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Differential Effects of Calorie Restriction and Rapamycin on Age-Related Molecular and Functional Changes in Skeletal Muscle

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Abstract

Aging is a multifactorial process associated with progressive degradation of physiological integrity and function. One of the greatest factors contributing to the deleterious effects of aging is the decline of functional ability due to loss of muscle mass, strength, and function, a condition termed sarcopenia. Calorie restriction (CR) has consistently been shown to extend lifespan and delay the onset and progression of various age-related diseases, including sarcopenia. Additional anti-aging interventions that are receiving scientific attention are CR mimetics. Of these pharmacological compounds, rapamycin has shown similar CR-related longevity benefits without the need for diet restrictions. To investigate the potential role of rapamycin as an anti-sarcopenic alternative to CR, we conducted a study in male and female C57BL/6 J mice to assess the effects of rapamycin on age-related gene expression changes in skeletal muscle associated with loss of muscle mass, strength, and function, relative to control. We hypothesize that the effects of rapamycin will closely align with CR with respect to physical function and molecular indices associated with muscle quality. Our results indicate CR and rapamycin provide partial protection against age-related decline in muscle, while engaging uniquely different molecular pathways in skeletal muscle. Our preclinical findings of the therapeutic potential of rapamycin or a CR regimen on geroprotective benefits in muscle should be extended to translational studies towards the development of effective strategies for the prevention and management of sarcopenia.

Keywords

calorie restriction; rapamycin; skeletal muscle; sarcopenia

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Declaration of competing interest

The authors declare there is no conflict of interest.

Introduction

Sarcopenia, defined as a geriatric syndrome and characterized by reduced muscle mass, strength and/or physical function, is a significant cause of physical disability, poor quality of life, and death (Santilli and others 2014). In the US, sarcopenia affects approximately 5-13% of adults 60-79 years and 11-50% of older adults 80 years and older (Rong and others 2020). Given the older adult population in the US is expected to double from current numbers by 2050, sarcopenia represents a major public health concern (Ortman and others 2014).

Although the precise molecular mechanisms associated with sarcopenia are unknown, emerging evidence suggests multiple pathophysiological processes are involved, including: a) reductions in growth factor signaling, particularly related to the insulin-like growth factor (IGF) pathway; b) infiltration of pro-inflammatory cytokines, including TNFα; c) oxidative damage due to the accumulation of reactive oxygen species (ROS); and catabolic processes induced by chronic activation of mechanistic target of rapamycin (mTOR) signaling (Bikle and others 2015; Fan and others 2016; Ferrucci and Fabbri 2018; Song and others 2013; Tang and others 2019b). These molecular changes, in turn, lead to reduced muscle quality and muscle quantity (Barclay and others 2019; Bian and others 2020). Muscle quality is defined as muscle strength (or power) per unit of muscle mass, and its loss is intimately linked to metabolic dysregulation, including reduction in insulin sensitivity, impaired oxidative defense, and decreased mitochondrial function (Nair 2005). Age-related loss of muscle quantity reflects an overall reduction of muscle mass to body weight ratio, or relative muscle mass. Reduced relative muscle mass is widely used as an index of sarcopenia and is an independent risk factor for loss of muscle function, adverse health outcomes, and increased mortality (Kalyani and others 2014; Lewandowicz and others 2020). Research efforts focused on understanding biological factors and molecular changes associated with age-related loss of muscle quality and quantity may facilitate the development of novel strategies for the prevention and/or treatment of sarcopenia.

One particularly interesting question under investigation considers if sarcopenia is amenable to intervention (Brook and others 2016). As such, one nonpharmacological intervention that seems promising is calorie restriction (CR) (Brook and others 2016). CR is a dietary intervention that reduces daily caloric intake without malnutrition and is the only intervention to date that consistently decreases the rate of biological aging and increases both mean and maximum lifespan (Redman and Ravussin 2011). The effects of CR are realized through multiple biological adaptations that are characterized by “synergistic and mutually non-exclusive” properties (Das and others 2017a). The net effect of these physiological changes is a reduction in oxidative damage, inflammation, autophagy, improved energy metabolism, insulin sensitivity, and mitochondrial metabolism (López-Lluch and Navas 2016). With respect to skeletal muscle, these multifactorial effects of CR on pathophysiology also contribute to the protective effects of CR against age-related skeletal muscle loss (López-Lluch and Navas 2016). While CR results in an initial loss of whole-body mass (muscle or lean mass and fat mass), this reduction is acute and remains stable in advanced age (Sohal and Forster 2014). In studies conducted in preclinical models (mice and nonhuman primates) and human clinical trials, the observed effect of CR on

muscle mass demonstrates that after an initial loss of absolute muscle mass, the proportion of muscle mass as a percentage of body mass (referred to as relative muscle mass) increases (Colman and others 2008; Das and others 2017b; Liu and others 2021; van Norren and others 2015). Increased relative lean mass is a potent preventative parameter for multiple metabolic diseases and a more meaningful marker of muscle preservation and function than absolute mass (Baumgartner and others 1998).

Understanding the multidimensional effects of CR on age-related muscle loss is the subject of much research. Notably, CR-induced changes on skeletal muscle occur at systemic, cellular, and molecular levels and are likely associated with gene expression changes (Swindell 2009). For example, gene expression analysis from CR studies conducted in nonhuman primates have provided clues to the metabolic connections between CR and age-related muscle loss. In a study conducted by Rhoads et al.,CR-induced changes in gene expression were associated with biological pathways involved in muscle RNA processing, protein synthesis and breakdown, and lipid synthesis (Rhoads and others 2020).

Additional anti-aging interventions that are receiving scientific attention are CR mimetics. These pharmacological compounds including resveratrol, metformin, and rapamycin have shown similar CR-related longevity benefits without requiring dietary limitations or restrictions (Madeo and others 2019). Of these pharmacological agents, overwhelming evidence suggests rapamycin is the most CR-comparable drug associated with universal anti-aging effects – that is, it extends lifespan in all models tested (yeast to mammals), delays the onset and progression of age-related disease, and protects several organs and tissues against age-related functional decline (Blagosklonny 2019; Lamming 2016; Partridge and others 2020). However, rapamycin's effect on aging skeletal muscle, has not been explored until recently. It has long been known that aging muscle is subject to chronic activation of mTOR signaling (a pathway responsible for protein synthesis and regenerative processes) (Tang and others 2019a). This overstimulation of mTOR is purported to increase mitochondrial oxidative stress and inhibition of mitophagy – pivotal mediators of mTOR-induced catabolism (Yoon 2017). These processes in turn lead to muscle fiber loss, atrophy, cellular damage, and deterioration of muscle strength and function (features of muscle quality) (Yoon 2017). Rapamycin, in complex with 12 kDa FK506-binding protein (FKBP12), acts by inhibiting mTOR signaling, and thus is hypothesized to protect aging muscle from atrophy and loss of muscle quality by reducing mitochondrial oxidative stress and cellular damage related to advanced age in skeletal muscle (Tang and others 2019a; Tang and others 2019b). Our objective was to investigate the potential role of rapamycin as an anti-sarcopenia alternative to CR, and to compare the effects of rapamycin versus CR on age-related changes in skeletal muscle associated with loss of muscle mass, strength, and function in mice.

