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Research Paper

Intermittent Methionine Restriction Reduces Marrow Fat Accumulation and Preserves More Bone Mass than Continuous Methionine Restriction

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Continuous methionine restriction (MR) is one of only a few dietary interventions known to dramatically extend mammalian healthspan. For example, continuously methionine-restricted rodents show less age-related pathology and are up to 45% longer-lived than controls. Intriguingly, MR is feasible for humans, and a number of studies have suggested that methionine-restricted individuals may receive similar healthspan benefits as rodents. However, long-term adherence to a continuously methionine-restricted diet is likely to be challenging (or even undesirable) for many individuals. To address this, we previously developed an intermittent version of MR (IMR) and demonstrated that it confers nearly identical metabolic health benefits to mice as the continuous intervention, despite having a relatively short interventional period (i.e., only three days per week). We also observed that female mice undergoing IMR show a more pronounced amelioration of diet-induced dysglycemia than continuously methionine-restricted counterparts, while male mice undergoing IMR retain more lean body mass as compared with continuously methionine-restricted controls. Prompted by such findings, we sought to determine other ways in which IMR might compare favorably with continuous MR.
While it is known that continuous MR has deleterious effects on bone in mice, including loss of both trabecular and cortical bone, we considered that mice undergoing IMR might retain more bone mass. Here, we report that, as compared with continuous MR, IMR results in a preservation of both trabecular and cortical bone, as well as a dramatic reduction in the accumulation of marrow fat. Consistent with such findings, mechanical testing revealed that the bones of intermittently methionine-restricted mice are significantly stronger than those of mice subjected to the continuous intervention. Finally, static histomorphometric analyses suggest that IMR likely results in more bone mass than that produced by continuous MR, primarily by increasing the number of osteoblasts. Together, our results demonstrate that the more practicable intermittent form of MR not only confers similar metabolic health benefits to the continuous intervention but does so without markedly deleterious effects on either the amount or strength of bone. These data provide further support for the use of IMR in humans.

Introduction

Continuous methionine restriction (MR) is known to confer improved metabolism and extended healthspan to a variety of organisms, including mammals. Rats fed a methionine-restricted diet are up to 45% longer-lived than controls, and the lifespan of methionine-restricted mice is also improved[1–3]. In addition to improving overall survival, MR confers many metabolic health benefits to rodents, including improved glycemic control, reduced fat accumulation, protection against hepatosteatosis, and altered levels of multiple hormones that regulate metabolism and healthspan[1,4–6]. Amazingly, the metabolic benefits of MR are so potent that this intervention totally protects animals against the diet-induced obesity that typically results from consumption of a high-fat diet[7].

Studies from our lab and others have suggested that MR is likely to confer healthspan benefits to humans similar to those observed for rodents and other organisms[8–12]. Indeed, the vegan diet is low in both total protein and free amino acids (including methionine and cysteine), meaning that MR is feasible for humans[13]. However, it is expected that long-term compliance with such a diet is likely to be challenging. Moreover, there are a few negative effects of continuous MR that some individuals might seek to avoid. To address these issues, we recently developed a more practicable intermittent version of MR (IMR) that features alternating periods of methionine repletion (four days) and methionine restriction (three days). When applied to mice, we found IMR to be similarly effective to the classical intervention at conferring a multitude of metabolic health benefits, including protection against both diet-induced obesity and hepatosteatosis, as

well as changes in the circulating levels of the nutrient-sensing hormones IGF-1, FGF-21, leptin, and adiponectin[14]. Although this study focused on the short-term effects of both continuous and intermittent MR (and thus did not assess the overall survival of mice), the fact that IMR reduces the circulating levels of IGF-1 strongly suggests that this intervention will also extend mouse lifespan. This is because (a) a preponderance of lifespan-extending dietary interventions are known to decrease IGF-1 signaling (e.g., calorie restriction, intermittent fasting, the ketogenic diet, and protein restriction)[15–21]; (b) dwarf mice with reduced IGF-1 signaling are long-lived; (c) continuous MR reduces IGF-1 levels, extends lifespan, and fails to further improve the extreme longevity of dwarf mice[1,4,7,22]; and (d) transgenic mice with increased IGF-1 activity show an impaired response to continuous MR[23]. When taken together, these results strongly suggest that continuous MR extends overall survival by maintaining low circulating IGF-1 levels and, furthermore, that IMR will share this ability. However, it is possible that IMR might not extend lifespan despite its effects on IGF-1. For example, a recent study found that feeding adult mice (7–12 months old) a methionine-restricted diet (containing 0.15% methionine) failed to extend their overall survival[24]. A possible explanation for this finding might be that continuous MR at a level of 0.15% methionine does not alter IGF-1 levels sufficiently in adult animals to produce robust longevity benefits. Unfortunately, it is not possible to say whether this is the case, as the authors did not measure circulating levels of IGF-1. In any case, until the long-term benefits of IMR can be assessed, it is clear that this novel intervention dramatically improves the short-term health of mice, but without any appreciable deleterious side effects[14].

Regarding such side effects, it has been previously demonstrated that continuous MR limits growth, body size, and the accumulation of lean muscle mass in rodents[1,2,7,25]. Consistent with such findings, continuously methionine-restricted rodents were found to have reduced bone volume, bone tissue density, and bone mineral content as compared with control-fed (CF) animals[7,25–27]. Not surprisingly, the bones of these animals were also weaker than those of controls. A possible explanation for the deleterious effects of continuous MR on bone architecture is altered osteoblast and osteoclast activity in response to MR-induced changes in FGF-21 and/or IGF-1 hormone levels. Overexpression of FGF-21, which is elevated by MR, has been reported to block cell growth and result in bone loss in mice[28]. Similarly, reduced IGF-1 activity is associated with shorter stature and smaller body sizes in a number of organisms, including rodents[29]. While the majority of the above experiments revealing the deleterious effects of continuous MR on both growth and bone formation were performed in young, still-developing rodents, nine-month-old adult male mice also suffered reduced bone mineral density and bone mineral content when subjected to this intervention[26]. Indeed, such a finding is consistent with the fact that adults who practice veganism (which, as mentioned above, can be considered a form of continuous MR) display an increased incidence of bone fractures as compared with non-vegans, suggesting that the former possess relatively weaker bones[30].

Given that we observe an amelioration of growth inhibition in intermittently methionine-restricted male mice, we considered the possibility that a transient reversal of FGF-21 and IGF-1 hormone levels during the methionine-replete phase of the IMR regimen might allow for relatively unimpaired bone development as compared with continuous MR. To test this hypothesis, we compared the long bones of mice that were intermittently methionine-restricted with those of mice that were either continuously methionine-restricted or maintained continuously on a methionine-replete control diet. Here, we show that, as compared with continuous MR, IMR results in both a preservation of bone mass and a dramatic reduction in the accumulation of marrow fat. In addition, mechanical testing revealed that the bones of intermittently methionine-restricted mice are significantly stronger than those of their continuously methionine-restricted counterparts. Our results also suggest that IMR likely results in more bone mass than that produced by continuous MR by increasing the number of osteoblasts. Taken together, our findings indicate that the more practicable IMR intervention is more beneficial to bone health than continuous MR.

Experimental Procedures

Animal care and feeding

All animal studies were approved by the Institutional Animal Care and Use Committee of the Orentreich Foundation for the Advancement of Science, Inc. (Permit Number: 0511MB). C57BL/6J mice (Stock number 000664) were purchased from The Jackson Laboratory (Bar Harbor, ME) and housed in a conventional animal facility maintained at 20 ± 2 °C, $50\% \pm 10\%$ relative humidity, with a 12/12-hour light/dark photoperiod. Mice were singly housed, and food and water were provided *ad libitum*. Upon arrival, animals were acclimatized for one week and fed Purina Lab Chow 5001 (Ralston Purina, Co.; St. Louis, MO). Mice were then given one of three isocaloric (5.3 kcal/gm) high-fat diets, comprising 12% kcal protein, 31% kcal carbohydrate, and 57% kcal fat (Research Diets; New Brunswick, NJ). These diets were essentially formulated as follows: (a) 0.86% methionine (control), (b) 0.12% methionine (methionine-restricted), and (c) 0% methionine (methionine-free), which were used to subject mice to CF, continuously methionine-restricted, and intermittently methionine-restricted dietary conditions. Additional details concerning diet compositions and feeding regimens are presented in **Supplementary Table 1** and **Figure S3**, respectively. To gain insight into the health of the mice during the experiment and confirm the specific pathogen-free status of the animal facility, groups of sentinel mice were exposed to spent bedding from study animals on a weekly basis. Skin swabs, as well as fecal and serological testing of sentinel mice thus exposed, were performed twice a year for the presence of specific viral and bacterial pathogens. No evidence of any pathogens was obtained.

