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Washington University in St. Louis

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WASHINGTON UNIVERSITY IN ST. LOUIS

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The Role of Obesity and Dietary Fatty Acid Content in Regulating Humeral Bone and Cartilage Homeostasis

by
Lauren Votava

A thesis presented to
The School of Engineering and Applied Science
of Washington University in
partial fulfillment of the
requirements for the degree
of Master of Science

May 2018
St. Louis, Missouri

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Lauren Votava

Washington University in St. Louis

May 2018

Dedicated to my parents and my grandmas,

Jane Votava and Ruth Pienkowski,

for their unwavering support.

Abstract

The Role of Obesity and Dietary Fatty Acid Content in

Regulating Humeral Bone and Cartilage Homeostasis

by

Lauren Votava

Master of Science in Biomedical Engineering

Biomedical Engineering

Washington University in St. Louis, 2018

Professor Farshid Guilak

Objective: The goal of this study was to investigate the effects of different dietary fatty acids in the context of diet-induced obesity on bone and cartilage in the humerus. It is known that obesity increases the severity of injury-induced osteoarthritis in the knee, however it is not fully understood what pathological changes have occurred due to diet alone[1]. Additionally, while it is known that shoulder osteoarthritis has a link to obesity, the alterations in this joint are incompletely described.

Methods: In order to examine diet-induced changes in both bone and cartilage, this research utilized mice that had been previously fed diets high in saturated fat (SF), omega-6 fatty polyunsaturated fatty acids (ω-6 PUFA) or omega-3 polyunsaturated fatty acids (ω-3 PUFA) for an earlier study[1]. Humeral heads were obtained for testing. Analysis for bone morphometry, bone mineral density, cartilage micro-scale mechanical properties using atomic force microscopy, and histological grading was performed.

Results: Differences in bone morphology and mineral density were seen between diet groups. The high-fat diets in general showed decreased bone quality with the ω-3 PUFA diet being partially protected. Micro-scale cartilage stiffness and overall modified Mankin scores showed no diet-dependence.

Discussion: This study showed that specific types of fatty acids differentially alter bone morphology and mineral density, with no observable changes in the articular cartilage. These findings suggest that in the shoulder, diet-induced obesity by itself may not be a risk factor for osteoarthritis, but may result in other musculoskeletal changes.

Chapter 1: Introduction

Osteoarthritis is a degenerative joint disease that is characterized by the thinning or loss of articulating cartilage surfaces, as well as altered bone remodeling and joint pain during movement[2]. There are currently no disease-modifying pharmacological treatments available for osteoarthritis (OA), and the standard of care is to prescribe painkillers until a total joint replacement is required[3]. One of the primary risk factors for OA is obesity; however, little research had been performed on this topic until recently, as historically OA was considered to be a disease of "wear and tear" or mechanical overloading. Patient data, including the link between obesity and hand and shoulder OA indicates that non-weight-bearing joints can be affected as well. Investigating this topic further, recent research has revealed that metabolic and inflammatory factors, including the content of dietary fatty acids, can significantly interact with mechanical factors to influence the pathogenesis of OA[1,4].

Pathogenesis of OA can affect more than just the articulating surface of cartilage. Increasingly this disease is known to be an whole-joint disease, and alterations in bone remodeling, bone mineral content, and synovitis, are known to occur[5]. In some studies, these changes have been shown to precede cartilage degradation markers of OA[5], although different types of OA may be initiated by differing mechanisms. While the precise timing of the bone changes leading to joint degradation have yet to be elucidated, several animal models of OA show an increase in subchondral trabecular separation and a decrease in bone volume fraction and mineral content within the affected joint[2,6]. Ultimately, these factors vary during the course of OA progression, but their direct influence on OA remains unknown. Furthermore, the biological factors associated

with obesity have also been found to contribute to bone formation and remodeling. Traditionally, the increased mechanical loading due to excess weight was thought to have an anabolic effect on bone, but studies have shown a decrease in overall bone quality with obesity[7]. This suggests that the increased incidence of OA in obese patients is not simply the result of increased mechanical forces.

Like bone, articular cartilage is sensitive to both mechanical and biological factors. Biological signals such as inflammatory cytokines can lead to upregulation of catabolic factors like matrix metalloproteinase (MMP) activity and decreases in anabolic cues like basic fibroblast growth factor (FGF)[8]. Both increased MMP activity and a decrease in FGF concentration point to amplified catabolic activity and, when accompanied by an upregulation of ADAMTS (a disintegrin and metalloproteinase with thrombospondin motifs), induce hallmarks of OA-like chondrocyte hypertrophy, osteocyte formation and cartilage matrix degeneration[8–10]. This degeneration can be seen on the microscale using atomic force microscopy to map the matrix stiffness in not only the bulk matrix, but also regions surrounding chondrocytes. Chondrocytes are surrounded by a region known as the pericellular matrix (PCM), which differs both in composition and mechanical properties to the bulk extracellular matrix (ECM). Previous studies have seen decreases in the stiffness of the PCM and the ECM with OA, as well as a decrease in the stiffness gradient between the softer PCM and stiffer ECM[11]. Decreases in the matrix stiffness would alter the mechanical environment of chondrocytes and are hypothesized to increase the forces sensed by the cell, thus leading to release of inflammatory mediators and more degeneration[12].

