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The Role of Osteocytic MMP13 in the Progression of Osteoarthritis in the Mouse TMJ



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The Role of Osteocytic MMP13 in the Progression of Osteoarthritis in the Mouse Temporomandibular Joint

by

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**THE ROLE OF OSTEOCYTIC MMP13 IN THE PROGRESSION OF OSTEOARTHRITIS IN THE MOUSE TMJ**

**Alena D. Larios**

Abstract

The Temporomandibular Joint (TMJ) is a robust complex in our body that contains networks of bone and cartilage working together to keep it in a healthy and functional state. The TMJ is subject to various pathological conditions, such as chronic inflammation seen in TMJ Osteoarthritis (TMJ OA), which negatively impacts the joint's ability to diffuse loads and work properly. This study was created to elucidate the role of osteocytic MMP13 in the progression of TMJ OA, which has signs of both cartilage and subchondral bone deterioration. Given that this model showed that MMP13 $^{OCY-/-}$ mice with OA in the knee had a more severe phenotype than their wildtype counterparts, we predicted the same results in our model. TMJ OA was induced using Monosodium Iodoactetate (MIA) in MMP13 $^{OCY-/-}$ mice and compared the effects to non-injured and saline injected mice. The histological results were remarkable for severe OA in the wildtype MIA group compared to the MMP13 $^{OCY-/-}$ mice, the opposite of what was hypothesized. Furthermore, microCT data was insignificant among all the parameters measured. Our data suggest that the presence of MMP13 has a protective effect on the TMJ cartilage in an osteoarthrtitic state and should be considered for future therapeutic modalities.

TABLE OF CONTENTS

**List of Figures**

## List of Tables

# CHAPTER I:

# REVIEW OF THE LITERATURE

## Introduction

Temporomandibular Joint Disorders (TMJDs) are a highly prevalent spectrum of conditions occurring in about 6 to 12% of the adult US population totaling over 10 million people and costing billions of dollars in health care and lost productivity. Patients with TMJDs frequently present with facial pain and headaches, joint sounds and functional limitations that significantly affect the quality of life due to its impact on critical functions such as eating and speech. Approximately 10-25% of patients with temporomandibular joint (TMJ) pain demonstrate features of TMJ osteoarthritis (OA).[1,2] TMJ OA is a condition that is classified as low-inflammatory arthritic condition, implying it's chronic and slow onset.[1] Patients with TMJ OA often present with other crippling co-morbidities such as chronic pain and fibromyalgia, and have also been reported to have elevated levels of suicidal ideation, depression, and anxiety as compared to the general population.[3,4] Therefore, new therapies to treat TMJD are clearly needed. While the etiologies of the TMJ OA remain unknown, due to the propensity of these disorders in adolescent females, an age group that coincides with orthodontic treatment, orthodontic therapy has often been attributed as a causative or predisposing factor for TMJ OA.[1,5] Furthermore, severe forms of these disorders such as idiopathic condylar resorption impact on the orthodontist's ability to deliver predictable treatment outcomes. Thus, understanding the causation or predisposing factors for TMJ OA are of critical importance to our profession.

Joint health and optimal function in the TMJ as well as in the appendicular skeleton is a product of healthy articular cartilage, soft connective tissues including ligaments surrounding the joint, and subchondral bone. Defects in any of these can disrupt the crosstalk among these tissue that can lead to disease. Though there is evidence to show that this interaction between the various tissues in the joint are important in joint health and disease, the cellular and molecular mechanisms of this crosstalk are less clear. We have recently found that osteocytes in subchondral bone play a critical causal role in the progression of joint disease. Specifically, we have found that osteocyte-mediated defects in subchondral bone contribute to the progression of OA of the knee joint. Given that the TMJ is a unique joint with distinct developmental origin, composition and function that are different from the knee and other appendicular joints, these findings may not be readily translatable to the TMJ.[6] Therefore, we propose to determine the extent to which defects in osteocyte function contribute to the progression and severity of TMJ OA.

Matrix metalloproteinases (MMPs) are a family of 25 enzymes that are characterized by their extracellular matrix substrate specificity, zinc-dependent activity, extracellular inhibition by tissue inhibitors of metalloproteinases (TIMPs), secretion as a zymogen and sequence similarities.[7] When MMPs are divided by functionality, MMP13 is identified specifically as a collagenase, with its primary substrates being helical collagens such as types I and II collagen, the predominant collagens found in bone and cartilage, respectively. Osteoblasts and chondrocytes express MMP13 and are directly involved with the degradation of collagen I and II.[2] Indeed, MMP13 expressed by chondrocytes is a key proteinase implicated in cartilage matrix degradation and propagation of OA.[6] In contrast to the known contributions of MMP13 to cartilage matrix loss, the effects of altered levels MMP13 expressed by bone cells particularly

osteocytes to OA remains largely unknown. This information is of critical importance due to the fact that altered subchondral bone phenotype is increasingly implicated in contributing to the pathogenesis of OA. Specifically, it has been shown that increased or decreased subchondral bone density leads to secondary adverse changes in overlying cartilage and the perpetuation of OA.[8–10] As bone cells are overloaded in OA, there are extensive changes to the structural framework of the subchondral bone. Some visible features include but are not limited to cartilage thickening, sclerosis, osteophytes, and bone marrow lesions. As the OA progresses, the cartilage layer thickens and becomes increasingly calcified, effectively reducing its primary function of diffusing loads. From these studies, it has been concluded that the subchondral bone and overlying cartilage effectively function together to promote distribution of mechanical loads. Thus, not only do perturbations in cartilage health induce or aggravate bone degenerative changes, but primary or secondary changes in bone or poor bone quality perpetuate cartilage degeneration.[8,9,11] This interplay between cartilage and bone make the understanding of the effect of one tissue on the other extremely critical to understanding and preventing OA.

In this context, our studies show that the MMP13 ablation results in defective bone matrix organization and impaired bone quality in long bones, defects that we trace to impaired osteocyte function. Similarly, the administration of glucocorticoid represses MMP13 expression in osteocytes in long bones and in the mandible in proximity to defects in bone matrix collagen and mineral organization in the perilacunar regions.[3,12] These lacunae house osteocytes and have a spanning network of canaliculi that have a variety of proposed functions including perilacunar remodeling (PLR).[3] PLR is a process where osteocytes housed in lacunae resorb and replace the local bone matrix to achieve stable systemic mineral levels. The canaliculi off-shooting from the lacunae depend on this remodeling to maintain connectivity of osteocytes to one another and to

the vascular supply. In the absence of PLR, there is observed degeneration of the osteocyte lacuno-canalicular network, collagen disorganization, and matrix hypermineralization. Our preliminary analysis of bone from mice with osteocyte-specific MMP13 ablation indicate that cell-intrinsic MMP13 is required for normal PLR and bone homeostasis in long bones (see Chapter 2). However, the consequences and contributions of the resulting changes in bone phenotype to fibrocartilage health and TMJ OA has not been elucidated. Our proposed studies will set the foundation to elucidate the contributions of subchondral osteocytes and bone quality to cartilage degeneration and OA progression in the TMJ.

## MURINE MANDIBULAR ANATOMY

The mandible develops by intramembranous ossification with the earlier developing Meckel's cartilage serving as a template. Nevertheless the condyle develops endochondrally as a secondary cartilage.[13] This is of significance because this secondary cartilage of the condyle serves as a growth site for the mandible, whereby chondrocytes proliferate to promote growth. Besides functioning as a growth plate, the condylar cartilage has an articular role that includes responding to functional and masticatory stresses. Following growth, the cartilage remains to continue serving as articular surface for the joint.[14] Additionally, the shape and location of the TMJ changes in murine and human models with age.[15] Liang et al., characterized the development of the murine TMJ from embryonic day 13.5 to post-natal day 180.[16] They characterize TMJ formation over three stages: initiation, growth, and cavitation. The condyle develops upwards into the glenoid fossa with which it articulates. Furthermore, through proliferation and apoptosis that is regulated by a variety of transcription factors and MMPs, the murine condyle develops into the skeletally mature state at the 3-month mark of the animal's

life.[16] The posterior portion of the condyle displaces posteriorly – lending to the up and back position of the condyle.[17]

## METHODS TO INDUCE TMJ OA – MONOSODIUM IODOACETATE (MIA)

MIA has been utilized for osteoarthritis induction for over 20 years, with its effects on joints characterized extensively. Localized injection of MIA into the synovial space of joints in the appendicular skeleton as well as in the rat TMJ results in a cascade of events affecting the function of chondrocytes. The metabolism of chondrocytes is impaired resulting in subsequent cartilage degeneration. Histological analysis has demonstrated this destruction is comparable to that of human OA.[18] While previous studies have used MIA in mediating mice knee joint OA and rat TMJ OA, no information is currently available currently in the dosing and effects of MIA in the mouse TMJ.[2,19–22] We used the dosing and outcomes data from these studies to test the calculated MIA dose bracket and temporal effects in inducing OA in the mouse TMJ. Current mouse TMJ OA models include dietary models, forced mouth opening, occlusal adjustments, and chemical induction. Dietary models are frequently used and present a non-invasive approach to discerning how changes in mechanical loading alter tissue phenotype. However, varying the pellet hardness (normal diet being hard pellets; experimental being soft) or size is often found to be insufficient in producing degenerative changes in the TMJ.[13] Additionally, the joint changes in this model are not thoroughly characterized. Forced mouth opening models present an aggressive model to induce TMJ changes which have notable outcomes such as subchondral bone thickening, increased expression of specific genetic markers, and chondrocyte irregularities.[23] However, custom designed springs on mice present logistical issues to maintain the spring, and variations in reproducibility of loading forces. Malocclusion models for TMJ OA such as placement of a unilateral bonded wire to irregularly load the TMJ are not well

characterized.[24] Studies using chemical approaches to induction of TMJ OA show reproducible findings, are well-characterized and present a reliable option for TMJ induction.[25] Current approaches include use of complete Freund's adjuvant and MIA. Freund's adjuvant-induced arthritis is highly inflammatory with features similar to that of rheumatoid rather than osteoarthritis.[26] In contrast, the MIA OA model has been used to successfully and reliably induce OA-like disease in rat and rabbit and in the rat TMJ.[2,19–22]

## ESSENTIAL PLR REGULATING ENZYMES

Osteocyte specific MMP13 null mice that will be used in these studies have been used to generate important preliminary data that points to the potential role of PLR in TMJ OA. The importance of osteocyte-mediated PLR in health and disease has been characterized by studying the effects of enzymes that regulate bone remodeling on this process. Several osteocyte derived proteins have been implicated in PLR including cathepsin K, MMP13, MMP14, MMP2, TRAP, carbonic anhydrase 2, and the Na/H+ exchanger.[12] Deficiency in each of these key enzymes impairs canalicular networks and yields other hallmarks of defective PLR. More specifically, dysregulation of bone remodeling caused by systemic or osteocyte-specific ablation of MMP13 significantly compromises bone quality (Figure 1). This includes aberrant collagen organization, hypermineralization and decreased resistance to fracture.[12]

## PLR SUPPRESSION IN HUMAN JOINT DISEASE

Although the abnormal subchondral bone of post-traumatic OA (PTOA) and osteonecrotic joints has been well-described, the integrity of PLR in these conditions has not previously been known.[27–29] We found that subchondral bone from patients with PTOA and osteonecrosis exhibits each of the classical hallmarks of defective PLR. Femoral head subchondral bone from patients with glucocorticoid- induced osteonecrosis has reduced levels of MMP13, disorganized collagen, truncated canaliculi, smaller lacunae, and hypermineralization (Figure 2). Our preliminary data indicate that the same hallmarks are present in PTOA tibial plateaus from veterans receiving knee replacements.[3] Among these, the most compelling evidence of PLR suppression is that canalicular length is reduced by 56% in PTOA bone, relative to bone from non-PTOA cadaveric donors ***(Fig. 3, p=0.003)***. Furthermore, canaliculi on the medial side of the tibial plateau, the most common site of PTOA, appear to be shorter than those on the relatively healthier lateral side of the same joint. Collectively, our findings strongly support the conclusion that PLR is suppressed in human osteonecrosis and in PTOA. However, analysis of tissues with terminal disease does not clarify whether PLR suppression contributes to disease progression, or is a result of the disease process. Thus, there is substantial value to performing *in vivo* mechanistic studies to understand the temporal relationship between PLR and OA and the direct contributions that aberrant PLR makes to OA.

### PLR suppression compromises subchondral bone quality and plays a causal role in PTOA

In addition to our findings on effects of dysregulated PLR on trabecular bone in long bones, we have found sclerotic bone and abrogated canalicular networks ***(Fig. 4)*** in subchondral bone in mice with either systemically ablated MMP13, or with osteocyte-specific knockout of MMP13.[12]

Our preliminary data further support the causal role of PLR suppression in joint disease. Using an established medial collateral ligament (MLI) injury model of knee joint PTOA in which the medial collateral ligament is transected and the medial meniscus is removed, we tested the hypothesis that PLR suppression would exacerbate the severity of PTOA.[30,31] While the analyses are still underway, the preliminary data support this hypothesis. Relative to the anticipated moderate degeneration of articular cartilage in MLI wild-type mice ***(Fig. 5B)***, the degeneration in an osteocyte-intrinsic model of PLR suppression (TBRII $^{OCY-/-}$) demonstrated full-thickness cartilage loss and severe bone sclerosis as visualized histologically ***(Fig. 5D)***. These findings support and extend the conclusions derived from the osteonecrosis model, in which glucocorticoid excess affected multiple cell populations. Specifically, we find that osteocyte-intrinsic suppression of PLR is sufficient to exacerbate the severity of joint degeneration. The extent to which similar processes participate in the degeneration of the TMJ remain to be determined and are the focus of this study.