Animal monitoring and tissue collection

Mice were randomly assigned to each diet group such that they had a similar average body mass (i.e., weight-matched). Body mass and food consumption were monitored at least once a week for the duration of the study. Prior to blood collection, animals were fasted for four hours to establish physiological baselines. Blood was then obtained from the retro-orbital plexus and collected using EDTA-K2-coated tubes (Milian Dutscher Group; Geneva, Switzerland), and the resulting plasma was stored at −80 °C until used for analysis. A portion of each blood sample was used for blood glucose determination using an Abbott Freestyle Lite glucometer and glucose strips (Abbott Diabetes Care, Inc.; Alameda, CA). At the end of the study, animals were fasted and bled, as described above, and sacrificed. Inguinal and perigonadal fat pads, as well as femurs, tibiae, and humeri, were harvested by

surgical resection and were either weighed or prepared for analyses, as described below.

Tissue preparation

After dissection, the left tibiae and right femurs of mice were fixed overnight in 70% ethanol and used for micro-computed tomography (micro-CT) analyses. Following micro-CT, left tibiae were prepared for histologic analyses using non-decalcified thin sections (5 μm) stained with toluidine blue. Right tibiae were fixed in 10% neutral-buffered formalin for 24 hours, rinsed in water, decalcified in 4% EDTA, and used for bone marrow fat measurements. Left femurs were wrapped in phosphate-buffered saline (PBS)-soaked gauze and stored at 4 °C for mechanical testing. Right humeri were stored at −80 °C for RNA isolation and qRT-PCR.

Blood hormone tests

Enzyme-linked immunosorbent assay kits were obtained commercially and used to measure the plasma levels of IGF-1 and FGF-21 (R&D Systems; Minneapolis, MN). All tests were performed according to the manufacturers' recommendations and quantified using a Molecular Devices SpectraMax M5 Microplate Reader (Molecular Devices LLC; San Jose, CA). Two technical replicates were performed for each sample.

Micro-CT

Femurs and tibiae were analyzed by micro-CT using a Scanco microCT-35 (Scanco Medical; Bruttisellen, Switzerland) at a maximum voxel size of 10 μm, an integration time of 500 ms, and an energy of 55 kVp. Renderings of the micro-CT data were generated with AltaViewer software, version 1.1.2 (Numira Biosciences; Salt Lake City, UT). Trabecular bone measurements include bone volume (BV, mm^3), total volume (TV, mm^3), BV/TV, connective density (Conn-dens, $1/mm^3$), trabecular number (Tb.N, 1/mm), trabecular spacing (Tb.Sp, mm), and trabecular thickness (Tb.Th, mm). Cortical bone measurements include cortical thickness (Ct.Th, mm), endosteal radius (mm), periosteal radius (mm), endosteal circumference (mm), periosteal circumference (mm), and polar moment of inertia (pMOI, mm^4).

Histomorphometry

Static histomorphometry was performed on left tibiae, as previously described[27]. Briefly, microscopic analysis of static parameters was performed using an Olympus microscope and the Osteomeasure system (Osteometrics; Atlanta, GA). Calculations of various parameters were based on the methods of Parfitt et al.[31]. All parameters were measured on trabecular bone beginning just under the growth plate in the primary spongiosa, excluding endosteal surfaces. The field size was 350 μm, and the area measured under the growth plate extended to 700 μm, where most of the trabecular bone is located.

Measurement of marrow fat

Formalin-fixed, decalcified tibiae were stained for neutral lipids using a 1:1 mixture of 2% aqueous osmium tetroxide and 5% potassium dichromate for 48 hours, as previously described[27]. Bones were then washed in tap water and imaged by micro-CT in water with an energy of 55 kVp, an integration time of 500 ms, and a maximum voxel size of 10 μm (i.e., the "high" resolution setting on a Scanco microCT-35 instrument). The selection of volumes of interest (VOIs) was performed so as to facilitate the visualization and quantitation of marrow fat. The results are presented as volumetric measurements, similar to the volumetric bone measurement, bone volume/total volume (BV/TV).

Mechanical testing

Femurs were loaded to failure using four-point bending. Tests were conducted by loading the femur in the anterior–posterior orientation, such that the posterior quadrant was subjected to tensile loads. The widths of the upper and lower supports of the four-point bending apparatus were 3 and 7 mm, respectively. Tests were conducted with a deflection rate of 0.05 mm/sec using a servohydraulic Instron model 8874 testing machine (Instron Corp.; Norwood, MA). The load and mid-span deflection measurements were acquired at a sampling frequency of 200 Hz. Load deflection curves were analyzed for stiffness, maximum load, and total work to fracture. Yield was defined as a 10% reduction in the secant stiffness relative to the initial tangent stiffness and used to determine the post-yield deflection, which was defined as the deflection at failure minus the deflection at yield. Tests were performed at room temperature, and bones were kept moist with PBS.

Bone marrow RNA isolation and qRT-PCR

To assess the relative expression of genes encoding factors involved in the regulation of osteoblast and osteoclast differentiation and activity, total RNA was harvested from humeral bone marrow using a modified version of a previously described protocol[27]. Briefly, soft tissue was removed from humeri and RNA was extracted using RNAzol RT (Molecular Research Center, Inc.; Cincinnati, OH) and a TissueLyser II bead homogenizer (Qiagen; Germantown, MD), according to the manufacturers' recommendations. One-step qRT-PCR was performed utilizing a QuantiNova SYBR Green RT-PCR kit (Qiagen) and a StepOne Plus Real-Time PCR System (Life Technologies; Carlsbad, CA), with an initial incubation of 50 °C for 10 min for cDNA synthesis, a 2-min incubation at 95 °C to activate the DNA polymerase, followed by 40 cycles of 95 °C for 5 sec and 60 °C for 10 sec. The primer sets used were: *Opg* 5'-GCCACGCAAAAGTGTGGAAT, 3'-TTTGGTCCCAGGCAAACTGT; *Rank* 5'-ACGTCAGGCCAAAGG ACAAA, 3'-GGGCCTACTGCCTAAGTGTG; *Runx2* 5'-CGCCCC TCCCTGAACTCT, 3'-TGCCTGCCTGGGATCTGT; and *Dmp1* 5'-CGCTGAGGTTTTGACCTTGTG, 3'-TGGTGACCCAGCCAAATCAC. Relative gene expression levels were determined using the comparative CT ($2^{-\Delta\Delta CT}$) method[32], with hypoxanthine guanine phosphoribosyl transferase (*Hprt* 5'-CTGGTGAAAAGGACCTCTCGAAG, 3'-CCAGTTTCACTAATGACACAAACG) as the reference gene.

Statistical analyses

Data were analyzed using the software package Prism 8 (GraphPad Software; La Jolla, CA). For longitudinal analyses of circulating analyte measurements, we performed an ordinary two-way ANOVA. For terminal analyses of all other measurements, we performed ordinary one-way ANOVA. *Post hoc* testing of ANOVA results was performed using Fisher's least significant difference tests and a family-wise significance level of 0.05 (95% confidence interval). In a separate analysis, multiple comparisons testing of ANOVA results was performed using the Tukey method for statistical hypothesis testing and a family-wise significance level of 0.05 (95% confidence interval). The results of the latter analyses are presented in **Supplementary File 1**. Where appropriate, statistically significant differences are indicated, as follows: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$.

Results

Both intermittent and continuous MR confer metabolic health benefits to mice

To test the effect, if any, of IMR on the bones of mice, we fed nine-week-old wild-type male C57BL/6J mice the following diets for a period of eight weeks: (a) continuous control diet (CF), (b) continuous methionine-restricted diet (MR), and (c) intermittent methionine-restricted diet (IMR). Nine weeks of age was selected because essentially all previous studies that reported deleterious effects of continuous MR on bone formation were performed on young animals[7,25–27], although one of these studies also reported similar impairment for older adult male mice[26]. As in our previous study characterizing the effects of IMR[14], the animal diets were formulated in a high-fat context (57% kcal from fat) in order to approximate the human Western diet. All diets were isocaloric and provided *ad libitum*. The IMR regimen was characterized by four days of the methionine-replete control diet, followed by three days of a diet devoid of methionine. The time-integrated concentrations of methionine in the diets were 0.86% (CF), 0.12% (MR), and 0.49% (IMR), respectively. As is standard for such experiments, none of the diets contained cysteine, thereby making methionine the sole dietary sulfur-containing amino acid. To confirm that the continuous and intermittent MR regimens were similarly effective to what we observed previously[14], we performed longitudinal measurements of body mass (**Fig. 1A**) and food consumption (**Fig. 1B**), as well as determinations of inguinal and perigonadal adiposity at the end of the feeding period (**Fig. 1C,D**). We also assessed the ability of continuous and intermittent MR to rescue diet-induced dysglycemia (**Fig. 1E**), as well as the effects of these interventions on the levels of the nutrient-sensing hormones FGF-21 and IGF-1 (**Fig. 1F,G**). As expected, we observed that continuous and intermittent MR robustly protected animals against both diet-induced weight gain (**Fig. 1A**) and the accumulation of inguinal and perigonadal fat (**Fig. 1C,D**). The latter is true even when the lesser overall weight of these animals is taken into consideration (**Fig. S1**). We also observed that both continuous and intermittent MR protected mice against dysglycemia, maintaining low fasting glucose levels (**Fig. 1E**). Interestingly, as we observed previously for female mice subjected to these interventions for 18 weeks, IMR was more effective than the classical intervention at maintaining low fasting glucose levels (61 vs. 83 mg/dl). That said, both interventions similarly elevated the plasma levels of the methionine-responsive hepatokine FGF-21 and maintained low circulating levels of IGF-1 (**Fig. 1F,G**). To confirm that these effects were due solely to MR rather than a combined effect of MR and any putative contribution from calorie restriction (should animals find the diets to be unpalatable), we compared the rate of food consumption for all three dietary regimens (**Fig. 1B, Fig. S2**). Consumption of the IMR regimen was at least equivalent to that of the control diet; consistent with previous studies, mice subjected to continuous MR consumed their food at an even greater rate. Indeed, these observations held true whether food consumption was considered in absolute terms or normalized to body mass (**Fig. 1B, Fig. S2**). It is thus clear that intermittently methionine-restricted mice were not calorie-restricted. Together, the above experiments confirmed that, as expected, both continuous and intermittent MR were effective at conferring metabolic health benefits to the current cohort of mice.

