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Effects of EGFR and CCR2 Signaling in Post-Traumatic Osteoarthritis

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Abstract

Background: Researchers demonstrated that inhibition of epidermal growth factor receptor (EGFR) and/or its downstream signal transducer, C-C motif chemokine receptor-2 (CCR2) blocked development of post-traumatic Osteoarthritis (PTOA) in rat model if applied from the time of injury. However, it is unclear how effective these drugs are if treatment is initiated after injury, a situation more translatable to the human condition.

Methods: Osteoarthritis (OA) was induced surgically in 4 groups. Treatment was initiated 4-weeks post-surgery where groups were given 50% DMSO solution(vehicle), AG1478(EGFR-blocker), RS504393(CCR2-blocker), and EGFR+CCR2 combinational inhibitor. Rats were sacrificed 7 and 10weeks after surgery. OA was examined histologically by staining the samples with Safranin-O-fast green and hematoxylin & eosin stain for assessing cartilage and synovial damage respectively.

Results: There are no significant differences seen in treated Osteoarthritis Research Society International (OARSI) and synovitis scores vs vehicle group in both timeline rats.

Conclusion: Thus, our results did not support our hypothesis and the described protective effects of these compounds require application at or immediately after injury.

Keywords: Epidermal Growth Factor Receptor, CCR2, CCL2, post-traumatic Osteoarthritis

Summary for Lay Audience

Osteoarthritis is a joint disorder that affects millions of people and is a leading cause of disability in Canada and around the globe. It is accompanied by severe pain in the affected joints such as hip, knee, and hands. Current treatment is limited to the suppression of symptoms or joint replacement surgery as there is no other treatment available to reverse the damage or prevent the further progression of disease. Scientists are making constant efforts to find out what actually is causing damage to the joint. Appleton et.al in 2015 have found out the inhibition of Epidermal Growth Factor Receptor (EGFR) and Chemokine receptor 2 (CCR2), to be beneficial for preventing further damage if treatment is given right after injury. We tested if the same treatment was given 1 month after causing injury to see if that would work for the circumstances that are more comparable to humans. We have given EGFR, CCR2 and combination of these inhibitors separately to rats and compared it with control groups. On comparison, we did not witness any protection against osteoarthritis. Hence, we concluded that inhibition of EGFR, CCR2, or both starting at 4 weeks after surgery does not protect from progression of post-injury OA, in contrast to administration from the time of injury.

Co-Authorship Statement

The animal work was done before the onset of this thesis by Dr. Michael Pest. All the data provided is compiled and interpreted by Jasleen Grewal. Ermina Hadzic and Garth Blacker helped with the scoring methods to analyze cartilage and synovial damage. Dr. Frank Beier assisted in developing this project and helped in formatting this thesis.

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List of Abbreviations

AC	Articular Cartilage
ACLT	Anterior Cruciate Ligament Transection
ADAMTS-5	A Disintegrin and Metalloproteinase with Thrombospondin Motifs
ANK-1	Ankyrin-1
AREG	Amphiregulin
ASK-1	Apoptosis Signal Regulating Kinase-1
BTC	Betacellulin
CCDSS	Canadian Chronic Disease Surveillance System
CJRR	Canadian Joint Replacement Registry
CCL2	C-C motif Chemokine Ligand-2
CCR2	C-C motif Chemokine Receptor-2
DMM	Destabilization of Medial Meniscus
DZ	Deep Zone
ECM	Extracellular Matrix
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor
EPGN	Epigen
EREG	Epiregulin
FMOD-	Fibromodulin

GAG	Glycosaminoglycans
GDP	Guanosine-5’-Diphosphate
GPCR	G-Protein Coupled Receptors
GTP	Guanosine-5’-Triphosphate
GWAS	Genome Wide Association Study
HA	Hyaluronic acid
HBEGF	Heparin-Binding Epidermal Growth Factor-like Growth Factor
IL	Interleukins
PI3K	Phosphoinositide 3-kinase
PKA	Phosphokinase-A
MAPK	Mitogen Activated Protein Kinase
MEK	Mitogen-Activated Extracellular Signal-Regulated Kinase
MFC	Medial Femoral Condyle
MIG-6	Mitogen-Inducible Gene-6
MMP	Matrix Metalloproteinases
MMT	Medial Meniscus Transection
MSC	Mesenchymal Stem Cells
MTP	Medial Tibial Plateau
MZ	Middle Zone
NF-κB	Nuclear Factor kappa B

NK	Natural Killer Cells
NSAIDS	Non-Steroidal Anti-Inflammatory Drugs
PG	Proteoglycan
PI3K	Phosphatidylinositol 3-Kinase
PKA	Phosphokinase A
pMM	Partial Medial Menisectomy
PRG4	Lubricin
PTOA	Post-traumatic Osteoarthritis
OA	Osteoarthritis
OARSI	Osteoarthritis Research Society International
SAR	Structure-activity Relationship
SF	Synovial Fluid
SLRP	Small Leucine Rich Proteoglycan
STZ	Superficial Zone
TACE	Tumor Necrosis Factor α Converting Enzyme
TGF-α	Transforming Growth Factor-α
TNF-α	Tumor Necrosis Factor-α
VCAM-1	Vascular Cell-adhesion Molecule-1

1. INTRODUCTION

Chapter 1

1 Osteoarthritis

Osteoarthritis (OA) is a progressive degenerative joint disease that affects nearly five hundred million people around the world. OA is not only the leading reason for disability in the Canadian population but also causes increased economic burden and decreased quality of life.[1] According to The Canadian Chronic Disease Surveillance System(CCDSS), the occurrence of this disease increases with increasing age and is 5% higher in females as compared to males.[1] Canadian Joint Replacement Registry (CJRR) mentioned in their annual report that more than 110,000 joint replacement surgeries were performed in the year 2020-2021 and the approximate cost of these surgeries was somewhere around $12,223 each, which indicates that more than $1.3 billion was spent on hospital cost in just one year.[2]

1.1 Risk Factors and Pathophysiology

OA is characterized by severe pain in different joints of body but mostly in hip, knee, hands, and backbone.[3] Risk factors for the disease include age, injury, sex hormones, genetics and obesity indicating OA to be a multi-factorial disease.[3]

Irregularity, surface fibrillation and development of focal erosions on articular cartilage are some of the earliest changes that occur in OA (Figure 1.1). [4][5]These focal erosions usually extend into the bone and continually enlarge to involve more surface area of the joint which leads to the formation of large bony lumps also termed as osteophytes.[4][5] After injury to cartilage, extracellular matrix is affected which is chiefly composed of type II collagen and proteoglycans.[6] Under usual conditions, this matrix undergoes dynamic remodeling where catabolic and anabolic processes are in balance to maintain a healthy articular cartilage.[6] Anabolic processes include formation of chondrocytes-specialized cells responsible for the production of collagen and extracellular matrix (ECM) while, catabolic processes include degradation of aggrecan, collage type-II, and other matrix proteins.[3][5] However, over-expression of matrix-degrading enzymes

disrupts this balance which correlates with phenotypic changes in the chondrocytes. In advanced diseases, subchondral bone thickening occurs which eventually causes the collapse of articular cartilage into the bone along with some degree of synovial damage and mesenchymal changes in the bone marrow.[3][4][5]

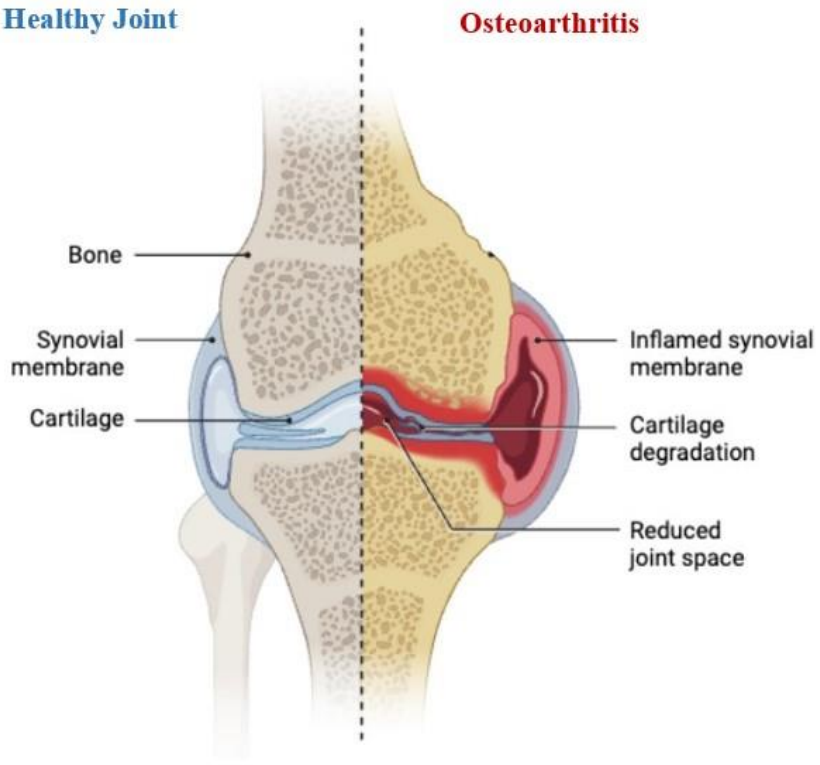


Figure 1.1: Healthy knee joint vs Osteoarthritis affected knee joint. Left side of image represents structure of healthy, non-affected knee joint while right side of image is a representative of a knee joint affected by Osteoarthritis showcasing degenerative changes in the articular cartilage, reduced joint space, inflammation in synovial membrane. Image is created using template from BioRender.com

1.2 Classification of Osteoarthritis

OA can be classified into primary and secondary categories.[5] Primary OA is idiopathic in nature can be further categorized based on the affected region such as hand, hip, knee OA.[5] Secondary OA, on the other hand, occurs due to some underlying condition such as congenital disorders like hip dysplasia or injury.[4][5] OA can also be categorized based on the risk factors present, such as sex-related, injury-related OA, age-related OA, OA due to some genetic predisposition. [7] Age related changes occurring inside the joint including cell and matrix changes, increased stiffness of ligament and tendons, degeneration of meniscus as well as outside the joint such as sarcopenia and repeated wear and tear predispose the older population to the risk of developing OA.[7] According to statistics provided by WHO in the year 2023, more than 73% of the population suffering from this OA are older than 55 years of age.[8] Injury-related or post-traumatic OA (PTOA) on the other hand, develops after acute joint trauma and instability in the joint.[10] Most common form of injuries of PTOA patients includes fractures. In a study conducted on 104 PTOA patients, it was found that 30% of people had knee fractures while 11% were with ligament injuries and 1% with patellar dislocation.[10] In contrast to injury and age-related OA which arise due to changes in the microenvironment of the joint, obesity related OA was believed to occur mainly as a result of increased forces on the weight bearing joints and altered biomechanics but some non-weight bearing joints such as hands are also found to be affected with obesity-related OA, which suggests that some metabolic factors playing a role, but these pathways are still unresolved.[11] Regardless of the type, people experience same pain index in PTOA as in primary OA and the disease looks similar at the histology level, at least at the end stage.[9][10][11]

1.3 Treatment of Osteoarthritis

Researchers have found that losing weight and mild exercise can help to maintain healthy joint and can be used as a preventative measure.[12] Even for people living with OA, milder forms of exercise are found to be useful in alleviating the symptoms of OA as it helps to keep the surrounding tissue around the affected joint, intact and healthy.[12]

Current management strategies are mainly conservative in nature and limited to end stage surgical treatment and suppression of the symptoms using drugs including non-steroidal anti-inflammatory drugs (NSAIDS).[4][5][12] At present times, no other pharmaceutical intervention is available for preventing the further progression of disease or to restore the damaged articular cartilage, as the underlying molecular mechanism causing OA is very complex and still unknown.