**Experiments to Induce TMJ OA and Assay TMJ Degeneration**

Our lab (Kapila et al., 1995) has previously developed and characterized the first reproducible animal model of inflammatory TMJ disease and were also the first to document the mandibulofacial growth changes in juvenile rheumatoid arthritis (JRA)-like disease of the TMJ in this animal model.[26] We have also characterized the MMPs expressed by TMJ cells, which provided the foundation of subsequent work by several investigators in deciphering the TMJ disease progression and markers of disease.[32] The preliminary data provided below demonstrates the technical and intellectual know-how to conduct the studies proposed in this application, which include histochemical and biochemical assays on changes in cartilage matrix macromolecules (Figure 6***)*** and μCT analyses of subchondral bone porosity (Figure 7).

**Summary and Significance**

A review of the literature and previously adopted methods led us to use MIA for inducing OA in the mouse TMJ. This required modifying approaches, doses and timelines that have previously been characterized in the mouse knee joint and rat TMJ to establish both the optimal conditions and to effectively administer MIA into the mouse TMJ to result in reproducible TMJ OA. Given the voids in our knowledge on the role of osteocyte-mediated bone remodeling in TMJ OA and because OA is a disorder that involves complex and as yet largely unknown crosstalk between cartilage and subchondral bone, we propose to explore the potential link between altered bone metabolism through loss of MMP13 in osteocytes and TMJ OA. Our overall goal is to understand the contribution of osteocyte-related bone phenotypic changes to the progression and severity of TMJ OA. Because the etiopathogenesis of TMJ OA is poorly understood, current treatments are largely palliative and designed to alleviate symptoms rather than rational and specific approaches to address cause(s) of these disorders. With increasing severity of the disease, it is treated with a spectrum of progressively invasive measures - from observation to full joint replacement. The proposed studies will not only provide fundamental information on osteo-chondral interactions and the role of each of these tissues to the initiation and / or progression of TMJ OA, but will be important in better understanding the pathogenesis of this disorder and in providing insights into potential therapeutic targets to specifically prevent or alleviate degenerative diseases of the TMJ. For example, if the effect of bone phenotype on fibrocartilage degeneration is demonstrated, then optimal treatment strategies for OA might include targeting both the bone and cartilage compartments. Also, by focusing on the TMJ rather than the hyaline cartilage joints, we will be able to decipher the specifics of fibrocartilage responses to altered bone quality, that will be critical to understanding disease progression that is

unique to the TMJ. Thus, the findings will be highly relevant to potential therapies that are specific for TMJ OA.

Previous studies have shown that systemic and osteocyte-specific ablation of MMP13 interferes with PLR in the mouse femur and tibia resulting in altered collagen matrix organization and hypermineralization in the trabecular compartment of long bones.[6] These defects manifest as bone sclerosis and canalicular network degeneration in subchondral bone and exacerbate the severity of joint degeneration in appendicular joints. Specifically, defective PLR results in full-thickness cartilage loss and severe bone sclerosis in the knee joint of MLI mouse model of OA as compared to moderate articular cartilage degeneration in MLI WT mice. Though our preliminary data support a causal role for osteocytic PLR in appendicular joint disease, the extent to which proteinases involved PLR such as MMP13 play a causal role in TMJ OA remains to be determined. Equally importantly, while we show the adverse effect PLR defects in subchondral bone on hyaline cartilage joint, it is not yet clear how these boney changes impact the progression of TMJ OA. Therefore, to evaluate the causal role of PLR in TMJ OA, we performed initial studies to evaluate the TMJ OA phenotype in MMP13$^{OCY-/-}$ mice in the MIA model using the optimal dose conditions.

# CHAPTER II:

# THE ROLE OF OSTEOCYTIC MMP13 IN THE PROGRESSION OF OSTEOARTHRITIS IN THE MOUSE TMJ


## ABSTRACT

The Temporomandibular Joint (TMJ) is a robust complex in our body that contains networks of bone and cartilage working together to keep it in a healthy and functional state. The TMJ is subject to various pathological conditions, such as chronic inflammation seen in TMJ Osteoarthritis (TMJ OA), which negatively impacts the joint's ability to diffuse loads and work properly. This study was created to elucidate the role of osteocytic MMP13 in the progression of TMJ OA, which has signs of both cartilage and subchondral bone deterioration. Given that this model showed that MMP13 $^{OCY-/-}$ mice with OA in the knee had a more severe phenotype than their wildtype counterparts, we predicted the same results in our model. TMJ OA was induced using Monosodium Iodoactetate (MIA) in MMP13 $^{OCY-/-}$ mice and compared the effects to non-injured and saline injected mice. The histological results were remarkable for severe OA in the wildtype MIA group compared to the MMP13 $^{OCY-/-}$ mice, the opposite of what was hypothesized. Furthermore, microCT data was insignificant among all the parameters measured. Our data suggest that the presence of MMP13 has a protective effect on the TMJ cartilage in an osteoarthrtitic state and should be considered for future therapeutic modalities.

# INTRODUCTION

The Temporomandibular Joint (TMJ) is a unique structure which, just like any other part in our body, can undergo pathologic changes. This spectrum of conditions, known as Temporomandibular Joint disorders (TMJD), is prevalent among 3.7% to 12% of the US population and results in symptoms such as facial pain and headaches.[42] A significant proportion of patients with TMJD also experience signs of clicking and popping upon mouth opening that is commonly associated with discomfort. More advanced and severe disease conditions afflicting the TMJ include osteoarthritis (OA) or degenerative joint disease, idiopathic or progressive condylar resorption (ICR/PCR) and rheumatoid arthritis.[1, 43] These inadvertently cause a disruption in mastication, deglutation and speech, which significantly impacts an individual's quality of life. Many patients seek the expertise of an orthodontist to re-establish their occlusion in the hopes that it will relieve their symptoms, and therefore it is within our profession's scope to understand and accurately diagnose TMJDs in our population. Nevertheless, the lack of known etiopathologic factors diminish the ability of orthodontists and other providers from providing rational, specific and efficacious therapies to mitigate TMJ dysfunction and diseases.

Approximately 10-25% of patients with TMD demonstrate features of TMJ osteoarthritis (OA).[1,2] What makes this condition unique among the others is that it is strongly linked to co-morbidities such as chronic pain and fibromyalgia, depression, anxiety, and suicidal tendencies.[3,4] It is furthermore characterized by its slow onset, chronic inflammatory state, and perplexing age and gender distribution. Thus, while the cause of this low-inflammatory arthritic condition is not known, we do know that it is highly prevalent among women in their reproductive age. Oftentimes this demographic develops TMJ OA symptoms while undergoing treatment, so orthodontics has been attributed to causing TMJ OA.[1,5,40, 43] While studies have

shown that estrogen levels are associated with the presence of TMD's, ultimately there are likely multiple factors at play and the research is unclear regarding any prominent cause. Patients with active forms of the disease often impede the orthodontist from obtaining predictable outcomes, and treatment is commonly delayed until the pathology is under control. Therefore, understanding the causation, predisposing factors for TMJ OA, and how to address them are of critical importance to our profession.

In order to comprehend how the TMJ degenerates during OA, it is imperative to first be familiar with its biology. The joint is part of a network of cells and ligaments that work together to keep it in a healthy and normally functioning state with specific proteins playing a role in maintaining homeostasis and / or contributing to disease. Key players of this network include a family of enzymes called matrix metalloproteinases (MMPs). Since MMP13 or collagenase-3 collagenase is known to degrade collagen types I and II, the two predominant types of collagen in bone and cartilage, respectively, it may be of critical and specific importance in the pathogenesis of the TMJ disorders. When MMP13 is overexpressed by chondrocytes, it leads to cartilage matrix degradation and propagation of OA.[6] Additionally, while several investigators have also shown noticeable changes in subchondral bone in OA the contribution of MMP13 particularly that derived from osteocytes to these degenerative changes and disease progression are not well delineated. While the contribution of MMP13 and its cell-specific source to subchondral bone degeneration has yet to be elucidated, it has been shown that increased or decreased subchondral bone density leads to secondary adverse changes in overlying cartilage and the perpetuation of OA.[8–10] These changes are often visualized as a thickened, calcified cartilage layer, sclerosis, presence of osteophytes, and bone marrow lesions which all prevent the TMJ from functioning properly. Given these observations, it is clear from these studies that

changes in the mechanical characteristics or biological signals from bone and cartilage, that are in communication with each other, determine the health of the TMJ. Furthermore, these findings suggest that cellular, matrix and physical changes in one tissue affects the other via cartilage-bone crosstalk in the progression of OA. However, the relative contributions of biological signals and mechanical tissue properties to joint degeneration are poorly understood and the mechanisms of OA progression remain unknown.

While little is yet known about MMP13's role in TMJ OA, in the knee joint, the loss of osteocytic MMP13 impairs the function of these cells and leads to an increase in the severity of OA primarily through affecting the perilacunar housing.[3,12] Osteocytes reside in the lacunae and communicate with neighboring osteocytes via the canaliculi extending from the lacunae. The oseocytes participate in perilacunar/pericanalicular remodeling (PLR), a process that is essential for healthy long bones.[3] During PLR, the bone resorbs and subsequently builds up again in a cyclical manner to keep the extracellular matrix intact. Without PLR, the osteocyte-canaliculi network is degraded and the cells lose their vascular supply. MMP13 is required for PLR in the long bones and is therefore necessary for healthy bone metabolism, but the question remains whether the process is the same in the TMJ.[39]

Osteocytic MMP13 in the joints of long bones plays an essential role in cartilage homeostasis and contributes to human joint disease. When MMP13 is blocked, PLR is suppressed and knee joint degeneration is the resulting effect. The TMJ is a very unique structure, but osteocytic MMP13 in this region has also shown to play an essential role in deciphering TMJ OA progression and markers of disease.[32] It would therefore be pivotal to understand the specific mechanisms in which the presence of osteocytic MMP13 affects TMJ OA. This knowledge is valuable because it could lead to new, specifically targeted therapeutic

strategies to combat OA of the TMJ. The purpose of this study is therefore to elucidate the contributions of subchondral osteocytes and bone quality to cartilage degeneration and OA progression in the TMJ. Given our knowledge about the knee joint, we hypothesized that the same would occur in the TMJ – that the loss of osteocytic MMP13 would lead to worsened TMJ OA.

# MATERIALS AND METHOD

## Selected Mice and Allocated Groups

Mice were generated by breeding homozygous MMP13-floxed mice that possess loxP sites flanking exons 3, 4, and 5, which encode the enzyme's active site with hemizygous -10kb-DMP1-cre$^{+/-}$ mice, which express Cre recombinase primarily in osteocytes.[33,34] Half of the mice from the resulting cross were DMP1-Cre$^{+/-}$; MMP13$^{flfl}$ (named MMP13 $^{OCY-/-}$) and half were DMP1-cre$^{-/-}$; MMP13$^{fl/fl}$ littermate controls (named WT mice), as confirmed by PCR genotyping. All experiments were initiated on 12-week old mice using animal procedures approved by the Institutional Animal Care and Use Committee of the University of California San Francisco and the Indiana University School of Medicine. A power analyses indicated that a sample size of four specimens per group will result in a statistical significance at the 0.05 level (two-tailed).

OA was induced using intraarticular injection of monosodium iodoacetate (MIA) previously established in mice knees and rat TMJs with appropriate adjustments in dosing to account for joint size and weight differences.[25] The studies were performed in three parts. In the initial two preliminary studies the methods of intraarticular administration of MIA was determined (see below) and appropriate dose of MIA was elucidated (described in Demirdji, MS Thesis). Mice used for preliminary dose determination studies were allocated as follows: four mice for the 0.10 mg (4 mg/kg) MIA dose, two for the 0.05 mg (2 mg/kg) MIA dose, and 3 for the saline group. The purpose was to define if the doses used resulted in reproducible histologic arthritic change to set the foundations for further studies using MMP13 conditional knockout mice. Using the appropriate MIA dose identified in the preliminary study which was 0.10 mg, we conducted the second definitive study to understand the contribution of osteocyte MMP13 on

MIA-induced TMJ arthritis. In this portion of the study, we used a minimum of 5 each of WT and MMP13$^{OCY-/-}$ male mice injected with PBS or MIA for histological analysis and a minimum of 3 each for microCT analysis. Tissue samples were retrieved at 28 days post-injection and MMP13 $^{OCY-/-}$ for these analyses as elaborated below. Only the male mice samples were used in order form a subset that could be analyzed fully within a limited amount of time.

**Induction of TMJ OA with Monosodium Iodoacetate (MIA)**

We used Monosodium Iodoacetate (MIA) as our method for inducing TMJ OA. While MIA has been previously used in mice knee joint OA and rat TMJs, but this study is the first to establish a sufficient dosing regimen and method of intraarticular injection in a mouse TMJ.[2,19-22] MIA works through suppression of chondrocyte metabolism and consequential cartilage degeneration that is histologically similar to the OA changes observed in humans.[18]

Preliminary studies were performed to optimize intraarticular injection of MIA on 12 week-old mice using 25-gauge 0.625 inch needle to administer Fast Green dye followed by euthanasia via lethal dose of $CO_2$. Surgical exposure of the TMJ confirmed the location of the dye (Figure 8C). These procedures were modified as needed and repeated until injections were noted to accurately and consistently deposit the dye into the joint. The final protocol for intra-articular injection was based on the length of the needle from the TMJ to the nose tip as well as the volume of the fluid to be injected. This involved inserting needle by placing it parallel to and directly inferior to the zygomatic arch, as medial as possible with the needle lying directly parallel to the mouse head in a coronal plane. The needle was then inserted until it hubs against nose of mouse and lateral to mouse molars (Figure 8A and 8B).