The complex interplay between biological signals and mechanical changes that occurs with OA is further complicated in obesity. Obesity is the result of excess adipose tissue that is now known to

be metabolically active and leads to systemic inflammation[4]. White adipose tissue releases both adipokines and cytokines that are implicated in OA progression[4]. The combination of obesity-induced pathophysiological loading and systemic inflammation from excess white adipose tissue can lead to cartilage degeneration, bone remodeling, and eventually symptomatic OA in multiple sites including the shoulder[8,13,14]. Research has shown correlations between adipokines upregulated in obesity and shoulder OA, but little is known about the link beyond observational studies[15]. To study the relationship between obesity and OA, animal models of obesity have been generated by feeding a high-fat diet[1,14,16–18]. Multiple studies have shown increased severity of surgically-induced OA for animals fed a high-fat diet, and the composition of the diet affects the severity of the disease[1,14]. High-fat diets composed of mainly saturated fatty acids (SFA) or omega-6 poly-unsaturated fatty acids (ω-6 PUFA) show considerably worse knee OA than weight-matched counterparts on a high-fat diet with omega-3 poly-unsaturated fatty acids (ω-3 PUFA) or a normal mouse chow diet[1]. These studies show that diets rich in ω-3 protect the knee from injury-induced OA while saturated fat and ω-6 PUFA seem to predispose the joint to increased damage after trauma.

While previous studies have shown OA progression to be dependent on dietary fatty acids after trauma to the joint, it is not fully understood whether diet-induced obesity causes OA in joints other than the knee. The presence of pathological changes in joint structure and stiffness due to diet alone may help to explain the increased degradation seen in certain types of diets and the protective effects of others. Additionally, most studies focus on knee or hip OA in the context of obesity, yet it is known that adipokine receptors are upregulated in chondrocytes from osteoarthritic shoulders and that adipokine concentrations in the joint correlate with body mass index (BMI)[15]. Shoulders are also the next most common site of OA after knees, with a link

between knee pain and shoulder pain[19]. Clinically, however, there is not a strong association between obesity and OA of the shoulder[20]. The goal of this investigation is to determine whether alterations in bone structure and cartilage integrity can be seen in the absence of trauma-induced OA to better understand the link between diet, obesity and predisposition to post-traumatic OA.

Chapter 2: Methods

2.1 Animal Model

All animal use procedures were approved by the local IACUC. Mice (C57BL/6) were fed diets high in saturated fat, omega-6 poly-unsaturated fatty acids (ω-6 PUFA), omega-3 poly-unsaturated fatty acids (ω-3 PUFA) or normal mouse chow for 24 weeks beginning at 4 weeks of age[1]. Initial reports on knee joints and full diet information can be found in Wu et al. 2014[1]. All animal specimens were stored at -20˚C following euthanasia. Prior to analysis, specimens were thawed at 4˚C, and humeral heads were isolated.

2.2 Micro-computed tomographical analysis of trabecular and cortical bone regions

Humeral heads were fixed in 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield PA) for 24 hours at room temperature. They were then dehydrated in ethanol and scanned in air using microCT at 9μm/pixel resolution, and an x-ray voltage of 55kV (Bruker SkyScan 1176). Trabecular regions began at the end of the subchondral plate and ended at the growth plate. These regions were thresholded and analyzed using the BoneJ extension in ImageJ[21]. Bone mineral density was calculated in both trabecular and cortical regions and was calibrated using hydroxyapatite phantoms obtained from Bruker. Trabecular bone volume fraction and trabecular thickness and separation, were calculated (n=12-15). Cortical bone regions were analyzed using the automated CTan software package (Bruker). Cortical bone cross-sectional area and thickness were calculated in two diaphyseal regions one 0.5mm from the humeral head and another at the end of the deltoid tuberosity (n=6-14).

2.3 Atomic Force Microscopy

Mechanical testing with an atomic force microscope was performed as described previously[22,23]. In brief, freshly dissected humeral heads were embedded in optimum cutting temperature (O.C.T. Tissue-Tek, Sakura Finetek USA, Inc. Torrance, CA) medium and 5μm thick cryosections of humeral head articular cartilage were obtained. Sections were briefly thawed and immunologically stained for collagen IV due to its exclusive presence in the PCM (Affinity Purified Rabbit Polyclonal Collagen Type VI Antibody; Fitzgerald Industries, Acton, MA, Goat Anti-Rabbit Alexa Fluor® 488; abcam, Cambridge, MA). Briefly, sections were washed in PBS, blocked in 10% normal goat serum (Life Technologies, Frederick, MD), and incubated with the primary antibody diluted 1:200 in blocking solution for 1 hour. Sections were rinsed with PBS and stained with Alexa-fluor488 conjugated goat anti rabbit antibody diluted at 1:200. Sections were maintained in PBS until analysis to preserve hydration. The local mechanical modulus was calculated using an atomic force microscope (MFP-3D, Asylum Research, Santa Barbara, CA) cantilever with a 5μm-diameter borosilicate spherical indenter (k = 5.4N/m; Novascan Technologies, Ames, IA) that was calibrated daily. Regions of interest were selected approximately 1-2 cells away from the outer edges of the sections to avoid edge artifacts while remaining out of deep-zone cartilage and subchondral bone. Each region was $10\mu m^2$ and an indentation was performed every 0.5μm to give a total number of 400 indentations per region. The sample was indented at a rate of 10μm/s as previous studies have shown little or no variation in modulus values for indentation speeds between 5 and 25μm/s[24]. The indentation was continued until the trigger force, 300nN, was reached.

