradiol replacement and resistance training increased mitochondrial biogenesis markers;
- 12 weeks of resistance training is effective to restore mitochondrial biogenesis markers
- 12 weeks of resistance training.

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Correspondence
***Dr. Marina Rodrigues Barbosa***
Education in Health
Universidade Federal de Sergipe
R. Padre Álvares Pitangueira, 288
49100-000 Lagarto
Brazil
Tel.: +55/799/91913 316
Fax: +55/793/0858 027
marinarbarbosa@yahoo.com.br

## Introduction

Estradiol (E2) reduction is associated with an increased risk of several metabolic abnormalities [33]. The use of ovariectomy (Ovx), i.e., the removal of the ovaries, as a model of human menopause is extensive [6] because data derived from this model have informed much of our fundamental understanding of E2 action in different organs in the body [6, 37–14]. Ovx in rats increases total body mass and body mass adiposity, at least in part due to higher energy intake, increases the levels of inflammatory markers and sarcopenia, and reduces bone mineral mass [43], suggesting that changes in energy metabolism might occur in E2-deficient animals [40]. Metabolic alterations in response to ovariectomy are well documented and occur within 3 weeks; however, adaptive responses continue throughout multiple systems over a long period of time [6].

Some studies have emphasized the role of E2 in the regulation of mitochondrial bioenergetics [31–23], and numerous signalling pathways that are regulated by E2 affect mitochondria [30]. There is evidence in the literature that the activation of nuclear respiratory factors (NRFs) and mitochondrial transcription factor A (TFAM) modulate the effects of E2 on the mitochondria. NRF1 and NRF2 are the primary regulators of mitochondrial transcription factors, such as TFAM, and of proteins that play crucial roles in the replication, transcription, and translation of mtDNA [11]. Experimental data have revealed

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ARTICLE

# Effects of resistance training and estrogen replacement on adipose tissue inflammation in ovariectomized rats

Maria Fernanda Cury Rodrigues, Fabiano Candido Ferreira, Natália Santanielo Silva-Magosso, Marina Rodrigues Barbosa, Markus Vinicius Campos Souza, Mateus Moraes Domingos, Gustavo Henrique Rigo Canevazzi, Uliana Sbeguen Stotzer, Sabrina Messa Peviani, Fábio Santos de Lira, Heloísa Sobreiro Selistre de Araújo, and Sérgio Eduardo de Andrade Perez

**Abstract:** Estrogen deficiency is directly related to central obesity and low-grade inflammation. Hormonal replacement and exercise training are both able to decrease fat accumulation and inflammation in postmenopausal women. However, the efficiency of resistance training (RT) and estrogen replacement (ER) in minimizing adiposity and inflammation in the visceral adipose tissue (VAT) of ovariectomized (OVX) rats has not yet been elucidated. In this study, Sprague–Dawley rats were divided into the following 6 groups: sham-operated sedentary (Sham-Sed), OVX-Sed, Sham-RT, OVX-RT, OVX-Sed-ER, and OVX-RT-ER groups. ER was performed by implanting silastic capsules containing 17β-estradiol. For RT, the animals were required to climb a 1.1-m vertical ladder with conical flasks containing weights attached to their tails for 12 weeks. Histological analyses were used to evaluate morphological changes. Gene expression levels were determined by quantitative real-time reverse transcriptase polymerase chain reaction, and protein concentrations were determined using Multiplex/Luminex assays. Ovariectomy increased the body mass (BM), adipocyte area, and inflammation in the VAT, the latter of which was indicated by reduced interleukin-10 (48%) and increased tumor necrosis factor (TNF)-α concentration (~3%). RT efficiently decreased BM, adipocyte area, and inflammation in the OVX groups. The combination of RT and ER decreased BM (19%) and the TNF-α concentration (18%) and increased the gene and protein expression levels of adiponectin (173% and 18%). These results indicate that RT and the combination of RT and ER are efficient strategies for reducing the BM and improving the inflammatory status of OVX rats.

