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The development and retention of articular cartilage, disrupted by metabolic stressors, is protected by low intensity vibration

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Abstract of the Dissertation

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Obesity and chronic alcohol consumption have been associated with an elevated risk of osteoarthritis (OA). We examined here whether the administration of metabolic stressors in the form of diet compromised cartilage morphology or biochemistry, and whether low intensity vibration could mitigate any of the degenerative effects observed. First, we investigated if high fat diet, in young mice, compromised the attainment of articular cartilage thickness. Further, we sought to determine if low intensity vibration (LIV) could enhance the formation of articular cartilage in a mouse model of diet induced obesity. Five-week-old, male, C57BL/6 mice were separated into 3 groups (n=10): Regular diet (RD), High fat diet (HF), and HF+LIV (HFv; 90Hz, 0.2g, 30 min/d, 5 d/w) with the diet administered for 8 weeks, and LIV for 6 weeks, which was considered short term diet and LIV administration. Additionally, an extended HF diet study was run for 6 months (LIV at 15m/d), and a separate short term HF diet study on older mice (17-weeks old C57BL/6 mice) to investigate the effects of short term diet and LIV on the mature knee joint. Lastly, we investigated the effects of chronic alcohol consumption on the young rat knee joint, and whether LIV could mitigate the degenerative effects observed. 19 four-week-old, female Wistar rats were separated into 3 groups (n=6-7/group): Control (C), Alcohol diet (A), or Alcohol +LIV, (A+LIV), with the diet and LIV administered for three weeks total. Articular cartilage and subchondral bone morphology, and sulfated GAG content were quantified using contrast agent enhanced μCT. For the young HF mouse study, gene expression

within femoral condyles was quantified using real-time polymerase chain reaction, and histology was used to measure chondrocyte cell density within the articular cartilage. After short and long term HF administration, beginning in young mice, HF cartilage thickness was not statistically different from RD, however, HF had a lower cartilage thickness to body weight ratio when compared to RD. In contrast, LIV increased cartilage thickness compared to HF, yielding a cartilage thickness to weight ratio not different from RD. Further, long term HF diet resulted in subchondral bone thickening, compared to RD, providing early evidence of OA pathology—LIV suppressed the thickening, such that levels were not significantly different from RD. HF diet and LIV administration, beginning in older mice, did not result in significant changes in articular cartilage thickness to body weight ratio, with no changes in subchondral bone thickness, however, incorporation of a refractory period stimulated increased cartilage thickness compared to RD controls. Looking at a different metabolic stressor, chronic alcohol consumption in young rats did not result in significant changes in articular cartilage thickness or sulfated GAG content. These data suggest that articular cartilage thickness failed to scale with increased body mass in HF diet mice, when the diet was administered to the animals at a young age. Dynamic loading, via LIV, stimulated an increase in cartilage formation, resulting in joint surfaces better suited to the risks of greater loading that parallel obesity. The data from older mice indicate that the aged joint was not responsive to high fat diet, and was only responsive to LIV with the incorporation of a rest period between bouts. Chronic alcohol consumption, within the short term, did not induce detectable degenerative changes in the developing rat knee. The lack of a cartilage response in the alcohol consumption model may be the result of there not being a weight gain in these animals, potentially eliminating the need for an adaptation of cartilage thickness. Age and the type of metabolic stressor will be important when considering a patient for LIV treatment for promoting joint health.

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Glossary

Abbreviation	Meaning
A	Alcohol group
A + LIV	Alcohol & low intensity vibration
Acan	Aggrecan
ADAMTS-5	A disintegrin and metalloproteinase with thrombospondin motifs 5
C	Control group
Cartilage th.	Cartilage thickness
CHAC	Chronic heavy alcohol consumption
Col2A	Collagen 2
d/w	days per week
EDTA	Ethylenediaminetetraacetic acid
EPIC-μCT	Equilibrium Partitioning of an ionic contrast agent for microcomputed tomography
g	Earth's acceleration due to gravitational field
GAG	glycosaminoglycans
HF	High Fat Diet group
HFv	High Fat Diet & low intensity vibration group
Hz	Hertz
IGF-1	Insulin-like Growth Factor 1
IL1α	Interleukin 1 alpha
Lat	Lateral
LIV	Low Intensity Vibration
Med	Medial
min/d	minutes per day
MMP-13	Metalloproteinase 13
MSCs	Mesenchymal Stem Cells
OA	Osteoarthritis
PBS/PI	phosphate buffered saline supplemented with proteinase inhibitor
RD	Regular Diet group
RHFv	High Fat Diet & Refractory low intensity vibration group
ROI	Region of interest
SB	Subchondral Bone
TNF-α	Tumor necrosis factor alpha
wks	weeks
μCT	Micro-computed tomography

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The time to submit my dissertation has finally arrived, and I have many people to thank for supporting me through this arduous journey. First, I must thank my father, Teera Pamon. Although he is not here today, I know he would be extremely proud of the challenges I endured to reach this milestone—I know this day would have meant the world to him. Thanks to my mother, Niyom, for never failing to make me laugh, lift my spirits, and being my personal on-call therapist, throughout my graduate studies.

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Lastly, I thank my wife, Grace, for always making me feel smarter and stronger than I really am. For always motivating me to reach my full potential, while at the same time, reminding me that life is also to be cherished and enjoyed. I cannot wait for our post graduate life together.

Global Hypothesis: The development and retention of articular cartilage, disrupted by metabolic stressors, is protected by low intensity vibration.

Hypothesis 1: Short term high fat diet will lead to thinner articular cartilage, and decreased proteoglycan content within the developing mouse knee, compared to regular diet animals, while low intensity vibration will mitigate these degenerative changes.

Specific Aim 1: *Assess the impact of short term high fat diet and low intensity vibration on the articular cartilage morphology and biochemistry in young mice.*
Five week old, male C57BI/6J mice will be fed a 45%kcal from fat diet for 8 weeks, to assess changes in articular cartilage morphology compared to regular diet controls. A subset of the high fat diet animals will be given low intensity vibration at the 2 week time point of diet administration, with the diet regimen continued for the remaining 6 weeks. Average cartilage thickness and relative sulfated GAG content, within the medial and lateral compartments of the mouse knee will be measured using contrast agent enhanced microCT. Cartilage matrix formation and degradative gene expression will be measured using RT-PCR. Average subchondral bone plate thickness will be quantified, in the medial and lateral compartments, using contrast agent enhanced microCT images.

Hypothesis 2: Long term high fat diet will lead to thinner articular cartilage, decreased proteoglycan content, and subchondral bone thickening, within the mouse knee compared to regular diet animals, while low intensity vibration will mitigate these degenerative changes.

Specific Aim 2: Assess the impact of long term DIO and low intensity vibration, into adulthood, on articular cartilage morphology and biochemistry.
Seven week old, male C57BI/6J mice will be fed a 45%kcal from fat diet for 6 months, to assess changes in articular cartilage morphology, after long term high fat diet administration, compared to regular diet controls. A subset of the high fat diet animals will be given low intensity vibration, with administration beginning the same time as the high fat diet. Average cartilage thickness, and relative sulfated GAG content, within the medial and lateral compartments of the mouse knee will be measured using contrast agent enhanced microCT. Average subchondral bone plate thickness will be quantified using the images obtained from the contrast agent enhanced microCT.

Hypothesis 3: Short term high fat diet and low intensity vibration, in skeletally mature mice will not lead to a change in articular cartilage thickness or proteoglycan content.

Specific Aim 3: Assess the impact of short term high fat diet, low intensity vibration, and incorporation of a refractory period into LIV treatment, beginning in adulthood, on articular cartilage morphology and biochemistry.
Seventeen week old male C57BI/6J mice will be fed a 45%kcal from fat diet for 8 weeks, to assess changes in articular cartilage morphology compared to regular diet controls. A subset of the high fat diet animals will be given low intensity vibration, and refractory low intensity vibration at the 2 week time point of diet administration, with the diet regimen continued for the remaining 6 weeks. Average cartilage thickness, and relative sulfated GAG content, within

the medial and lateral compartments of the mouse knee will be measured using contrast agent enhanced microCT. Average subchondral bone plate thickness will be quantified, in the medial and lateral compartments, within the same contrast agent enhanced microCT images.

Hypothesis 4: Short term chronic heavy alcohol consumption will lead to thinner articular cartilage, and decreased proteoglycan content within the developing rat knee, compared to controls, while low intensity vibration will mitigate these changes.

Specific Aim 4: Assess the effects of chronic heavy alcohol consumption and low intensity vibration on articular cartilage morphology and biochemistry in young rats.
Four week old Wistar rats will be fed a modified Lieber-DeCarli diet ad libitum, for three weeks containing 35% ethanol-derived calories to assess changes in cartilage morphology and biochemistry compared to control animals. Average cartilage thickness, and relative sulfated GAG content, within the medial and lateral compartments will be measured using contrast agent enhanced microCT.

Chapter 1: Background and Significance

Osteoarthritis

Osteoarthritis is a degenerative joint disease characterized by pain, reduced mobility, and cartilage loss. Although OA is often considered mainly a disorder affecting cartilage tissue, with articular cartilage legions and thinning commonly observed in OA patients, the disease also largely involves abnormal changes in the underlying bone. Osteoarthritis, is usually diagnosed by radiographic criteria, with the appearance of osteophytes, or bony protrusions, subchondral bone mineralization or thickening, narrowing of the joint space, or the manifestation of typical symptoms, such as joint pain and decreased range of motion[1]. Generally, the joint degeneration progresses gradually over years, largely affecting the middle-aged and elderly; it is the most frequent cause of physical disability among older adults[2]. However, some younger individuals have also exhibited osteoarthritis, although these cases are primarily a consequence of joint injuries resulting from trauma, such as ligament tears within the joint [3].

Articular cartilage composition and structure

Articular cartilage is the specialized hyaline cartilage of synovial joints, which facilitates the transmission of loads, and provides a lubricated surface for reduced friction during joint articulation[4]. Healthy cartilage tissue is avascular, with nutrients and wastes exchanged mainly through diffusion, which is largely assisted through cartilage compression from day to day loading[5]. The tissue is sparsely populated by chondrocytes, the cartilage cells that secrete and maintain the extracellular matrix (ECM) [6]. The cartilage ECM is primarily composed of water, collagen, and proteoglycans, which are critical to providing the tissue with its functions as a shock absorber, and a bearing surface during motion[6]. As will be described in greater

detail, proteoglycans provide the tissue with the ability to swell with water, while the collagen meshwork counters the swelling with tensile forces, providing a stable structure that can resist compressive and torsional joint loads.

Proteoglycans are large protein-polysaccharide molecules, containing a protein core, to which one or more glycosaminoglycans (GAGs) are attached. Aggrecan is the largest and most abundant GAG present in articular cartilage, and possesses chondroitin sulfate and keratin sulfate chains that provide a high density of anionic charge, and interact with hyaluronic acid to form multimolecular aggregates with even greater size. The sulfate and carboxyl groups present on the GAG chains are major contributors to the overall anionic charge of the proteoglycan complexes. As these chains electrostatically repel each other, they contribute to the osmotic properties needed for cartilage to imbibe the surrounding synovial fluid, and swell, to resist compressive and loads [7].

Collagen accounts for approximately two thirds of the dry weight of adult articular cartilage, forming a fibrillar network that entraps the proteoglycan aggregates, and provides the tissue with tensile strength. Collagen II is the principal type present within articular cartilage, however, collagens III, VI, IX, X, XI, XII, and XIV all contribute to the mature cartilage matrix [8]. In articular cartilage, these fibers have a diameter of 25-40nm[9], and as will be discussed later, their orientation varies depending on the layer of articular cartilage. Collagen II produces a fibrous ultrastructure within the tissue; the extensive cross-linking of collagen fibers, and the orientation of the fibers contributes to the tissue's material strength[8]. Collagen fibers, however, offer minimal resistance to compressive loads, they work concurrently with the swelling properties of proteoglycans to yield a stable structure.

Articular cartilage layer organization

Mature articular cartilage is separated into 4 zones: the superficial zone, middle (transitional) zone, deep zone, and calcified zone---each zone is characterized by factors such as collagen fiber orientation, proteoglycan content, and chondrocyte morphology[6] (Figure 1). The thin superficial zone is in contact with the surrounding synovial fluid, and the collagen fibers of this zone run parallel to the articular surface. The chondrocytes within this region have a flattened morphology, and the cell density is relatively higher in this region. Below the superficial zone is the middle (transitional) zone, and contains proteoglycans and thicker collagen fibrils. The collagen fibers in this region are oriented obliquely, or in a slantwise fashion. The deep zone of cartilage consists of collagen fibrils with the largest diameter, oriented in the radial direction, or perpendicular to the articular surface. Chondrocytes in this region are arranged in columns, perpendicular to the joint line. The tidemark separates the deep zone from the calcified cartilage. The calcified cartilage region is a mineralized portion of articular cartilage that provides an attachment of cartilage to bone. Within this region, chondrocytes are hypertropic [10]. Below the calcified cartilage region, is the subchondral bone.

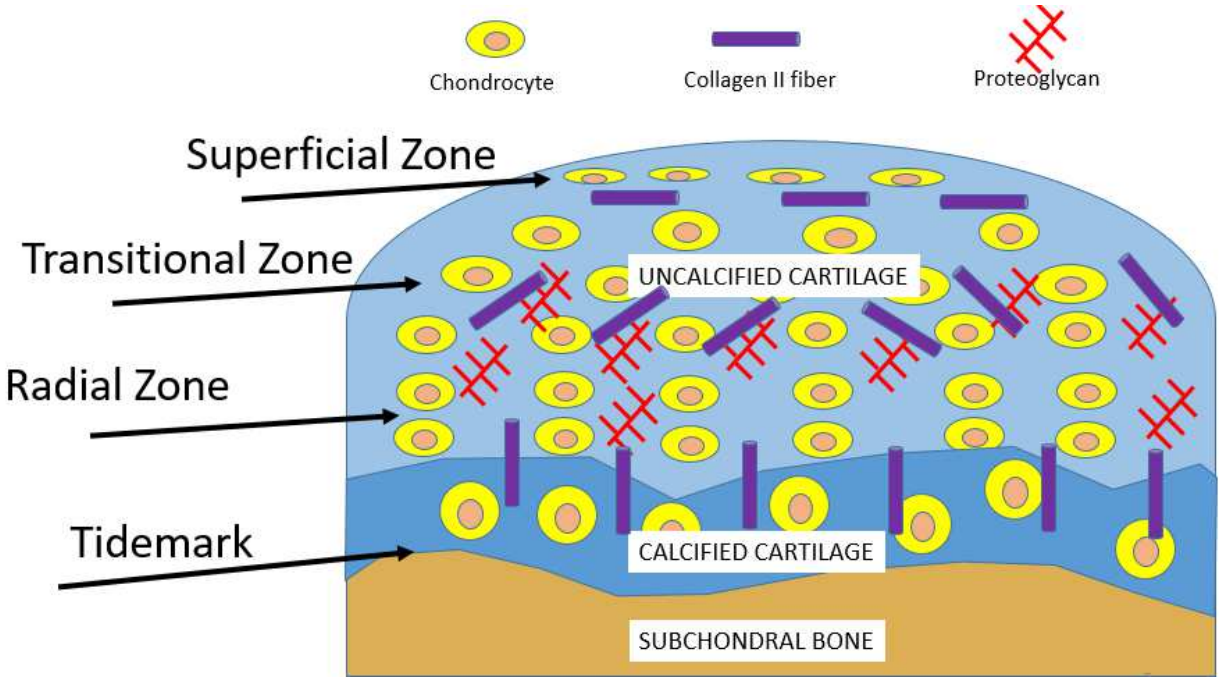

Figure 1: Schematic of articular cartilage layers, including general distribution of proteoglycans and orientation of collagen II fibers. Adapted from Hardin et al. [11]

Subchondral Bone

The bone beneath the articular cartilage can be separated into the subchondral bone plate, which is largely cortical bone, and the subchondral trabecular bone. The subchondral bone plate is the bone layer that separates the calcified cartilage from the epiphyseal marrow space [12, 13]. Within the subchondral bone plate exists vascular channels originating from the marrow space, that extend into the calcified cartilage layer, providing nutrients to the deep cartilage layers that are not nourished by the diffusion of synovial fluid[13]. Subchondral bone supports articular cartilage in its function as a shock absorber, and its mineralization and geometry is maintained as the bone remodels under physiological joint loading conditions [14], however, as will be discussed later, subchondral bone can thicken during later stage OA, leading to altered biomechanics within the joint and further degeneration.

Articular Cartilage Development

Postnatally, there is a period of cartilage development, in which significant changes in articular cartilage composition and structure occur, eventually yielding mature cartilage, which is highly anisotropic, and separated into different layers, as described previously. In New Zealand white rabbits, an animal model used for articular cartilage studies, one month after birth, superficial zone chondrocytes were flat and oriented parallel to the articular cartilage surface, however, cells were arranged more isotropically in the underlying zones. Also, the collagen network is arranged randomly, throughout the matrix. At 2-3 months after development, chondrocyte cells start to orient themselves more within the different cartilage layers, and the overall height of the articular cartilage layer begins to decrease, also the calcified cartilage region becomes more mineralized, with decreased invasion of the vasculature within the deep layers of the calcified region, and the collagen network becomes more anisotopic, resembling the alignment seen in mature cartilage. From four months, onward, the articular cartilage thickness, chondrocyte and collagen fiber orientation remain relatively constant[15].

Aging Articular Cartilage

Mature articular cartilage, in healthy individuals, has a very limited ability to maintain itself and repair, and with the natural process of aging, these functions further decline [6]. Aging chondrocytes have been shown, for example, to exhibit decreased proliferation rates and synthesis of cartilage matrix proteins when grown in culture[16]. Likewise, aging chondrocytes, when exposed to anabolic stimuli, such as growth factor IGF-1, responded with less production of proteoglycans compared to chondrocytes isolated from younger individuals[17]. Therefore, a

decrease in cartilage matrix formation is likely in aging tissue. Electron microscopy has also revealed changes in composition of the aging cartilage matrix, such as decreased length of chondroitin sulfate-rich regions of aggrecan, and elevated levels of keratin sulfate change[18]---all of which alter the density of anionic charges of the tissue, impairing its ability to draw in water and resist compression.

Aging, by itself, however, does not inevitably lead to osteoarthritis; additional factors can exacerbate cartilage degeneration to the point of joint disease, the work in this thesis will explore metabolic stressors, in the form of diet, as potential factors that can contribute to accelerated degeneration of articular cartilage tissue morphology and biochemistry. Joint disease, particularly osteoarthritis, is diagnosed by radiographic criteria, with the appearance of osteophytes, joint space narrowing, subchondral bone sclerosis, or the manifestation of typical symptoms, such as joint pain and decreased range of motion[1]. As articular cartilage tissue thinning begins, there is an initial repair attempt by chondrocytes, and remodeling and sclerosis of the subchondral bone[6]. However, the joint is eventually unable to recover, leading to the crippling disease.

Osteoarthritis Progression

Osteoarthritic changes in animal models are often observed through histology; a loss of proteoglycans is an early sign of joint degeneration, which as stated earlier, can result in impaired swelling capabilities, compromising articular cartilage mechanical properties. More severe joint changes include fibrillation of the articular cartilage surface, cartilage tears that extend through multiple cartilage regions, vascular invasion from the subchondral bone into the calcified cartilage region, and increased subchondral bone mineralization or thickening [19].

The bone resulting from subchondral plate thickening has been described as coarse fiber woven bone, similar to the primitive bone matrix initially secreted after bone fracture[20].

OA progression is thought to involve both the articular cartilage and subchondral bone, although the sequence in which these tissues degenerate, or the mechanisms in which these two regions interact during OA progression, is still unclear[14]. Subchondral bone plate thickening, alterations in the microarchitecture of subchondral trabecular bone and the formation of osteophytes at the joint margins are all bone changes associated with osteoarthritis progression. Studies using isotope labeled agents and radiography indicated that subchondral bone changes can occur early in OA, sometimes preceding detectable cartilage thinning and joint space narrowing[12, 21-23]. A recent study demonstrated that shortly after ligament transection, mesenchymal stem cells (MSCs) residing in the subchondral bone secrete TGF-β, which contributes to accelerating the degeneration of articular cartilage; blocking this signaling pathway in this cell population had a protective effect on articular cartilage [24, 25].

High fat diet and articular cartilage health

Metabolic stressors in the form of diet, can affect chondrocyte function, and contribute to the progression of OA. High fat diet induced obesity, for example, leads to the accumulation of adipose tissue, and is associated weight-related increases in joint loading. Increased functional demands placed on cartilage with obesity have been suggested as a major contributor of cartilage degeneration[26]. While at first this makes sense, it is counterintuitive, as the obese are typically less active[27], and may put new lower loads on the cartilage. A clinical study in obese individuals with high OA risk revealed that a combination of modest weight loss plus moderate exercise produced overall improvements in measures of function, pain and mobility

compared with either intervention alone [26], while another study demonstrated that a reduction in body fat (not body weight) exhibited the strongest correlation to symptomatic relief of knee OA, suggesting that joint load is not the only contributor to increased OA risk[28].