1.4 Joint at Cellular and Molecular Level

1.4.1 Cartilage

Cartilage is a form of connective tissue that primarily provide smooth lubricated surfaces for better articulation along with providing resilience and flexibility to bony areas.[13] Cartilage is avascular in and receives its nutritional supply from the surrounding tissues such as the synovium.[13] Due to the lack of blood supply in the cartilage, its intrinsic capacity to heal is relatively low and it takes a long time to heal once injured.[14] It also lacks neuronal supply, and thus, there is no sensation of pain when its damaged.[14] It is composed of 10% cells (chondrocytes) and 90% matrix which includes water, proteoglycans and collagen.[13]

1.4.1.1 Chondrocytes

Chondrocytes are highly specialized and are the only type of cells present in articular cartilage (AC).[15] Their chief function is to maintain joint homeostasis within the articular cartilage joint by secreting collagen and other ECM, thus, forming the cartilaginous tissue.[15] Apart from being the key component of AC, chondrocytes also play role in endochondral ossification.[15][16] Histologically, they are clearly visible when GAGs are stained with Safranin O Fast Green dye. Chondrocytes in mature articular cartilage remain quiescent and no longer undergo proliferation.[15][16] Since, chondrocytes play chief role in maintaining joint homeostasis, any disruption of balance from external stimuli such as hormones or cytokines can have the destructive effects on

articular cartilage and may lead to the development of osteoarthritis.[16][17] Loss of chondrocytes in the articular cartilage is considered as the main parameter according to the guidelines recommended by Osteoarthritis Research Society International (OARSI) while scoring for cartilage damage in osteoarthritis.[15]

1.4.1.2 Collagen

Collagen is the chief protein present in the body and forms two-third of the dry weight of articular cartilage.[18] Around 28- 29 collagens have been identified belonging to the class of glycoproteins and the most abundant types are collagen in articular cartilage are type II, IX, XI.[19] Collagen type II is found to be the main component of articular cartilage and is constitutes about 95% of the total collagen in this tissue. It is a fibrillar collagen which provide three-dimensional architecture to tissues.[19][20] It assembles with collagen type IX and forms covalent cross-links with type XI and engages with small leucine-rich proteoglycans (SLRPs).[20] Any mutation in *COL2A1*, the gene encoding type-II collagen, can lead to the development of pre-mature OA.[21] The main function of collagen is to provide strength and integrity to the articular cartilage.[19] The enzymes responsible for the cleavage of collagen are known as collagenases and belong to the family of metalloproteinase enzymes including matrix metallopeptidase-3 (MMP-13).[18][19]

1.4.1.3 Proteoglycans

Proteoglycans (PGs) such as aggrecan, decorin, and fibromodulin are macromolecules that are found in the ECM and have complex structures composed of core protein to which glycosaminoglycan (GAGs) chains are attached.[22] Chondroitin sulfate, and keratan sulfate are attached to the core protein to form the proteoglycan monomer while hyaluronic acid forms the backbone and can associate around 80 PG units to form larger aggregates.[23]

Aggrecan is most frequently found in the articular cartilage and has a molecular weight of more than 2500 kDa.[23] This core protein has more than 100 GAG chains attached to it.[23] Aggrecan also plays key roles in chondrocyte-chondrocyte and chondrocyte-matrix interactions because of its capacity to associate with hyaluronan link-protein.[24]

Contrary, decorin is smaller in size (90-140 kDa) and produced by fibroblast cells, smooth muscle cells, and endothelial cells.[25] Fibromodulin (FMOD) is also smaller in size and a leucine-rich proteoglycan (SLRP) that binds to collagen type-1 and prevents cell adhesion.[26] The proteoglycan function is based on the physicochemical properties of GAG molecules, to attract water and provide hydration and swelling to withstand the compressional forces and act as a cushion to absorb shock.[23] Anchoring cells to the ECM, providing structural integrity, and lubricating joint are other important functions served by PGs.[24][25]

1.4.1.4 Types of Cartilage

Based on the cell organization, relative amount of macromolecules and tissue function, cartilage is categorized as i) fibrocartilage ii) articular cartilage iii) elastic cartilage and iv) epiphyseal plate cartilage.[27] Fibrocartilage is present in the meniscus of the knee joint, intervertebral disks and at the insertions of ligament and tendons.[27] Elastic cartilage tends to provide elasticity to the tissues such as the pinna of the ear, larynx, and eustachian tube.[27] Epiphyseal plate cartilage mediates the longitudinal growth of long bones. [27] When the bone growth is complete, epiphyseal cartilage gets replaced by bone. Out of all 4 cartilages, articular cartilage is majorly involved in the etiology and pathophysiology of osteoarthritis.[28]

1.5 Articular Cartilage

In adults, composition of articular cartilage includes a low number of chondrocytes (less than 5% in volume), high content of water (60-85%), 10-20% collagen and 4-7% proteoglycans that impart unique tensile properties to articular cartilage.[29] Articular cartilage is divided into 4 zones- superficial, middle, deep and calcified zone.[29] The composition of ECM and the pattern of distribution of chondrocytes are different for each zone.(Figure 1.2) [33] The superficial zone (SZ), chondrocytes are sparsely distributed, they are relatively higher in number and more flattened in shape as compared to the chondrocytes in middle (MZ) which are more spherical in shape, while the chondrocytes in the deep zone (DZ) and calcified zone are stacked as pile of coins.[28][30] Similarly,

the pattern of distribution of collagen fibers differs in these zonal areas.[28][29][30] The greatest resistance to compression comes from the deep zone, where collagen fibrils lie perpendicular to the articular surface.[28][30]Also, the calcified layer plays a crucial role of anchoring collagen fibers from deep zone to subchondral bone while the collagen fibers in the middle and superficial zone are more obliquely arranged.[32] The loosely arranged collagen fibrils in a felt-like pattern impart articular cartilage to withstand high shear and compressive forces in the weight-bearing joints.[30][31]

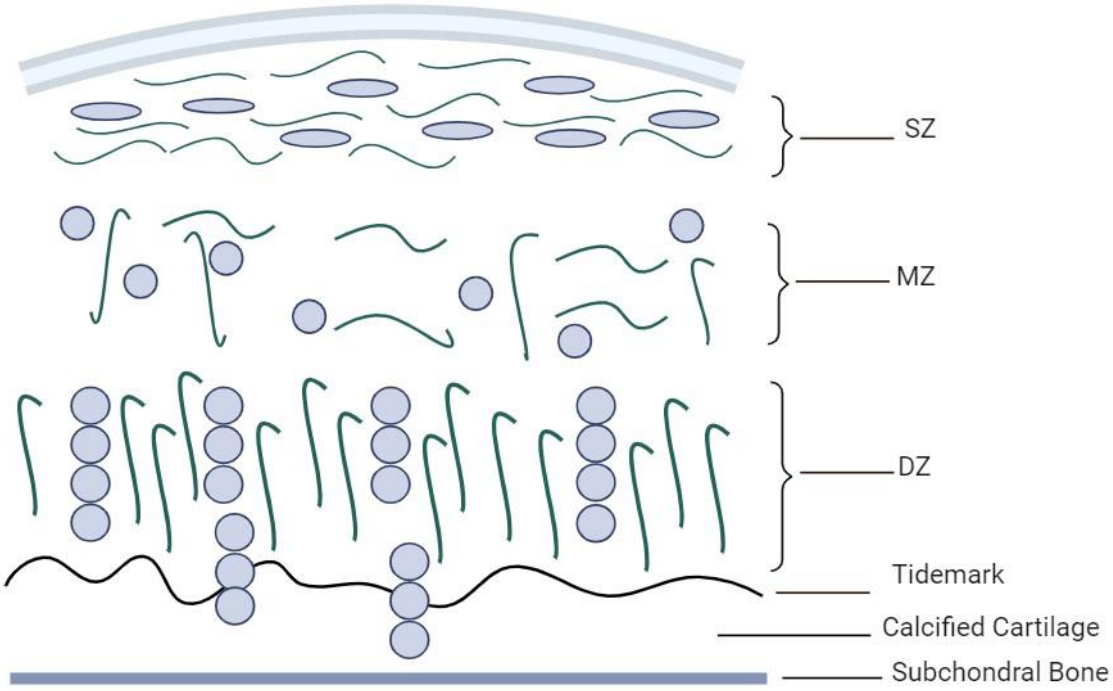


Figure 1.2 Different zones of Articular Cartilage. SZ- Superficial zone where chondrocytes are flattened in shape; MZ- middle zone; DZ- deep zone. The image was created with the help of BioRender.com.

1.5.1 Articular Cartilage and Osteoarthritis

OA develops when chondrocytes fail to keep the balance between the catabolic and anabolic processes occurring in the joint.[30] Catabolic processes can be the release of degradation enzymes such as metalloproteinases including collagenase and aggrecanase while anabolic processes include the synthesis of proteoglycans and collagen type-II. In a healthy joint, both degradation and repair of cartilage are balanced processes, which means that rate of degradation and rate of repair are similar.[32] However, it is not yet known what actually triggers this imbalance occurring in OA. Increasing age, trauma and obesity are the main risk factors for causing OA.[32][33]

After trauma, inflammation in the joint leads to secretion of pro-inflammatory cytokines that attract the macrophages and monocytes at the site. [33] In addition, chondrocytes are stimulated to release degrading enzymes which leads to the breakdown of collagen and proteoglycans (Figure 1.3).[34] This further attracts more macrophages and some inflammatory cytokines such as TNF-alpha, EGFR ligands, chemokines such as CCL2, some interleukins (ILs).[35] These cytokines then binds to receptors on chondrocytes and block the synthesis of collagen and proteoglycans and increase the release of metalloproteinases such as MMP-13, MMP-3.[34][35] As a result, there is sharp decline in the collagen and proteoglycans in the cartilage which weakens the cartilage and leads to death of chondrocytes. Ultimately, all these events contribute to the development of this degenerative joint disorder.[34][35]

Changes that occur in the articular cartilage as a result of OA includes fibrillation and fragmentation of articular cartilage extending into subchondral bone, initial increase proliferative activity of chondrocytes, decreased cartilage thickness or even complete loss, formation of osteophytes in an attempt to repair tissue, increased water content along with depletion of PGs and collagen, increased concentrations of fibronectin, loss of tensile strength with increased permeability and ultimate death of chondrocytes.[35][36]

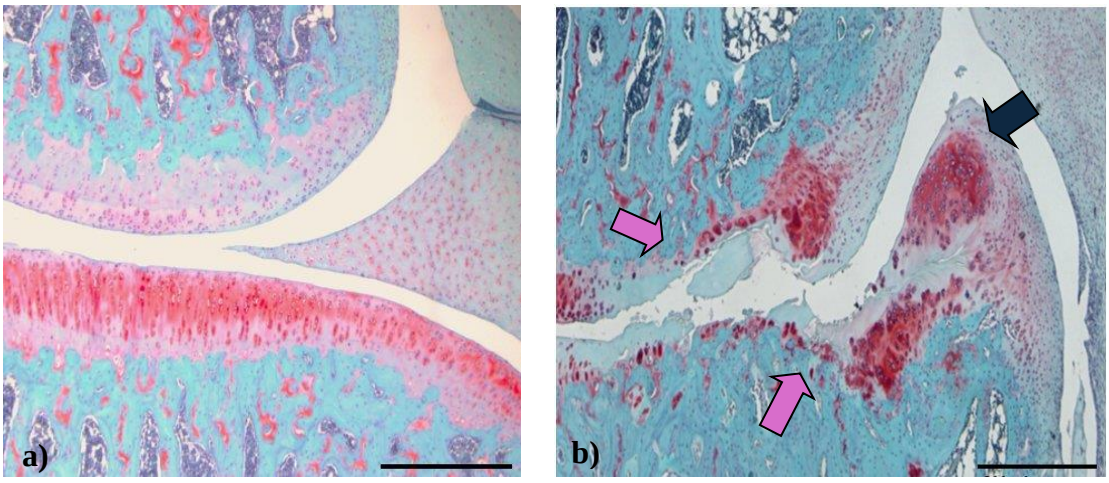


Figure 1.3 Articular Cartilage with Safranin O Fast Green Stain in a) Healthy & b) OA Rat Knee Joint. Purple arrows indicate loss of proteoglycans with chondrocyte cell death that causes advanced form of OA. Blue arrow marked the presence of big osteophyte. Scale bar 200 µm