*Key words:* estrogen, inflammation, cytokines, resistance training, rats, adipose tissue.

**Résumé :** La carence en œstrogène est directement associée à l'obésité centrale et à l'inflammation de faible intensité. Le traitement hormonal substitutif et l'entraînement physique peuvent diminuer l'accumulation de gras et l'inflammation chez les femmes postménopausées. Toutefois, l'efficacité de l'entraînement contre résistance (« RT ») et de l'hormonothérapie de remplacement (« ER ») pour minimiser l'adiposité et l'inflammation dans les tissus adipeux viscéraux (« VAT ») chez des rates ovariectomisées (« OVX ») n'est pas encore claire. Dans la présente étude, on divise les rates Sprague–Dawley en 6 groupes : sédentaire-chirurgie simulée (Sham-Sed), OVX-Sed, Sham-RT, OVX-Sed-ER et OVX-RT-ER. On effectue ER en implantant des capsules en silastique renfermant du 17β-estradiol. Dans la condition RT, les animaux montent durant 12 semaines à une échelle verticale de 1,1 m avec des flacons coniques attachés à la queue. On effectue des analyses histologiques pour l'évaluation des variations morphologiques. On détermine le taux d'expression génique par la technique quantitative de transcription inverse et amplification en chaîne par polymérase en temps réel et, de plus, on détermine les concentrations protéiques par des dosages Multiplex/Luminex. L'ovariectomie suscite une augmentation de la masse corporelle (BM), de la surface adipocytaire et de l'inflammation dans le VAT; cette dernière modification est révélée par la diminution des concentrations de l'interleukine-10 (48 %) et du facteur de nécrose tumorale alpha (TNF-α, ~3 %). RT suscite une diminution efficace de BM, de la surface adipocytaire et de l'inflammation dans les groupes OVX. La combinaison de RT et ER suscite une diminution de BM (19 %) et de la concentration de TNF-α (18 %) et suscite, en outre, une augmentation des taux d'expression génique et protéique de l'adiponectine (173 % et 18 %). Ces résultats indiquent que RT et la combinaison de RT et ER sont des stratégies efficaces pour diminuer BM et améliorer le statut inflammatoire des rates OVX. [Traduit par la Rédaction]

*Mots-clés :* œstrogène, inflammation, cytokines, entraînement contre résistance, rats, tissu adipeux.

## Introduction

Menopause is associated with increased body weight, fat deposition, and metabolic dysfunction (Maltais et al. 2009). Postmenopausal women have a greater propensity for developing abdominal adiposity associated with increased systemic levels of inflammatory cytokines, indicating that estrogen can modulate both body adiposity and systemic inflammation (Skurk et al. 2007). Rodent ovariectomy is widely used as a means of investigating the mechanisms

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**M.F.C. Rodrigues, F.C. Ferreira, N.S. Silva-Magosso, M.V.C. Souza, M.M. Domingos, G.H.R. Canevazzi, U.S. Stotzer, S.M. Peviani, H.S. Selistre de Araújo, and S.E.A. Perez.** Department of Physiological Sciences, Federal University of São Carlos, UFSCar, São Carlos, São Paulo 13565-905, Brazil.
**M.R. Barbosa.** Department of Health Education, Federal University of Sergipe, Lagarto, Sergipe 49400-000, Brazil.
**F.S. de Lira.** Exercise and Immunometabolism Research Group, Department of Physical Education, University State Estadual Paulista, UNESP, Presidente Prudente, São Paulo 19060-900, Brazil.
**Corresponding author:** Maria Fernanda Cury Rodrigues (email: mafe_cury@hotmail.com).