The TMJ OA was induced by injection of monosodium iodoacetate (MIA) (Sigma I2512) dissolved in 10 µl saline into both TMJs of 12 [KS1] week-old mice as described above under anesthesia without surgical exposure. For our pre-liminary experiment, in establishing and characterizing OA TMJ model in mice, we performed preliminary low and high dose-response analysis of 0.05mg (2mg/kg) and 0.10mg (4mg/kg) of MIA in saline administered into mouse TMJs to generate reproducible OA in the mouse TMJ. The previous studies on mouse knee and rat TMJ have also demonstrated joint changes characteristic of OA within 3 to 21 days of intraarticular administration of MIA. For sham controls, we injected 10 µl saline into TMJs of a small sample of mice and histologically assayed the TMJs at 28 days following administration of each dose of MIA or saline. Mice were euthanized at 16 weeks of age and the TMJs were harvested for further evaluation. After establishing the appropriate dose for establishing OA model, we randomly assigned 12 week old mice into three groups: MIA, Sham, and un-injected Controls.

We used un-injected control animals that did not receive anesthesia or analgesics at age 13 weeks (corresponding to 7 dpi sham and MIA mice) and 16 weeks (corresponding to 7 dpi sham and MIA mice) for baseline data. All animals were allowed unrestricted activity, food, and water. At 28 days post injection (dpi), animals were euthanized via lethal dose of $CO_2$, followed by cervical dislocation and hemi-mandibles were harvested for histological and radiographic analyses. The left hemi-mandibles were frozen for future microCT studies. The right hemi-mandible was fixed in 10% neutral buffer formalin (NBF), decalcified in 10% EDTA, sectioned, stained with Safranin-O/Fast Green stain and OA severity determined through modified Mankin scoring. Samples at these time points for male and female WT and MMP13 $^{OCY-/-}$ were collected.

Of these five samples each from WT and MMP13$^{OCY-/-}$ 16-week old male mice (controls for 28 dpi) were analyzed per group at 0.10mg (4mg/kg) euthanized at 28 days post injection for

the final study, while the rest were banked for ongoing studies. All animals were allowed unrestricted activity, food, and water. Mice were euthanized via lethal dose of $CO_2$, followed by cervical dislocation.

**MIA Dosage**

From the observable OA histologic change in the TMJ at 0.10 mg MIA dose based on the work from Demirdji et. al, it was determined that this would serve as an effective dose to successfully and reproducibly induce OA (Figure 9, 10). While we observed some effect on histologic changes in the TMJ with 0.05 mg MIA dose, this lower dose did not result in consistent joint changes and may be useful in providing a more sensitive dose range in studying its combined effects of with other modulators of OA including joint injury with or without genotype effects on the severity of OA. Given the above findings, only mice injected with 0.10 mg MIA were used for subsequent studies to determine the role of osteocyte MMP13 on the progression and severity of OA.

**Histology and OA Quantitation**

The hemi-mandibles were dissected and fixed in 10% neutral buffered formalin and then decalcified in 10% EDTA until fully decalcified, followed by serial ethanol dehydration and paraffin embedding. Coronal sections (6 □m thick) were generated using a microtome (Leica Microsystems, Buffalo Grove, IL) for Safranin-O/Fast Green stain. The staining protocol was adapted from University of Rochester (University of Rochester Center for Musculoskeletal Research. Safranin O/Fast Green stain for Cartilage. https://www.urmc.rochester.edu/musculoskeletal-research/core-services/histology/protocols.aspx. Published 2017. Accessed May 1, 2018). Briefly, paraffin

sections were deparaffinized, rehydrated, and stained in Weigert's Iron Hematoxylin for 5 minutes then washed in water and differentiated in 1% acid-alcohol. Slides were then incubated in 0.02% Fast Green for 15 minutes, followed by staining with 1.0% Safranin-O for 20 minutes and subsequently dehydrated, cleared, and mounted. Images were captured using a Nikon Eclipse E800 bright-field microscope for OA quantitation.

A minimum of 5 sections of each condyle taken as close as possible to the midcoronal location of the joint were used to quantitate OA using a Modified Mankin Score that is scored from a minimum aggregate score of 6 for normal cartilage to 25 for aggressive cartilage destruction as summarized below and in Table 1. [36,37,40] The Modified Mankin Score has six subcategories contributing to the overall score for TMJ OA. The criteria were modified for this study by the addition of criteria number six, which was custom added by the graders. The grading criteria was discussed among the three graders and definitions were agreed to as follows:

1. Cartilage Erosion – Presence of an intact, eroded, and/or fibrillation on the surface of the condylar cartilage.
2. Chondrocyte Periphery Staining – intensity in Safranin O stain in the surrounding region of the chondrocytes.
3. Spatial arrangement of chondrocytes – Diffuse hypercellularity was noted as a clear increase in the general number of chondrocytes, while clustering was agreed upon as three or more cells congregated together. Hypocellularity was chosen when the cartilage had areas lacking any chondrocytes.
4. Background Staining Intensity – Intensity of the counterstain, Fast Green.

5. Subchondral Bone Deterioration – Anatomy of the subchondral bone as identified with Fast Green staining adjacent to the cartilage (Safranin O stain) to observe erosion and sclerotic changes.
6. Demarcation –the boundary between the cartilage and the subchondral bone. Examples of a well-demarcated versus a vaguely demarcated is shown below. The sample that is not demarcated has finger-like projections from the cartilage infiltrating into the bone.

Illustrations demonstrating the above can be found in Figure 11A-D.

Histologic analysis was performed using the modified Mankin OA score.[37, 40] The panel of images were scored by 3 independent blinded graders. Intra-operator reliability was determined by calibration of the graders and statistical analysis. Histological samples were placed on a screen and the graders reviewed the characteristics of each category in the Modified Mankin Score Table with relation to the displayed image. This discussion occurred with multiple images until all graders were able to score in the same manner for each subcategory and had a uniform understanding of what constituted a high versus a low score. One section was presented per sample and each sample was shown at 20x and 40x magnification.

**Microcomputed Tomography Analysis**

The harvested left TMJ of the mouse mandible was analyzed by microcomputed tomography. Specimens were immersed in 70% ethanol and sent to the University of Michigan School of Dentistry's microCT Core, funded in part by NIH/NCRR S10RR026475-01**.** Specimens placed in a 19 mm diameter specimen holder and scanned over the entire width of the mandible using a microCT system (μCT100 Scanco Medical, Bassersdorf, Switzerland). Scan settings were as follows: voxel size 12 μm, 70 kVp, 114 μA, 0.5 mm AL filter, and an

integration time 500 ms. Analysis was performed using the manufacturer's evaluation software to isolate the articular surface of the condyle, and a fixed global threshold of 20% (200 on a grayscale of 0–1000) was used to segment bone from non-bone. The region of interest was the subchondral bone of the mouse TMJ, which is demarcated with the green line in Figure 12. The ANOVA for bone volume fraction, bone mineral density, tissue mineral density, trabecular number, trabecular thickness, and trabecular spacing was completed using a level of significance of $p<0.05$ and a $n = 26$ using the Prism Software. Post hoc Tukey tests were completed for pairwise comparison among groups.

**Statistical Analyses and Data Management**

Power analysis was performed using conservative assumptions and indicates an 80% chance of reaching statistical significance at the 0.05 level (two-tailed) with a sample size of 4 specimens per group. q1 = 0.500 [MMP13$^{OCY-/-}$ mice with TMJ OA]; q0 = 0.500 [DMP1 mice with TMJ OA]; Alpha = 0.05 (2-sided); Beta = 0.20 (80% power); E = 1.3 - 0.5 = 0.80; S = 0.4. Five WT and five MMP13 $^{OCY-/-}$ mice at 16 weeks old (28dpi) were analyzed to assess for differences in MIA-mediated OA severity and the contributions of osteocyte MMP13 to any observed changes. Using Prism Graphpad software and Microsoft Excel, the descriptive statistics (mean, standard deviation, standard error) were calculated for each parameter for all groups. All data was also analyzed for outliers using Grubb's test. The microCT and histological data were analyzed using one way analysis of variance (ANOVA). The level of significance was set at $p<0.05$. A Tukey post-hoc test was used to analyze pairwise difference between groups with a significance level set at $p<0.05$.

# RESULTS

## Histological Findings

Given that our preliminary studies demonstrated that 0.1 mg of MIA reliably induced TMJ OA, we used this dose of MIA for all subsequent studies. The non-injected baseline Cre-control mice had significantly greater mean Mankin Score than the non-injected Cre+ or both genotypes of mice that were administered intraarticular PBS (Fig. 13). This likely resulted from generational differences in the mice, or as a result of the inflammatory response due to the injection. Because of this, further comparisons were only made between PBS and MIA-injected WT and osteocyte-specific MMP13 null mice. Inter-rater reliability was calculated to be 12.4%.

Histologic analyses of WT and $MMP13^{OCY-/-}$ mice unexpectedly revealed that while MIA administration resulted in significant increase ($p<0.05$; N= 5 per group) in aggregate Mankin score in WT mice at 28 dpi, the absence of osteocyte MMP13 mitigated this response (Fig. 13). In further characterizing the criteria that contributed to MIA-mediated arthritic changes in the WT group, we found significantly enhanced chondrocyte periphery staining, altered spatial arrangement of chondrocytes with increased in diffuse hypocellularity and more vague demarcation of cartilage in the MIA WT group compared to the sham WT and both sham and MIA $MMP13^{OCY -/-}$ samples (Fig 15). In contrast, the sections from $MMP13^{OCY -/-}$ mice show similar degrees of Safranin-O intensity staining, spatial arrangement of chondrocytes, and little to no cartilage erosion between the PBS sham and MIA-injected groups. Finally, within the WT group, the MIA-administered mice showed significantly greater ($p<0.05$) staining than the PBS sham mice. The statistical analysis using ANOVA with at least a n=5 per group phenotypic findings from the histology sections and demonstrates a significant difference between sham and

MIA treated WT mice, but not between the two treatments in the MMP13$^{Ocy\ -/-}$ mice (Fig, 14). Thus, the subcategories of Modified Mankin scoring revealed that the chondrocyte periphery staining, spatial arrangement of chondrocytes, background staining intensity, and demarcation between the cartilage and bone were the primary factors which drove the differences in the overall Mankin Scoring numbers (Figure 15).

**Micro-computed Tomography Subchondral Bone Analyses**

The normal control, sham-treated, and MIA treated groups were analyzed statistically for changes in bone quality and quantity. Normal controls were excluded due to the statistical difference when evaluated with a student t-test compared to the sham-treated controls as discussed previously (Fig. 13). There were no significant differences for any of the measured parameters listed, namely subchondral bone volume fraction, bone mineral density, tissue mineral density, trabecular number, trabecular thickness, and trabecular spacing between WT and MMP13$^{OCY\ -/-}$ sham and MIA mice and between these two genotypes (Figure. 16). However, microCT images in Figure. 17 illustrate that MMP13$^{OCY\ -/-}$ samples have qualitatively denser bone and less trabecular spaces below the subchondral bone and MIA appears to increase spaces within the condylar bone in WT MIA mice than in sham WT mice confirming the histologic observations. These images provide greater detail about the condylar bone, which was not captured in the quantitative microCT analysis conducted for this study.

# DISCUSSION

The initial aims of this study were completed by Demirdji et. al who established the duration, dosage, and protocol for MIA injection. Using 0.10 mg MIA determined by these experiments in generating reproducible and observable TMJ OA, we were able to then move forward with testing the hypothesis that the loss of osteocytic MMP13 increases the severity of MIA-induced TMJ osteoarthritis in male mice at 28 dpi. In this study, we confirmed that injection of MIA effectively induces TMJ OA in a mouse model, and also discovered that lack of osteocytic MMP13 does not lead to worsened TMJ OA. Histopathologically, $MMP13^{OCY\ -/-}$ did not show potentiated degradation of cartilage or subchondral bone erosion compared to WT mice. The subchondral bone also did not show any significant difference in bone quality/quantity parameters when analyzed with microCT. Our findings are opposite of what is seen in osteoarthritic conditions of the knee, where loss of osteocytic MMP13 increases the severity of the disease. Not only did the TMJ show normal phenotype in $MMP13^{OCY\ -/-}$ mice (unlike the knee) but also this deficiency protected the TMJ from a serious insult/injury, such that the MIA $MMP13^{OCY\ -/-}$ mice TMJs were indistinguishable from the non-injured even though MIA induced a sever defect in the WT. This suggests that TMJ OA, unlike the knee, may benefit from therapies that inhibit MMP13 activity in osteocytes.

We examined the cartilage phenotype using Mankin Scoring for each sample and saw a distinct baseline phenotypic difference between the uninjured and the sham treated models as noted in Figure 1. This could be explained by generational differences of the uninjured mice, which were collected at a different time point than the sham and MIA treated samples. Yet it is also highly probable that this difference can be attributed to the saline injection causing an

inflammatory response and effectively normalizing their phenotype. The loss of a genotype-dependent difference has also been seen in studies on TbRII$^{OCY\ -/-}$ mice, but the significance has yet to be fully understood [41]. During the Mankin scoring, the independent graders were calibrated and reviewed various features of OA in order to achieve a consistent assessment of the photomicrographs. The general scoring protocol was modified for the purposes of this study. The subchondral bone subcategory from Wang et. al was extrapolated due to its relevance to the mouse TMJ, and the demarcation category was discussed among the graders and added since all histologic samples had a clear anatomic border (or lack thereof) between the cartilage and the bone. Overall, we see that the chondrocyte periphery staining, spatial arrangement of chondrocytes, background staining intensity, and demarcation between the cartilage and bone were the primary factors which drove the differences in the overall Mankin Scoring numbers. The subscore with the most influence on the overall Modified Mankin scoring was the spatial arrangement of chondrocytes, likely because it was very clear to denote among the graders and is therefore the most reliable indicator. Although the significance of the demarcation is not known, it is possible that the findings from this specification may be applicable to future knowledge as the TMJ continues to be studied. The other three factors are known markers of a diseased condition, however the analysis of the overall scores still illustrates that TMJ OA did not worsen when MMP13 was not present.

