ORIGINAL ARTICLE

JBMR®

Skeletal Protection and Promotion of Microbiome Diversity by Dietary Boosting of the Endogenous Antioxidant Response

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ABSTRACT

There is an unmet need for interventions with better compliance that prevent the adverse effects of sex steroid deficiency on the musculoskeletal system. We identified a blueberry cultivar (Montgomerym [Mont]) that added to the diet protects female mice from musculoskeletal loss and body weight changes induced by ovariectomy. Mont, but not other blueberries, increased the endogenous antioxidant response by bypassing the traditional antioxidant transcription factor Nrf2 and without activating estrogen receptor canonical signaling. Remarkably, Mont did not protect the male skeleton from androgen-induced bone loss. Moreover, Mont increased the variety of bacterial communities in the gut microbiome (α-diversity) more in female than in male mice; shifted the phylogenetic relatedness of bacterial communities (β-diversity) further in females than males; and increased the prevalence of the taxon *Ruminococcus1* in females but not males. Therefore, this nonpharmacologic intervention (i) protects from estrogen but not androgen deficiency; (ii) preserves bone, skeletal muscle, and body composition; (iii) elicits antioxidant defense responses independently of classical antioxidant/estrogenic signaling; and (iv) increases gut microbiome diversity toward a healthier signature. These findings highlight the impact of nutrition on musculoskeletal and gut microbiome homeostasis and support the precision medicine principle of tailoring dietary interventions to patient individualities, like sex. © 2020 American Society for Bone and Mineral Research (ASBMR).

KEY WORDS: NUTRITION; GENETIC ANIMAL MODELS; SEX STEROIDS; OSTEOPOROSIS; THERAPEUTICS—OTHER

Introduction

Musculoskeletal deterioration upon sex steroid deficiency or aging is a major cause of bone fractures worldwide. Current therapies have poor compliance, are not suitable for all patients, and exhibit increased risk of damaging side effects.[1–6] Lower medication adherence is also influenced by socioeconomic factors, including high costs and insufficient medical insurance.[1,3] Further, the mechanisms underlying bone loss, therapeutic response, and overall morbidity/mortality may differ between women and men.[7–11] Thus, there is an unmet need for interventions with better compliance, fewer side effects, and tailored to individualities, like sex, to meet the goals of precision medicine.

Accumulation of reactive oxygen species (ROS) in bone underlies the skeletal fragility ensuing with loss of sex steroids and aging.[12,13] ROS stabilizes the transcription factor *Nrf2* (nuclear factor, erythroid derived 2, like 2), which triggers an endogenous antioxidant response (EAR) in an attempt to mitigate cellular effects of ROS.[14–16] The EAR is genetically controlled by the antioxidant enzymes that degrade ROS, thioredoxin reductase 1 (*Txnrd1*), and superoxide dismutase 1 (*Sod1*) and by the phase II detoxifying enzymes that indirectly neutralize ROS by

Received in original form July 16, 2020; revised form December 9, 2020; accepted December 9, 2020. Accepted manuscript online December 14, 2020.
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Additional Supporting Information may be found in the online version of this article.
Journal of Bone and Mineral Research, Vol. 00, No. 00, Month 2020, pp 1–11.
DOI: 10.1002/jbmr.4231

conjugating xenobiotics, heme oxygenase 1 (*Hmox1*), ferritin light chain 1 (*Ftl1*), and glutathione S-transferase phosphate 1 (*Gstp1*).

The fundamental impact of nutrition on skeletal health is fully accepted.[17–22] However, how dietary components regulate skeletal homeostasis is far from being elucidated. Beyond minerals and vitamins, polyphenolic compounds found in natural plant products might benefit the skeleton by mechanisms associated with their antioxidant properties. In particular, diets enriched in blueberries have been shown to protect against bone loss in some[23–26] but not all studies.[27] In addition, the degree of protection markedly varies among subjects. Therefore, the factors controlling why some diets, but not others, are effective and why some individuals, but not all, are fully responsive remain unknown.

To address these unsolved issues, we utilized here the preclinical model of gonadectomy in the mouse that recapitulates the effects of sex steroid deficiency in humans, genetically modified mice lacking the Nrf2 transcription factor, and diets supplemented with dry extracts of three different cultivars of blueberries. The current study identifies a cultivar of blueberries (Montgomery [Mont]) that, unlike two other blueberry types, when incorporated into the diet fully protected female mice from the damaging effects of estrogen loss on bone, muscle, and body composition leading to peripheral fat accumulation. Only the Mont diet effectively increased the skeletal EAR and the underlying mechanism appears to be unique as it is independent of the traditional antioxidant transcription factor Nrf2 and does not result from activation of canonical estrogenic signaling. These benefits were linked to a healthier gut microbiome signature characterized by increased diversity of bacterial communities as quantified by richness, evenness, and phylogenetic relatedness. In contrast to the effects in females, Mont diet was minimally effective in protecting the skeleton from androgen deficiency and in increasing microbiome diversity in males. These findings provide an explanation for the varied effectiveness of dietary interventions and emphasize the importance of adapting therapeutic approaches to patient individualities, such as sex. Further, they strongly suggest that skeletal EAR and the gut microbiome signature are suitable predictors of individual responses to interventions targeting the musculoskeletal system.

Materials and Methods

Mice and diets

Wild-type (WT) and Nrf2 knockout (KO) littermate mice were generated by breeding Nrf2 heterozygous mice (B6.129X1-Nfe2/2^{tm1Ywk}/J) from Jackson Laboratory (Bar Harbor, ME, USA), and genotyping was performed by PCR, using the primers and the nucleotide-free UltraPure distilled water (ref #10977–023, Invitrogen, Carlsbad, CA, USA) with controls as published earlier.[28] Skeletally mature, 4-month-old mice were sham operated (SHAM), ovariectomized (OVX) or orchidectomized (ORX), and fed a control diet (Modified AIN-93 M Rodent Diet With Corn Oil, product #D00031602, Research Diets Inc., New Brunswick, NJ, USA) or a control diet containing 10% lyophilized (freeze-dried) Wild Blueberry, Ira, or Montgomery blueberries obtained by the North Carolina State University, Plants for Human Health Institute (Kannapolis, NC, USA). Wild Blueberry is a composite of lowbush wild blueberries (*V. angustifolium*, Aiton) harvested from sites, as earlier.[29] Ira and Montgomery are two different cultivars of rabbiteye blueberries (*V. asheii*, Reade). Supplemental Tables S1 to S3 shows the polyphenolic concentrations and Supplemental Table S4 compares diets' nutritional contents. All diets contained the recommended levels for rodents of 0.5% calcium and 0.2% phosphorus. Mice received food and water *ad libitum*, and food was replaced every 2 to 3 days. Animals were maintained on a 12-hour light/dark cycle in polycarbonate cages. The study was ended when statistically significant decreases in bone mineral density (BMD) upon gonadectomy were detected in control-fed animals (6 weeks for females and 4 weeks for males). Euthanasia was performed by sedation with 2% isoflurane (Abbott Laboratories, Chicago, IL, USA), followed by cervical dislocation. All animal procedures were approved by the Institutional Animal Care and Use Committee of Indiana University School of Medicine, and animal care was carried out in accordance with institutional guidelines.

