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FGF21, not GCN2, influences bone morphology due to dietary protein restrictions

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ABSTRACT

Background: Dietary protein restriction is emerging as an alternative approach to treat obesity and glucose intolerance because it markedly increases plasma fibroblast growth factor 21 (FGF21) concentrations. Similarly, dietary restriction of methionine is known to mimic metabolic effects of energy and protein restriction with FGF21 as a required mechanism. However, dietary protein has been shown to be required for normal bone growth, though there is conflicting evidence as to the influence of dietary protein restriction on bone remodeling. The purpose of the current study was to evaluate the effect of dietary protein and methionine restriction on bone in lean and obese mice, and clarify whether FGF21 and general control nonderepressible 2 (GCN2) kinase, that are part of a novel endocrine pathway implicated in the detection of protein restriction, influence the effect of dietary protein restriction on bone.

Methods: Adult wild-type (WT) or *Fgf21* KO mice were fed a normal protein (18 kcal%; CON) or low protein (4 kcal%; LP) diet for 2 or 27 weeks. In addition, adult WT or *Gcn2* KO mice were fed a CON or LP diet for 27 weeks. Young New Zealand obese (NZO) mice were placed on high-fat diets that provided protein at control (16 kcal%; CON), low levels (4 kcal%) in a high-carbohydrate (LP/HC) or high-fat (LP/HF) regimen, or on high-fat diets (protein, 16 kcal%) that provided methionine at control (0.86%; CON-MR) or low levels (0.17%; MR) for up to 9 weeks. Long bones from the hind limbs of these mice were collected and evaluated with microcomputed tomography (μCT) for changes in trabecular and cortical architecture and mass.

Results: In WT mice the 27-week LP diet significantly reduced cortical bone, and this effect was enhanced by deletion of *Fgf21* but not *Gcn2*. This decrease in bone did not appear after 2 weeks on the LP diet. In addition, *Fgf21* KO mice had significantly less bone than their WT counterparts. In obese NZO mice dietary protein and methionine restriction altered bone architecture. The changes were mediated by FGF21 due to methionine restriction in the presence of cystine, which did not increase plasma FGF21 levels and did not affect bone architecture.

Conclusions: This study provides direct evidence of a reduction in bone following long-term dietary protein restriction in a mouse model, effects that appear to be mediated by FGF21.

1. Introduction

Dietary restrictions of nutrients, including protein, have been utilized to treat various disorders in humans, including chronic kidney disease, for hundreds of years (Aparicio et al., 2012; Mandayam and Mitch, 2006). Dietary restriction also extends life span in both humans

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and animals (Leto et al., 1976; Mair and Dillin, 2008); however, the benefit of these diets with respect to one organ system is at times offset by detrimental effects found in other organ systems. Protein provides essential amino acids for the synthesis of bone collagen matrix, and dietary protein restriction has been associated with a reduction in bone density in humans (Ammann et al., 2000) and in animal models (Bourrin et al., 2000; Huang et al., 2014; Mardon et al., 2008). In an elderly population, protein malnutrition has been attributed to a reduction in bone mass and subsequent osteoporosis (Rizzoli and Bonjour, 2004; Rizzoli, 2008; Rizzoli et al., 2014). Similarly, a positive correlation between bone mineral density (BMD) and both energy and protein intake have been reported, especially in the aged population (Devine et al., 2005; Hannan et al., 2000). Recently a gap has been identified in the literature evaluating the involvement of dietary nutrient restrictions, including protein restriction, on bone quality, indicating that the majority of studies focus on evaluating only BMD, which does not provide a broad picture of bone health (Huang and Ables, 2016). While BMD is still the "gold standard" diagnostic test for evaluating overall bone health and fracture risk in humans (Fonseca et al., 2014; NIH Consensus Development Panel on Osteoporosis Prevention, Diagnosis, 2001), in animal models there have been advances in the field of imaging of bone and surrounding tissue which have significantly enhanced the evaluation of volumetric BMD as well as bone geometry in both trabecular and cortical bone in numerous locations. Specifically, micro-computed tomography (μCT) allows complete evaluation of these parameters in rodents, providing an opportunity to fully evaluate the potential impact of these dietary restrictions on bone health (Bouxsein et al., 2010). In addition, a large portion of the literature reporting impacts of insufficient protein on skeletal tissue has been conducted in either animal models of bone growth, or in cohorts that represent the impacts on aged bone remodeling (Ammann et al., 2000; Brennan-Speranza et al., 2011; Rouy et al., 2014). There are very few studies that evaluate these changes in animal models that do not have confounding comorbidities associated with advanced age, or outside of young, skeletally immature animals that are potentially more sensitive to changes in nutrient restriction.

Dietary restrictions, such as methionine restriction, have been shown to regulate metabolism through inconsistent mechanisms (Wanders et al., 2014). Fibroblast growth factor 21 (FGF21) is expressed in numerous tissues, especially the liver (So and Leung, 2016), and an increase in circulating FGF21 has been associated with metabolic responses to protein restrictions (Laeger et al., 2016; Laeger et al., 2014a) and fasting (Inagaki et al., 2008; Guan et al., 2016). General control nonderepressible 2 (GCN2) kinase has been reported as an upstream regulator of FGF21 during a short-term but not long-term dietary protein restriction (Laeger et al., 2016; Laeger et al., 2014a). GCN2 phosphorylates the eukaryotic initiation factor 2 alpha in response to depletion of cellular amino acids (Wek et al., 1995), which results in the activation of the activating transcription factor 4, which binds to the FGF21 promoter that contains amino acid response elements (Laeger et al., 2016; De Sousa-Coelho et al., 2012). FGF21 has also been implicated in the regulation of bone homeostasis following nutrient restriction, including protein, in humans (Fazeli et al., 2015; Lee et al., 2013). In patients afflicted with anorexia nervosa, a disorder in which individuals experience self-induced starvation, a significant inverse correlation has been found between circulating FGF21 levels and bone mass (Fazeli et al., 2015). In mouse models evaluating FGF21 effects on bone, transgenic mice with FGF21 overexpression and mice with high circulating levels of FGF21 secondary to methionine restriction have been identified as having low bone density (Ables et al., 2012; Wei et al., 2012). On the contrary, a study in humans has shown a strong positive correlation between plasma FGF21 levels and BMD in healthy women (Lee et al., 2013). Similarly, a recent report suggests that increased circulating FGF21 does not regulate bone homeostasis in either direction (Li et al., 2016a). Thus, there appears to be conflicting evidence regarding the precise role of FGF21 in bone homeostasis, and whether it plays a similar role in the presence of dietary protein restriction and/or comorbidities. In addition, the upstream regulator of FGF21, GCN2, has not been explored as a possible mediator in this process. Therefore, the purpose of the current study was to systematically evaluate the impact of dietary protein and methionine restriction on bone quality in adult and young mice, and clarify whether there is an involvement of FGF21 and GCN2 in this process. Lastly, obesity is known to affect bone, particularly during development, however the specific impacts and mechanisms are unclear (Farr and Dimitri, 2017; Shapses et al., 2017). Therefore, we tested the effect of these diets in an obese mouse model to identify whether obesity influenced the impact of a protein restricted diet.