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# Irisin Signaling Pathway is up Regulated by Resistance Training in Ovariectomized Rats

**Silva-Magosso NS[1*], Barbosa MR[2], Tomaz LM[3], Rodrigues MFC[3], Magosso RF[4], Canevazzi GH[3], Souza MVC[3], Assis RP[4], Selistre-de-Araujo HS[5] and Sergio Eduardo Perez[3]**

[1]*Department of physical education and human motricity, Federal University of São Carlos, São Carlos/SP, Brazil*
[2]*Department of education and health, Federal University of Sergipe, Lagarto/SE, Brazil*
[3]*Department of physiological sciences, Federal University of São Carlos, São Paulo/SP, Brazil*
[4]*Postgraduate education in Human Motricity Sciences, Department of physical education, São Paulo State University, Rio Claro/SP, Brazil*
[5]*Department of Clinical Analysis, School of Pharmaceutical Sciences, São Paulo State University, Araraquara/SP, Brazil*

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***Corresponding author:** : Natalia Santamelo Silva Magosso, Department of physical education and human motricity, Federal University of São Carlos, São Carlos/SP Brazil. Tel: +55 16 99770-5343; E-mail: nataliasantamelo@gmail.com*

## Abstract

Exercise is known to increase the concentrations of irisin, a remarkable myokine that may play an important protective role against metabolic disorders.

**Propose:** This study investigated the effects of irisin signaling pathway induced by Resistance Training (RT) in ovariectomized (Ovx) rats.

**Methods:** Thirty-two Holztman rats were randomly distributed to four experimental groups: Sham-Sedentary (Sed); Ovx-Sed; Sham-RT and Ovx-RT. The RT protocol demanded from the animals a vertical climb. Each session consisted of 4 to 12 climbs with 2 min. of rest during 12 weeks. To quantify mRNA expression the ΔΔCt method was applied, protein expression was verified by Western Blotting and the analysis of irisin was determined by ELISA. When group averages were different ($p \leq 0.05$), a Tukey post-hoc test was applied.

**Results:** The Ovx-RT group had higher expression of PGC1α FNDC5, irisin levels, and UCP1 compared to Ovx-Sed.

**Conclusion:** RT was led to higher expression of the irisin signaling pathway in the Ovx group showing that the RT seems to be an excellent strategy to counteract the ovariectomy-induced metabolic disorders.

**Keywords:** PGC1-A; FNDC5; Adipose Tissue; UCP1; Resistance Training

## Introduction

Reduction of estrogen levels, a physiological status of female aging, causes problematic deleterious effects, such as loss of muscle mass, changes in fat mass, with the possibility of developing obesity [1-3]. This is a very important issue for public health, once obesity is associated with increased risk of cardiovascular disease and type 2 diabetes [3-5]. In experimental studies with rats, menopause is simulated by ovariectomy, which leads to effects such as loss of muscle mass and increased body and fat mass [6-8].

Physical exercise can be used as an intervention to improve the health of postmenopausal women given that that Resistance Training (RT) has been associated to decreases in fat mass and increases in lean body mass to prevent sarcopenia, cardiovascular diseases, type 2 diabetes and obesity [9-14]. These benefits occur in part because skeletal muscle is an endocrine organ and exercise stimulates skeletal muscle to produce and release myokines, which have endocrine functions [15]. Irisin is a remarkable myokine and its concentration seems to increase in response to both endurance training and RT [16-20]. During physical exercise, the release of this moykine is induced by the activation of PPARγ co-activator 1 alpha (PGC1-α), which stimulates the fibronectin type III domain containing 5 (FNDC5) to cleave and release irisin into the blood [16].

Irisin is bound to unidentified receptors in the surface of white fat adipocytes and positively regulates the release of UCP1 (Uncoupling Protein 1), which provokes uncoupling in mitochondrial respiration and loss of energy in the form of heat and browning of the White Adipose Tissue (WAT) [16,21,22]. In addition, irisin could stimulate energy expenditure through modulation of hypothalamic neuropeptides and neurotransmitters involved in feeding control [23]. Thus, the thermogenic changes in white adipose tissue may play an important protective role against metabolic disorders, such as cardiovascular diseases, type-2 diabetes and obesity [24,25]. In view of these benefits, the activation of the irisin pathway could play an important protective role against the deleterious effects caused by reduced levels of estrogen. However, to date there are no studies that demonstrate the effect of RT on the irisin signaling pathway in Ovariectomized (Ovx) rats. Our hypotheses in the present study were: 1) the irisin signaling pathway would be lower in Ovx rats, and 2) the Sham and Ovx rats would have greater irisin signaling pathway after RT.