Cantilever deflection and z-piezo movement were obtained from the Asylum Research software and analyzed using a custom MATLAB script (The MathWorks, Natick, MA). For determination

of the elastic modulus, a modified Hertz model was utilized as it has been previously applied to atomic force microscope (AFM) analysis of cartilaginous tissue[22,24–27]. Contact point extrapolation was used to determine the point at which the cantilever contacted the surface as previously described[28]. A threshold was applied to the fluorescent images to be used as an overlay for the stiffness maps to assign modulus values to either ECM or PCM. Analysis points within the cell body were excluded from further analysis. Data points were excluded if they exceeded 2.5 times the surrounding values and were replaced with the mean of the average value of the adjacent points. To plot the radially changing modulus, the cell region was thresholded in MATLAB and average moduli in increasing 0.5μm thick rings out from the edge of the cell were calculated. Due to the close proximity of other chondrocytes in murine cartilage, the data was truncated at its maximum value to avoid including the edges of PCM from the neighboring chondrocytes.

2.4 Histology

Humeral heads were fixed in 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield PA), decalcified using Calex II (Fisher) and embedded in paraffin. The samples were sectioned at 8μm and stained using safranin-O, fast green and hematoxylin. The cartilage phenotype was graded using established procedures for OA grading including a modified Mankin scoring system. This consisted of the following categories: cartilage surface structure (0-11), tidemark duplication (0-3), Safranin-O staining (0-8), chondrocyte clones in uncalcified cartilage (0-2), hypertrophic chondrocytes in calcified cartilage (0-2), and subchondral bone thickness (0-2). The total score possible was 28.

2.5 Statistical Analysis

Significance between diet groups for bone and AFM outcomes were determined by one-way ANOVA and Tukey post-hoc tests. For discrete histological grading scores, significance was determined using a Kruskal-Wallis test. Data is shown as mean ± standard deviation. Outliers were determined using a ROUT test (Q=1%). All statistics were performed in GraphPad Prism 7.03.

Chapter 3: Results

3.1 Epiphyseal bone microstructure

Micro-computed tomography (microCT) analysis of the humeral epiphyseal trabecular region indicated in Figure 3.1A showed no changes in the bone mineral density of the trabeculae (Figure 3.1B) or the trabecular thickness (Figure 3.1D). Bone volume fraction (Figure 3.1C) and trabecular separation (Figure 3.1E) were significantly different between diet groups. Bone volume fraction was significantly decreased in both the ω-6 PUFA and saturated fat diet groups whereas trabecular separation increased for the saturated fat diet group alone compared to control diet group.