2. Methods

2.1. Animals & study design

All animal procedures with mice on C57BL/6 background and NZO/HIBomDife mice were approved by the Pennington Biomedical Research Center Institutional Animal Care and Use Committee and by the animal welfare committees of DIfE and local authorities (Landesamt für Umwelt, Gesundheit und Verbraucherschutz, Brandenburg, Germany), respectively. Male C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME, USA) were used, and *Fgf21*-deficient mice on a C57BL/6 background were provided by Dr. Steven Kliewer (University of Texas Southwestern) as previously described (Potthoff et al., 2009). *Gcn2*-deficient mice on the C57BL/6 background were purchased from Jackson Laboratory (Stock #008240). NZO/HIBomDife mice were bred in house at the DIfE. All mice were single housed in a 12:12 h light:dark cycle with ad libitum access to food or water unless otherwise noted.

To identify the long-term impact of dietary protein restriction, two separate experiments were conducted with *Fgf21*-deficient (*Fgf21*-KO) or *Gcn2*-deficient (*Gcn2*-KO) and a group of wild-type (WT) mice. Mice were initially adapted to a control diet for five days, and at 14–15 weeks of age, mice of each genotype were divided into two groups and were placed on either a normal protein (CON; outlined below in Section 2.2) or low protein (LP; outlined below in Section 2.2) diet. The *Fgf21* long term study included n = 10/group, with a total of 40 animals; the *Gcn2* long-term study included n = 8/group, with a total of 32 animals. Animals were maintained on these diets for 27 weeks, at which point they were sacrificed, and tissues were collected for analysis.

To evaluate whether there was a short-term effect of dietary protein restriction on bone architecture, *Fgf21*-KO and WT mice were initially adapted to a control diet for five days. At 10 weeks of age, mice of each genotype were divided into two groups (n = 10/group, total of 40 animals) and placed on either a normal protein (CON) or low protein (LP) diet. Animals were maintained on these diets for 14 days, at which point they were sacrificed and bones were collected for analysis.

In addition, to evaluate the effect of dietary protein and methionine restriction on bone under obese conditions, after weaning male New Zealand obese (NZO) mice, a model for polygenic obesity and type 2 diabetes, were placed on high-fat diets (outlined below in Section 2.2) that provided protein at control (CON) levels, low protein in the context of either high-carbohydrate (LP/HC) or high-fat (LP/HF) regimen, or on high-fat diets without lowering the protein content that provided methionine at control (CON-MR) with cystine, or low levels (MR) with cystine, and MR without cystine for up to 9 weeks. At the end of the study mice were sacrificed and tissues were collected for analysis.

Intact hind limbs were collected at necropsy and fixed in 10% neutral-buffered formalin. Femurs (FGF21 long-term study, GCN2 long-term study) and tibiae (FGF21 short-term study) were disarticulated and dissected free from soft tissue for subsequent micro-computed tomography (μCT) scanning and histology, as outlined below.

A detailed dietary composition and data related to changes in body weight and composition, blood glucose, plasma insulin, plasma FGF21,

energy expenditure, and food intake have been previously published for all these mice (Laeger et al., 2016; Laeger et al., 2014a; Laeger et al., 2018; Castaño-Martinez et al., 2019).

2.2. Dietary compositions

Control (normal protein, CON) and low protein (LP) diets were formulated and produced by Research Diets (New Brunswick, NJ, USA), and were designed to be isocaloric by equally varying protein and carbohydrate content while keeping fat constant. The CON diet contained 18% casein (by weight) as the protein source (18 kcal% protein, 60 kcal% carbohydrate, 22 kcal% fat), while the LP diet contained 5% casein (4 kcal% protein, 74 kcal% carbohydrate, 22 kcal% fat). A detailed dietary composition has been previously published (Laeger et al., 2016).

High fat diets were formulated as outlined: control (CON; 16 kcal% protein, 51 kcal% carbohydrate, 33 kcal% fat), low protein levels in a high-carbohydrate (LP/HC; 4 kcal% protein, 63 kcal% carbohydrate, 33 kcal% fat) or low protein levels with high-fat (LP/HF; 4 kcal% protein, 49 kcal% carbohydrate, 47 kcal% fat) regimen. High-fat diets (16 kcal% protein, 52 kcal% carbohydrates, 32 kcal% fat) provided methionine at control (CON-MR; 0.86%) or low levels (MR; 0.17%) with cystine (0.3%) or without.

2.3. Micro-computed tomography (μCT)

For studies involving mice on the C57BL/6 background, samples were placed in holders with appropriate fluid (70% ETOH) for scanning by μCT (Scanco Model 40; Scanco Medical AG, Bassersdorf, Switzerland). The samples were scanned in a coronal plane at 55 kV, 0.3-second integration time, with a 10 μm voxel size in plane and a 10 μm slice thickness. The lengths of the bones were determined from the scans. The greater trochanter in the mouse extends further proximally than the femoral head, therefore to remain consistent in measurements, the bones were measured using transverse μCT slices from the proximal tip of the greater trochanter to the distal edge of the femoral condyles. The regions of interests (ROIs) for tissue analysis in the femur were determined to be the distal 30% of the femur to the distal physis for trabecular bone, and midshaft (50% of the length of the femur) for cortical bone. The ROIs for tissue analysis in the tibia were determined to be the proximal 30% of the tibia to the proximal physis for trabecular bone, and midshaft (50% of the length of the tibia) for cortical bone. The proper thresholds for both trabecular and cortical bone were tested and the same thresholds were used throughout the study. For trabecular bone, total volume (TV, mm^3), bone volume (BV, mm^3), BV/TV, bone surface (BS, mm^2), BS/BV, tissue density, apparent density, trabecular number (Tb.N, 1/mm), trabecular thickness (Tb.Th, mm), trabecular spacing (Tb.Sp, mm), connectivity density (Conn.D, 1/mm^3), structural model index (SMI), and degree of anisotropy (DA) were evaluated. For cortical bone, total cortical area (Tt.Ar, mm^2), cortical bone area (Ct.Ar, mm^2), bone area/total area (BA/TA), thickness (Ct.Th, mm), porosity (Ct.Po, %), marrow area (Ma.Ar, mm^2), polar moment of inertia (J, mm^4), and bone mineral density (BMD, mg HA/ccm) were evaluated. All variables use established published procedures and nomenclature (Bouxsein et al., 2010).