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*Original Article (short paper)*

# Resistance training and hormone replacement increase MMP-2 activity, quality and quantity of bone in ovariectomized rats

Markus V.C Souza[1*], Anderson D. S Lino[2], Leandro G. D Ruffoni[2], Mateus M. Domingos[2], Marina R. Barbosa[2], Maria F C. Rodrigues[2], Fabiano C. Ferreira[2], Luciane M. Tomaz[2], Gustavo H. R. Canevazzi[2], Natália S. Silva[2], Jeferson A. A. Teixeira[2], Richard D. Leite[3], Gilberto E. Shiguemoto[2], Sérgio E. A. Perez[2]

[1]*Universidade Federal do Triângulo Mineiro, UFTM, Uberaba, MG, Brazil;*
[2]*Universidade Federal de São Carlos, UFSCar, São Carlos, SP, Brazil;*
[3]*Universidade Federal do Sergipe, Aracaju, SE, Brazil;*
[4]*Universidade Federal do Espírito Santo, UFES, Vitória, ES, Brazil.*

**Abstract — Aims**: The aim of the present study was to investigate the influence of resistance training (RT) and hormone replacement (HR) on MMP-2 activity, biomechanical and physical properties bone of ovariectomized (OVX) rats. **Methods**: Sprague-Dawley female rats were grouped into six experimental groups (n = 11 per group): sham-operated sedentary (SHAM Sed), ovariectomized sedentary (OVX Sed), sham-operated resistance training (SHAM RT), ovariectomized resistance training (OVX RT), ovariectomized sedentary hormone replacement (OVX Sed-HR), and ovariectomized resistance training hormone replacement (OVX RT-HR). HR groups received implanted silastic capsules with a 5% solution of 17β-estradiol (50 mg 17β-estradiol/ml of sunflower oil). In a 12-week RT period (27 sessions; 4–9 climbs) the animals climbed a 1.1 m vertical ladder with weights attached to their tails. Biomechanical and physical bone analyses were performed using a universal testing machine, and MMP-2 activity analysis was done by zymography **Results**: Bone density and bone mineral content was higher in the RT and HR groups. The MMP-2 activity was higher in the RT and HR groups. The biomechanical analysis (stiffness, fracture load and maximum load) demonstrated better bone tissue quality in the RT associated with HR. **Conclusion**: The RT alone as well as when it is associated with HR was efficient in increasing MMP-2 activity, biomechanical and biophysical properties bone of ovariectomized rats.

Keywords: osteoporosis, resistance training, ovariectomy, hormone replacement and metalloproteinase-2 (MMP-2).

## Introduction

Osteoporosis is one of the most serious health problems of postmenopausal women, and occurs in approximately 20% of them[1]. Estrogen deficiency is related with decline in bone mass and disruption of the micro architectural arrangement of bone tissue. Previous studies show that the ovariectomy results in an increased bone turnover with osteoclastic resorption exceeding the osteoblastic formation[2-4]. This condition contributes to increased urinary calcium excretion[5, 6], bone mineral content and bone density reduction, loss in mechanical strength and increased fracture risk. Bone quality is influenced by many factors, such as alterations on extracellular matrix (ECM), modifications at organic composition (collagen and non-collagenous matrix proteins), inorganic composition (hydroxyapatite) and activity of matrix metalloproteinases (MMPs)[3].