Animal models of high fat diet induced obesity have also displayed osteoarthritis pathology, including loss of proteoglycans, thickening of subchondral bone, and the appearance of osteophytes at the joint margins [29-31]. In a long term high fat diet study in mice, signs of cartilage degeneration were only observed in the medial side of the knee, generally a region considered to endure higher loads[32], suggesting that local structural or biomechanical factors contributed to OA severity[29].

However, animal studies also provide evidence that body weight is not the only contributor of increased osteoarthritis risk. Extreme obesity due to impaired leptin signaling, in leptin deficient (ob/ob), and leptin receptor deficient (db/db) mice did not result in OA progression. Despite the increased weight due to adipose tissue accumulation, the study suggests leptin signaling also plays an important role in osteoarthritis progression.

In addition to biomechanical factors, researchers have proposed that increased cartilage catabolism is, to a degree, a byproduct of excess adipose tissue and the concurrent elevated secretion of adipokines and hormones, establishing a chronic state of low grade inflammation[29], with the infrapatellar fat pad being suggested as a possible local source of adipokines[33].

Currently, there is considerable evidence that proinflammatory cytokines can contribute to cartilage tissue degeneration. For example, synovitis, or inflammation of the synovial tissue is commonly observed in early and late stage OA patients. Mononuclear cells infiltrate the

synovial tissue, producing proinflammatory cytokines interleukin 1β (IL-1β) and tumor necrosis factor alpha (TNF-α)[34]. IL-1 and TNF-α has been shown to stimulate chondrocytes to secrete proteases, including matrix metalloproteinases 1, and 13, (MMP-1, and MMP-13), that degrade both collagen and proteoglycans[35, 36]. Cartilage samples taken from osteoarthritic animals have exhibited increased presence of TNF-alpha and its receptors, suggesting an association between this inflammatory cytokine and cartilage degeneration[37]. Inhibition of cytokine activity has become a source of several new drug targets for the treatment of OA[38], which could potentially reduce osteoarthritis risk in obese patients.

Alcohol consumption and cartilage health

Another metabolic stressor investigated in this proposal is chronic heavy alcohol consumption (CHAC). Although CHAC is widely known to promote fat storage in the liver, several epidemiological studies have reported positive associations between alcohol consumption and body weight and abdominal obesity, as measured by a waist-to-hip ratio[39]. Additionally, alcohol has also been shown to promote inflammation in tissues besides the liver, such as the pancreas[40] and brain[41]. The accumulation of adipose tissue, and elevated inflammatory environment promoted by alcohol consumption, suggests that this metabolic stressor could also contribute to osteoarthritis development and progression.

Exercise and Mechanical loading effects on cartilage health

Exercise is commonly prescribed by clinicians to alleviate the symptoms of osteoarthritis[26], however, the intensity of the exercise can be a concern because extreme loads can have detrimental consequences on cartilage health. Strenuous treadmill running in rats has been shown to induce loss of proteoglycans, chondrocyte apoptosis, and increased

expression of inflammatory cytokines[42]. On the other end, the absence of mechanical stimulation, such as in the case of extended bedrest, has been shown to induce cartilage thinning within the knee[43]. Moderate running, in animal models have led to increased glycosaminoglycan content, and increased cartilage thickness, compared to sedentary controls, the anabolic effects being localized to regions of the knee viewed as bearing higher loads[44, 45]. In humans, moderate exercise, has been associated with a decreased risk of severe knee OA, suggesting that mechanical signals falling within this range could have a protective effect against cartilage degeneration [46, 47]. A clinical study investigating a regimen of supervised moderate exercise, consisting of cycling, jogging, and rope jumping, 3 times a week for a period of 4 months, on patients at high risk of developing OA (had previously undergone partial medial meniscus resection), demonstrated an increase in cartilage GAG content[45].

While direct measurements of in vivo stresses experienced in the human knee joint is challenging, studies have investigated the *ex vivo* cartilage tissue response in more controlled loading environments. Confirming the idea of overloading being detrimental to cartilage, after testing impact loads ranging from 0.5 to 65 MPa in bovine cartilage explants, there was a critical threshold of 15-20MPa that caused cell death and rupture of the collagen fiber matrix, with other studies showing degradation at an even lower range of 7-12MPa [47-49]. Cartilage tissue is also responsive to strain rate—bovine cartilage did not exhibit any changes in biosynthetic activity or mechanical properties, when compressed at a strain rate of 0.01/sec to a final strain of 50%, although peak stresses were reached (12MPa) that were high enough to induce cell death[47, 49]. In contrast, higher strain rates on the order of 0.1 and 1 per second, and peak stresses of about 18-23 MPa resulted in reduction of cartilage matrix protein production and

compressive and shear stiffness[47, 50]. Controlled mechanical loading has also been explored *in vivo*, cyclic tibial loading of 4.5N and 9.0N peak loads (1200 cycles at 4Hz, 5 days/wk) in mice over a period of 1-6 weeks induced cartilage degeneration, particularly with longer duration of loading. Cyclic loading induced articular cartilage thinning and subchondral bone thickening, and with the 9.0N loading regimen, the formation of osteophytes was observed[51].

Mechanical stimulation regimens have also been shown to have protective effects on cartilage, depending on the amplitude and frequency of the signal. For example, low-strain-amplitude (1-4%) unconfined compression at low frequency (<0.001 Hz) of cartilage explants did not result in altered biosynthesis, while, low strain amplitudes at higher frequencies (0.01-1Hz) stimulated aggrecan and protein synthesis[52, 53].

Mechanical stimulation in the form of low intensity ultrasound, has been investigated as a potential chondroprotective agent for patients at risk for developing OA. LIPUS (low intensity ultrasound; 30mW/cm2, 1.5MHz) has been shown, *in vitro*, to promote the synthesis of aggrecan, collagen type II, and collagen type IX, and *in vivo,* mitigated cartilage degeneration in an experimental rat OA model [47].

Cartilage Tissue response to mechanical forces

Experiments have confirmed that compression of cartilage causes chondrocyte and matrix deformation, hydrostatic pressure gradients, fluid flow, and changes in matrix water content, fixed charge density, ion concentrations and osmotic pressure, all of which can potentially influence cartilage matrix metabolism[53]. At the tissue level, studies using autoradiography to image cartilage explants revealed that stimulation of proteoglycan production seems to correspond to areas of increased intratissue fluid flow and matrix deformation. In addition to

physical deformation of the matrix and fluid flow, there is a transport of solutes within the articular cartilage tissue due to dynamic mechanical loading, including growth factors, nutrients, cytokines, degradative enzymes, etc. Previous studies have indicated that dynamic loading parameters, particularly amplitude frequency, can affect the transport of solutes via fluid convection. When testing peak-to-peak compression amplitudes from 5-50% and frequencies from 0.0006-0.1Hz, maximum increases in solute transport were seen with 10-20% compression, and the highest frequency of loading[54].

In terms of gene expression, articular chondrocytes have displayed anabolic responses after exposure to dynamic mechanical stimulation, such as intermittent hydrostatic pressure, and dynamic compression *in vitro*, including increased expression of aggrecan (Acan) and collagen II (Col2a) [55]. Additionally, dynamic mechanical loading of chondrocytes *in vitro* has been associated with a decrease in expression of catabolic genes including matrix metalloproteinases MMP-3 and MMP-13, enzymes associated with the degradation of collagens and cartilage proteoglycans[56, 57]. Also, an enzyme known as a disintegrin-metalloprotienases with thrombospondin motifs-5 (ADAMTS-5), cleaves proteoglycans, and is upregulated in patients exhibiting OA symptoms[58], however, fewer studies have observed noticeable changes in ADAMTS expression due to mechanical stimulation.

Low intensity Vibration and Cartilage

Mechanical stimulation, in the form of low intensity vibrations (LIV), will be investigated as a potential therapeutic, to protect cartilage development and retention, in the presence of high fat diet, and heavy consumption of alcohol. LIV, serving as a surrogate to exercise, has been shown to be anabolic to bone[59], while suppressing adiposity, in animals fed a high fat diet,

partly through the biasing of bone marrow mesenchymal stem cells towards osteoblast over adipogenic lineages [60]. LIV has also been shown to slow bone loss due to disuse, in adult female rats, and has also demonstrated the ability to protect bone quality in ovariectomized mice, a model of postmenopausal osteoporosis. In addition to inducing anabolic effects in bone tissue, LIV has been reported to mitigate the suppression of muscle satellite cell populations[61] in skeletal muscle due to ovariectomy. Further, LIV has displayed protective effects on intervertebral disc morphology and biochemistry [62], in a rat disuse model of hindlimb unloading.

In humans, LIV has also induced anabolic responses in bone, mitigating bone loss in postmenopausal women[63] and children with disabling conditions[64]. Additionally, administration of LIV has been shown to preserve postural control in healthy subjects undergoing 90 days of bedrest, and helped retain flexion strength in the knee and ankle, reinforcing animal data that reported an anabolic effect in muscle tissue due to LIV[65]. Administration of LIV has also attenuated intervertebral disc swelling in subjects after 90 days of bed rest, and 7 days of reambulation[66], demonstrating a potential anabolic effect of LIV in cartilage tissue.

The magnitude of forces imparted by LIV are orders of magnitude below what would be experienced with exercise[67], therefore, we predict the signal will not produce degenerative effects in cartilage tissue, but instead, would fall within the category of moderate mechanical stimulation, inducing an anabolic effect in cartilage tissue.

Overall Objective and hypothesis

In this study presented here, we seek to determine whether metabolic stressors, in the form of high fat diet, and chronic alcohol consumption, disrupt the development and retention of articular cartilage, and should that happen, if low intensity vibration can protect development and retention, challenged by these stressors.

CHAPTER 2:

Articular cartilage thickness, failing to scale with obesity, in young mice, achieves body mass appropriate morphology, when stimulated by low magnitude mechanical signals

Specific Aim 1: ***Assess the impact of short term high fat diet and low intensity vibration on the articular cartilage morphology and biochemistry in young mice.***

Abstract: Obesity is a risk factor for osteoarthritis, a degenerative joint disease characterized by joint pain and immobility. The elevated risk has been attributed to higher joint loading due to body weight. Since obesity has been associated with the disruption of bone formation, it is possible that cartilage formation is also impeded. Recent evidence has shown that low intensity vibration (LIV) protects mesenchymal stem cell (MSC) lineage selection towards the formation of higher order connective tissues, and we sought to determine if these signals could protect the formation of AC in spite of the obese phenotype. C57BL/6 male, 5w mice were separated into 3 groups (n=10): Regular diet (RD), High fat diet (HF), and High fat diet + LIV (HFv). 2w of high fat diet were given to HF and HFv. LIV (90Hz, 0.2g, 30 min/d, 5 d/w) was then administered to HFv for 6 wks, with all groups continuing their respective diets. The R tibias were extracted, and the AC and subchondral bone (SB) of the tibial plateau were visualized using contrast agent enhanced μCT. AC and SB thickness were measured within a 486um ROI, centered at the midpoint of the medial condyle. Means and SD are reported, and the one-way ANOVA was used to detect differences between groups; a p-value<0.05 was considered statistically significant. After 8 wks of high fat diet, AC thickness between RD and HF animals was not statistically different. However, HF may still require thicker AC, compared to RD, to support a 17.2% increase in body weight (p<0.01). Within the medial tibia, HF exhibited a lower thickness/weight ratio compared to RD (-27.3%, p=0.04). In contrast, LIV led to increased AC thickness, compared to the HF (62.0+%, p<0.001), and exhibited a higher thickness/weight ratio than HF (+66.3%, p<0.01), to levels n.s.d. from RD. SB thickness and mineralization was not

different between groups. While HF did not result in a thinning of cartilage, LIV induced an increase in cartilage thickness, which brought the ratio of cartilage thickness to body weight to levels not significantly different from the RD group. Although no join degeneration was detected with this short term study, in the longer term, the elevated articular cartilage thickness of the HFv group could provide a more suitable joint surface to endure the increased joint loads due to obesity.

Introduction:

Obesity is a recognized risk factor for osteoarthritis, the elevated evidence of osteoarthritis which parallels obesity is often presumed to result from tissue ablation as might be caused by weight-related increases in joint loading[68], exacerbated by an inability of cartilage to positively adapt to the elevated mechanical demands. While at first glance this makes some sense, the increased functional demands placed on cartilage with obesity, it is also counterintuitive, as the obese typically are less active[27], and may actually put net lower loads on cartilage, making it difficult to conclude that osteoarthritis is a result of cartilage abuse rather than inadequate cartilage thickness to start with. Aside from increased body weight, others have proposed that increased cartilage catabolism is – to a degree - a byproduct of excess adipose tissue and the concurrent elevated secretion of adipokines and hormones, establishing a chronic state of low-grade inflammation[29]

In adult animal models, high fat diet has been associated with advanced osteoarthritis, as measured by lower proteoglycan content within the articular cartilage, thickening of subchondral bone, and increased osteophyte formation within the knee joint [29-31] **.** Fewer

studies, however have investigated the consequences of high fat diet on the growth and attainment of articular cartilage in young, developing animals, to determine if a propensity to form adipose tissue in some way compromises the formation of articular cartilage. One study performed in young, obese mice reported that a high fat diet induced the formation of cartilaginous osteophytes, which was paralleled by elevated infrapatellar fat pad size, vascularization, and secretion of adipokines, and inflammatory cytokines--- the fat pad was suggested as playing a pivotal role in the formation of osteophytes [33].However, whether high fat diet in these young animals hindered cartilage development, or elevated catabolic activity is still unclear.

In parallel with dietary consequences on cartilage morphology, extreme loading demands on the joint can have detrimental consequences on cartilage health. In animals, strenuous treadmill running in mature small animals has been shown to induce osteoarthritic changes in the joint, such as reduced chondroitin sulfate and hyaluronic acid levels, increased chondrocyte apoptosis and elevated inflammatory cytokine expression [42]. Defined high peak loads applied cyclically in young and older mice resulted in decreased articular cartilage thickness, with incidences of periarticular osteophyte formation [51]. In humans, extreme demands on joints, as might result from long distance running [69] or weight lifting results in a higher incidence and earlier onset of osteoarthritis[70].

Curiously similar to the damage caused by extreme loading, the *absence* of mechanical challenges to the joint as also can be detrimental to cartilage tissue. In humans, extended bedrest has led to decreased cartilage thickness within the knee joint [43].

In contrast to the deleterious impact of strenuous loading or marked unloading, moderate exercise in the form of cycling, rope skipping, and light jogging, has been shown to improve knee cartilage GAG content in patients at high risk of developing knee OA [45], emphasizing that *some* mechanical signals are better than none. In a hamster model of osteoarthritis, daily wheel running was shown to mitigate the cartilage fibrillation, and proteoglycan loss that was exhibited in the sedentary group[44] , despite previous observations that exercising hamsters tend to be even heavier than sedentary ones due to greater muscle mass. Moderate treadmill running in dogs was shown to enhance cartilage GAG content and thickness compared to non-running controls [71]. Likewise, daily running in high fat diet fed mice mitigated the cartilage GAG loss and subchondral bone thickening[29], without any changes in bodyweight compared to the sedentary group, suggesting that dynamic mechanical signals may promote cartilage health while static mechanical signals are unable to mitigate OA progression. Looking at the cellular level, chondrocytes cultured *in vitro* generally increased total synthesis of GAGs when exposed to dynamic compression when compared to chondrocytes under static compression [72]. While these data suggest that, excessive or inadequate loading may be deleterious to cartilage, there appears to be a 'window' where some degree of exercise is beneficial to the tissue. While moderate exercise is generally recommended by physicians to mitigate osteoarthritis symptoms [26], the challenge is that compliance with a daily exercise regimen is typically very poor, particularly in the morbidly obese[73].

As a surrogate for exercise, low intensity vibration (LIV) delivers a relatively high frequency (10-100Hz), low magnitude (<1.0g, where 1g is Earth's gravitational field), mechanical signal,

resulting in a challenge to the musculoskeletal system that is several orders of magnitude below that which arises during strenuous activity [67], but has been shown to be anabolic to both bone [59] and muscle[61], but suppress adiposity[60]. Using rodent models of diet-induced obesity, recent evidence has shown that LIV biases mesenchymal stem cell (MSC) lineage selection towards the formation of higher order connective tissues such as bone, and away from adipogenesis[59-61, 67, 74]. In the study reported here, we sought to determine if high fat feeding disrupted the formation of cartilage and thus contributed to articular surfaces being under-engineered for the challenges of obesity, and should that happen, if LIV could protect the formation of articular cartilage otherwise challenged by the obese phenotype.

Methods

Animals and Experimental Design

This study was reviewed and approved by the Institutional Animal Care and Use Committee of Stony Brook University. To investigate the effects of high fat diet and low intensity vibration on knee joint changes in growing mice, 30 five-week old male C57BL/6J mice (The Jackson Laboratory, Bar Harbor, ME, USA) were weight-matched, and assigned to 3 groups (n=10/group): regular diet (RD), high fat diet (HF), and high fat diet +LIV (HFv). At baseline, mice were fed either a regular or high fat diet (45% kcal from fat; van Heek Series 58V8; TestDiet, Richmond, IN, USA) *ad libitum*. Food consumption was monitored and animals were weighed once a week. At the 2w time-point, the HFv group was subjected to LIV (0.2g, 90 Hz sine wave, 30 min/d, 5 d/w; where g is Earth's gravitational field, or 9.8m/s^2). Each day, all mice in RD and

HF were placed on an inactive LIV platform for the same duration as HFv. Each group continued their respective diets for the following 6 weeks. At 8w, at the end of the protocol, all animals were euthanized, and thus data for the short term protocol are presented for mice at 13w of age.

Assessment of articular cartilage thickness using a contrast agent based microcomputed tomography technique.

At tissue harvest, right tibias from each animal were extracted, bisected at the midshaft, and the proximal end stored in phosphate buffered saline supplemented with proteinase inhibitor (PBS/PI), and temporarily stored at 4^{o}C. To enhance image intensity of the articular cartilage, the proximal region of each tibia was incubated in a solution of 15% Ioxaglate (Hexabrix, Mallinckrodt Inc., St Louis, MO) and 85% PBS/PI at 37^{o}C for 30 min, as described by Kotwal et. al [75]. Hexabrix is a negatively charged hexaiodinated dimer, which when diluted appropriately, has x-ray attenuation properties that are distinct from the underlying bone, allowing for segmentation of the articular cartilage. Also, due to the electrochemical interactions between both negatively charged Hexabrix and proteoglycans of the cartilage, the equilibrated concentrations of the contrast agent is inversely related to the proteoglycan concentration within the tissue [21]. Samples were then patted dry to remove adherent fluid droplets, and scanned in air using high resolution micro computed tomography (ex vivo µCT 40; Scanco Medical, Bassersdorf, Switzerland), using 6-µm isotropic voxels at 45kVp, 177 µA, and 300ms integration time.

The 3D grayscale image of each sample was rotated to obtain sagittal sections of the tibial plateau, and contained the subchondral bone, cartilage, and surrounding air, while

excluding the marrow space. An appropriate global threshold was identified from attenuation histograms of representative samples from each group, to isolate the articular cartilage from the subchondral bone---one global threshold was then applied for all animals (RD, HF, HFv).

The regions containing the thickest cartilage were isolated within the medial and lateral plateau (Figure 2), which we inferred as being high weight bearing regions. The medial and lateral tibial ROIs were centered at the midline of each condyle, each spanning a length of 486um (Figure 2). The width of the medial ROI was defined at the midpoint slice of the medial plateau, using a dip in the growth plate, and the posterior end of the tibia as landmarks, and the resulting width was maintained across the entire ROI (Figure 3). For the lateral plateau, the width was defined on the slice closest to the lateral edge---the anterior half of the flat surface of the lateral plateau was isolated (Figure 4).

Subchondral bone was isolated from the same ROIs described above, using a separate global threshold for bone, applied to all samples. As with cartilage, a distance transformation algorithm within the Scanco software was used to calculate average subchondral bone plate thickness for the medial and lateral tibial plateau.

