

Original article

# Correlation network analysis shows divergent effects of a long-term, high-fat diet and exercise on early stage osteoarthritis phenotypes in mice

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## Abstract

**Background:** Obesity increases knee osteoarthritis (OA) risk through metabolic, inflammatory, and biomechanical factors, but how these systemic and local mediators interact to drive OA pathology is not well understood. We tested the effect of voluntary running exercise after chronic diet-induced obesity on knee OA-related cartilage and bone pathology in mice. We then used a correlation-based network analysis to identify systemic and local factors associated with early-stage knee OA phenotypes among the different diet and exercise groups.

**Methods:** Male C57BL/6J mice were fed a defined control (10% kcal fat) or high fat (HF) (60% kcal fat) diet from 6 to 37 weeks of age. At 25 weeks, one-half of the mice from each diet group were housed in cages with running wheels for the remainder of the study. Histology, micro computed tomography, and magnetic resonance imaging were used to evaluate changes in joint tissue structure and OA pathology. These local variables were then compared to systemic metabolic (body mass, body fat, and glucose tolerance), inflammatory (serum adipokines and inflammatory mediators), and functional (mechanical tactile sensitivity and grip strength) outcomes using a correlation-based network analysis. Diet and exercise effects were evaluated by two-way analysis of variance.

**Results:** An HF diet increased the infrapatellar fat pad size and posterior joint osteophytes, and wheel running primarily altered the subchondral cortical and trabecular bone. Neither HF diet nor exercise altered average knee cartilage OA scores compared to control groups. However, the coefficient of variation was  $\geq 25\%$  for many outcomes, and some mice in both diet groups developed moderate OA ( $\geq 33\%$  maximum score). This supported using correlation-based network analyses to identify systemic and local factors associated with early-stage knee OA phenotypes. In wheel-running cohorts, an HF diet reduced the network size compared to the control diet group despite similar running distances, suggesting that diet-induced obesity dampens the effects of exercise on systemic and local OA-related factors. Each of the 4 diet and activity groups showed mostly unique networks of local and systemic factors correlated with early-stage knee OA.

**Conclusion:** Despite minimal group-level effects of chronic diet-induced obesity and voluntary wheel running on knee OA pathology under the current test durations, diet and exercise substantially altered the relationships among systemic and local variables associated with early-stage knee OA. These results suggest that distinct pre-OA phenotypes may exist prior to the development of disease.

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**Keywords:** Inflammation; Knee; Mouse; Obesity; Wheel running

## 1. Introduction

Obesity is one of the most clinically significant and preventable risk factors for developing osteoarthritis (OA).<sup>1,2</sup> There are no disease-modifying treatments for OA, and current pain medications such as opioids and nonsteroidal anti-inflammatory drugs have limited long-term efficacy as well as

adverse side effects.<sup>3</sup> Obesity increases OA risk in both knee and hand joints, although the impact is greatest for the knee, where risk is elevated  $>2$ -fold.<sup>4–6</sup> Consequently, the average onset of disease occurs at a younger age in obese individuals,<sup>7</sup> resulting in an increase in the duration of disability and additional health risks before patients undergo joint replacement surgery.<sup>7,8</sup> The health concerns of OA extend beyond increased pain and disability; recent data show that painful knee OA is a risk factor for cardiovascular disease, all-cause mortality, and cardiovascular disease-linked death.<sup>9,10</sup>

Indeed, OA clusters with numerous metabolic conditions collectively referred to as the metabolic syndrome.<sup>11–14</sup>

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Understanding how these systemic metabolic conditions, such as excess adiposity, insulin resistance, hypertension, and dyslipidemia, contribute to OA pathogenesis and disease progression remains a significant gap in knowledge.<sup>15,16</sup> One of the barriers to addressing this knowledge gap is the lack of adequate experimental and analytical approaches to differentiate the disease-modifying role of systemic metabolic factors from local joint factors, such as altered mechanical loading. For example, significant weight loss improves metabolic and inflammatory outcomes and lowers OA risk; however, it also reduces joint forces.<sup>17–19</sup> A recent study sought to differentiate the contribution of obesity *per se* from serum leptin using a statistical regression approach that incorporated a factor mediation analysis.<sup>20</sup> The results suggested that approximately one-half of the effect of obesity (i.e., body mass index) on knee OA risk is due to serum leptin.<sup>20</sup> However, body mass index is not an accurate surrogate for joint loading, and approaches that comprehensively integrate biomechanical, inflammatory, and metabolic factors remain incomplete.<sup>21</sup>

Animal models provide a useful approach for testing the independent effects of obesity-related factors in OA pathogenesis. For example, animal studies have shown that obesity-related factors increase knee OA even in the absence of substantial weight gain. Specifically, high-fat (HF) diets, diets with a high ratio of n-6/n-3 polyunsaturated fatty acids, and high circulating triglycerides increase knee OA even when weight is not increased.<sup>22–26</sup> Leptin-deficient obesity,<sup>27</sup> microbiome alterations,<sup>28</sup> and altered dietary carbohydrates<sup>29</sup> are additional approaches that illustrate how obesity-related factors can be isolated from body weight to modify OA pathogenesis. We previously showed that voluntary exercise in young adult mice fed a very HF diet protected against early-stage knee OA despite no reduction in body mass or body fat or changes in the circulating levels of inflammatory cytokines.<sup>30</sup> An intriguing finding from this study was that, although the absolute levels of serum adipokines and cytokines were not altered with exercise, the correlations among proinflammatory circulating factors and other indices of metabolic disease (e.g., fasting blood glucose and adiposity) were greatly disrupted with exercise.<sup>30</sup> This finding suggests that the beneficial effects of exercise may operate on a systems-level scale that fundamentally alters how biological factors signal and evoke tissue-specific responses at different levels of biological organization.<sup>31</sup>

We recently reported that feeding C57BL/6J male mice a very HF diet from 6 to 52 weeks of age is sufficient to increase knee OA without introducing a joint injury.<sup>32</sup> Given that our prior study focused on a short-term exercise treatment (4 weeks) in young mice fed a very HF diet from 12 to 24 weeks,<sup>30</sup> we decided to test the effects of an HF diet and exercise in older animals. C57BL/6J male mice were fed a very HF diet from 6 to 25 weeks, and then one-half of the mice from each diet group were housed with running wheels until the end of the study at 37 weeks. The initial goal of the study was to determine the effect of exercise on systemic and local OA-related outcomes after the extended development of diet-induced obesity. The second goal was to test the effect of

diet-induced obesity and exercise on the associations between systemic factors (i.e., metabolic, inflammatory, and biomechanical outcomes) and local knee structural and OA-related variables. Characterizing the systemic and local factor networks associated with early-stage knee OA may reveal unique “pre-OA” phenotypes associated with the onset and progression of disease. Advances in bioinformatics, such as correlation-based network analyses, have made it easier to identify variables associated with disease outcomes under different experimental conditions.<sup>33,34</sup> Exercise is among the safest and most effective interventions to reduce OA pain and improve joint mobility and function.<sup>3,35,36</sup> Therefore, identifying how exercise alters an obesity-related OA network may improve our understanding of disease pathogenesis that lead to new therapeutic strategies.