For studies involving NZO mice μ-CT analysis was conducted with LaTheta LCT-200 (Hitachi-Aloka) using manufacturer's pre-defined parameters for isolated bone measurements of bone slices with 24 μm thickness. A region of interest was scanned 25 slices from the distal growth plate extending 100 slices proximally towards the diaphysis. For trabecular bone, trabecular area ratio (%), total bone mineral density (mg/cm^3), plane bone mineral density (g/cm^2), and polar moment (mg x cm) were evaluated. For cortical bone, thickness (mm) and cortical area ratio (%) were evaluated.

2.4. Histopathology

Following μCT analysis, femurs from both studies (*Fgf21*-KO and *Gcn2*-KO) that were fed the LP diet for 27 weeks were decalcified with 10% EDTA and prepared for routine histological processing. Two midcoronal sections for each femur were prepared and stained with hematoxylin & eosin (H&E). Care was taken to include the distal epiphysis, physis, and metaphysis of each bone within the section for evaluation by a board-certified veterinary pathologist. Sections were evaluated to qualitatively confirm μCT findings and characterize any potential changes identified within the distal physis for potential impacts of dietary protein restriction and/or *Fgf*21 deficiency on growth. In addition, any other abnormalities in surrounding tissue (marrow contents, vasculature) were noted.

2.5. Statistics

Data were analyzed using software Prism 6 (GraphPad Software, San Diego, CA, USA) applying two-way ANOVA (with Bonferroni post-hoc analyses) or unpaired two-tailed *t*-test. All data are expressed as means ± SEM, with a probability value of 0.05 considered statistically significant.

3. Results

3.1. FGF21 is required for long-term low-protein-induced changes in femoral cortical morphology, but not trabecular morphology

At week 27 on a diet, compared to CON WT mice, plasma levels of FGF21 were robustly increased in LP WT mice (Laeger et al., 2016). Neither WT nor *Fgf21* KO mice showed a diet effect in regard to the femur length (Fig. 1A) upon 27-week dietary protein restriction. LP WT mice showed a significantly lower femoral cortical thickness, cortical total area (TA), cortical bone area/total area (BA/TA), cortical polar moment of inertia (pMOI), and significantly increased cortical bone porosity in comparison to CON WT mice (Fig. 1B–F). All these effects were lost in the *Fgf21* KO mice (Fig. 1). In regard to the femoral trabecular bone, LP WT mice showed significantly decreased bone volume/total volume (BV/TV), a lower trabecular number, and lower trabecular connectivity density compared to CON WT mice, dietary effects which could be observed in the *Fgf21* KO mice on LP diet as well (Fig. 1H, I, K), without a significant interaction found between strain and diet. The trabecular thickness was only lower by number in WT mice on a LP diet compared to CON WT mice (Fig. 1J). This change was also evident in qualitative histopathological evaluation; the amount of trabecular bone was markedly decreased in quantity in the *Fgf21* KO on LP diet compared to all other groups (data not shown). Due to variability in plane of section, more detailed histologic evaluation of the trabecular bone could not be performed. The physes of all sections were inactive and there were no discernable histologic abnormalities in any section. Neither WT nor *Fgf21* KO mice showed a dietary impact on femoral trabecular total volume (Fig. 1G). In summary, a LP diet resulted in both cortical and trabecular bone loss, with FGF21 being required for the effects of LP diet on cortical but not trabecular bone.

3.2. Short-term protein restriction does not induce changes in tibia morphology

In contrast to the 27-week diet data outlined above, no diet effects in the tibia could be observed in WT and *Fgf21* KO mice after 2 weeks on a LP diet in respect to cortical thickness, cortical TA, cortical BA/TA, cortical pMOI, cortical bone porosity, and marrow area (Fig. 2A–F), or tibial length, despite increased plasma FGF21 levels in WT LP mice (Laeger et al., 2014b). However, there appeared to be a potential early, though insignificant, effect of a reduced protein diet on trabecular bone volume in WT mice, where the BV/TV was lower in LP vs. CON fed mice