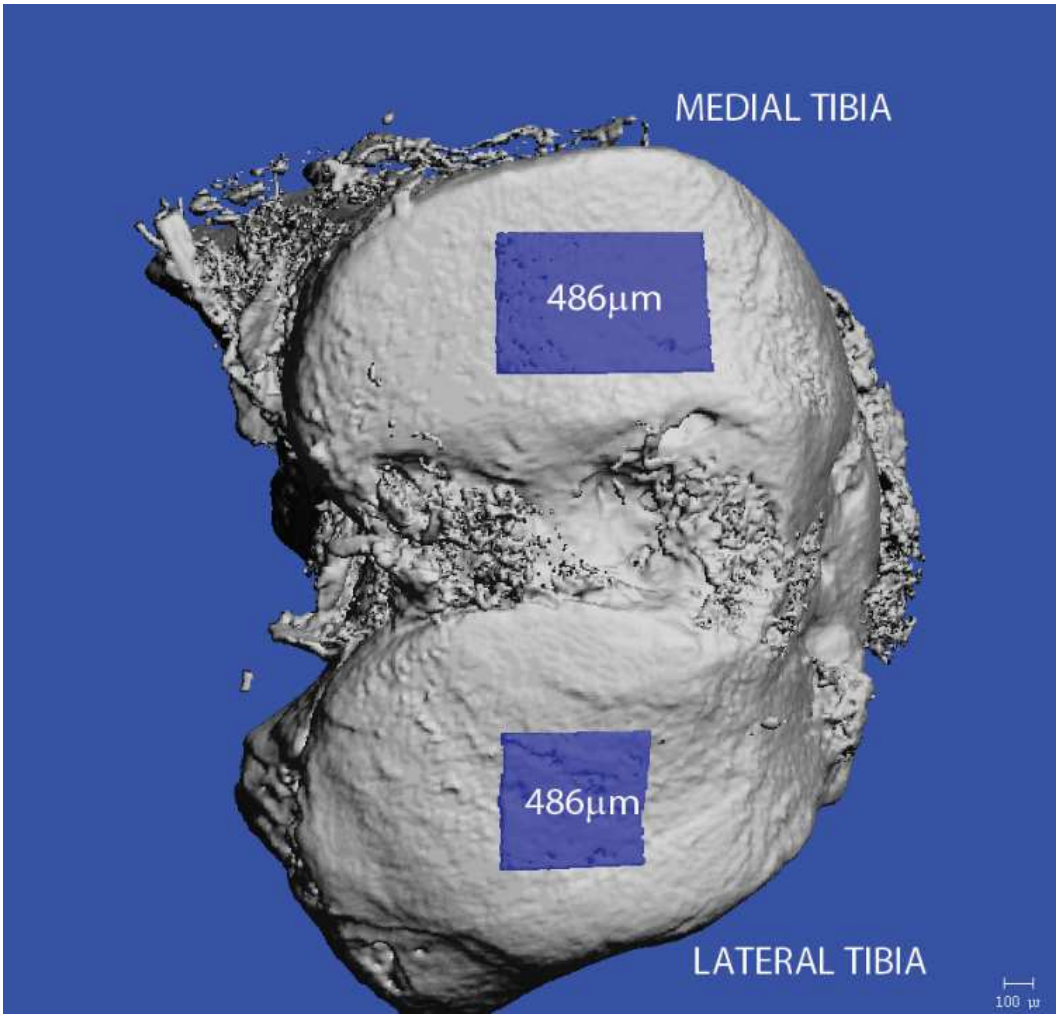

Figure 2: Representative regions of interest, for articular cartilage thickness and x-ray attenuation calculations, for medial and lateral tibia.

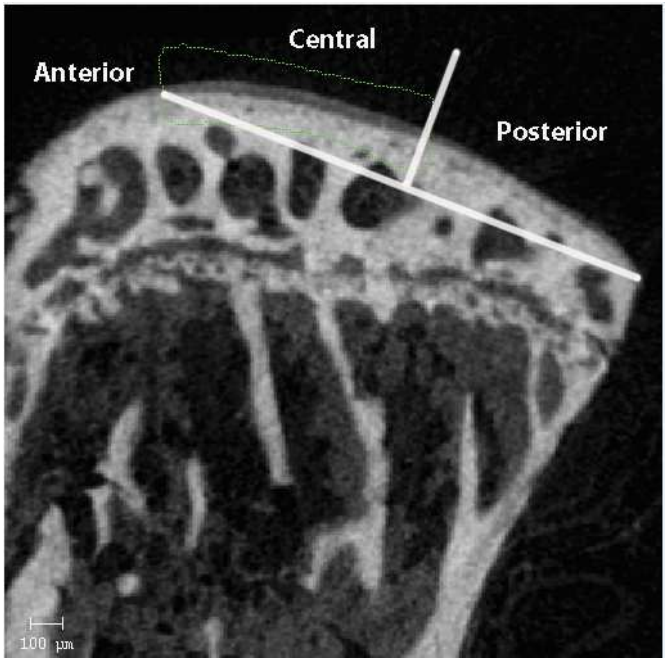

Figure 3: Medial tibia sagittal contouring to isolate central region of medial surface

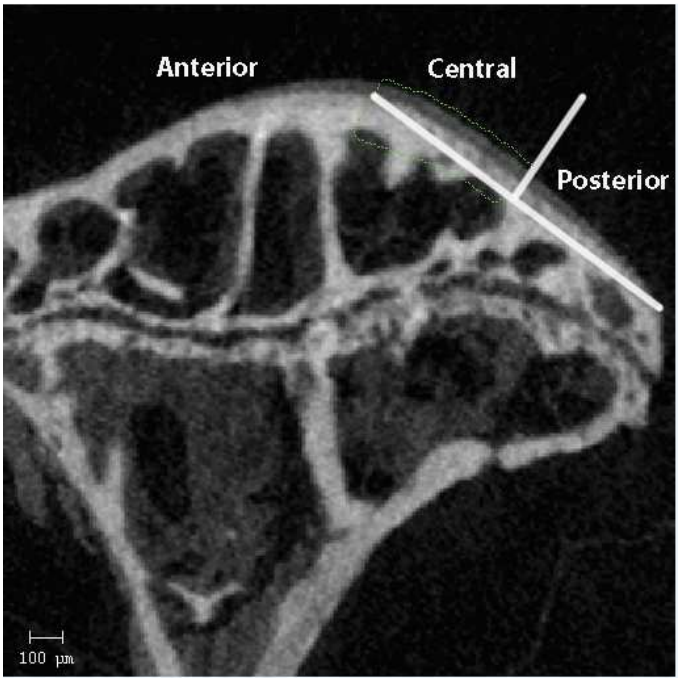

Figure 4: Lateral tibia sagittal contouring to isolate central portion of lateral surface

Measuring relative proteoglycan content using contrast agent enhanced microCT

Relative sulfated GAG content, was measured on a 3-dimensional scale, by calculating the average x-ray attenuation of the medial and lateral cartilage ROIs obtained from the EPIC-μCT. Hexabrix has a net negative charge, which repels the negative charges of the sulfated GAGs associated with the large proteoglycan aggrecan. Therefore, the conditions of electrochemical equilibrium predict that Hexabrix distribution within the tissue would be inversely related to the amount of sulfated GAGs present at a particular location; a sample having a lower average attenuation value was therefore, interpreted as having lower relative sulfated GAG content.

Measuring chondrocyte density using histological staining methods

Histological staining was used to assess chondrocyte cell density. Intact left knee joints, including the distal femur and proximal tibia from each mouse were decalcified in 14% EDTA, dehydrated and embedded in paraffin. Sagittal 6-micron sections were collected at the approximate midpoint of the medial and lateral condyles of the femur and tibia, stained with Toluidine blue, a cationic dye that has a high affinity for proteoglycans, and whose staining intensity has been associated with proteoglycan content, and counter stained with fast green. Using IMAGE J, within a single section of the medial and lateral tibia, the total number of cells were counted within an area of the uncalcified cartilage region---density was calculated as the (total # of cartilage cells/total area of interest).

Characterizing chondrocyte anabolic and catabolic activity via RT-PCR

At sacrifice, the distal right femur, dissected to include articular cartilage, bone, and the entirety of the growth plate, was extracted and stored in RNAlater. To extract RNA, the distal

femur was first cleaned to eliminate surrounding muscle tissue, and crushed using a handheld homogenizer, and further homogenized with steel beads in the Bullet Blender in Qiazol reagent to lyse cells. Chloroform separation was used to isolate RNA in an aqueous phase, and was then purified using a Qiagen RNeasy kit, including DNase digestion of contaminating DNA. The concentration of each RNA sample was measured using the Nanodrop ND-1000 spectrophotometer, which were then diluted to 5ng/uL, and converted to cDNA using the StepOnePlus RT-PCR system. Expression levels of cartilage matrix genes, Aggrecan (Acan) and Collagen II (Col2A), and cartilage catabolic genes: Matrix Metalloproteinase 13 (MMP-13) and a disintegrin and metalloproteinase with thrombospondin motifs 5 (ADAMTS5) were measured using Taqman gene expression assays, with mouse GAPD used as a housekeeping gene.

Statistics

All data are shown as means ± SD. GraphPad Prism 6 (GraphPad Software Inc, CA, USA) was used for all statistical analyses. To determine differences between groups, a 1-way analysis of variance (ANOVA) with a Tukey *post hoc* analysis test was used. Differences were considered statistically significant if $p \leq 0.05$. For linear regression analysis, $p \leq 0.05$ was considered statistically significant. Grubb's test, with an alpha=0.05, was used to determine if data points were considered outliers.

Results:

Short term high fat diet promoted the obese phenotype

Following 8w of high fat diet, the young HF and HFv groups exhibited a 17.2% and 15.4% increase in total body weight, respectively, as compared to that realized by the regular diet group over the same time period (**Fig. 5**, $p \leq 0.01$). The total abdominal fat pad mass of the

young HF and HFv groups were 168% and 159% greater, respectively, than the regular diet group ($p \leq 0.003$). There were no significant differences in body weight or abdominal fat pad mass between the young HF and HFv groups.

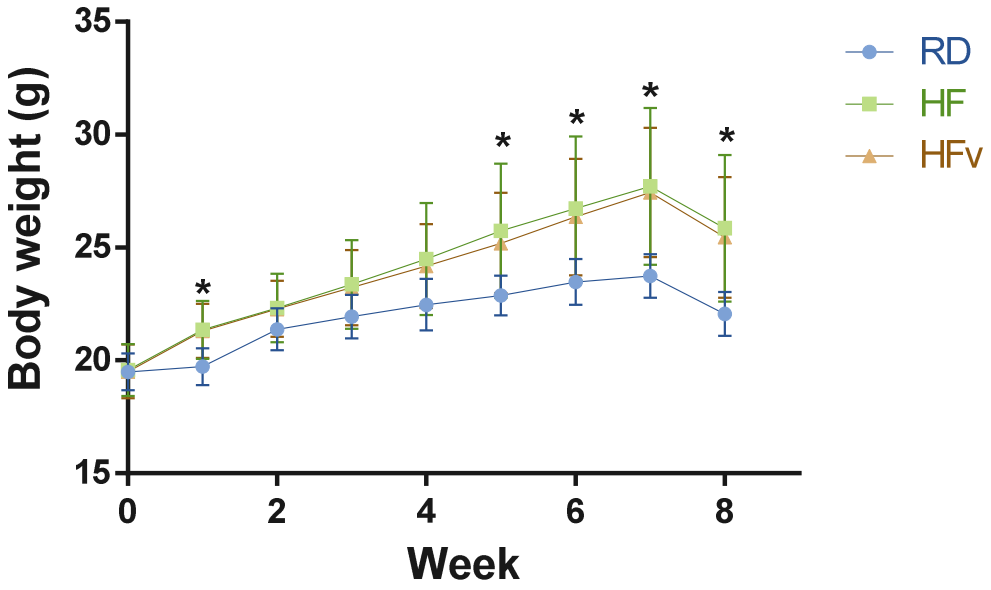

Figure 5: Weekly body weights of young mice receiving short term diet/LIV. HF and HFv exhibited significantly higher body weights compared to RD, but were not significantly different from each other (* $p<0.05$ HF and HFv compared to RD).

Cartilage thickness fails to scale with weight in young obese mice

There were no significant differences in cartilage thickness between young RD and HF ($p=0.45$) **(Fig 6 and 7)**, despite the 17% difference in body mass. However, while body weight correlated strongly with medial tibial cartilage thickness for young RD mice (R-square = 0.5144, $p=0.02$), this relationship was disrupted in young HF and HFv mice **(Fig 8).**

The absence of differences between cartilage thickness of RD and HF was also evident within the lateral tibial plateau, (+7.1%, $p= 0.83$) **(Figure 6)**.

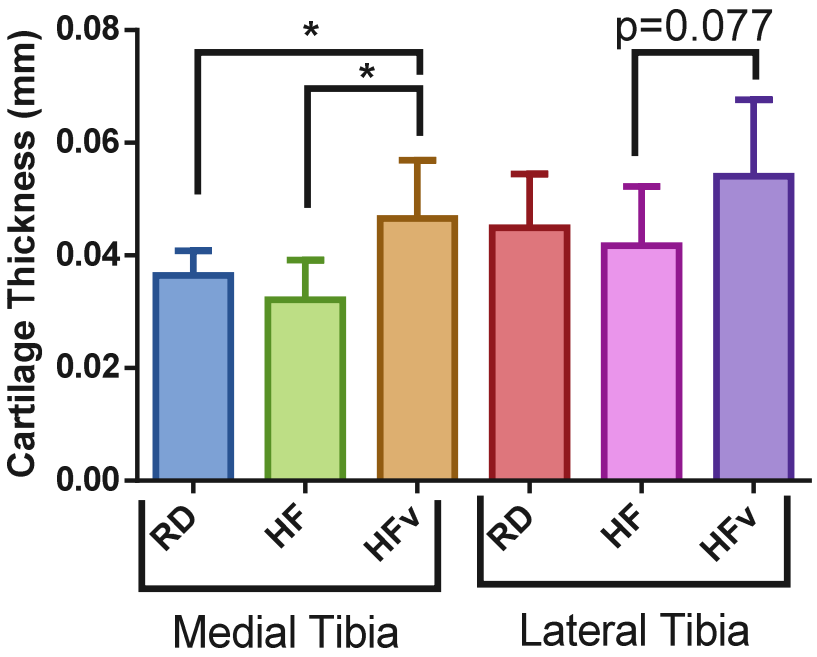

Figure 6: Medial and lateral articular cartilage thickness measured using EPIC-uCT in young mice receiving short term diet/LIV. HFv exhibited increased articular cartilage thickness compared to both RD and HF groups in the medial side, with a trend of thicker cartilage due to LIV in the lateral compartment (*$p<0.05$ compared to HFv).

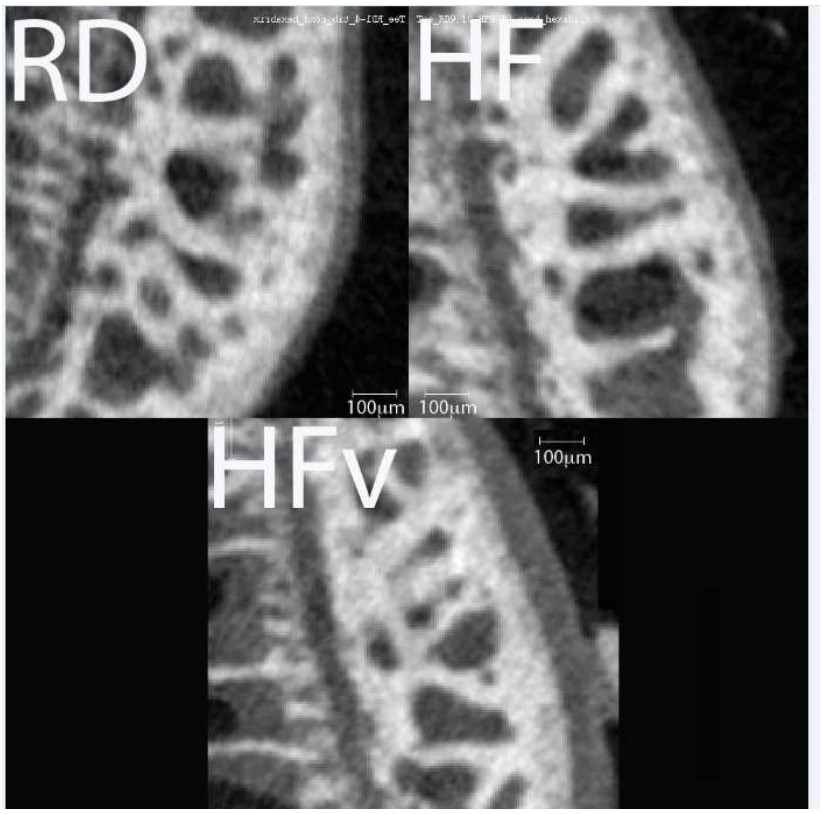

Figure 7: Representative images of medial tibia cartilage from RD, HF and HFv groups in young mice receiving short term diet/LIV. HFv group exhibited significantly thicker cartilage compared to both RD and HF groups.

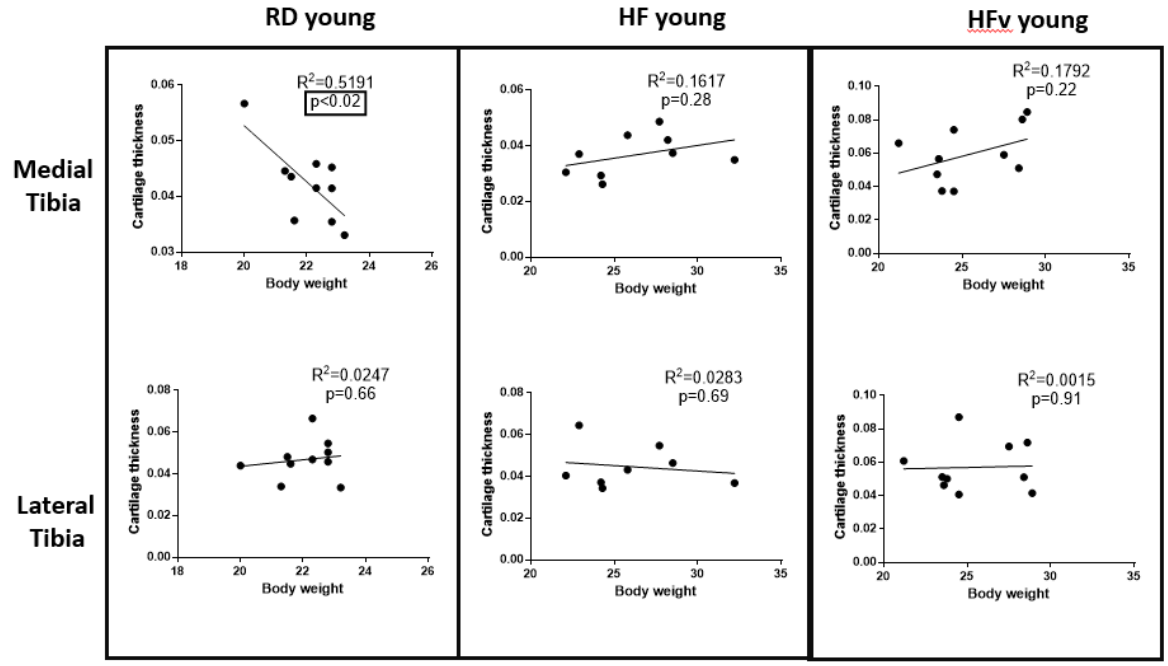

Figure 8: Linear regression analysis of articular cartilage thickness to body weight in young mice receiving short term diet/LIV, for the medial and lateral tibia. RD animals exhibited a linear relationship between cartilage thickness and bodyweight, although in all other groups, there was no linear relationship between cartilage thickness and body weight

Short term LIV promotes thicker cartilage, preserving the cartilage thickness to body weight ratio

LIV stimulated increased cartilage thickness within the medial condyle, in young HFv mice, as compared to HF (+33.1%, $p<0.001$). Young HFv animals exhibited cartilage thickness that exceeded the young RD group (+27.8%, $p<0.02$) **(Figure 6)**.

Within the lateral tibial plateau, young HFv exhibited a trend of increased cartilage thickness (+29.6%, $p<0.08$) compared to young HF **(Figure 6)**. In contrast, no difference in lateral tibial cartilage thickness was measured when comparing HF to HFv (+0.32%, $p= 0.58$). HFv lateral tibial cartilage thickness was not significantly different from RD (+20.4%, $p=0.19$).

Neither diet nor LIV perturb proteoglycan content in cartilage

In the medial and lateral tibial plateau cartilage, there were no significant differences in x-ray attenuation, measured via EPIC-µCT, between the young RD, HF, and HFv groups **(Figure 9)**, indicating that there was no detectable difference in sulfated GAG content between the three groups.