Materials and Methods

Animals

Eight-month-old male (n = 45) and female (n = 45) C57BL/6J mice were purchased from the Jackson Laboratory (Sacramento, CA) and acclimated for ~8 weeks, which allowed the animals to become fully adjusted to the environment and diet. During the acclimation

period, 2 female mice were euthanized due to neurologic illness. The remaining mice (n = 43 female, n = 45 male) were included in the study. When testing began, mice were 10 months of age, a developmental phase that is operationally defined in mice as middle-aged (Hagan 2017). We selected this age to identify the physiologic changes in balance and coordination that occur in the transition from middle age to advanced age. Mice were singly housed in standard Techniplast Green Line cages (floor area = 501 cm^2/77.6 in^2) (Tecniplast USA, Inc., West Chester, PA) and provided with Crink-l'Nest α-dry bedding, crinkle paper enrichment, (The Andersons, Inc., Goldsboro, NC), and a polycarbonate mouse housing tent (Datesand Limited, United Kingdom). Mice were maintained on a 12:12 light:dark cycle with lights on at 0700 and lights out at 1900 and had free access to water and (during the acclimation period) a purified control diet (D12450J) formulated by Research Diets, Inc., New Brunswick, NJ. All procedures concerning the use and care of animals were approved and monitored by the Institutional Animal Care and Use Committee at University of North Carolina at Chapel Hill.

Diet Intervention

After the acclimation period, mice were randomized to one of three diet groups: 1) control diet (D20011502), provided ad libitum; 2) Calorie restricted (CR) diet (D20011503) providing 70% of the average daily caloric intake of the control mice and administered as a daily aliquot between 9-11 am; or 3) Rapamycin diet (D20011501) containing an enteric, encapsulated form of rapamycin (eRAPA) at a concentration of 14 mg/kg, provided ad libitum. The dose of eRapa was selected based on previous studies showing chronic, low dose (14 mg/kg) enteric eRapa is sufficient to enhance age-related decline in male and female mice (Halloran and others 2012; Wilkinson and others 2012). Both the control and CR diets contained the vehicle compound, Eudragit, a copolymer of methyl acrylate used to encapsulate eRAPA. The micronutrient, fat and protein content of the CR diet was adjusted to provide 100% of all vitamins, minerals, fatty acids, and amino acids, but 70% of total energy, consumed by the controls. Mice were maintained on respective diets for 14 weeks, given ad libitum access to water, and food intake for control and rapamycin mice was monitored weekly. All diets were formulated by Research Diets, Inc.

Anthropometric and Physical Function Procedures

Body weight was recorded weekly. Physical strength and function were assessed using grip strength, inverted screen, and balance beam performance tests (Orenduff and others 2021). On the day of functional testing sessions, mice were transferred in their home cages from the mouse housing room to the experimental room 30 minutes prior testing. This allowed mice to fully awaken and acclimate to the experimental environment. Functional testing was conducted between the hours of 10:00 am and 2:00 pm at baseline and endpoint (14 weeks after diet onset, time of euthanasia).

Grip strength

Grip strength test is used to measure forelimb grip strength. The grip-strength meter is a calibrated grip strength apparatus (Panlab), consisting of a grasping trapeze connected to a force transducer. Strength measurements included a set of three maximum effort repetitions.

Mean grip strength and normalized grip strength ([unit of force (newtons) per body mass (g)]) were determined from the three trials.

Inverted screen test

Inverted screen test is an assessment of coordination and whole body strength. Mice were placed individually on top of a square (7.5 cm x 7.5 cm) wire screen that was secured within a wooden frame. The screen was inverted and suspended 1 meter from the floor. Bedding was placed under the screen to prevent injury when the mice fell from the screen. The test concluded when the mouse fell from the screen and the maximum hang time was recorded. To account for body mass, holding impulse (body mass multiplied by maximum hang time and converted to newtons) was calculated.

Balance beam

Balance beam test assesses agility, balance, and coordination. The balance beam apparatus was composed of two smooth wooden beams, each 1 meter in length and 6 mm or 12 mm in width. The beams were securely suspended 50 cm from the floor using sturdy trestles. Enclosed safe houses were placed at the escape ends of each of the two beams and bedding was added to encourage the mice to enter. To prevent injury to animals should they fall off the beam, a net was tethered to the trestles 25 cm below the beam. A distance of 80 cm was marked on the beam, indicating the length at which the animal is tested, with an extra distance of 10 cm in front of the starting line to allow a space for the mouse to be placed on the beam and 10 cm after the finish line in case the mouse paused or hesitated right before entering the safe house. Mice were placed on the beam and a stopwatch was used to time the mice from the start line to the finish line. Mice that paused or hesitated were retested up to 3 times. Latency to cross the balance beams (6mm and 12 mm) was the mean of the 3 trials.

Muscle Tissue Samples—The right and left quadriceps (quad), gastrocnemius (gastroc), and tibialis anterior (TA) muscles were extracted at time of euthanasia (following 14 weeks of diet/drug intervention and within 5 days after the last functional test). Harvested muscles were carefully weighed, flash frozen in liquid nitrogen, and stored at −80°C for future analysis. The combined weight of the right and left muscles was used to calculate absolute muscle mass for each muscle group (quad, gastroc, and TA). For subsequent molecular profiling, the quad muscle was selected as the representative muscle group for gene expression analyses due to its common use in assessments of muscle quantity and quality in humans (Byrne and others 2019).

mRNA Extraction

Sections of flash frozen skeletal muscle (quadriceps) (~50 mg) were homogenized using bead-based Qiagen TissueLyser II in l mL TRIzol™ Reagent (Invitrogen™ ThermoFisher #15596026). Following homogenization, mRNA was extracted using Qiagen RNeasy Mini Kit (Qiagen #74104) and stored at −80°C.

Affymetrix Gene Expression Microarray Analysis

The Functional Genomics Core at The University of North Carolina at Chapel Hill performed the expression profiling and genotyping using an Affymetrix Clariom™ S microarray. The Affymetrix assay plates were read on a GeneTitan Instrument from Affymetrix. Gene Expression Analysis was conducted for each sex separately using Transcriptome Analysis Console (TAC 4.0, Applied Biosystems). Unsupervised hierarchical clustering analysis and principal component analysis (PCA) plots were generated using the 500 most variably expressed genes from each sex (defined by highest overall F statistic) in R (ggplot 3.1.1 and ggbiplot 0.55 packages, respectively).

Gene set enrichment analysis (GSEA)

GSEA was performed using GSEA software (version 4.2.1) from the Broad Institute using MSigDB Hallmark gene sets (Liberzon and others 2015; Subramanian and others 2005). GSEA was performed using 1,000 gene set level permutations for each comparison and results were considered significant where FDRq <0.05. Leading edge analysis was performed to determine core enrichments for all significant gene sets for each pairwise comparison. Leading edge results were visualized using chord diagrams in R (circlize 0.4.13). Differentially expressed genes identified with TAC 4.0 that also contributed to the core enrichment of at least one gene set were overlaid on each chord diagram. Differentially expressed genes were considered significant where FDRq<0.05 and fold change >1.5.

Statistical Analysis

Analyses were performed using a 2 (sex) by 3 (diets: control, CR, and rapamycin) block design with two repeated measures for some measures (e.g., performance and weight), and single measures for others (including post-mortem gene and muscle measures). For the repeated measures, the pre- and post-outcome measures were transformed to change scores (post-baseline) and analyzed by OLS regression. Following good statistical clinical trial practice (2004), baseline was incorporated into the analytic structure as a covariate. With the exception of the baseline covariate in repeated measures, the analytic structure was constant across each of the primary and secondary outcomes. For each outcome, ANOVA assessed main effects and the diet group by sex interactions. If statistically significant, follow-up contrasts were computed to assess where the sex and diet differences existed. If the test of the interaction was nonsignificant, only main effects of sex and diet were tested (1 and 2 df, respectively) with pairwise comparisons of the difference in change for the 3 diets. Additional statistical analysis performed using GraphPad Prism 7 for Windows, version 7.0c (GraphPad Software Inc.). Significance was determined by $p<0.05$.