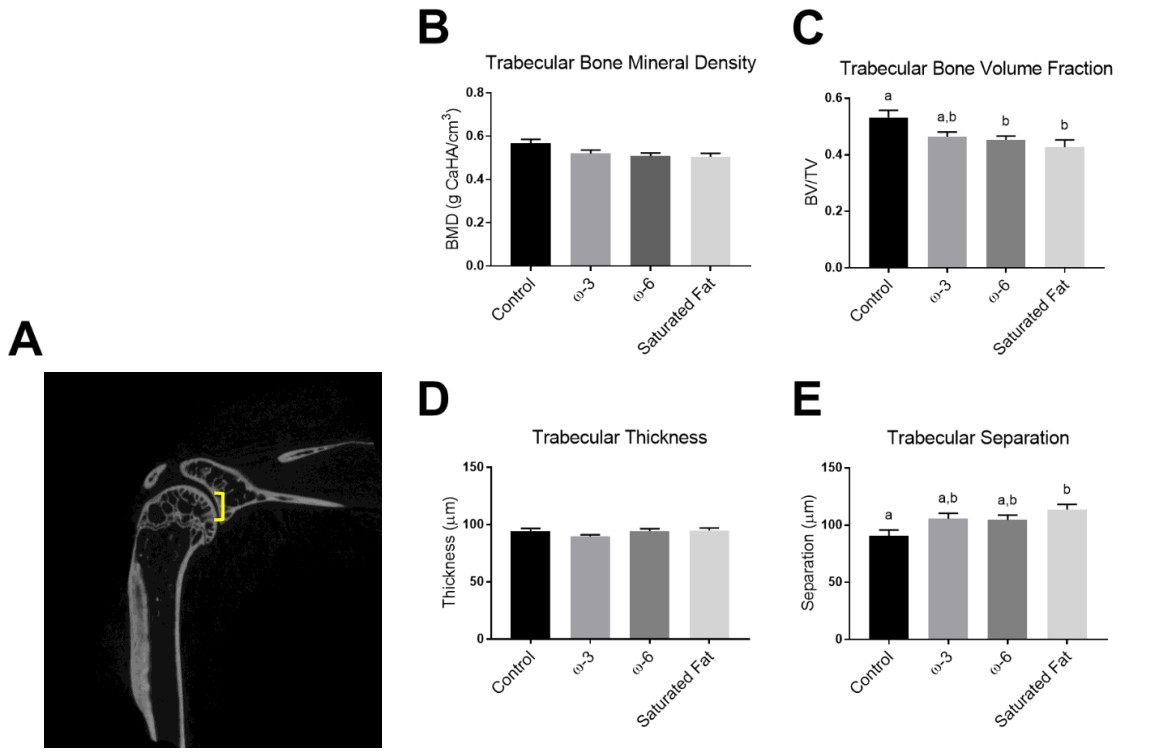

Figure 3.1: Trabecular region micro-computed tomography results. A) Visual of the trabecular region analyzed. The region began at the end of the subchondral plate and ended at the growth plate. B) Trabecular bone mineral density. No significant differences were seen due to diet. C) Bone volume fraction (bone volume/total volume). Decreases were seen in the ω-6 PUFA and saturated fat diet groups but the ω-3 PUFA diet group was not different from control. D) Trabecular thickness. Trabecular thickness was not dependent on diet. E) Trabecular separation. The saturated fat diet group showed an increase in trabecular separation as compared to the control diet. n=12-15 per diet group. Different letters are significantly different from each other. Statistical significance was determined using two-way ANOVA, $p<0.05$. All data is presented as mean ± standard error of the mean.

3.2 Cortical bone microstructure

In the cortical bone, two regions were analyzed and are shown as the upper cortical region and lower cortical region in Figure 3.2A. More pronounced changes in the bone mineral density can be seen in the lower cortical region, but significant differences due to diet are seen in both (Figure 3.2B and 3.2C). Interestingly, the saturated fat diet shows a lower bone mineral density in the upper region, but not in the lower region. Figure 3.2D and 3.2E show the average total cross-sectional area for each diet group. In the lower region, all high-fat diets result in a decrease in the total cross-sectional area, however in the upper region, only the ω-3 PUFA diet group had a decrease in area. The upper region cortical thickness in the ω-3 PUFA group was also decreased from all other diets, but the lower region thickness showed no diet-dependence.