We see that the MIA model is successful to induce osteoarthritis in this study and others [40]. In the knee joint, MMP13$^{OCY\ -/-}$ induces a more severe osteoarthritic outcome [39]. In the TMJ, however, we see the opposite indicating a protective effect of MMP13. The anatomic as well as biological differences between the knee and TMJ could explain these differences. The knee joint has a thicker subchondral bone plate than the TMJ which could decrease the diffusivity of

MMP13 from the bone and, as a result, its influence on the cartilage. The lack of bone mass in the subchondral bone of the TMJ may allow osteocytic MMP13 MIA induced cartilage degeneration when it is present, however this theory needs to be studied. Another explanation may be that the expression of MMP13 depends on the age of the mouse and differs significantly between the TMJ and knee cartilage[44]. Yet Lam et. al discovered that overexpression of MMP13 is related to an OA phenotype in the TMJ's of mice lacking type IX and type XI collagen [45]. In our study, we see that loss of MMP13 causes a loss of proteoglycan and increased fibrillation on the surface in the WT but not in the knockout, suggesting that osteocyte derived MMP13 is part of the mechanism which is driving the degeneration of the TMJ cartilage. It also illustrates the highly compartment-specific effects of MMP13. The studies of the knee joint illustrate the biomechanical influence of MMP13, yet the MIA-induced TMJOA in mice suggests that soluble factors are predominating in this model for the reasons suggested above.

The microCT analysis of the subchondral bone was unremarkable for bone volume fraction, bone mineral density, tissue mineral density, trabecular number, trabecular thickness, and trabecular spacing. This was not considerably surprising given that the subchondral bone is a very indistinct part of the TMJ. The histological sections also corroborate the impression that there may be changes in the bone that are more likely to be detected in the condylar portion as opposed to concentrated directly below the cartilage. The region of interest for this data was the subchondral bone, therefore it is possible that this region should be altered in future analyses to include the larger condylar bone area which underlies the subchondral bone. Cross-sectional images of this portion of the TMJ showed denser bone with less trabecular spaces in the $MMP13^{OCY\ -/-}$ group, indicating a possible mechanism occurring in this section of the TMJ that prevents the osteocytic MMP13 from cross-talking with the MMP13 in the cartilage. This would

offer a different hypothetical suggestion for osteocytic MMP13's role in the progression of TMJOA. microCT analyses will be performed at a future time for this region to better understand how the various bone parameters compare to that of the subchondral bone and the data obtained for this study.

Limitations of this study include the exclusion of female mice and the use of only one time point. In humans, women tend to show more signs and symptoms of TMJ, yet since the mouse model is not completely reflective of humans it is possible that the results would have been similar. Additionally, TMJ OA is a progressive disease and understanding the phenotypic changes and how they occur along its temporal spectrum would enhance the way in which we decide which therapies to employ and when. As with many studies, a larger sample size could have benefitted the robustness of the overall results as well. We were unable to use our non-injured control due to inconsistencies that could not be determined yet use of these additional samples could have also contributed to the overall results and our understanding of TMJ OA. Ideally, future researchers in this field can apply what we know from this study to humans and establish a protocol that we can use directly to benefit our population. It was also difficult to be consistent with the staining process, since not all samples could be stained simultaneously. As a result, the samples had variable hues that could not be ruled out by any single factor. Furthermore, we tried to select sections that appeared to be taken as close as possible to the midcoronal location of the joint but cannot confirm whether this was achieved. We were ultimately restricted by trying to select sections that were both robust and of uniform color, which proved to be nearly impossible. Therefore, the Modified Mankin scoring results could have been affected from these inconsistencies.

# CHAPTER III

## FUTURE DIRECTIONS AND CONCLUSIONS

This study established the foundations for investigation the presentation of OA in WT and MMP13$^{OCY-/-}$ specific to the TMJ. It was postulated that the osteocyte specific MMP13 knockout would result in an increased qualitative expression of OA due to the disorganization the canaliculi projecting from the osteocytes or to differences in subchondral bone structure and mechanics, as seen in the knee.[3] However, here we see the opposite. The TMJ is different than long bones because there is no clearly delineated boundary between the subchondral bone plate and cartilage, which could possibly allow for a more continuous interface and crosstalk.

In the longer term, we intend to decipher whether the progression and severity of OA in response to subchondral bone defects are different between joints with hyaline articular cartilage versus the fibrocartilage-lined TMJ. Additional future directions of study include determining the localization of MMP13 in control and MIA-treated TMJ, in control and in mice with an osteocyte-intrinsic defect in MMP13 expression. This will reveal if MMP13 protein is more highly and broadly present in the control TMJ in the presence of MIA than in the mutant mice. In addition, future studies test the ability of molecular interventions to rescue osteocyte function to mitigate the severity of TMJ OA. Thus, the work in this study, as well as in future proposals will establish the model system and show proof of concept for the role of osteocytes in TMJ disease, providing the foundation needed for future studies intended to identify new therapeutic interventions. Finally, the data collected from this study will be critical to seeking NIH funding to undertake more thorough mechanistic studies on the interactions between subchondral bone

and fibrocartilage in contributing to progression of OA and the specificity of this response to the TMJ as opposed do appendicular hyaline cartilage joints.

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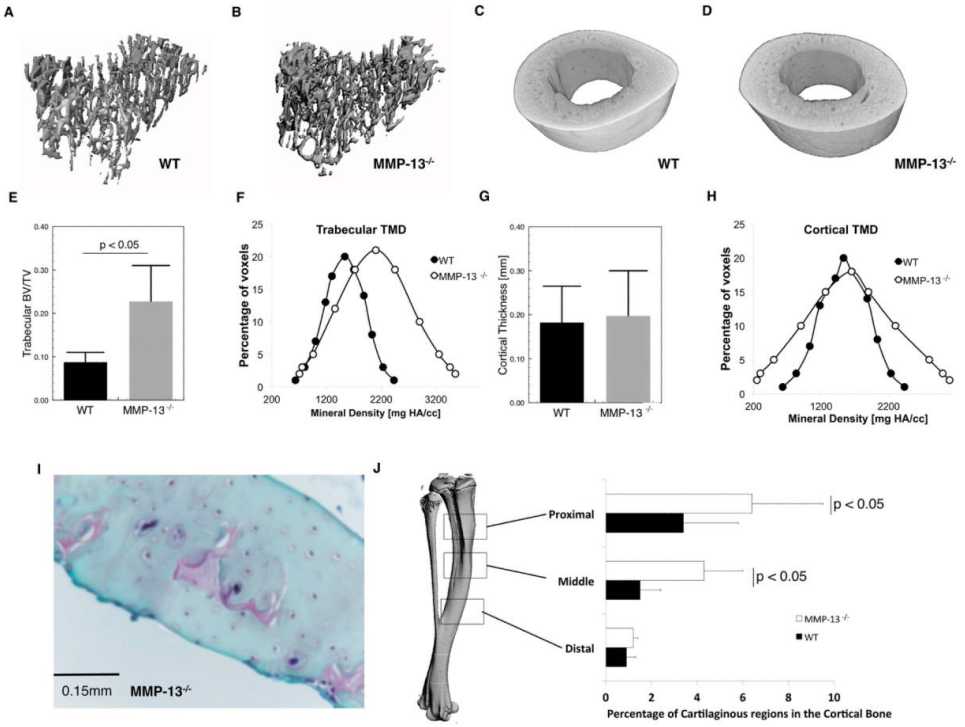



**Figure 1.1** MMP-13-deficiency results in increased trabecular bone volume and the altered distribution cortical bone mineralization. The trabecular bone contoured from the proximal region of the tibia confirms previous observations that MMP13$^{-/-}$ bones have significantly increased trabecular bone volume fraction (BV/TV; $p < 0.05$) (A, B, E, F). MMP13-deficiency did not affect the geometric structure of the cortical bone (cortical thickness; $p = 0.52$) (C, D, G). Nonetheless, a histographic plot of the cortical bone tissue mineral densities (Cortical TMD) reveals that MMP13 $^{OCY-/-}$ mice have significantly altered TMD distribution ($p < 0.001$), characterized by an increased incidence of lower and higher TMD compared to the WT (H). This can in part be explained by the osteoid remnants, which stain red with Safranin-O, that are predominantly observed in the middle and proximal aspects of the tibia (I). However there were no detectable differences in Safranin-O staining in the distal region near the tibiofibular junction where bulk of the subsequent analyses were performed (J).

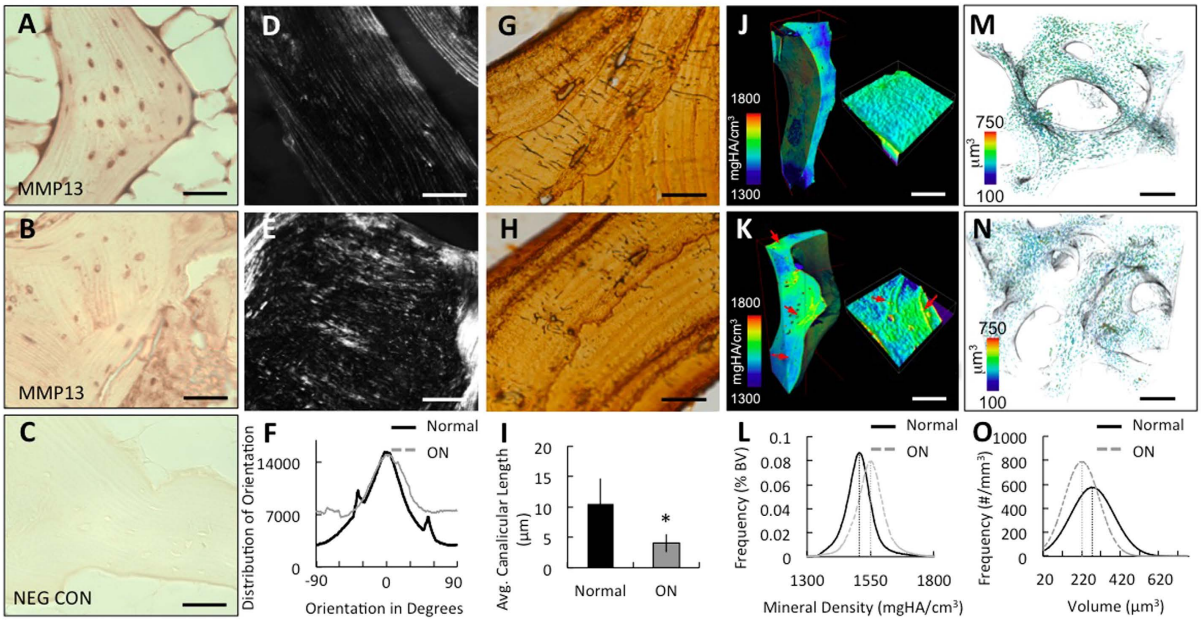



**Figure 1.2.** PLR suppression in human osteonecrosis. Trabeculae from the sclerotic regions of human femoral heads (B,E,H,K,N) show hallmarks of defective PLR relative to those distant from the lesion (A,D,G,J,M). These include reduced MMP13 expression (IHC, A–C) (scale bar, 20 µm), defects in collagen organization (picrosirius red stain, D–F) (scale bar, 50 µm), and reduced canalicular length (silver nitrate stain, G–I). Bar graph represents mean ± SEM of $n \geq 3$ regions from human cadaveric or human osteonecrotic bone samples, * p-value $\leq 0.05$ compared to control. Xray tomographic microscopy shows hypermineralization (L, red arrows) and reduced lacunar size (O) in osteonecrotic bone (K, N) relative to trabeculae that are more distant (J,M). Vertical lines (L, O) signify peak volumes.