## 2. Materials and methods

### 2.1. Animal housing and treatments

We purchased male C57BL/6J mice from The Jackson Laboratory (Bar Harbor, ME, USA) through its Diet-Induced Obese Mouse service. This service randomizes animals to one of 2 irradiated, purified open-source diets (Research Diets Inc., New Brunswick, NJ, USA) beginning at 6 weeks of age: (1) control-fat diet (Control) containing 10% kcal fat (D12450Bi) or (2) HF diet containing 60% kcal fat (D12492i). In total, 20 control mice and 20 HF mice were delivered to the Oklahoma Medical Research Foundation (OMRF). The shipment contained 5 mice from each diet that were 12, 13, 14, and 15 weeks of age. The ages were staggered so that behavioral and metabolic tests could be conducted on aged-matched animals. The mice were housed ( $\leq 5$  animals per cage) in ventilated cages in a temperature-controlled room maintained at  $22^{\circ}\text{C} \pm 3^{\circ}\text{C}$  on 14/10-h light/dark cycles with *ad libitum* access to food and water. We weighed animals and replaced diets weekly, and OMRF vivarium staff conducted daily health inspections and routine veterinary assessment. At 25 weeks of age, mice were individually housed in cages with or without a stainless steel running wheel 4.5 inches in diameter (Mini Mitter; STARR Life Sciences Corp., Oakmont, PA, USA). Running activity was monitored continuously throughout the study in 1-min intervals using an automated computer acquisition system (VitalView; STARR Life Sciences Corp.). The mice were provided access to running wheels for 12 weeks. Wheels were locked 48 h before the end of the study at 37 weeks of age. The mice were humanely killed by rapid decapitation using a guillotine device to minimize tissue metabolic alterations caused by exposure to carbon dioxide or anesthesia agents. All experiments were conducted in accordance with protocols approved by the Association for Assessment and Accreditation of Laboratory Animal Care-accredited Institutional Animal Care and Use Committee at OMRF.

### 2.2. Metabolic and behavioral phenotype testing

Tactile sensitivity, glucose tolerance, and infrapatellar fat pad (IFP) size measurements were conducted before and after

wheel-running exposure. Tactile sensitivity was evaluated using von Frey filaments testing procedures.<sup>37</sup> In this study, withdrawal responses were recorded for a series of von Frey hairs (3.22, 3.84, 4.17, 4.56, and 4.93; Stoelting, Wood Dale, IL, USA) applied perpendicularly to the plantar surface of the hind foot, with applications separated by at least 1 min. The number of positive responses for each filament size was recorded over 5 trials. A weighted tactile sensitivity response was calculated by multiplying the provided bending force of each filament by the number of positive withdrawal responses for that filament size, summing all force-response values, and dividing this sum by the total number of positive responses. Thus, animals that were more sensitive had a smaller response score. Von Frey testing was conducted at 23 and 34 weeks of age. Fasting blood glucose and glucose tolerance testing (GTT) were conducted as previously described.<sup>38</sup> Briefly, mice were fasted overnight from 5:00 p.m. to 9:00 a.m. Mice were weighed, and fasting blood glucose was measured from the tail tip using a glucose meter (Ascencia, Bayer, Parsippany, NJ, USA). Filter-sterilized D-glucose (G8270; Sigma-Aldrich, St. Louis, MO, USA) (37°C, 200 mg/mL) was injected intraperitoneally at 2 mg/g body mass in normal saline. Blood glucose was then measured at 30, 60, 90, and 120 min after glucose administration. The blood glucose area under the curve (AUC) was evaluated relative to the fasting pre-injection level. GTT was conducted at 23 and 34 weeks of age. IFP volume was measured using water-suppressed magnetic resonance imaging at 24 and 35 weeks of age, as previously described.<sup>32,39</sup> Changes in outcomes between the 2 time points were quantified as the change in post- versus pre-exercise values expressed as a percent of the pre-exercise value. Finally, grip strength and body fat were measured at 36 weeks of age, as previously described.<sup>40</sup>

### 2.3. Serum analysis

Animals were transported to the laboratory for a period of 1–2 h before death between 8:30 a.m. and 9:30 a.m. Animals had to be killed over a period of several days to maintain a consistent time of euthanasia, with 1–2 animals per treatment group humanely killed per day. Animals were decapitated and blood was collected from the carotid arteries and allowed to clot in microvette tubes (CB 300Z; Sarstedt, Sarstedt, Germany) at room temperature for 20 min before centrifugation at 10,000g for 5 min. Serum was aliquoted and frozen at –80°C until analysis. Samples were analyzed in the OMRF Proteomics Core following manufacturer instructions. Concentrations of Interleukin-1 $\beta$  (IL-1 $\beta$ ), Interleukin-6 (IL-6), C-C motif chemokine ligand 2 (CCL2), and tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) were measured using a Mouse Luminex 4-plex kit (R&D Systems, Minneapolis, MN, USA), and leptin, adiponectin, tissue inhibitor of metalloproteinases 1 (TIMP-1), insulin-like growth factor-1 (IGF-1), and vascular cell adhesion molecule 1 (VCAM-1) were measured by enzyme-linked immunosorbent assay (R&D Systems). The lowest standard for each analyte was 60.31 pg/mL for IL-1 $\beta$ , 10.68 pg/mL for IL-6, 73.86 pg/mL for CCL2, 7.12 pg/mL for TNF- $\alpha$ , 63.24 pg/mL for leptin, 157.45 pg/mL for

adiponectin, 37.37 pg/mL for TIMP-1, 31.21 pg/mL for IGF-1, and 309.07 pg/mL for VCAM-1. IL-1 $\beta$  and TNF- $\alpha$  were not detected at levels above the lowest standard in any sample and were excluded from further analysis. CCL2 was not detected in 11 of 24 samples and was also excluded.

### 2.4. Histological analysis

After death, the right hind limb was isolated, skinned, wrapped in 1 $\times$  phosphate-buffered saline (Gibco™ 10010023; ThermoFisher Scientific, Waltham, MA, USA) soaked gauze in anatomic position, and frozen at –80°C until high-resolution micro-computed tomography (CT) scanning. Immediately before micro-CT analysis, limbs were thawed and placed in a holding tube in 10% buffered formalin. Knees were scanned using a viva CT 40 scanner (Scanco Medical, Basserdorf, Switzerland), as previously described.<sup>27</sup> After scanning, joints were prepared by removing muscles by gross dissection, rinsing in phosphate buffered saline, and decalcifying in Cal-Ex™ Decalcifier (CS510-1D; ThermoFisher Scientific) for 3 days at 4°C. Knees were then dehydrated in an ethanol gradient before paraffin embedding and sagittal sectioning. Slides were stained with hematoxylin, Fast Green, and Safranin-O for histological grading, as described previously.<sup>33</sup> Two experienced graders evaluated multiple stained sections from the medial and lateral joint compartments. Slides were organized by knee joint sample, randomized by diet and exercise treatment, and assigned a temporary identification code to blind graders to group assignment and minimize any order effect. Modified Mankin OA grading scores were assigned separately for the medial tibia, medial femur, lateral tibia, and lateral femur independently by each grader. Additional pathological changes, such as osteophyte severity and IFP collagen content, were evaluated as previously described.<sup>32</sup> Scores were averaged for each grader and then for each location.

### 2.5. Correlation network analyses

Correlation-based networks were built as previously described<sup>33</sup> under R-environment<sup>41</sup> and visualized in Cytoscape.<sup>42</sup> Considering the different nature of outcome parameters tested and consequently large differences in variable scales, Spearman's rank correlation was chosen for the network construction. The correlation threshold was chosen based on a  $p$  value of <0.05 for global networks, and a  $q$  of <0.05 for exercise networks. Individual missing values were imputed using “mice”-package under R-environment<sup>43</sup> ahead of the correlation matrices computation.

### 2.6. Statistical analyses

Samples size calculations were based on detecting the effects of diet and exercise on OA histopathology. Based on prior studies, a group size of  $n = 10$  is estimated to provide >80% power to detect a 30% difference in mean modified Mankin scores with a significance level of  $p = 0.05$ . Our secondary outcomes were the effects of diet and exercise on body habitus, serum metabolic biomarkers, grip strength, tactile

sensitivity, and structural changes in the tibial bone and IFP. Sample sizes of  $n=6$  per group for serum inflammatory biomarkers were due to insufficient sample availability. One animal in the exercise control diet group died from complications during the first GTT; therefore, the sample size for this group was  $n=9$ . One animal in the exercise HF group died from complications during the 2nd magnetic resonance imaging scan at 35 weeks, although the joint was retained and included in histopathology analyses. Pre-exercise diet treatments were analyzed by two-tailed Student's  $t$  test, and diet and exercise treatment effects were evaluated by two-way analysis of variance. Data that did not meet test assumptions for homoscedasticity or normality of residuals were log-transformed. Tests showing a significant effect of diet, exercise, or interaction

effects ( $p < 0.05$ ) were followed up with multiple-comparison *post hoc* tests to identify individual group differences as specified in figure legends. Statistical tests were conducted using the software Prism Version 8.01 for Mac OS X (GraphPad Software, San Diego, CA, USA). Data were expressed as mean  $\pm$  SD, unless otherwise stated. The  $n$  indicates animal numbers per group, and  $p < 0.05$  was considered significant.