1.6 Histological Evaluation of Cartilage Damage

1.6.1 Background

Earlier systems to grade cartilage damage in OA developed by Collins and Mankin poses various challenges that question the consistency and reliability on these systems for grading disease histologically.[37][38][39] Collins developed a system emphasized on the macroscopic features of OA pathology and labeled the changes as ‘lesser’ and ‘more advanced’ form of OA. For grading, he gave score I to IV based on morphological changes seen after autopsy such as bony changes, lesion size and texture of cartilage surface.[40] The Mankins system on the other hand is more based on the microscopic analysis of decalcified sections using Safranin O staining.[37] He graded the extent of severity of disease from 1 to 14 based on cellular changes. However, both these systems were formed on the basis of samples from very advanced form of osteoarthritis and thus, questions the validity and reliability on these systems for scoring mild or earlier phases of disease.[37][40]

1.6.2 OARSI

In order to standardize histopathological assessment of OA, a more novel and reliable, OARSI scoring system was developed based on histological features of articular cartilage during progression of osteoarthritis.[41] This system was developed with an intent to standardize the radiologic, clinical and arthroscopic evaluation of OA.[42] It is developed keeping in mind the five principles for an ideal histopathological assessment of disease which are as follows:

i. Simplicity: recommendations are simple and easy such that it can be used by all analyst having different levels of histological experiences.[41][42]
ii. Utility: They can be equitably used for experimental OA models and clinical assessment of disease. Along with the morphological changes, system should also be able to define the changes occurring at molecular level during OA progression [42]
iii. Scalability: They are scalable that it should provide clear association between the morphological and histological changes
iv. Extendibility: system should be capable of extending to more detailed grading or staging of disease
v. Comparability: system should be comparable to other diseases associated with cartilage [42]

One particular feature of OARSI over other scoring systems is its ability to identify the differences within early OA.[41][42] OARSI guidelines put great emphasis on the extent of cartilage damage over the articular surface through a ‘stage’ component which is based on the extent of area, surface, and volume of articular cartilage engaged in OA, while the grade component is based on the extent of pathology into the depth of cartilage (such as grading 0 to 5 for subchondral bone damage with 0 being no damage while higher score indicates more aggressive OA progression).[41][43] OARSI has recommended few parameters in order to assess the OA, out of which three main parameters are-

1.6.2.1 Cartilage degeneration:

One of the primary parameters to score the damage in articular cartilage based on the percentage of area affected by the disease. Loss of chondrocytes is the primary determinant of this score. Loss of proteoglycans without chondrocyte cell death has been excluded from areas of cartilage where chondrocytes are intactly present.[41] In rats, for scoring cartilage degeneration, the medial tibia plateau (MTP) is divided into 3 equal zones so that pathology of different load-bearing areas can be assessed with zone 1 to be assigned on the side of meniscus. [41] Each zone is graded from 0 to 5 depending upon the percentage of area involved. A score of 0 is given when less than 5% of the area is involved and referred to as no degeneration. Score 1 is specified when 5-10% of total cartilage area is affected by matrix and chondrocyte loss indicating minimal degeneration. Similarly, when 11-25% of affected areas show degeneration, a mild form of 2 is granted. Likewise for 51-75% and more than 75% area affected in degeneration, Scores 4 and 5 are given, respectively, indicating more severe disease.[41] Similarly, the medial femoral condyle is also divided into three equal zones and each zone is graded from out of 5. All three zones are summed by adding the values obtained from each zone and a maximum 3 zone sum is considered to be 15 for both MTP and MFC which would be in the case where entire tibial or femoral cartilage is lost.

1.6.2.2 Osteophyte Measure:

Osteophytes are the bony lumps (‘bony spur’) on the margins of joints affected by OA. Osteophyte score is based on the size of osteophyte (Table 1.1). If more than one osteophyte is present, then the largest osteophyte is measured from edge to base at the thickest point.[41][43]

Table 1.1: Osteophyte Score based on Size (in mm) as recommended by OARSI Guidelines

Score 0	marginal changes but size is <200 µm
Score 1	smaller in size (200-299 µm)
Score 2	moderate in size (300-399 µm)
Score 3	large in size (400-499 µm)
Score 4	very large in size (>500 µm)

1.6.2.3 Subchondral Bone Damage:

This parameter assesses the changes in subchondral bone and mineralized cartilage associated with OA such as increased basophilia at the tidemark, mesenchymal changes in marrow, and cartilage collapse into the bone.[41] Score 0 is assigned when there are no changes in the subchondral bone. Score 1 is given where there is slightly increased basophilia at the tide mark. Score 2 is assigned when there is minimal fragmentation in mineralized cartilage, and $1/4^{th}$ of subchondral bone region has undergone mesenchymal changes in bone marrow in addition to increased basophilia at the tide mark. Similarly, for score 3, mesenchymal changes involved in more than 75% region of subchondral bone but the articular cartilage has not collapsed into the bone. Score 4 is given if in addition to above marrow changes, articular cartilage has collapsed into bone into a depth of 250 µm. Likewise, if articular cartilage has collapsed into bone into a depth of more 250 µm, score 5 is given.

1.7 Synovium

Synovium is vascularized connective tissue that lines the inner surface of diarthrodial joints, tendon sheaths, fat pads and bursae.[44] Along with bone, articular cartilage, ligaments and tendons, a healthy synovium forms the integrated joint structure.[44] Synovium has many important functions which include the production of lubricin and hyaluronic acid, providing required nutrients to chondrocytes and playing sentinel role in immune system defense (responsible for detecting infections in the joint). Thus, the synovium plays a key role in maintaining joint health.[45] The cells of synovium are termed synoviocytes.[46] Unlike articular cartilage, synovium is abundant in blood supply and is a highly vascularized structure.[46] The fluid present in the synovial cavity is called synovial fluid and serves important functions like providing lubrication to the joint and preventing friction during articulation.[45][46] It is made from the ultrafine filtrate of human blood plasma and about 2ml of this ultrafine blood plasma is present in healthy joint.[46] Synovial fluid contains essential components such as HA, lubricin, prostaglandins, collagenases, other proteases etc.[44][45]

Furthermore, synovium consists of two chief subtypes of synoviocytes, namely type-A and type-B cells.[46] Type-A cells are macrophage-like cells derived from hematopoietic cell-lineage that have a key role in removing waste and debris through the process of phagocytosis and located on the inner surface of joint.[45][46] Type-B cells are fibroblast-like cells that in involved in the synthesis and release of synovial fluid and hyaluronic acid, lubricin, and major components of ECM.[46][47]

The synovium is composed of two layers. The outer layer is just one cell thick and is called the intimal layer having both type-A and type-B cells. The layer beneath the intima is the sub-intimal layer which is composed of multiple layers of connective tissue and is abundant in type-1 collagen and blood supply, fat cells and fibroblasts.[44][45][46]

1.7.1 Histology of Healthy Synovium

On the basis of structure and cellular composition of the sub-intima seen through histological studies, synovium is divided into 3 types- areolar, fibrous, and adipose.[47] Of all three types, areolar synovium is the most specialized type that appears like folds or ridges when stained with hematoxylin & eosin (Figure 1.4).[48] These folds often vanish when stretched. Just below the cells of sub-intima (2-3 layers thick), lies the network of capillaries, blood vessels, lymphatic vessels, and nerve innervation. In contrast, in adipose synovium, the intimal layer is positioned directly on the cells of adipose tissue.[48] However, between intima and adipocytes, substratum is present that is rich on collagen.[49] Fibrous synovium, on the other hand, is very hard to define as it resembles fibrocartilage and is often seen in middle layers of ligament and tendons.[49] All three types contain matrix/substratum abundant in type-1 collagen. However, fibrous synovium has dense collagen network while areolar synovium shows loose collagenous-type arrangement.[48][49]

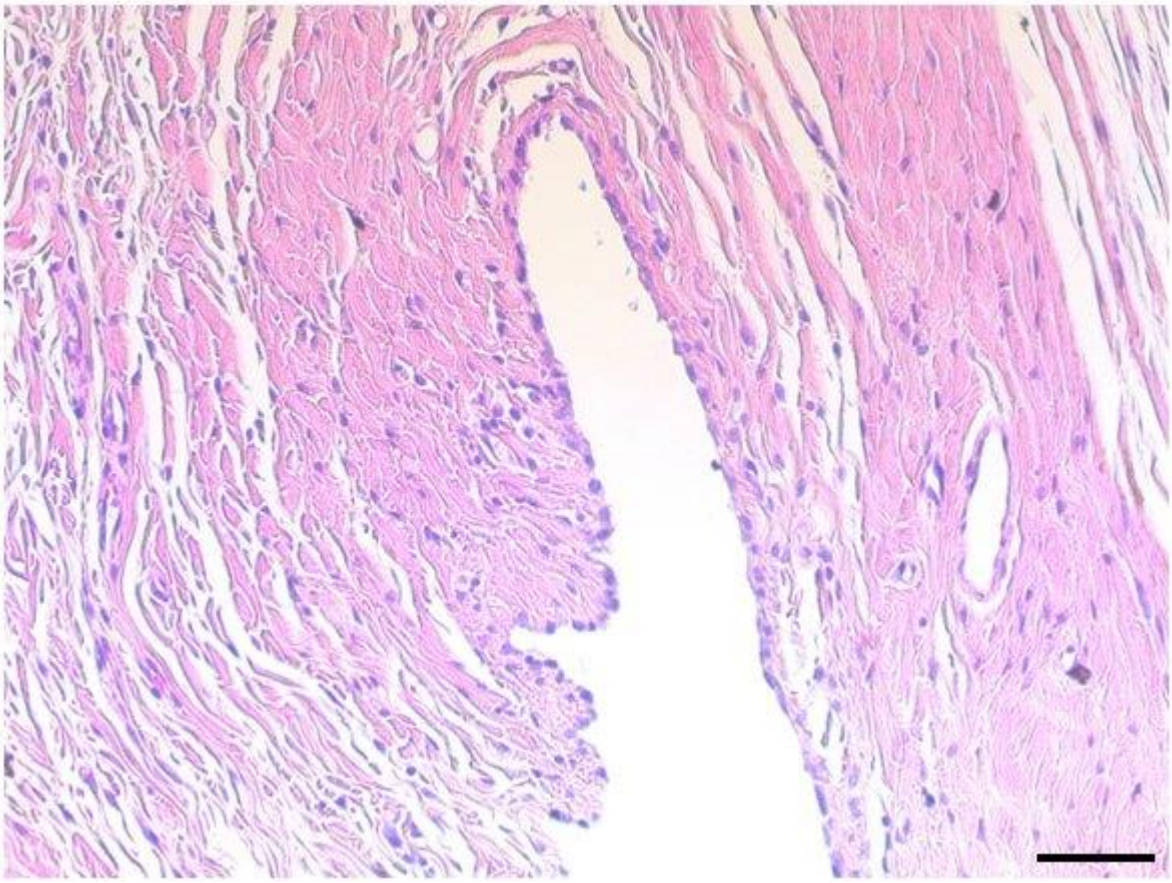


Figure 1.4 Healthy synovium stained with Hematoxylin & Eosin. Yellow arrows represent single-celled intimal layer in superior medial compartment in 7-week-old healthy naïve rat group. Scale bar- 100µm.