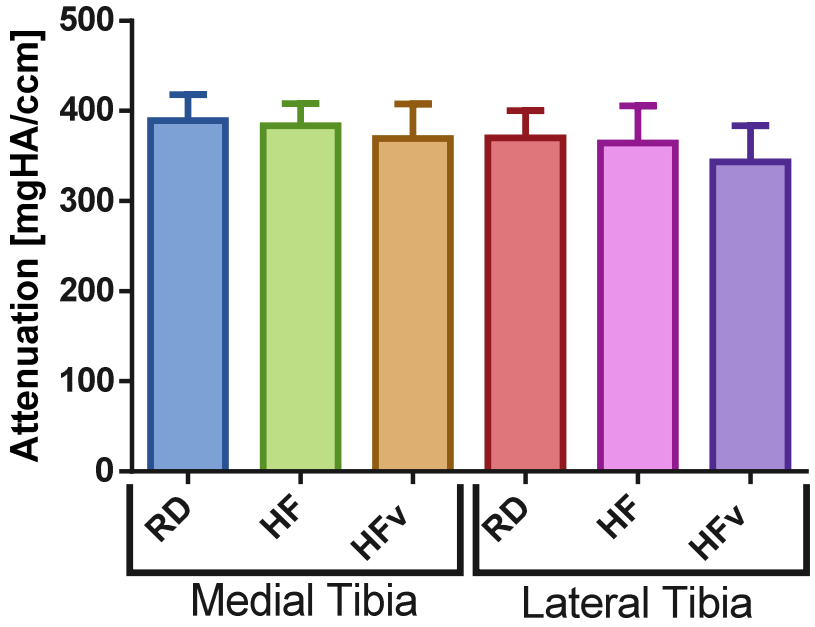

Figure 9: Medial and lateral tibial cartilage x-ray attenuation in young mice receiving short term diet/LIV , interpreted as relative proteoglycan content. No differences were observed between RD, HF, and HFv groups.

Articular cartilage cell density maintained after diet and LIV

There was no difference in articular cartilage cell density within the medial or lateral tibia between RD and HF groups. Despite an increase in cartilage thickness in the LIV group, cell density was maintained **(Figure 10).**

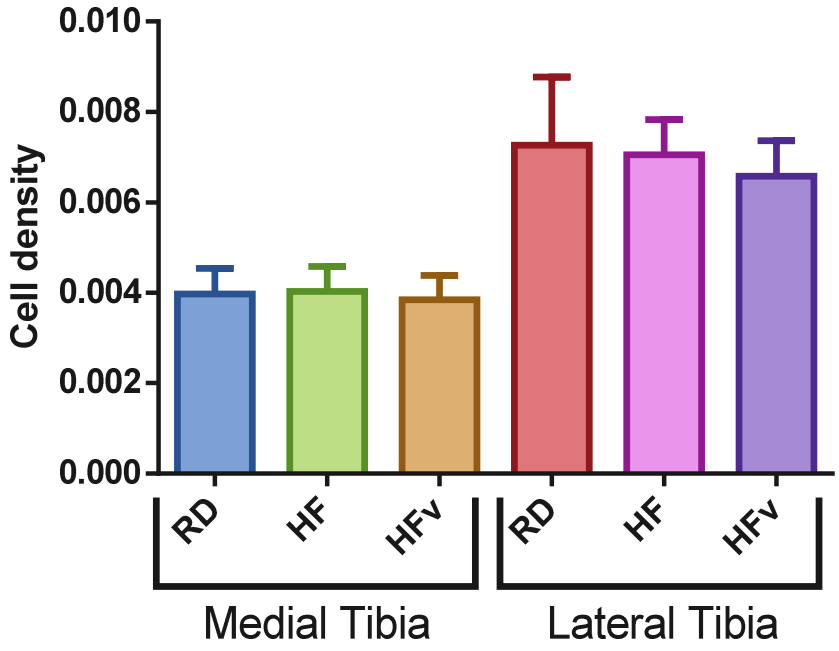

Figure 10: Medial and lateral tibial articular cartilage cell density, determined through histological staining with Toluidine Blue/Fast Green, in young mice receiving short term diet/LIV. No difference was observed in articular cartilage cell density between groups.

No changes in expression of anabolic or catabolic cartilage genes detected, although LIV decreased the level of inflammatory cytokine expression.

The entire femoral condyle included a pooled samples of the articular cartilage, subchondral bone, and growth plate cartilage. Within this pooled sample, there was no detectable difference in gene expression of anabolic genes: aggrecan (Acan) and collagen 2 (Col2A), or catabolic genes MMP-13, or ADAMTS-5 **(Figure 11)**. However, LIV did result in a significant decrease in expression of inflammatory cytokine, TNF-a relative to the HF group, and a trend of lower expression compared to the RD group **(Figure 12)**.

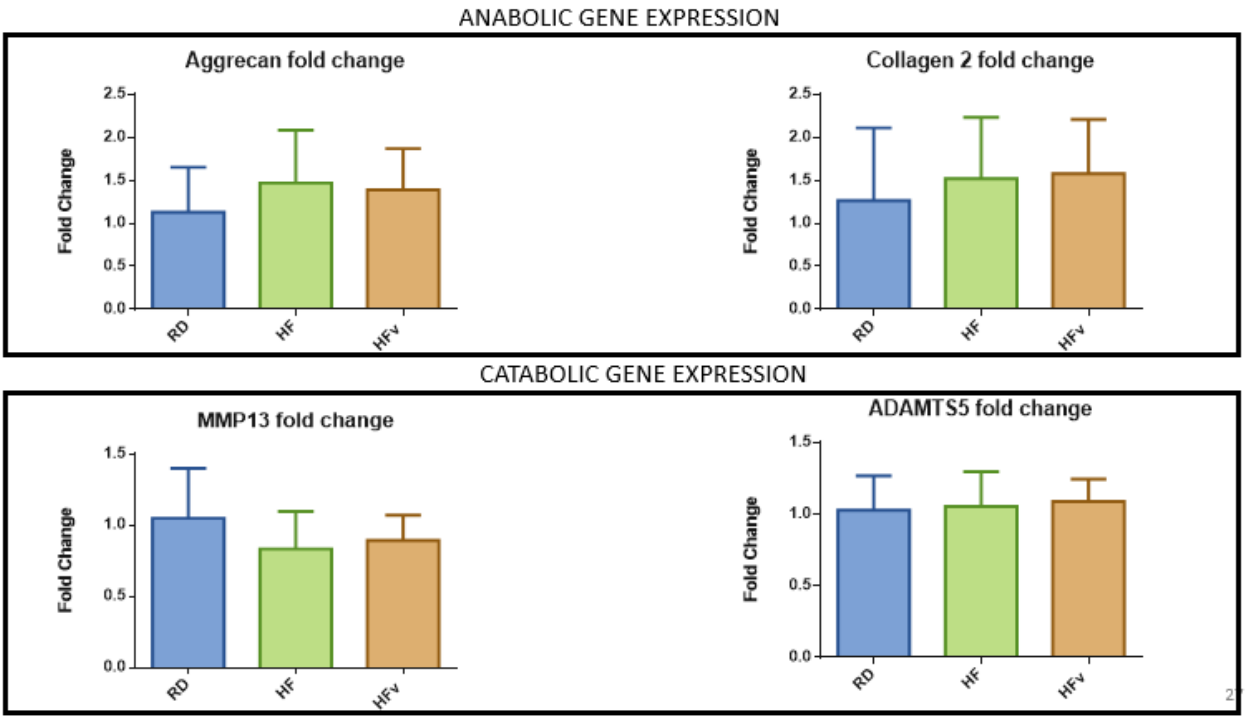

Figure 11: Relative gene expression, determined through RT-PCR, of cartilage matrix proteins, aggrecan and collagen II, and catabolic genes, ADAMTS-5 and MMP-13 within the distal femur of young mice receiving short term diet/LIV. No difference in the relative gene expression was observed between groups.

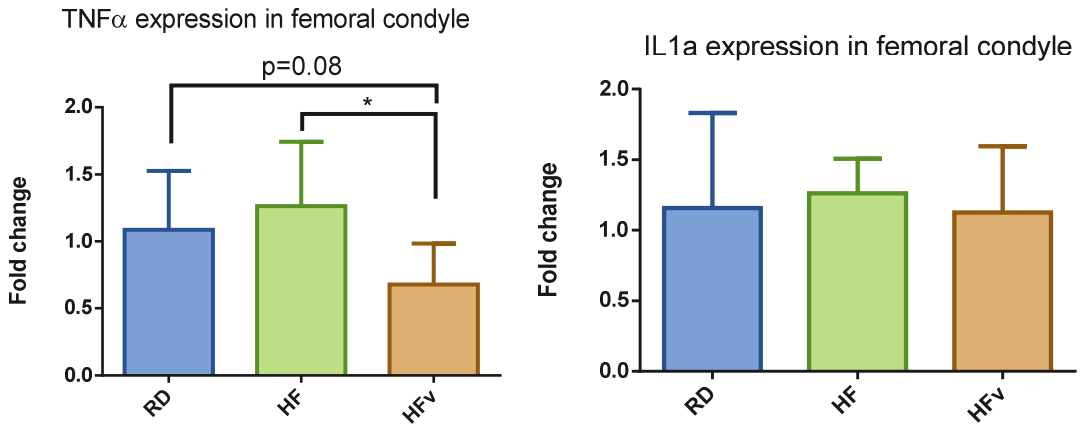

Figure 12: Relative gene expression of inflammatory cytokines, determined through RT-PCR, of TNF-a and IL-1a, within the distal femur of young mice receiving short term diet/LIV. HFv exhibited a significant reduction in expression of TNF-a compared to the HF group, and a trend of lower expression compared to RD.

Short term high fat diet and LIV did not result in changes in subchondral bone thickness, in both the medial and lateral tibia **(Figure 13)**.

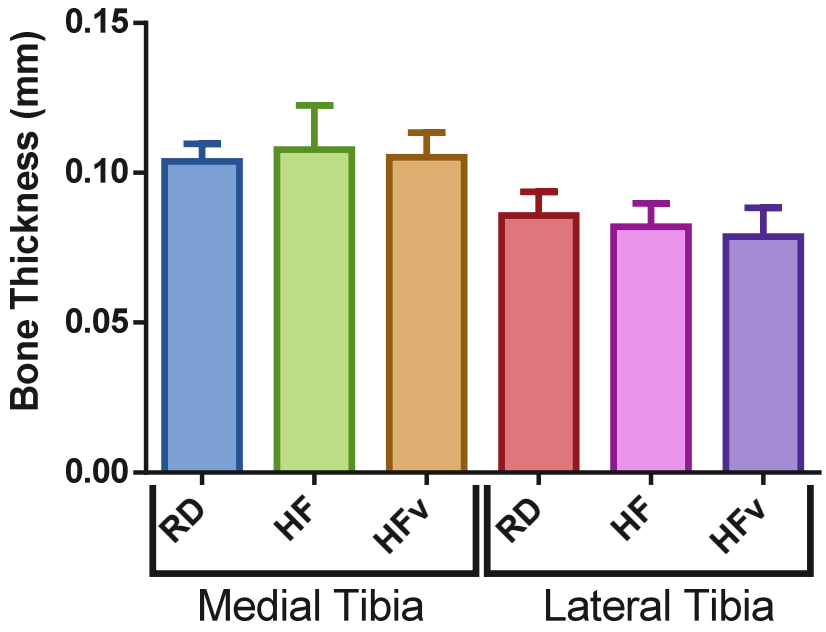

Figure 13: Medial and lateral tibial subchondral bone plate thickness determined from EPIC-uCT. No differences in SB thickness was detected between groups in both the medial and lateral tibia.

Discussion

Osteoarthritis is a crippling disease, the incidence of which is certain to increase as the obesity crisis escalates in both children and adults. While the articular cartilage degeneration that parallels the escalation of our population's Body Mass Index is most often considered a consequence of weight-bearing abuse of the joint surface, the work here was designed to determine if – rather than ablation – if a decrease in the *formation* of cartilage was in part responsible for inadequate bearing surface. Further, considering the salutary influence of

exercise on bone anabolism, to determine if low intensity vibration – serving as a surrogate for exercise – could protect cartilage thickness from the consequences of obesity.

Counter to our 'developmentally suppressed' hypothesis, eight weeks of high fat diet in young, growing mice did not result in thinner articular cartilage within the knee joint as compared to control animals fed a regular diet. These data indicate that combatting obesity during this critical period of musculoskeletal system development– in and of itself – is not a factor in cartilage thinning. What was striking, however, was that cartilage thickness failed to increase in response to the greater weight-bearing demand. Cartilage failed to adapt to the increased demands of weight bearing inherent in obesity. This compromised structure, seemingly appropriate for an animal of much lower weight, would presumably be at a greater lifetime risk of osteoarthritis, with ablation caused by chronic, abusive challenges to the joint. These findings are particularly pertinent to the growing proportion of the juvenile population that is obese: it isn't so much that obesity damages cartilage *per se*, but results in a cartilage system that is under-engineered for the increased demands that obesity brings.

While cartilage failed to adapt to the functional challenge of obesity, short term LIV did stimulate cartilage growth, significantly increasing cartilage thickness in obese mice. LIV was introduced here as a surrogate, not a replacement for exercise. That said, what these data suggest is that brief, daily exposure to mild mechanical signals have the capacity to stimulate cartilage adaptation towards a morphology more structurally adequate for the increased weight-bearing challenges of obesity. In the context of the juvenile obesity crisis, these data suggest that inactivity –while not catabolic to cartilage – results in an under-structured bearing

surface, a consequence that is alleviated by introducing some form of mechanical signal as an anabolic signal to cartilage, thus protecting and preserving the joint for a longer period.

The significant increases in cartilage thickness due to LIV were specific to those regions identified as having greater load-bearing responsibility (medial tibial plateau), while only trends in increased thickness were measured in regions with less load bearing contact (i.e., lateral tibial plateau). The site-specificity of the adaptive response emphasizes that the salutary influence of the low magnitude mechanical signals was realized only in those regions that 'received' the stimulus, while those adjacent areas remained at a morphology consistent with RD and/or HF mice. This local response to a focal stimulus suggests a mechanically-mediated mechanism of perception and response that is regional, rather than a systemic adaptation to these mechanical signals.

Sulfated GAG content was not significantly different between young RD, HF, and HFv animals in both the medial and lateral compartments, as determined by Toluidine Blue staining intensity and Hexabrix staining intensity. Although previous studies have reported decreased sulfated gag content with high fat diet[29], and increased sulfated GAG production with moderate exercise [29, 68, 71], our results support the idea that neither short term high fat diet nor low magnitude mechanical signals, significantly disrupts or promotes sulfated gag content in cartilage compared to young RD controls.

The additional static load commensurate with the increased body weight in obese animals failed to initiate an adaptation appropriate for increased mass. In distinct contrast, the incorporation of a *dynamic* signal stimulated an anabolic response in the cartilage, leading to increased articular thickness scaled to the ratio defined by the regular diet mice. These data

support previous in vitro research which shows that cartilage plugs – unresponsive to static loading - increase GAG synthesis when subject to cyclic compressive loading [76].

Our results contrast with recent research which reports that repeated exposure to low intensity vibration induced marked degeneration of murine knee joints, including meniscal tears and focal damage to the cartilage surface within the medial joint compartment [77]. Of the numerous small animal models that we have used in our LIV studies ([61, 78-82]), as well as other human studies in the literature ([65, 66, 83-85]), we have not observed damage to connective tissues, whether bone, cartilage, tendon, IVD, muscle or ligament. It is important to note that this research group used CD-1 mice in their study, however, when repeating the study in C57BL/6 mice, the same strain used in our studies, no joint degeneration due to LIV was detected[86]. Therefore, differences in strain of mouse may be a source of the contrasting results, however, the degenerative effects in the CD-1 mice will be important to investigate further.

The mechanical signal amplitudes administered in this study are well below those that occur during locomotion [87]. That said, it is also important to recognize that vibration, at high magnitudes (greater than 1.0g), is capable of causing significant damage to a number of physiologic systems, and is a recognized contributor to ailments such as low-back pain, circulatory disorders, and neural dysfunction, however, the signal used here is considered safe by ISO for up to four hours each day[88].

Our study is limited in that we do not study the causes of OA, but whether high fat diet can influence the growth of cartilage during development. Additionally, although we attempted to assess cartilage biochemistry through histology and contrast agent enhanced microCT, only

sulfated GAG content was examined--- the integrity of the collagen II network has yet to be investigated. Furthermore, joint morphology was investigated in limited joint surfaces--- OA is characterized by localized cartilage legions, and a more in depth characterization of the knee joints of both hindlimbs would ensure that all cartilage abnormalities were accounted for.

This current study provides evidence for negative consequences of high fat diet on articular cartilage development in young individuals; the tissue is unable to adapt to the elevated loading resulting from increased 'static' body weight, and in adulthood, was associated with a thickening of the underlying subchondral bone---an early sign of osteoarthritic changes. The administration of low intensity vibration however, appears to stimulate the formation of cartilage tissue, resulting in tissue that is better suited to support the increased body weight, and the elevated cartilage thickness remained present in adulthood, without a thickening of subchondral bone. Future studies are needed to investigate the biological mechanism/ signaling pathway (or lack thereof) in which cartilage fails to adapt to increases in static loads, but that dynamic mechanical signals stimulate increases in thickness. Also, in vitro studies are needed to investigate how low intensity vibration affects chondrocyte proliferation, differentiation, and matrix secretion. Additionally, studies should investigate whether LIV plays a role in reducing joint inflammation, since mechanical stimulation in the form of exercise has been previously reported to reduce the expression of inflammatory cytokines, which would be a possible explanation for the beneficial effects of vibration that were observed in this study. These studies ultimately may provide the bases for a non-drug approach to preventing and/or treating osteoarthritis, and disease that is likely to increase with obesity.

CHAPTER 3:

Articular cartilage thickness fails to achieves body mass appropriate morphology, and subchondral bone thickens, with long term high fat diet administration, while low magnitude mechanical signals mitigate these degenerative effects

Specific Aim 2: Assess the impact of long term DIO and low intensity vibration, into adulthood, on articular cartilage morphology and biochemistry.

Abstract: Obesity is associated with an elevated risk of osteoarthritis (OA). We examined here whether high fat diet administration, starting in young mice, and extending into adulthood, compromised the attainment of articular cartilage thickness. Further, we sought to determine if low intensity vibration (LIV) could protect the maintenance of articular cartilage thickness in a mouse model of diet induced obesity. Seven-week-old, male, C57BL/6 mice were separated into 3 groups (n=8-10): Regular diet (RD), High fat diet (HF), and HF+LIV (HFv; 90Hz, 0.2g, 15 min/d, 5 d/w) administered for 6 months. Articular cartilage and subchondral bone morphology, and sulfated GAG content were quantified using contrast agent enhanced μCT. HF cartilage thickness was not statistically different from RD, however, HF had a lower cartilage thickness to body weight ratio when compared to RD. In contrast, LIV increased cartilage thickness compared to HF, yielding a cartilage thickness to weight ratio not different from RD. Further, long term HF diet resulted in subchondral bone thickening, compared to RD, providing early evidence of OA pathology—LIV suppressed the thickening, such that levels were not significantly different from RD. These data suggest that articular cartilage thickness failed to scale with increased body mass in HF diet mice. Dynamic loading, via LIV, stimulated an increase in cartilage formation, resulting in joint surfaces better suited to the risks of greater loading that parallel obesity.

Introduction:

Long term high fat diet induced obesity has been associated with increased risk for osteoarthritis progression [29, 31]. The joint pain and impaired mobility often lead to decreased overall physical activity, which can lead to a cascade of health complications in adulthood. Increased joint loading due to elevated body weight has been suggested as a major contributor to tissue degeneration, with exercise shown to improve physical function, pain, and mobility in obese adults with OA[26]. However, obese individuals are typically less active, and may actually

put net lower loads on cartilage, and osteoarthritis can also develop in non-weight bearing joints, such as in the hands[89], making it unclear as to whether increased loading is the main contributor to cartilage degeneration.

In adult animal models, high fat diet has been associated with advanced osteoarthritis, as measured by lower proteoglycan content within the articular cartilage, thickening of subchondral bone, and increased osteophyte formation within the knee joint [29-31]**.** The elevated formation of subchondral bone, in OA, has been described as an attempt at tissue repair since histologically, osteoarthritic subchondral bone has been described as coarse fiber woven bone, similar to the bone matrix secreted shortly after bone fracture [90]. There is increased metabolism of collagen I, and an overall change in collagen composition [91], which is expected to result in overall weakened subchondral bone, contributing to further degeneration of the joint.

Moderate exercise in the form of light jogging, cycling etc. has been shown to improve knee cartilage GAG content in patients at high risk of developing knee OA [45], emphasizing that *some* mechanical signals are better than none. Daily running in high fat diet fed mice mitigated the cartilage GAG loss and subchondral bone thickening [29], without any changes in bodyweight compared to the sedentary group, suggesting that dynamic mechanical signals may promote cartilage health while static mechanical signals are unable to mitigate OA progression. While excessive or inadequate loading may be deleterious to cartilage, there appears to be a middle ground, where some degree of mechanical loading is beneficial to the tissue. Although moderate exercise is generally recommended by physicians to mitigate osteoarthritis

symptoms [26], the challenge is that the morbidly obese generally exhibit poor compliance with a daily exercise regimen [73].