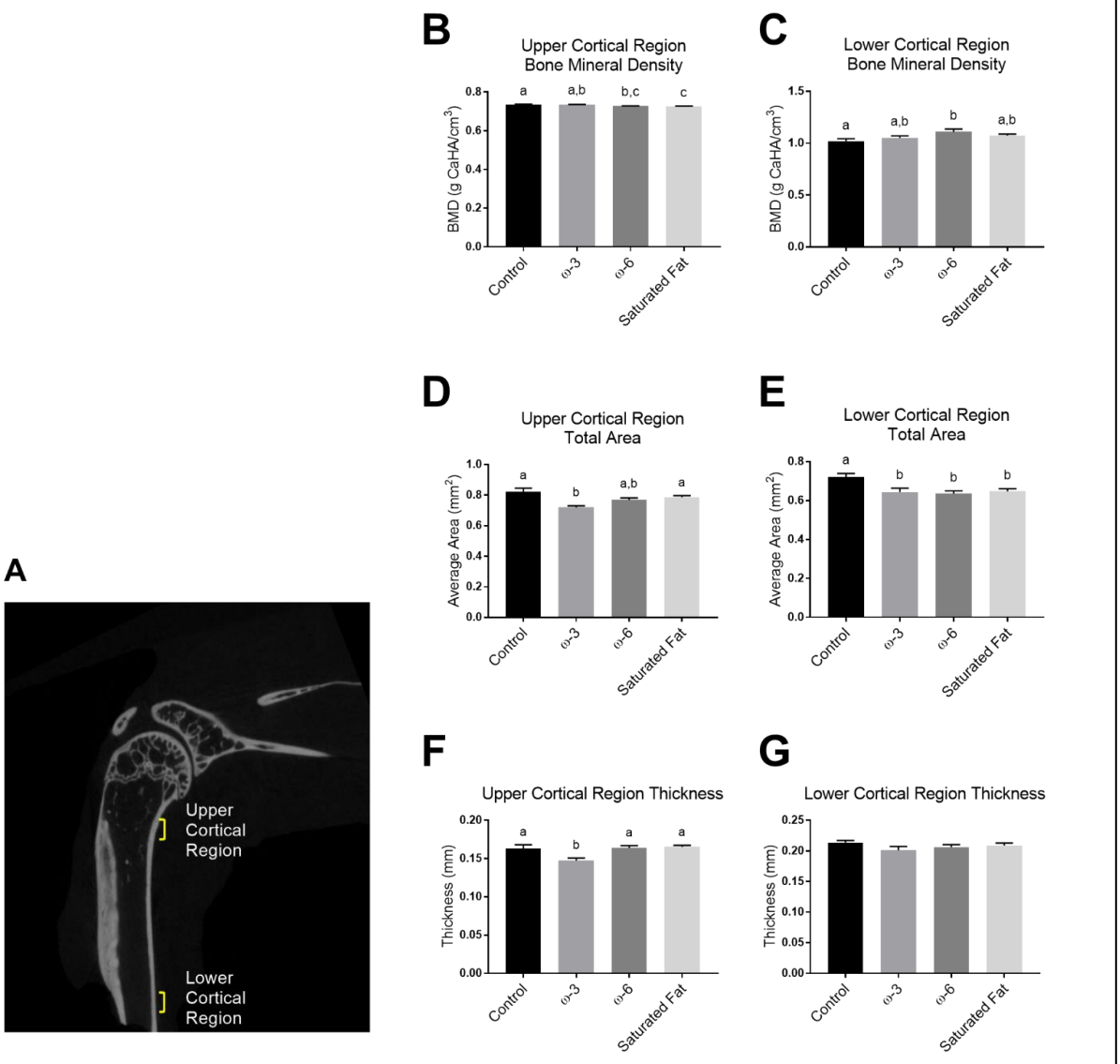

Figure 3.2: Cortical bone micro-computed tomography results. A) Visual of the two diaphyseal regions analyzed. The upper region begins 0.5mm below the end of the humeral head and is 0.5mm thick. The lower region begins after the deltoid tuberosity and is 0.4mm thick. B) Upper cortical region bone mineral density. The ω-6 PUFA and saturated fat diet groups were significantly different from the control diet, and the saturated fat diet was significantly different from the ω-3 PUFA diet as well. C) Lower cortical region bone mineral density. Contrary to what was seen in the upper region, the ω-6 PUFA diet group showed an increase in mineral density in this region as compared to the other diet groups. D) The upper cortical region cross-sectional area. A significant decrease in the cross-sectional area is seen in the ω-3 PUFA diet group. E) Lower cortical region cross-sectional area. Decreases in the cross-sectional area are seen for all high-fat diets as compared to control. F) Upper cortical region thickness. The ω-3 PUFA diet group showed a decrease in the cortical thickness as compared to all other diets. G) Lower cortical region thickness. No significant differences were seen between diet groups. All statistical significance was determined using a two-way ANOVA. $p<0.05$, different letters are significantly different from each other. All data is presented as mean ± standard error of the mean.

3.3 Atomic Force Microscopy

Immunofluorescence-guided atomic force microscopy mechanical testing showed no changes in the modulus values either in the bulk ECM (Figure 3.3A) or the PCM surrounding the cell (Figure 3.3B). The progression of the modulus from softer PCM to stiffer ECM was unchanged due to diet (Figure 3.3C). An example stiffness map is shown in Figure 3.3D. This map shows the softer PCM surrounding the cell and the gradual progression out to stiffer ECM.