1.7.2 Synovial Inflammation

Some cytokines such as interleukin-1 (IL-1), IL-6, and TNF-alpha are usually detected in the healthy synovium, but the amount of these inflammatory cytokines is far less than the levels seen in diseases like osteoarthritis.[50] Synovial inflammation can occur in response to degrading cartilage fragments and matrix which further causes the activation of certain transcription factors including nuclear factor kappa B (NF-κB).[51] This leads to release of pro-inflammatory cytokines and chemokines CCL2, TNF-alpha, many inter-leukins including IL-1 and IL-6, and increased expression of cell-adhesion molecules

such as vascular cell adhesion molecule-1 (VCAM-1). As a result, there is increased influx of monocytes and leukocyte from the surrounding blood stream.[52] Certain anti-inflammatory cytokines are also produced at the inflammation site but pro-inflammatory cytokines usually utweigh the role of anti-inflammatory cytokines in OA.[53] Increased cellular stress also induces the activation of macrophages which further favors catabolic processes including the release of MMP-3 and other metalloproteases. All this suggests that the innate immunity plays a key role in aggravating synovial inflammation.[53]

Histologically, synovial inflammation can be characterized by the thickening of synovial cell-lining aka synovial hyperplasia (Figure 3.12), increased infiltration of monocytes and leukocytes (Figure 3.14), stromal vascularization (Figure 3.18), presence of fibrin (in response to the influx of macrophages and T-cells) (Figure 3.16), formation of fibrotic tissue (Figure 3.20) in more severe grade of inflammation, and perivascular edema.[54]

1.8 Role of Metalloproteinases in OA

Matrix metalloproteases are potent matrix degrading enzymes which belong to the class of zinc-based endopeptidases that are catabolic in nature. In articular cartilage, a total 7 types of MMPs are found to be expressed depending on the circumstances.[55][56] MMP-1, also called interstitial collagenase, has the capacity to degrade collagen type-I, II, and III. Although it is not seen to be expressed under normal circumstances, levels are up-regulated in response to conditions such as OA. [55] MMP-2 is known to be active during wound repair and has the capacity to cause the break-down of collagen type-IV.[57] MMP-3 which is also known as stromelysin-1, is causing degradation of extracellular matrix including proteoglycans, collagen type-II,III,IX and X along with the deterioration of fibronectin and elastin.[58] It also has the capacity to up-regulate the expression of other metalloproteases suggesting that it has some transcription factor like activity.[58] High MMP-3 expression is seen in the regions involving inflammation such as in synovitis, osteoarthritis etc.[57][58] MMP-8 on the other hand plays a principle role in tissue remodeling, and thus, might have a beneficial effect on the cartilage rather than causing harm even in conditions like osteoarthritis.[59] Indeed, absence of MMP-8 does

have deterioratory effects on the joint health.[59] MMP-13 is the most extensively studied metalloprotease by researchers because it targets type-II collagen which is the principle form of collagen present in the articular cartilage. Also, MMP-13 is found to be over-expressed under inflammatory situations.[60] It also cleaves other matrix proteins such as aggrecan, osteonectin, percelan, type-IX collagen etc. Researchers have found so many genes and signaling pathways that are regulating the expression of MMP-13.[60] MMP-9 plays a role during embryonic development and MMP-14 can cleave cadherin and aggrecan.[55]

However, expression of MMPs alone does not explain OA development as other factors contribute in the pathology of the disease too.[55][56] A balance of all these MMPs and anabolic processes is required for maintaining healthy joint physiology and any disruption to this balance may lead to development of chronic diseases including osteoarthritis.

1.8.1 ADAMTS-5

A disintegrin and metalloproteinase with thrombospondin motifs-5 (ADAMTS-5) is an aggrecan-degrading enzyme that is encoded by *Adamts-5* gene.[61] It belongs to the family of zinc metallo-endoproteases and is actively involved in the pathogenesis of OA.[61] It is also found to be present in low levels in heart, lungs, tendons and ligaments.[62] Direct inhibition of ADAMTS-5 has not found to be beneficial as it over-activates the anabolic events and leads to the accumulation of proteoglycans in major organs such as heart and disrupts the aortic wall framework leading to valve disease.[62][63]

1.9 TGF-alpha

Transforming growth factor-alpha is an important growth factor that is encoded by *TGFA* gene and is known to be involved in cell-proliferation.[64] The effects of TGF-alpha are carried out through epidermal growth factor receptor (EGFR) signaling pathway where it

binds to EGF-receptor either as soluble ligand or as transmembrane bound-ligand.[65] Thus, its physiological functions of are similar to EGF and initiate events like cell-proliferation and differentiation of chondrocytes.[64][66] It is abundantly expressed in cells such as macrophages, cells of gastric mucosa, keratinocytes, brain cells etc. and known to be activated by TNF-alpha converting enzyme (TACE), and metalloproteinase-17.[67][68]

1.9.1 Role of TGF-alpha in OA

Appleton, T. et.al (2007) conducted thorough research on the role of TGF-alpha in osteoarthritis and he demonstrated through microarray analysis of mRNA of synovial samples from OA affected population, that TGF-alpha levels were up-regulated in a subset population. Further, in-vitro studies on TGF-alpha confirmed the its role in disrupting the cytoskeleton of chondrocytes leading to cell-death.[69] It was also found out that TGF-alpha is promoting catabolic activities such as increased expression of MMP-13, and cathepsin-C, and down-regulates anabolic activities such as expression and activity of Sox-9, a transcription factor essential for chondrogenesis.[69][70] TGF-alpha is also seen to be up-regulating the expressions of chemokines such as CCL2, and certain metalloproteases including MMP-13.[69] Another study conducted by Usmani et.al demonstrated significantly lower levels of cartilage damage in young TGF-alpha knockout mice using destabilization of medial meniscus (DMM) surgery.[71] However, this protection was not seen in older mice. Also, young TGF-alpha null mice were not protected from spontaneous OA during suggesting that TGF-alpha is imparting context-specific protection.[71]

1.10 Examination of Relevant Literature

1.10.1 EGFR

Epidermal growth factor receptor is a type of transmembrane glycoprotein that belongs to the protein kinase superfamily (erbB receptor family) and has strong binding affinity towards epidermal growth factor family ligands.[72] There are four subtypes of erbBs that are structurally closer to each other which includes erbB-1, erbB-2, erbB-3 and erbB-4.[72] Out of these four, EGFR belongs to erbB-1 subtype receptors. erbB is named after viral oncogene (erythroblastic leukemia viral oncogene) which is homologous to these receptor types.[73] Stanley Cohen received a Nobel Prize in Medicine for the discovery of epidermal growth factor and its receptors.[74] On activation of EGFR by its ligand, it undergoes structural transition to form a homodimer. There is some evidence that suggests that EGFR may remain inactivated even in homodimer form and couple with other receptors from the erbB family to form an activated heterodimer.[73][75] This dimerization further causes intracellular protein-tyrosine kinase activity which results in the autophosphorylation process of tyrosine residues on the C-terminal domain of the receptor and activates downstream signaling.[75] This downstream signaling further initiates a series of cascade reactions through ras/raf/mitogen activated kinase (MAPK), phosphatidylinositol-3 kinase (P13K)/akt and Rho-GTPase, JAK/STAT pathways leading to cellular responses including DNA synthesis and proliferation.[73][74][75]

1.10.1.1 EGFR Ligands

Researchers have identified a total of seven ligands that bind to and activate epidermal growth factor receptor signaling.[73] These ligands are synthesized as transmembrane precursors type-1 which are cleaved to form more soluble ligands that stimulates the EGF receptors.[75] Epidermal growth factor (EGF), transforming growth factor-α (TGF- α), amphiregulin(AREG), epiregulin(EREG), betacellulin (BTC), heparin-bining epidermal growth factor-like growth factor (HBEGF), epigen (EPGN) are the seven ligands that bind and activate EGF receptors.[75][76] Out of these seven ligands, 3 ligands (HBEGF, BTC, EREG) are also known to bind to other subtype ebrB-4 family members while the 4

bind solely to EGFR receptors.[76] No ligand is known to bind to erbB-2 and erbB-3 subtype receptors.[75] The 4 ligands that solely bind and activate EGFR do not have same biological activities but so far there is no explanation for the differences in downstream signaling that change the biological responses. An attempt was made by Jennifer L. Macdonald-Obermann et.al (2014) to identify these differences in biological outcomes and their results established a relationship between the formation of homodimers and heterodimers by different ligands on activation of EGFR.[77] EGF and TGF- α forms more heterodimers (EGFR:ERBB-2) than to form EGFR:EGFR homodimers while AREG and BTC forms homodimers equally for both types. They concluded that EGFR ligands induce different receptor dimerization patterns, which is possible since EGFR can cross-induce other types which makes the study of these receptors and ligands even more complex and difficult.[75][76][77]

1.10.1.2 Role of EGFR on Articular Cartilage

Studies have suggested that EGFR is expressed in the superficial layer of articular cartilage along with its ligands.[78] TGF-alpha and HBEGF are two EGFR ligands that are most abundantly present in the human articular cartilage with OA.[79] Cartilage specific (Col2/Cre) or skeletal specific (Prx1/Cre) deletion of Mig-6, an endogenous inhibitor of EGFR, have caused increased chondrocyte proliferation and thickened articular cartilage.[80][81] M. Pest also showed that cartilage specific knockout of mitogen-inducible gene-6 (Mig-6) mice have resulted in formation of chondro-osseous nodules and increased thickness of articular cartilage in knee, ankle and elbow.[82] This suggests that EGFR is important for promoting proliferation and survival of chondrocytes.[82] Moreover, Fang et.al have demonstrated EGFR activation promotes lubrication by increasing Prg-4(lubricin) and hyaluronic acid (HA) (tested on bovine cartilage explant cultures), which is important for joint health and provide protection to the superficial zone of articular cartilage and confers protection against any 87insult during initial stages of OA progression.[83] Contrary to this, EGFR signaling also has catabolic action on chondrocytes as EGFR increases the expression of MMPs, particularly, MMP-9, MMP-13, and MMP-14 that play active roles in ECM degradation

in cartilage.[84] It is also found that EGFR is important for RANKL (receptor activator of nuclear factor kappa-B ligand) expression, thus, promoting osteoclastogenesis. In addition, it also inhibits the expression of SOX-9 transcription factor and suppresses the synthesis of cartilage matrix proteins, collagen-II and aggrecan.[85] Thus, it can be concluded that EGFR signaling pathway can either provide cartilage protection or promote cartilage degradation, thus, can have both protective and deleterious effects on articular cartilage depending on the context.

1.10.1.3 Role of EGFR in OA

So far, we know that TGF-alpha and HB-EGF are two ligands that are expressed more abundantly than any other ligands during OA initiation and progression.[75][86]In addition to this, Castano-Betancourt MC et.al 2016 conducted genome-wide association study (GWAS) for articular cartilage thickness and have revealed that TGF-alpha is amongst the genes associated with the articular cartilage thickness in hip region in human OA.[87] Infact, in post-traumatic OA models, mice undergoing anterior-cruciate ligament surgery (ACL) or destabilization of medial meniscus (DMM), have found to have elevated levels of TGF-alpha.[86] Since TGF-alpha and HB-EGF bind to and initiate EGFR signaling, it suggests that EGFR and its ligand are involved in the pathogenesis of osteoarthritis.[88] Also, the fact that EGFR is expressed in superficial layer of cartilage and plays a context specific role in articular cartilage biology, points towards the involvement of EGFR signaling in osteoarthritis.[78] However, the results of EGFR inhibition on animal models are contradicting and further support a context-specific role of this pathway.[69[83][86]