Low intensity vibration (LIV), serving as a surrogate for exercise, delivers a relatively high frequency (10-100Hz), low magnitude (<1.0g, where 1g is Earth's gravitational field), mechanical signal, resulting in a mechanical stimulus to the musculoskeletal system that is several orders of magnitude below that which arises during strenuous activity [67], but has been shown to be anabolic to both bone [59] and muscle[61], but suppress adiposity[60]. Recent studies have shown that LIV biases mesenchymal stem cell (MSC) lineage selection towards the formation of higher order connective tissues such as bone, and away from adipogenesis[59-61, 67, 74]. In the study reported here, we sought to determine if long term high fat diet administration, starting during cartilage development and lasting into adulthood, impairs cartilage retention and thus contributes to articular surfaces being under-engineered for the challenges of obesity, and should that happen, if LIV could protect the retention of articular cartilage otherwise challenged by the obese phenotype.

Methods

Animals and Experimental Design

This study was reviewed and approved by the Institutional Animal Care and Use Committee of Stony Brook University. To investigate the effects of long term diet and low intensity vibration on knee joint cartilage and subchondral bone, seven-week old male C57BL/6J mice were separated into RD (n=7), HF (n=7), and HFv (n=10) groups. At baseline, each group received

their respective regular or fat diets, as described previously, and vibration treatment (0.2g, 90 Hz sine wave, 15 min/d, 5 d/w) was administered to the LIV group, while the other two groups were handled in an identical fashion but placed on an inactive, sham, device. Each group continued their diets for 6 months, and at the end of the protocol, all animals were euthanized, and thus data for the long-term protocol are presented are for mice at 31 weeks of age.

Assessment of articular cartilage and subchondral bone thickness using a contrast agent based microcomputed tomography technique.

At tissue harvest, right tibias from each animal were extracted, and placed in 70% ethanol for long term storage. The proximal tibia was then separated from the rest of the bone, for microCT scanning. To enhance image intensity of the articular cartilage, the proximal region of each tibia was incubated in a solution of 15% Ioxaglate (Hexabrix, Mallinckrodt Inc., St Louis, MO) and 85% PBS/PI at 37^{o}C for 30 min, as was described in Specific Aim 1. Hexabrix is a negatively charged hexaiodinated dimer, which has x-ray attenuation properties that allow for segmentation of the articular cartilage from the underlying subchondral bone--- the average x-ray attenuation values will also be used to assess relative sulfated GAG content within the tissue. Samples were then patted dry to remove adherent fluid droplets, and scanned in air using high resolution micro computed tomography (ex vivo µCT 40; Scanco Medical, Bassersdorf, Switzerland), using 6-µm isotropic voxels at 55kVp, 177 µA, and 300ms integration time for the older mice.

As in Specific Aim 1 (see Chapter 2) the 3D grayscale image of each sample was rotated to obtain sagittal sections of the tibial plateau, and contained the subchondral bone, cartilage, and surrounding air, while excluding the marrow space. An appropriate global threshold was

identified from attenuation histograms of representative samples from each group, to isolate the articular cartilage from the subchondral bone---one global threshold was then applied for all animals (RD, HF, HFv).

Cartilage thickness was measured within ROIs centered at the midline of the medial and lateral condyles, spanning 480um in the medial and lateral condyles **(Figure 2)**. As in Specific Aim 1, articular cartilage thickness was measured in the central portion of the medial condyle, and within the inner edge of the lateral ROI, the lateral condyle was separated into a central and posterior half--- cartilage thickness was measured in the central half.

From the same contrast agent enhanced microCT images of the tibial plateau, and contours containing cartilage, subchondral bone, and air, another global threshold was used to isolate the underlying subchondral bone from the cartilage. Average thickness of the 3D ROI of subchondral bone was calculated in the same fashion as articular cartilage.

Proteoglycan content quantification using contrast agent enhanced microCT

To assess relative sulfated GAG content, on a 3D scale, we calculated the average x-ray attenuation of the 3D cartilage ROIs (medial and lateral compartments calculated separately) obtained from the Hexabrix enhanced microCT images, used previously to calculate cartilage thickness. The Hexabrix distribution within the cartilage tissue is inversely related to the amount of sulfated GAGs present at a given location, as stated in Specific Aim 1. Therefore, lower average attenuation values interpreted as lower relative sulfated GAG content.

Statistics

All data are shown as means ± SD. GraphPad Prism 6 (GraphPad Software Inc, CA, USA) was used for all statistical analyses. To determine differences between groups, a 1-way analysis of

variance (ANOVA) with a Tukey *post hoc* analysis test was used. Differences were considered statistically significant if $p \leq 0.05$. For linear regression analysis, $p \leq 0.05$ was considered statistically significant. Grubb's test, with an alpha=0.05, was used to determine if data points were considered outliers.

Results:

Long term high fat diet promoted the obese phenotype

Following 24w of high fat diet, both older HF and HFv groups were significantly heavier than the older regular diet controls (+27.3% and +17.3%, respectively, $p<0.004$) **(Figure 14)**, while there were no significant differences in body weight between the older HF and HFv groups. HF and HFv had significantly heavier epididymal fat pads, as compared to RD (+123% and 119%, respectively, $p<0.0001$), while there no significant differences between HF and HFv (+1.5%, $p>0.05$).

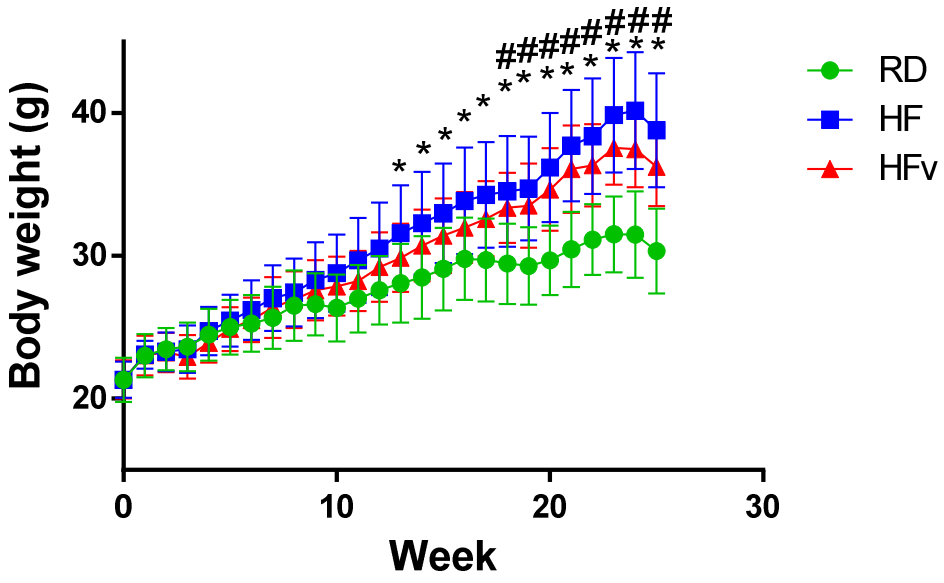

Figure 14: Weekly body weight measurements of mice during administration of long term diet/LIV.

* $p<0.05$ HF compared to RD, # $p<0.05$ HFv compared to RD.

Cartilage thickness fails to scale with weight in older obese mice

Cartilage thickness and body weight did were not linearly related for all older diet and vibration groups (RD, HF, HFv) **(Figure 15)**. Long-term high fat diet did not result in significant differences in cartilage thickness to that measured in older RD, within the medial tibia **(Figure 16)**. Within the lateral tibial plateau, there also was no difference in cartilage thickness between RD and HF groups (+6.5%, p= 0.67) **(Figure 16)**.

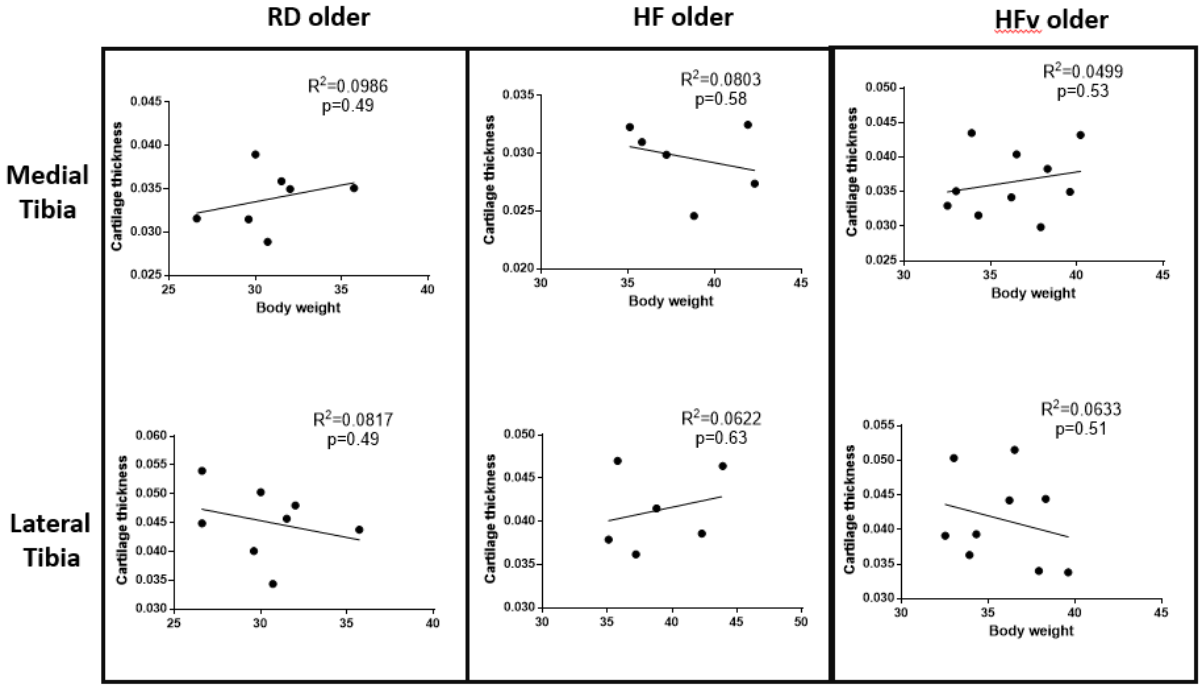

Figure 15: Bodyweight to cartilage thickness linear regression analysis, in mice receiving long term diet/LIV, into adulthood.

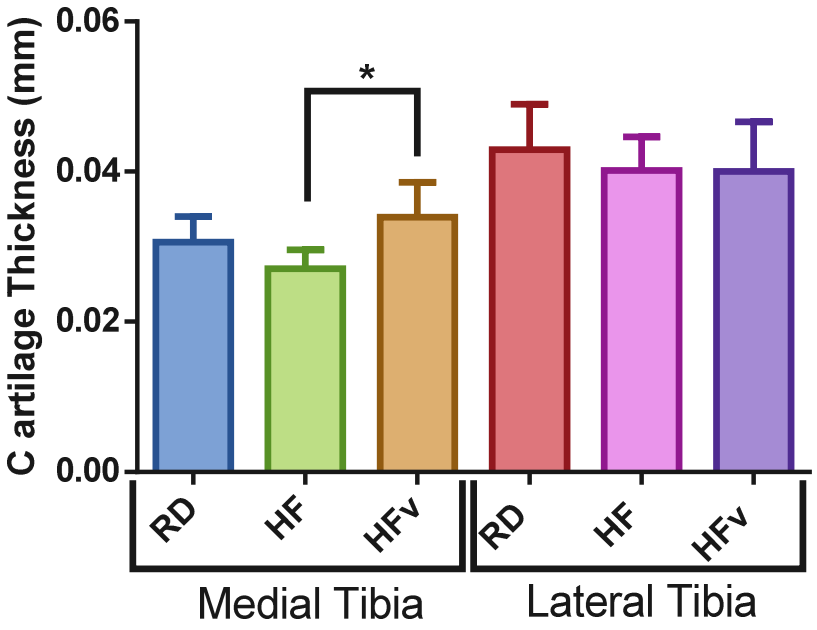

Figure 16: Medial and lateral articular cartilage thickness, determined from EPIC-uCT in mice receiving long term diet/LIV. HFv exhibited thicker cartilage compared to HF in the medial side, whereas no differences in cartilage thickness were detected in the lateral compartment. * p <0.05 HF compared to HFv.

LIV promotes thicker cartilage, preserving cartilage to body weight ratio

LIV stimulated increased cartilage thickness within the medial condyle, in older HFv mice, as compared to HF (+25.2%, p≤ 0.007 in older mice), to levels not significantly different from RD (+10.8%, p=0.22) **(Figure 16)**. Within the lateral tibial plateau, no difference in lateral tibial cartilage thickness was measured when comparing HF to HFv (+0.32%, p= 0.58) or thickness normalized to weight (-8.3%, p= 0.75) in older animals **(Figure 16)**. Additionally, HFv lateral tibial cartilage thickness was not significantly different from RD (+6.79, p= 0.58) **(Figure 16)**.

Proteoglycan content not perturbed by LIV, although a trend of decreased proteoglycan content in HF animals

Long-term high fat diet resulted in a trend of higher x-ray attenuation, or Hexabrix staining intensity, compared to the RD group (+10.8%, p= 0.06), in the lateral tibia, indicating a trend of lower relative proteoglycan content, whereas HFv average attenuation was not significantly different from RD controls (-8.0%, p = 0.14) **(Figure 17)**. Within the medial tibia however, no significant differences in proteoglycan content were observed between all groups **(Figure 17)**.

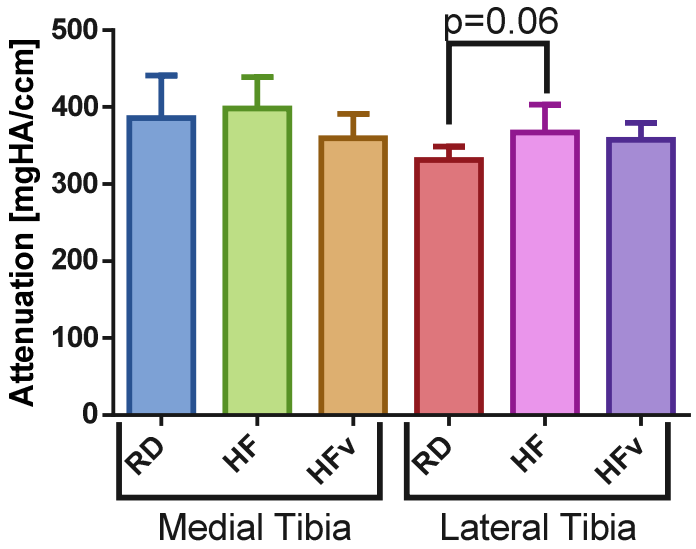

Figure 17: Medial and Lateral articular cartilage x-ray attenuation, in long diet/LIV animals, measured using EPIC-uCT and used as a relative measure of proteoglycan content. HF exhibited a trend of lower proteoglycan content, or higher cartilage attenuation, within the lateral compartment.

Long term high fat diet promotes subchondral bone thickening, and was mitigated with LIV

Twenty-four weeks of HF resulted in a significant thickening of subchondral bone within the medial tibial plateau as compared to RD controls (+19.7%, p=0.02) **(Figure 18)**. However,

subchondral bone in HFv was 8.9% thinner than HF (p=0.33), such that the thickness was not significantly different from older RD (+9.0%, p=0.23) **(Figure 18).**

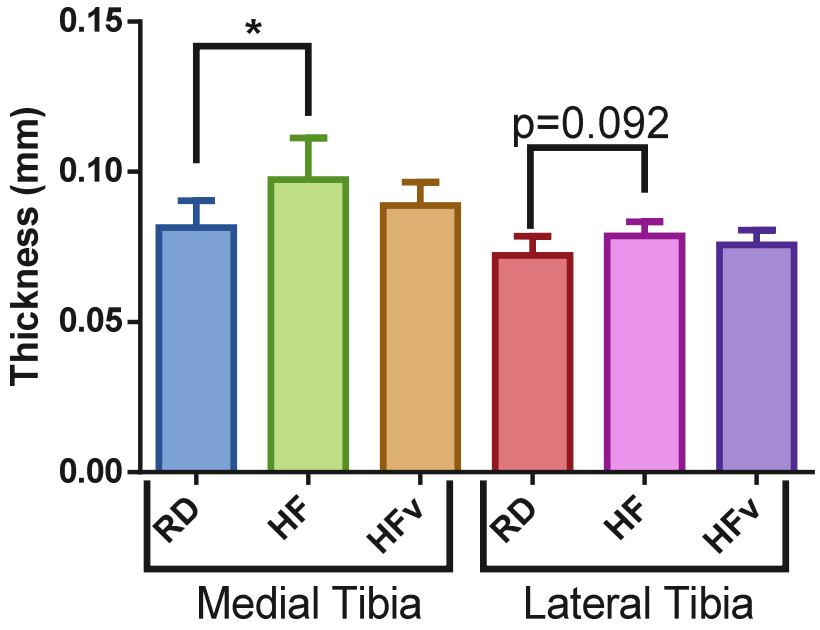

Figure 18: Medial and lateral tibial subchondral bone thickness, determined using EPIC-µCT, in mice receiving long term diet/LIV. HF diet induced subchondral bone thickening in the medial side, while LIV mitigated these changes. * p<0.05 HF compared to RD

Discussion

Osteoarthritis is a debilitating disease, the incidence of which is likely to increase as the obesity crisis escalates in both children and adults. While increased weight-bearing of the joint surface is often attributed as a main contributor to obesity induced disease progression, the work here was designed to determine if long term high fat diet, beginning during cartilage

development and continuing into adulthood, would have lasting effects on the retention of the mature cartilage tissue. Further, considering that exercise has been shown to promote bone anabolism, to determine if low intensity vibration – serving as a surrogate for exercise – could protect cartilage thickness from the consequences of obesity, into adulthood.

Previously, we have shown that young developing mice failed to adapt cartilage thickness to greater load bearing demands with six weeks of high fat diet, resulting in cartilage that was thinner than the vibration treated animals (Chapter 2). In this study, after chronic obesity (24w), contrary to our hypothesis, high fat diet animals did not have thinner cartilage compared to regular diet animals and thus it does not appear that excess weight ablates cartilage thickness. However, older HF mice, when considering their increased body weight, these animals may still require thicker cartilage than their RD counterparts—the articular cartilage of HF animals may have failed to adapt to increased structural demands.

Long term LIV led to retention of augmented cartilage thickness, normalizing the cartilage thickness to body weight ratio to that achieved in older regular diet animals. LIV was introduced here as a surrogate, not a replacement for exercise. That said, what these data suggest is that brief, daily exposure to mild mechanical signals have the capacity to stimulate cartilage adaptation towards a morphology more structurally adequate for the increased weight-bearing challenges of obesity, with an enhanced thickness lasting into adulthood.

In terms of biochemical composition, long-term high fat diet led to a trend of proteoglycan loss compared to older RD, within the lateral tibial compartment, evidenced by a trend of higher cartilage x-ray attenuation with contrast agent enhanced microCT (+10.8%, $p= 0.06$, Figure 24). LIV protected against proteoglycan loss within this region, which is important for the

osmotic properties that allow cartilage to swell and resist compression. In Specific Aim 1 (Chapter 2), we demonstrated that LIV stimulated an increase in articular cartilage thickness, forming an appropriate matrix that appears to be maintained, even into adulthood.

Consistent with early signs of osteoarthritis [90], long term obese mice demonstrated a significant thickening of the subchondral bone. Previous research has shown increased subchondral bone thickness has been associated with increased severity of cartilage legions, with recent studies demonstrating that MSCs residing in the subchondral bone secrete TGF-β, that contributes to the articular cartilage degeneration that occurs after ligament transection [20]. Also, thicker subchondral bone can alter the biomechanical environment within the joint, further encouraging cartilage degeneration. In concert with LIV's capacity to serve as an anabolic signal to chondrogenesis, these low magnitude mechanical signals also mitigated changes in subchondral bone thickness, such that the ratio of articular cartilage to subchondral bone thickness is maintained

This current study provides evidence for negative consequences of long term high fat diet on articular cartilage adaptation to the increased load burden. HF animals were unable to positively adapt to the elevated loading resulting from increased 'static' body weight, and in adulthood, was associated with a thickening of the underlying subchondral bone---an early sign of osteoarthritic changes. The administration of low intensity vibration however, appears to stimulate the formation of cartilage tissue such that it was weight normalized to that seen in RD, resulting in tissue that is better suited to support the increased body weight, and the elevated cartilage thickness remained present in adulthood, without a thickening of subchondral bone.

Future studies are needed to investigate the biological mechanism/ signaling pathway (or lack thereof) in which cartilage fails to adapt to increases in static loads, but that dynamic mechanical signals stimulate increases weight normalized appropriate thickness. Also, in vitro studies are needed to investigate how low intensity vibration affects chondrocyte proliferation, differentiation, and matrix secretion. In general, chondrocytes are sensitive to the intensity and frequency of the mechanical signal, with overloading generally encouraging catabolic processes, while moderate loads encouraging anabolic responses[92-94]. Our data suggests that LIV would fall in the category of mechanical signals that would encourage anabolic responses in chondrocytes. Additionally, studies should investigate whether LIV plays a role in reducing joint inflammation, since mechanical stimulation in the form of exercise has been previously reported to reduce the expression of inflammatory cytokines, which would be a possible explanation for the beneficial effects of vibration that were observed in this study.