BMD measurement and micro-CT analysis

Lean body mass and BMD of the total body (excluding head and tail), lumbar spine (L_1 to L_6), and femur were measured by dual-energy X-ray absorptiometry (DXA) using a PIXImus II densitometer (GE Medical Systems, Lunar Division, Madison, WI, USA). Initial BMD was taken 3 days before and 6 or 4 weeks after gonadectomy for female or male mice, respectively. Mice were randomized at the beginning of the study based on initial spinal BMD to assure no statistical differences among experimental groups. BMD was expressed as percent change per month using the following equation: % change in BMD/mo = 30d × [100 × [(Final BMD – Initial BMD) / Initial BMD] / d]. Total body weight and fat mass were also expressed as percent change per month. For micro-CT analysis, soft tissue was removed, and bones were fixed in 10% neutral-buffered formalin and stored in 70% ethanol until scanned at 10-μm resolution (SCANCO 35, SCANCO Medical, Brüttisellen, Switzerland). Cancellous bone measurements were taken for the entire vertebral body excluding the 60-μm region adjacent to the growth plates, and cortical bone measurements were taken in a 0.2-mm region located at the femoral midpoint.[30,31] Nomenclature follows the recommended guidelines.[32]

Serum biochemistry

Blood was collected from the jugular vein of 3-hour fasted mice. C-terminal telopeptides of type I collagen (CTX) and N-terminal propeptide of type I procollagen (P1NP) was measured using enzyme-linked immunosorbent assays with the positive and negative controls provided by the manufacturers (Immunodiagnostic Systems Inc., Gaithersburg, MD, USA).[30,33]

Quantitative PCR

Total RNA was extracted from vertebral lumbar bones (L_6) that were carefully cleaned from soft tissues and qPCR was performed as earlier.[33] Briefly, cDNA was synthesized using high-capacity cDNA reverse transcription (Applied Biosystems Inc., Foster City, CA, USA). Primer and probe sets were from Applied Biosystems or Roche Applied Science (Indianapolis, IN, USA). Relative mRNA expression was quantified and normalized to *Gapdh* expression using the ΔCt method. Ratios are expressed as fold change versus WT SHAM mice of the corresponding sex fed the control diet. No statistical differences were detected in bone mRNA expression levels for *Gapdh* in SHAM, OVX, ORX, WT, and Nrf2 KO mice fed with any of the diets. Similar results we found when CT values were normalized by a different housekeeping gene (*Rplp2*, a ribosomal protein).

Microbiome analysis

Fecal gDNA was extracted using FastDNA Spin (MP Biomedicals, Santa Ana, CA, USA). Quality was evaluated by Nanodrop 1000 spectrophotometry (Thermo Fisher Scientific, Wilmington, DE, USA) and agarose gel analysis. DNA was quantified using a Nanodrop 3300 fluorospectrometer after Hoechst dsDNA dye staining. The 16S rRNA gene was PCR amplified using primers targeting region V3 to V4: 343-forward TAC GGR AGG CAG CAG and 804-reverse CTA CCR GGG TAT CTA ATC C.[34,35] Primers with dual index tags were used to differentiate multiple samples in a single run (Illumina, San Diego, CA, USA), as earlier.[36] Reactions were carried out using ~10 ng of template DNA in Q5 High Fidelity DNA Polymerase 2X master mix (New England Biolabs, Ipswich, MA, USA). PCR amplicons were purified using AxyPrepMag PCR clean-up kit (Axygen Scientific, Big Flats, NY, USA) and quantified using a Nanodrop 3300 fluorospectrometer after staining with the QuantiFluor dsDNA System (Promega, Madison, WI, USA). Equimolar amounts of amplicons from each sample were combined and sequenced using a MiSeq Illumina system (Purdue Genomics core facilities). Sequences were analyzed using the QIIME 2 pipeline (version 2017.6.0),[37,38] and included quality check, denoising, and merging of paired end reads, and amplicon sequence variants (ASV) were selected using DADA2.[39] Taxonomic assignments were made using the Silva data set (version 132_99).[40] The lowest number of reads among the samples was chosen to rarefy data sets to use equal number of reads for all community comparisons. α-diversity measurements were used for richness and evenness (Shannon diversity).[41,42] Completeness of ASV representation was estimated by Good's coverage, and ranged from 99.999% to 100%, indicating that the analysis included almost all taxa. Differences in α-diversity metrics were determined using nonparametric ANOVA equivalent, Kruskal–Wallis test with 999 permutations. β-diversity measures were calculated using principal coordinate analysis (PCoA) of weighted phylogenetic UniFrac distances[43] and nonphylogenetic Jaccard distance.[44] Significant differences in β-diversity among communities were determined using 999 permutations of analysis of similarity (ANOSIM). Potential taxa differentiating diet and sex was determined using analysis of composition of microbiomes (ANCOM).[45]

Statistical analysis

Scientific rigor was achieved by performing analyses in a blinded manner and validating the results by at least a second investigator. Data are expressed as means ± standard deviation (SD). Statistical analysis was performed using SigmaPlot 12.0 (Systat Software Inc., San Jose, CA, USA). Previous studies showed that spinal BMD reductions after ovariectomy in mice are on the order of 1.5 standard deviations and that at least seven mice per group are required to confer an 80% power to detect a difference in means of this size using a one-sided significance test at the 0.05 level. Three-way ANOVA was performed for the independent variables of diet (control, Wild Blueberry, Ira, Montgomery), genotype (WT, KO), and operation (SHAM, OVX, ORX), followed by a Tukey post hoc test when appropriate. Two-way ANOVA was used when the three-way ANOVA detected interactions between independent variables, followed by a Tukey post hoc test when appropriate. Each main effect and interaction in the ANOVAs was tested using a significance level of 0.05. Outliers were identified by the 1.5 interquartile range rule for BMD[46] and the two SD range rule for other measurements.

Results

A diet containing Montgomery blueberries fully prevents bone loss and architectural deterioration induced by estrogen deficiency but not by androgen deficiency in mice

We examined the skeletal effect of three different diets containing 10% dry weight of three distinct cultivars of blueberries fed to WT skeletally mature female or male mice. Overall there was not a genotype effect on BMD, with exception for an increase in spinal BMD of Nrf2 KO, SHAM-operated females fed the Ira diet compared with the corresponding WT mice (Supplemental Fig. S1). Mice fed a control diet exhibited the expected loss of BMD upon gonadectomy, as quantified by DXA and expressed as % change BMD/mo (Fig. 1). Only one of the three blueberry-enriched diets, the one containing berries from the Mont cultivar, prevented bone loss induced by estrogen deficiency in OVX mice at all sites (total, spinal, and femoral) (Fig. 1*A*; Supplemental Fig. S1*A*). However, OVX mice fed the other blueberry-enriched diets (Ira and Wild Blueberry) exhibited reductions in BMD similar to OVX mice fed the control diet versus the respective SHAM-operated mice, although mice fed the Wild Blueberry diet or WT, but not Nrf2 knockout, mice fed the Ira diet showed protection in the spine (Supplemental Fig. S1*A*). In contrast, none of the berry-containing diets prevented the bone loss induced by ORX in male mice (Fig. 1*B*; Supplemental S1*B*), except for a minimal effect of the Mont diet on femoral BMD observed in the WT mice, but not in the Nrf2 KO mice. Further, the response of Nrf2 KO either female or male mice was indistinguishable from that of the respective littermate WT mice regarding the effect of gonadectomy and diets (Fig. 1*A*, *B*; Supplemental Fig. S1*A*, *B*).