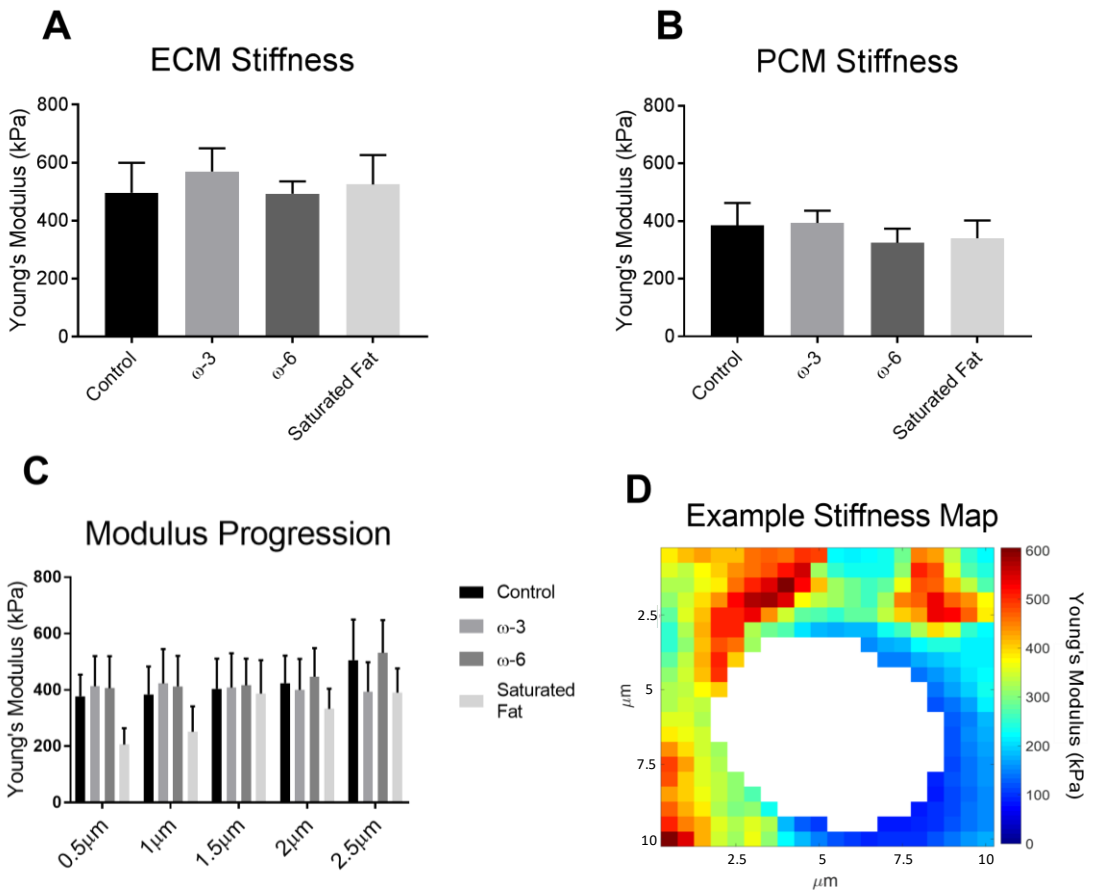

Figure 3.3: Atomic force microscopy mechanical mapping results. A) Average ECM Young's modulus. No significant differences were seen due to diet. B) Average PCM Young's modulus. No significant differences were seen due to diet. C) Young's modulus progression out from the cell in concentric rings of 0.5µm. High variability between mice was seen and no significant difference in slope due to diet was observed. D) An example stiffness map. Each pixel represents one force plot for a total of 400 indentations for each 10µm^2 area. Statistical significance was tested using a two-way ANOVA. All data is shown as mean ± standard error of the mean.

2.3 Histology

Example histology images are shown in Figure 3.4. A full humeral head is shown in Figure 3.4A and enlarged images of the cartilage surface are shown in Figure 3.4B. Full modified Mankin score results are shown in Figure 3.5.

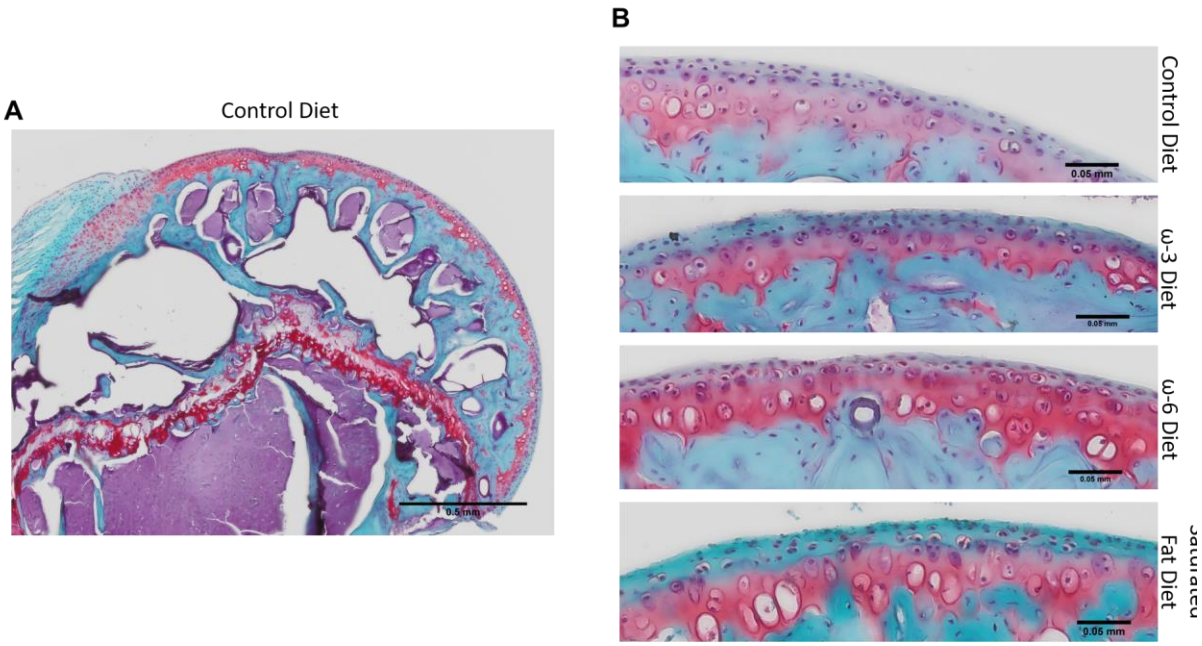

Figure 3.4: Representative histology images. A) Full humeral head from a mouse on the control diet. B) Zoomed-in images of representative cartilage surfaces for each diet group.

While an overall Mankin score showed no differences with diet (Figure 3.5A), two sub-scores that form part of the Mankin scoring system varied with diet. The number of hypertrophic chondrocytes in calcified cartilage (Figure 3.5B) was increased in the ω-6 PUFA and saturated fat groups and the number of chondrocyte clones in uncalcified cartilage (Figure 3.5C) was decreased in the ω-6 PUFA diet. Corroborating the absent cartilage phenotype, the thickness of the uncalcified cartilage (Figure 3.5D) was independent of diet, while the thickness of the calcified cartilage shows statistically insignificant trends (Figure 3.5E). Possibly due to the same

mechanisms that caused the decrease in cortical thickness in Figure 3.2F, the thickness of the subchondral plate was significantly decreased in the ω-3 PUFA diet (Figure 3.5F).