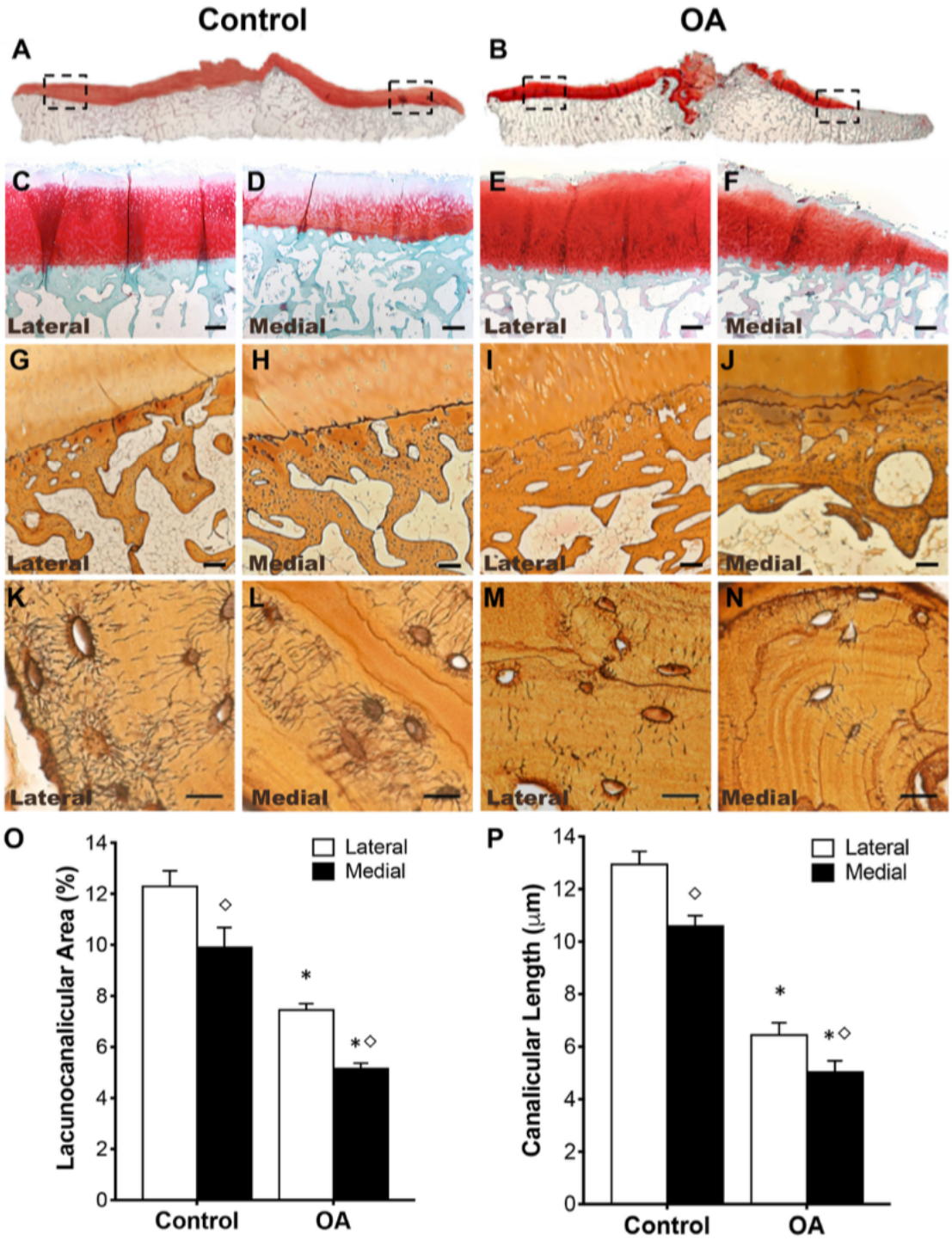



**Figure 1.3.** Disrupted lacunocanalicular networks in human OA subchondral bone**.** Control cadaveric (A, C, D) and OA (B, E, F) specimens stained with Safranin-O/ Fast Green and imaged at 0.5x (A, B) or 10x (C-F) magnification displayed differences in articular cartilage and subchondral bone morphology on the lateral and medial sides of the tibial plateau. Subsequent analyses compared the indicated regions of interest (black boxes in A, B) between control and

OA specimens, and between the less affected lateral side with the more severely degraded medial side. These identified regions of interest in Ploton silver stained sections were evaluated at low (4x, G-J) and high (100x, K-N) magnification to visualize the lacunocanalicular network of subchondral bone. Quantification of lacunocanalicular area normalized to bone area (O) and canalicular length (P) revealed significant OA-dependent reductions in both parameters (n=5). Scale bars are 400 μm in C-F, 200 μm in G-J, and 20 μm in K-N. *p<0.05 compared to respective regions of control specimens, p<0.05 between regions by Holm-Sidak post-hoc tests.

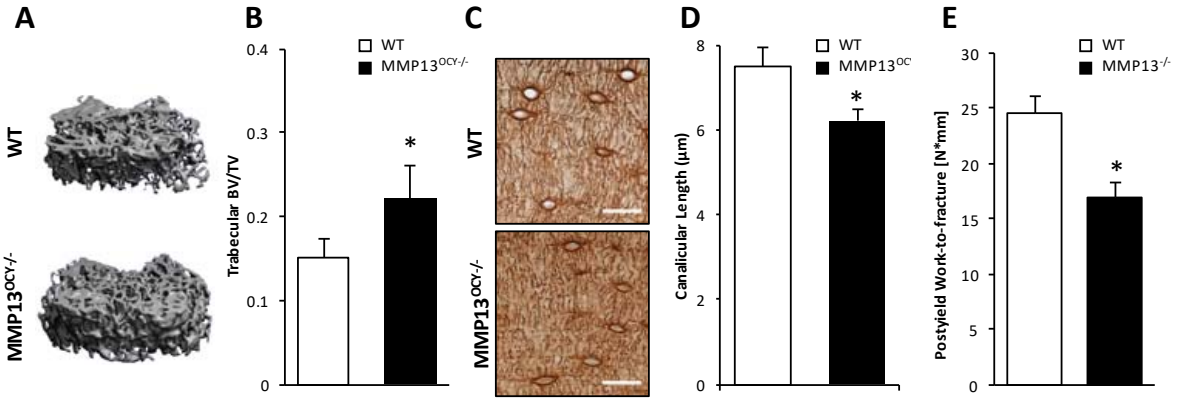



**Figure 1.4.** Osteocyte-intrinsic ablation of the PLR enzyme MMP13 causes subchondral bone sclerosis and canalicular network degeneration. Osteocyte-specific ablation of MMP13 results in trabecular bone sclerosis (A,B) and reduced canalicular length (silver nitrate stain, C,D). These findings complement those in human PTOA and in systemic MMP13-deficient mice, which also show poor bone quality with significantly reduced work- to-fracture (E). * p-value ≤ 0.05 compared to control.

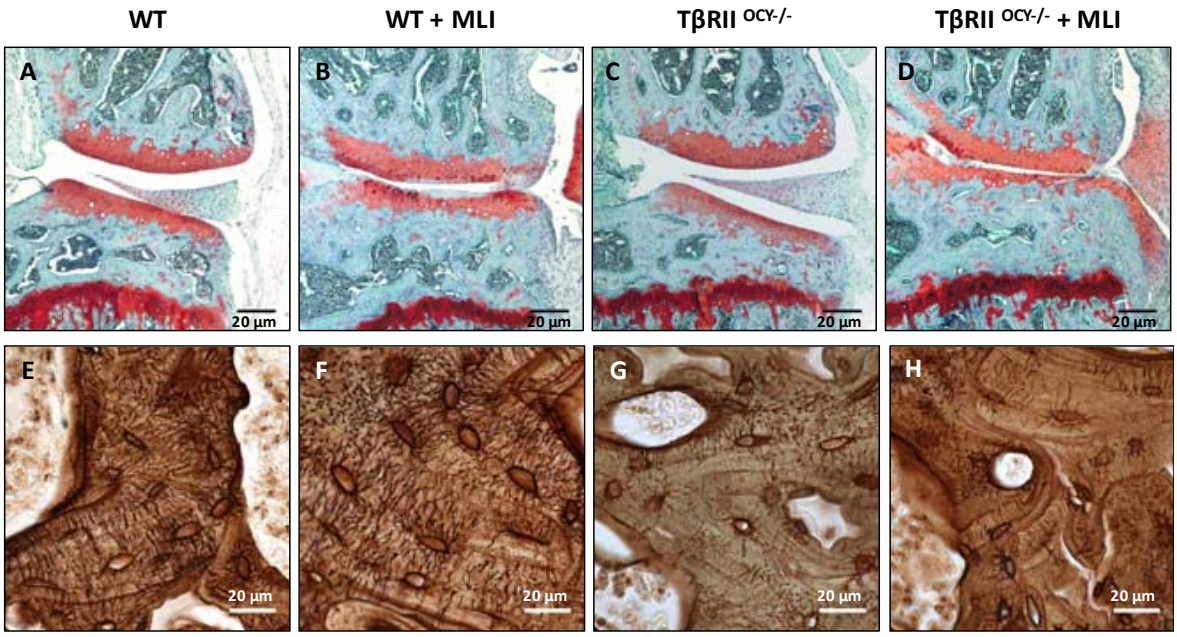



**Figure 1.5.** Increased PTOA severity in TβRIIOCY-/- mice shows critical role of osteocytes in joint disease. Meniscal/ligamentous injury (MLI) in WT mice results in a PTOA phenotype with loss of proteoglycan staining and subchondral bone sclerosis (Safranin- O/Fast Green stain, B) compared with non- injured controls (A) (scale bar = 20 µm). Defective PLR, induced by osteocyte-specific ablation of TβRII, exacerbates cartilage degeneration and subchondral bone sclerosis in the presence of injury (D) compared with uninjured TβRII $^{OCY-/-}$. Canalicular networks are disrupted in TβRII $^{OCY-/-}$ mice (G, H) compared with WT mice (E, F). Blinded reviewers consistently ranked the joints of the MLI TβRII $^{OCY-/-}$ mice as the most severe PTOA phenotype. This qualitative preliminary data is representative of N=5 mice/group.

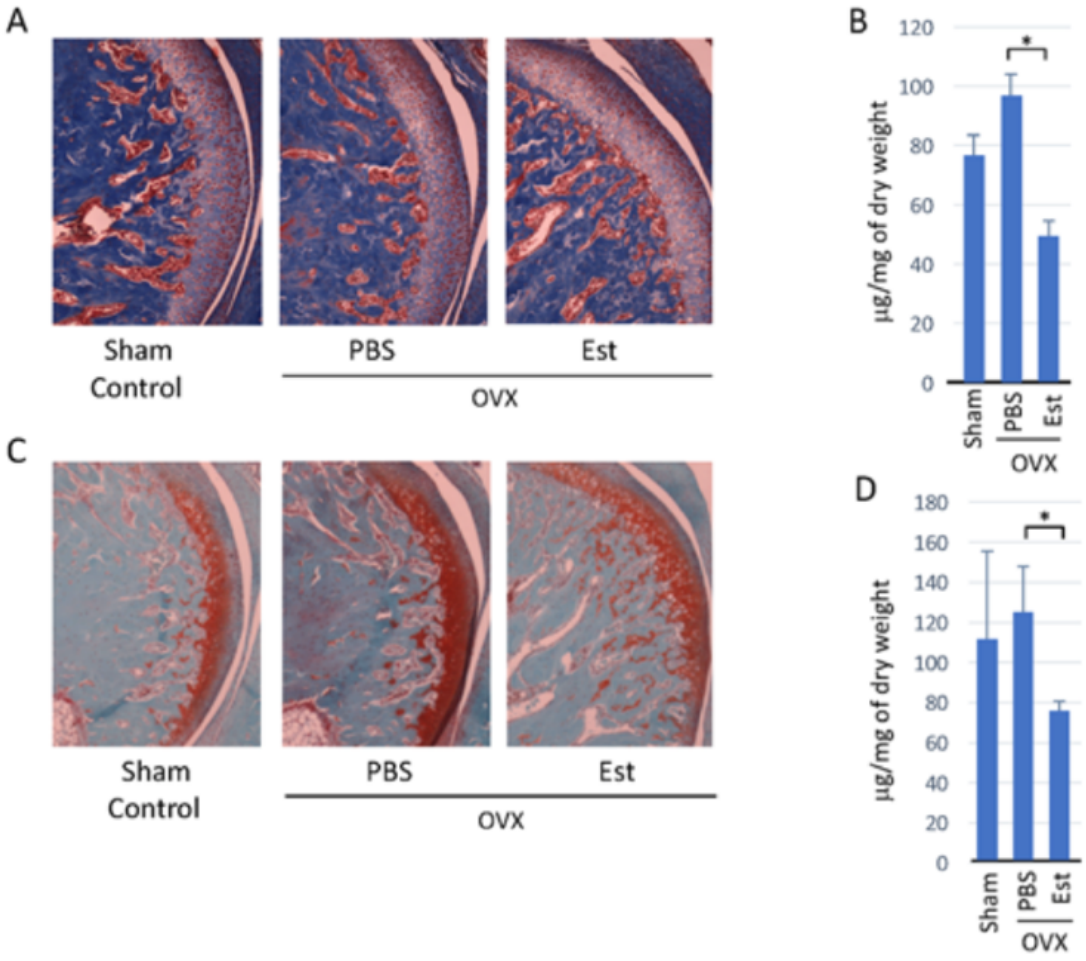



**Figure 1.6.** In Vivo Effects of Estrogen (Est) on Collagen and Glycosaminoglycan (GAG) content of TMJ Fibrocartilage. Ovariectomized (OVX) young female mice were administered PBS or estrogen for seven days and TMJ or fibrocartilage harvested for histochemical staining (A and C) or biochemical assays for collagen (B) or GAG (D), respectively. (A) Masson's trichome staining showing diminished collagen staining (blue) in TMJ fibrocartilage from estrogen treated mice relative to controls, that corresponds with loss of collagen as determined by hydroxyproline assay (B). (C) Safranin-O staining demonstrates decreased staining for GAG (orange) in TMJ fibrocartilage from estrogen treated mice relative to PBS control mice, which is confirmed quantitatively by Alcian Blue assay (D). Sham-operated mice were used as controls ($P < 0.05$).