Microarchitectural deterioration induced by estrogen deficiency was also prevented in female mice fed the Mont diet, as quantified by micro-CT (Fig. 2*A*; Supplemental Fig. S2*A*, *B*). The reduction in both cancellous and cortical bone volume (BV/TV and Ct.Ar/Tt.Ar,[32] previously named BA/TA, respectively), the trabecular and cortical thinning (Tb.Th and Ct.Th, respectively), and the decrease in trabecular number (Tb.N) exhibited by OVX mice (either WT or KO) was prevented in mice fed the Mont diet (Supplemental Fig. S2*A*, *B*). Although no statistical differences were detected in trabecular spacing (Tb.Sp) by three-way ANOVA, a tendency for increased Tb.Sp in OVX WT and KO mice fed the control diet was observed. Further, a main effect of OVX increasing the size of the marrow cavity was detected in mice fed the control diet but not in mice fed the Mont diet (Supplemental Fig. S2*B*). Thus, the average marrow area (Ma.Ar) in OVX (WT and KO) mice fed the control diet was increased compared with the average Ma.Ar of SHAM (WT and KO) mice. Moreover, this difference became significant in the KO mice when WT or KO mice were compared individually. In contrast, ORX induced similar microarchitecture deterioration of bone (in both WT and KO mice) fed with either control or Mont diet (Supplemental Fig. S5*A*–*C*). Nevertheless, and similar to the small effect of the Mont diet found in the femoral BMD (Fig. 1*B*), ORX mice fed the Mont diet lost less bone compared with ORX mice fed the control diet (Supplemental Fig. S5*A*–*C*).

Taken together, these findings indicate that the female, but not the male, skeleton of mice fed the Mont diet was fully protected from loss of bone mass and architectural deterioration induced by sex steroid deficiency, through a mechanism independent of the transcription factor Nrf2.

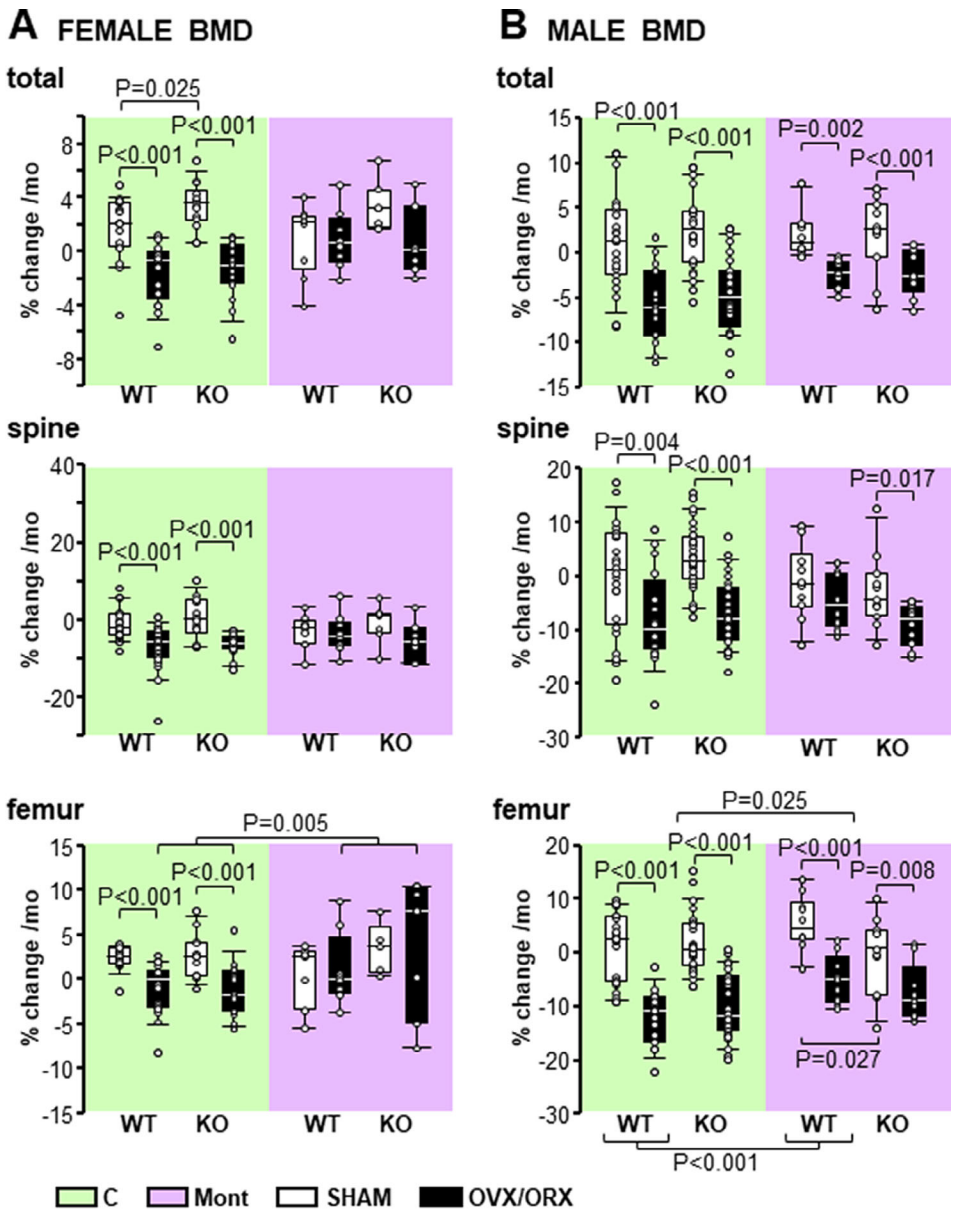

Fig 1. Mont diet fully prevents bone loss induced by estrogen deficiency but not by androgen deficiency in mice. Percent change in BMD/mo in gonadectomized WT and Nrf2 KO mice fed with the indicated diets. C = control diet; Mont = Montgomery diet; Ira = Ira diet; Wild = Wild Blueberry diet; OVX = ovariectomized mice; ORX = orchiectomized mice; SHAM = SHAM-operated mice. The *p* values are comparisons versus the corresponding C-fed mice by three-way ANOVA, Tukey post hoc test. (*A*) N values for WT-SHAM, WT-OVX, KO-SHAM, and KO-OVX are for C: 20, 19, 14, and 15; for Mont: 9, 9, 7, and 7. (*B*) N values for WT-SHAM, WT-ORX, KO-SHAM, and KO-ORX are for C: 26, 16, 29, and 28; for Mont: 10, 10, 11, and 10.