Also, while we predicted that long term high fat diet would have more severe consequences on articular cartilage thickness, however, we have yet to see late stage osteoarthritis, mainly cartilage thinning. Therefore, the model of OA that we chose to investigate has the limitation of not fully developing the disease. Ligament transection and meniscus destabilization models generate more severe symptoms of OA, however, these models are specific to injury models of OA. However, our data suggests that LIV has the potential to stimulate increased cartilage thickness, and further studies, in larger animal models, and eventually humans ultimately may provide the bases for a non-drug approach to preventing and/or treating osteoarthritis, and disease that is certain to increase with obesity.

Chapter 4:

Short term high fat diet in adult animals produced adaptations in cartilage morphology at a lower weight bearing site, while mature cartilage was only responsive to low intensity vibration with the incorporation of a refractory period

Specific Aim 3: Assess the impact of short term high fat diet, low intensity vibration, and low intensity vibration with a refractory period, beginning in adulthood, on articular cartilage morphology and biochemistry.

Abstract: Obesity has been shown to increase the risk of developing osteoarthritis, a degenerative joint disease characterized by thinning of articular cartilage. Our previous data showed that administration of low Intensity Vibration (LIV) augmented cartilage thickness in a young high fat diet model, potentially leaving these animals better equipped for potential joint degeneration with high fat diet and aging. However, the effect of high fat diet and mechanical stimulation in older animals is still unclear. Taking into account the decreased responsiveness of adult chondrocyte to external stimuli, we hypothesized that while high fat diet would decrease cartilage thickness in adult mice, incorporation of a refractory period would be necessary for LIV to mitigate these degenerative changes. Thirty-four 17wk old C57BL/6J mice were divided into 4 groups: regular diet (RD) (n=7), high-fat diet (HF) (n=7), high-fat diet receiving LIV (HFv) (n=9), and high fat diet animals receiving refractory LIV (RHFv) (n=8). Each of the 4 groups were fed their respective diets for 2 wks before beginning the administration of LIV (90Hz, 0.2g, 30 min/d, 5 d/wk) or RHFv (90Hz, 0.2g, 2x15 min/d, 5 hour rest between bouts, 5 d/wk, which was carried out for an additional 6 wks. Tibias were harvested and articular cartilage was visualized using contrast agent enhanced MicroCT. Articular cartilage thickness data were collected within a 480μm region of interest, centered at the midpoints of the medial and lateral plateaus. Relative articular cartilage sulfated GAG content was determined by quantifying the x-ray attenuation of the cartilage tissue in the microCT scans. After 8 weeks of high fat diet, a non-significant increase in cartilage thickness was observed between RD and HF (+12.1%, p=0.58). Likewise, a non-significant increase in thickness was observed between HF and HFv (+3.0%, p=0.95), and there was no significant difference between RD and HFv cartilage thickness (+15.5%, p=0.37). Diet also did not result in any difference in cartilage attenuation, indicating no difference in sulfated GAG content between RD and HF (-4.3%, p=0.62), and no significant difference between RD and HFv (-9.7%, p=0.09). However, with the incorporation of a refractory period, cartilage thickness signficiantly increased compared to RD in the medial tibia (+34.0%, p=0.029). These data suggest that the articular cartilage of older animals is less responsive to the anabolic effects of low intensity vibration, and incorporation of a refractory period may be necessary to stimulate an increase in cartilage thickness. This study highlights the importance of age in the response of articular cartilage tissue to low intensity vibration, and a possible method of enhancing the response of cartilage tissue in the aging population.

Introduction

Aging has been shown to induce changes articular cartilage molecular composition, structure, organization, and mechanical properties, increasing the risk of developing OA[95]. These changes have largely been attributed to an age related decrease in chondrocyte proliferation, and synthesis of cartilage matrix proteins [96]. For example, when chondrocytes were isolated from cartilage biopsies of human samples of different age groups, and expanded in CTR medium, cell proliferation rates, were markedly reduced in samples from individuals older than 30, although introduction of growth factor combinations (TGFB1, FGF-2, PDGF-BB) were able to mitigate these age-related changes [97]. Aging chondrocytes have also been shown to exhibit decreased responsiveness to anabolic factors, such as insulin-like growth factor I [16, 17, 98], evidenced by a decrease in proteoglycan production, which was a similar response exhibited in chrondrocytes isolated from individuals with osteoarthritis.

Electron microscopy has revealed age related changes in articular cartilage proteoglycan structure, including a decrease in the length of chondroitin sulfate-rich region of aggrecan, and increasing variability in aggrecan protein core length [18, 99, 100], and elevated levels of keratin sulfate chains, which, in theory, would affect the rheological properties of cartilage. Decreasing the density of anionic GAG side chains would impair the ability of cartilage to draw in water, compromising its function of resisting compressive loads Atomic force microscopy (AFM) measurement of cartilage tissue, after proteoglycan digestion, revealed an age-dependent thickening of collagen fibrils, and when testing aging native cartilage in aging mice, AFM revealed an age-dependent increase in nanostiffness, concomitant with a decrease in GAG content[101]. This change in composition, structure, and mechanical properties could lead to

cartilage tissue that is less equipped to endure compressive loads, potentially leading to aging related cartilage tissue ablation.

On the macroscale, aging contributes to decreases in articular cartilage thickness, with a study indicating that aging in healthy "reference" individuals, accounted for a significant linear decrease in cartilage thickness in both lateral and medial weight bearing femoral cartilage in both knees, but failed to account for significant changes in thickness at other cartilage sites[102], and another study, using MRI in asymptomatic volunteers revealed an age related increase in cartilage T2, indicating changes in cartilage hydration and collagen network anisotropy[103]. Taken together, aging can contribute to changes in cartilage tissue morphology, composition, and structure, possibly leaving the aging joint more susceptible to OA.

As discussed in previous sections, high fat diet has been associated with increased osteoarthritis risk, with increased joint loading, and elevated secretion of inflammatory cytokines and adipokines both suggested as contributors to cartilage tissue degeneration. Loss of proteoglycan content, the appearance of cartilage lesions, and subchondral bone sclerosis have all been observed with high fat diet consumption in small animal models. However, in these studies, high fat diet was administered for extended periods of time, in the range of 20-50 weeks [29-31]; the short term effects of high fat diet on cartilage health is currently unclear.

While exercise has been shown to be beneficial to joint health, with an enhancement of proteoglycan synthesis, increased cartilage thickness, and reduced pain [26, 44]--- aging has been reported to have a negative effect on cellular mechanotransduction, diminishing the tissue response to mechanical stimuli[104]. For example, in skeletal muscle, human and animal

studies have demonstrated that aged muscles can undergo hypertrophy in response to resistance training or mechanical loading, however, the capacity of aged tissue to respond is diminished, with a decrease in baseline levels of important muscle growth factors, impaired mitogen-activated protein kinases, and impaired Akt signaling being possible explanations for the reduced response. Likewise, aging bone cells, particularly osteoblasts, have been shown to have diminished calcium influx following mechanical stimulation via fluid-flow and another study showed aging osteoblasts have a reduced ability to simulate osteoprogenitor formation after exposure to IGF-I[105]. However, there is limited data on the effects of aging on the ability of chondrocytes to respond to mechanical signals.

Considering the reduced sensitivity of aging cells to mechanical signals, it may be necessary to tweak the administration of low intensity vibration in a way that may "prime" the adult cells to be more responsive to the signal. Optimizing LIV to produce an anabolic response in adult cartilage is important considering more than a third of adults in the United States are obese[106], with a significant portion of this population having elevated OA risk.

One strategy that has been shown to enhance cellular responses to low intensity mechanical loading is the insertion of a refractory period, or a time interval of rest, between loading cycles. A study investigating cyclic mechanical loading of turkey ulnas (100 cycles, 1-Hz) and mouse tibias (100 cycles/day of 0.25N peak load, revealed that insertion of a 10 second rest period between each of the 100 loading cycles significantly enhanced the osteogenic effects of the signal[107]. Insertion of 10-15s refractory periods between cycles of oscillatory fluid flow (after every 10 loading cycles) resulted in augmented intracellular Ca2+ signaling in osteoblastic cells[108]. Further, researchers investigating the incorporation of a refractory period with low

intensity vibration treatment of mesenchymal stem cells revealed that putting at least a 1 hour rest period between LIV bouts augmented the suppression of adipogenic gene expression (driving differentiation away from fat, and presumably towards bone lineages). Also, extending the rest period to 3 hours further reduced adipogenic gene expression compared to 1 hour rest[74]. The insertion of a refractory period has been viewed as a method of allowing cells to "reset" their responsiveness to mechanical signals, leading to an augmented response overall.

In this study, we are investigating whether consumption of a high fat diet in mature adult mice induces degenerative effects on cartilage tissue morphology and biochemistry, and if so, whether low intensity vibration mitigates these changes. Considering the diminished "responsiveness" of aged musculoskeletal tissue to various stimuli, we hypothesize that neither short term high fat diet nor low intensity vibration will result in changes in articular cartilage thickness or proteoglycan content, however incorporation of a refractory period into LIV treatment will increase articular cartilage thickness and proteoglycan content.

Methods:

Animals and Experimental Design

This study was reviewed and approved by the Institutional Animal Care and Use Committee of Stony Brook University. To investigate the effects of initiating short term diet and low intensity vibration in adults, on knee joint cartilage and subchondral bone, seventeen-week old male C57BL/6J mice were separated into RD (n=7), HF (n=7), HFv (n=9), and RHFv (HF animals receiving refractory LIV) (n=8) groups. The diet and LIV was administered as described in Specific Aim 1; at baseline, each group received their respective regular or fat diet, and 2 weeks

later, vibration treatment (0.2g, 90 Hz sine wave, 30 min/d, 5 d/w) was administered to the LIV group, for 6 weeks. RHFv animals received two bouts of vibration treatment with a refractory period in between (0.2g, 90Hz sine wave, 2x15 min/d, 5 hour rest between bouts, 5 d/w). As described previously, the RD and HF groups were handled in an identical fashion but placed on an inactive, sham, device.

Assessment of articular cartilage thickness using a contrast agent based microcomputed tomography technique.

As described in Specific Aim 1, at tissue harvest, right tibias from each animal were extracted, bisected at the midshaft, and the proximal end stored in phosphate buffered saline supplemented with proteinase inhibitor (PBS/PI), and stored at 4^{o}C. Articular cartilage was imaged using EPIC- μCT, as described by Kotwal et al.[75], incubating the samples in 15% Ioxaglate (Hexabrix, Mallinckrodt Inc., St Louis, MO) and 85% PBS/PI at 37^{o}C for 30 min; Samples were scanned in air using high resolution micro computed tomography (ex vivo μCT 40; Scanco Medical, Bassersdorf, Switzerland), using 6-μm isotropic voxels at 45kVp, 177 μA, and 300ms integration time.

Also, as described in Specific Aim 1, Chapter 2, the 3D grayscale image of each sample was rotated to obtain sagittal sections, and contoured to isolate the subchondral bone, cartilage, and surrounding air, while excluding the marrow space. A global threshold was used to segment the articular cartilage, while a separate global threshold was used to segment subchondral bone.

The regions of interest for measurement of articular cartilage and subchondral bone thickness are the same as described in Specific Aim 1; centered at the midpoints of the medial and lateral plateaus, and spanning 480μm (Figure 2).

Measuring relative proteoglycan content using contrast agent enhanced microCT

Relative sulfated GAG content, was measured on a 3-dimensional scale, by calculating the average x-ray attenuation of the medial and lateral cartilage ROIs obtained from the EPIC-μCT. Hexabrix has a net negative charge, which repels the negative charges of the sulfated GAGs associated with the large proteoglycan aggrecan. Therefore, the conditions of electrochemical equilibrium predict that Hexabrix distribution within the tissue would be inversely related to the amount of sulfated GAGs present; a sample having a lower average attenuation value was therefore, interpreted as having lower relative sulfated GAG content.

Statistics

All data are shown as means ± SD. GraphPad Prism 6 (GraphPad Software Inc, CA, USA) was used for all statistical analyses. To determine differences between groups, a 1-way analysis of variance (ANOVA) with a Tukey *post hoc* analysis test was used. Differences were considered statistically significant if $p \leq 0.05$.

Results

Short term high fat diet, starting at an older age, promoted an obese phenotype

After 8w of high fat diet, the HF and HFv groups exhibited a 28.6% and 27.2% increase in total body weight, respectively, compared to the regular diet group ($p< 0.01$). The RHFv group did not exhibit a significant difference in body weight compared to RD (+17%, p=0.10) , HF (-8.8%, p=0.42) or HFv (-7.8%, p=0.48) **(Figure 19)**.

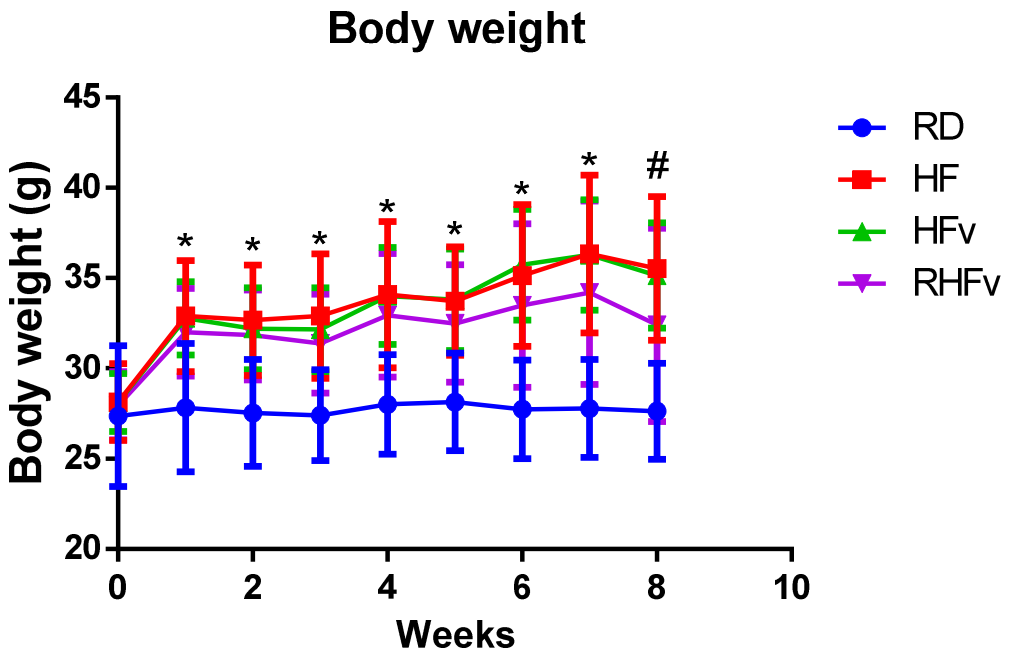

Figure 19**:** Weekly body weight of mice receiving short term diet, LIV , and refractory LIV, starting at adulthood. *p<0.05 HF, HFv, and RHFv compared to RD, #p<0.05 HF and HFv compared to RD.

Cartilage thickness not compromised by high fat diet, when initiated in older mice

There were no significant differences in medial cartilage thickness between RD and HF animals (-12.1%, p= 0.73) **(Figure 20)**, in the medial tibia, despite a 28.6% increase in body mass **(Figure 20)**.

Within the lateral tibia, however, there was a significant increase in cartilage thickness in the HF group (+29.3%, p=0.03) **(Figure 20)** compared to RD. Additionally, no linear relationship was observed between cartilage thickness and body weight for RD, HF and HFv groups, in both the medial and lateral tibia **(Figure 21)**.

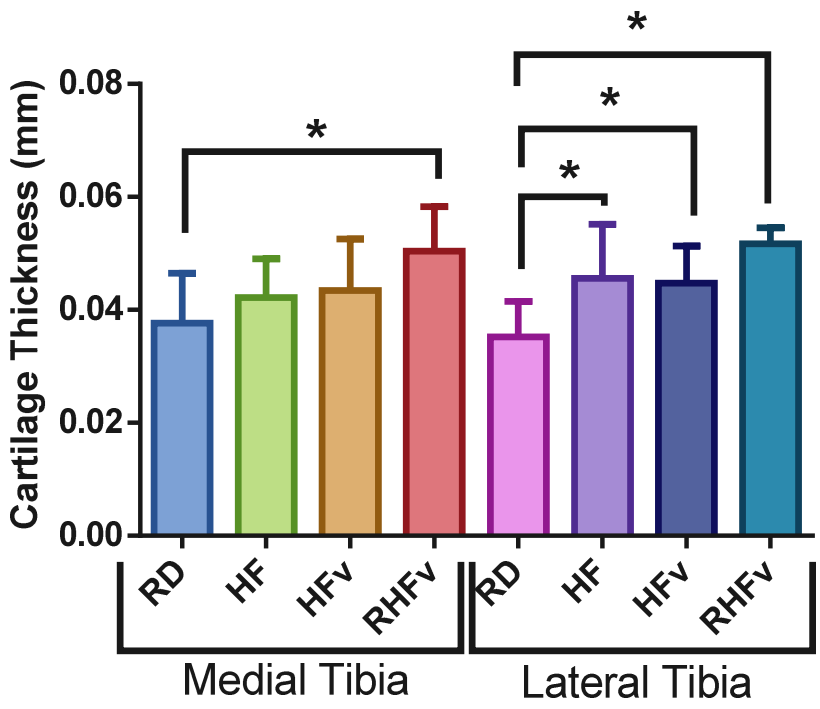

Figure 20: Medial and lateral tibial cartilage thickness, measured using EPIC-uCT, in mice receiving short term high fat diet, LIV, and refractory LIV starting at adulthood.

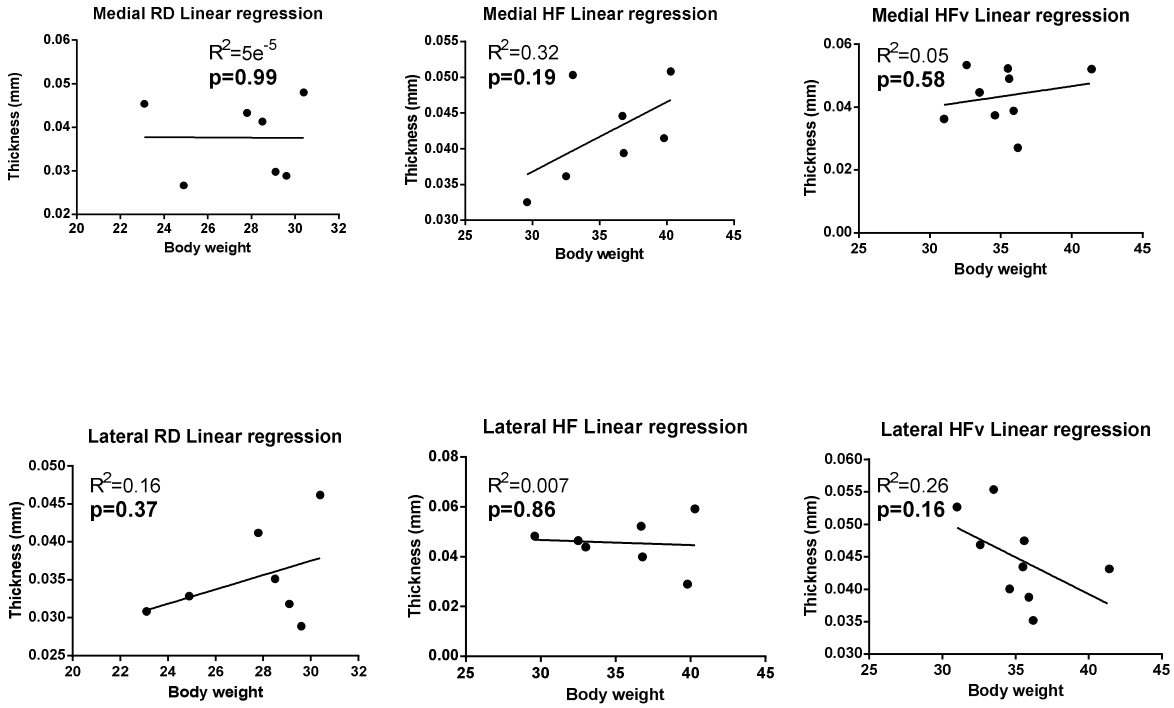

Figure 21: Linear regression analysis of articular cartilage thickness to body weight in young mice receiving short term diet, LIV, and refractory LIV, for the medial and lateral tibia.