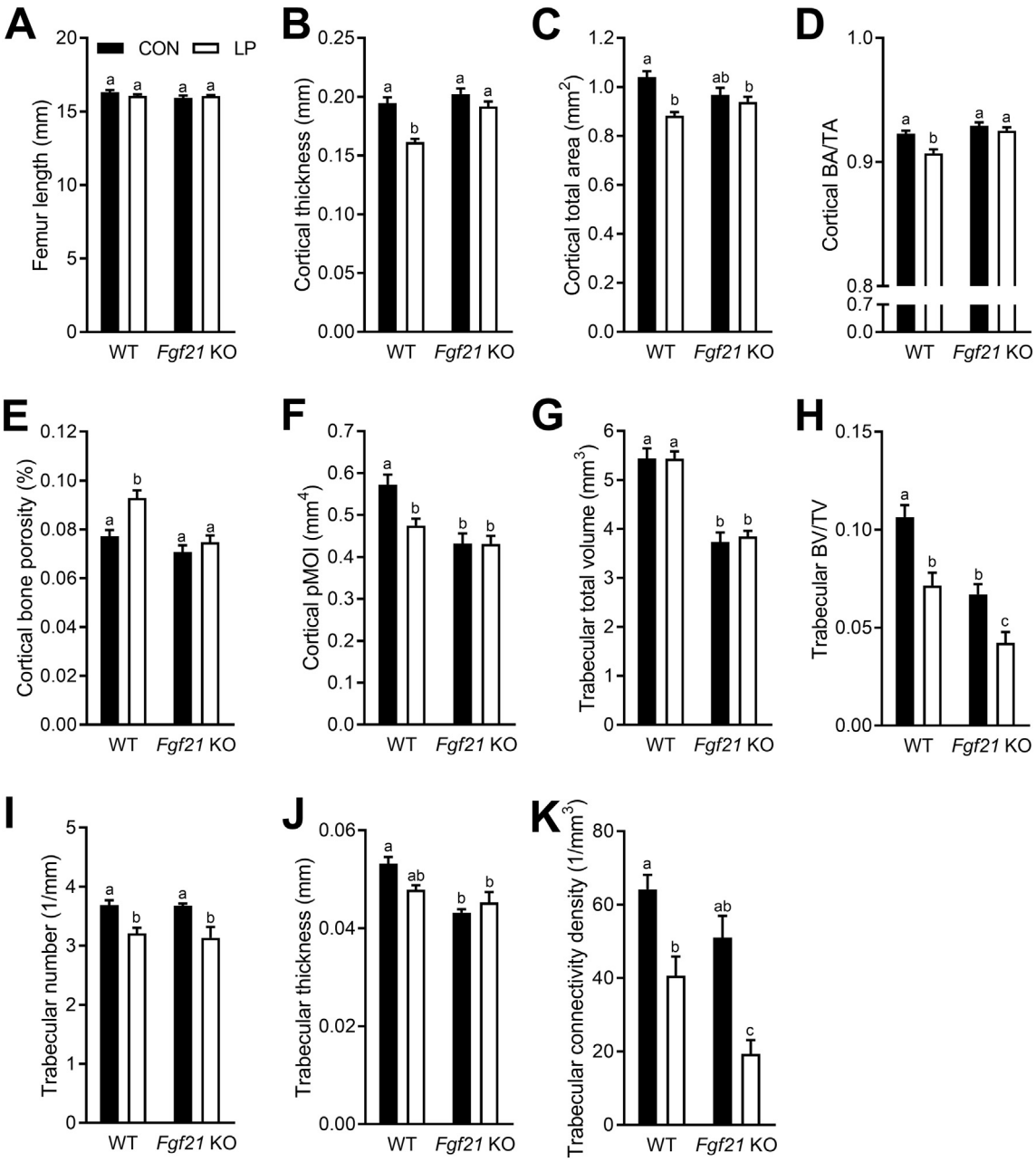

Fig. 1. FGF21 is required for long-term low-protein-induced changes in femoral morphology. At 14–15 weeks of age, wild-type (WT) and FGF21-deficient (*Fgf21* KO) mice were placed on a control (CON) diet for 5 days, at which point a random subgroup of mice was transferred to the low-protein (LP) diet for 27 weeks. (A) Femur length. Femoral cortical (B) thickness, (C) total area, (D) BA/TA, (E) bone porosity, and (F) pMOI. Femoral trabecular (G) total volume, (H) BV/TV, (I) number, (J) thickness, and (K) connectivity density. Data are presented as means ± SEM (n = 10/group). Differences were calculated by using two-way ANOVA. Mean values with different lowercase letters differ with $P \leq 0.05$.

(Fig. 2H). Other trabecular parameters were not different between CON and LP fed WT or *Fgf21* KO mice (Fig. 2G, I, J, K).

3.3. GCN2 is not required for long-term low-protein-induced changes in femoral morphology

A similar study to the chronic LP study using WT and *Fgf21* KO mice was conducted in *Gcn2*-deficient mice, and a summary of these data is presented in Fig. 3. In contrast to *Fgf21* KO mice, the *Gcn2* KO mice responded in the same way as the WT mice on a LP diet. Also, circulating FGF21 levels were increased in *Gcn2* KO mice on a low protein diet (Laeger et al., 2016). In detail, femoral cortical thickness, cortical TA, cortical BA/TA, and cortical pMOI were significantly lower in both strains (WT and *Gcn2* KO) as a response to a LP diet (Fig. 3B, C, D, F). The cortical bone porosity was significantly increased in both WT and *Gcn2* KO mice on a LP diet (Fig. 3E). In respect to the trabecular bone, the total volume, BV/TV, trabecular number, trabecular thickness, and connectivity density were significantly lower in both WT and *Gcn2* KO mice on a LP diet compared to mice on a CON diet (Fig. 3G–K). Qualitative histopathologic evaluation of the trabecular bone was similar for all WT mice regardless of diet, however the *Gcn2* KO mice fed the control (CON) diet had a noticeable decrease in trabecular bone as compared to both of the WT groups, and the *Gcn2* KO mice fed the low protein (LP) diet had decreased trabecular bone as compared to both the WT groups and the *Gcn2* KO CON group (data not shown). Again, due to variability in plane of section, more detailed histologic