Preservation of the female skeleton by the Montgomery diet is mediated by inhibiting bone resorption without activating canonical estrogenic signaling

Consistent with the recognized pro-resorptive effect of sex steroid deficiency, circulating levels of the bone resorption marker CTX were elevated in OVX (WT and KO) mice fed the control diet (Fig. 2*B*). However, OVX mice fed the Mont diet exhibited similar CTX levels as SHAM-operated mice. No significant differences in circulating P1NP levels were detected by OVX, Nrf2 deficiency, or the Mont diet (Fig. 2*C*). However, a main Mont diet effect was detected compared with control diet (Fig. 2*C*). The effect of resorption parallels the preservation of bone microarchitecture in OVX mice fed the Mont diet (shown in Fig. 2*A* and Supplemental Fig. S2*A*, *B*) and is consistent with the notion that thinning of trabecular and cortical bone is a recognized consequence of increased resorption. In contrast and consistent with the systemic bone loss induced by ORX in mice fed with any diet (Fig. 1*B*; Supplemental Fig. S5*A*–*C*), CTX was elevated to a similar extent in ORX mice fed either control or Mont diet, as detected statistically by a main group effect (all sham versus all ORX) (Supplemental Fig. S5*D*).

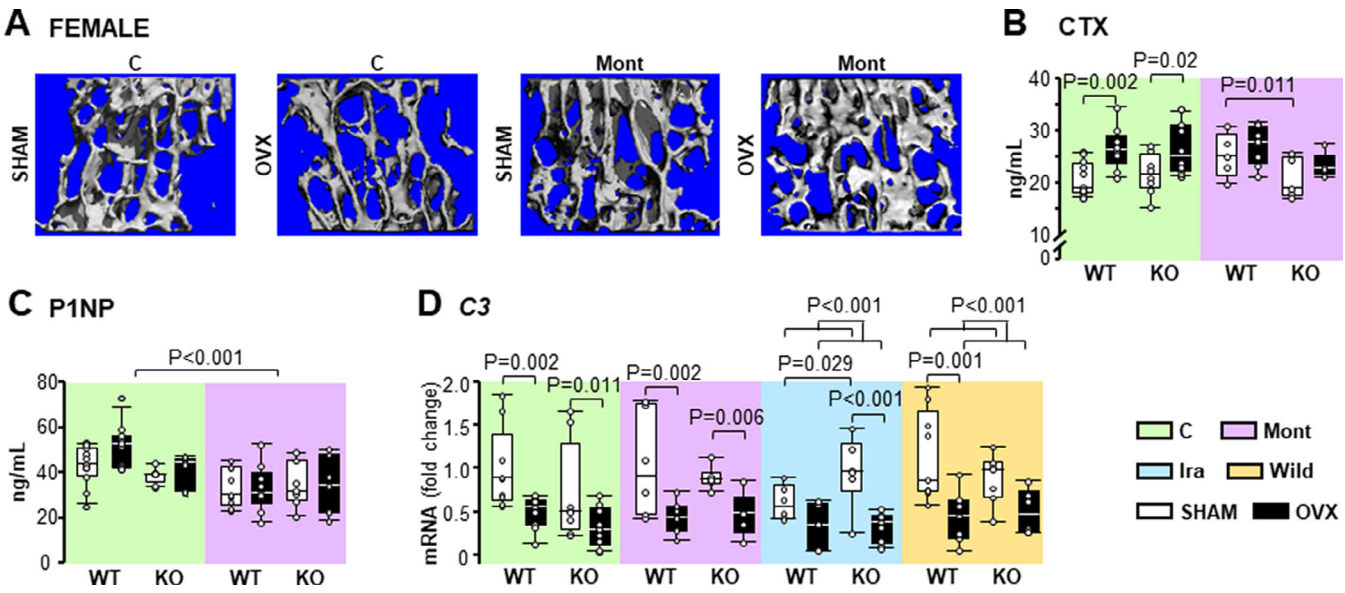

Fig 2. Mont diet protects against architectural deterioration and increased resorption induced by estrogen deficiency without activating canonical estrogenic signaling in bone. (*A*) Representative 3D reconstruction images of L_4 lumbar vertebral samples with the calculated closest absolute values to the mean for the corresponding experimental groups are shown, analyzed by micro-CT. Serum bone resorption marker CTX (*B*) and bone formation marker P1NP (*C*) after 6 weeks of operation. (*D*) Expression of *C3* gene in lumbar vertebra (L_6). C = control diet; Mont = Montgomery diet; Ira = Ira diet; Wild = Wild Blueberry diet; OVX = ovariectomized mice; SHAM = SHAM-operated mice. The *p* values are comparisons versus the corresponding C-fed mice by three-way ANOVA, Tukey post hoc test. (*B*, *C*) N values for WT-SHAM, WT-OVX, KO-SHAM, and KO-OVX are for C: 13, 12, 11, and 11; for Mont: 9, 9, 7, and 7. (*D*) N values for WT-SHAM, WT-OVX, KO-SHAM, and KO-OVX are for C: 12, 12, 12, and 12; for Mont: 8, 9, 6, and 6; for Ira: 8, 8, 8, and 9; and for Wild: 11, 12, 11, and 9.

Skeletal protection by certain dietary components (like soy) is accompanied by activation of similar signaling pathways as those triggered by estrogen, with the potential increased risk of negative side effects in other tissues.[47–49] In contrast, the protective bone effect of Mont diet was not accompanied by increased estrogenic canonical signaling. Indeed, OVX (WT and KO) mice fed control diet showed the expected lower expression of the estrogen response element (ERE)-containing gene *Complement component 3* (C3) in bone compared with SHAM-operated mice (Fig. 2*D*). C3 expression remained low in OVX mice fed with Mont diet (as well as in mice fed any of the other diets). Similarly, bones from ORX (WT and KO) mice exhibited lower expression of the androgen response element (ARE)-containing gene *Reproductive homeobox 5* (*Rhox5*), which was not altered by any of the diets (Supplemental Fig. S5*E*).

The EAR in bone is regulated by Nrf2 and sex steroid deficiency only in the female skeleton, and it is amplified by the Montgomery diet

Our earlier studies demonstrated that the skeletal EAR (antioxidant and phase II detoxifying enzymes) is controlled by Nrf2 in a distinct manner depending on sex.[28] Specifically, whereas EAR expression in bone is reduced in both growing and skeletally mature female Nrf2 KO mice, EAR expression is low only in growing KO male mice. Consistent with this evidence, in the current study using skeletally mature mice, only female, but not male, Nrf2 KO mice fed control diet exhibited lower EAR in bone (Fig. 3*A*, *B*; Supplemental Fig. S3*A*, *B*). Further, gonadectomy increased the EAR in bone only in females but not in males fed control diet (Fig. 3*A*, *B*; Supplemental Fig. S3*A*, *B*). In addition, the Mont diet increased EAR in both female and male mice combined, whereas the Ira and Wild Blueberry diets reduced EAR expression in bone as detected by main group effects (Fig. 3*A*, *B*; Supplemental Fig. S3*A*, *B*).