Matrix metalloproteinases (MMPs) are responsible for the breadown of ECM components that contributes to normal tissue regulation and remodeling and the matrix metalloproteinases 2 (MMP-2), which are important for the integrity of the ECM at the bone[3, 7]. The increase of MMP-2 activity is indicative of matrix degradation, a process necessary for tissue growth[3]. Previous studies showed that the reduction in MMP-2 causes destruction of bone tissue favoring osteoporosis; this fact demonstrated the importance of MMP-2 in the development and maintenance of bone tissue[3, 8].

Ovariectomy is associated with a reduction in the production of the ovarian hormones, which decreases the activity of MMP-2[9-11]. The reduction of these hormones causes many effects on the body, including the increase in body fat[10, 12], which favors the concentrations of circulating inflammatory markers such as tumor necrosis factor alpha (TNF-α) and interleukin 1 and 6[13, 14]. These markers increase the activity of MMP-2[14], and increase bone remodeling. This physiological change causes changes in the morphology and function of the bones, which negatively affect the skeleton[14].

The bone demineralization and collagen decrease are associated with the higher osteoclastic activity in the absence of estrogen, thereby promoting reduction in the biophysical and biomechanical properties of bones[3]. Thus, the hormone replacement therapy is considered to minimize the ovariectomy effects[10].

Hormone replacement promotes tissue remodeling, favoring the biomarkers' activation that reflect the: 1) bone resorption; 2) number of osteoclasts, and 3) bone formation. Markers of bone formation are divided into two categories: 1) increased proteins that induces the osteoblast differentiation, and 2) pro collagen fragments that are released during incorporation of collagen in the bone matrix[1]. Thus, the development of pharmacological and non-pharmacological therapies is needed to reduce the effects caused by the absence of ovarian hormones.

Exercise has generally been considered to have positive influence on the skeleton with mechanical loading being the

## 12.2 Book chapters published during the PhD

FERREIRA, F. C.; **CANEVAZZI, G. H. R.**; CONCEICAO FILHO, J. C.; TOMAZ, L. M.; MAGOSSO, N. S. S. Molecular Effects of Strength Training on Skeletal Muscle In: Strength Training: Methods, Health Benefits and Doping.1 ed. New York: Nova Science Publishers, 2016, p. 47-70. Additional references: United States / English. Means of Disclosure: Printed, ISBN: 9781634841566.

Home page: https://www.novapublishers.com/catalog/product_info.php?products_id=56557&osCsid=dd44fec15e08ab71d0e4e9aad3a12ed7

In: Strength Training: Methods, Health Benefits and Doping ISBN: 978-1-63484-156-6
Editors: C. Ferraresi and D. Rodrigues Bertucci 

*Chapter 4*

# MOLECULAR EFFECTS OF STRENGTH TRAINING ON SKELETAL MUSCLE

***Fabiano Candido Ferreira,***
***Gustavo Henrique Rigo Canevazzi,***
***Júlio César Conceição Filho*,***
***Luciane Magri Tomaz***
***and Natalia Santanielo Silva Magosso***
Federal University of São Carlos, São Carlos - SP, Brazil

## ABSTRACT

Every day new researches report muscular adaptations at the molecular level stimulated by exercise. Thus, this chapter aims to bring new and relevant information on the subject. This chapter will initiate with a brief introduction giving some basic concepts of classic gene expression. Then it will present some signaling pathways and molecular adaptations in skeletal muscles induced by exercise in general and others specifically induced by resistance training. Finally some signaling pathways and molecular adaptations on skeletal muscle tissue induced by resistance training in different populations.

**Keywords:** exercise, signaling pathways for muscle hypertrophy, skeletal muscle molecular adaptations, strength training, exercise alterations and molecular alterations

### 12.3 Abstracts published in international conferences during the PhD

**Canevazzi G. H. R.**, Nguyen D., Soleimani V. D., Bergeron R. Effets de la délétion de REST du muscle squelettique sur la fonction musculaire. ***Journée de la Recherche en Kinésiologie*** – Université de Montréal, 2018, Montréal, Canada.