### 3. Results

#### 3.1. Systemic metabolic outcomes and IFP

We began tracking the body mass of animals at 15 weeks of age, which was 9 weeks after the start of control and HF diet treatments (Fig. 1A). By this age, HF fed mice weighed 15%

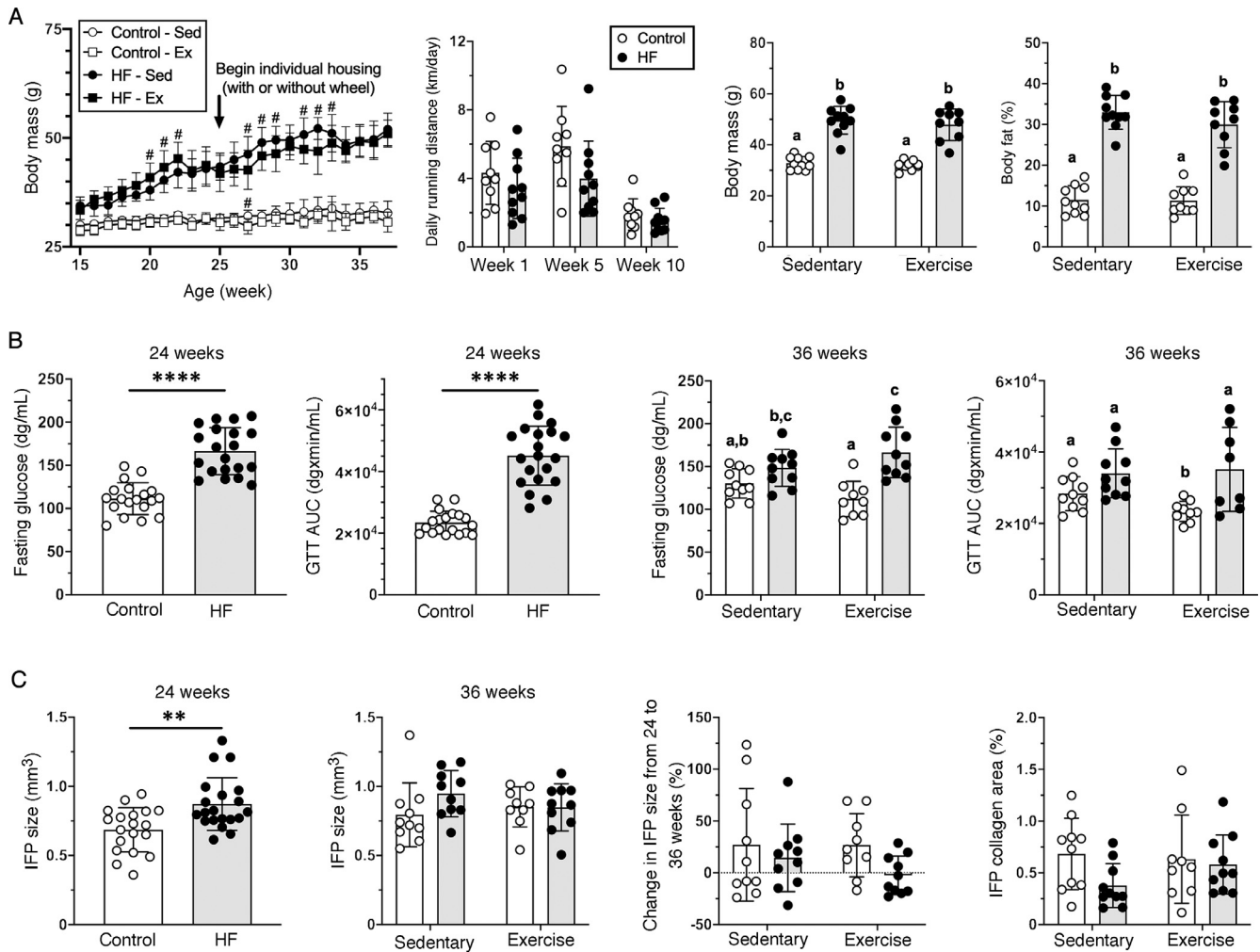


Fig. 1. Effect of HF diet and exercise on body habitus, glucose tolerance, and IFP structure. (A) Body mass versus age, running distance, and end-of-study body mass and body fat in animals according to their diet and activity group assignments. Voluntary wheel-running exercise began at 25 weeks. Body mass gain slowed in HF animals during behavior testing and imaging periods (i.e., 23–25 weeks and 34–36 weeks). Exercise caused modest age-dependent reductions in body mass as indicated by hash marks between 25 and 37 weeks of age ( $^{\#}q < 0.05$ , sedentary vs. exercise in diet and age-matched groups; false discovery rate correction by two-stage linear step-up procedure of Benjamini, Krieger, and Yekutieli (Prism 8.0.1)). (B) Fasting blood glucose and GTT AUC values at pre-exercise and post-exercise time points. (C) MRI-measured IFP sizes before and after exercise as well as Sirius red-based collagen content analysis. Change in IFP size (%) =  $((V_{T2} - V_{T1}) / V_{T1}) \times 100\%$ .  $V$ : volume of IFP size;  $T1$ : 24 weeks;  $T2$ : 36 weeks. For all graphs, values are mean  $\pm$  SD. Diet and age or activity comparisons were analyzed by two-factor analysis of variance. In tests with significant factor effects, a *post hoc* analysis was conducted to identify group differences using Holm-Sidak's multiple comparisons test. Bars not sharing a lowercase letter are significantly different from one another ( $p < 0.05$ ).  $^{**}p < 0.01$ ;  $^{****}p < 0.0001$ ;  $^{\#}q < 0.05$ . AUC = area under the curve; Ex = exercise; GTT = glucose tolerance testing; HF = high fat; IFP = infra-patellar fat pad; MRI = magnetic resonance imaging; Sed = sedentary.

more than control mice ( $p < 0.001$ ), and this difference grew throughout the duration of the study to 62% by the endpoint at 37 weeks ( $p < 0.001$ ). After animals assigned to the exercise group were housed with running wheels beginning at 25 weeks, body weight dropped modestly and sporadically in diet-matched runners vs. non-runners. Notably, despite the increase in body mass of HF-fed mice, diet did not alter the average daily wheel-running distance measured after 1, 5, or 10 weeks of access to wheels (Fig. 1A). Animals in both diets ran less during week 10 compared to weeks 1 or 5. Upon completion of the study, an HF diet caused significant increases in body mass and body fat, but neither outcome was altered by exercise (Fig. 1A). Before exercise, an HF diet caused significant increases in fasting blood glucose and glucose intolerance, as indicated by a >2-fold increase in the glucose AUC obtained during a GTT (Fig. 1B). By 36 weeks, fasting blood glucose and GTT AUC values were elevated in control sedentary mice compared to 24-week values, which diminished differences with sedentary HF animals. In contrast, glucose values in 36-week exercise control animals remained similar to 24-week values, resulting in significant differences from either sedentary or exercise HF animals (Fig. 1B). We evaluated size changes in the IFP resulting from diet and exercise treatments. Similar to our prior report,<sup>32</sup> we observed a significant increase in IFP size with HF feeding at 24 weeks (Fig. 1C). By 36 weeks, this diet effect was absent, although IFP volume changed less from 24 to 36 weeks in HF versus control mice ( $p=0.035$ ). There were no significant effects of exercise on either the absolute IFP volume or the change in volume from 24 to 36 weeks (Fig. 1C). IFP collagen content did not increase in HF animals as previously observed at an earlier time point,<sup>32</sup> and exercise did not alter collagen content (Fig. 1C).