Taken together, these results indicate that Nrf2 regulates EAR in a sex-dependent manner under physiological and sex steroid–deficient conditions. Thus, loss of Nrf2 downregulates EAR in the female but not male skeleton, and sex steroid deficiency only upregulates EAR in the female skeleton. Remarkably, even though the Mont diet increases the EAR in both female and male bone, Mont only protects the female skeleton. These findings suggest that EAR is not part of the skeletal response to androgen deficiency and that increased EAR by the diet is not sufficient to achieve full protection of the male skeleton.

The Montgomery diet also protects against weight gain, fat mass accumulation, and loss of muscle mass induced by estrogen deficiency

Female mice (both WT and KO) exhibited changes in body composition upon OVX, with increased weight and accumulation of peripheral fat mass, but only when fed control, Ira, or Wild Blueberry diets (Fig. 4). In contrast, OVX mice fed the Mont diet were protected from these changes. In addition, OVX mice fed the control diet showed reductions in the wet weight of the gastrocnemius muscle (Fig. 4), whereas mice fed the Mont (or Wild Blueberry) diet were protected. In contrast, the decreased body weight exhibited by male mice that underwent ORX were not altered by any of the diets (although the decrease in body weight in WT mice fed the Ira diet did not reach significance) (Supplemental Fig. S6*A*). In addition, fat mass was not altered

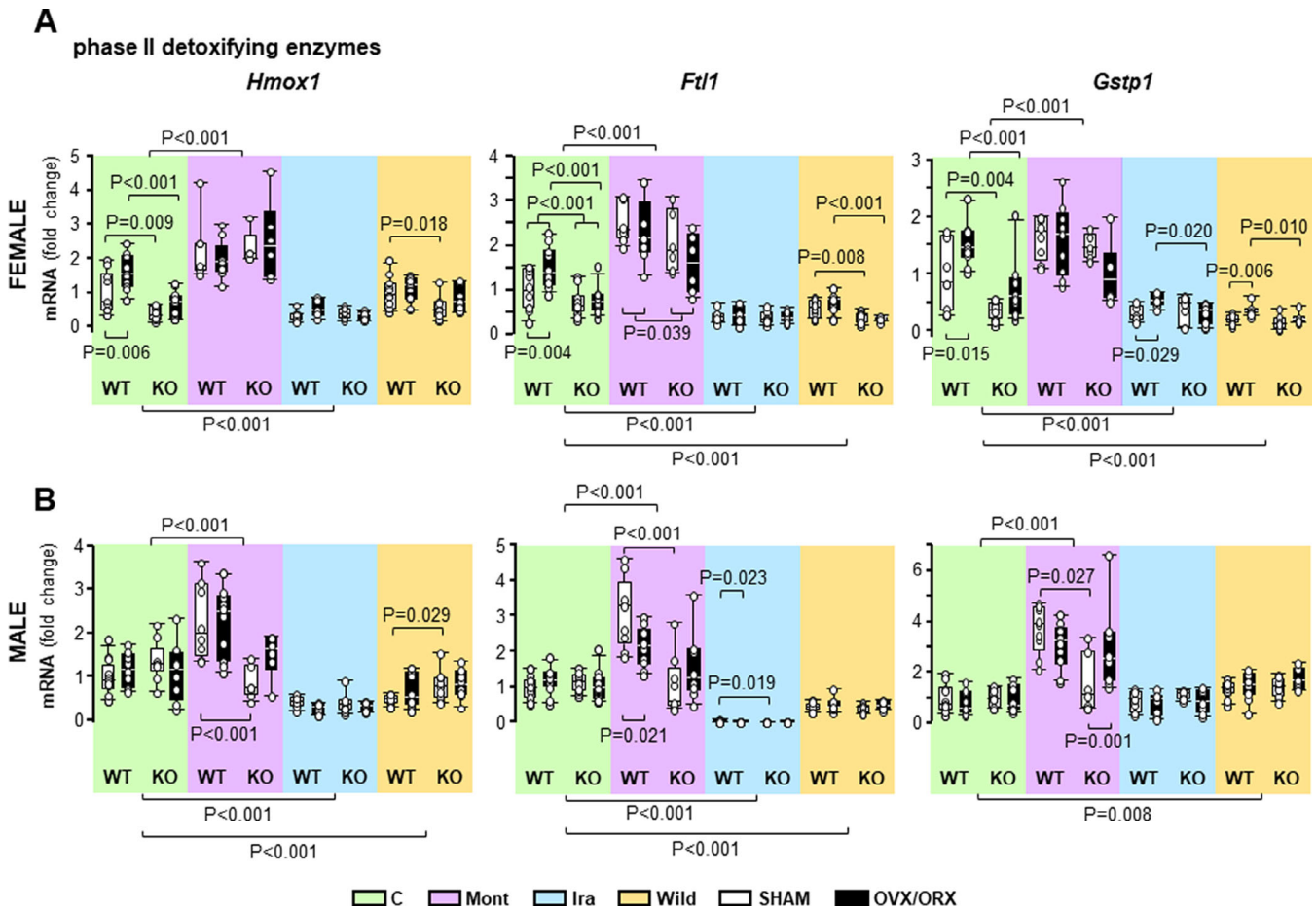

Fig 3. The endogenous antioxidant response (EAR) in bone is regulated by Nrf2 and sex steroid deficiency only in the female skeleton, and it is amplified by the Mont diet. (*A*, *B*) EAR gene expression in lumbar vertebral L_6 bones from female and male mice fed the indicated diets. C = control diet; Mont = Montgomery diet; Ira = Ira diet; Wild = Wild Blueberry diet; OVX = ovariectomized mice; ORX = orchiectomized mice; SHAM = SHAM-operated mice. The *p* values are comparisons versus the corresponding C-fed mice by three-way ANOVA, Tukey post hoc test. (*A*) N values for WT-SHAM, WT-OVX, KO-SHAM, and KO-OVX are for C: 12, 12, 12, and 12; for Mont: 8, 9, 6, and 6; for Ira: 8, 8, 8, and 9; and for Wild: 11, 12, 11, and 9. (*B*) N values for WT-SHAM, WT-ORX, KO-SHAM, and KO-ORX are for C: 14, 12, 12, and 12; for Mont: 9, 10, 9, and 9; for Ira: 9, 9, 9, and 9; and for Wild: 11, 11, 11, and 9.

by androgen deficiency overall, except for a decrease in KO mice fed control diet (Supplemental Fig. S6*B*). Regarding changes in muscle weight, ORX increased the % muscle weight to total body weight, although this effect was driven by the loss of body weight rather than by an effect of androgen deficiency on muscle (Supplemental Fig. S6*C*). Further, the only diet-related action in males was an overall increase in muscle mass induced by the Mont diet, detected as a main effect compared with control-fed mice.