IMR preserves more trabecular bone than continuous MR

To assess the amount and quality of bone present in the long bones of mice fed the various diets, animals were sacrificed at

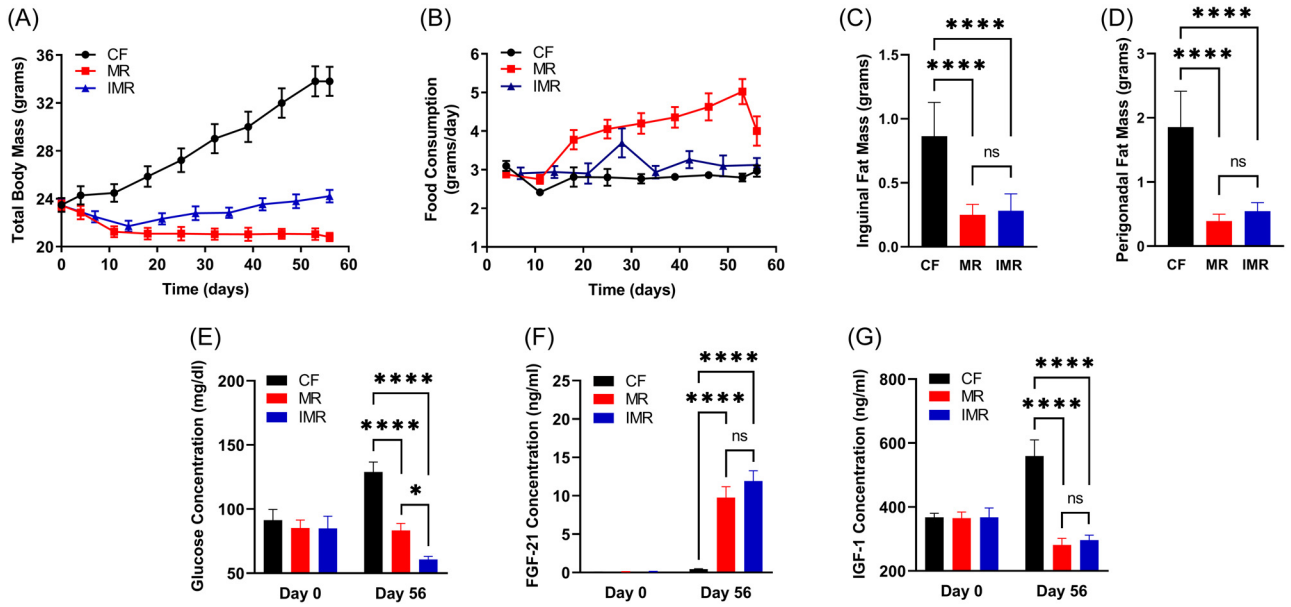

Figure 1. Both intermittent methionine restriction (IMR) and continuous MR confer metabolic health benefits to mice. Comparisons over time of average values are shown for **(A)** total body mass and **(B)** food consumption for control-fed (CF; black circles), continuously methionine-restricted (MR; red squares), and intermittently methionine-restricted (IMR; blue triangles) mice. Also shown are average values at the conclusion of the experiment (eight weeks) for the mass of **(C)** inguinal and **(D)** perigonadal fat pads. In addition, average values for the circulating levels of **(E)** fasting glucose, **(F)** plasma FGF-21, and **(G)** plasma IGF-1 are also depicted. For panels (E)–(G), IMR values were obtained following a period of methionine-restricted feeding. For all panels, error bars denote (SEM). For panels (C)–(G), statistically significant differences are either indicated (*, $p < 0.05$; ****, $p < 0.0001$) or absent (ns). N = 8 for all groups.

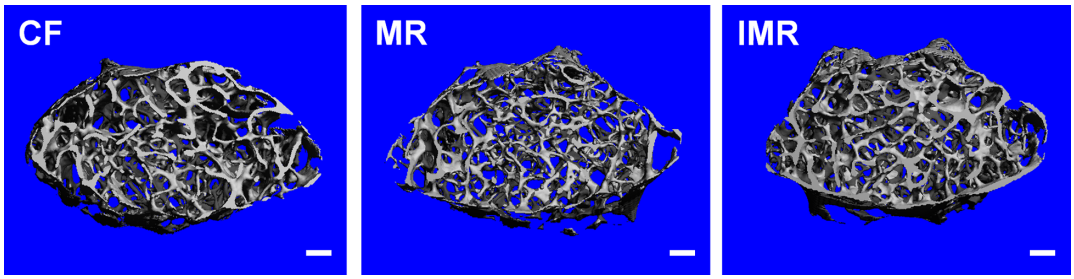

Figure 2. Imaging of trabecular bone by micro-computed tomography (micro-CT). Representative images are shown depicting trabecular bone in femurs harvested from control-fed, continuously methionine-restricted (MR), and intermittently methionine-restricted (IMR) mice at the conclusion of the experiment (eight weeks). Bars denote 200 μm.

the end of the feeding period (eight weeks), and their tibiae and femurs were surgically resected and processed for subsequent analyses. In order to measure the microarchitecture of trabecular bone, tibiae and femurs were analyzed by micro-CT. From the resulting images (examples of which are shown in Fig. 2), it is apparent that continuous MR produces a loss of trabecular bone mass as compared with the control diet. In contrast, trabecular bone mass appears to be relatively unaffected by IMR. Quantitation of such images revealed that, in tibiae, trabecular bone volume, connective density, trabecular number, and trabecular thickness were all reduced by continuous MR (25%, 15%, 13%, and 5%, respectively; Fig. 3A–D). Consistent with these effects, we found that this intervention increased trabecular spacing (20%; Fig. 3E). Interestingly, and as hypothesized, we observed that IMR resulted in a marked amelioration of all of the aforementioned deleterious effects on tibial trabecular bone. For trabecular bone volume and connective density, values for animals undergoing IMR did not differ from those of controls (Fig. 3A,B). For trabecular number, thickness, and spacing, all values were significantly improved as compared with those of mice undergoing continuous MR (Fig. 3C–E). Surprisingly, IMR resulted in an average trabecular thickness that was improved even as compared with that of control-fed animals (6%; Fig. 3D).

Regarding the maintenance of trabecular bone in femurs, continuous MR also had negative effects on bone volume and connective density (35% and 25%; Fig. 3F,G), as well as trabecular number and thickness (14% and 10%; Fig. 3H,I). As expected, these effects were associated with an increase in trabecular spacing (20%; Fig. 3J). Similar to the results for tibiae, IMR resulted in a dramatic amelioration of trabecular bone loss in femurs, maintaining connective density and trabecular thickness at levels equivalent to those of controls (Fig. 3G,I). For all other measurements, values were improved as compared with those of continuously methionine-restricted mice (Fig. 3F,H,J). Taken together, these results clearly demonstrate that, in contrast to their continuously methionine-restricted counterparts, mice undergoing IMR experience very little trabecular bone loss, if any.

IMR preserves more cortical bone than continuous MR

Given that cortical bone, like trabecular bone, is known to be lost in animals undergoing continuous MR[25–27], we made use of micro-CT to image cortical bone in the long bones of mice. Representative images are shown (Fig. 4) that demonstrate the qualitative effects of the diets on cortical (as well as trabecular) bone. These images reveal that continuous MR negatively impacts cortical bone mass as compared with the control diet. They also confirm the loss of trabecular bone that is similarly caused by continuous MR, as described above (Figs. 2, 3). In contrast, these images show a clear preservation of both cortical and trabecular bone as a result of IMR (Fig. 4).

Quantitation of such images revealed that, as compared with control values, cortical thickness, periosteal radius, periosteal circumference, and polar moment of inertia were all reduced in the tibiae of animals undergoing continuous MR (9%, 7%, 7%, and 22%, respectively; Fig. 5A,C,E,F). Indeed, these findings are consistent with those of previous studies[25–27]. No differences were observed for endosteal measurements (Fig. 5B,D). Interestingly, IMR resulted in a preservation of cortical bone in tibiae, producing an average cortical thickness not only greater than that of continuously methionine-restricted mice (11%; Fig. 5A), but essentially the same as that of controls. For all other measurements, there were no significant differences between continuous and IMR (Fig. 5B–F).