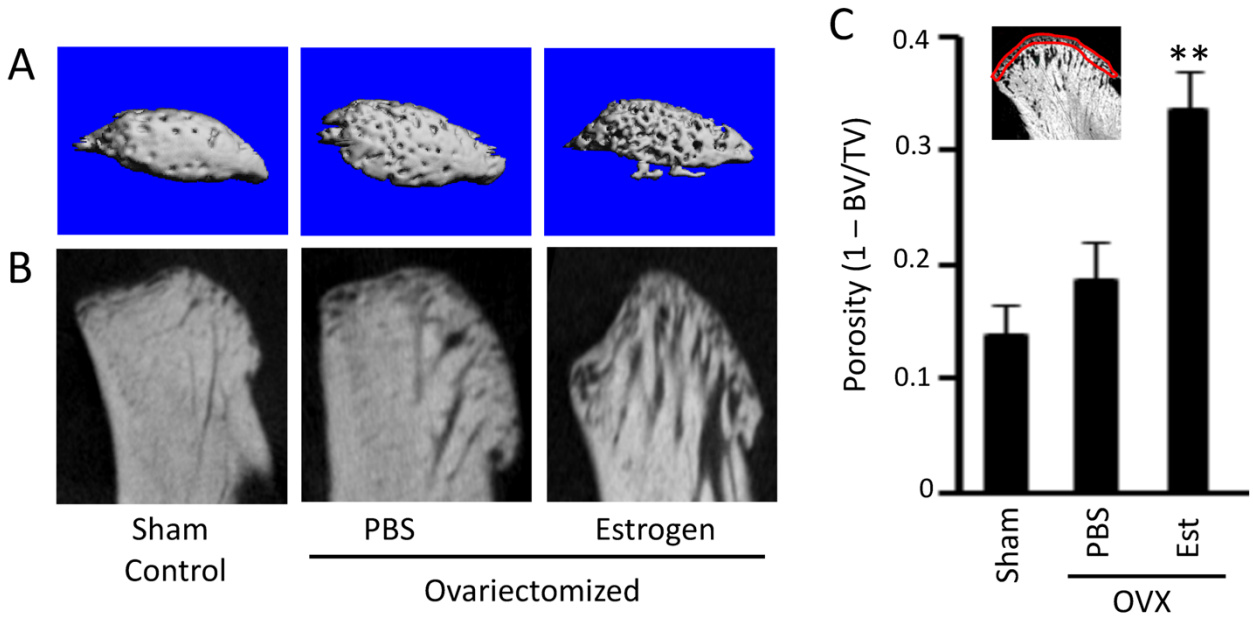



**Figure 1.7.** In Vivo Effects of Estrogen (Est) on Subchondral Bone Porosity**.** Ovariectomized (OVX) young female mice were administered PBS or estrogen and TMJs retrieved for microCT after 28 days. Three-dimensional reconstruction (A) and coronal sections (B) of representative condyles show increased subchondral porosity. (C) This finding was confirmed quantitatively by analyzing the porosity in the subchondral zone (demarcated by the red line in the panel) and found to be statistically significant in mice administered estrogen relative to sham and PBS control mice (**P < 0.01).

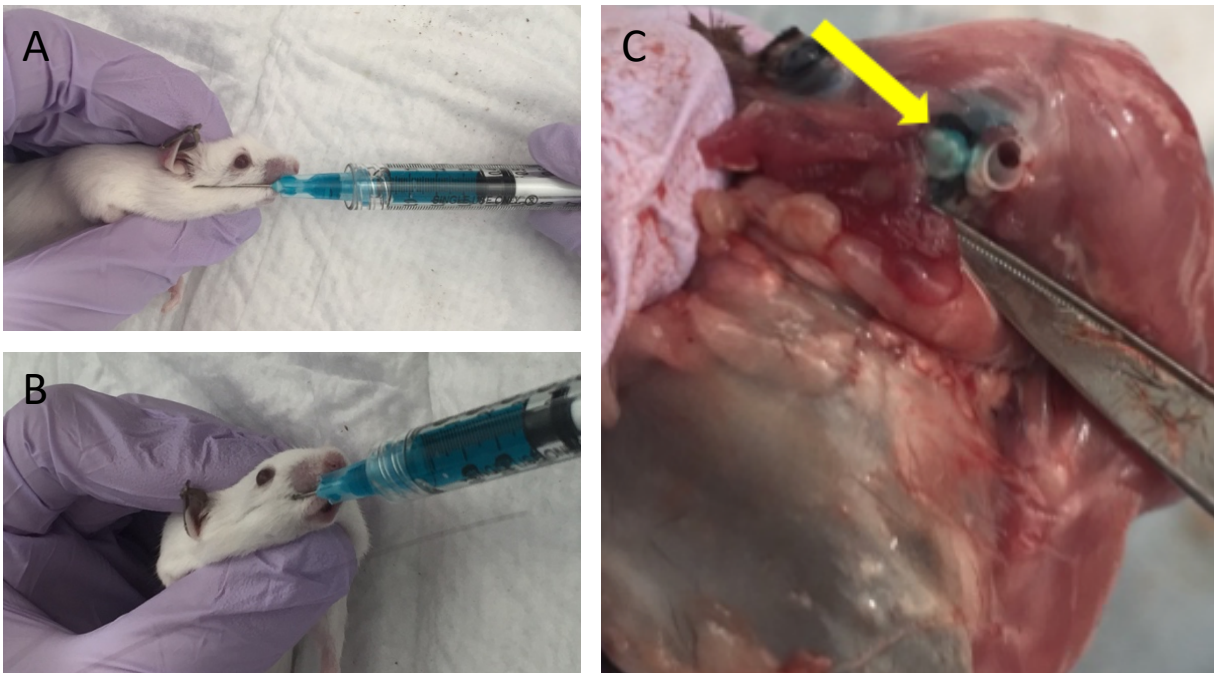



**Figure 1.8**. Optimizing TMJ Injection Protocol. Characterizing and verifying an optimal method for intra-articular TMJ injection in the mouse. Lateral (A) and anterior (B) views showing direction of needle insertion. (C) Dissected mouse head showing Fast Green staining of condylar head and TMJ fossa (arrow).

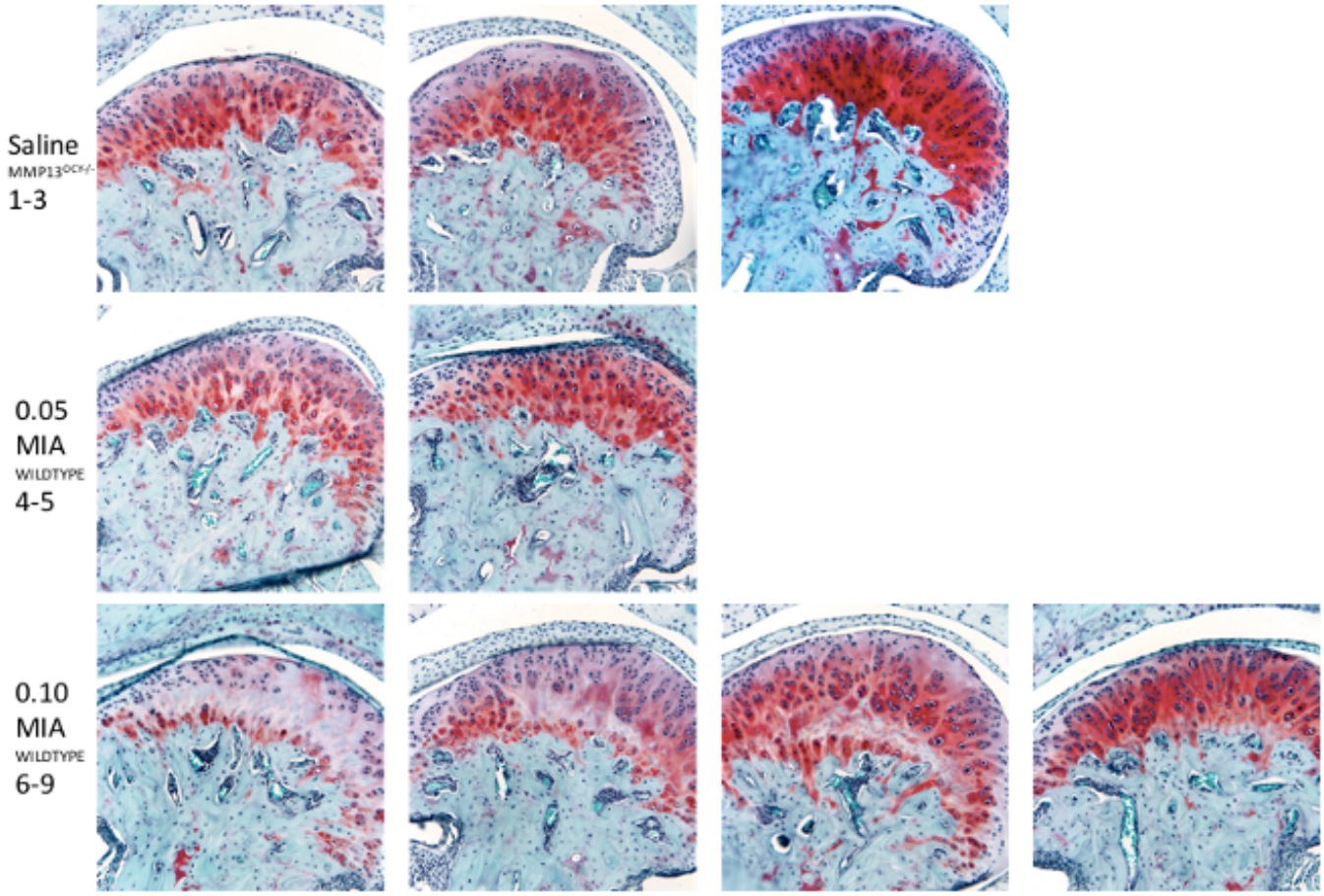



**Figure 1.9.** Coronal sections of TMJs from control PBS MMP13OCY$^{-/-}$ vs 0.05 mg MIA WT and 0.10 mg MIA WT mice at 28 dpi (20x magnification).

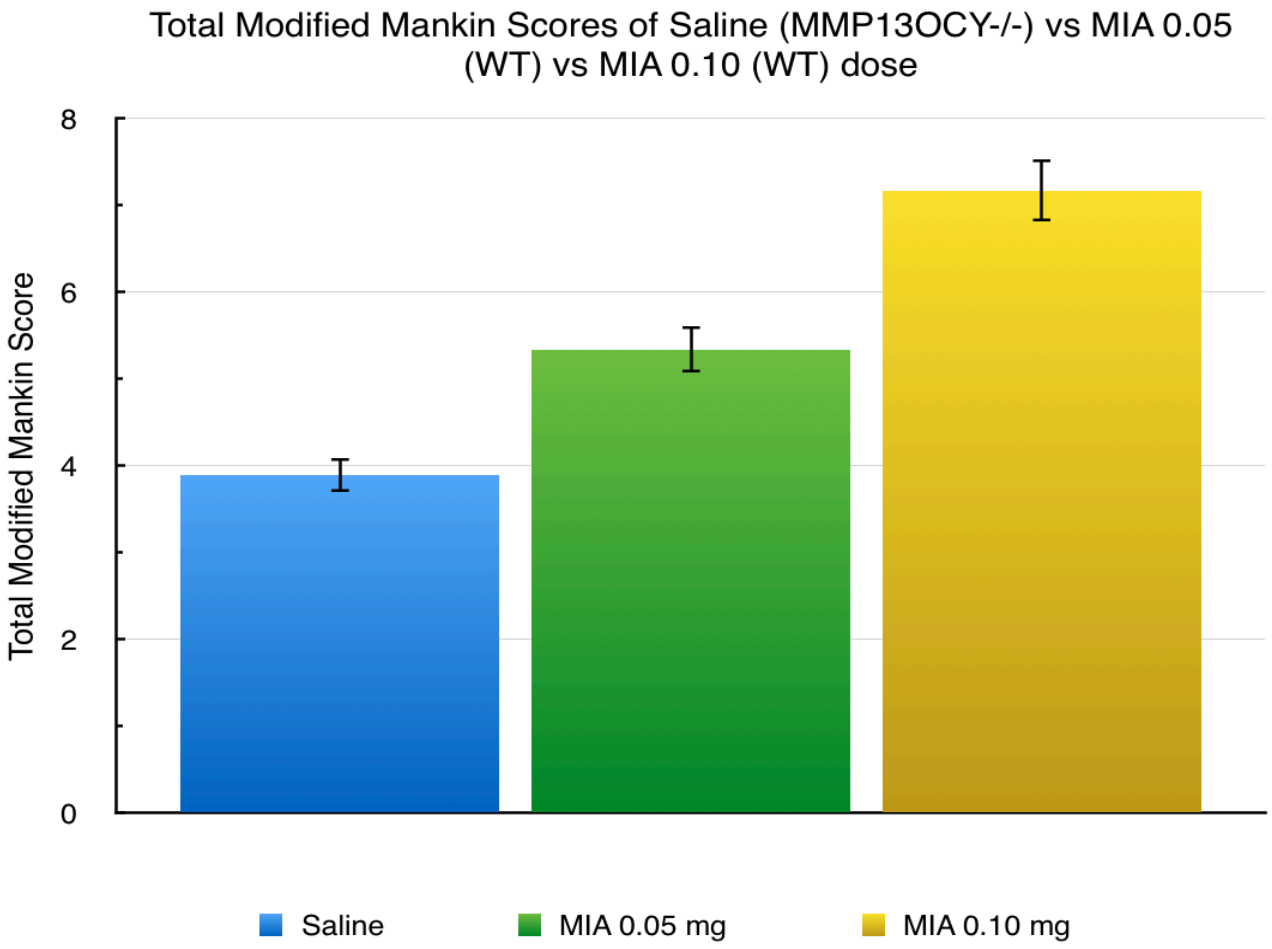



**Figure 1.10.** Modified Mankin Scores of TMJs from PBS control MMP13$^{OCY-/--}$ vs 0.05 mg MIA WT and 0.10 mg MIA WT mice. 0.10 mg MIA demonstrated a significantly higher Modified Mankin Score than the 0.05 mg counterpart.