Protection from musculoskeletal loss and peripheral fat accumulation by the Mont diet is linked to higher gut bacterial diversity—a hallmark of healthy intestinal microbiome

Hormonal changes as well as dietary components might impact the gut microbiome, which in turn could affect homeostasis of several tissues. In the current study, loss of sex steroids (OVX or ORX versus respective SHAM) did not induce significant changes in the variety of bacterial communities, named α-diversity and measured by the Shannon index,[41] in mice fed either control or Mont diet (Supplemental Fig. S4*A*). Because of the lack of impact of sex steroid status, all female (OVX and SHAM) or male (ORX and SHAM) mice were pooled to examine the potential effect of the diet on the microbiome (Fig. 5*A*–*D*; Supplemental Fig. S4*A*–*C*). The Mont diet increased gut microbiome α-diversity compared with the control diet (Fig. 5*A*), and the response was of higher magnitude in female than in male mice. In addition, Mont diet shifted the relatedness among bacterial communities, named β-diversity, further in female than in male mice (Fig. 5*B*; Supplemental Fig. S4*B*). This Mont diet effect was detected when relatedness was assessed by PCoA, accounting for both the absence or presence of taxa (Jaccard distance)[44] (Fig. 5*B*) and by the relative taxa abundance (weighted UniFrac)[43] (Fig. 5*C*; Supplemental Fig. S4*B*). Detailed analysis of the phylogenetic diversity using ANCOM[45] detected lower prevalence of the bacterial communities *Bifidobacterium* and *Coriobacteriaceae UCG-002* in Mont-fed female, but not male, mice (Supplemental Fig. S4*C*). Further, females exhibited even higher abundance of both of these taxa than corresponding males in control diet–

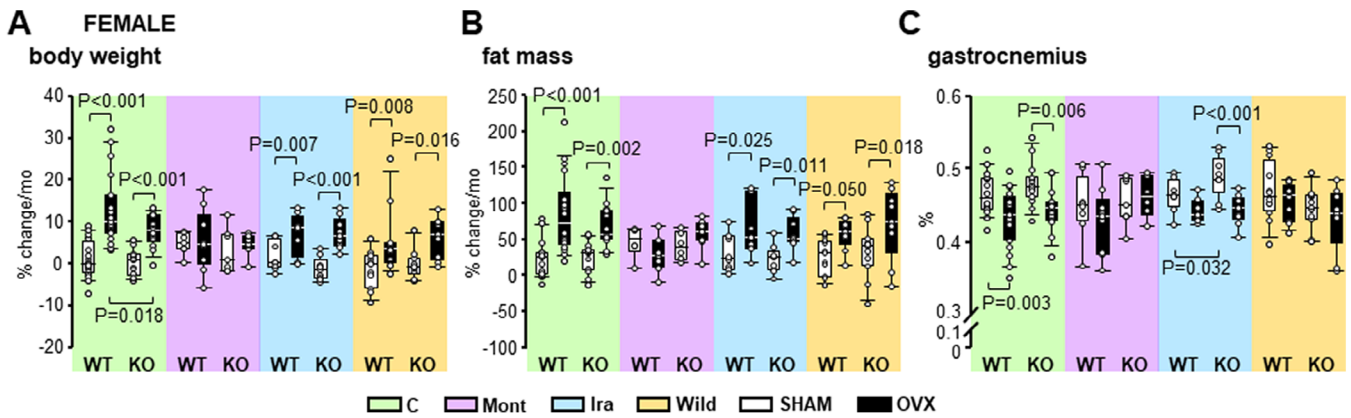

Fig 4. Mont diet prevents weight gain, fat mass accumulation, and loss of muscle mass induced by estrogen deficiency. (*A*) Body weight and (*B*) fat mass expressed as percent change by month. (*C*) Wet weight of gastrocnemius muscles normalized by total body weight. C = control diet; Mont = Montgomery diet; Ira = Ira diet; Wild = Wild Blueberry diet; OVX = ovariectomized mice; SHAM = SHAM-operated mice. The *p* values are comparisons versus the corresponding C-fed mice by three-way ANOVA, Tukey post hoc test. (*A–C*) N values for WT-SHAM, WT-OVX, KO-SHAM, and KO-OVX are for C: 20, 19, 14, and 15; for Mont: 9, 9, 7, and 7; for Ira: 8, 8, 9, and 9; and for Wild: 12, 12, 11, and 9.

fed mice, indicating sex-specific distinctions in gut microbiome biodiversity. ANCOM also detected a main effect of the Mont diet inducing higher prevalence of the bacterial communities *Ruminococcus 1* (starch fermenters), *Prevotellaceae UCG-001*, and the unclassified taxon *Coriobacteriales Incertae*, when male and female Mont-fed mice were analyzed all together (Fig. 5*D*). In addition, the magnitude of the increased prevalence induced by the Mont diet was higher in females than in males for *Ruminococcus 1* and *Prevotellaceae UCG-001* bacterial communities.

Discussion

The negative impact of musculoskeletal frailty on the health and quality of life of the aging population is fully recognized. However, there is a substantial treatment gap for diseases of bone and muscle mainly because the current pharmacologic standard of care presents varying effectiveness, low compliance, and high costs. The present study identifies a nutritional intervention that protects bone, skeletal muscle, and body composition from the undesirable effects of estrogen deficiency. The underlying protective mechanism of the diet is sex-specific, involves stimulation of non-traditional/Nrf2-independent antioxidant responses, and does not encompass activation of canonical estrogenic signaling (Fig. 6). Further, musculoskeletal protection was linked to healthier manifestations in the gut microbiome signature with higher diversity in number and phylogenetic relatedness of bacterial communities. Because dietary interventions might have higher compliance[50] and lower costs compared with pharmacologic agents, nutrition-based approaches could be more accessible than pharmacological interventions and contribute to narrowing the treatment gap for musculoskeletal diseases.

Diet- and sex-specific musculoskeletal protection

The cause for the effective musculoskeletal protection and prevention of peripheral fat accumulation with the Mont diet, but not with other blueberry types, is not fully understood and is the subject of ongoing studies. Differences in either composition or component bioavailability may explain the distinct properties of Mont compared with the other blueberry cultivars. Indeed, the total phenolic and anthocyanin content by weight of the Mont diet is lower compared with the other berries used in our study. However, bioavailability of some of the Mont components is three- to eightfold higher as indicated by the levels of anthocyanins found in the circulation in a rat study.[51] In any event, the disparities among berry types in this study might explain the varying response and effectiveness to dietary supplements reported in human populations as well as in preclinical animal models.[23–27,52–54] Furthermore, the Mont diet unexpectedly did not protect against androgen deficiency, providing an additional source of variation in the reported response to dietary interventions. Taken together, these considerations highlight the importance of tailoring therapies to patient individualities, including sex.

The ROS/Nrf2/EAR axis in bone

Cellular increases in ROS activate Nrf2, which in turn mounts an EAR consisting of increased expression of enzymes that neutralize ROS effects.[14–16] It remains unknown whether there is a direct relationship between ROS levels and the EAR, or whether the ROS/Nrf2/EAR axis is regulated in a similar or different manner dependent on sex, and particularly in bone. The current findings shed light on these unanswered questions. ROS levels are increased in the bone/bone marrow compartment with loss of sex steroids in both female and male mice.[12] In the current study, we found that enhanced EAR in bone is only triggered by depletion of estrogens in females, whereas depletion of androgens in males did not elicit changes in skeletal EAR. In addition, although the Mont diet increased EAR in bone from both female and male mice, it only protected the female skeleton from gonadectomy-induced bone loss. These findings raise the possibility that the susceptibility of the skeleton to hormonal changes, age, or even therapies might not depend on ROS levels in the bone/bone marrow microenvironment as proposed earlier, but depend rather on expression levels of EAR enzymes in the skeleton, which are also altered by individual qualities, like sex.