Tomaz L. M., Barbosa M. R., Ferreira F. C., Domingos M. M., Souza M. V. C., Silva N. S., Lagoeiro C. G., Rodrigues M. F. C. Perez S. E. A., **Canevazzi G. H. R.** 12-weeks resistance training is better than hormone replacement to prevent skeletal muscle mass reduction induced by ovariectomy. ***Canadian Society for Exercise Physiology*** – CSEP, 2015, Hamilton, Canada.

Souza M. V. C., Domingos M. M., Rodrigues M. F. C., Barbosa M. R., Tomaz L. M., Ferreira F. C., **Canevazzi G. H. R.**, Silva N. S., Lagoeiro C. G., Perez S. E. A. Resistance training and hormone replacement increase MMP-2 activity and improve boné properties in ovariectomized rats. ***American College of Sports Medicine***, 2015, San Diego – California, USA.

Tomaz L. M., Barbosa M. R., Ferreira F. C., Domingos M. M., **Canevazzi G. H. R.**, Souza M. V. C., Silva N. S., Lagoeiro C. G., Rodrigues M. F. C., Farahnak Z., Lavoie J. M., Perez S. E. A. Effects of ocariectomy in rats submitted or not to a 12-weeks resistance training programme on hepatic GLUT2 gene expression.. ***Canadian Society for Exercise Physiology*** – CSEP, 2014, St. Johns, Canada.

Barbosa M. R., Tomaz L. M., Ferreira F. C., Domingos M. M., **Canevazzi G. H. R.**, Souza M. V. C., Silva N. S., Lagoeiro C. G., Rodrigues M. F. C., Perez S. E. A. Effects os resistance training on skeletal muscle of ovariectomized rats. ***American College of Sports Medicine***, 2014, Orlando, USA.

Domingos M. M., Rodrigues M. F. C., Barbosa M. R., Tomaz L. M., Ferreira F. C., **Canevazzi G. H. R.**, Souza M. V. C., Silva N. S., Lagoeiro C. G., Perez S. E. A. Resistance training increase gene expression of biomarkers to mitochondrial biogenesis in brain on ovariectomized rats. ***Integrative Physiology of Exercise***, 2014, Miami – Florida, USA.

## 12.4 Articles in submission process

**CANEVAZZI G. H. R.**, TOMAZ L. M., DOMINGOS M. M., RODRIGUES M. F. C., FERREIRA F. C., BARBOSA M. R., LAGOEIRO C. G., SILVA N. S., SOUZA M. V. C., PEREZ S. E. A. 12-weeks resistance training is better than hormone replacement to prevent skeletal muscle mass reduction induced by ovariectomy. **Submitted in Motriz, 2018**.

STOTZER U. S., PISANI G. F. D., **CANEVAZZI G. H. R.**, SHIGUEMOTO G. E., DUARTE A. C. G. O., PEREZ S. E. A., SELISTRE-DE-ARAÚJO H. S. Benefits of resistance training on body composition and glucose clearance are inhibited by long-term low carbohydrate diet in rats. **Submitted in PLOS ONE, 2018**.

## 12.5 Projects developed during the PhD

### 12.5.1 Project 1

**Title: Effects of skeletal muscle REST deletion on muscle function of C57BL/6 mice.**

**Members:** PhD student Gustavo Henrique Rigo Canevazzi, Prof. Dr. Duy Nguyen, Prof. Dr. Vahab Soleimani and Prof. Dr. Raynald Bergeron.