With respect to cortical bone measurements, results in femurs were broadly similar to those in tibiae. Compared with control feeding, continuous MR reduced both cortical thickness and polar moment of inertia (18% and 18%, respectively; Fig. 5G,L). It is interesting to note that there was a lesser effect of diet on endosteal and periosteal measurements in femurs than in tibiae, with all three dietary regimens resulting in essentially the same values (Fig. 5H–K). Nevertheless, IMR resulted in a greater average cortical thickness as compared with that of continuously methionine-restricted animals (11%; Fig. 5G). Overall, these results demonstrate that IMR preserves more cortical bone than continuous intervention.

IMR results in both improved osteoid formation and bone resorption

To further examine the physiological changes occurring in the bones of mice fed the various dietary regimens, we performed static histomorphometric analyses of tibiae. These analyses were critical in that they allowed us to explore the effects of continuous and intermittent MR not only on the formation of bone-precursor osteoid but also on the cell populations involved in the production and turnover of bone. Consistent with the micro-CT results described above, we observed that bone volume (BV/TV (%)) was negatively affected by continuous MR (53%), but preserved by IMR (Fig. 6A). Identical effects were observed for osteoid volume (OV/TV (%)), trabecular thickness, and osteoid thickness, with these attributes being reduced dramatically in the bones of continuously methionine-restricted mice (57%, 28%, and 32%, respectively), but maintained at control levels by IMR (Fig. 6B–D). Consistent with its negative effects on bone mass, continuous MR resulted in bones with not only fewer osteoblasts (which produce bone) than controls (44%; Fig. 6E), but also more

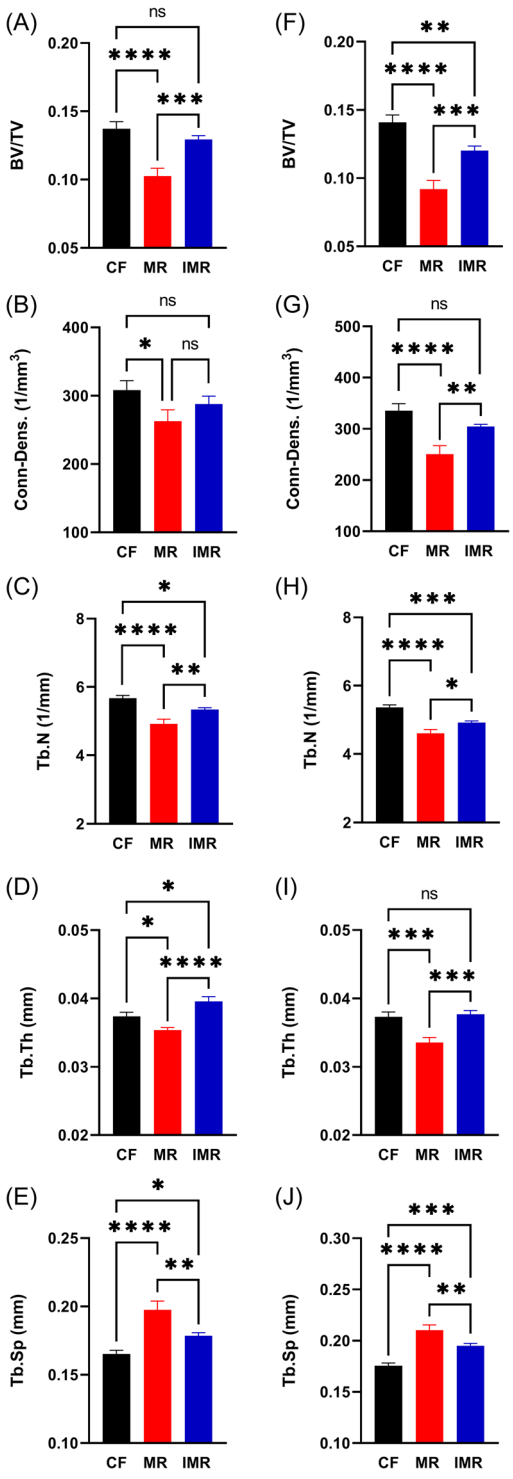

Figure 3. IMR preserves trabecular bone. Average values at the conclusion of the experiment (eight weeks) are shown for **(A,F)** trabecular bone volume/total volume (BV/TV), **(B,G)** connective density, **(C,H)** trabecular number (Tb. N), **(D,I)** trabecular thickness (Tb. Th), and **(E,J)** trabecular spacing (Tb. Sp). Values were determined by micro-CT analyses of tibiae (panels (A)–(E)) and femurs (panels (F)–(J)), respectively. Bars denote SEM. Statistically significant differences are either indicated (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$) or absent (ns). N = 8 for all groups.

osteoclasts (which degrade bone) (60%; Fig. 6F). However, the effect of IMR on these cell populations is strikingly different. We observed that IMR increased the number of osteoblasts in tibiae, with average values being far greater than those of continuously methionine-restricted animals (288%; Fig. 6E). Interestingly, this value also reflected a 61% increase in osteoblast number as compared with that of control-fed animals (Fig. 6E). We were also surprised to observe an increase in the number of osteoclasts in the bones of intermittently methionine-restricted mice (87%; Fig. 6F). Although IMR increases osteoclastogenesis, bone mass is maintained in animals undergoing this intervention. Our results indicate that this is achieved through a concomitant increase in the number of osteoblasts.

IMR alters the expression of factors that regulate bone structure

Regarding the molecular mechanisms by which IMR confers a relative preservation of bone mass, we considered the possibility that recurrent periods of methionine repletion (four days per week) might produce a reversal of MR-dependent changes in the expression of factors involved in osteoblast and osteoclast differentiation and activity, and consequently, bone development. For example, we previously found that continuous MR decreases expression of *Runx2*, *Dmp1*, *Opg*, and *Rank* in bone marrow harvested from continuously methionine-restricted mice[27], likely explaining the observed changes in osteoblast and osteoclast numbers. To gain insight into the mechanisms underlying the relative benefits of IMR, we performed similar experiments assessing the levels of these transcripts in humeral bone marrow harvested from mice subjected to control-feeding, continuous MR, and IMR. Consistent with our previous results, we found that continuous MR reduced the levels of *Runx2* as compared with control-feeding (52%; Fig. 7). Although the relative expression levels for *Opg*, *Rank*, and *Dmp1* in continuously methionine-restricted animals were also lower than those of control-fed animals (25%, 7%, and 57%, respectively), these differences were not statistically significant. In contrast, the relative levels of all four transcripts were greater for mice undergoing IMR. In the case of *Opg* and *Rank*, transcript abundance was greater (87% and 84%, respectively) than for mice undergoing continuous MR. For *Runx2* and *Dmp1*, the relative increases in expression were 16% and 136%, respectively, although these differences were not statistically significant. It is important to note that the bone marrow samples used for these analyses were harvested after a three-day period of methionine-deficient feeding. Thus, it is possible that changes that trend upward in intermittently methionine-restricted animals (i.e., *Runx2* and *Dmp1*) might be found to be significantly higher after a period of methionine-replete feeding. In any case, it is clear that the overall expression pattern of factors influencing osteoblast and osteoclast differentiation and function differs in intermittently methionine-restricted animals as compared with continuously methionine-restricted counterparts.

IMR prevents bone marrow adipogenesis

Bone marrow adipogenesis increases with age[33,34] and is associated with reduced osteoblast activity and weaker bones[35]. Previous studies have demonstrated that continuous MR fails to prevent the accumulation of marrow fat and, in some cases, even exacerbates it[27]. To test the effects of continuous MR on bone marrow adipogenesis, tibiae were decalcified, stained with osmium to

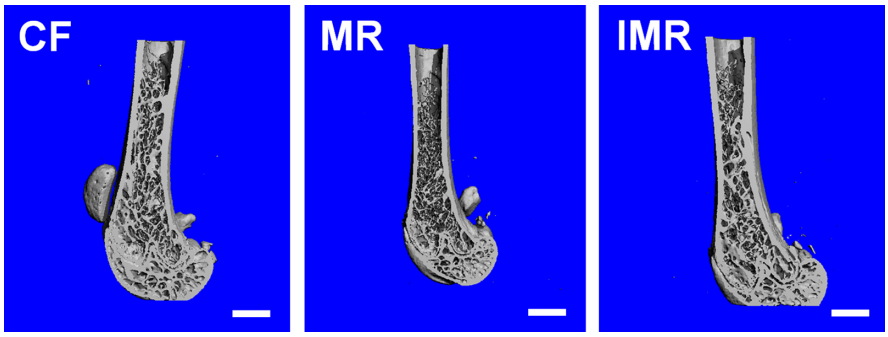

Figure 4. Imaging of cortical and trabecular bone by micro-CT. Representative images are shown depicting both cortical and trabecular bone in femurs harvested from control-fed, continuously methionine-restricted (MR), and intermittently methionine-restricted (IMR) mice at the conclusion of the experiment (eight weeks). Bars denote 1 mm.