Results

Chronic rapamycin treatment does not affect body weight or fasting blood glucose compared with control

Data analysis included male (n=45) and female (n=43) C57BL/6 J mice. At 10 months of age, mice were randomized to one of 3 diet groups: control (n=15 males, n=14 females), rapamycin (eRapa) (n=15 males, n=14 females) or CR (n=15 males, n=15 females). Body

weights and weekly food intake are reported in Figure 1A-B and Supplementary Figure 1, respectively. As expected, CR mice consistently exhibited lower body mass compared with mice fed control or rapamycin-supplemented diets. There were no differences observed in body weight or food intake between control and rapamycin animals. Fasting blood glucose (FBG) was significantly lower in CR mice, relative to control and rapamycin groups in both sexes (Figure 1C-D). These results align with expected results for these interventions and indicate that the low dose of rapamycin provided was not sufficient to alter blood glucose or body weight in these animals, relative to control.

CR improves body composition in male mice

The impact of rapamycin treatment and CR-mediated effects on muscle mass (absolute and relative) were assessed using three major muscle groups, the quadriceps (quad), gastrocnemius (gastroc), and tibialis anterior (TA) muscles. For CR male and female mice, absolute mass of the quad, gastroc, and TA muscles was significantly reduced compared with control-fed and rapamycin treated male and female mice (Figure 2A-F). There were no differences observed for absolute muscle mass between control and rapamycin treated mice for the muscle groups tested. When accounting for body mass, we observed relative muscle mass (g/g body mass) increased in CR males for each muscle type, compared with control and Rapamycin (Figure 2G-I). Relative muscle mass for CR female mice did not differ between diet/treatment groups for any muscle type tested (Figure 2J-L). Female mice in the CR group lost both lean and fat mass at similar rates.

CR and rapamycin provide partial protection against loss of muscle strength and function in middle aged mice

In addition to muscle mass (muscle quantity), muscle force generation is derived through muscle strength and function (muscle quality). Hence, we conducted a series of functional assays in these mice at baseline and endpoint (14 weeks post diet/treatment onset) to disentangle the CR and rapamycin treatment effects on these features of muscle quality (function and strength). Metrics of peak force and strength (both mean force/strength and normalized force/strength) were assessed using grip strength. Despite reduced absolute muscle mass (quads, gastroc, and TA muscles), mean grip strength did not differ in CR males and females compared with control and rapamycin (Figure 3A-B). To determine relative force and strength, given differences in body weight between groups (CR mice weighed ~30% less than control and Rapamycin mice), grip strength was normalized to body weight. Relative to control, CR mice had increased normalized grip strength, for both males and females (Figure 3C-D). For rapamycin treated mice, females displayed greater mean and normalized strength compared with control female mice (Figure 3B, D). Together, these results suggest CR exerts effects on grip strength (i.e. higher force/strength capacity), relative to control, while the effects of rapamycin on grip strength are sex-specific.

To investigate agility, balance, and coordination, a balance beam test was performed. This test determines the speed with which a mouse will cross a narrow beam (6/12 mm), with faster times indicating higher agility. Overall, across both beams (6 mm and 12 mm), we found mice in the intervention groups (CR and rapamycin) were significantly faster than

Control mice (Figure 3E-H). These results suggest CR and rapamycin treatment prevented age-related decline in function, with respect to balance and agility.

Whole body strength and coordination were assessed with the inverted screen test. Maximum hang time for both females and males was significantly increased in CR compared with Control and Rapamycin (Figure 3I-J). To determine if the increased hang time observed in the CR mice could be explained by weight differences between groups, we computed holding impulse to correct for the effects of body mass on hang time. Results indicate hang time for CR females remained significantly greater than control and rapamycin females (Figure 3K). For males, hang time was not different than control or rapamycin (Figure 3L).

Differential effects of CR and rapamycin on gene expression profiles in quadricep muscle

To assess the effects of chronic CR and rapamycin treatment on transcriptional changes in skeletal muscle, we conducted global transcriptomic analysis of mRNA isolated from quadricep muscle and analyzed male and female mice independently. PCA and unsupervised hierarchal clustering of the 500 most variably expressed genes in quadricep muscle from male mice revealed that all three groups clustered separately, with gene expression profiles of muscle from control diet-fed and rapamycin treated mice being more similar to each other than to CR (Figure 4A-B). To identify candidate biological pathways and processes modulated by a chronic CR diet or rapamycin treatment, we performed GSEA using the MSigDB curated Hallmark gene sets. Two gene sets were enriched in CR mice relative to control mice (‘G2M checkpoint’, ‘E2F targets’; FDRq <0.05) while two other gene sets were suppressed in the CR group (‘Epithelial mesenchymal transition’, ‘Coagulation’; FDRq <0.05) (Figure 4C). Rapamycin treatment resulted in the suppression of seven gene sets relative to control, and ‘Coagulation’ was the only gene set suppressed by both CR and rapamycin relative to control (Figure 4D). ‘mTORC1 signaling’, ‘Adipogenesis’, and ‘Cholesterol homeostasis’ gene sets were all enriched in quadriceps from CR mice relative to rapamycin treated mice (Figure 4E).

A subsequent leading edge analysis identified shared and unique genes amongst the significantly enriched gene sets within each pairwise comparison. This analysis indicated that a minimal number of genes were shared between gene sets, with the most overlapping genes observed between ‘E2F targets/G2M checkpoint’ and ‘Epithelial mesenchymal transition’/ ‘Coagulation’ (Figure 4F-H). These findings highlight that CR and rapamycin treatment promote distinct pathways which are underpinned by disparate gene expression profiles.

Like males, quadricep gene expression in the female mice was distinct between all groups but control and rapamycin treated mice had a more similar profile relative to CR mice (Figure 5A-B). GSEA using Hallmark gene sets revealed stark differences between CR and rapamycin treated mice. When compared with control fed mice, CR led to the suppression of three gene sets (‘Cholesterol homeostasis,’ ‘Interferon gamma response,’ ‘Epithelial mesenchymal transition’; FDRq <0.05) while rapamycin treatment promoted enrichment of five separate gene sets (‘Xenobiotic metabolism,’ ‘Coagulation’, ‘Bile acid metabolism,’ ‘Peroxisome,’ and ‘Fatty acid metabolism’; FDRq <0.05) (Figure 5C-D). Moreover, when

compared directly with CR, rapamycin treatment drove activation of seven gene sets while CR did not enrich for any, suggesting that rapamycin treatment promoted a set of biological processes and pathways distinct from CR (Figure 5E). Leading edge analysis revealed an analogous picture where very few genes across gene sets overlapped for the CR versus rapamycin comparision. Col6a3 was the only gene that was both differentially expressed (FDRq <0.05) and present in the leading edge of a significantly enriched gene set ('Epithelial mesenchymal transition' for CR vs. rapamycin) (Figure 5F-H).

Discussion

The anti-aging, anticancer, and other positive effects of CR on human health have led to the development of CR mimetic agents which offer the benefit of a CR diet, without reducing caloric intake. Of these compounds, rapamycin has been shown to protect several tissues from age-related functional decline, however its effects on aging muscle are incompletely understood (Johnson and others 2013; Schinaman and others 2019). This study assessed the impact of an enteric form of rapamycin versus a 30% CR regimen on age-related changes in muscle of middle aged mice.

In male mice, we found CR improved body composition (increased relative muscle mass), a key CR-induced response; and provided partial protection against age-related functional decline. Our findings are consistent with results in other laboratories conducted in male mice (van Norren and others 2015) and align with our results of the molecular analysis of muscle tissue. Relative to control, the pathways enriched by CR in male mice support muscle maintenance and repair (G2M Checkpoint and E2F targets) and suppress processes associated with aging muscle (EMT and coagulation). Collectively, these CR-induced transcriptional adaptations may have a collateral effect to prevent age-related loss of muscle quantity and quality (Santos and others 2019). While we did not observe improvements for whole body strength, assessed via inverted screen test, CR had a clear impact on functional performance with respect to balance, coordination, and grip strength in males, relative to control.

Female CR mice, despite having significantly less muscle quantity (mass) and no difference in relative muscle mass, performed distinctly better than control females for all functional measures tested. While these findings are likely the product of a complex milieu of molecular interactions, our results suggest the higher functional capabilities of CR females may be more dependent on improved muscle quality rather than muscle quantity. Transcriptional analysis revealed that CR resulted in suppression of EMT and pro-inflammatory signaling pathways that may be responsible for improved muscle quality, and in turn increased function in females exposed to CR (Dalle and others 2017; Santos and others 2019).