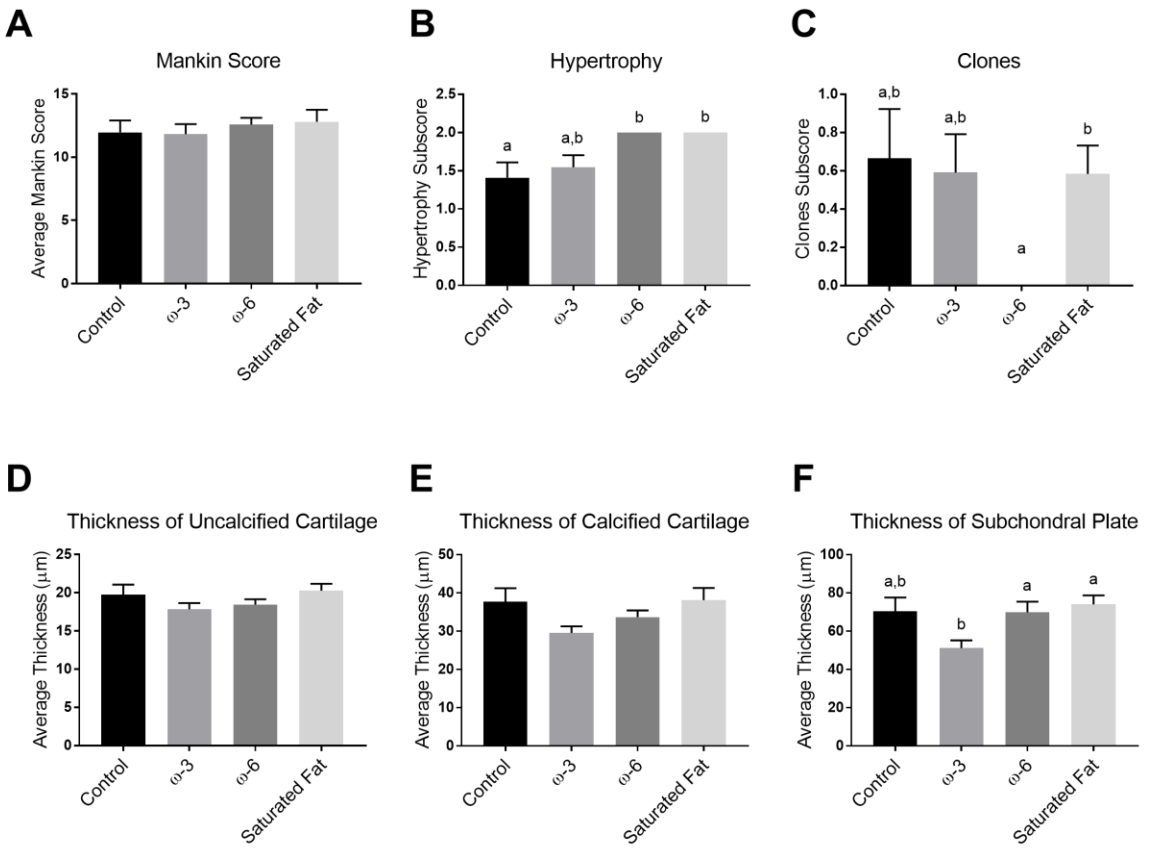

Figure 3.5: Histological scoring and thickness results. A) Overall Mankin score. No differences were seen in the total Mankin score due to diet. B) Hypertrophy subscore. The hypertrophy subscore of the total Mankin score showed a significant increase in the number of hypertrophic chondrocytes in the calcified cartilage in the ω-6 PUFA and saturated fat diet groups. C) Clones subscore. The clones subscore of the total Mankin score showed a significant decrease in the number of chondrocyte clones in the uncalcified cartilage in the ω-6 PUFA diet group as compared to the saturated fat diet. D) Average thickness of uncalcified cartilage. No significant difference was seen due to diet. E) Average thickness of calcified cartilage. No significance was seen due to diet but trends are beginning to appear. F) Average thickness of subchondral plate. A significant reduction in the thickness of the subchondral plate in the ω-3 PUFA diet group was seen. Outliers were eliminated using a ROUT test (Q=1%). Statistical significance for the Mankin scores and subscores was determined using a Kruskal-Wallis test, $p<0.05$. Statistical significance for average thicknesses was determined using a two-way ANOVA, $p<0.05$. All data is shown as mean ± standard error of the mean.