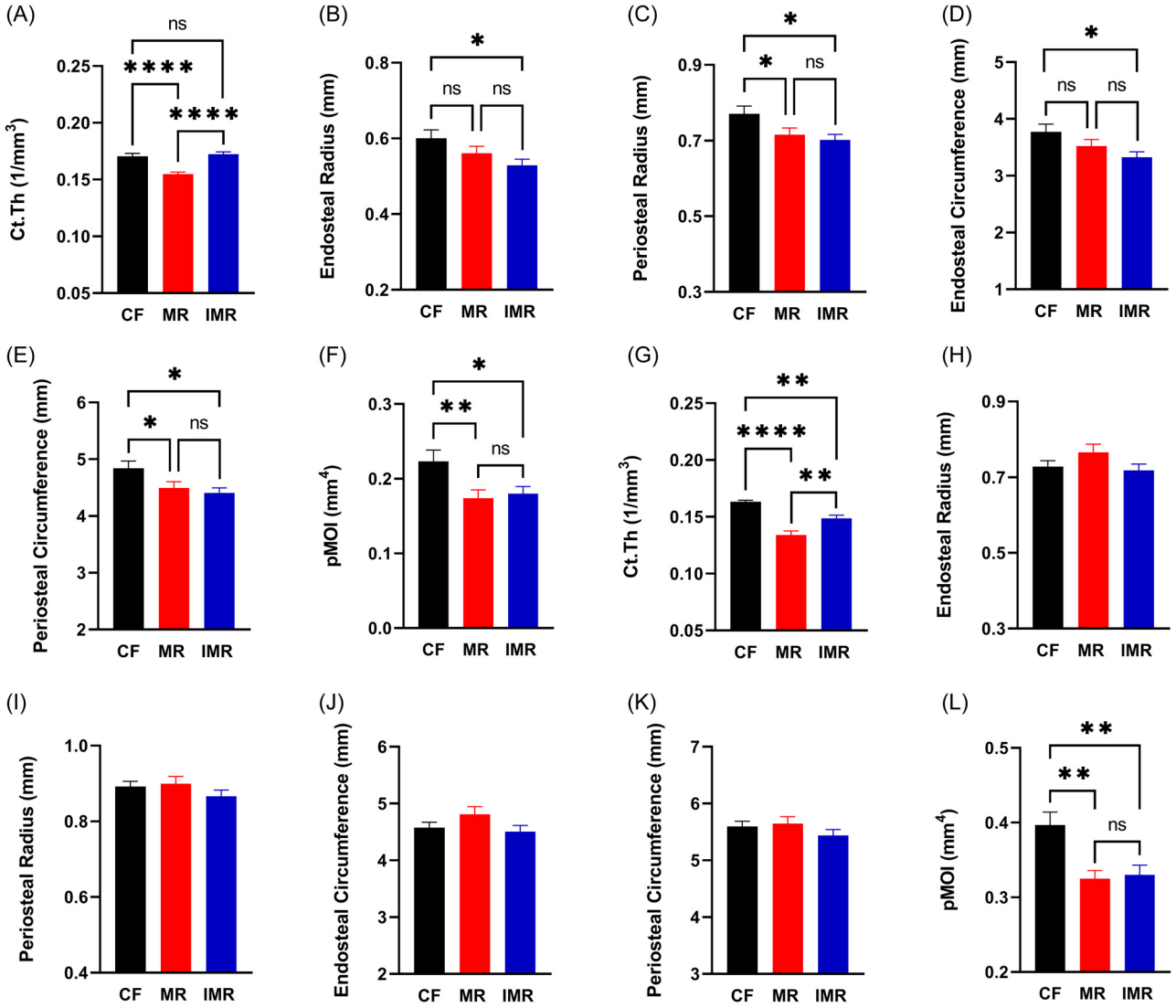

Figure 5. IMR preserves cortical bone. Average values at the conclusion of the experiment (eight weeks) are shown for **(A,G)** cortical thickness (Ct. Th), **(B,H)** endosteal radius, **(C,I)** periosteal radius, **(D,J)** endosteal circumference, **(E,K)** periosteal circumference, and **(F,L)** polar moment of inertia (pMOI). Values were determined by micro-CT analyses of tibiae (panels (A)–(F)) and femurs (panels (G)–(L)), respectively. Bars denote SEM. Statistically significant differences are either indicated (*, $p < 0.05$; **, $p < 0.01$; ****, $p < 0.0001$) or absent (ns). N = 8 for all groups.

detect marrow fat, and imaged using micro-CT. The relative amounts of marrow fat were measured in four regions, termed "volumes of interest" (VOIs). These regions correspond to the epiphyseal (VOI1), metaphyseal (VOI2), metaphysis (VOI3), and diaphysis (VOI4) of the long bone and are indicated diagrammatically (Fig. 8A). With the exception of the VOI4 region, continuous

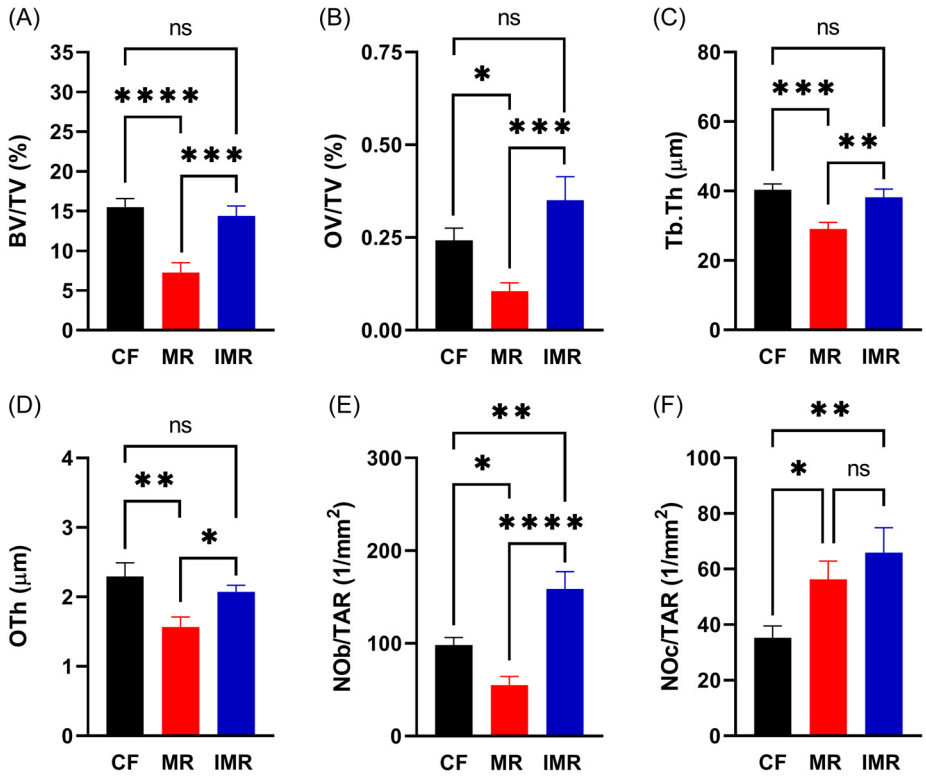

Figure 6. IMR results in both improved osteoid formation and bone resorption. Average values at the conclusion of the experiment (eight weeks) are shown for **(A)** trabecular bone volume/total volume (BV/TV; %), **(B)** osteoid volume/total volume (OV/TV; %), **(C)** trabecular thickness (Tb. Th), **(D)** osteoid thickness (OTh), **(E)** number of osteoblasts/total area (NOb/TAR), and **(F)** number of osteoclasts/total area (NOc/TAR). Measurements were obtained by static histomorphometric analyses of tibiae. Bars denote SEM. Statistically significant differences are either indicated (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$) or absent (ns). N = 8 for all groups.

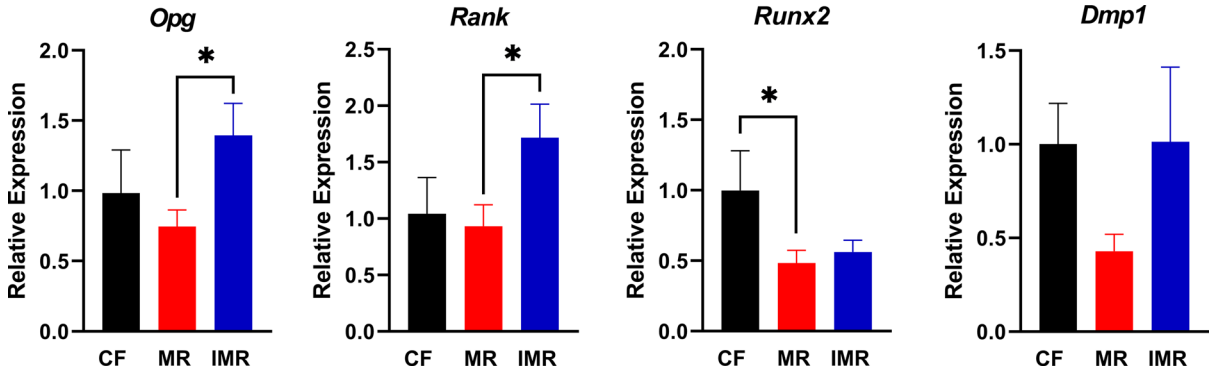

Figure 7. Relative expression of factors involved in the differentiation and activity of osteoblasts and osteoclasts. Average relative expression is shown for genes encoding factors that regulate the differentiation and activity of osteoclasts (*Opg* and *Rank*) and osteoblasts (*Runx2* and *Dmp1*). Values represent the relative abundance of these transcripts in humeral bone marrow at the conclusion of the experiment (eight weeks) for animals fed the indicated diets. Bars denote SEM. Statistically significant differences are indicated (*, $p < 0.05$). N = 8 for all groups.