The concept that muscle quality plays a pivotal role in prevention of age-related decline in function, independent of muscle quantity, is recapitulated in mice treated with rapamycin, albeit to a lesser extent. However, as GSEA revealed, the functional improvements observed for rapamycin mice were associated with vastly different metabolic pathways compared with CR. Our findings suggest, for males treated with rapamycin compared with control,

downregulation of metabolic, nutrient-sensing pathways, including mTORC1, resulted in increased balance and coordination, while having no impact on muscle quantity (despite mTOR's key role in maintaining muscle mass), at least in the timeframe of our study. These results support a role for rapamycin in maintaining features of muscle function without a consequential loss of muscle mass. For females treated with rapamycin, the molecular changes associated with rapamycin treatment were strikingly different than observed for males, yet yielded increased function compared with female controls for measures of balance, coordination, and grip strength. While rapamycin treatment in females did not translate to significant mTORC1 inhibition, (potentially due to the pharmacodynamics of rapamycin or sex differences in regulation of mTOR signaling independent of rapamycin, for example estrogen has been shown to activate mTOR and downstream signaling by increasing Rheb activity, a potent activator of mTORC1), the drug did result in a relative increase in cellular immune response (complement, interferon alpha/gamma response) and antioxidant defense (xenobiotic metabolism), compared with control, which are important anti-aging and anti-sarcopenic signaling pathways (Strong and others 2020; Takahara and others 2020; Yu and Henske 2006). Taken together, while these results provide insight about the effects of CR and rapamycin on muscle-specific functional and molecular indices, future studies are needed to fully understand the link between these biological pathways and physiological findings in both male and female mice.

Our study was subject to several limitations. First, while we performed our study with a large cohort of male and female mice, our transcriptional analysis was limited to a sample size of 5 males and ≤5 females per group. As the effects of CR and rapamycin are sex dependent, investigating the effects with a larger sample size per sex and group may provide a more in-depth understanding of the transcriptional changes associated with CR and rapamycin interventions. Additionally, we appreciate the value that body composition imaging, such as quantitative magnetic resonance, would have contributed to our study. Unfortunately, due to COVID-related closures of core facilities, we were unable to obtain these measures. Also, in light of our findings that suggest both CR and rapamycin provide protection to aging muscle, albeit to varying degrees and result in distinctly different molecular profiles, combination regimens would have been informative, but were beyond the scope of this study. Future testing of a combination regimen of CR + rapamycin, and extending these studies to include other forms of CR (such as intermittent CR) and CR mimetic agents, would provide insight into the additive potential of diet + drug. Finally, given mice experience a progressive decline in physical function (beginning as early as 6 months of age) the age of mice for this study (10 months) was strategically selected to capture the transitional changes that occur in skeletal muscle at late middle age (Yanai and Endo 2021). However, it is important to note that the intervention ended while the mice were at a fairly healthy age (~14 months), and perhaps before more profound changes associated with age related loss of muscle mass and function could be observed.

In conclusion, the increase in the world's aging population and the impact this is having on morbidities associated with advanced age, such as sarcopenia, has led to an urgent need to better understand the mechanisms underlying age-related effects on muscle and identify countermeasures. Our preclinical findings establish that CR and rapamycin provide partial protection against age-related functional decline in skeletal muscle while engaging uniquely

different molecular pathways. These findings provide an impetus for further translational exploration of the mechanistic targets and functional effects of CR, rapamycin, and related dietary and pharmacologic interventions, for the mitigation and prevention of age-related sarcopenia.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

Committee for Proprietary Medicinal Products (CPMP): points to consider on adjustment for baseline covariates. Stat Med. 23:701–709; 2004 [PubMed: 14981670]

Barclay RD; Burd NA; Tyler C; Tillin NA; Mackenzie RW The Role of the IGF-1 Signaling Cascade in Muscle Protein Synthesis and Anabolic Resistance in Aging Skeletal Muscle. Front Nutr. 6:146; 2019 [PubMed: 31552262]

Baumgartner RN; Koehler KM; Gallagher D; Romero L; Heymsfield SB; Ross RR; Garry PJ; Lindeman RD Epidemiology of sarcopenia among the elderly in New Mexico. Am J Epidemiol. 147:755–763; 1998 [PubMed: 9554417]

Bian A; Ma Y; Zhou X; Guo Y; Wang W; Zhang Y; Wang X Association between sarcopenia and levels of growth hormone and insulin-like growth factor-1 in the elderly. BMC Musculoskeletal Disorders. 21:214; 2020 [PubMed: 32264885]

Bikle DD; Tahimic C; Chang W; Wang Y; Philippou A; Barton ER Role of IGF-I signaling in muscle bone interactions. Bone. 80:79–88; 2015 [PubMed: 26453498]

Blagosklonny MV Rapamycin for longevity: opinion article. Aging (Albany NY). 11:8048–8067; 2019 [PubMed: 31586989]

Brook MS; Wilkinson DJ; Phillips BE; Perez-Schindler J; Philp A; Smith K; Atherton PJ Skeletal muscle homeostasis and plasticity in youth and ageing: impact of nutrition and exercise. Acta Physiol (Oxf). 216:15–41; 2016 [PubMed: 26010896]

Byrne CA; McNeil AT; Koh TJ; Brunskill AF; Fantuzzi G Expression of genes in the skeletal muscle of individuals with cachexia/sarcopenia: A systematic review. PLoS One. 14:e0222345; 2019 [PubMed: 31498843]

Colman RJ; Beasley TM; Allison DB; Weindruch R Attenuation of sarcopenia by dietary restriction in rhesus monkeys. J Gerontol A Biol Sci Med Sci. 63:556–559; 2008 [PubMed: 18559628]

Dalle S; Rossmeislova L; Koppo K The Role of Inflammation in Age-Related Sarcopenia. Frontiers in Physiology. 8; 2017

Das SK; Balasubramanian P; Weerasekara YK Nutrition modulation of human aging: The calorie restriction paradigm. Mol Cell Endocrinol. 455:148–157; 2017a [PubMed: 28412520]

Das SK; Roberts SB; Bhapkar MV; Villareal DT; Fontana L; Martin CK; Racette SB; Fuss PJ; Kraus WE; Wong WW; Saltzman E; Pieper CF; Fielding RA; Schwartz AV; Ravussin E; Redman LM Body-composition changes in the Comprehensive Assessment of Long-term Effects of Reducing Intake of Energy (CALERIE)-2 study: a 2-y randomized controlled trial of calorie restriction in nonobese humans. Am J Clin Nutr. 105:913–927; 2017b [PubMed: 28228420]

Fan J; Kou X; Yang Y; Chen N MicroRNA-Regulated Proinflammatory Cytokines in Sarcopenia. Mediators Inflamm. 2016:1438686; 2016 [PubMed: 27382188]

Ferrucci L; Fabbri E Inflammageing: chronic inflammation in ageing, cardiovascular disease, and frailty. Nat Rev Cardiol. 15:505–522; 2018 [PubMed: 30065258]

Hagan C When Mice are Considered Old? The Jackson Laboratory; 2017

Halloran J; Hussong SA; Burbank R; Podlutskaya N; Fischer KE; Sloane LB; Austad SN; Strong R; Richardson A; Hart MJ; Galvan V Chronic inhibition of mammalian target of rapamycin by rapamycin modulates cognitive and non-cognitive components of behavior throughout lifespan in mice. Neuroscience. 223:102–113; 2012 [PubMed: 22750207]

Johnson SC; Rabinovitch PS; Kaeberlein M mTOR is a key modulator of ageing and age-related disease. Nature. 493:338–345; 2013 [PubMed: 23325216]