### 3.2. Serum adipokines and inflammatory mediators

We further evaluated systemic markers of inflammation and metabolic regulation by measuring serum adipokines, inflammatory mediators, and tissue repair and growth factors at the study endpoint (Fig. 2). An HF diet caused a robust increase in leptin (>10-fold,  $p < 0.0001$ ), which was not altered by exercise. Adiponectin was not altered by either diet or exercise, and IL-6 showed a nonsignificant trend ( $p=0.12$ ) for being elevated by an HF diet (Fig. 2). VCAM-1 also showed a nonsignificant trend ( $p=0.12$ ) for increase by an HF diet, which was primarily due to a trending reduction in the control exercise group. Both TIMP-1 and IGF-1 were elevated in mice fed an HF diet independent of exercise treatment ( $p < 0.001$ ).

### 3.3. Mechanical tactile sensitivity and grip strength

The development of OA in spontaneous and joint injury models is associated with mechanical allodynia and reduced grip strength. We assessed mechanical allodynia indirectly by calculating a weighted mechanical sensitivity score in response to von Frey filament testing. Animals that respond more frequently to the application of smaller sized filaments generate lower scores. Thus, a lower score or a reduction in score indicates a greater level of mechanical sensitivity. At 24

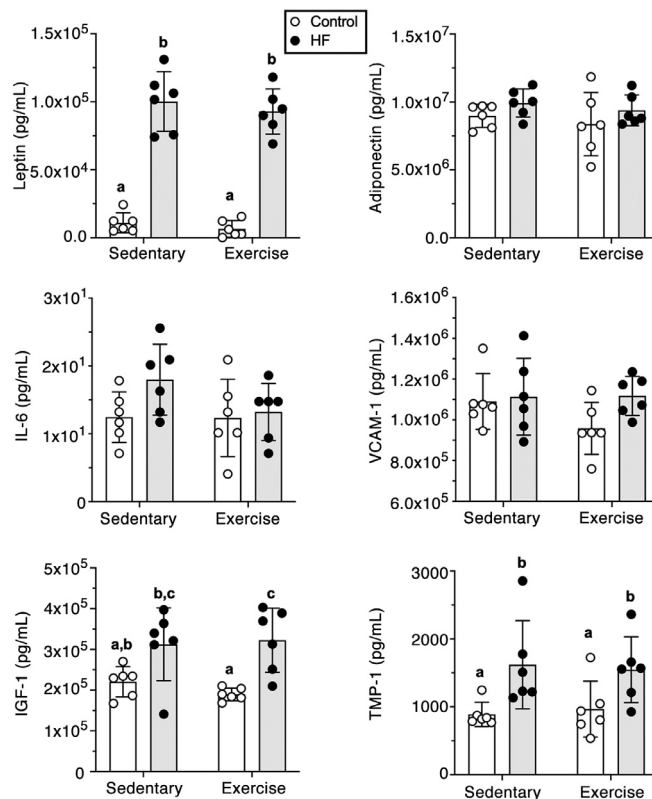


Fig. 2. Effect of HF diet and exercise on serum adipokines, inflammatory mediators, and growth and repair factors. Serum biomarkers were measured in a subset of animals ( $n=6$ /group) at the study endpoint by Luminex and ELISA-based assays. IL-1 $\beta$ , tumor necrosis factor  $\alpha$ , and CCL2 were largely below the level of detection and therefore are not shown. HF diet effects were observed for leptin, IGF-1, and TIMP-1 by two-factor analysis of variance of log-transformed data ( $p < 0.05$ ). Bars not sharing a lowercase letter are significantly different from one another ( $p < 0.05$ ; Holm-Sidak's multiple comparisons test). For all graphs, values are mean  $\pm$  SD. CCL2 = C-C motif chemokine ligand 2; ELISA = enzyme-linked immunosorbent assay; HF = high fat; IGF-1 = insulin-like growth factor 1; IL-1 $\beta$  = Interleukin-1 $\beta$ ; IL-6 = Interleukin-6; TIMP-1 = tissue inhibitor of matrix metalloproteinase 1; VCAM-1 = vascular cell adhesion molecule 1.

weeks, there were no differences in mechanical sensitivity due to an HF diet (Fig. 3A). At 36 weeks, an HF diet increased the weighted response score ( $p=0.008$ ) and exercise lowered it ( $p=0.0435$ ) (Fig. 3A). Compared to 24 weeks, exercise was associated with an increase in mechanical sensitivity in control diet mice by reducing the weighted response score ( $p=0.028$ ) (Fig. 3A). Neither HF diet nor exercise altered the absolute grip strength in the mice (Fig. 3B). However, when normalized to body weight, an HF diet caused a significant reduction in grip strength independent of exercise ( $p < 0.001$ ) (Fig. 3B).

### 3.4. OA-related and structural changes in knee cartilage and bone

We evaluated OA-related joint changes by semi-quantitative modified Mankin scoring and osteophyte severity scoring. The overall modified Mankin scoring (maximum score = 24), averaged throughout the whole joint, was not altered by HF diet or exercise (Fig. 4A). Furthermore, subcomponent scoring, which

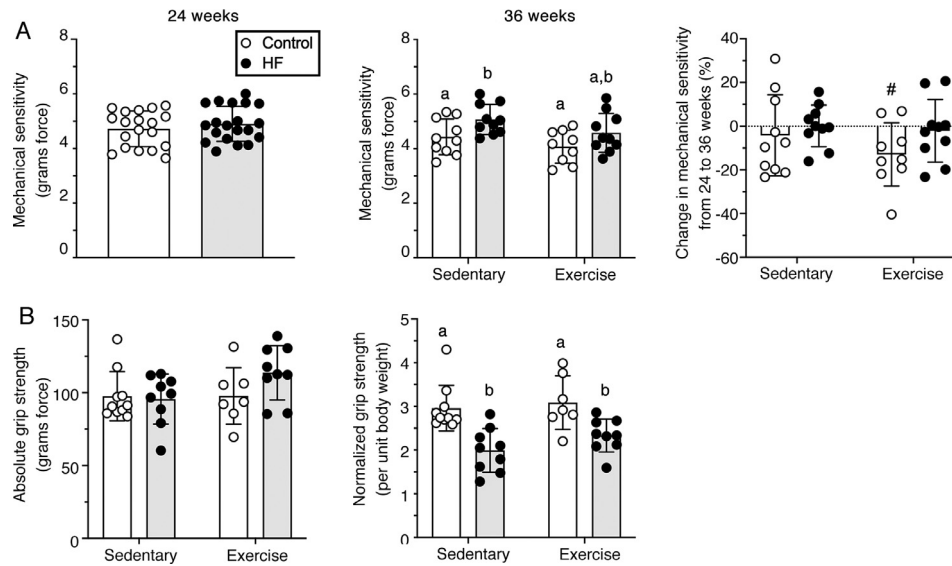


Fig. 3. Effect of HF diet and exercise on mechanical sensitivity and grip strength. (A) Mechanical sensitivity was evaluated by measuring a weighted withdrawal response score to the standardized application of a graded series of von Frey hairs. The score was calculated by multiplying the number of positive responses by the corresponding hair size, summing these response–force values for each hair, and dividing this summed value by the total number of positive responses. Thus, a lower score or a reduction in score indicates a greater level of mechanical sensitivity because it indicates that an animal responded more frequently to the application of smaller-sized filaments. Diet did not alter mechanical sensitivity at 24 weeks, but at 36 weeks mechanical sensitivity was increased in the sedentary and exercise control groups compared to sedentary HF. Change in mechanical sensitivity (%) =  $((F_{T2} - F_{T1}) / F_{T1}) \times 100\%$ . F: force; T1: 24 weeks; T2: 36 weeks. (B) Diet and exercise did not alter the absolute grip strength in mice, but when normalized to body weight, grip strength was weaker in HF animals. For all graphs, values are mean  $\pm$  SD. Bars not sharing a lowercase letter are significantly different from one another ( $p < 0.05$ ; Holm-Sidak's multiple comparisons test). #  $p < 0.05$  versus a hypothetical value of 0. HF = high fat.