MR fails to reduce the high levels of marrow fat present in tibiae (Fig. 8B–D). In stark contrast, IMR results in a dramatic inhibition of marrow adipogenesis in tibiae, with all four VOI regions possessing massively reduced levels of marrow fat as compared with controls (64%, 70%, 67%, and 88%, respectively; Fig. 8B–E). Thus, these results clearly demonstrate that, of the two interventions, only IMR is effective at preventing bone marrow adipogenesis.

IMR results in stronger bones than continuous MR

Given that IMR both preserves more bone mass (trabecular and cortical) and reduces the accumulation of marrow fat as compared with continuous MR, it would be expected that the bones of intermittently methionine-restricted animals would be stronger than those of their continuously methionine-restricted counterparts. To test this prediction, four-point bending tests were performed on the femurs of mice fed the control diet as well as animals fed the continuous and intermittent regimens. The stiffness, max load, and total work performed were all found to be significantly reduced by continuous MR (33%, 36%, and 37%, respectively; Fig. 9A–C). Although the average post-yield deflection of bones was also determined, this value was not altered by either MR regimen (Fig. 9D). As predicted, IMR improved both the max load and

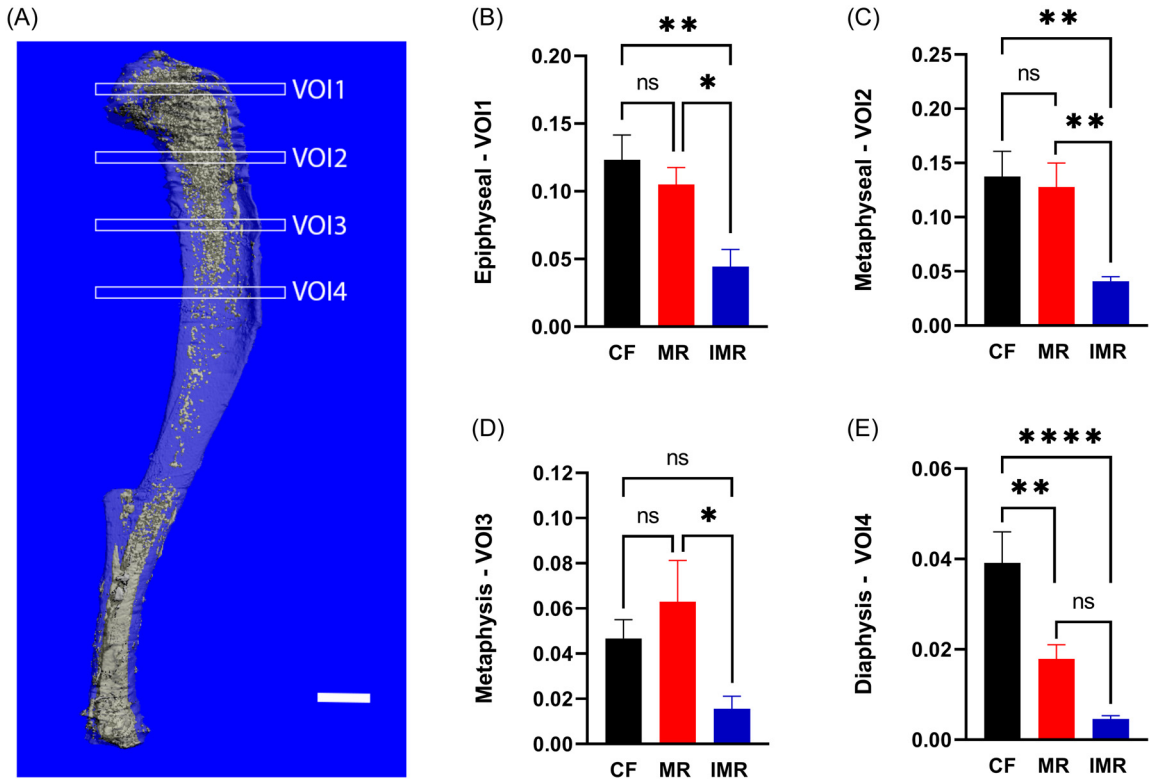

Figure 8. IMR prevents bone marrow adipogenesis. (A) Positioning of the volumes of interest (VOIs) in a representative micro-CT image of an osmium-stained tibia from a control-fed mouse at the conclusion of the experiment (eight weeks; light blue, bone; gray scale, marrow fat). Average values are shown for the proportions of marrow fat present in the tibiae of mice fed the indicated diets. Values are for fat located in the **(B)** epiphyseal (VOI1), **(C)** metaphyseal (VOI2), **(D)** metaphysis (VOI3), and **(E)** diaphysis (VOI4). For panel (A), bar denotes 1 mm. For panels (B)–(D), bars denote SEM. Statistically significant differences are either indicated (*, $p < 0.05$; **, $p < 0.01$; ****, $p < 0.0001$) or absent (ns). $N = 8$ for all groups.

total work performed relative to continuous MR (31% and 54%, respectively; Fig. 9B,C). Although IMR did not improve the stiffness of femurs, the total work performed was actually equivalent to that of bones from control-fed animals (Fig. 9A,C). Taken together, these findings confirm that the long bones of intermittently methionine-restricted mice are stronger than those of their continuously methionine-restricted counterparts.

Discussion

Although the benefits of continuous MR to mammalian healthspan are well documented[1–3], we previously developed a more practicable version of this intervention to help translate the health benefits of MR to humans[14]. Not only does IMR have a much shorter interventional period than continuous MR (three vs. seven days per week), but it is arguably superior in other ways as well. For example, while we have often observed that continuous MR fails to rescue the high fasting glucose levels that characterize female mice maintained on a high-fat diet[14,36], IMR rescues all aspects of diet-induced dysglycemia in both males and females[14]. In addition, while continuous MR is known to inhibit overall growth and the development of lean body mass, we have observed a trend toward the preservation of musculoskeletal mass in intermittently methionine-restricted male mice. As a result, we considered the possibility that IMR might not have the same deleterious effects on bone formation as continuous MR. As the latter intervention has increasingly become the subject of clinical trials aimed at translating the metabolic health benefits of MR to humans, the reduced bone density and strength observed for animals (and people) subjected to long-term continuous MR are clear causes for concern. However, if mice undergoing IMR were found to preserve more bone mass and retain stronger bones than their continuously methionine-restricted counterparts, then such findings would represent yet additional evidence that IMR is likely to produce better health outcomes for human subjects than the classical intervention.

In this study, we report that, as compared with continuous MR, IMR preserves both trabecular and cortical bone in tibiae and femurs. In addition, unlike continuous MR, the intermittent intervention is very effective at preventing the accumulation of marrow fat. As would be expected, given the observed preservation of bone mass and inhibition of marrow adipogenesis, direct mechanical testing of femurs demonstrated that IMR results in measurably stronger bones than continuous MR. Regarding the mechanism by which such benefits are engendered, static histomorphometric analyses revealed an elevated number of osteoblasts in the femurs of intermittently methionine-restricted mice, as well as a concomitant increase in both the osteoid volume and thickness. This is in contrast to the results for bones from continuously methionine-restricted animals, which show a reduction in osteoblast number as compared with controls. The latter finding is consistent with the results of our previous study, wherein deleterious changes in the bone microarchitecture of continuously methionine-restricted mice were found to involve altered miRNA and *Runx2* expression, leading to changes in the activity of osteoblast precursors[27]. Indeed, an independent study reported that continuous MR alters