Kalyani RR; Corriere M; Ferrucci L Age-related and disease-related muscle loss: the effect of diabetes, obesity, and other diseases. Lancet Diabetes Endocrinol. 2:819–829; 2014 [PubMed: 24731660]

Lamming DW Inhibition of the Mechanistic Target of Rapamycin (mTOR)-Rapamycin and Beyond. Cold Spring Harb Perspect Med. 6; 2016

Lewandowicz A; Sławiński P; Kądalska E; Targowski T Some clarifications of terminology may facilitate sarcopenia assessment. Archives of Medical Science. 16:225–232; 2020 [PubMed: 32051727]

Liberzon A; Birger C; Thorvaldsdóttir H; Ghandi M; Mesirov JP; Tamayo P The Molecular Signatures Database (MSigDB) hallmark gene set collection. Cell Syst. 1:417–425; 2015 [PubMed: 26771021]

Liu P; Li Y; Ma L Caloric Restriction May Help Delay the Onset of Frailty and Support Frailty Management. Frontiers in Nutrition. 8; 2021

López-Lluch G; Navas P Calorie restriction as an intervention in ageing. J Physiol. 594:2043–2060; 2016 [PubMed: 26607973]

Madeo F; Carmona-Gutierrez D; Hofer SJ; Kroemer G Caloric Restriction Mimetics against Age-Associated Disease: Targets, Mechanisms, and Therapeutic Potential. Cell Metab. 29:592–610; 2019 [PubMed: 30840912]

Nair KS Aging muscle. The American Journal of Clinical Nutrition. 81:953–963; 2005 [PubMed: 15883415]

Orenduff MC; Rezeli ET; Hursting SD; Pieper CF Psychometrics of the Balance Beam Functional Test in C57BL/6 Mice. Comp Med. 71:302–308; 2021 [PubMed: 34311810]

Ortman J; Velkoff V; Hogan H An Aging Nation: The Older Population in the United States. Population Estimates and Projections. U.S. CENSUS BUREAU: U.S. Department of Commerce Economics and Statistics Administration; 2014

Partridge L; Fuentealba M; Kennedy BK The quest to slow ageing through drug discovery. Nat Rev Drug Discov. 19:513–532; 2020 [PubMed: 32467649]

Redman LM; Ravussin E Caloric restriction in humans: impact on physiological, psychological, and behavioral outcomes. Antioxid Redox Signal. 14:275–287; 2011 [PubMed: 20518700]

Rhoads TW; Clark JP; Gustafson GE; Miller KN; Conklin MW; DeMuth TM; Berres ME; Eliceiri KW; Vaughan LK; Lary CW; Beasley TM; Colman RJ; Anderson RM Molecular and Functional Networks Linked to Sarcopenia Prevention by Caloric Restriction in Rhesus Monkeys. Cell Syst. 10:156–168.e155; 2020 [PubMed: 31982367]

Rong S; Wang L; Peng Z; Liao Y; Li D; Yang X; Nuessler AK; Liu L; Bao W; Yang W The mechanisms and treatments for sarcopenia: could exosomes be a perspective research strategy in the future? J Cachexia Sarcopenia Muscle. 11:348–365; 2020 [PubMed: 31989804]

Santilli V; Bernetti A; Mangone M; Paoloni M Clinical definition of sarcopenia. Clin Cases Miner Bone Metab. 11:177–180; 2014 [PubMed: 25568649]

Santos F; Moreira C; Nóbrega-Pereira S; Bernardes de Jesus B New Insights into the Role of Epithelial-Mesenchymal Transition during Aging. Int J Mol Sci. 20; 2019

Schinaman JM; Rana A; Ja WW; Clark RI; Walker DW Rapamycin modulates tissue aging and lifespan independently of the gut microbiota in Drosophila. Scientific Reports. 9:7824; 2019 [PubMed: 31127145]

Sohal RS; Forster MJ Caloric restriction and the aging process: a critique. Free Radic Biol Med. 73:366–382; 2014 [PubMed: 24941891]

Song YH; Song JL; Delafontaine P; Godard MP The therapeutic potential of IGF-I in skeletal muscle repair. Trends Endocrinol Metab. 24:310–319; 2013 [PubMed: 23628587]

Strong R; Miller RA; Bogue M; Fernandez E; Javors MA; Libert S; Marinez PA; Murphy MP; Musi N; Nelson JF; Petrascheck M; Reifsnyder P; Richardson A; Salmon AB; Macchiarini F; Harrison DE Rapamycin-mediated mouse lifespan extension: Late-life dosage regimes with sex-specific effects. Aging Cell. 19:e13269; 2020 [PubMed: 33145977]

Subramanian A; Tamayo P; Mootha VK; Mukherjee S; Ebert BL; Gillette MA; Paulovich A; Pomeroy SL; Golub TR; Lander ES; Mesirov JP Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. Proc Natl Acad Sci U S A. 102:15545–15550; 2005 [PubMed: 16199517]

Swindell WR Genes and gene expression modules associated with caloric restriction and aging in the laboratory mouse. BMC Genomics. 10:585; 2009 [PubMed: 19968875]

Takahara T; Amemiya Y; Sugiyama R; Maki M; Shibata H Amino acid-dependent control of mTORC1 signaling: a variety of regulatory modes. Journal of Biomedical Science. 27:87; 2020 [PubMed: 32799865]

Tang H; Inoki K; Brooks SV; Okazawa H; Lee M; Wang J; Kim M; Kennedy CL; Macpherson PCD; Ji X; Van Roekel S; Fraga DA; Wang K; Zhu J; Wang Y; Sharp ZD; Miller RA; Rando TA; Goldman D; Guan KL; Shrager JB mTORC1 underlies age-related muscle fiber damage and loss by inducing oxidative stress and catabolism. Aging Cell. 18:e12943; 2019a [PubMed: 30924297]

Tang H; Shrager JB; Goldman D Rapamycin protects aging muscle. Aging (Albany NY). 11:5868–5870; 2019b [PubMed: 31454792]

van Norren K; Rusli F; van Dijk M; Lute C; Nagel J; Dijk FJ; Dwarkasing J; Boekschoten MV; Luiking Y; Witkamp RF; Müller M; Steegenga WT Behavioural changes are a major contributing factor in the reduction of sarcopenia in caloric-restricted ageing mice. J Cachexia Sarcopenia Muscle. 6:253–268; 2015 [PubMed: 26401472]

Wilkinson JE; Burmeister L; Brooks SV; Chan CC; Friedline S; Harrison DE; Hejtmancik JF; Nadon N; Strong R; Wood LK; Woodward MA; Miller RA Rapamycin slows aging in mice. Aging Cell. 11:675–682; 2012 [PubMed: 22587563]

Yanai S; Endo S Functional Aging in Male C57BL/6J Mice Across the Life-Span: A Systematic Behavioral Analysis of Motor, Emotional, and Memory Function to Define an Aging Phenotype. Frontiers in Aging Neuroscience. 13; 2021

Yoon M-S mTOR as a Key Regulator in Maintaining Skeletal Muscle Mass. Frontiers in Physiology. 8; 2017

Yu J; Henske EP Estrogen-Induced Activation of Mammalian Target of Rapamycin Is Mediated via Tuberin and the Small GTPase Ras Homologue Enriched in Brain. Cancer Research. 66:9461–9466; 2006 [PubMed: 17018601]