Chapter 4: Discussion

This study showed that a high-fat diet in the absence of joint injury was sufficient to cause detrimental changes in bone remodeling in the humerus both in the trabecular region as well as the cortical region. The changes seen involved alterations both in bone mineral density as well as structural changes that have been linked to an increase in fracture risk like cortical area. Unlike bone, the properties of the cartilage were mostly unaffected by a high-fat diet, even on the microscale. Matrix stiffness and the gradient between softer PCM and stiffer ECM were unchanged. Both stiffness and gradient values have been shown to decrease with OA, and the lack of a statistically significant difference in stiffness measurements on this length scale indicate the relative mechanical integrity of the cartilage. Corroborating the AFM findings, no significant differences in histological scoring for traditional signs of cartilage degeneration were found. Two subscores from the modified Mankin scoring system showed significant differences between diet groups: the number of hypertrophic chondrocytes in the calcified cartilage and the number of chondrocyte clones in the uncalcified region, but the cartilage overall showed few signs of alterations. Another histological measurement, the average thickness of the subchondral plate, was significantly reduced in the ω-3 PUFA diet group compared to the ω-6 PUFA diet and saturated fat diet.

Similar to the controversy that has surrounded the impact of obesity on OA, the effect of obesity on bone quality is currently under investigation. An increase in body weight is expected to lead to an increase in bone strength to support the extra weight during daily activities[29]. However obesity has been linked to a site-specific increase in fracture risk, including an increased risk in the proximal humerus[30]. This risk has been partially attributed to a decrease in mineral content, but the relationship between obesity and bone mineral density is incompletely understood. Some

clinical studies indicate a positive relationship between body mass index (BMI) and bone mineral density (BMD), but others show the opposite or no change[29]. These differences are due in part to the differences in age, sex, genetics, and selection of bone region to analyze.

Differences are also seen in animal models of obesity with some studies showing an increase in bone formation and others a decrease[31,32]. Our results correlate with a study by Cao et al. using the same mouse strain and a diet slightly lower in fat (45% as compared with 60%) which saw a decrease in cancellous bone mass but not cortical bone mass in the tibia[33]. This previous study showed a decrease in trabecular bone volume fraction as well as trabecular separation as was seen in our data[33]. However Cao et al. show no significant effects of high-fat feeding on tibial cortical thickness or total area[33]. Differences in the diet composition, age of the mice, length of the study and tibia versus humerus could all account for the variation in outcomes. It has been hypothesized that obesity initially leads to overall bone formation due to the increase in body weight, but eventually the systemic inflammation results in a decrease in bone formation[34]. This process has been hypothesized to occur at different rates in different bones, stressing the need for further investigation into the response of humeral bone to obesity[35].

While obesity has clearly been shown to affect bone, the type of fat present in the diet has also been shown to alter the response of bone to a high-fat diet. ω-3 PUFA have been associated with positive cardiovascular health outcomes among others, but a study found a decrease in cortical area and maximal load with high levels of supplementation in developing rabbits[36]. This decrease in cortical area agrees with our results in the upper cortical region. The decrease in bone volume for the ω-3 PUFA diet has been hypothesized to be caused by the upregulation of adiponectin in these mice as reported previously[1]. Adiponectin, acting through FoxO1 has been shown in some situations to decrease osteoblast proliferation and promote apoptosis[37]. Under what conditions ω-

3 PUFAs result in catabolic or anabolic affects is still an active area of research, but many studies report protective effects of ω-3 PUFA supplementation on bone[29]. Although awareness of ω-3 PUFA benefits is increasing, the average American diet has proportionally more ω-6 PUFA and saturated fats[38]. The relationship between ω-6 PUFA and bone is under investigation, but prostaglandin E_2, a downstream metabolite of a prevalent ω-6 PUFA, is linked to an increase in inflammation and may inhibit bone formation at high doses of ω-6 PUFA[31,35]. Intermediate doses of ω-6 PUFA show conflicting results, with clinical studies finding a positive correlation between ω-6 PUFA consumption and overall BMD in a study of post-menopausal women[39]. Saturated fatty acid intake does not show the conflicting results that ω-6 PUFA consumption does, with most but not all studies concluding that saturated fats lead to detrimental bone changes[31,32]. An *in vitro* study showed that saturated fat led to an increase in osteoclast survival and an *in vivo* study showed a decrease in bone volume fraction and an increase in trabecular separation after a high-fat diet[40,41]. These in vivo results correspond with our findings in this study.

The effects of a high-fat diet on cartilage and OA are also investigated in the literature. A series of studies showed that dietary supplementation with lard altered the progression but not the incidence of OA in mice[42,43]. Other studies have determined an increase in OA score due to a high-fat diet alone, while other studies saw no difference in Mankin scores between a chow diet and a high-fat diet[17,44]. This study found no difference in overall OA scores due to diet alone, indicating that, at least in the shoulder, bone changes occur before cartilage degeneration or that cartilage degeneration requires an initial trauma to the joint. This was corroborated by the lack of microscale cartilage stiffness changes that are known to occur with OA[11]. The lack of macro- or microscale cartilage alterations could be due to a number of factors. Changes may occur on a

longer time scale than 14 weeks on a high fat diet, and this time scale as well as the degree of damage may be joint-specific.

In this study, we found diet-specific changes in bone due to obesity in the absence of trauma, while the cartilage surface remained relatively unchanged. Studies have shown joint-specific differences in response to obesity-related OA, with correlations being seen in knee and hand OA but with little or no correlation for hip OA[4,15]. Literature on shoulder OA is relatively sparse, and future work may shed more light on the pathogenesis of shoulder OA as well as the link between it and obesity.

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