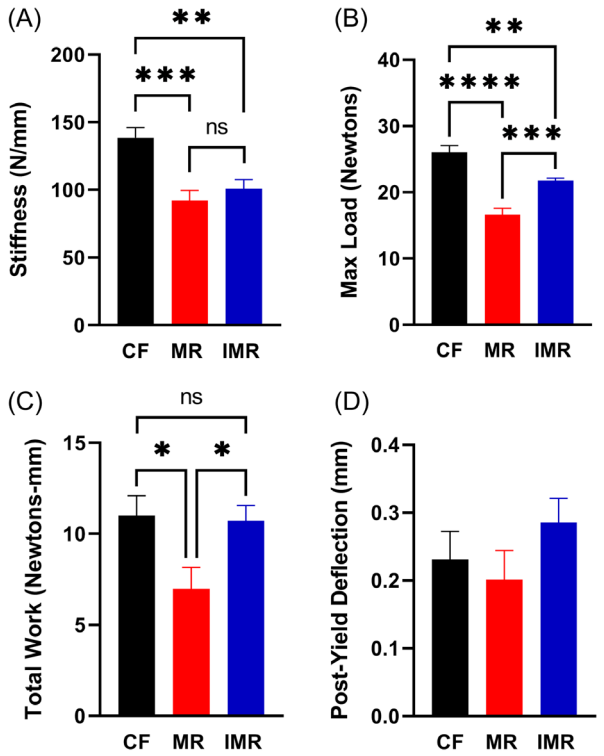

Figure 9. IMR preserves bone strength. Average values at the conclusion of the experiment (eight weeks) are shown for **(A)** bone stiffness, **(B)** maximum load, **(C)** total work required to fracture bone, and **(D)** post-yield deflection. Values were determined by four-point bending tests of femurs from mice fed the indicated diets. Bars denote SEM. Statistically significant differences are either indicated (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$) or absent (ns). N = 8 for all groups.

the expression of *Runx2* in a number of settings[26]. Combined with an observed increase in the average number of osteoclasts, the decrease in osteoblast number that we observe suggests that the bone loss resulting from continuous MR is due to both decreased bone formation and increased bone resorption. However, unlike the continuous intervention, IMR elevates both osteoblast and osteoclast cell numbers. While the latter observation is somewhat surprising, it may be that any increase in the rate of bone resorption in these animals is offset by an even greater rate of bone formation. Should this be the case, intermittently methionine-restricted mice would be expected to maintain significant amounts of high-quality bone, with bones also becoming stronger and more robust over time. Suggestive of such a possibility, we find that the increase in osteoblast number (60.37/mm^2; Fig. 4E) is twice as great as the increase in osteoclast number (30.66/mm^2; Fig. 6F). However, given that the regulation of osteoblast/osteoclast activity is more complex than mere cell numbers, future experiments will be necessary to definitively determine whether the bones of intermittently methionine-restricted mice do, in fact, become stronger the longer that animals are on the diet. Relatedly, a weakness of this study is that the effects of the various dietary interventions on bone formation were tested only in young, still-developing mice. In future studies, it will be informative to test whether IMR is similarly effective at sparing adult animals the bone loss that is engendered by continuous MR.

As discussed above, bone marrow expression of *Runx2* is reduced by continuous MR (Fig. 7)[27]. Given that RUNX2 controls the differentiation and maturation of osteogenic precursors, this decrease likely contributes to the reduced osteoblast number observed in the bones of continuously methionine-restricted mice (Fig. 6E). Similarly, our 2017 study also reported that continuous MR decreases the expression of *Dmp1*, a gene involved in the regulation of both osteoblast differentiation and bone mineralization. We have also found that continuous MR reduces the expression of *Opg*, which encodes a decoy protein that inhibits osteoclast differentiation through the RANK pathway. Collectively, these effects likely explain the observed changes in the number of both osteoblasts (44% less than controls) and osteoclasts (60% more than controls) caused by continuous MR (Fig. 6E,F). In contrast, the bones of animals undergoing IMR show a different pattern of expression for such genes. In the case of *Runx2* and *Dmp1*, expression of these genes trend higher for intermittently methionine-restricted mice than their continuously methionine-restricted counterparts, although we did not find these differences to be statistically significant. However, we observed much more robust effects for *Opg* and *Rank*, with IMR dramatically increasing the levels of these transcripts as compared with continuous MR (87% and 84%, respectively; Fig. 7). Although OPG functions as a decoy for the RANK receptor, it may be that increased expression of RANK is more than sufficient to promote osteoclastogenesis through this pathway, thereby resulting in the increased number of osteoclasts observed for animals undergoing IMR. In any case, it is probable that reduced expression of *Runx2*, *Dmp1*, *Opg*, and *Rank* underlies the altered osteoblast and osteoclast numbers observed in animals undergoing continuous MR, which in turn results in bone loss. In contrast, the levels of these transcripts are either maintained or elevated for animals undergoing IMR, beneficially altering the number and activity of osteoblasts and osteoclasts, thereby preserving bone. Regardless of the specific mechanisms at play, it is clear from our results that IMR is a much kinder intervention for bones than continuous MR. Indeed, this finding is consistent with a previous study suggesting that continuous low-level methionine intake can be less than ideal with respect to multiple physiological outcomes[37]. When taken together with its more practicable nature, robust metabolic health benefits, and reduced undesirable side effects, the relative benefits of IMR to bone bolster the argument that this intervention is not only a superior alternative to classical MR but an ideal candidate strategy for improving human healthspan. It is possible that IMR might even be used to provide metabolic health benefits for older individuals who are at risk of developing osteoporosis and/or sarcopenia, whereas continuous MR would likely promote or exacerbate these conditions. We look forward to future clinical studies that will determine whether (and to what extent) IMR produces similar healthspan benefits for humans as it does for rodents.

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Author Contributions

J.E.J. and M.C.H. designed the study, collected samples, and analyzed the data. M.C.H. oversaw bone analyses. J.D.P. performed animal care and feeding, animal monitoring, sample collection, data analysis, and measurement of circulating analytes. J.E.J. wrote the article. J.D.P. and M.C.H. read, commented on, and approved the article.

Declaration of Interests

The authors declare no competing conflicts of interest.

Data Availability Statement

All data associated with this study are present in this article or as supplementary information.

Supplementary Materials

Supplemental information can be found online at https://doi.org/10.59368/agingbio.20230019.