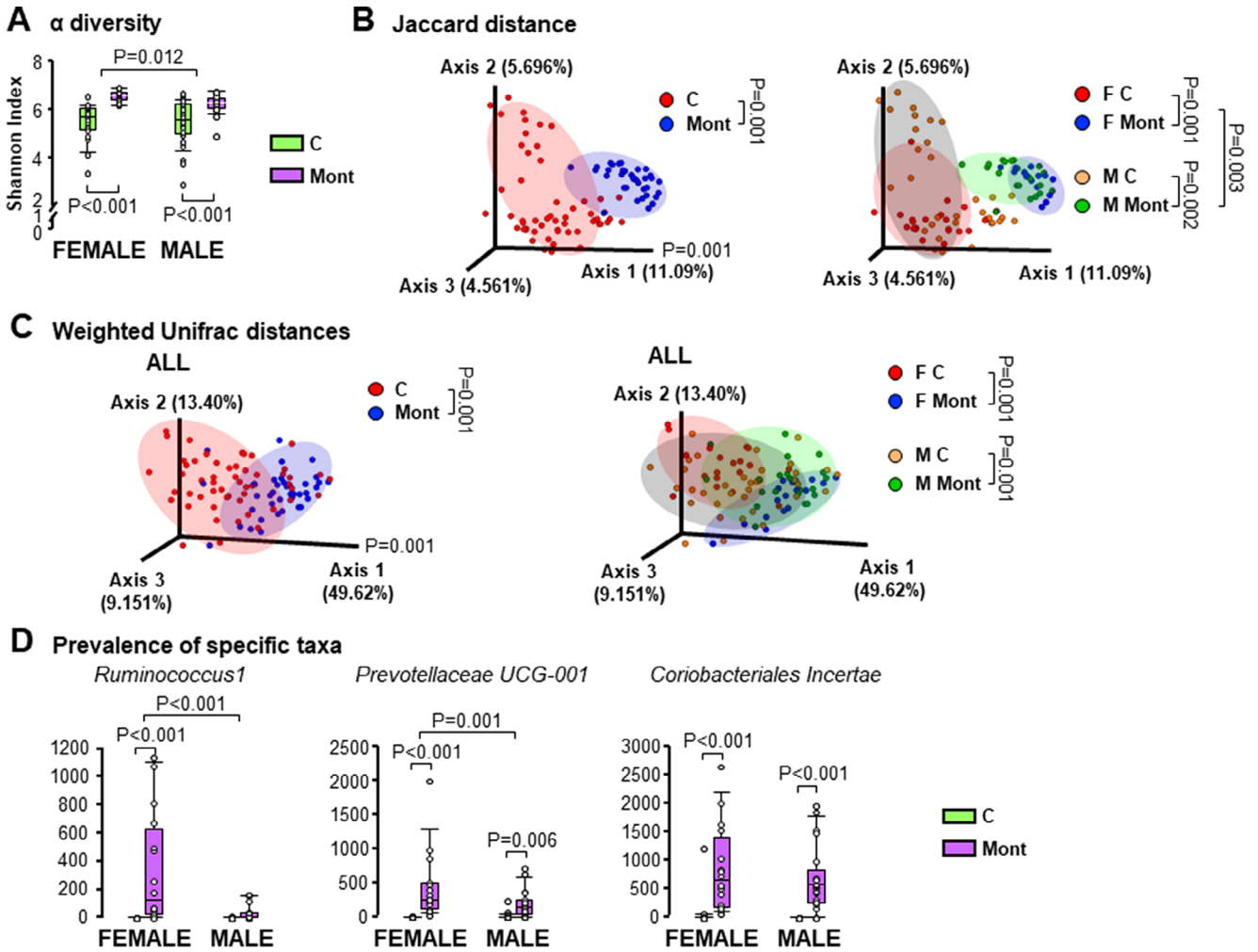

Fig 5. Mont diet–fed WT mice exhibit higher gut bacterial diversity in a sex-specific manner. α-diversity quantified by the Shannon index (*A*). β-diversity calculated by principal coordinate analysis (PCoA) of (*B*) Jaccard distance or (*C*) weighted UniFrac distances. (*D*) Prevalence of specific taxa in bacterial communities, detected by ANCOM analysis of microbiome composition. C = control diet; Mont = Montgomery diet. (*A*–*D*) N values for female WT for C: 20; for female WT Mont: 16. N values for male WT for C: 35; for male WT Mont: 20. The *p* values are comparisons versus the corresponding C-fed mice by Kruskal–Wallis for *A*, by ANOSIM for *B* and *C*, and by ANCOM analysis of microbiome composition for *D*.

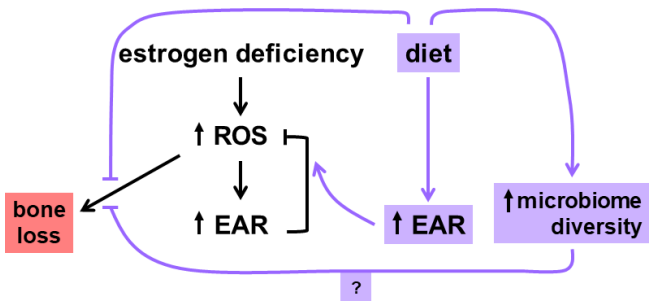

Fig 6. Dietary protection from estrogen deficiency–induced bone loss is achieved by boosting the endogenous antioxidant response (EAR) and is linked to promotion of gut microbiome diversity. Nutritional intervention with the Montgomery blueberry diet protects bone, skeletal muscle, and body composition from the undesirable effects of estrogen deficiency by heightening the EAR. The underlying protective mechanism is sex-specific, independent of the classical antioxidant transcription factor Nrf2, and unrelated to canonical estrogenic signaling. Furthermore, diet-induced musculoskeletal benefits are linked to higher α- and β-diversity, hallmark characteristics of a healthier gut microbiome signature.

Our previous findings demonstrated that the EAR was regulated by the transcription factor *Nrf2* in both female and male skeletons and in growing and adult skeletons.[28] Taken together these findings demonstrate that the EAR in bone is indeed responsive to the traditional antioxidant transcription factor *Nrf2* but that in the frame of estrogen deficiency and dietary interventions, *Nrf2* is not required to elicit a skeletal response, either for the bone loss due to estrogen deficiency or for the bone protection by the diet. Thus, the underlying protective mechanism is not only sex-specific but also involves activation of nontraditional, *Nrf2*-independent defense responses.

Dietary protection and independence from canonical estrogenic signaling

Another remarkable feature of the Mont diet's skeletal protection is the lack of canonical estrogenic activation. These findings contrast with other dietary supplements that elicit skeletal protection by mimicking classical estrogenic signaling.[47,55] Estrogen receptor signaling has protective effects on the skeleton; however, it also causes deleterious side effects in other organs, including the uterus and breast. Therefore, the independence from canonical estrogenic signaling by the Mont diet in preventing bone loss induced by estrogen deficiency represents an important advantage compared with hormone replacement therapies. Future studies beyond the scope of the current study will address whether the skeletal protection by the diet is a consequence of activation of non-classical ER-dependent mechanisms in bone cells (such as kinase signaling or anti- versus pro-apoptotic signaling in osteoblasts/osteocytes versus osteoclasts, respectively) or results from ER-independent compensatory actions.