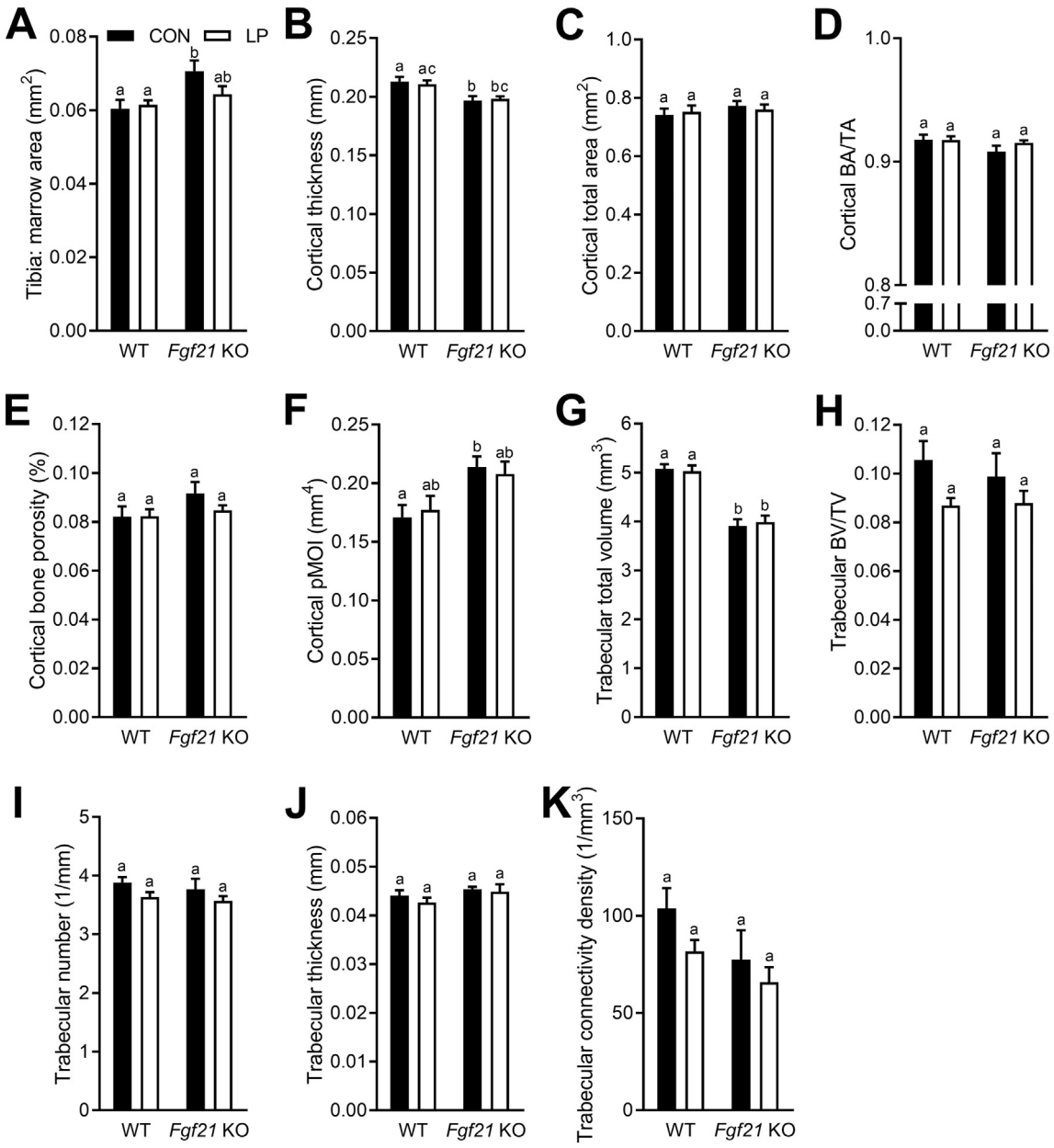

Fig. 2. Short-term protein restriction does not induce changes in tibia morphology. At 10 weeks of age, wild-type (WT) and FGF21-deficient (*Fgf21* KO) mice were placed on a control (CON) diet for 5 days, at which point a random subgroup of mice was transferred to the low-protein (LP) diet for 2 weeks. (A) Tibial marrow area. Tibial cortical (B) thickness, (C) total area, (D) BA/TA, (E) bone porosity, and (F) pMOI. Tibial trabecular (G) total volume, (H) BV/TV, (I) number, (J) thickness, and (K) connectivity density. Data are presented as means ± SEM (n = 10/group). Differences were calculated by using two-way ANOVA. Mean values with different lowercase letters differ with $P \leq 0.05$.

evaluation of trabecular bone could not be performed. The physes of all sections were inactive and there were no discernable abnormalities in any section. In summary, unlike in the chronic *Fgf21* KO study, *Gcn2* depletion did not blunt these changes, and a reduction in cortical integrity was still identified in the *Gcn2* KO mice on LP diet compared to CON diet.

3.4. Dietary protein restriction alters bone architecture in New Zealand Obese mice

To evaluate the effect of dietary protein restriction on bone under obese conditions, NZO mice, a model for polygenic obesity, were placed on high-fat diets that provided protein at control (CON); low protein levels with high levels of carbohydrates (LP/HC); or a low-protein, high-fat (LP/HF) regimen for 8 weeks. Both dietary protein restrictions increased plasma FGF21 concentrations (Laeger et al., 2018). Interestingly, only LP/HF mice showed a significant reduced femur length compared to CON mice (Fig. 4A). The tibia length was reduced, albeit insignificantly, in LP/HF mice compared to CON mice (Fig. 4A). Interestingly, the femoral cortical thickness was significantly higher in the LP/HC and lower in the LP/HF mice compared to CON mice (Fig. 4B). The femoral cortical area ratio was significantly higher in LP/HC compared to CON and LP/HF mice, whereas the trabecular area ratio was not different between the 3 groups (Fig. 4C). The analysis of the BMD showed a significantly lower cortical BMD in the LP/HF vs LP/HC group, without differences in the cancellous BMD, total BMD, and plane BMD between all 3 groups (Fig. 4D–E). In respect to the min moment, both LP/HC and LP/HF was lower, although insignificantly, vs CON mice (Fig. 4F) and the polar moment was significantly lower in both LP fed groups compared to CON mice (Fig. 4G). In summary,