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References

1. Miller R.A., Buehner G., Chang Y., Harper J.M., Sigler R., & Smith-Wheelock M. (2005). Methionine-deficient diet extends mouse lifespan, slows immune and lens aging, alters glucose, T4, IGF-I and insulin levels, and increases hepatocyte MIF levels and stress resistance. *Aging Cell* **4**(3), 119–125. PMID: 15924568; doi: 10.1111/j.1474-9726.2005.00152.x.
2. Orentreich N., Matias J.R., DeFelice A., & Zimmerman J.A. (1993). Low methionine ingestion by rats extends life span. *J. Nutr.* **123**, 269–274. PMID: 8429371; doi: 10.1093/jn/123.2.269.
3. Richie J.P. Jr., Leutzinger Y., Parthasarathy S., Malloy V., Orentreich N., & Zimmerman J.A. (1994). Methionine restriction increases blood glutathione and longevity in F344 rats. *FASEB J.* **8**, 1302–1307. PMID: 8001743; doi: 10.1096/fasebj.8.15.8001743.
4. Malloy V.L., Krajcik R.A., Bailey S.J., Hristopoulos G., Plummer J.D., & Orentreich N. (2006). Methionine restriction decreases visceral fat mass and preserves insulin action in aging male Fischer 344 rats independent of energy restriction. *Aging Cell* **5**(4), 305–314. PMID: 16800846; doi: 10.1111/j.1474-9726.2006.00220.x.
5. Malloy V.L., Perrone C.E., Mattocks D.A., Ables G.P., Caliendo N.S., Orentreich D.S., & Orentreich N. (2013). Methionine restriction prevents the progression of hepatic steatosis in leptin-deficient obese mice. *Metabolism* **62**(11), 1651–1661. PMID: 23928105; doi: 10.1016/j.metabol.2013.06.012.
6. Yang Y., Wang Y., Sun J., Zhang J., Guo H., Shi Y., … Le G. (2019). Dietary methionine restriction reduces hepatic steatosis and oxidative stress in high-fat-fed mice by promoting H2S production. *Food Funct.* **10**(1), 61–77. PMID: 30534793; doi: 10.1039/C8FO01629A.
7. Ables G.P., Perrone C.E., Orentreich D., & Orentreich N. (2012). Methionine-restricted C57BL/6J mice are resistant to diet-induced obesity and insulin resistance but have low bone density. *PLoS One* **7**(12), e51357. PMID: 23236485; doi: 10.1371/journal.pone.0051357.
8. Dong Z., Sinha R., & Richie J.P. Jr. (2018). Disease prevention and delayed aging by dietary sulfur amino acid restriction: Translational implications. *Ann. N. Y. Acad. Sci.* **1418**(1), 44–55. PMID: 29399808; doi: 10.1111/nyas.13584.
9. Gao X., Sanderson S.M., Dai Z., Reid M.A., Cooper D.E., Lu M., … Locasale J.W. (2019). Dietary methionine influences therapy in mouse cancer models and alters human metabolism. *Nature* **572**(7769), 397–401. PMID: 31367041; doi: 10.1038/s41586-019-1437-3.
10. Johnson J.E., & Johnson F.B. (2014). Methionine restriction activates the retrograde response and confers both stress tolerance and lifespan extension to yeast, mouse and human cells. *PLoS One* **9**, e97729. PMID: 24830393; doi: 10.1371/journal.pone.0097729.
11. Olsen T., Ovrebo B., Haj-Yasein N., Lee S., Svendsen K., Hjorth M., … Vinknes K.J. (2020). Effects of dietary methionine and cysteine restriction on plasma biomarkers, serum fibroblast growth factor 21, and adipose tissue gene expression in women with overweight or obesity: A double-blind randomized controlled pilot study. *J. Transl. Med.* **18**(1), 122. PMID: 32160926; doi: 10.1186/s12967-020-02288-x.
12. Richie J.P. Jr., Sinha R., Dong Z., Nichenametla S.N., Ables G.P., Ciccarella A., … Orentreich D. (2023). Dietary methionine and total sulfur amino acid restriction in healthy adults. *J. Nutr. Health Aging* **27**, 111–123. PMID: 36806866; doi: 10.1007/s12603-023-1883-3.
13. McCarty M.F., Barroso-Aranda J., & Contreras F. (2009). The low-methionine content of vegan diets may make methionine restriction feasible as a life extension strategy. *Med. Hypotheses* **72**(2), 125–128. PMID: 18789600; doi: 10.1016/j.mehy.2008.07.044.
14. Plummer J.D., & Johnson J.E. (2022). Intermittent methionine restriction reduces IGF-1 levels and produces similar healthspan benefits to continuous methionine restriction. *Aging Cell* **21**(6), e13629. PMID: 35570387; doi: 10.1111/acel.13629.
15. Boden G., Sargrad K., Homko C., Mozzoli M., & Stein T.P. (2005). Effect of a low-carbohydrate diet on appetite, blood glucose levels, and insulin resistance in obese patients with type 2 diabetes. *Ann. Intern. Med.* **142**(6), 403–411. PMID: 15767618; doi: 10.7326/0003-4819-142-6-200503150-00006.
16. Breese C.R., Ingram R.L., & Sonntag W.E. (1991). Influence of age and long-term dietary restriction on plasma insulin-like growth factor-1 (IGF-1), IGF-1 gene expression, and IGF-1 binding proteins. *J. Gerontol.* **46**(5), B180–B187. PMID: 1716275; doi: 10.1093/geronj/46.5.B180.
17. Dunn S.E., Kari F.W., French J., Leininger J.R., Travlos G., Wilson R., & Barrett J.C. (1997). Dietary restriction reduces insulin-like growth factor I levels, which modulates apoptosis, cell proliferation, and tumor progression in p53-deficient mice. *Cancer Res.* **57**, 4667–4672. PMID: 9354418.
18. Fontana L., Weiss E.P., Villareal D.T., Klein S., & Holloszy J.O. (2008). Long-term effects of calorie or protein restriction on serum IGF-1 and IGFBP-3 concentration in humans. *Aging Cell* **7**(5), 681–687. PMID: 18843793; doi: 10.1111/j.1474-9726.2008.00417.x.
19. Fraser D.A., Thoen J., Bondhus S., Haugen M., Reseland J.E., Djoseland O., … Kjeldsen-Kragh J. (2000). Reduction in serum leptin and IGF-1 but preserved T-lymphocyte numbers and activation after a ketogenic diet in rheumatoid arthritis patients. *Clin. Exp. Rheumatol.* **18**, 209–214. PMID: 10812493.
20. Rahmani J., Kord Varkaneh H., Clark C., Zand H., Bawadi H., Ryan P.M., … Zhang Y. (2019). The influence of fasting and energy restricting diets on IGF-1 levels in humans: A systematic review and meta-analysis. *Ageing Res. Rev.* **53**, 100910. PMID: 31116995; doi: 10.1016/j.arr.2019.100910.
21. Scarth J.P. (2006). Modulation of the growth hormone-insulin-like growth factor (GH-IGF) axis by pharmaceutical, nutraceutical and environmental xenobiotics: An emerging role for xenobiotic-metabolizing enzymes and the transcription factors regulating their expression. A review. *Xenobiotica* **36**(2–3), 119–218. PMID: 16702112; doi: 10.1080/00498250600621627.
22. Brown-Borg H.M., Rakoczy S.G., Wonderlich J.A., Rojanathammanee L., Kopchick J.J., Armstrong V., & Raasakka D. (2014). Growth hormone signaling is necessary for lifespan extension by dietary methionine. *Aging Cell* **13**, 1019–1027. PMID: 25234161; doi: 10.1111/acel.12269.
23. Brown-Borg H.M., Rakoczy S., Wonderlich J.A., Borg K.E., & Rojanathammanee L. (2018). Metabolic adaptation of short-living growth hormone transgenic mice to methionine restriction and

supplementation. *Ann. N. Y. Acad. Sci.* **1418**, 118–136. PMID: 29722030; doi: 10.1111/nyas.13687.

24. Thyne K.M., & Salmon A.B. (2023). Sexually dimorphic effects of methionine sulfoxide reductase A (MsrA) on murine longevity and health span during methionine restriction. *Geroscience* **45**(5), 3003–3017. PMID: 37391679; doi: 10.1007/s11357-023-00857-8.
25. Huang T.H., Lewis J.L., Lin H.S., Kuo L.T., Mao S.W., Tai Y.S., … Yang R.S. (2014). A methionine-restricted diet and endurance exercise decrease bone mass and extrinsic strength but increase intrinsic strength in growing male rats. *J. Nutr.* **144**(5), 621–630. PMID: 24647387; doi: 10.3945/jn.113.187922.
26. Ouattara A., Cooke D., Gopalakrishnan R., Huang T.H., & Ables G.P. (2016). Methionine restriction alters bone morphology and affects osteoblast differentiation. *Bone Rep.* **5**, 33–42. PMID: 28326345; doi: 10.1016/j.bonr.2016.02.002.
27. Plummer J., Park M., Perodin F., Horowitz M.C., & Hens J.R. (2017). Methionine-restricted diet increases miRNAs that can target RUNX2 expression and alters bone structure in young mice. *J. Cell. Biochem.* **118**(1), 31–42. PMID: 27191548; doi: 10.1002/jcb.25604.
28. Wei W., Dutchak P.A., Wang X., Ding X., Wang X., Bookout A.L., … Wan Y. (2012). Fibroblast growth factor 21 promotes bone loss by potentiating the effects of peroxisome proliferator-activated receptor gamma. *Proc. Natl. Acad. Sci. U. S. A.* **109**(8), 3143–3148. PMID: 22315431; doi: 10.1073/pnas.1200797109.
29. Bartke A. (2005). Minireview: Role of the growth hormone/insulin-like growth factor system in mammalian aging. *Endocrinology* **146**(9), 3718–3723. PMID: 15919742; doi: 10.1210/en.2005-0411.
30. Tong T.Y.N., Appleby P.N., Armstrong M.E.G., Fensom G.K., Knuppel A., Papier K., … Key T.J. (2020). Vegetarian and vegan diets and risks of total and site-specific fractures: Results from the prospective EPIC-Oxford study. *BMC Med.* **18**(1), 353. PMID: 33222682; doi: 10.1186/s12916-020-01815-3.
31. Parfitt A.M., Drezner M.K., Glorieux F.H., Kanis J.A., Malluche H., Meunier P.J., … Recker R.R. (1987). Bone histomorphometry: Standardization of nomenclature, symbols, and units. Report of the ASBMR Histomorphometry Nomenclature Committee. *J. Bone Miner. Res.* **2**(6), 595–610. PMID: 3455637; doi: 10.1002/jbmr.5650020617.
32. Livak K.J., & Schmittgen T.D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* **25**(4), 402–408. PMID: 11846609; doi: 10.1006/meth.2001.1262.
33. Breault S.R., Heye T., Bashir M.R., Dale B.M., Merkle E.M., Reiner C.S., … Gupta R.T. (2013). Quantitative dynamic contrast-enhanced MRI of pelvic and lumbar bone marrow: Effect of age and marrow fat content on pharmacokinetic parameter values. *AJR Am. J. Roentgenol.* **200**(3), W297–W303. PMID: 23436875; doi: 10.2214/AJR.12.9080.
34. Roldan-Valadez E., Pina-Jimenez C., Favila R., & Rios C. (2013). Gender and age groups interactions in the quantification of bone marrow fat content in lumbar spine using 3T MR spectroscopy: A multivariate analysis of covariance (Mancova). *Eur. J. Radiol.* **82**(11), e697–e702. PMID: 23938236; doi: 10.1016/j.ejrad.2013.07.012.
35. de Paula F.J., de Araujo I.M., Carvalho A.L., Elias J. Jr., Salmon C.E., & Nogueira-Barbosa M.H. (2015). The relationship of fat distribution and insulin resistance with lumbar spine bone mass in women. *PLoS One* **10**(6), e0129764. PMID: 26067489; doi: 10.1371/journal.pone.0129764.
36. Plummer J.D., Postnikoff S.D., Tyler J.K., & Johnson J.E. (2021). Selenium supplementation inhibits IGF-1 signaling and confers methionine restriction-like healthspan benefits to mice. *Elife* **10**. PMID: 33783357; doi: 10.7554/eLife.62483.
37. Forney L.A., Wanders D., Stone K.P., Pierse A., & Gettys T.W. (2017). Concentration-dependent linkage of dietary methionine restriction to the components of its metabolic phenotype. *Obesity (Silver Spring)* **25**(4), 730–738. PMID: 28261952; doi: 10.1002/oby.21806.