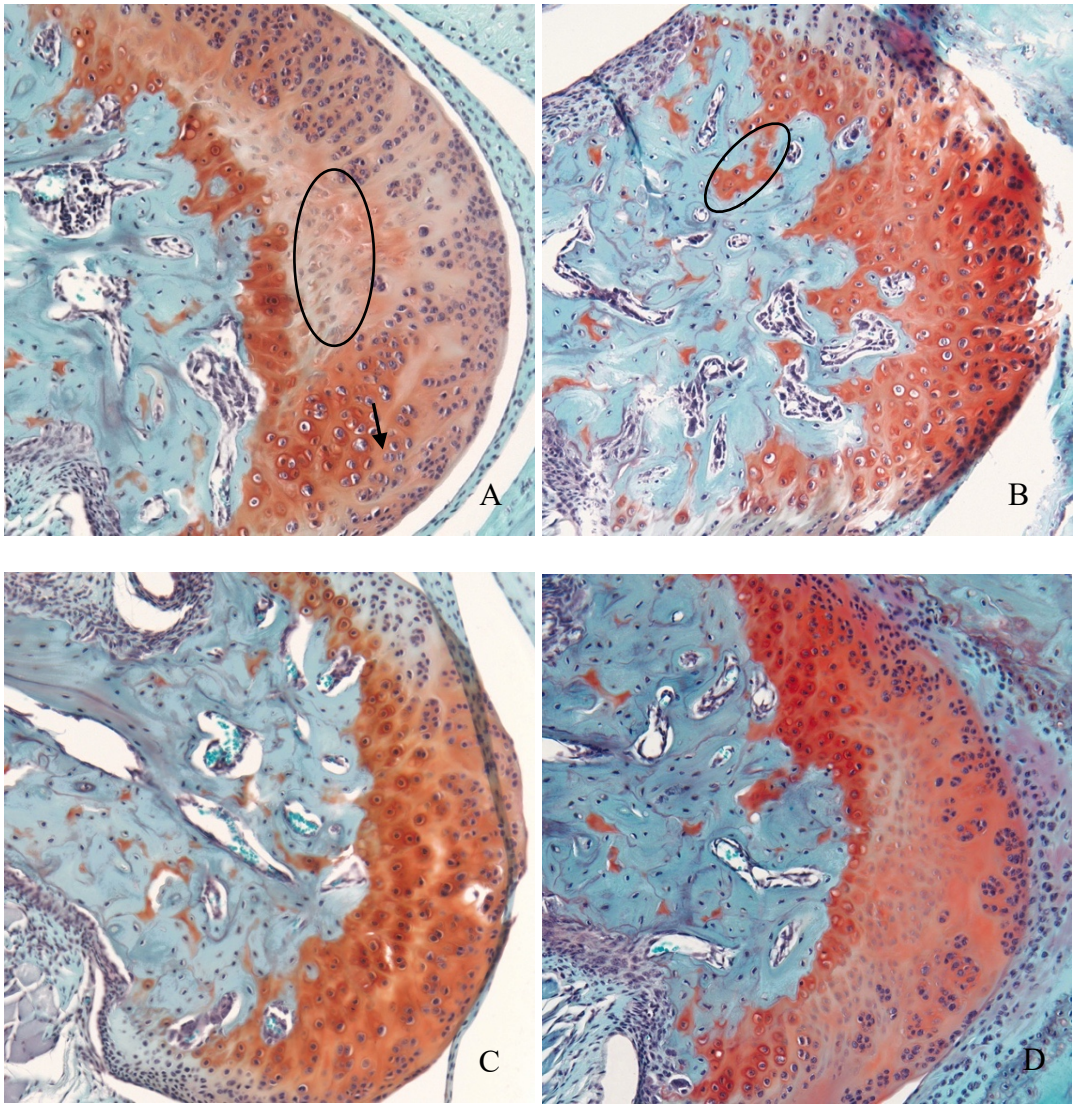



**Figure 2.1.** (A) Sample with a high CPS score and hypocellularity as shown by black arrow and black circle, respectively. (B) A wildtype MIA induced TMJOA with subchondral bone deterioration. (C/D) Sham treated MMP13$^{OCY-/-}$ TMJ compared to a wildtype MIA treated TMJ, which has more evidence of Safranin-O entering the underlying bone.

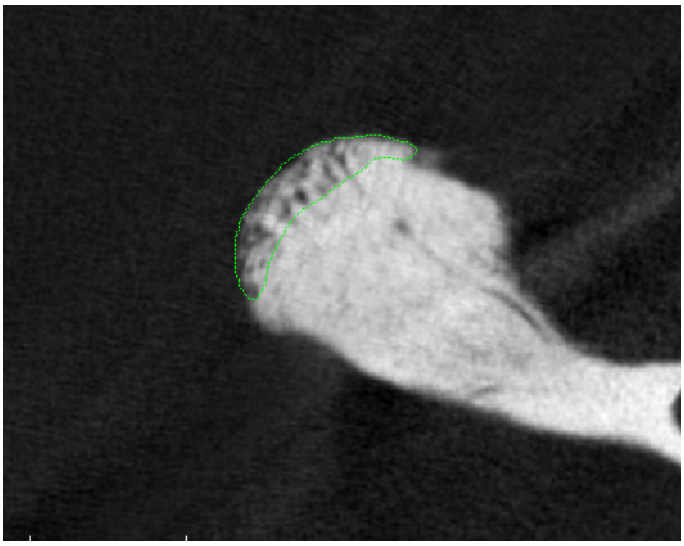


**Figure 2.2.** ROI used for microCT analysis. This is the subchondral bone of the mouse TMJ.

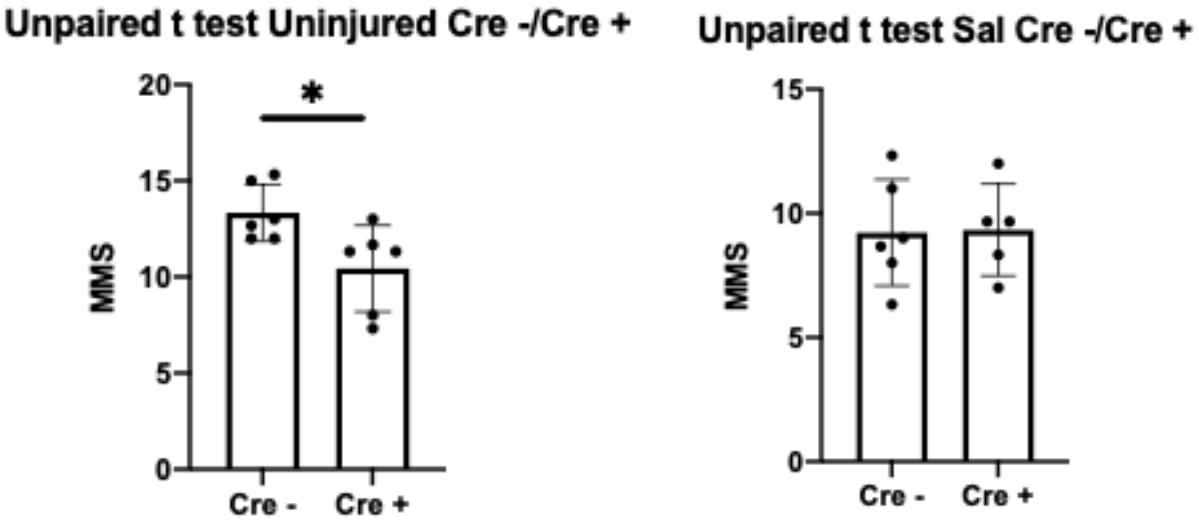



**Figure 2.3.** Unpaired t-tests of Uninjured Cre-/Cre+ (N=6/N=6) displayed on the left and Saline injected (N=5/N=6) displayed on the right. A significant difference is noted between the KO and WT in the uninjured model ($p<0.05$).

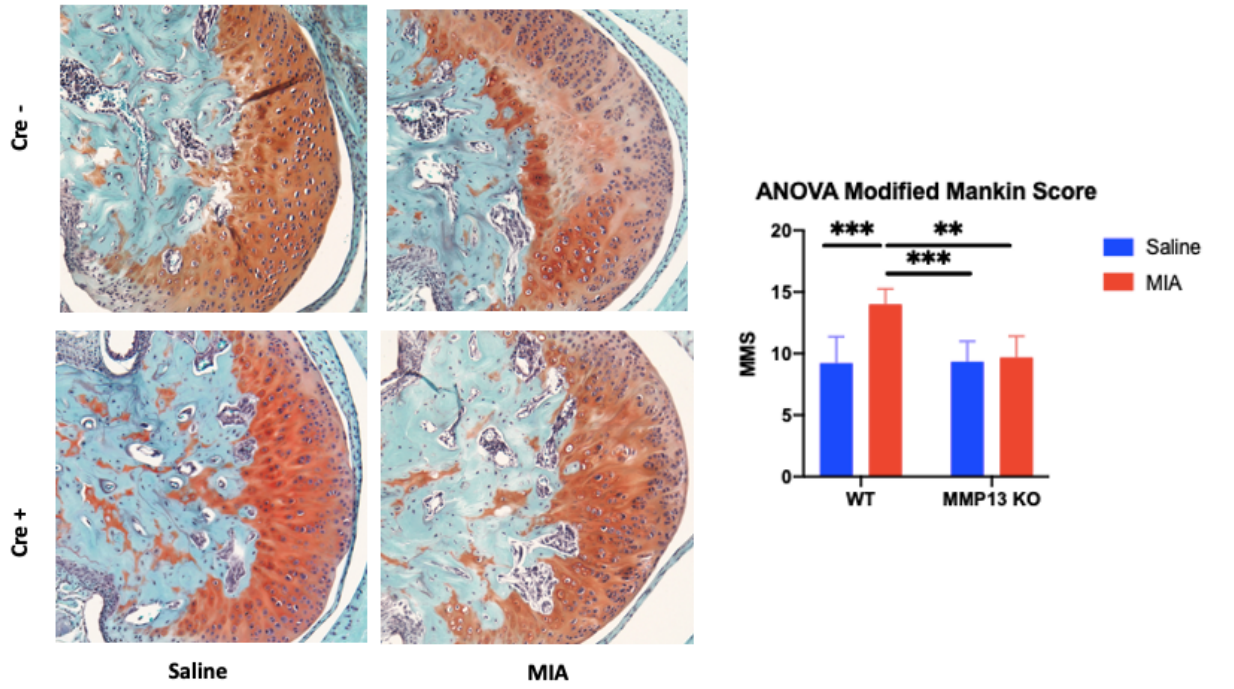



**Figure 2.4.** Representative samples from each group with the complementary statistical analysis. Cre – signifies the WT, Cre + signifies the knockout. MMS is the notation for Modified Mankin Score. A significant increase is noted among the MIA WT compared to all other groups ($p<0.05$).

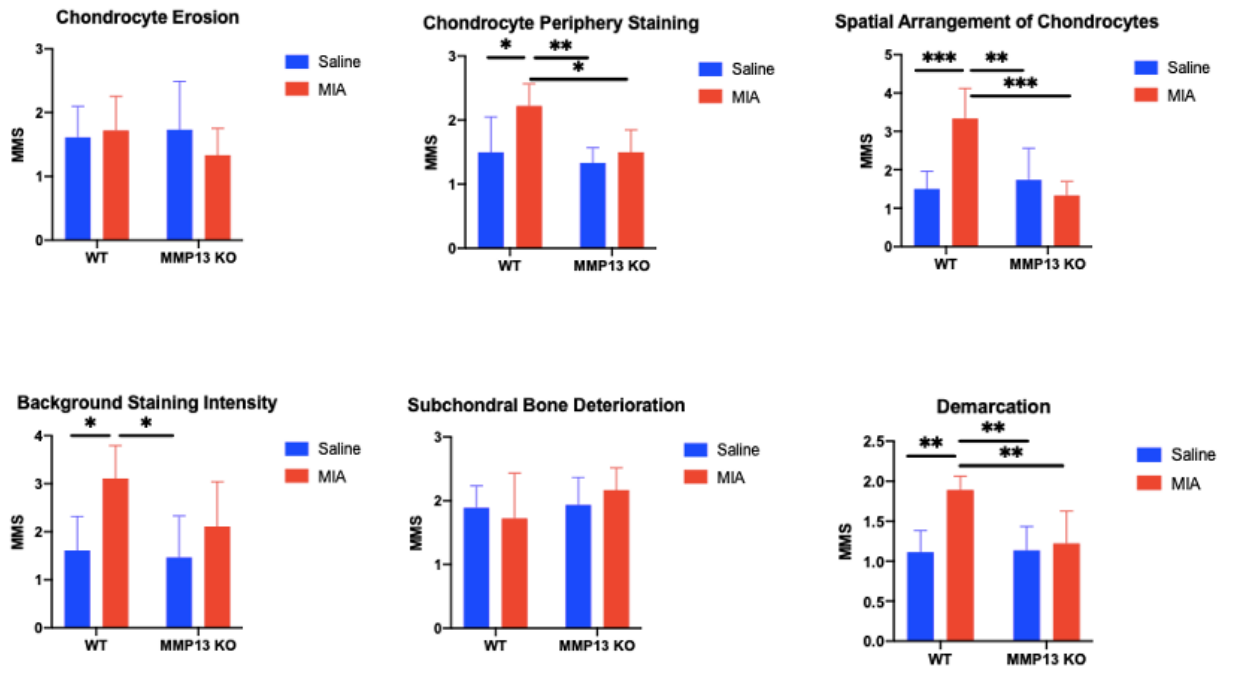



**Figure 2.5.** Individual ANOVA analysis of each subcategory listed in the Modified Mankin Score Table. Significant differences in Modified Mankin Scoring (MMS) were demonstrated among chondrocyte periphery staining, spatial arrangement of chondrocytes, background staining intensity, and demarcation between the cartilage and bone were the primary factors which drove the differences in the overall Mankin Scoring numbers ($p<0.05$).