1.10.2 Chemokines

Chemokine (conserved-cysteine motif) ligand 2 belongs to the group of chemo-attractant cytokines that modulate the biology of monocytes and macrophages.[89] Broadly chemotactic cytokines are heparin-binding, small protein structures formed from 60-100

amino acids.[89] Gene that mainly encodes for C-C motif chemokines are residing on chromosome number 17 in humans.[90] Chemokines are released in response to signals from pro-inflammatory cytokines and start recruiting monocytes, neutrophils and macrophages to the site of inflammation. The C-C structure is formed of 3 domains i) N-terminal cysteine ii) loop that leads to β-pleated sheets iii) α-helical structure lies over these sheets.[90][91] Clark-Lewis have disclosed in their structure-activity relationship (SAR) studies that the N-terminal domain of these chemokines is essential for the binding to their receptors.[92] So far, researchers have identified more than 50 chemokines that play role in inflammation or in maintaining homeostasis with some of them having dual activities in both homeostatic and inflammatory role.[89] The inflammatory chemokines performs the function of recruiting monocytes and macrophages to the site of inflammation and injury while homeostatic chemokines involved more in functions like immune surveillance and modulate leukocytes to lymphoid organs and bone marrow to monitor the invasion of pathogens, thus, plays a chief role in maintaining these housekeeping functions.[91] CCL2 is an example that belongs to the class of inflammatory chemokines and bind to its receptor called Chemokine receptor-2 (CCR2). These chemokine receptors are G-protein coupled receptors (GPCR) and give their response through initiating the conversion of guanosine diphosphate (GDP) to guanosine triphosphate (GTP) on Gα subunit of G-protein.[93] This further initiates the intrinsic signaling pathway including adenylyl cyclase (AC), cyclic adenosine monophosphate (cAMP), and protein kinase A (PKA).[93] They can also activate phospholipase C and phosphodiesterase signaling based on ligand-receptor interaction. Studies also confirmed that more than 1 ligand can bind to the same receptor and sometimes the same ligand can bind to different receptors.[94]

1.10.2.1 CCL2

CCL2 (made up of 76 AA and 36kDa in size) is the most potent chemotactic agent that has principle role in inflammation and injury and is released by different cells including monocytes, fibroblasts, microglial cells, epithelial cells, smooth muscles.[91] It can be released constitutively or induced depending upon the cytokines involved, level of stress

and presence of different growth factors.[90][91] CCL2 modulate migration pattern of T-cells, natural killer cells, monocytes and macrophages. Due to the involvement of CCL2 in many diseases including osteoarthritis, rheumatoid arthritis, cancer, insulin-resistance, nephropathy, it is extensively being researched and can be a potential target used as an intervention of various diseases.

1.10.2.2 CCR2

As mentioned earlier, different ligands can bind to the same receptor and same ligand can bind to different receptors.[90] This poses a line of complexity while evaluating the downstream signaling of these CC receptors. CCL2 binds to CCR2 receptor but other chemokine ligands such as CCL-7, CCL-8, CCL-12, CCL-13 and CCL16 are known to bind to CCR2 as well.[94] Out of all these ligands, only CCL-16 falls under the sub-category of homeostatic chemokines, while all other ligands are known to be of inflammatory nature.[89][94] Thus, it is clear that CCR2 has a dual role and can have pro-inflammatory or anti-inflammatory response.[89][94] Apart from this, CCR2 exists in two isomeric forms- CCR2A and CCR2B. Mononuclear cells and smooth muscle cells predominantly expresses CCR2A while CCR2B is seen to be expressed in monocytes, and natural killer cells (NK cells).[95]

1.10.3 Association of CCL2/CCR2 with Pain-Related Behavior

Liang Li, et.al 2015 explored whether serum or synovial fluid (SF) levels of CCL2 have any association with self-reported pain and severity in primary OA patients (n=161) (healthy control=138). They revealed that only SF concentrations have a positive cor-relation with a pain-index (WOMAC pain ratings and total WOMAC score) and affected quality life of OA patients. They also found out that CCL2 increases VCAM-1 expression in human OA.[96]

Ishihara S. et.al 2021 made an attempt to describe the presence of CCR2 in intra-articular sensory neurons and their association with knee hyperalgesia in experimental OA in a

mouse model. Ca2+ imaging showed that CCL2 and CCR2 levels were up regulated in dorsal root ganglia (DRG) and caused knee hyperalgesia in WT naïve mice after DMM surgery but not in CCR2 null mice.[97] They also demonstrated the absence of pain in WT naïve mice after DMM surgery when intra-articular injection of CCR2 receptor antagonist was given but the same effect was absent in vehicle treated mice.[97]

Rachel E. Miller et.al 2016 tested ADAMTS-5 specific monoclonal-antibody in DMM mouse model and their study suggested that CCL2 and CCR2 expression were significantly reduced with the use of anti-ADAMTS-5 antibody along with reversed mechanical allodynia.[98]

They also demonstrated that monocyte chemo-attractant protein (MCP-1 aka CCL2) and CCR2 are central to the development of pain in knee OA.[99]

All these findings suggest a strong relationship between CCL2/CCR2 signaling and OA-associated pain.

1.10.4 Role of CCL2 in Inflammation

It is found that CCL2 regulates the nuclear factor kappa-B(NF-κB) downstream signaling pathway to increase the expression of MMP-3 and ADAMTS-5 and consequently lesions in articular cartilage and subchondral bone and loss of proteoglycans and GAG molecules in the ECM.[100] Wen-Ting Hu has shown the blockade of CCL2/CCR2 expression with the use of NF-κB blocker.[100]

Appleton (2015) has shown an increased *Ccl2* transcription 4 weeks after surgery in PTOA.[101]Similar to this, Yuchen Zhang (2023) in his review article published that post-injury, levels of CCL2 increased and recruited CCR2+ monocytes.[91] Moreover, Harris et.al suggested that CCL2 expression hinders the chondrogenesis in mesenchymal stem cells (MSCs) recruited to OA lesions that otherwise could be regenerative.[102] On the contrary, studies from Jablonski et.al indicating that cartilage regeneration after

injury is inhibited by CCR2 while CCL2 is required for the differentiation of MSCs and have a protective role in cartilage regeneration.[103]

Hsi-Kai Tsou et,al 2012 has successfully demonstrated the up-regulation of CCL2 expression induced by TGF-alpha via ANK-1 (ankyrin-1) signaling pathway.[104] In addition to this, Appleton T. also indicated through immunofluorescence detection of CCL2 protein that CCL2 expression is increased by TGF-alpha which is inhibited by a MEK-inhibitor (mitogen-activated protein kinase inhibitor).[101] He found out that CCL2 is responsible for inducing high *Mmp-3* and *Tnfα* expressions when they expose rat chondrocytes to either CCL2 or TGF-alpha for 48 hours.[101] However, CCL2 or TGF-alpha fail to produce *Tnfα* or *Mmp-3* expression when used in the presence of CCR2 inhibitor (RS504393).[101] Also, L.Longobardi et.al (2017) did a similar study using the DMM model and revealed that CCR2 inhibitor (RS504393) confers protection against cartilage damage in injury-induced OA which suggest that CCL2/CCR2 axis is critical downstream mediator in inflammation involved post-injury and providing enough evidence for targeting CCL2/CCR2 axis as a potential intervention to be used in inflammation and OA.[105] Yet, the contradicting data also suggests that CCL2/CCR2 downstream signaling has a context specific role based on the type of injury, site of inflammation, disease state, microenvironment, type of cells involved and thus requires further investigations.

1.11 Animal Models of OA

The use of animal models in studying OA has been increased in the last decades. Study of naturally occurring OA in humans poses certain challenges such as slow disease progression and that symptoms often appear in the later stages.[106] In order to counter these difficulties, animal models have been recognized and many animal models have been developed since then.[106] Smaller animals offer numerous advantages such as their ease of handling, low cost, shorter life span and faster disease progression. The major disadvantage of animal models is the disparity in the structure of tissue and different

biomechanics of joints, compared to patients. Mice, rats, guinea pigs, rabbits are often used in OA research.[106]

Rats have thicker cartilage (1mm in thickness) as compared to mice, thus offer a wider area, and allow to study the full thickness defects. Thus, rats are often used for investigating the promising targets that can be used to treat OA before trial runs in larger animals such as horses.[41] However, older rats develop more aggressive OA as compared to younger rats post-surgery.[107] It is also seen that older rats who have undergone sham surgery or even control rats develop milder forms of OA changes.[106]

These animals can develop spontaneous OA (naturally occurring) or can be induced using surgical methods or intra-articular injections of certain chemicals (e.g. iodoacetate).[41] The surgical methods are generally used to study post-traumatic OA (PTOA) and can include using destabilization of medial meniscus (DMM), partial (pMM) or complete medial meniscus transection (MMT), anterior cruciate ligament transection (ACLT), or posterior cruciate ligament transection (PCLT).[41][107] Due to the difference in the level of severity of OA lesions, OARSI have also recommended guidelines to choose the right type of surgical model for variable OA studies.[41] For instance, in rats it is recommended to use MMT model in the case of development of potential intervention for symptomatic OA as it was recognized as a joint pain model (reduction in thresholds for paw withdrawal and reduced weight bearing capacity in the surgical leg). It is also recommended to use the combination of pMM and ACLT as ACL transection alone results in less severe form of lesions, small or absence of osteophytes, and mild cartilage damage accompanied by slow progression of disease.[41][107] In contrast, ACLT+pMM provokes cartilage degeneration withing 4-12 weeks post-surgery. The damage in cartilage is seen mostly in 1/3 part of tibia and femur on meniscal side. Osteophytes also grow on the outer edges of MTP and MFC within 4-6 weeks of operation and sclerosis of subchondral bone is also evidently seen in this post-traumatic model.[41][107]

1.12 Rationale

Epidermal Growth Factor Receptor (EGFR) signaling plays an important role in maintaining joint homeostasis through multiple pathways. It plays dual action on articular cartilage: i) It plays an anabolic action by stimulating chondrocyte proliferation and survival while promoting lubrication which is important for healthy joint mobility. ii) EGFR has catabolic action by suppressing the expression of S0X-9 which is a key transcription factor for the synthesis of collagen II, aggrecan and matrix proteins.[83] TGF-alpha, a ligand for EGFR is dysregulated in OA and suppresses the chondrocyte phenotype and induces expression of the chemokine CCL2 that further attracts macrophages and causes damage to articular cartilage [101]. Harris et.al demonstrated that CCL2 inhibits chondrogenesis of mesenchymal stem cells that might otherwise be regenerative.[102]

As of now, we know that EGFR plays both catabolic and anabolic role in joint homeostasis. Appleton et.al 2015 have successfully demonstrated that inhibition of EGFR and/or the receptor for CCL2, CCR2, blocked development of post-traumatic OA in rat model if applied from the time of injury.[101]. However, it is not known how effective these drugs are if treatment is initiated after injury, a situation more translatable to human condition.

1.13 Hypothesis

We **hypothesized** that pharmacological inhibition of CCR2 and/or EGFR after onset of OA development will result in reduced severity of Post-traumatic Osteoarthritis.

1.14 Objectives

Our focus is to demonstrate if pharmacological inhibition of EGFR and CCL2 reduces the severity of PTOA by :

1. Examining the damage in the cartilage and bone using OARSI scoring [41]
2. Measurement of cartilage area using OsteoMeasure
3. Examining the damage in the synovium using 6 parametric Synovitis scoring [108]

2.0 METHODS

Chapter 2

2.1 Experimental Design

The experiment was conducted in sixty male Sprague-Dawley rats (weighed approx. 350gm) that were randomly divided into 6 distinct groups (n=10 for each group). All rats were bred and housed according to Animal Care and Use Guidelines of Western University. OA was induced by anterior cruciate ligament transection (ACLT) and partial medial meniscectomy (pMM) in four groups which were later treated with vehicle, EGFR, CCR2 and a combinational treatment of both EGFR and CCR2 blockers.