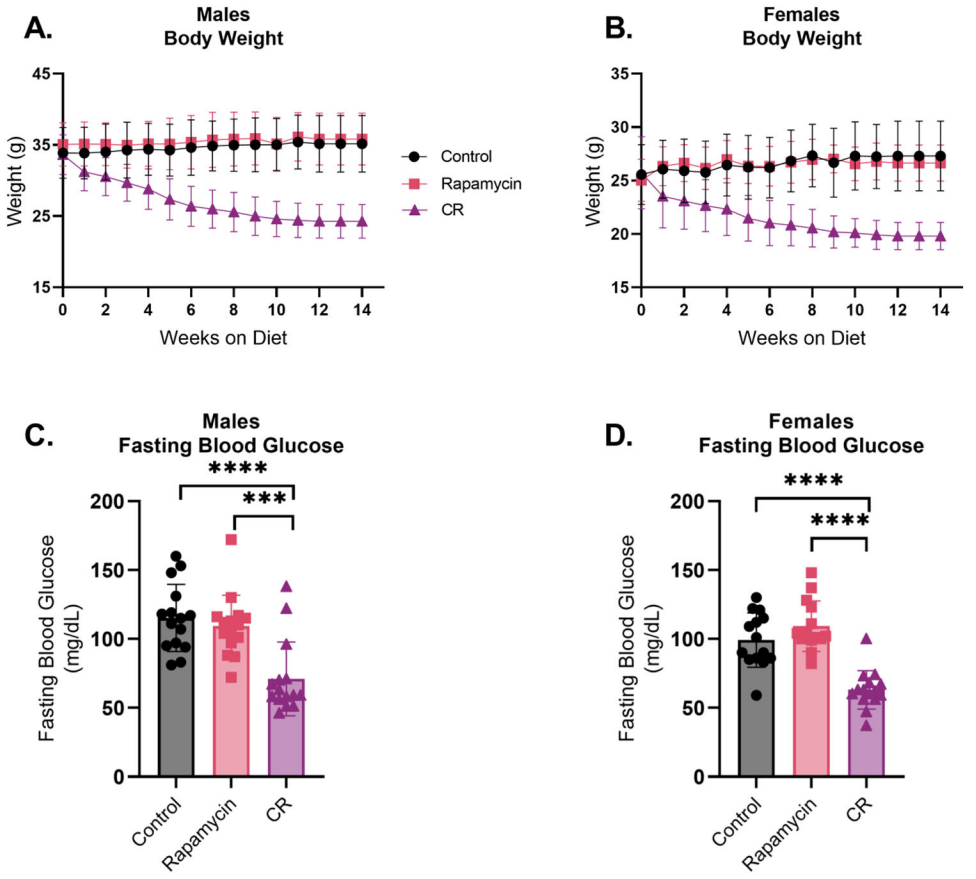


Figure 1: Body weight and fasting blood glucose.

Body weight was recorded weekly throughout the 14 weeks for (A) males and (B) females. Fasting blood glucose (6 hours) was obtained at time of euthanization for (C) males and (D) females. Weight is presented in grams (g) and fasting blood glucose is in mg/dL. Sample size: control (n=15 males) (n=14 females); Rapamycin (n=15 males) (n=14 females); CR (n=15 males) (n=15 females), ***p<0.005, ****p<0.0001. Values are means SD. Statistical tests: (C) and (D) one-way ANOVA and Tukey's multiple comparison post hoc test.

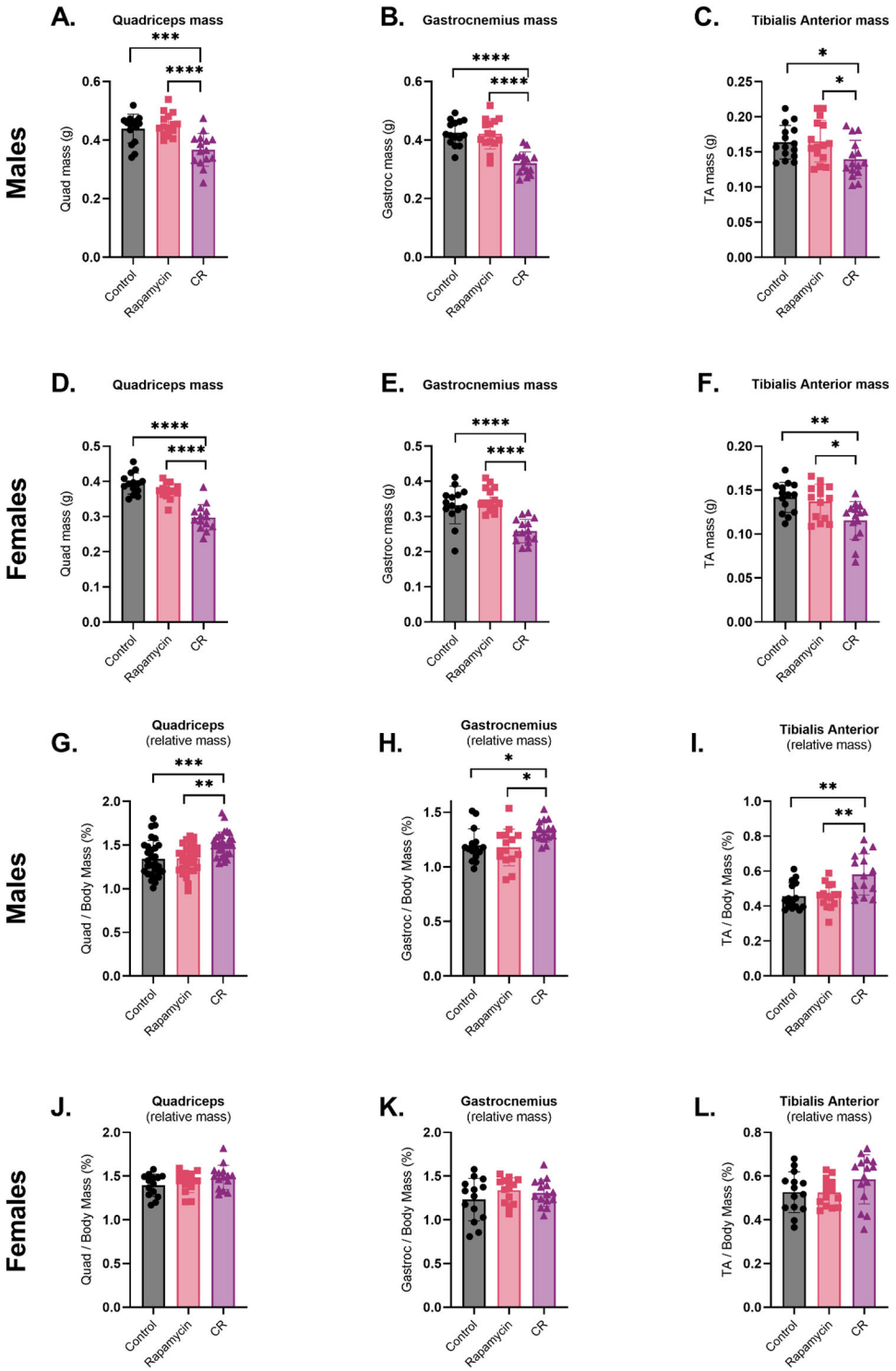
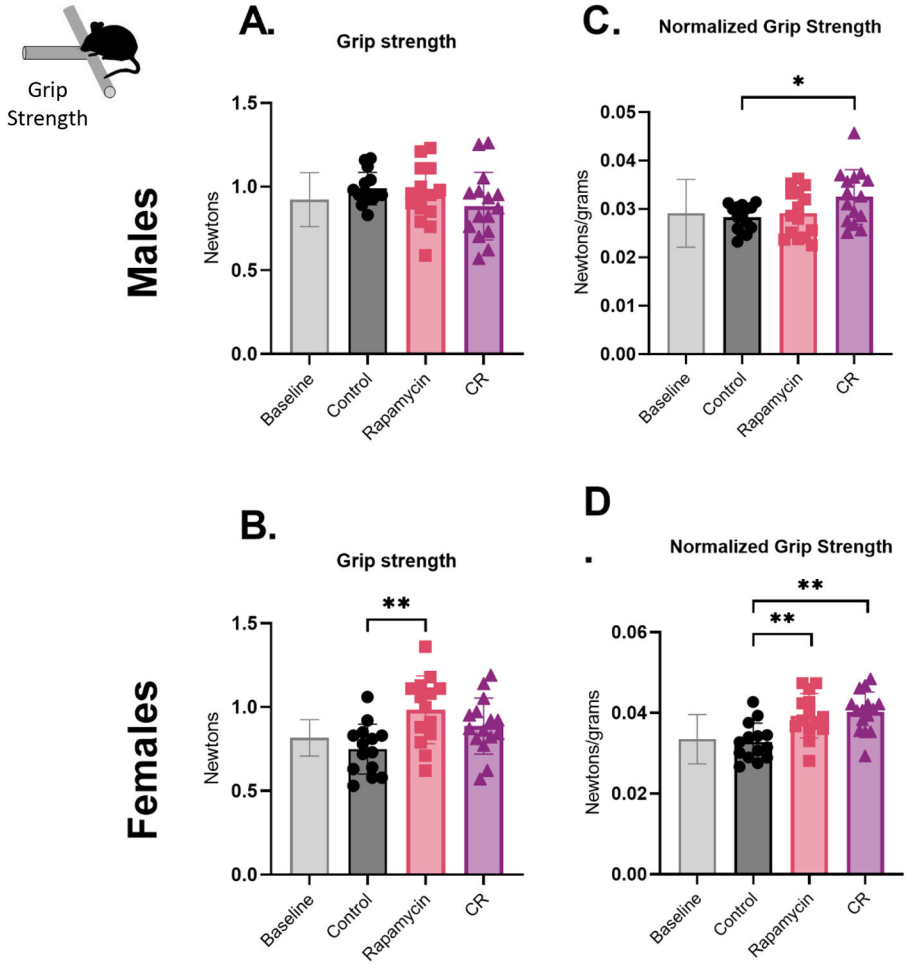
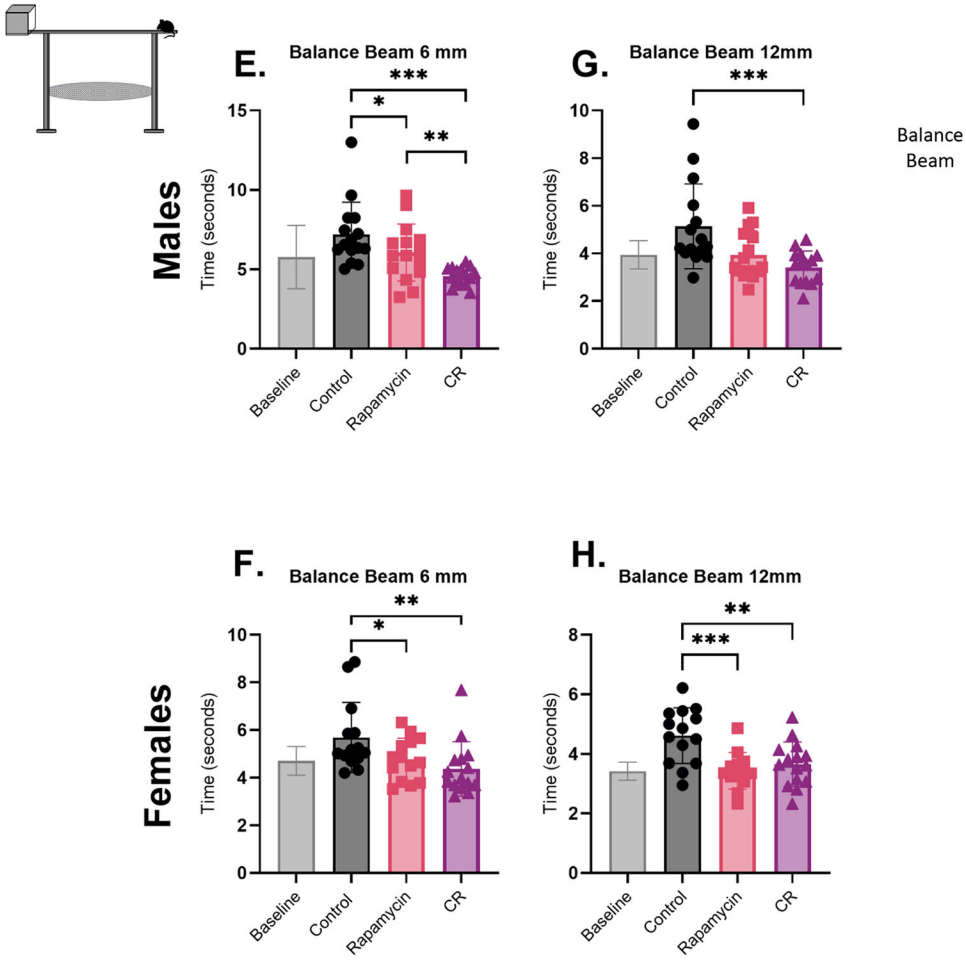