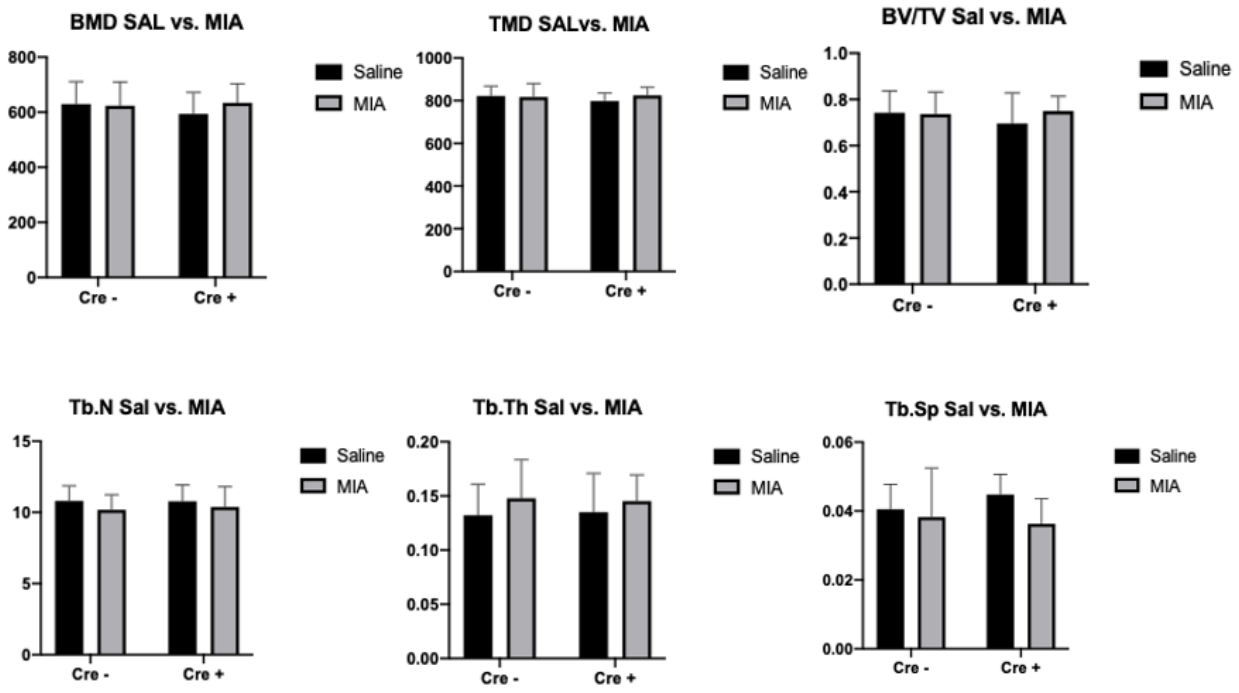
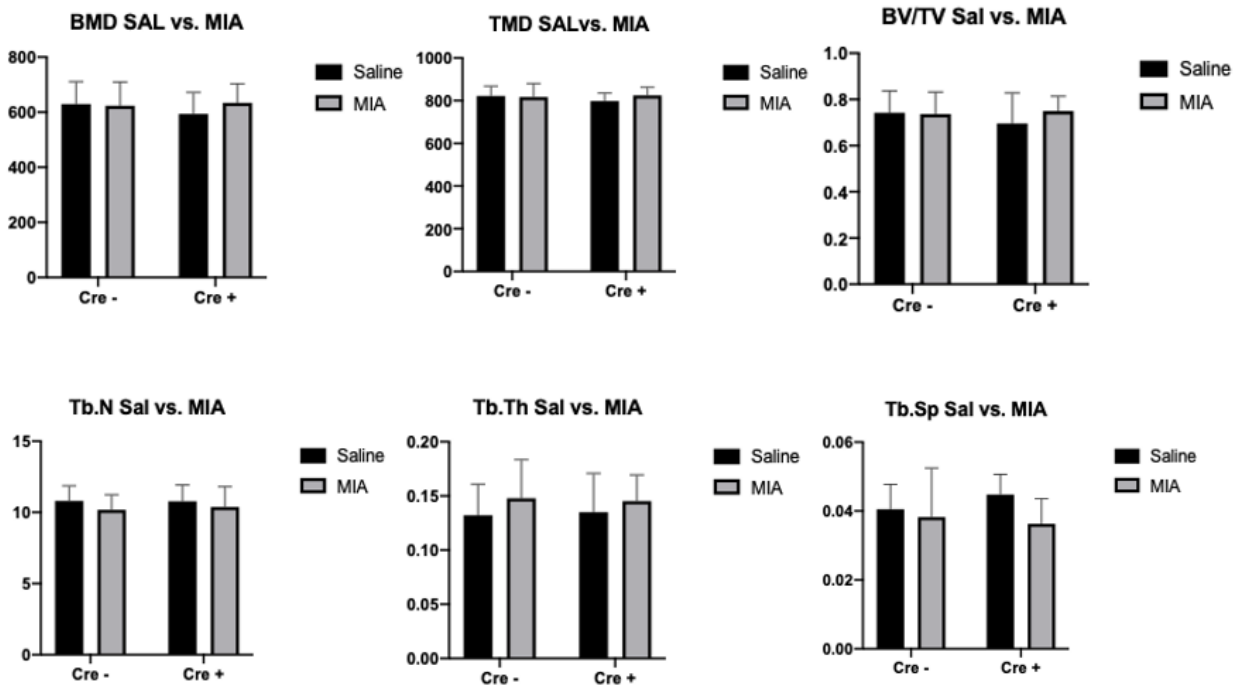
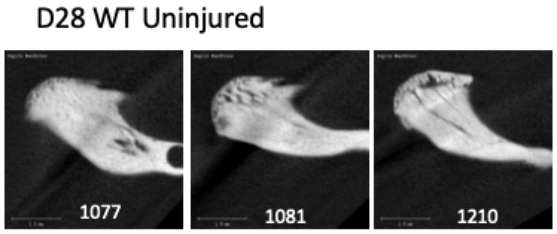
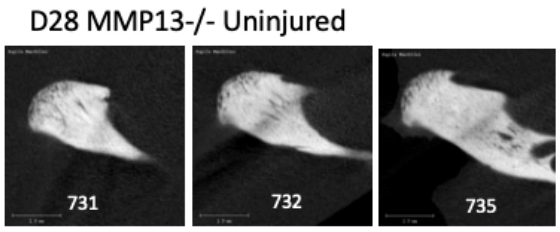
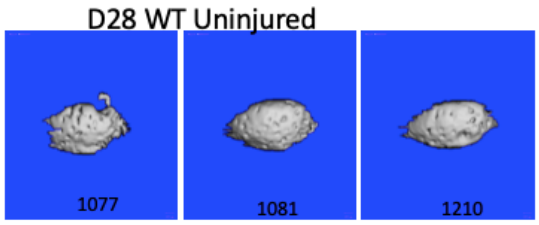


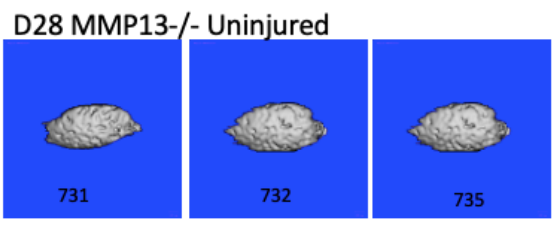

**Figure 2.6.** ANOVA for bone quality and bone quantity in the indicated ROI. No parameters displayed significant differences ($p<0.05$).


BMD SAL vs. MIA
800
600
400
200
0
Cre -
Cre +
Saline
MIA
TMD SALvs. MIA
1000
800
600
400
200
0
Cre -
Cre +
Saline
MIA
BV/TV Sal vs. MIA
1.0
0.8
0.6
0.4
0.2
0.0
Cre -
Cre +
Saline
MIA
Tb.N Sal vs. MIA
15
10
5
0
Cre -
Cre +
Saline
MIA
Tb.Th Sal vs. MIA
0.20
0.15
0.10
0.05
0.00
Cre -
Cre +
Saline
MIA
Tb.Sp Sal vs. MIA
0.06
0.04
0.02
0.00
Cre -
Cre +
Saline
MIA


D28 WT Uninjured
1077
1081
1210


D28 MMP13-/- Uninjured
731
732
735


D28 WT Uninjured
1077
1081
1210


D28 MMP13-/- Uninjured
731
732
735

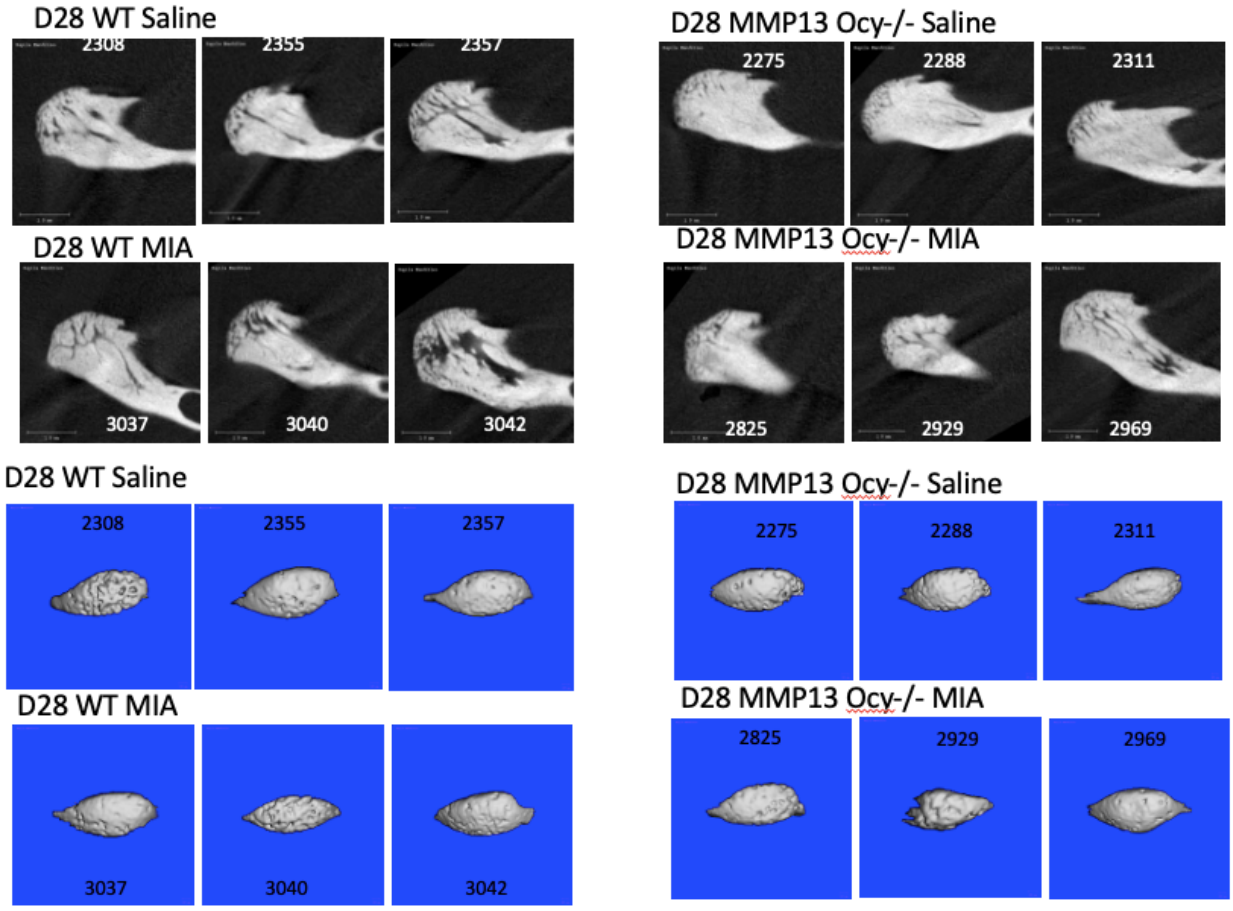



**Figure 2.7.** 3D images of representative samples extracted from microCT evaluated the condylar bone quality and quantity. The numbers on the images reflect three mice per group, WT samples are on the left and knockouts are on the right, which show qualitatively denser bone and less trabecular spaces below the subchondral bone. MIA appears to increase spaces within the condylar bone in WT MIA mice than in sham WT mice

**Table 2.1.** Modified Mankin Score used to quantify severity of TMJ OA as per previous studies.[37, 40]

| | |
|---|---|
| 1. Cartilage Erosion Scoring Criteria | |
| a. Smooth non-eroded cartilage | 1 |
| b. Rough non-eroded cartilage | 2 |
| c. Superficial fibrillation | 3 |
| d. Separation of uncalcified from calcified cartilage | 4 |
| e. Erosion of uncalcified cartilage only | 5 |
| f. Erosion extending into calcified cartilage | 6 |
| g. Erosion down to subchondral bone | 7 |
| 2. Chondrocyte periphery staining | |
| a. Normal | 1 |
| b. Slightly enhanced | 2 |
| c. Intensely enhanced | 3 |
| 3. Spatial arrangement of chondrocytes | |
| a. Normal | 1 |
| b. Diffuse hypercellularity | 2 |
| c. Clustering | 3 |
| d. Hypocellularity | 4 |
| 4. Background staining intensity | |
| a. Normal | 1 |
| b. Slight reduction | 2 |
| c. Moderate reduction | 3 |
| d. Severe reduction | 4 |
| e. No dye noted | 5 |
| 5. Subchondral bone deterioration | |
| a. Normal | 1 |
| b. Local bone defect: single/superficial | 2 |
| c. Local bone defect: multiple/single deep | 3 |
| d. Bone sclerosis | 4 |
| 6. Demarcation | |
| a. No | 1 |
| b. Yes | 2 |

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