Treatment was given via model 2ML4 Alzet osmotic mini pumps (lot-10232-09) starting 4 weeks after surgery. Group 1 was given 50% DMSO in deionized water that makes the vehicle solution. Group 2 was administered AG1478 (50% Inhibition concentration [IC_{50}] 0.07-0.2µM) (lot-5A/110926) as a treatment that acted as potent inhibitor on EGF receptor. Group 3 was treated with RS504393 (IC_{50} = 0.33 µM) (lot-5A/110926) which is pharmacological inhibitor of CCR2. RS504393 precipitated immediately when dissolved in water and in order to re-ionize the drug, 2ul 2M HCl solution was added to the mixture of RS504393 and distilled water. Group 4 was given a combinational treatment of AG1478 and RS504393. Group 5 and 6 are the non-surgical healthy rat groups where the former had undergone sham surgery while latter group constitutes healthy naïve rats. Pumps were filled slowly using a 20ml syringe in 0.22uM with a DMSO resistant filter and supplied filling tube. Each pump was weighed with regulator before and after loading solution to ensure the solution weight lies within the tolerance range. After all pumps were filled, 25ml normal saline was added and pumps were incubated overnight in 37ºC cell culture incubator. Pumps were implanted subcutaneously in the scruff of neck using isoflurane gas anesthesia. The treatment was given at a constant rate of 2.5uL/hour providing continual administration of AG1478 (21nmoles/kg/hour) and RS50496 (200nmoles/kg/hour). The dose targets were chosen based on the published literature and work done by previous lab member where plasma

and cartilage concentrations of AG1478 and RS504393 were demonstrated after 3 days of continual delivery by subcutaneous mini-osmotic pumps using liquid chromatography multiple reaction monitoring. Plasma concentrations for AG1478 and RS504393 were found to be 11.6 ± 3.1 µM and 2.5 ± 0.7 µM, respectively and cartilage concentrations were found to be 3.8 ± 1.3 µM for AG1478 and 1.4 ± .08 µM for RS504393.

2.2 Animal Harvesting

Rats were sedated with Isoflurane gas and were sacrificed with CO2 gas 7weeks (n=5 for each group) and 10 weeks (n=5 each) after surgery. Excess muscle was dissected off while removing the right (ipsilateral) and left limbs (contralateral). Also, surfeit bone was trimmed away using a diamond tipped rotary tool while meticulously maintaining the knee joint capsule. Isolated joints were placed in 4% PFA and stored at 4º Celsius for 24 hours and then transferred for decalcification process in Formical-2000. Knees were then bisected coronally using a razor blade. Knee samples were embedded in paraffin blocks and sectioned at Robarts Research Institute (Western University, London, ON, Canada)

Please note that steps 2.1 and 2.2 were performed by previous student (M. Pest) prior to this thesis and samples were stored.

2.3 Histological Assessment

Samples were processed for histology and stained in 1.5% Safranin O/ 0.01% fast green to examine cartilage and bone damage. The cartilage, and glycosaminoglycans produce orange stain while nuclei and cytoplasm stained in black and bluish-green color. Slides were deparaffinized by dipping into xylene followed by different strengths of alcohols (from 100% to 70%) and were finally hydrated to distilled water. After hydration, the

slides were kept in fresh Weigert's Iron Hematoxylin solution for 5 minutes. After repeated washing with water to remove the excess dye, slides were then rinsed in 0.01% fast green, 1% acetic acid and 1.5% Safranin O solution, respectively. At each step, slides were drained with water to prevent the solution from carrying over. For the dehydration process, samples were rinsed into ethyl alcohol with increasing strength order (70%, 95% and 100%) and then finally with xylenes. Slides were finally mounted with coverslip using xylene based SHURMount solution.

Slides were also stained for Hematoxylin and Eosin (0.25%) to further analyze inflammation in synovial membrane. Similar to Safranin-O-Fast Green staining, samples were first deparaffinized with the help of xylenes and then hydrated by dipping into alcohols in decreasing strength order. Next, slides were treated with Harris hematoxylin for nuclear staining (8mins) followed by the differentiation process with acid dip to remove excess background stain. Further, slides were dipped into 0.2% ammonium solution for bluing process that converts soluble orange color of hematoxylin in the nucleus to an insoluble blue color. Next step involves the treatment with eosin (0.25%) for cytoplasmic staining and then finally slides were dehydrated with the help of graded series of alcohol and xylene.

Both staining procedures were conducted in the fume hood for all the sample slides in accordance with the standard staining protocol of Western University.

2.4 OARSI Scoring

Cartilage and bone damage was scored for 3 parameters based on OARSI recommendations for histological assessment of OA in the rat knee [7] on medial femoral condyle (MFC) and medial tibial plateau (MTP). Scoring was done on Safranin-O-Fast Green stained, coronally embedded knee sections. A total of 4-7 serial samples were considered for each animal and a minimum of three animals were used for each treatment group. Images were taken and stitched together with the help of Leica Application Suite Version 4.13 using an objective lens with magnification power of 4X to score cartilage and subchondral bone damage while, for the measurement of osteophyte size, ImageJ software was used. All images were blinded using Random Names application and

scoring was performed by an additional experienced and blinded observer for the first fifty slides. Kappa analysis was conducted to check the level of agreement between two observers.

2.5 Measurement of Cartilage Area

Additionally, cartilage area (in mm^2) was measured using OsteoMeasure bone histomorphometry software to further check articular cartilage damage. Medial-tibial plateau (MTP) and Medial Femoral Condyle (FMC) were divided into three zones according to the recommendations provided by OARSI for scoring cartilage damage in rat knees. However, area was measured only in zone 2 as zone 1 and 3 has large osteophytes that otherwise would potentially interfered with the results.[8] M. Nomura et,al. 2017 performed similar zone wise comparison for measuring cartilage area.[109]

Cartilage area was measured separately for mineralized and unmineralized cartilage on both MFC and MTP. A minimum of 5 and up to 8 samples were taken into account for each animal. Total area was also traced for 20 samples and plotted against the sum of mineralized and unmineralized cartilage area using Bland-Altman plot with the help of GraphPad Prism version 8.0.1 software to ensure consistency while measuring cartilage (Figure 3.9).

2.6 Synovitis Scoring

Slides were also stained for Hematoxylin and Eosin to further analyze inflammation in the synovium. Synovitis was scored following KRENN Scoring where in OA, synovial inflammation is assessed by the following 6 parameters i) synovial hyperplasia ii) presence of fibrin iii) infiltration of non-endothelial cells iv) vascularization v) perivascular oedema and vi) presence of fibrous tissue.[9] Scoring was done on 6 distinct compartments which include the lateral-parapatellar compartment, medial para-patellar compartment, superior-lateral compartment, superior-medial compartment, inferior-lateral compartment and inferior-medial compartment. All six parameters were graded

from 0 to 3 with 0 indicating absence of pathology, 1 suggesting mild, 2 suggesting moderate and 3 referring to severe pathology. Using this score, we analyzed >400 samples in total (6-8 serial samples for each animal) For synovitis scoring, the first 20 slides were scored by two independent observers in a blinded manner and results were evaluated by kappa analysis. Before initiating the actual scoring, healthy slides were used for calibration purposes. For all groups, a minimum of 5 and up to 8 samples for each animal were considered and then averaged score was counted and plotted for statistically significant differences.

2.7 Statistical Analysis

Data for all parameters were examined using GraphPad Prism version 8.0.1 for normality with Shapiro-Wilk normality test where N<4 for all groups. For the datasets that follow a normal distribution curve, One-way ANOVA was run along with Dunnett's multiple comparison test where each treated and non-treated groups are compared for statistical significance with respect to the vehicle treatment group. The groups that did not follow a normal distribution curve, went through non-parametric analysis using Kruskal-Wallis test along with Dunn's multiple comparison testing, comparing vehicle treatment group against all treatment and non-treatment groups, separately for both 7 and 10week timepoints. Data was presented as mean + standard deviation (SD) and mean + 95% confidence intervals for parametric and non-parametric analysis, respectively. Also, the sham group from the 7-week timepoint was excluded due to artifacts including thick and thin areas, and folding and over-lapping of tissue on slides which makes the scoring difficult for this group. Thus, only healthy naïve rats were compared to vehicle treated group by performing unpaired t-test with Welch's correction and descriptive statistics for each dataset in case of parametric analysis or unpaired t-test with Mann-Whitney test. For cartilage area, data sets for both mineralized and unmineralized were analyzed separately on FMC and MTP. A minimum of three animals per group were used in all statistical analyses.

3.0 Results

3.1 Three-Parametric Cartilage Scoring

To analyze the extent of damage in the cartilage, 3 parameters are considered as per the recommendations suggested by OARSI for the OA histopathology in rat (Figure 3.1).[43] The first parameter examined was cartilage damage which provides insights into overall pathology of cartilage and involve parameters such as chondrocyte cell death and fibrillation of matrix.[43] One-way ANOVA analysis and Kruskal-Wallis test has been conducted for the data obtained from scoring cartilage damage based on the normal distribution curve. Tibial and femoral bone were compared separately for both 7-week & 10-week timeline groups to ensure capturing the total knee for scoring (Figure 3.2, 3.3, 3.4). Healthy naïve rat group & sham group were compared separately with the vehicle treatment group to compare surgically induced OA-models with non-OA groups. The second parameter was scored based on the size of osteophyte present. The third parameter assessed was the damage in the subchondral bone which helps to evaluate the outcomes of different treatments on OA-dependent changes in the bone beneath cartilage.[43]

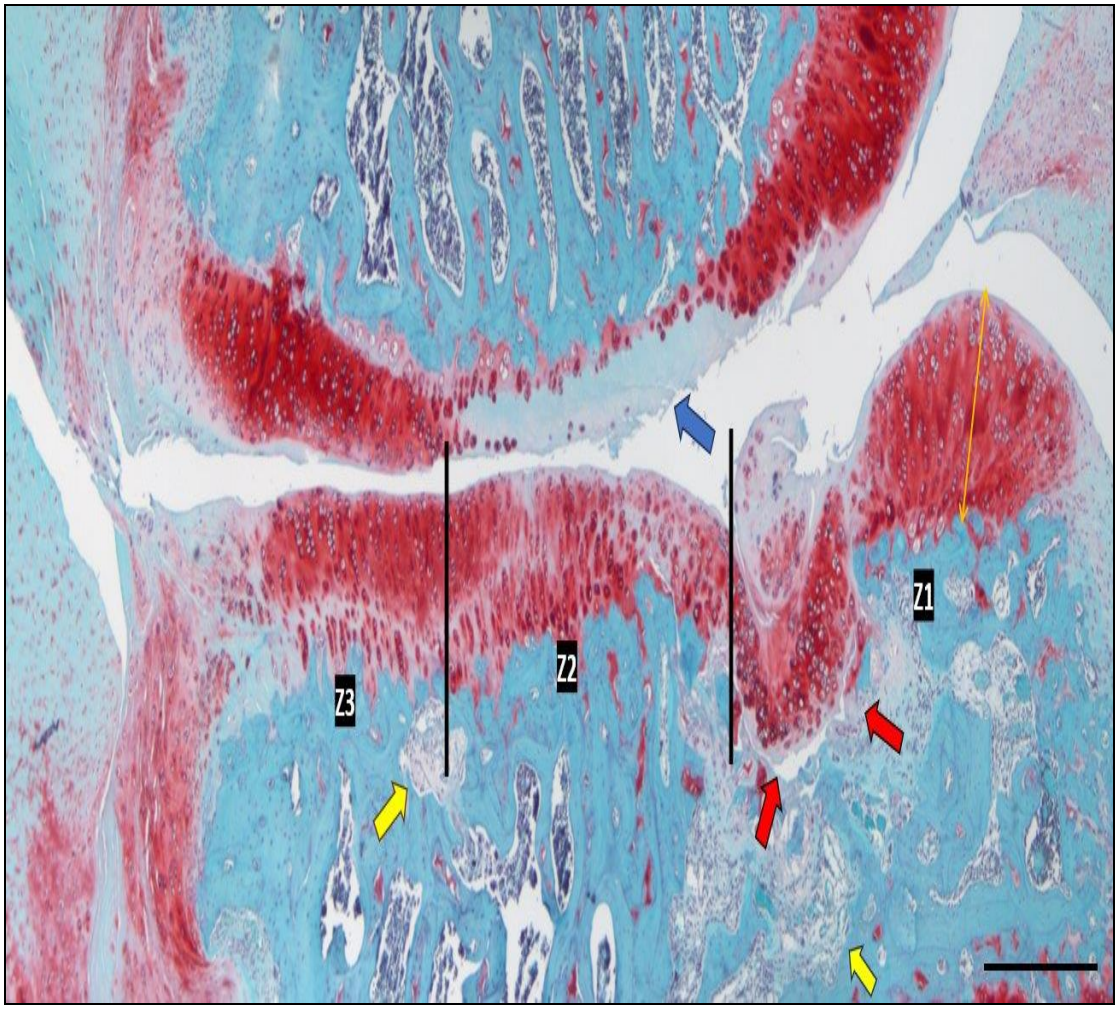


Figure 3.1 Three-parametric cartilage scoring using medial side of rat knee joint with Safranin O Fast Green staining. Blue arrow indicates the damage in the articular cartilage where loss of proteoglycan is witnessed along with chondrocyte cell death in zone 2 while double-headed yellow arrow indicates the presence of an osteophyte whose size can be measured using ImageJ software. Red and yellow arrows represent damage in subchondral bone where yellow arrows pointing towards mesenchymal changes in the bone marrow while red arrow specify the collapse of articular cartilage into the bone. Scale bar-200µm.