The microbiome, diets, and the musculoskeletal system

Protection of the musculoskeletal system from the effects of estrogen loss by the Mont diet was linked to changes of the gut microbiome signature toward higher diversity—a hallmark of intestinal health. An association between gut microbiome and skeletal changes has been shown to involve leakage of the intestinal blood barrier and increased circulating endotoxin in estrogen deficiency, glucocorticoid excess, and parathyroid hormone–induced bone anabolism.[56–58] Whether the intestinal barrier is affected in mice fed the Mont diet is the subject of current studies and would support a connection between activation of anti-inflammatory pathways and nutrition. Future studies in humans are warranted to examine the effectiveness of blueberry diets on the skeleton, the linkage with microbiome diversity, as well as the sexual dimorphism found in mice in the current study.

Disclosures

Service on advisory boards: MGF: Florida Department of Citrus (Scientific Advisory Committee 2017–present); Clorox Company (Scientific Advisory Committee 2020). MAL: GSK Pfizer CMI-NA Strategic Advisory Council (2019–present); Enviotic Scientific Advisory Board (2019–present); Clorox/RenewLife Woman & Wellness Science Council (2019–present); International Scientific Advisory Council of ICPH2017 (International Conference on Polyphenols and Health) (2017–2021). DBB: Fibrous Dysplasia Foundation (Scientific Advisory Board). CHN: Michigan State Institute for Integrative Toxicology National Institute of Environmental Health Sciences (NIEHS) Superfund Program. CMW: California Plum Board; International Life Sciences Institute; Food and Drug Administration Science Board; Yogurt in Nutrition (YINI) Board.

Service on boards of directors: MGF: Sensient Technologies Corporation (Independent Director; 2015–present); International Life Science Institute North America – ILS-NA (Trustee; 2016–present). MAL: Editorial Board/Associate Editor, Nutrition & Healthy Aging (2019–present); Editorial Board, Annual Reviews Food Science & Technology (2017–2021). TB: ASBMR President; ORS Musculoskeletal Biology Workshop Co-Chair; Endocrine Fellows Forum Co-Director.

Other consulting: MGF: Juice Products Association (2019–2020). MP: Calcilyix Incorp Inc. DBB: Agnovos (orthopedic hip implants); Parker Waichman (Gadolinium). CMW: Tate & Lyle; Bayer.

Serving as an expert witness or consultant in litigation for commercial entities: MGF: Kleinfeld, Kaplan and Becker LLP (2019).

Honoraria or royalties for books or publications or for lectures (speaker fees) or participating in a speakers bureau: MGF: books: John Wiley & Sons, Inc.; Elsevier speaker honoraria: Unilever; Welchs; Council for Responsible Nutrition; Biofortis; International Food Information Council. MAL: FFAR (Foundation for Food & Agricultural Research) honoraria in 2020 for serving on their grant review panel. DBB: Elsevier; Springer; Japan Implant Practice Society. CHN:Invited guest speaker at universities or research institutes.

Research grants, direct salary support or other financial support from commercial entities: AYS: NIH: T32-AR065971. MGF: Pepscio; Welch's; Newell Brands. MAL: MAL and Giuseppe Valacchi, in vitro Study to Compare Liquids in a Cell Culture (Nrf2 Activation), Reoxcyn Discoveries Group; Formulation of Versatile, Stable, Highly Functional Protein-Polyphenol Colloidal Aggregate Ingredients, With Attenuated Allergenicity and Capacity for Broad-Spectrum Incorporation Into Whole Food Matrices, Standard Process, Inc. DBB: NIH: research grants and salary support; Department of Defense (PR191841); National Basketball Association and GE Healthcare Orthopedics and Sports Medicine Collaboration, activities and factors influencing the magnitude and distribution of tibial bone strains in basketballers. CHN: gift grant: NanoChem Solutions; previous support: Tate & Lyle. TB: NIH and VA: research grants and salary support; PharmaMar: research grants.

Stock holdings and/or stock options in pharmaceutical, medical device, or diagnostic companies: TB: Radius Health Inc.

Partnerships, warrants, royalties for inventions (licensing revenues), or other ownership interest: MAL: US Provisional Application 62/438,246, filed December 22, 2016, "Polyphenol-protein compositions and methods of making," which more broadly covers functional food applications of a semi-viscous liquid preparation of protein-polyphenol stabilized colloidal aggregate particles. Licensed by Sinnovatek, 2016. MAL: Hypoallergenic food-grade protein matrix for immunotherapy applications. Disclosure assigned file number 12120, managed by Kultaran Chohan. December 2011. Provisional patent application #61/669353 "Hypoallergenic food-grade protein matrices and uses thereof," filed July 9, 2012. Conversion of patent July 9, 2013. 61/669,353 NCSU ref 12–120; USDA ref. 0057.12; UNC-CH ref not known; EG ref NS12004WO. US Provisional Application No. 62/076,978 filed November 7, 2014, "Methods and compositions for attenuating allergenicity in protein products," which describes the masking of allergenic epitopes on edible proteins (peanut, milk, egg, soy) via irreversible and reversible binding of fruit/plant proanthocyanidins, in order to attenuate allergenicity. Licensed by Sinnovatek, 2016.

Acknowledgments

The authors acknowledge EG Atkinson for technical support. The Wild Blueberry composite lyophilized powder was donated by the Wild Blueberry Association of North America. This research was supported by the National Institutes of Health (R01-AT008754 and O2S1-AT008754 to CMW and TB; R01-AR059357 to TB; T32-AR065971 to AYS) and the Veterans Administration (I01 BX002104 and IK6BX004596 to TB). The contents do not represent the views of the US Department of Veterans Affairs or the United States Government.

Authors' roles: TB and CMW: conceptualization, experimental design, resources, formal analysis, supervision, project administration. AYS and TB: writing. AYS, GGP, RBC, MM, OJ, MC, KM: study execution, data acquisition, and skeletal phenotyping. CHN: microbiome analysis and statistics. MGF and MAL: diet provision and analysis. LDM and GPM: statistical analysis. MP and DBB: data interpretation and manuscript editing.

Author contributions: AYS: Formal analysis; investigation; visualization; writing-original draft; writing-review and editing. GGP: Investigation. MC: Investigation. KM: Investigation. RBC: Investigation. MM: Investigation. OJ: Investigation. LDM: Formal analysis. CPM: Formal analysis. MGF: Resources; writing-review and editing. MAL: Resources; writing-review and editing. MP: Writing-review and editing. DBB: Writing-review and editing. CHN: Formal analysis; investigation; writing-review and editing. CMW: Conceptualization; formal analysis; funding acquisition; project administration; resources; supervision. TB: Conceptualization; formal analysis; funding acquisition; project administration; resources; supervision; writing-original draft; writing-review and editing.

Peer Review

The peer review history for this article is available at https://publons.com/publon/10.1002/jbmr.4231.

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