Only LIV with a refractory period induced increased cartilage thickness in medial tibia

After 6wks of LIV treatment (without refractory period), there was no significant difference in cartilage thickness compared to the RD (+15.5, p=0.51), or HF groups (+3.0%, p=0.99), within the medial tibia. In the lateral tibia, LIV exhibited increased cartilage thickness relative to RD (+26.9%, p=0.04), but there was no difference with respect to the HF group (-1.8%, p=0.99). However, incorporation of a refractory period, with the RHFv group, resulted in a significant increase in articular cartilage thickness in the medial tibia, relative to RD (+46.6%, p=0.03). In

the lateral side, RHFv exhibited thicker cartilage than RD (46.6%, p<0.001), but not HF (-13.4%, p=0.30) or HFv (-15.5%, p=0.16).

Refractory LIV exhibits trend of increased proteoglycan content in older animals

X-ray attenuation, which was used as a relative measure of proteoglycan content, was not significantly different in HF and HFv, compared to RD in the medial tibia , nor was it different in lateral tibia, in HF and HFv compared to RD **(Figure 22)**. However, incorporation of refractory LIV resulted in a trend of decreased proteoglycan content compared to the RD group **(Figure 22)**.

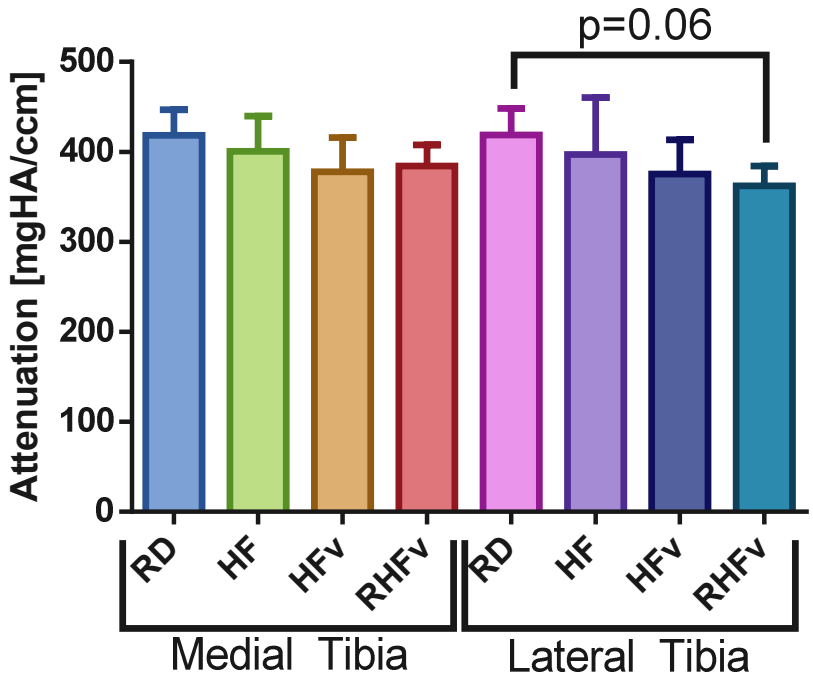

Figure 22: Medial and lateral tibial cartilage x-ray attenuation, measured using EPIC-uCT, and assessed as relative proteoglycan content, in mice receiving short term diet, LIV, and refractory LIV starting at adulthood.

No changes in subchondral bone observed, due to high fat diet or LIV, in older animals

Within both the medial and lateral tibia, there were no significant differences in subchondral bone thickness between RD, HF, and HFv groups **(Figure 38)**.

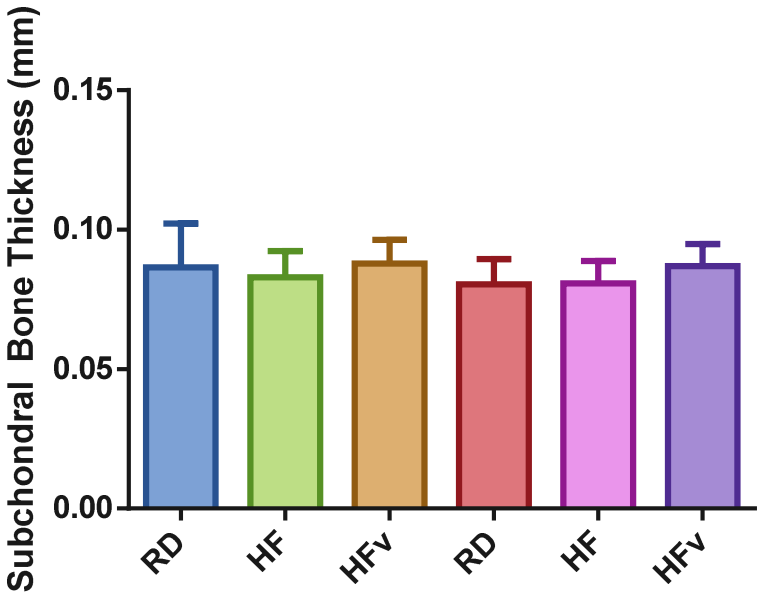

Figure 23: Medial and lateral tibial subchondral bone plate thickness determined from EPIC-uCT. No differences in subchondral bone thickness was detected between groups in both the medial and lateral tibia.

Discussion

Osteoarthritis is a crippling disease, significantly reducing the quality of life of the elderly[109], and its incidence is likely to increase as the adult obese population expands [110]. This study was designed to investigate whether short term high fat diet, beginning after skeletal maturity, had detrimental effects on articular cartilage morphology and biochemistry within the adult knee, and if so, whether low intensity vibration mitigated those changes.

Previously, we have shown that young developing mice failed to adapt cartilage thickness to increased load bearing demands with six weeks of high fat diet and consequent increase in body mass, resulting in cartilage that was understructured relative to the regular diet animals.

In contrast, vibration treated, high fat diet fed mice exhibited increased articular cartilage thickness, resulting in a knee joint that was better equipped to endure the increased loads due to obesity, which was confirmed when HFv animals did not undergo subchondral bone thickening, with long term HF diet, whereas the HF group did. Further, we showed that extended administration of high fat diet, starting in skeletally immature animals and extending into adulthood, also resulted in cartilage that was thinner than animals that were treated with vibration. The elevated articular cartilage in the HFv group provided a joint surface that was better equipped to endure the increased loads due to obesity. This was confirmed with the HF group exhibiting subchondral bone thickening, whereas the HFv animals maintained subchondral bone thickness that was not significantly different from RD.

In this study, short term high fat diet did not result in a thinning of articular cartilage in the adult mouse knee, particularly in the medial compartment, however, again, HF animals were not able to increase cartilage thickness in response to the elevated body weight. Within the lateral compartment of the knee, however, high fat diet animals had thicker cartilage than the regular diet controls. High fat animals were able to adapt cartilage thickness to the increased body weight in this lower weight bearing region (compared to the medial compartment).

Short term LIV, like high fat diet, did not result in significant changes in articular cartilage thickness within the medial knee. However, incorporation of a refractory period, in high fat animals, resulted in articular cartilage that was thicker than RD controls. These data are in agreement with previous research indicating an augmented response of mesenchymal stem cells, as well as osteoblasts, to mechanical signals with the incorporation of a refractory period[74, 107]. Researchers have reported the importance of the cytoskeletal adaptations in

sensing of mechanical signals. The signaling cascade involved with cytoskeletal adaptation has been discussed as saturating quickly, presumably requiring a rest period to "reset" the process, allowing for reactivation with successive bouts of mechanical stimulation[74]. However, the signaling pathway in which chondrocytes respond to low intensity vibration is likely different from how mesenchymal stem cells respond, therefore, the mechanism in which chondrocytes respond to LIV needs to be further investigated. Nonetheless, articular cartilage tissue in mature animals appear to be more responsive to LIV administered with a rest period.

In the lateral compartment, LIV and refractory LIV, similar to high fat diet, exhibited increased cartilage thickness compared to RD. Mechanical stimulation did not hinder adaptation in the lateral compartment, nor did it stimulate increases in cartilage thickness in this region. Additionally, no changes in relative proteoglycan content, measured by x-ray attenuation after EPIC-uCT imaging, were detected. The lack of response to mechanical stimulation in the lateral compartment could be due to the signal not being "received" as intensely in this region, considering the lateral side of the knee endures less of a weight burden than the medial compartment. The differing response in the two compartments suggests that local biomechanical factors interact with LIV to elevate articular cartilage thickness.

Although high fat diet has been shown to increase OA risk in adults, fewer studies have examined the short-term effects of diet consumption. Adult animals are still able to adapt cartilage thickness, at least in the less load bearing region of the knee, suggesting that the effects of high fat diet are not as detrimental in adults as it is in younger individuals (in S.A. 1, the younger animals were not able to adapt cartilage thickness in either region of the knee). Our results also show that articular cartilage is not responsive to low intensity vibration in adult

mice—anabolic responses due to LIV were only observed when a refractory period was inserted between bouts of stimulation. As stated earlier, a rest period has been previously shown to enhance cellular responses to mechanical stimulation, although this has not been closely investigated in chondrocytes. The refractory period presumably allows chondrocytes to "reset" their mechanosensitivity to LIV, augmenting the response to stimulation. Although the pathway is largely unclear, in mesenchymal stem cells, the transient formation and breakdown of focal adhesions, and adaptation of the cell cytoskeleton is thought to be involved in the resetting process[111].

An increase in cartilage thickness within the lateral tibia in older mice high fat diet mice was surprising because generally, data suggests that high fat diet increases osteoarthritis risk. We postulate that HF animals were able to adapt cartilage thickness in the lateral side as opposed to the medial compartment, because the lateral side experiences less of the load burden, providing an environment that is less challenging to stimulate articular cartilage thickness. Another possible explanation for an increase in cartilage thickness in mature animals would be a degeneration of the collagen II tensile network, which, would allow cartilage to swell abnormally. However, the organization of the collagen network was not quantified in our study, and remains a limitation of our research. Future studies should examine the effect of high fat diet and low intensity vibration on the collagen II network.

Our results are consistent with literature indicating that aging chondrocytes are less responsive to anabolic stimuli. As stated earlier, aging chondrocytes have been shown to exhibit decreased responsiveness insulin-like growth factor I, usually an anabolic signal [16, 17, 98], resulting in lower production of proteoglycans compared to younger controls---this effect

was also seen in individuals with osteoarthritis. The reduced responsiveness of cartilage tissue is not surprising, as bone and muscle have also been shown to exhibit diminished responses to anabolic signals, such as mechanical stimuli. However, inclusion of a rest period between bouts of vibration enhanced the cartilage response in mature animals, producing a detectable elevation in cartilage thickness compared to RD controls.

While aging cartilage tissue did not appear to be negatively affected by short term high fat diet in our study, considering HF animals were able to adapt cartilage thickness in the lateral side, extended administration of high fat diet could begin to induce degenerative effects. Refractory LIV was able to produce elevated cartilage thickness in the medial side, presumably leaving these animals better equipped for a potential onset of cartilage degeneration in the future. This study highlights the potential of refractory periods to enhance cartilage responses to LIV, which may be necessary for the treatment of obese patients who have already reached skeletal maturity.

Chapter 5: Short term heavy alcohol consumption, in young rats, does not induce degenerative changes in cartilage thickness or biochemistry

Specific Aim 4: Assess the effects of chronic heavy alcohol consumption and low intensity vibration on articular cartilage morphology and biochemistry in young rats.

Abstract

Chronic heavy alcohol consumption (CHAC), while being a major contributor to the development of liver disease, has also been explored as having degenerative effects on the musculoskeletal system. Alcohol consumption has been associated with increased fat accumulation and inflammation and has been investigated as a potential risk factor for osteoarthritis. In this study, we investigated the short-term effects of CHAC on articular cartilage thickness and biochemistry in young rats. 19 four-week-old Wistar rats were randomized into 3 groups (n=6-7): C (control diet), A (alcohol diet), or L (alcohol diet + LIV), with the diet and vibration protocol lasting for 3 weeks. The R proximal tibias were scanned using contrast agent enhanced microCT (EPIC-μCT), and articular cartilage thickness and relative proteoglycan content were measured. While Chronic Heavy Alcohol Consumption has been associated with increased osteoarthritis risk, our results indicated that 3 weeks of CHAC did not induce cartilage thinning or any detectable changes in proteoglycan content. Likewise, 3 weeks of low intensity vibration did not induce any changes in cartilage morphology or biochemistry. These results are in stark contrast with our high fat diet studies, in which LIV had an stimulatory effect on cartilage thickness in young animals. One important difference between the two disease models was that, unlike the HF diet models, CHAC did not induce an increase in body weight, therefore, an adaptation of cartilage thickness may have not been necessary, since the biomechanical environment would be more similar in the CHAC mice relative to the controls. However, it is important to note that CHAC was administered for a very short time period, and an extended diet administration may have been necessary to see either diet or LIV effects.

Introduction:

Chronic heavy alcohol consumption is a major contributor to the development of chronic liver disease, over time increasing risk for liver cirrhosis and cancer[112]. Most chronic heavy drinkers develop fatty livers, which has been associated with increased inflammation and can eventually lead to tissue scarring. Alcohol ingestion suppresses the oxidation of fat, possibly contributing to increased fat storage. In addition to increased fat storage in the liver, several epidemiological studies have reported positive associations between alcohol consumption and body weight and abdominal obesity, as measured by a waist-to-hip ratio[39], although others have reported no relationship. Type of alcoholic

beverage (i.e. beer vs wine), drinking pattern, and gender have all been suggested as factors that could differentially affect how alcohol contributes to adiposity[113].

There is increased evidence that osteoarthritis progression is associated with increased systemic inflammation. Alcohol has been shown to promote inflammation in multiple organs including the liver[112], brain[41], and pancreas[40]. This taken together with the idea that the accumulation of adiposity has also been considered a risk factor of osteoarthritis development, suggests that chronic heavy alcohol consumption could contribute to osteoarthritis development and progression.

One study in which an alcohol diet was administered in adult mice, for 8 weeks, demonstrated increased OA progression in mice fed alcohol, which was associated with increased expression of pro-inflammatory markers such as NF-kB and ERK-1/2 compared to control mice, and increased catabolic enzyme expression in the knee, such as matrix metalloproteinase 13 (MMP-13, human collagenase 13) and ADAMTS-5 [114], a major aggrecanse in mouse cartilage. However, considering the significant amount of adolescents becoming involved with heavy drinking [115], the short term effects of chronic heavy alcohol consumption on cartilage development are still unclear. We hypothesize that the degenerative effects of chronic alcohol consumption will be evident after short term diet administration.

We investigated whether low intensity vibration could mitigate any the degenerative effects on the articular cartilage of short-term, chronic heavy alcohol consumption induced cartilage degeneration. LIV, serving as a surrogate to exercise, has been shown to be anabolic to bone and muscle, while suppressing adiposity--- biasing mesenchymal stem cell (MSC) lineage selection toward the formation of higher order tissues. In this study here, we seek to determine whether short term chronic heavy alcohol consumption disrupts the formation of articular cartilage, and should that happen, if LIV can protect the formation of articular cartilage challenged by alcohol consumption.

Methods

Animals and Experimental Design

This study was reviewed and approved by the Institutional Animal Care and Use Committee of Stony Brook University. To investigate the effects of chronic heavy alcohol consumption on knee joint changes in growing rats, 19 four-week-old Wistar rats were randomized into 3 groups (n=6-7): C (control diet), A (alcohol diet), or L (alcohol diet + LIV). Alcohol animals were fed a modified Lieber-DeCarli diet ad libitum containing 35% ethanol-derived calories, while control animals received an isocaloric liquid diet with maltose-dextrin, for 3 weeks. L group was subjected to LIV, immediately at the start of the diet regimens, on a vertically oscillating plate, for 3 weeks (90Hz, 0.3g peak acceleration, 30min/d, 7d/wk). All rats were sacrificed at 7 weeks of age.

Assessment of articular cartilage thickness and relative proteoglycan content using a contrast agent based microcomputed tomography technique.

To enhance image intensity of the articular cartilage, the proximal region of each rat tibia were incubated in a solution of 40% Ioxaglate (Hexabrix, Mallinckrodt Inc., St Louis, MO) and 60% PBS at 37^{o}C for 30 min, as described by Xie et al, at 45kVp, 177uA, 200ms integration time with a voxel size of 12um. Samples will be patted dry to remove adherent fluid droplets, and scanned in air using high resolution micro computed tomography

For the cartilage thickness analysis, the 3D grayscale image of each sample were rotated to obtain sagittal sections of the tibial plateau, and contained the subchondral bone, cartilage, and surrounding air, while excluding the marrow space. An appropriate global threshold was identified from attenuation histograms of representative samples from each group, to isolate the articular cartilage from the subchondral bone---one global threshold was then applied be for all animals (C, A, A+LIV). As stated in previous aims, separate ROIs were isolated in the medial and lateral tibia plateau, each of

which was centered at the midpoint of each region, each spanning a distance of 732um, for the young rats.

Also, as stated in previous aims, Hexabrix is a negatively charged hexaiodinated dimer, which when diluted appropriately, has x-ray attenuation properties that are distinct from the underlying bone, allowing for segmentation of the articular cartilage. The equilibrated concentrations of the contrast agent is inversely related to the proteoglycan concentration within the tissue [21]; as in the previous aims, average x-ray attenuation was measured in the medial and lateral compartments, and used as a relative measure of sulfated GAG content.

Statistics

All data are shown as means ± SD. GraphPad Prism 6 (GraphPad Software Inc, CA, USA) was used for all statistical analyses. To determine differences between groups, a 1-way analysis of variance (ANOVA) with a Tukey *post hoc* analysis test was used. Differences were considered statistically significant if $p \leq 0.05$.

Results:

Short-term CHAC induced heavier liver, without significant changes in body weight

Three-weeks of CHAC did not result in any significant changes in bodyweight (Fig 1A), although the liver weights of A and A+L groups were both significantly higher than the control group (+23.3%, $p<0.01$; and +22.3%, $p<0.013$, respectively) **(Figure 24)**.

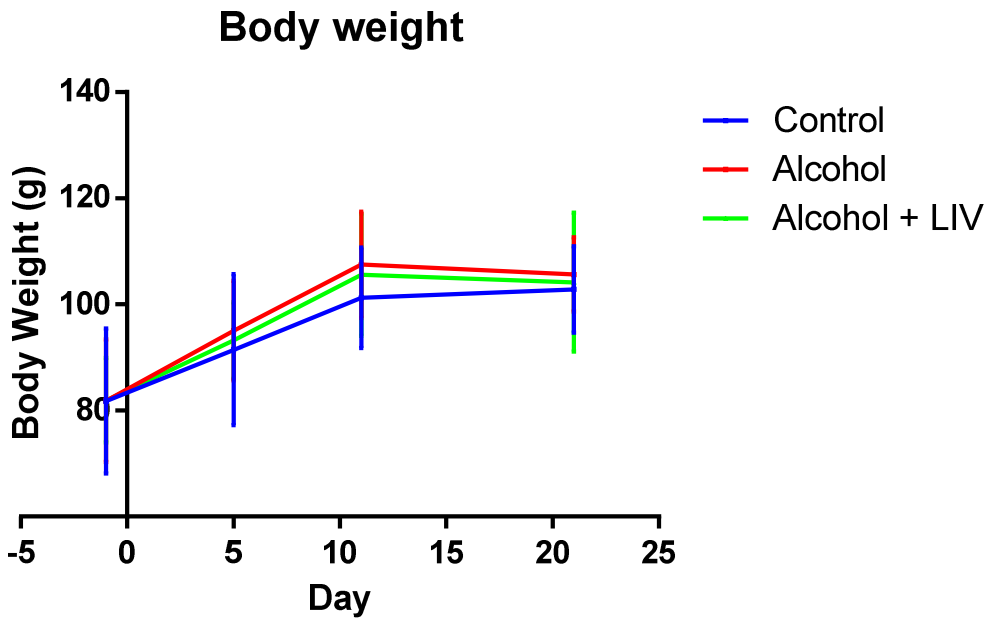

Figure 24: Weekly body weight of rats receiving CHAC diet and LIV for a period of 3 weeks. No significant differences in body weight were detected.

Articular cartilage thickness and relative proteoglycan content not affected by chronic alcohol consumption nor low intensity vibration

Three weeks of chronic alcohol consumption resulted in no changes in articular cartilage thickness within the medial plateau (+0.4%, p>0.90) **(Figure 25)**, nor in the lateral compartment (+10.8%, p=0.12) **(Figure 25)**, compared to the control group. Likewise, no differences in x-ray attenuation of articular cartilage was detected in the medial side (+2.9%, 0.90) **(Figure 26)** nor in the lateral side (-0.79%, 0.97) compared to the control **(Figure 26)**.

Similarly, three weeks of low intensity vibration resulted in no detectable changes in medial articular cartilage thickness compared to the A and C groups (-3.9%, p= 0.62 and -3.6%, p=0.70 ,respectively) **(Figure 25)**, nor were there differences in lateral articular cartilage thickness compared to the A and C groups (+1.1%, p= 0.97 and +12.2%, p=0.09) **(Figure 25)**. Furthermore,

there were no changes in x-ray attenuation with LIV, compared to the C and A groups **(Figure 26)**.