included cartilage damage (maximum score = 11), Safranin-O staining loss (maximum score = 8), tidemark duplication (maximum score = 3), and hypertrophic chondrocyte abundance (maximum score = 2), was also not altered by diet or exercise (Fig. 4A). We also evaluated modified Mankin scoring (maximum score = 24) specifically for the tibia and femur in the medial and lateral compartments, and there were no differences caused by diet and exercise treatments (Fig. 4B). An HF diet and exercise did not alter the severity of osteophytes (maximum score = 3) along the anterior-medial margin of the tibia, although an HF diet did increase osteophyte severity along the posterior-medial joint margin in exercised mice ( $p = 0.0094$ ) (Fig. 4C). We next evaluated tibial subchondral and trabecular bone mineral density for changes in structural composition. For most locations, an HF diet and exercise did not alter bone density. However, both an HF diet ( $p = 0.031$ ) and exercise ( $p = 0.022$ ) caused a modest change in bone mineral density in the lateral femur subchondral cortical bone, where an HF diet reduced density and exercise increased it. The effect was most pronounced in control exercised mice compared to sedentary HF diet mice (Fig. 4D). Several additional changes were observed in the trabecular bone within the proximal tibial epiphysis. As with bone density in the lateral femur, the relative bone volume (i.e., bone volume to total volume) was reduced with an HF diet ( $p = 0.0048$ ) and increased with exercise ( $p = 0.024$ ) (Fig. 4E). This effect was associated with significant changes in trabecular separation, which was increased with an HF diet ( $p = 0.038$ ) and showed a trend for reduction with exercise ( $p = 0.06$ ). An HF diet was also associated with a trend for thinner trabecula ( $p = 0.053$ ) (Fig. 4E). Altogether, these findings

show that bone was more responsive to an HF diet and exercise treatments compared to cartilage.

### 3.5. Diet-dependent exercise correlation networks

Wheel running is generally an effective method of eliciting an exercise treatment response in mice because mice voluntarily run long distances on wheels. In this study, wheel access was provided at 25 weeks of age, which is an older age than typical wheel-based exercise studies. We observed high variance in the average nightly running distance in both control and HF mice. After 5 weeks of wheel access, when the average daily running distances were greatest (Fig. 1A), the coefficient of variation in nightly running was 40% in control mice and 55% in HF mice. This high degree of variation lends itself well to a correlation network analysis, which we conducted in control and HF exercise mice to evaluate outcomes that were associated with nightly running distance. We also evaluated correlations with running phase, which we defined as the percent of the total daily running distance that occurred during the dark phase. Mice are primarily active during the dark phase; thus, reductions in running phase would be associated with disruptions in the circadian clock and metabolic dysregulation.<sup>44,45</sup> We did not observe diet-dependent differences in running phase at any time point, although it was lowest at Week 1 ( $83.4\% \pm 10.3\%$ , mean  $\pm$  SD for both diet groups) and greatest at Week 5 ( $94.8\% \pm 5.8\%$ ). The coefficient of variation ranged from 12.3% at Week 1 to 6.1% at Week 5.

We included all experimental endpoint outcomes presented in Figs. 1–4 as potential running distance or phase correlates (i.e.,

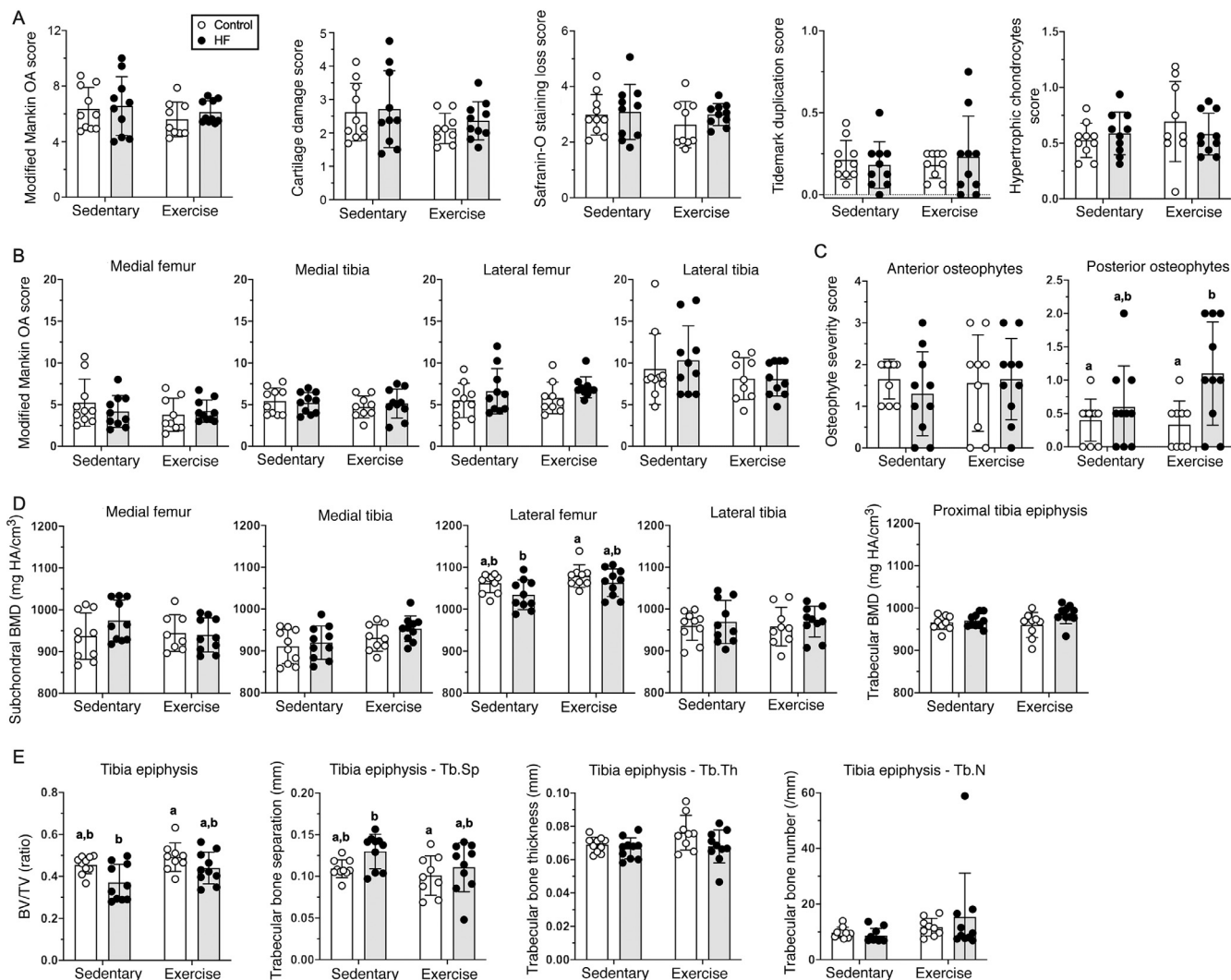


Fig. 4. Effect of HF diet and exercise on knee cartilage and bone structural and OA-related changes. (A) Neither HF diet nor exercise altered the modified Mankin scoring for knee cartilage OA pathology. Sub-component scores were not altered either. (B) Knee site-specific modified Mankin scoring was also not altered by diet or exercise. (C) Semi-quantitative osteophyte scores for anterior and posterior tibial joint margins in the medial compartment. HF diet increased posterior osteophytes under exercise conditions. (D) Subchondral cortical bone mineral density for site-specific locations and trabecular bone mineral density for the proximal tibial epiphysis. The lateral femur subchondral density was lower in sedentary HF animals. (E) Structural trabecular bone changes in the proximal tibia epiphysis. Control diet exercise mice had a higher relative BV/TV and smaller Tb.Sp compared to sedentary HF mice. For all graphs, values are mean  $\pm$  SD. Diet and age comparisons were analyzed by two-factor analysis of variance. In tests with significant factor effects, group-specific differences were determined by Holm-Sidak's multiple comparisons test. Bars not sharing a lowercase letter are significantly different from one another ( $p < 0.05$ ). BMD = bone mineral density; BV/TV = bone volume/total volume; HF = high fat; OA = osteoarthritis; Tb.N = trabecular number; Tb.Sp = trabecular separation; Tb.Th = trabecular thickness.