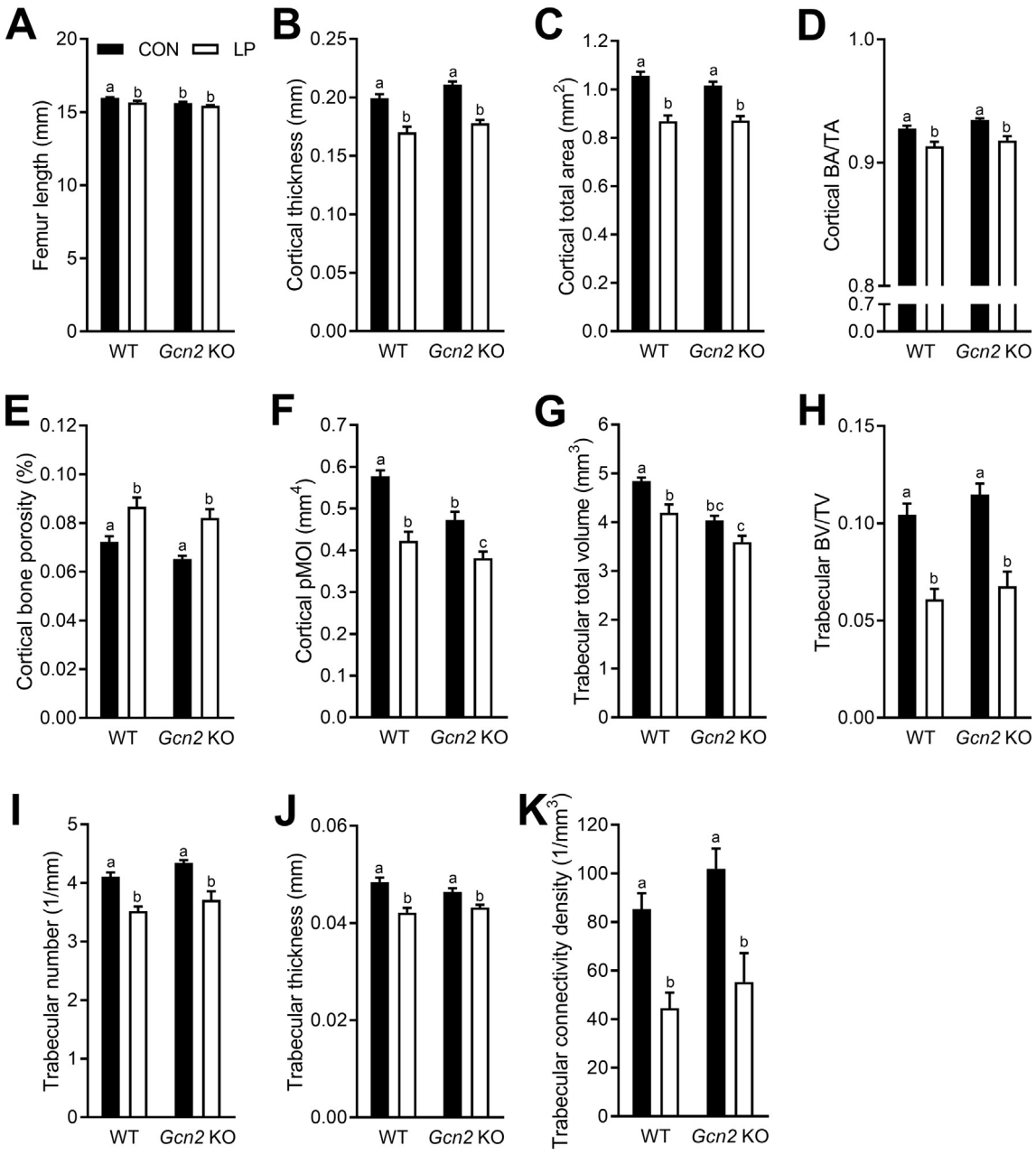

Fig. 3. GCN2 is not required for long-term low-protein-induced changes in femoral morphology. At 14–15 weeks of age, wild-type (WT) and GCN2-deficient (*Gcn2* KO) mice were placed on a control (CON) diet for 5 days, at which point a random subgroup of mice was transferred to the low-protein (LP) diet for 27 weeks. (A) Femur length. Femoral cortical (B) thickness, (C) total area, (D) BA/TA, (E) bone porosity, and (F) pMOI. Femoral trabecular (G) total volume, (H) BV/TV, (I) number, (J) thickness, and (K) connectivity density. Data are presented as means ± SEM (n = 8/group). Differences were calculated by using two-way ANOVA. Mean values with different lowercase letters differ with $P \leq 0.05$.

protein restriction led to negative impacts on the morphology of bones under high-fat diet conditions.

3.5. Dietary methionine restriction alters bone architecture in New Zealand Obese mice

To evaluate the effect of dietary methionine restriction on bone under obese conditions, NZO mice were placed on high-fat diets with a normal protein content that provided methionine at control (CON) or low levels (MR) with or without cystine for up to 9 weeks. Only the MR feeding without cystine (-Cys) increased plasma FGF21 concentrations (Castaño-Martinez et al., 2019). In general, only the MR (-Cys) mice showed alterations in bone architecture compared to CON (-Cys) mice (Fig. 5). All the effects were lost in MR mice fed with cystine, which did not increase plasma FGF21 levels (Fig. 5). In detail, the femur length did not differ between the groups, but the tibia length was significantly shorter in MR vs CON mice without cystine (Fig. 5A–B). The femoral cortical thickness (Fig. 5C), femoral cortical area ratio (Fig. 5D), and femoral trabecular area ratio (Fig. 5E) were significantly lower in MR (-Cys) compared to CON (-Cys) mice. The total BMD and the polar moment was significantly reduced in MR (-Cys) vs CON (-Cys) mice, without difference between the groups in the plane BMD (Fig. 5F–H). In summary, a methionine restriction led to bone loss and a higher fragility under high-fat diet conditions only in the absence of cysteine when FGF21 was increased in the circulation.

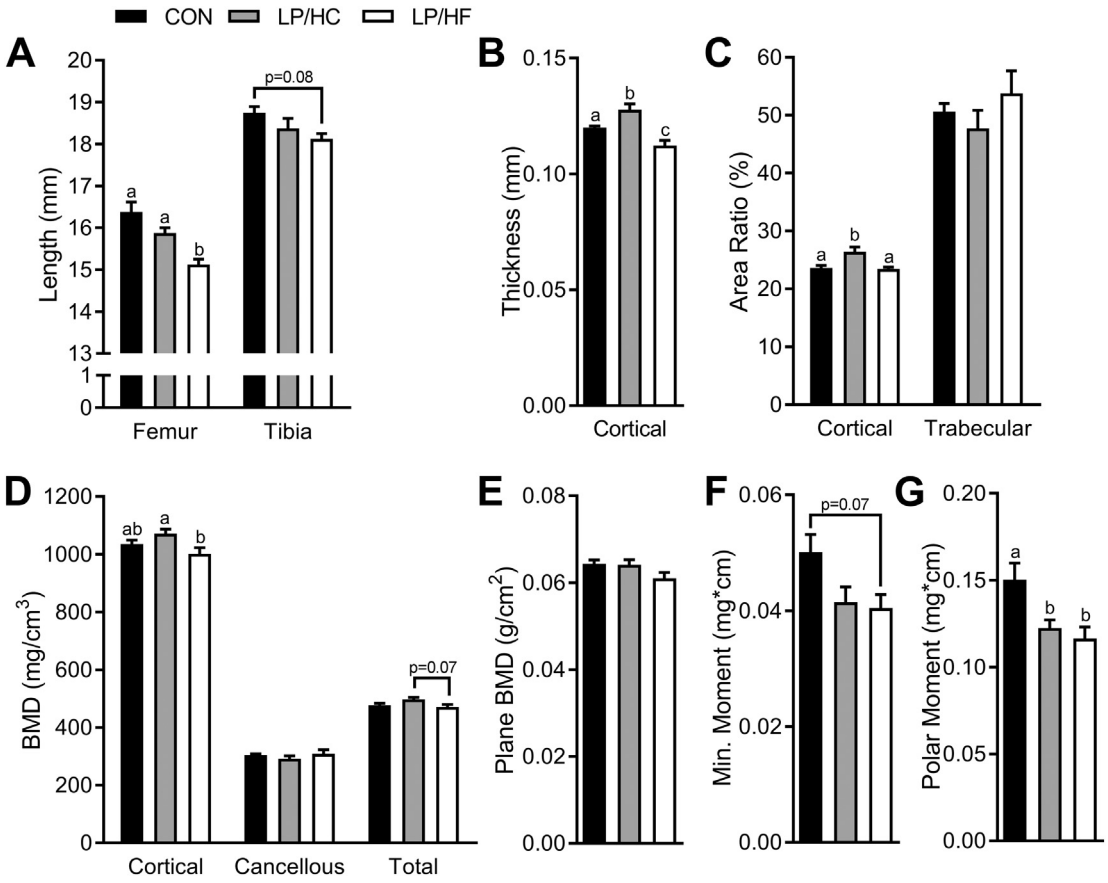

Fig. 4. Dietary protein restriction alters bone architecture in New Zealand Obese mice. At 3 weeks of age, NZO mice were placed on a high-fat (CON) diet for 1 week, at which point two random subgroups of mice were transferred to the LP/HC or LP/HF diet for 8 weeks. (A) Femur and tibia length. (B) Femoral cortical thickness and (C) cortical and trabecular area ratios. (D) Femoral bone mineral density (BMD), (E) plane BMD, (F) min moment, and (G) polar moment. Data are presented as mean ± SEM (n = 4/group). Differences between groups were calculated by one-way ANOVA. Mean values with different lowercase letters differ with $P \leq 0.05$.