3.1.1 EGFR and CCR2 inhibition do not protect cartilage against damage in Osteoarthritis

The treatments given in our research do not protect the articular cartilage against damage. Safranin O fast green stain was performed to determine the extent of disease. Upon AG1478, RS504393 or combinational treatment of AG1478 and RS504393, no significant difference was observed when these groups compared with the vehicle treatment group in both 7-week & 10-week timeline groups. We have witnessed significant chondrocyte cell death, proteoglycan loss and fissures in articular cartilage (Figure 3.2). Combination treatment of AG1478 and RS504393 have resulted in complete loss of cartilage in zone 1 and zone 2 (Figure 3.2). The OARSI scores for this group were more than 11 for the cartilage damage on tibial bone in the 7-week timepoint group (Figure 3.3). Although, statistically, there was no significant difference between vehicle and AG1478+RS504393 treatment group.

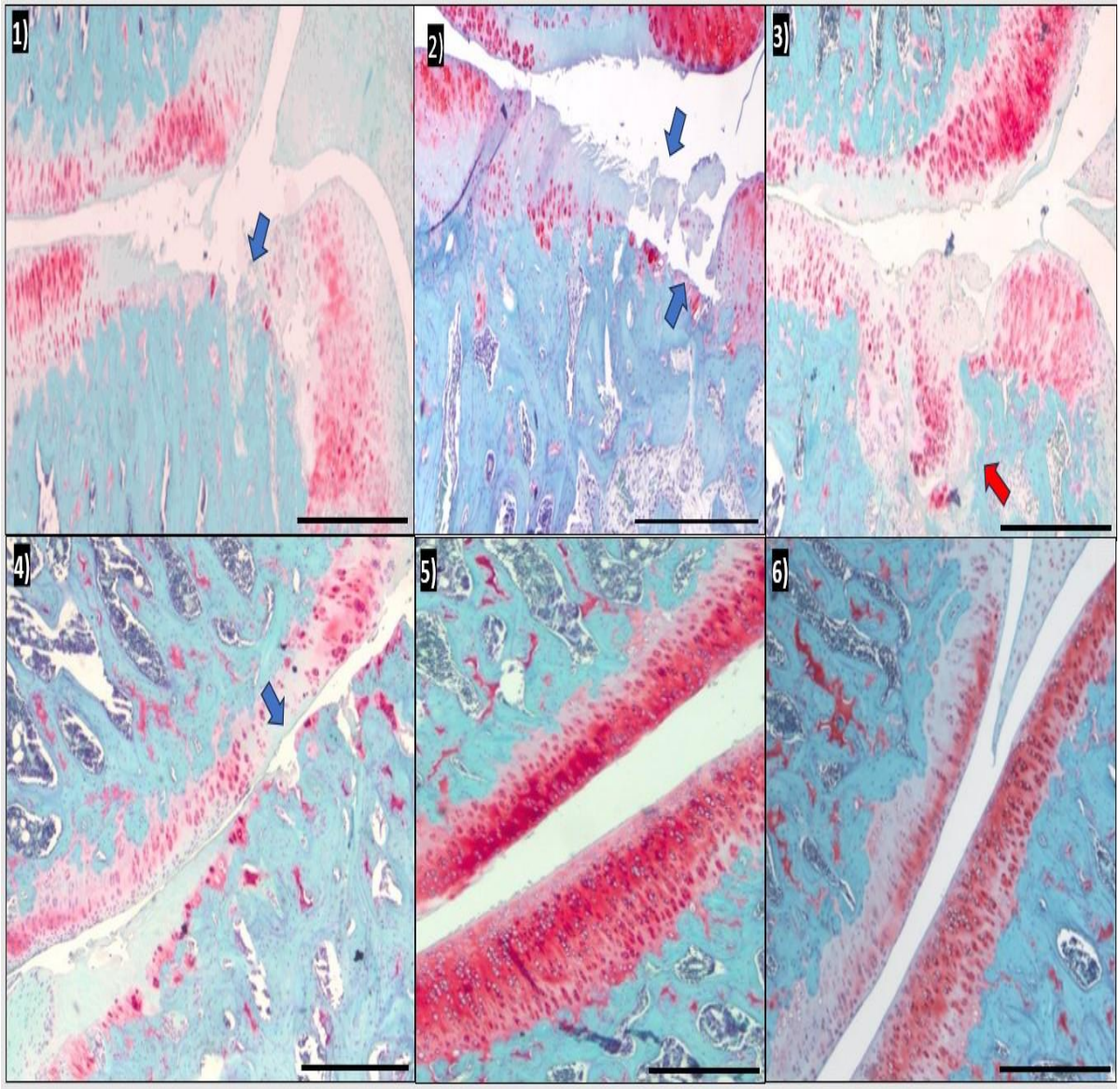


Fig.3.2 Blocking EGFR & CCR2 does not offer protection against cartilage damage. Image 1) Vehicle treatment group (50% DMSO in deionized water): shows significant damage in articular cartilage as indicated by blue arrow 2) AG1478 treated group: degenerative articular cartilage changes seen evidently. Blue arrows indicate cracks and fibrillation on cartilage surface 3) RS504393 treated group: no protection is seen against cartilage damage. Red arrows indicate the collapse of articular cartilage into subchondral bone. 4) AG1478+RS504393 treated group: shows significant cartilage damage 5) Sham-operated group: cartilage seems healthy with no proteoglycan loss or chondrocyte cell death 6) Healthy naïve rats: cartilage is intact and healthy. Scale bar-200µm.

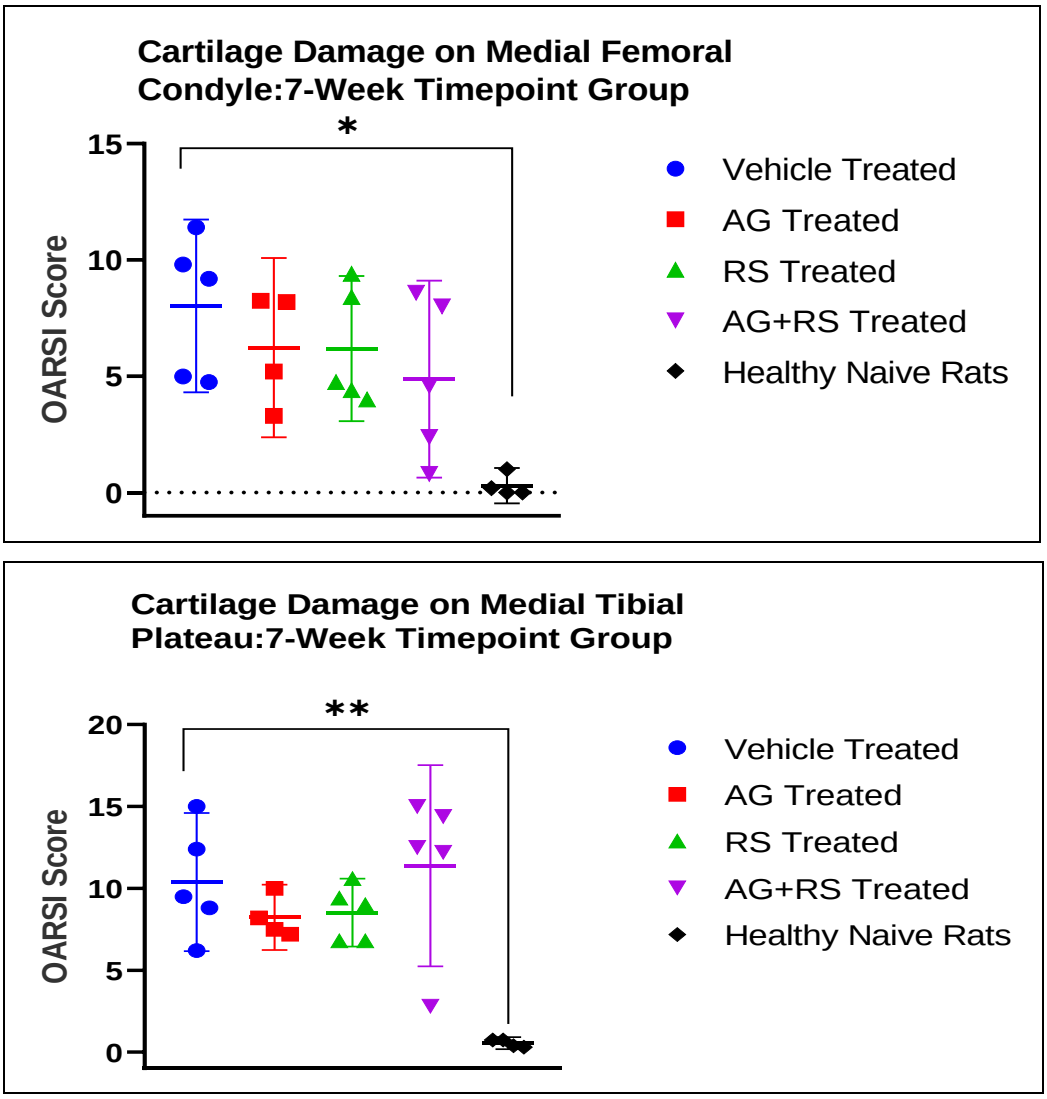


Figure 3.3 Histological scoring of cartilage damage in accordance with the recommendations provided by OARSI in 7-week timepoint group. One-way ANOVA analysis and Kruskal-Wallis test was performed for tibial and femoral cartilage degeneration scores along with Dunnet's multiple comparison tests in 7w rat groups. All treated and non-treated groups are compared with vehicle treatment groups separately for 7-week and 10-week timelines. No significant difference is seen between vehicle and other treated groups after surgery (p-value>0.05). Sham-operated rats are excluded in 7-week timepoint group due to presence of artifacts on sample slides. N>3