38 variables), which resulted in 16 variables with  $\geq 1$  significant correlation for control diet mice and 11 variables for HF diet mice, not including correlations among running distance and phase variables themselves (Fig. 5). Out of the 27 total variables that correlated with running distance or phase in both diets, 4 variables were present in both diet networks: serum leptin, medial tibia subchondral bone mineral density, tibial epiphysis trabecular thickness, and hypertrophic chondrocyte abundance. Leptin was negatively correlated with running distance in both diets. Subchondral bone mineral density was negatively correlated with distance and phase in control mice and phase in HF mice. Trabecular thickness was negatively correlated with running phase in both diets, and hypertrophic chondrocyte abundance was positively correlated with running distance in both diets.

The additional 19 unique variables that correlated with exercise parameters, 12 in control and 7 in HF animals, indicate that a range of systemic and local factors were associated with exercise in a diet-dependent manner. Some of the notable associations include negative correlations between running distance and serum biomarkers, glucose tolerance AUC, and change in IFP size in control diet mice. In addition, running distance was positively correlated with lateral femur modified Mankin OA score in control mice. In HF mice, running distance was negatively correlated with loss of Safranin-O cartilage staining, which is consistent with our prior study showing that short-term exercise protected against loss of Safranin-O staining in young diet-induced obese mice.<sup>30</sup> Notably, in HF diet mice, running phase showed a much greater number of systemic and local

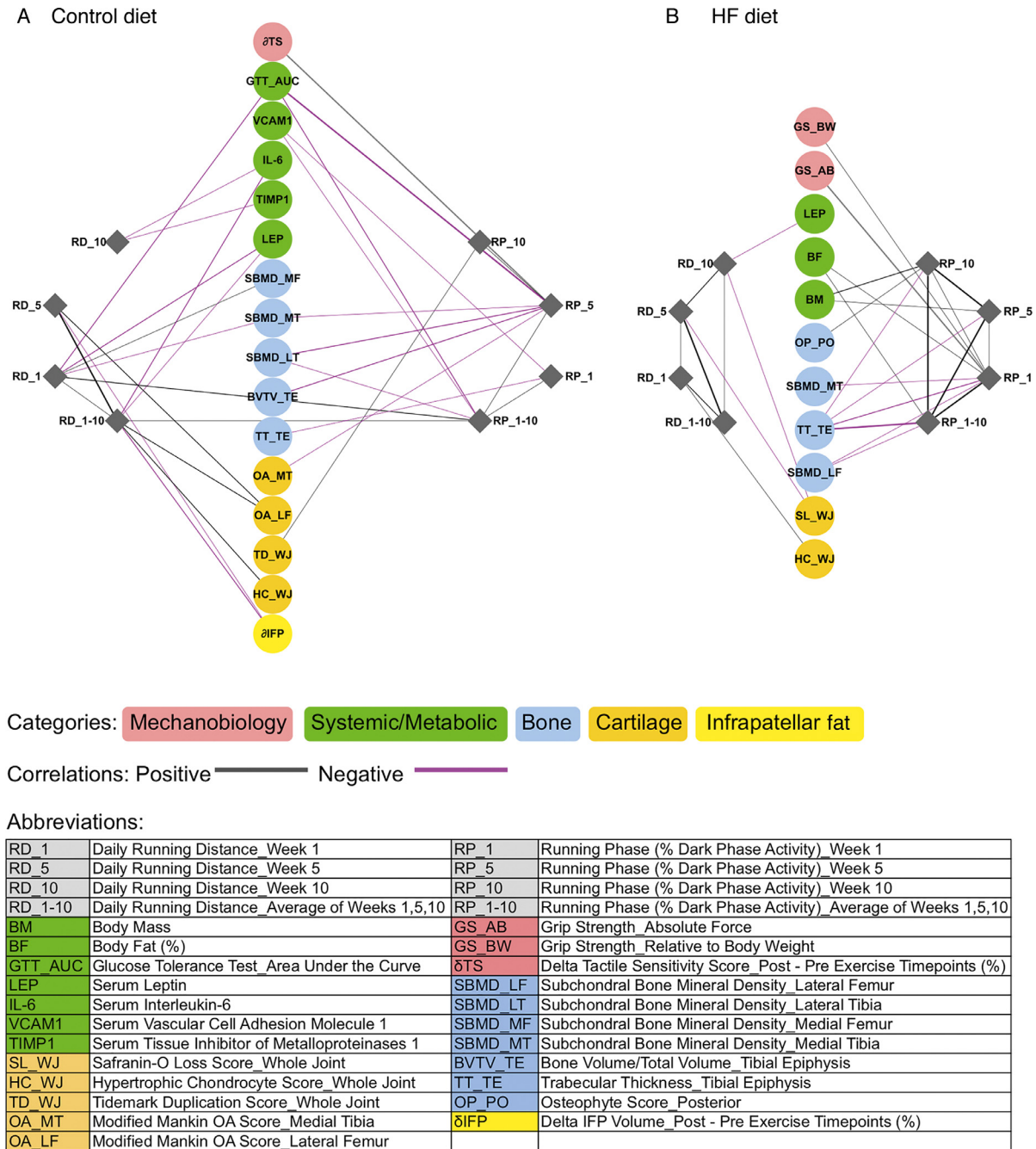


Fig. 5. Exercise correlation networks in control and HF diet mice. Correlation network analysis of (A) control and (B) HF diet exercise groups. Running distance and running phase parameters were correlated to 38 outcome variables falling into 5 categories: mechanobiology, systemic/metabolic, bone, cartilage, and infrapatellar fat (color coded on the plot). Lines show correlations based on Spearman’s rank analysis with FDR-corrected significance of  $q < 0.05$ , with line thickness proportional to the correlation strength ( $|r| > 0.6$ ). Correlations between non-running variables are not included in the network. FDR = false discovery rate; HF = high fat.

correlations compared to running distance, suggesting a more substantial role for circadian-related factors.

### 3.6. Effect of an HF diet and exercise on OA-related network correlations

We further investigated the integrated effect of exercise under different dietary regimes by building a separate correlation-based network for each diet and activity group using the 38 local and systemic endpoint outcomes presented in

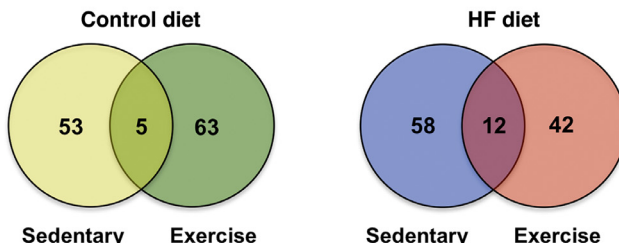
Figs. 1–4 (Fig. 6). While the sizes of the networks were comparable among the different groups, there were substantial differences when comparing sedentary and exercise networks under different diets. For example, exercise had the opposite effect on the network density based on the diet. In the control diet group, density increased with exercise from 0.08 to 0.11, whereas in the HF diet group, exercise reduced density from 0.12 to 0.09 (Fig. 6A). In addition, the ratio of positive to negative correlations was much greater in HF mice, especially under exercise conditions.