4. Discussion

This study presents a systematic evaluation of the impact of dietary protein restriction on bone morphology in adult, skeletally mature but not elderly mice, and tests the role of FGF21 and GCN2 as mediators of this effect. The data indicate that prolonged dietary protein restriction results in a significantly negative impact on both trabecular and cortical bone parameters, and these results were consistently reproduced in two independent animal cohorts. These results also suggest that deletion of FGF21 not only affects bone morphology as compared to controls, but also appears to aggravate the effects of protein restriction in trabecular bone while protecting against diet-induced changes in cortical bone. GCN2 deletion, in contrast, was largely without effect. This effect can be explained by the delayed but robustly induced FGF21 increase in these *Gcn2* KO mice (Laeger et al., 2016).

It is well known that deficiency in dietary energy and various nutrients (e.g. calcium, phosphorous) negatively impact bone growth and remodeling in humans and animal models (Talbott et al., 1998; O'uchi et al., 1999; Riedt et al., 2005; Talbott et al., 2001). Protein has been demonstrated to be critical for development and bone health in both humans and animal models. Insufficient dietary protein during development has resulted in impaired bone growth and poor bone quality in adults (Orwoll et al., 1992; Jones et al., 2011; Mardon et al., 2009), and energy and protein restriction in growing rats impairs peak bone mass acquisition, resulting in decreased skeletal strength (Mardon et al., 2009). Moderate dietary protein restriction has also been shown to reduce bone growth in mice, with the source of protein (casein vs. soy) differentially impacting the negative changes in bone growth (Rouy et al., 2014). Our findings in the current study support the previous data indicating the importance of dietary protein in bone health. However, the studies presented in the current project were performed in mice that were nearing skeletal maturity at the onset of protein restriction, which indicates an impact of dietary protein restriction on bone remodeling processes as opposed to bone development. However, taking into account the effect of protein restriction on cortical thickness and area, appositional cortical growth may be impaired, and this effect may be mediated by FGF21. In addition, we were able to obtain these data using high-resolution μCT, which provides an extensive evaluation of both cortical and trabecular microarchitecture that can be used to aid in assessment of overall bone health. We were also able to demonstrate a negative impact of dietary protein restriction on bone in normal, wild-type mice, whereas other studies have only found this reduction in bone remodeling in the presence of comorbidities, such as osteoporosis secondary to ovariectomy or in aged animals (Bourrin et al., 2000).

Our results demonstrating FGF21 deficiency results in reduced bone contradict a previous study demonstrating that transgenic mice overexpressing FGF21 exhibit low bone density and low trabecular bone, while bone was increased in the same line of FGF21 deficient mice used in the study by Wei et al (Wei et al., 2012). However, this study provides no data regarding the age of the mice that were analyzed, and a more recent follow up study by another group was unable to re-create the aforementioned decrease in bone density secondary to FGF21 overexpression (Li et al., 2016a). A separate study identified a decrease in bone density in a setting of high circulating levels of FGF21 secondary to methionine restriction; however, this study was conducted in mice that were only 7 weeks of age (Ables et al., 2012), indicating an effect of protein restriction on bone growth and not only bone remodeling as outlined in the present study. Conversely, high protein

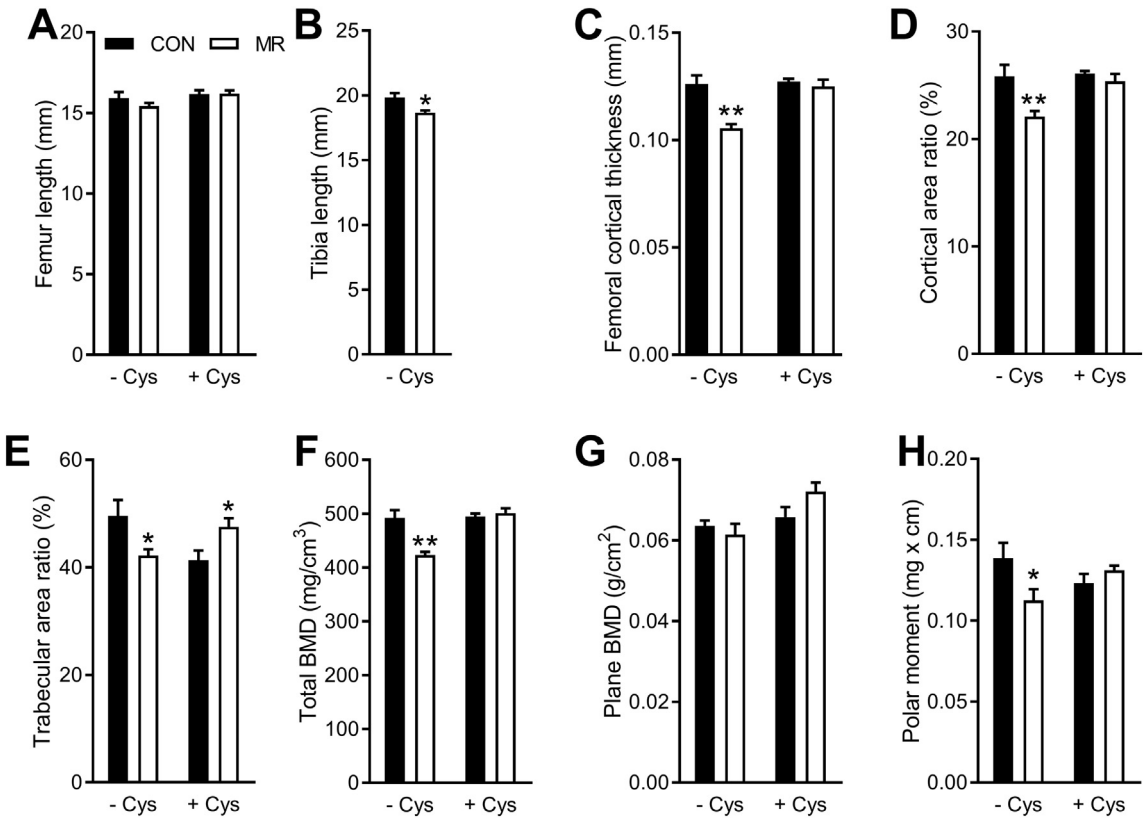

Fig. 5. Dietary methionine restriction alters bone architecture in New Zealand Obese mice. At 3 weeks of age, NZO mice were placed on a high-fat diet that provided methionine at control level (CON) for 1 week, at which point a subgroup of mice was transferred to a methionine-restricted (MR) diet without cystine, MR diet with cystine, or control diet with cystine for 9 weeks. (A) Femur and (B) tibia length from –Cys group only. (C) Femoral cortical thickness, (D) cortical and (E) trabecular area ratios. (F) Femoral bone mineral density (BMD), (G) plane BMD, and (H) polar moment. Data are presented as mean ± SEM (n = 5–6/group). Differences between groups were calculated by two-tailed *t*-test. *$p \leq 0.05$; **$p \leq 0.01$.

diets have been shown to attenuate bone loss, including in the presence of energy restriction, and rats fed high protein diets for four weeks showed an increased bone turnover and volumetric bone mineral density (Gaffney-Stomberg et al., 2014). Thus, while a large volume of data support relationships between dietary protein intake on bone health, contradictory data exist regarding the role of FGF21.