Figure 2. Absolute and relative muscle mass for male and female mice.

(A-C) Absolute muscle mass for males (n=15) per diet group. (D-F) Absolute muscle mass for females (n=14) for control and rapamycin diet groups and (n=15) for the CR diet group. (G-I) Relative muscle mass for males. (J-L) Relative muscle mass for females. Relative muscle mass is derived by dividing muscle mass by whole body mass, *p<0.05, **p<0.01,

***p<0.005, ****p<0.0001. Values are means SD. Statistical tests: one-way ANOVA and Tukey's multiple comparison post hoc test.





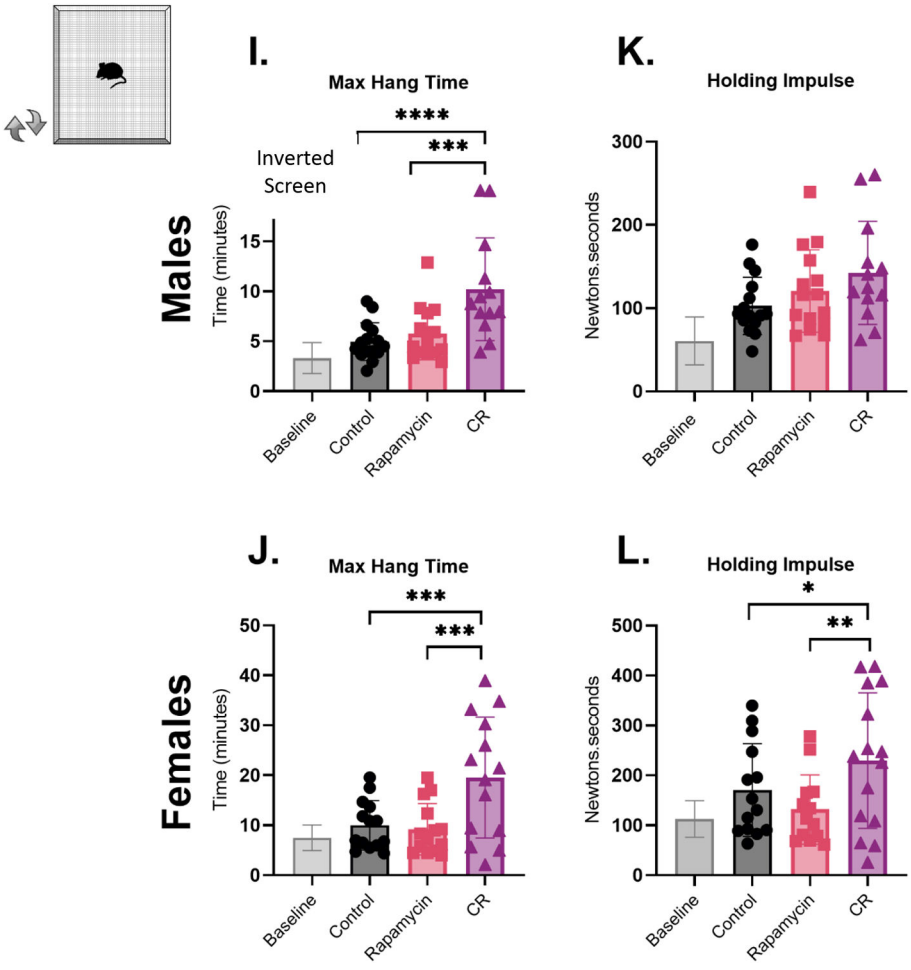


Figure 3. Functional tests:

Measures of muscle strength, function, balance, and coordination were assessed. Schematic of grip strength: (A-B) Mean grip strength (derived from the three replicated measurements) for males and females. (C-D) Relative grip strength [(derived from dividing unit of force (newtons) by body mass (g)]. Schematic of balance beam: Latency to cross the balance beam presented as the mean of three trials for the 6mm beam (E-F) and 12mm beam (G-H). Schematic of inverted screen test: (I-J) Maximum hang time for males and females and (K-L) holding impulse. Holding impulse was calculated by multiplying body mass by maximum hang time and converting to newtons, *p<0.05, **p<0.01, ***p<0.005, ****p<0.0001. Values are means SEM. Statistical tests: two-way ANOVA and Tukey's multiple comparison post hoc test. In the graph for holding impulse for females, the highest score was truncated to reduce the influence of an extreme outlier

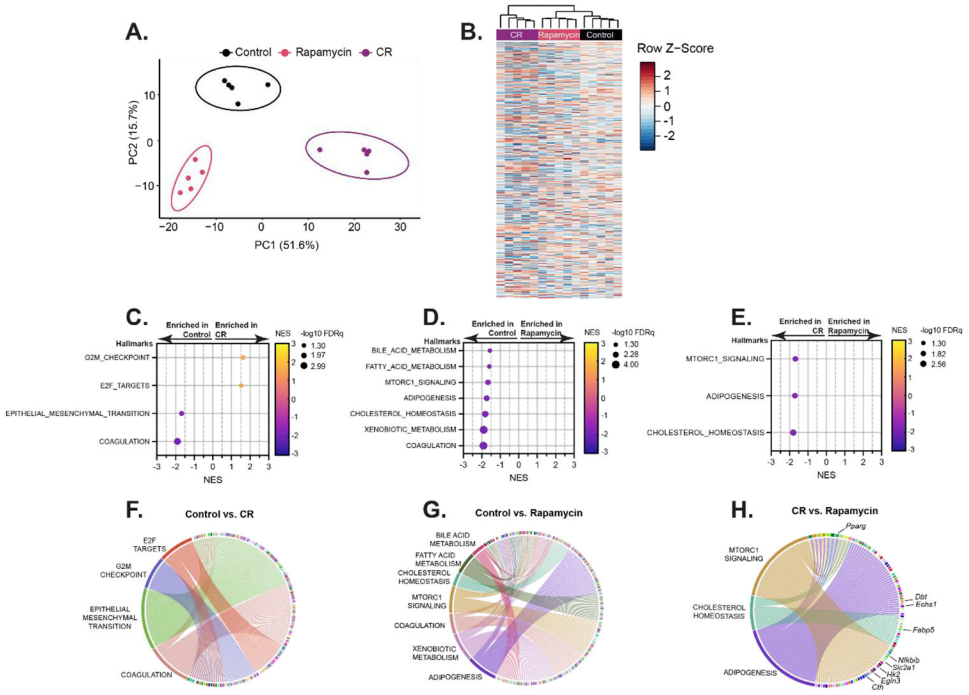


Figure 4. Effect of CR and rapamycin treatment on the gene expression profile of quadricep muscle in male mice.

(A) Principal component analysis of the top 500 differentially expressed genes from quadricep muscle in male mice. **(B)** Heat map showing unsupervised hierarchical clustering of the top 500 differentially expressed genes from quadricep muscle in male mice. **(C-E)** GSEA MSigDB Hallmark gene sets significantly enriched in quadricep muscle from either control fed, CR, or rapamycin treated male mice. **(F-H)** Leading edge analysis of the significantly enriched gene sets. The chord diagrams visualize the significantly enriched gene sets (long sectors), the genes encompassed within the leading edge of the gene sets (short sectors), and the number of times the gene shows up in a gene set (links between a gene set and the specific gene). Labeled genes signify that the gene was both significantly differentially expressed for that pairwise comparison and was detected in the leading edge of at least one significantly enriched gene set. n=5/group; PC1 = principal component 1; PC2 = principal component 2; NES = normalized enrichment score; significance was defined as FDRq<0.05 by PERMANOVA for PCA analysis and FDRq<0.05 for GSEA and differentially expressed gene analyses.

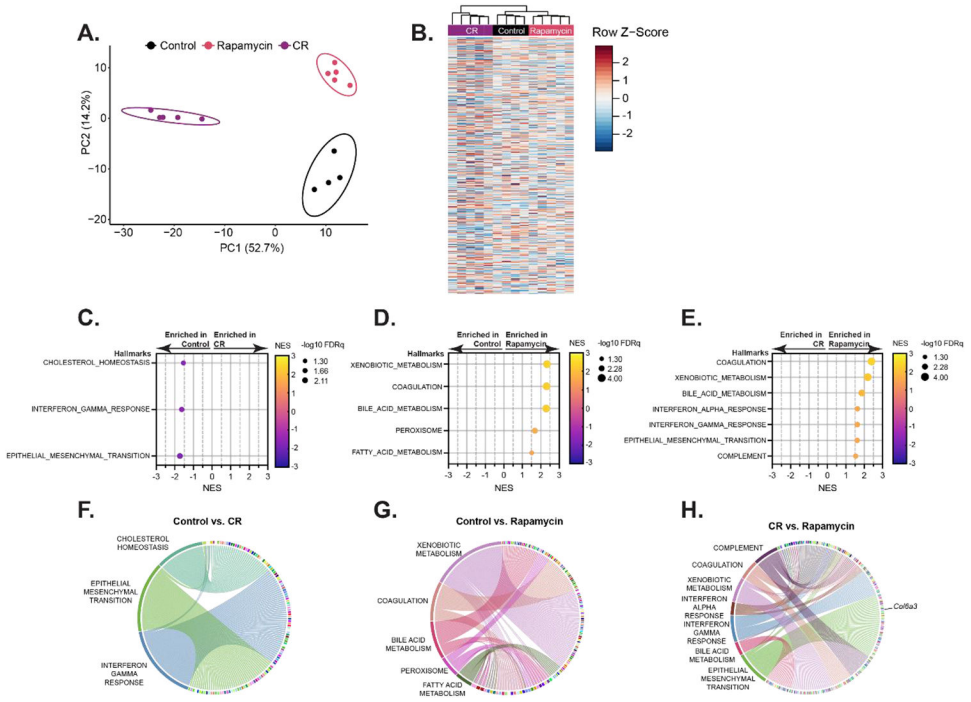


Figure 5. Effect of CR and rapamycin treatment of the gene expression profile of quadricep muscle in female mice.

(A) Principal component analysis of the top 500 differentially expressed genes from quadricep muscle in female mice. **(B)** Heat map showing unsupervised hierarchical clustering of the top 500 differentially expressed genes from quadricep muscle in female mice. **(C-E)** GSEA MSigDB Hallmark gene sets significantly enriched in quadricep muscle from either control fed, CR, or rapamycin treated female mice. **(F-H)** Leading edge analysis of the significantly enriched gene sets. The chord diagrams visualize the significantly enriched gene sets (long sectors), the genes encompassed within the leading edge of the gene sets (short sectors), and the number of times the gene shows up in a gene set (links between a gene set and the specific gene). Labeled genes signify that the gene was both significantly differentially expressed for that pairwise comparison and was detected in the leading edge of at least one significantly enriched gene set. n=4-5group; PC1 = principal component 1; PC2 = principal component 2; NES = normalized enrichment score; significance was defined as FDRq<0.05 by PERMANOVA for PCA analysis and FDRq<0.05 for GSEA and differentially expressed gene analyses.