A Network parameters

Categories	Control-Sed	Control-Ex	HF-Sed	HF-Ex
Number of variables (“nodes”) with $\geq 1$ significant correlation	38	35	35	36
Total number of significant correlations (“edges”)	58	68	70	54
Network density	0.08	0.11	0.12	0.09
Ratio of positive/negative correlations (“edges”)	2.6	2.8	3.9	6.0

B Diet-specific unique and shared correlations (“edges”)



C Primary (“first neighbor”) networks for whole-joint modified Mankin OA score

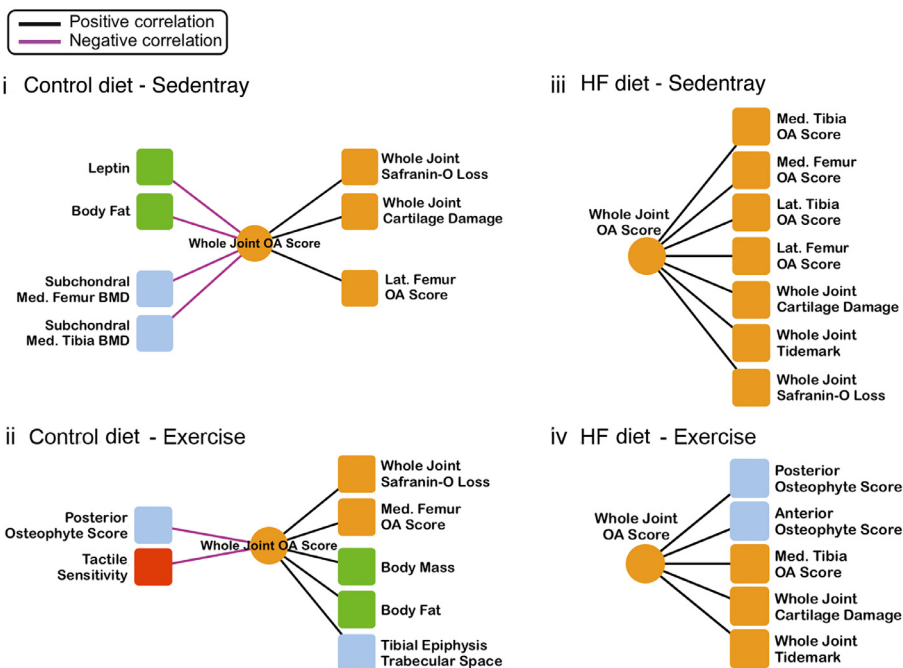


Fig. 6. Analysis of overall correlation networks within each diet and activity group. (A) Local and systemic effects of HF diet and exercise were evaluated by building correlation-based networks for each diet and activity condition. Thirty-eight outcome variables falling into 5 categories (mechanobiology, systemic/metabolic, bone, cartilage, and infrapatellar fat) were evaluated by Spearman’s rank correlation analysis, and variables (i.e., “nodes”) with at least 1 significant correlation (i.e., “edge”;  $p < 0.05$ ) were included in the network (see Appendix 1 for a list of network variables). (B) Venn diagrams comparing unique and shared correlations (edges) between sedentary and exercise cohorts in control diet and HF diet groups. Few correlated outcome variables were shared between sedentary and exercise cohorts in either diet. (C) Primary “first neighbor” network of whole-joint modified Mankin OA scores in control and HF diet animals under sedentary and exercise conditions. Note that the only variables correlated with the whole-joint OA score in the HF diet group were local-joint variables. Ex = exercise; HF = high fat; Med. = medial; Lat. = lateral; Sed = sedentary; OA = osteoarthritis.

In control diet mice, the specific pairs of correlated variables (i.e., “edges”) were almost entirely different between sedentary and exercised groups, with only 5 common correlated pairs (Fig. 6B). The common pairs were primarily *a priori* associated variables, such as the Safranin-O loss score and the whole-joint modified Mankin OA score or body mass and

body fat. To better understand potential links to knee OA and how they varied with exercise, we focused on the primary (i.e., “first neighbor”) network of the whole-joint modified Mankin OA score. In sedentary control diet mice, knee OA was primarily associated with cartilage damage and Safranin-O staining loss as well as the OA score for the lateral femoral

compartment (Fig. 6B). Surprisingly, knee OA was negatively associated with serum leptin, body fat, and medial femur and tibia subchondral bone density. With exercise, knee OA was also correlated with body fat, although the relationship flipped to become positive. Knee OA was also positively correlated with body mass, tibia trabecular spacing, Safranin-O staining loss, and medial femoral OA. Knee OA was negatively associated with the tactile sensitivity withdrawal force and posterior osteophytes.

In HF diet mice, as in control diet mice, the pairs of correlated variables were mostly different between sedentary and exercise groups, although the number of common correlated variables was greater than in control mice (Fig. 6C). These common correlates were also primarily *a priori* associated variables, such as body mass and body fat or cartilage damage and whole-joint knee OA scores. However, additional serum correlates were also observed, including IL-6 and IGF-1, IL-6 and TIMP-1, and leptin and TIMP-1 (all positive). Interestingly, unlike in control diet mice, there were no systemic variables within the primary “first neighbor” network of the whole-joint modified Mankin OA score (Fig. 6C). In sedentary HF mice, whole-joint knee OA was associated with site-specific OA scores in all 4 locations (i.e., medial and lateral, tibia and femur) indicating that OA changes occurred throughout the joint. Whole-joint knee OA was also associated with whole-joint cartilage damage, tidemark duplication, and Safranin-O staining loss. In exercise HF mice, whole-joint knee OA remained positively correlated with cartilage damage, tidemark duplication, and medial tibia OA. In addition, knee OA was positively correlated with anterior and posterior osteophytes. Thus, although HF sedentary mice developed a range of metabolic pathologies associated with obesity, the variation associated with these conditions was not directly associated with knee OA pathology.

#### 4. Discussion

An initial goal of this study was to determine the effect of voluntary wheel-running exercise on systemic and local OA-related outcomes after an extended duration of diet-induced obesity. Although an HF diet did not cause an overall increase in OA pathology in this study, it caused numerous metabolic alterations. These changes included elevated body mass, increased body fat, elevated fasting blood glucose and glucose intolerance, increased IFP size, elevated serum biomarkers (i.e., leptin, TIMP-1, and IGF-1), elevated von Frey withdrawal forces, and reduced weight-normalized grip strength. Exercise reduced body mass in HF-fed animals for several weeks after the initiation of wheel running, but this effect diminished by the end of the study. Exercise did not alter any other HF diet-induced changes, although other metabolic and functional outcomes were modified by exercise in control diet mice (e.g., reduction in the GTT AUC and increased mechanical sensitivity). Thus, the fact that most OA-related outcomes did not change after 31 weeks of HF feeding or with exercise indicates that the timescale of obesity-related OA pathogenesis is slower than systemic

metabolic and functional changes. This interpretation is supported by a recent study also showing an extended lag time between metabolic changes and OA pathology.<sup>46</sup>

Among the joint structural factors that were altered by obesity and exercise, our findings showed that bone was more sensitive to change than cartilage (Fig. 4). Trabecular bone in the proximal tibial epiphysis was particularly sensitive to differences between sedentary HF diet animals and exercise control diet animals. Sedentary HF animals had reduced relative bone volume (BV/TV) and increased trabecular spacing compared to exercise control diet animals. Subchondral bone mineral density in the lateral femur was also reduced in sedentary HF animals compared to exercise control diet animals. Osteophyte formation was also sensitive to diet and exercise. In this case, an HF diet and exercise increased the development of osteophytes in the posterior joint region compared to sedentary or exercise control diet animals. Altered joint biomechanical loading is linked to osteophyte formation,<sup>47</sup> and we only observed this effect through a combination of HF diet and wheel running. Interestingly, a relationship between osteophyte severity and cartilage pathology was only observed in exercise animals, although the nature of this relationship differed between animals fed a control or HF diet. In control diet exercise animals, posterior osteophyte severity was negatively correlated with the cartilage modified Mankin score. In contrast, both posterior and anterior osteophyte severity were positively correlated with cartilage modified Mankin score in HF diet exercise mice (Fig. 6). These findings suggest that diet alters the relationship between bone and cartilage structural changes that precede the development of more severe OA.

These intriguing findings of contrasting diet-dependent relationships between cartilage and bone pathologies show how alternative analytical approaches may provide new insight for identifying distinct “pre-OA” phenotypes that modify OA risk. We explored this by using correlation network analysis to determine the effect of diet-induced obesity and exercise on the associations between systemic factors (i.e., metabolic, inflammatory, and biomechanical outcomes) and local knee structural changes during early-stage OA. Correlation network analysis takes advantage of within-group variation to integrate data across different levels of biological organization and function and identify relationships among multiple groups of variables. As previously mentioned in Section 3.5., we observed high variance in the average nightly running distance in both control and HF mice. The variance was also moderate for modified Mankin OA scores and body fat levels within a given diet with coefficients of variation between 16% and 30%. Thus, we took advantage of this variance to gain insight into relationships among local and systemic variables by constructing correlation-based networks.