**ABSTRACT:** In order to elucidate the role of skeletal muscle REST (*RE1 Silencing Transcription Factor*) protein on body composition and muscle functionality, we aimed at evaluating the effects of the specific deletion of REST from skeletal muscle (REST MKO) on mouse morphology, *in vivo* muscle strength and locomotor activity, and *in vitro* mechanical properties of isolated fast and slow skeletal muscles. Twenty-five mice were used in our study: 12 wild type (WT; 6 males and 6 females) and 13 REST MKO (8 males and 5 females). After 2 weeks of acclimation, the animals were housed in cages mounted with a running wheel (Day 0) so as to assess the level of voluntary physical activity. Subsequently (Day 4), the animals returned to standard cages and 3 days later (Day 7) grip strength and body composition were assessed. Euthanasia was carried out in the following two to four days (Days 9, 10 and 11; n = 2-4 mice/day) during which slow-twitch soleus (SOL) and fast-twitch extensor digitorum longus (EDL) muscles were collected to assess their mechanical properties during electrical-stimulation protocols. No statistical differences between REST MKO and WT mice were found in female mice. Based on this observation, only the data obtained in male mice were used. REST MKO had no significant effect on body weight, relative grip strength and voluntary physical activity; however, it increased fat (+9%) and decreased lean (-9%) masses significantly. Muscle relative weight was 31% higher in SOL and 19% smaller in the EDL of the REST MKO as compared to WT group (both $p<0,05$). Evaluation of isolated skeletal muscles mechanical properties during electrostimulation protocols indicated that REST MKO had no effect on maximal relative force in both the SOL and EDL. Regarding the muscle effects, REST MKO promoted greater force development and reduced fatigability in the EDL during the fatigue protocol. In contrast, in SOL muscles the deletion of REST led to a decrease in force production at low frequencies of stimulation and greater fatigue. We have shown that skeletal

muscle REST protein seems to be important for the maintenance of lean mass and its loss leads to functional implications in different skeletal muscle tissues.

### 12.5.2 Project 2

**Title: Effects of resistance training and long-term low carbohydrate diet on skeletal muscle system in rats.**

**Members:** PhD student Gustavo Henrique Rigo Canevazzi, Profa. Dra. Grazielle Pereira de Oliveira, Profa. Dra. Uliana Sbeguen Stotzer, Profa. Dra. Heloisa Sobreiro Selistre de Araújo and Prof. Dr. Sergio Eduardo de Andrade Perez.

**ABSTRACT:** Diets associated with resistance training are the most effective manner to promote weight loss. Low-carbohydrate high-fat diet gained attention and popularity due to the evidence that this type of diet facilitates the reduction of body weight, although some studies have shown that these diets increase the adiposity even without increasing of body mass. However, to our knowledge the effects of long-term low carbohydrate diet associated to resistance training on skeletal muscle remodeling remain unknown. The objective of this project was to evaluate the effects of 21 weeks of low-carbohydrate high-fat diet associated, in the last 10 weeks, with resistance training, on skeletal muscle remodeling and body composition of rats. Male Sprague Dawley rats were initially divided into two groups (n = 18 / group), one group received low-carbohydrate high-fat diet and the control group received standard diet (low fat and high carbohydrate). After 10 weeks each group was divided: low-carbohydrate high-fat diet sedentary (n = 9), standard diet sedentary (n = 9), resistance training low-carbohydrate high-fat diet (n = 9) and resistance training standard diet (n = 9). The resistance training groups performed for 11 weeks a stair climbing program with progressive loads, 3 times a week. Resistance-trained groups presented similar and progressive increase in maximal workload throughout the resistance training period. Resistance training reduced body weight and improved body composition (increasing lean mass and reducing fat mass). In animals fed with low-carbohydrate high-fat diet, resistance training did not alter body composition, with exception for bone mineral density that was lower in resistance training animals. These groups fed with low-carbohydrate high-fat diet, sedentary and trained, had higher visceral fat mass percentage, lower lean and skeletal muscle mass percentage and lower bone mineral density. Interventions did not alter the amount of muscle glycogen. We are analyzing the effects of resistance training and high-fat diet low-carbohydrate on muscle cross-sectional area, fiber types and expression of mTOR, p70S6K, MuRF1 and MAFbx in soleus and plantaris muscles (analisis in progress).