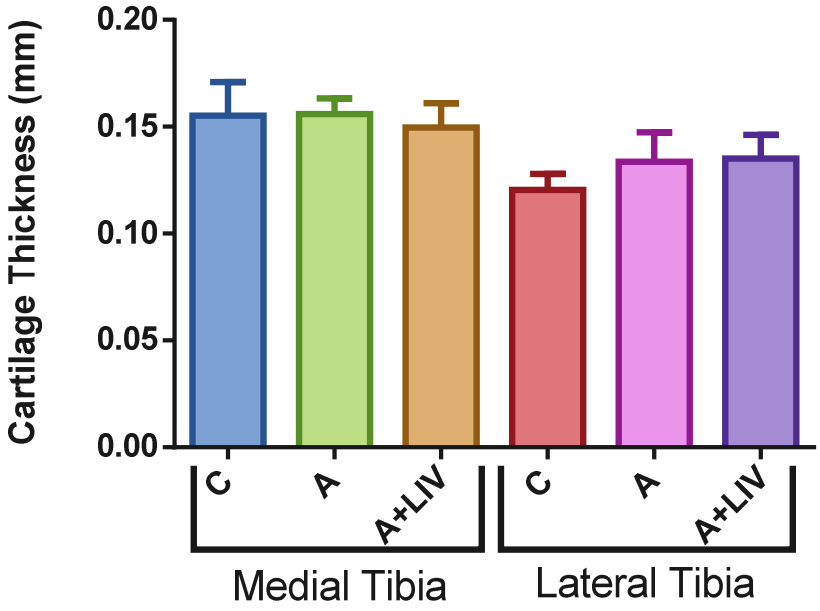

Figure 25: Medial and lateral articular cartilage thickness of CHAC study rats, measured from EPIC-µCT scans.

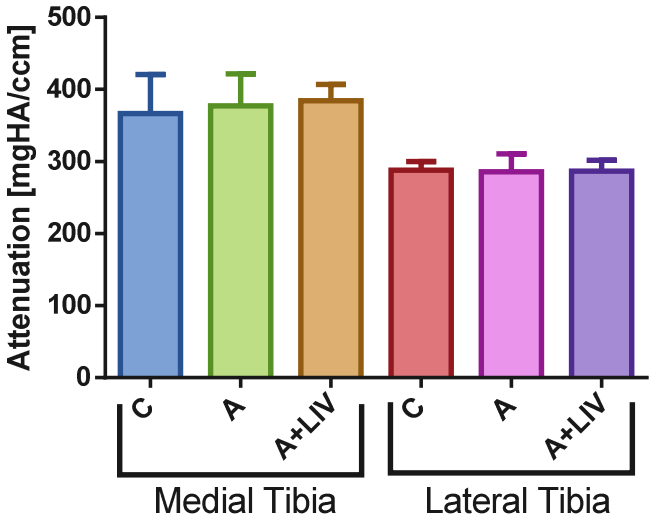

Figure 26: Medial and lateral tibial articular cartilage-ray attenuation, interpreted as relative proteoglycan content, measured from EPIC-µCT scans.

Discussion:

The work here was conducted to examine the early effects of chronic alcohol consumption on the developing knee joint. A female Wistar rat model was used because it is an established model for alcohol induced bone loss, however, the effects on articular cartilage in this model are still largely unknown. While Chronic Heavy Alcohol Consumption has been associated with increased osteoarthritis risk, our results do not indicate cartilage thinning or any detectable changes in proteoglycan content, from our measurements using EPIC-uCT. Like high fat diet, alcohol consumption has been associated with increased accumulation of fat tissue, particularly abdominal adiposity, as measured through waist-to-hip ratio. However, it is important to note that there were no significant differences in body weight between the control, alcohol, and alcohol + LIV groups. Unlike in the high fat diet studies described previously, where feeding was given *ad libitum* for all groups, the animals in the chronic alcohol study were calorie matched with controls; control animals were fed an equivalent amount of calories based on

the quantity the alcohol animals consumed. The biomechanical environment that these animals experienced therefore, is likely more similar, between experimental and control animals, than in the high fat diet studies, and possibly a reason why differences in cartilage thickness were not observed in our study.

Our results are in contrast with a previous chronic alcohol consumption study that indicated increased proteoglycan loss and surface fibrillation in young mice (7-9 weeks of age), with increased expression of catabolic genes MMP-13 and ADAMTS-5, after 8 weeks on the diet. The work reported here was conducted in Wistar rats that were only 4 weeks old, and the alcohol diet was administered for a significantly shorter time period of 3 weeks. Our results suggest that an extended duration of alcohol consumption may be needed to observe phenotypic changes in articular cartilage morphology and biochemistry. This however does not eliminate the possibility that there were detectable change in articular chondrocyte gene expression in response our short term administration of the alcohol diet---RT-PCR analysis of cartilage anabolic and catabolic genes was not done, and remains a limitation of this study.

Data from the previous aims suggested that the articular cartilage of young animals is more receptive to mechanical signals than in skeletally mature adults. It was surprising that LIV did not elevate cartilage thickness in the A+L group, considering they are relatively young animals. However, considering that A and A+L groups were not heavier than C animals, it is possible that an 'adaptation' of thicker cartilage was not necessary. If alcohol animals were not calorie matched with control animals, but instead allowed to get heavier than the controls, it is possible that we would see an anabolic effect of the LIV signal.

Our study is limited in that only EPIC-uCT was used to examine cartilage morphology and biochemistry. Histology, and grading the knee joint based on the Mankin Score is still the gold standard

of assessing OA progression, and this data is not presented here. Additionally, cartilage thickness and relative proteoglycan content were examined in specific regions of the knee joint that contained the thickest cartilage, and inferred as being the most high weight bearing regions; it is possible that we overlooked potential alcohol and LIV effects within other regions of the joint. Also, we lack *in vitro* studies that examine the direct effect of alcohol on chondrocyte gene and protein expression. Also, our study lacks a control + LIV group, to examine whether LIV has effects on healthy cartilage tissue. However, it is important to note that CHAC and LIV administration was very short term in this study, and an extended period of diet and mechanical stimulation may be needed to yield detectable effects on cartilage morphology and biochemistry.

Chapter 6: Global Discussion

Global Discussion

The objective of this dissertation was to investigate the degree to which high fat diet and obesity impact articular cartilage morphology and biochemistry, and determine whether low intensity vibrations would mitigate any alterations that might arise. Counter to our hypothesis, high fat diet administration in mice did not lead to any decrease in articular cartilage thickness, regardless of whether the high fat diet was administered in young or skeletally mature animals, or whether the diet was administered short (8 weeks) or long term (6 months). However, we observed a significant decrease in the articular cartilage thickness to body weight ratio in high fat diet animals compared to the regular diet controls, in short and long term studies in which high fat diet was administered, beginning at a young age; this was not the case when high fat diet administration began in adulthood. In terms of biochemistry, proteoglycan content remained unchanged when high fat diet was administered short term, in both young and older animals, however, long term high fat diet administration, beginning in young mice and extending into adulthood, resulted in a trend of reduced proteoglycan content, particularly within the lateral compartment. Loss of proteoglycan content has been reported in response to extended periods of high fat diet consumption, in small animal models, and has been suggested as an initial sign of joint degeneration, and if left unchecked, leading eventually to cartilage thinning, lesions, and bone sclerosis—characteristics of osteoarthritis. Proteoglycans provide the osmotic properties necessary for cartilage tissue to imbibe and swell with the surrounding synovial fluid, allowing it to resist compressive forces[116]; a significant loss of proteoglycans has been associated with changes in mechanical properties, such as a reduction in compressive and shear moduli [117], compromising the tissue's functional properties.

Articular cartilage thinning was not observed in any of our high fat diet studies, however, cartilage thickness to body weight ratio was lower in high fat animals compared to regular diet animals, where high fat diet was administered in young animals, for both short and long term, suggesting that the articular cartilage tissue may be less equipped to withstand the increased loads induced by high fat diet. Long term administration of high fat diet, which we predicted would lead to the most severe degenerative effects, induced subchondral bone thickening compared to regular diet controls—a sign of early osteoarthritis progression.

Subchondral bone provides articular cartilage with mechanical support during movement or joint loading, and can undergo modeling and remodeling in response to changes in the mechanical environment [118]. The proximity of subchondral bone to the articular cartilage combined with subchondral vascular invasion in the calcified zone, which increases with OA progression, allows for the tissues to communicate on a chemical level [119]. Although it is still unclear whether cartilage degeneration precedes subchondral bone changes, or vice versa, recent studies have suggested that TGF-β signaling in the mesenchymal stem cells of subchondral bone is heavily involved with the progression of osteoarthritis in a ligament transection model, and that this elevated signaling precedes visible cartilage damage [24]. Our results also show significant subchondral bone changes that precede changes in articular cartilage morphology. However, TGF- β expression within the subchondral bone was not measured in our studies, but should be investigated since changes in subchondral bone morphology were already observed.

Exercise is often prescribed by clinicians to alleviate the symptoms of osteoarthritis, including inflammation and pain, however, studies indicate that patients should be wary of the

intensity of the mechanical loads that their joints endure. Strenuous running in mature rats for example, reduced levels of chondroitin sulfate and hyaluronic acid within the cartilage matrix. Also, long distance running and weight lifting have been associated with higher incidence and earlier onset of osteoarthritis. On the other extreme, absence of mechanical loads, as occurs extended bedrest, have also been viewed as detrimental to articular cartilage health. In our studies, we investigated the use of low intensity vibration as a surrogate for exercise, with the idea that the intensity and frequency of our mechanical signal would fall within a therapeutic 'window', that would mitigate some of the degenerative effects due to the metabolic stressors.

Low intensity vibration administration in high fat diet animals, beginning at a young age, resulted in articular cartilage that was thicker than the high fat diet groups after six weeks, and this elevated thickness was maintained, as LIV and diet administration was extended for six months. The increased thickness normalized the articular cartilage thickness to body weight ratio to levels not significantly different from regular diet animals.

Furthermore, long term LIV mitigated changes in subchondral bone thickness, to levels not significantly different from the regular diet group. Also, proteoglycan content loss was mitigated with LIV, in the lateral tibia, compared to the HF group, to levels not significantly different from the RD group. Taken together, HF diet, when administered starting at a young age (5-7weeks), resulted in articular cartilage thickness that did not scale up with respect to body weight, and long term consumption of the high fat diet induced initial joint degeneration, while administration of LIV maintained elevated cartilage thickness, and in the long term, concurrently mitigated changes in subchondral bone thickness and proteoglycan content,

resulting in a joint surface that is potentially more equipped to withstand the increased loading associated with obesity.

It seems that dynamic nature of LIV initiated HFv articular cartilage to adapt in a way that prevented subchondral bone thickening from occurring. The articular cartilage of older mice, within the medial tibia, was less responsive to high fat diet and low intensity vibration. Eight weeks of high fat diet, and six weeks of low intensity vibration did not lead to significant changes in articular cartilage thickness, nor was there a significant difference in articular cartilage thickness to body weight ratio, and no detectable changes in proteoglycan content. However, the incorporation of a refractory period into the LIV treatment regimen produced elevated articular cartilage thickness within the medial tibia, compared to RD, whereas this adaptation was not observed in the LIV group. This data is in agreement with previous research indicating an augmented cellular response to mechanical signals with the incorporation of a rest period [74], with researchers reporting the importance of the cytoskeletal network in sensing of the mechanical signal, and the signaling cascade involved with cytoskeletal adaptation saturates quickly, requiring a rest period to essentially "reset" the process, allowing for reactivation with successive bouts of mechanical stimulation [111].

Within the lateral tibia however, HF exhibited a significant increase in articular cartilage, and the HFV group exhibited a statistical trend of increased cartilage thickness, compared to RD, with no significant differences in articular cartilage thickness to body weight ratio. Like the medial tibia, no changes in proteoglycan content were detected, and no changes in subchondral bone thickness were observed. Taken together, short term high fat diet does not appear to have degenerative effects on articular cartilage in the medial or lateral compartment, and their

articular cartilage and subchondral bone also seem to be unresponsive to low intensity vibration. Adult chondrocytes seem to be unresponsive to the dynamic mechanical signal, unlike the chondrocytes of young animals, as in Specific Aim 1. This is not surprising to us, as previous research has reported aging chondrocytes to be less responsive to anabolic stimuli.

An increase in cartilage thickness within the lateral tibia in older mice high fat diet mice was surprising because generally, increases in cartilage thickness are not reported in skeletally mature animals, and high fat diet is generally reported as accelerating cartilage degeneration. Studies in rabbits, for example, show that articular cartilage growth declined and ceased from three to four months of age, and stayed relatively the same until the time of skeletal maturity (8 months of age). Furthermore, cell studies showed that aging chondrocytes grown in culture, are shown to secrete less proteoglycans in response to anabolic stimuli, such as insulin-growth factor I. It is possible that HF animals were still able to adapt cartilage thickness in the lateral side, since it has less of a load burden, compared to the medial side. Also, a possible explanation for an increase in cartilage thickness in mature animals would be a degeneration of the collagen II mesh within the cartilage matrix, which counteracts the swelling osmotic pressure produced by proteoglycans—a degenerated tensile network, would allow cartilage to swell abnormally. Future studies should examine the effect of high fat diet and low intensity vibration on the collagen II network.

Overall, while high fat diet did not lead to articular cartilage thinning as was originally hypothesized, long term high fat diet did eventually show signs of joint degeneration through an increase in subchondral bone thickness, which was mitigated with administration of low intensity vibration. Low intensity vibration appeared to induce increased cartilage thickness in

young animals, which was maintained into adulthood. Together with mitigating the changes in the underlying bone, low intensity vibration appears to protect the knee joint from the degenerative effects of high fat diet. In older animals however, incorporation of a refractory period was necessary to induce an increase in cartilage thickness compared to controls. Since aging chondrocytes are generally less responsive, the rest period may have been necessary to produce a detectable effect, and should be considered when treating older patients in the clinic.

A possible mechanism through which LIV increased articular cartilage thickness could be the suppression of inflammation. In the short term high fat diet study in S.A. 1, LIV reduced the gene expression of TNF-α within the knee, which is an inflammatory cytokine commonly detected in the synovial fluid of patients with early and later stage osteoarthritis[120]. Cytokines such as TNF- α and IL-1 have been shown to significantly upregulate matrix metalloproteinases[121], which contribute to the catabolic activity leading to cartilage matrix degeneration. Previous research has demonstrated that mechanical loading, applied as cyclic compressive loads (0.5 MPa peak stresses, sinusoidal waveform 0.5 Hz for 3 days), inhibited the IL-1 induced aggrecan loss in bovine articular cartilage explants[122]. Future *in vitro* studies investigating the effect of LIV on chondrocyte and synoviocyte production of inflammatory cytokines would help us understand the effect of LIV on inflammation in the knee, and potentially highlight its potential use in other diseases exacerbated by inflammation of the joint, such as rheumatoid arthritis.

It is important to note however that mechanical vibrations have also been reported to be detrimental to cartilage health. High intensity, low frequency vibrations are commonly

experienced by truck drivers, and has been associated with both spinal degeneration and low back pain. The mechanical signal that we investigated here, however, is orders of magnitude below the loads experienced during exercise, and our results do not show signs of low intensity vibration inducing degenerative changes within the knee. Other research groups however, have reported vibrations of similar frequency and magnitude to our signal, as being detrimental to articular cartilage health in relatively young mice (10 weeks of age), with reports of meniscal tears and focal cartilage damage due to the vibration treatment. We are surprised by this result because, of the many small animal models that we have used in our LIV studies ([61, 78-82]), as well as other human studies in the literature ([65, 66, 83-85]), we have not observed damage to connective tissues, whether cartilage, bone, tendon, IVD, muscle or ligament. It is worth noting that this research group used CD-1 mice in their study, however, when repeating the study in C57BL/6 mice, the same strain used in our studies, no joint degeneration due to LIV was detected[86]. Therefore, differences in strain of mouse may be a source of the contrasting results, however, the degenerative effects in the CD-1 mice will be important to investigate further. The mechanical signal amplitudes administered in this study are well below those that occur during locomotion, and are considered to be safe by the ISO for up to four hours [87].

The anabolic response in cartilage that we see with low intensity vibration may provide insights on how chondrocytes sense mechanical signals. Cartilage tissue is considered a viscoelastic material, with a porous permeable collagen—proteoglycan matrix, filled with interstitial fluid that flows through the matrix as cartilage is compressed. Due to the tissue's viscoelastic nature, its response to mechanical loading greatly depends on the frequency at which mechanical signals are applied. While cartilage tissue's response to physiologic loading

has been largely characterized, at loading frequencies ranging from 0.001 – 1.0 Hz, exhibiting dynamic moduli that have both an elastic and fluid component[123], the cartilage response to high frequency loading i.e. LIV applied at 90Hz, is still largely unclear. Computational "biphasic" models that incorporate the solid and fluid phases of the tissue, and experimental data from unconfined cartilage compression tests, have predicted that at high compression loading frequencies (10Hz and above) the dynamic modulus will reach a maximum magnitude, with the phase angle reaching a minimum, meaning that the tissue will behave largely as an incompressible solid[124]—the fluid component of cartilage is largely absent from the mechanical response of the tissue. Considering these predictions, during LIV administration, cartilage is likely to stiffen with minimal fluid flow occurring around the chondrocytes. Considering that cartilage tissue produced an anabolic response to LIV, our studies suggest that chondrocytes are responsive to deformation of the matrix, rather than interstitial fluid flow. Studies comparing the response of articular cartilage to different loading frequencies from the range of 0.001Hz-100Hz would help reveal the optimal mechanical environment to stimulate chondrocyte activity, with lower frequencies exposing chondrocytes more to fluid flow, and higher frequencies favoring matrix deformation.

Our studies are limited in that we focus mainly on contrast agent enhanced microCT to examine cartilage morphology and biochemistry. Additionally, our analysis was limited to the proximal tibia; the distal femur also contains high weight bearing regions, which our analysis did not cover. Particularly in the case of measuring proteoglycan content, more direct and quantitative assays exist, such as the dimethylmethylene blue assay, which uses a colorimetric dye and spectrophotometry to quantify proteoglycan content. However, we were constrained

by lack of tissue available in the mouse knee; isolating sufficient articular cartilage tissue to achieve concentrations within the detection limits of the assay is a challenge. Additionally, collagen II is another major component of the cartilage matrix which was not measured in our studies, again as with the assays for proteoglycans, quantitative collagen II assays would be challenging to carry out with the sparse amount of mouse cartilage tissue.

Immunohistochemistry and histological staining techniques also exist to measure collagen II content, however, these methods would provide qualitative data, and especially in a high fat diet model, where the degeneration in cartilage was expected to be more gradual compared to joint injury models, more quantitative assays were necessary to detect differences between groups. Carrying out our high fat diet studies in a larger animal model, such as a New-Zealand Rabbit, would have made these quantitative assays more feasible to conduct, however, we chose to use the C57BL/6 mouse because it is a highly established model for high fat diet induced obesity.

Our studies are also limited in that we report articular cartilage morphological changes resulting from exposure to LIV, however, a mechanism of action was not provided. Although we attempted to probe the expression level of anabolic genes, aggrecan and collagen II and catabolic genes (ADAMTS-5 and MMP-13), no significant differences in expression were detected. Additionally, serum markers of cartilage degradation, such as hyaluronic acid, and cartilage oligomeric matrix protein, could also reveal whether catabolic processes were affected by diet or LIV. In vitro studies, investigating the effect of LIV on cultured chondrocytes may help us better understand how these low magnitude mechanical signals may affect cell proliferation, or secretion of cartilage matrix proteins.

Additionally, translating our results to human subjects will be challenging, as rodents are quadruped, therefore, the gait and joint loading patterns are significantly different from those experienced in bipedal humans; the high weight bearing regions of the tibia that we focused our analysis to would be different in the human knee joint. Computational methods would have helped model the force distribution across the rodent mouse surface, as well as fluid flow/perfusion within the cartilage tissue, which would allow more accurate determination of high and low load bearing regions. This would help in finding regions of the knee at high risk for degeneration in response to the increased loads due to obesity. Currently there is a lack of published data on rodent knee force distributions; this should be pursued in future studies.

In conclusion, high fat diet, while not inducing cartilage thinning, does induce subchondral bone thickening when administered long term, which may be the result of cartilage tissue not scaling up in thickness to the increasing body weight. LIV, on the other hand, stimulated increased cartilage thickness in young animals, which was maintained into adulthood, and concurrently mitigated the subchondral bone thickening that was seen in HF animals. Overall our data suggests that high fat diet can have a detrimental effect on the developing knee joint, especially with long term diet consumption, and that LIV may have the potential to stimulate cartilage tissue formation, leaving the knee joint better equipped to deal with the increased loads due to obesity.

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