Our results showed that an HF diet and exercise significantly altered “pre-OA” networks, which were composed of metabolic, inflammatory, biomechanical, and OA pathology variables. Running activity was associated with variables across all categories, consistent with broad systemic and local effects of exercise. When comparing control and HF diet running networks (Fig. 5),

the smaller network size in the HF diet group suggests that diet-induced obesity may dampen the effects of exercise because animals ran a similar amount in both diet groups. In addition, we observed more links to light/dark phase running patterns compared to running distance in HF-fed mice (Fig. 5B), suggesting that circadian-related factors may be especially important in mediating OA pathology in the context of obesity.

It is striking to observe how distinct each network is among the different diet and exercise conditions (Fig. 6). For example, the majority of edges in the exercise groups are not present in the sedentary groups, suggesting that the biological relationships among variables are substantially altered with exercise. An excellent example of this observation in the context of OA is the relationship between knee OA and body fat in control diet animals. Sedentary control animals have a negative relationship between OA and fat, whereas the relationship is positive in exercised control animals. Prior studies have reported a positive relationship between body fat and OA in a population that includes lean and obese individuals or animals.<sup>40,48,49</sup> However, the link between body fat and OA in the current study was only observed in control mice, which were generally lean, with body fat levels between 7% and 18%. These data suggest that systems involved in body fat regulation, even in the absence of obesity, are associated with OA pathology and that the level of physical activity fundamentally alters this association. The mechanism responsible for this difference remains to be determined, although it was not associated with changes in the size of the IFP, a potential mediator of OA progression.<sup>50</sup>

A limitation of this study was the absence of information about joint-level metabolic and inflammatory variables. An HF diet has previously been shown to induce TNF expression in the synovium of mice.<sup>51</sup> The effect of TNF on OA was linked to synovial insulin resistance, suggesting that joint inflammation may be an early-stage driver of disease related to obesity and metabolic syndrome.<sup>15</sup> An analysis of synovial fluid metabolites and pro-inflammatory mediators would also provide a valuable resource for evaluating the effects of diet and exercise, especially if the same variables were tested in serum so that the relationship between systemic and local changes could be more directly compared. Additionally, we only studied male mice, although we have previously established numerous local and systemic links to OA in an HF diet female mouse model.<sup>40</sup> Clinical data also suggest that obesity-associated metabolic conditions, such as diabetes or dyslipidemia, may increase OA risk in a gender-specific manner.<sup>16</sup> Thus, future studies that compare male and female animals may reveal sex-specific relationships linking metabolic regulation to OA progression. Finally, our study was limited to early-stage or “pre-OA” phenotypes because the HF diet treatment did not markedly increase OA pathology. Given that the same type of HF diet is sufficient to increase knee OA in male C57BL/6J mice when fed from 6 to 52 weeks of age, the time period between 37 and 52 weeks is likely associated with more rapid HF diet-induced OA progression.

An additional variable for us to consider compared to our earlier acute study<sup>30</sup> is that we used a defined control diet rather than chow as the control diet. The carbohydrate composition of chow diets differs substantially from defined

diets,<sup>52,53</sup> and the lack of soluble fiber in the defined diet we used has been associated with gut atrophy and microbiome alterations.<sup>54,55</sup> We recently showed that defined low-fat diets that differ in carbohydrate composition alter OA pathology without changing body mass or body fat.<sup>29</sup> Whether or not OA pathology is altered in mice fed a defined low-fat control diet compared to a chow diet remains to be tested.

## 5. Conclusion

We found that a chronic model of diet-induced obesity and voluntary wheel running in male mice substantially altered the relationships among systemic and local OA-related variables using correlation network analyses. These changes occurred despite minimal overall effects of HF diet and exercise on OA pathology under the current test durations. The network analyses suggest that systemic metabolic factors may be more involved in mediating idiopathic knee OA under normal body weight conditions than previously appreciated. This work supports the use of correlation network analysis as a discovery-based method for integrating large biological data sets to generate novel hypotheses for studying OA pathophysiology.

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## Authors' contributions

TMG was responsible for the study conceptualization, methodology development and validation, formal analysis, manuscript writing (preparation and editing), data visualization, study supervision, and funding acquisition; AB was responsible for software development, formal analysis, writing (preparation and editing), and data visualization; JH was responsible for study methodology development and validation, formal analysis, study investigation, visualization, and project administration; EBPL was responsible for study investigation, formal analysis, and writing (editing). All authors have read and approved the final version of the manuscript, and agree with the order of presentation of the authors.

## Competing interests

The authors declare that they have no competing interests.

## Appendix 1. Network abbreviations.

Variable name	Abbreviation category	
Osteophyte score_anterior	OP_AN	Bone
Osteophyte score_anterior posterior average	OP_AP	Bone
Osteophyte score_posterior	OP_PO	Bone
Subchondral bone mineral density_lateral femur	SBMD_LF	Bone
Subchondral bone mineral density_lateral tibia	SBMD_LT	Bone
Subchondral bone mineral density_medial femur	SBMD_MF	Bone
Subchondral bone mineral density_medial tibia	SBMD_MT	Bone
Tibial Epiphysis_bone mineral density	BMD_TE	Bone
Tibial Epiphysis_bone volume/total volume	BTVV_TE	Bone
Tibial epiphysis_trabecular number	TN_TE	Bone
Tibial epiphysis_trabecular spacing	TS_TE	Bone
Tibial epiphysis_trabecular thickness	TT_TE	Bone
Cartilage damage score_whole joint	CD_WJ	Cartilage
Hypertrophic chondrocyte score_whole joint	HC_WJ	Cartilage
Modified Mankin OA score_lateral femur	OA_LF	Cartilage
Modified Mankin OA score_lateral tibia	OA_LT	Cartilage
Modified Mankin OA score_medial femur	OA_MF	Cartilage
Modified Mankin OA score_medial tibia	OA_MT	Cartilage
Modified Mankin OA score_whole joint	OA_WJ	Cartilage
Safranin-O loss score_whole joint	SL_WJ	Cartilage
Tidemark duplication score_whole joint	TD_WJ	Cartilage
Change in IFP volume_post - pre exercise timepoints (%)	ΔIFP	IFP
Infrapatellar fat pad fibrosis (% area)	IFP_F	IFP
Infrapatellar fat pad volume	IFP_V	IFP
Change in tactile sensitivity_post - pre exercise timepoints (%)	ΔTS	Mechanobiology
Grip strength_absolute force	GS_AB	Mechanobiology
Grip strength_relative to body weight	GS_BW	Mechanobiology
Tactile sensitivity withdrawal force	TS	Mechanobiology
Daily running distance_average of Weeks 1, 5, 10	RD_1-10	Running activity
Daily running distance_Week 1	RD_1	Running activity
Daily running distance_Week 10	RD_10	Running activity
Daily running distance_Week 5	RD_5	Running activity
Running phase (% during dark phase)_average of Weeks 1, 5, 10	RP_1-10	Running activity
Running phase (% during dark phase)_Week 1	RP_1	Running activity
Running phase (% during dark phase)_Week 10	RP_10	Running activity
Running phase (% during dark phase)_Week 5	RP_5	Running activity
Body fat (%)	BF	Systemic/metabolic
Body mass	BM	Systemic/metabolic
Fasting blood glucose	FBG	Systemic/metabolic
Glucose tolerance test_area under the curve	GTT_AUC	Systemic/metabolic
Serum adiponectin	APN	Systemic/metabolic
Serum insulin-like growth factor 1	IGF-1	Systemic/metabolic
Serum interleukin-6	IL-6	Systemic/metabolic
Serum leptin	LEP	Systemic/metabolic
Serum tissue inhibitor of metalloproteinases 1	TIMP-1	Systemic/metabolic
Serum vascular cell adhesion molecule 1	VCAM-1	Systemic/metabolic

Abbreviations: IFP = infra-patellar fat pad; OA = osteoarthritis.

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