We also tested the impact of methionine restriction on bone morphology in young, skeletally immature mice, and demonstrated a role of methionine restriction on bone morphology. Skeletally immature mice were used in this study as the authors wished to prevent type II diabetes from confounding the experiment; it is difficult to maintain healthy normoglycemic adult NZO mice because they rapidly develop a hyperglycemia as soon as they are placed on a carbohydrate-containing diet. It is known that vegan and vegetarian diets demonstrate a lower methionine content, therefore methionine intake is decreased compared to omnivores (McCarty et al., 2009; Schmidt et al., 2016). Individuals adhering to a vegan or vegetarian diet may induce FGF21 levels; indeed, human plasma FGF21 levels have been demonstrated to be higher in vegetarians and vegans compared to omnivores, and a short-term change in diet to vegetarianism increased plasma FGF21 levels in omnivores (Castaño-Martinez et al., 2019). As meta-analyses have suggested that vegetarian diets, and in particular vegan diets (Ho-Pham et al., 2009), are associated with lower BMD, it can be speculated that these changes in bone may be associated with higher FGF21 levels.

A strength of this work is that it demonstrates the essential contribution of FGF21 to bone remodeling as a consequence of protein restriction, effects that we observed in different murine genetic lines. This impairment was found to be independent of the method by which protein or methionine restriction was achieved, as long as FGF21 was ultimately increased. Inclusion of both LP/HC and LP/HF diets in these studies demonstrates that independent of the substituent for protein (i.e. FGF21 is increased), the bone architecture is altered, indicating that FGF21 has profound effects. A limitation of this study, however, is the absence of direct measurements of bone remodeling parameters using dynamic histomorphometry. Thus, we are unable to directly link a diet restricted in protein with a decrease in bone remodeling in mice. In addition, we were not able to directly evaluate the effect of the protein-restricted diet on biomechanical strength of the bones, though the data demonstrate significant effects on pMOI and total cortical area, which strongly indicate an effect on overall bone strength. Lastly, the data obtained demonstrating the dietary effects in skeletally mature mice were derived from two different sites; both long-term studies utilized femora, whereas the FGF21 short-term data was derived from tibiae. However, both sites are acceptable for evaluating trabecular and cortical bone (Bouxsein et al., 2010), and develop using endochondral ossification at very similar times during embryonic development. In addition, both bones undergo the same remodeling processes found in long bones throughout the body. Additionally, as both bones are located in the same limb, they experience very similar biomechanical loads. Taking these limitations into consideration, future work could be designed with these skeletal endpoints in mind, including consistent sampling methods, histological preparations for histomorphometry, and setting aside specific samples for biomechanical testing such as three-point bending or torsional testing. Histological sections were evaluated by a boarded veterinary pathologist for any abnormalities in physes or boney tissue, and while the reduction in trabecular bone volume in some of the more severely affected treatment groups was confirmed, no differences in appearance of growth plates were observed. However, it should be noted that all growth plates were inactive at the timepoints examined. Additional experiments examining growth

plates of younger WT mice with active growth plates may allow for evaluation for alterations in bone growth. We also did not evaluate the mechanical properties of bone, which will ultimately provide the likelihood of fracture that is an important factor in overall bone health in humans.

The mechanisms for the decrease in bone identified in the present study are currently unknown. Further, the mechanism by which systemically elevated FGF21 is protecting against long-term diet-induced changes in cortical bone is unclear. It has been shown that circulating IGF-1 levels were reduced in mice fed a low protein diet (Brennan-Speranza et al., 2011). GH is a well-known stimulator of IGF-1, and both GH and IGF-1 have significant roles in regulating bone growth (Olney, 2003). GH directly, as well as through IGF-1, stimulate osteoblast proliferation and activity, promoting bone formation. Indeed, both have been implicated as successful in therapies for osteoporosis and factures (Locatelli and Bianchi, 2014). In turn, FGF21 has been shown to inhibit growth hormone (GH) signaling (Inagaki et al., 2008), as has protein undernutrition (Ammann et al., 2000). Therefore, there is a potential pathway that FGF21, by negatively impacting GH/IGF-1 signaling, would have a negative impact on bone remodeling in adult mice. Particularly, overexpression of IGF-1 in mouse osteoblasts was able to negate the effects of protein restriction for eight weeks in elderly mice (Brennan-Speranza et al., 2011). Future studies would involve evaluating whether the GH/IGF-1 pathway is directly involved in FGF21-mediated bone remodeling. Another potential mechanism is through osteoblastic differentiation. In vascular smooth muscle cells it has been shown that FGF21 is suppressing bone morphogenic protein-2 (BMP-2) mRNA expression (Cao et al., 2017a; Cao et al., 2017b), which is a potent osteogenic protein required for osteoblast differentiation and bone formation (Cai et al., 2012). FGF21 inhibits osteoblast differentiation and vascular calcification in vitro by the BMP-2/Smad signaling pathway (Liu et al., 2018), and could inhibit the osteogenic differentiation in human bone mesenchymal stem cells (hBMSCs) under hyperglycemic conditions (Li et al., 2016b). Future work could include in vitro studies to further clarify the mechanisms responsible for the effect on bone outlined herein.

5. Conclusion

Overall, this study demonstrates a negative impact of dietary protein restriction on bone mass and architecture in adult mice in the absence of comorbidities, as well as in the NZO mouse model that is affected by hyperglycemia and insulin resistance. We also identified that short-term interventions of dietary restriction do not appear to impair the bone architecture, and that reduced bone strength identified in humans engaging in vegetarian or vegan diets may be the consequence of increased FGF21 levels. Therefore, we conclude that FGF21 is implicated in the protein-restriction-mediated modulation of bone remodeling.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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