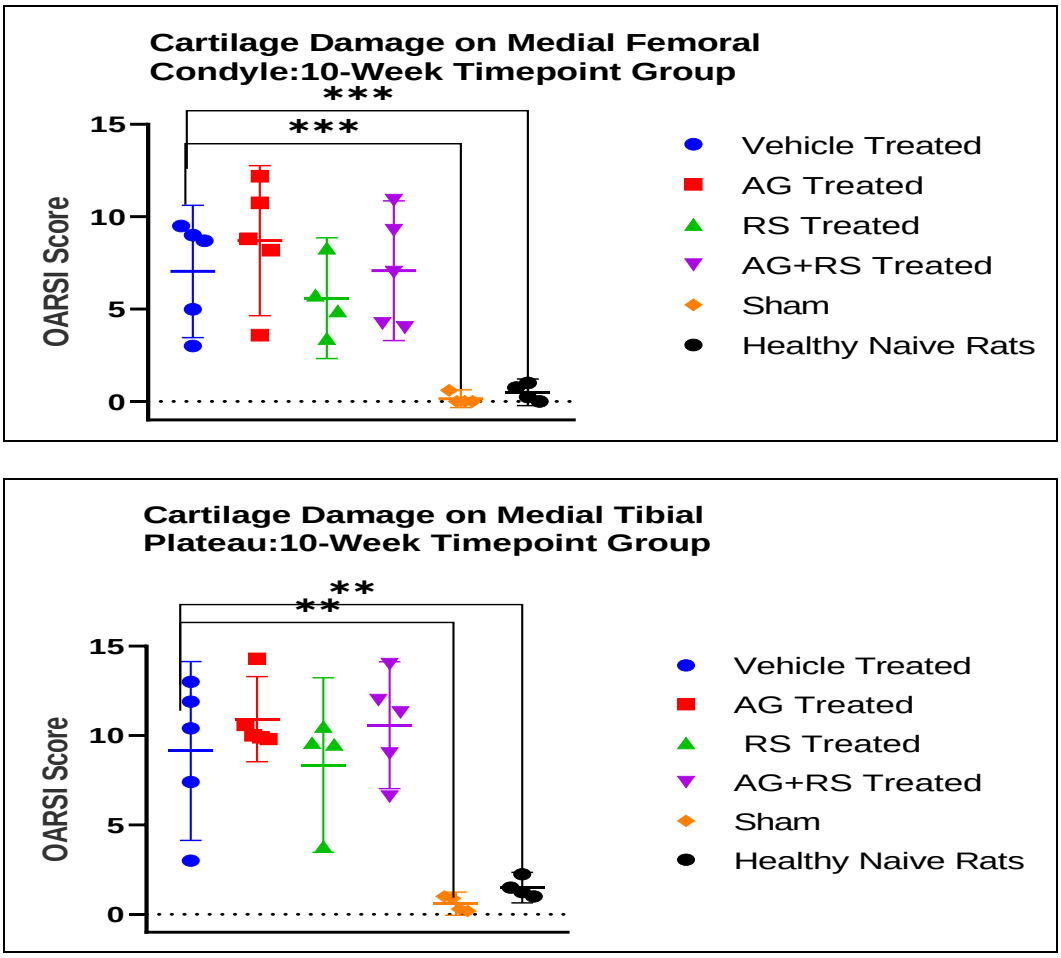


Figure 3.4 Histological scoring of cartilage damage in accordance with the recommendations provided by OARSI in 10-week timepoint group. One-way ANOVA analysis and Kruskal-Wallis test was performed for tibial and femoral cartilage degeneration scores along with Dunnet's multiple comparison tests in 10w rat groups for statistical significance. Although, no significant difference is seen between vehicle and other treated groups after surgery (p-value>0.05). N>3

3.1.2 AG1478 treatment proves to be efficient in reducing osteophyte size in both 7- week and 10-week time points

We witnessed a significant increase in osteophyte size in RS504393 and AG1478+RS504393 treatment groups (Figure 3.5). However, in the 7-week timeline group, AG1478 showed a significant difference ($p \leq 0.01$) when compared with the vehicle group, while in the 10-week timepoint group, the p-value is ≤ 0.05 (Figure 3.5, 3.6). The OARSI score for osteophyte size remained ≤ 1 for the 7-week timepoint and ≤ 1.5 for 10-week time point group. Yet, other groups on comparison with control group (vehicle group) have not shown any differences indicating that RS504393 and combinational treatment have not provided any protection against the formation of osteophytes.

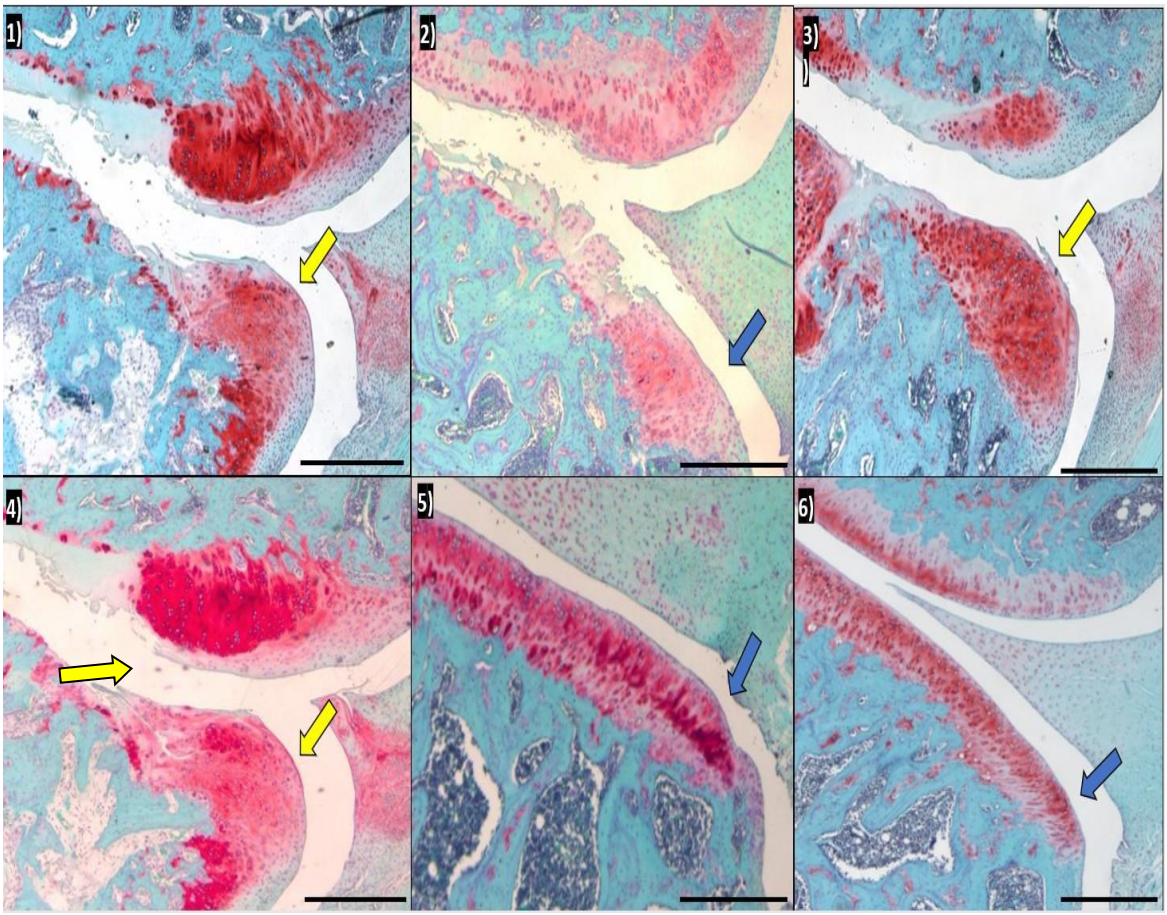


Figure 3.5 Coronally embedded knee sections with Safranin-O Fast Green Stain represents the presence of osteophyte in 10-week timeline group. Image 1) Vehicle treatment (50% DMSO in deionized water 2) AG1478 treated group 3) RS504393 treated group 4) AG1478+RS504393 treated group 5) Sham-operated group 6) Healthy naïve rats. Yellow arrows indicate the presence of large osteophytes in Vehicle, RS504393, combinational blockade of AG1478+RS504393 treatment groups while blue arrow shows the absence of osteophytes on the edges of the articular cartilage in AG1478 treated, sham-operated and healthy naïve rat groups. Scale bar-200µm.

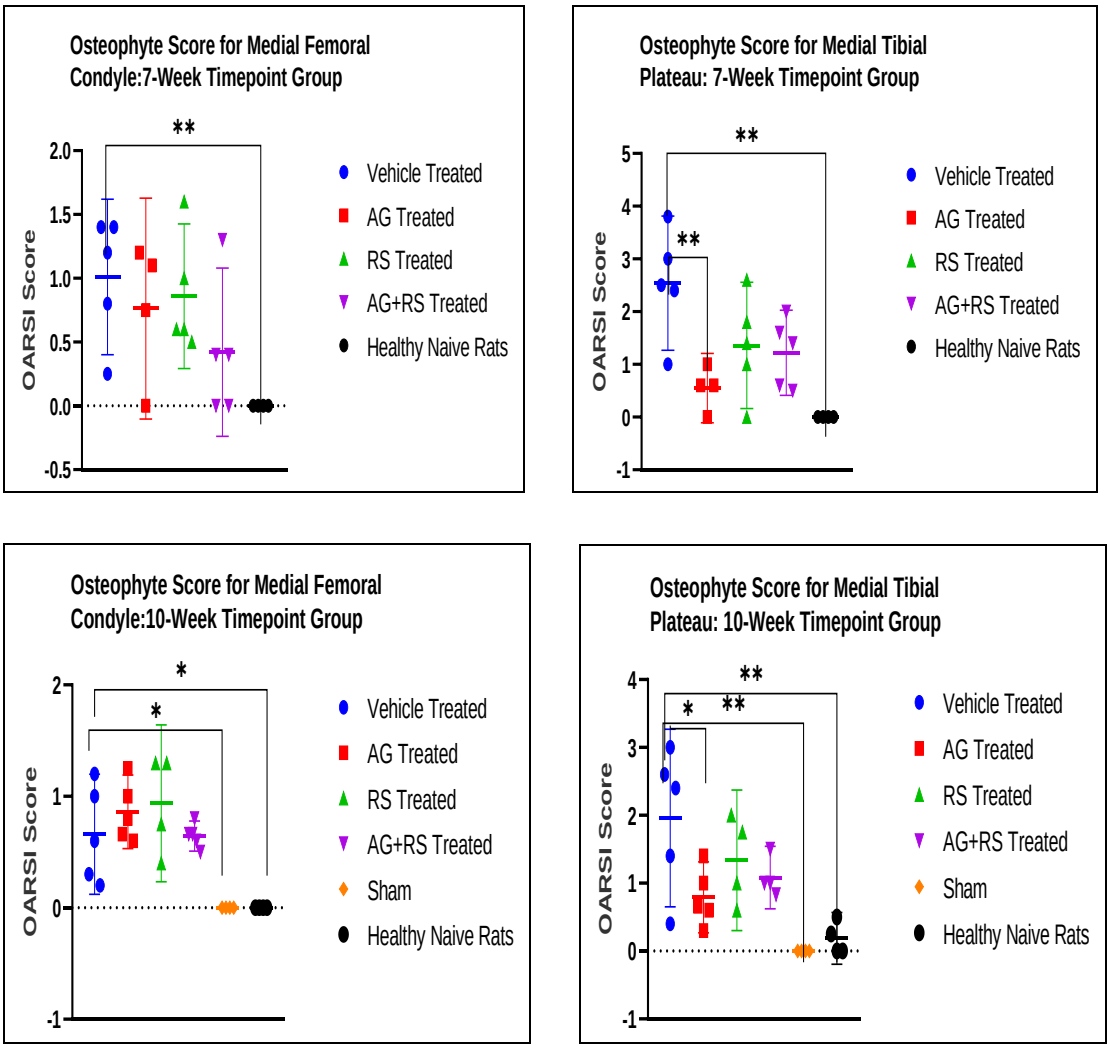


Figure 3.6 Histologically assessed OARSI scores for the presence of Osteophyte. Osteophyte scores have been calculated using ImageJ software. For statistical testing, One-way ANOVA and Kruskal Wallis test was performed with Dunnett's multiple comparison testing for 7-week and 10-week timepoints. AG1476 treated group on tibial bone show a significant reduction in osteophyte size in comparison with vehicle treated group ($p \leq 0.01$). N>3

3.1.3 EGFR/CCR2 inhibition do not protect subchondral bone damage

Safranin-O-stained samples revealed prominent damage in subchondral bone. We witnessed mesenchymal changes in the bone marrow along with the collapse of articular cartilage into the bone (Figure 3.7). However, these changes are seen majorly in the tibial bone, while OARSI score was consistently low for the subchondral bone damage in medial femoral condyle (FMC) for both timeline groups (Figure 3.8).

One-way ANOVA and Kruskal-Wallis test was conducted along with multiple comparison testing. There were no significant differences seen on comparing vehicle with other treatment groups suggesting that AG1478, RS504393 and combinational treatment of both agents do not have the protective effects against the changes in bone associated with OA (Figure 3.7, 3.8).

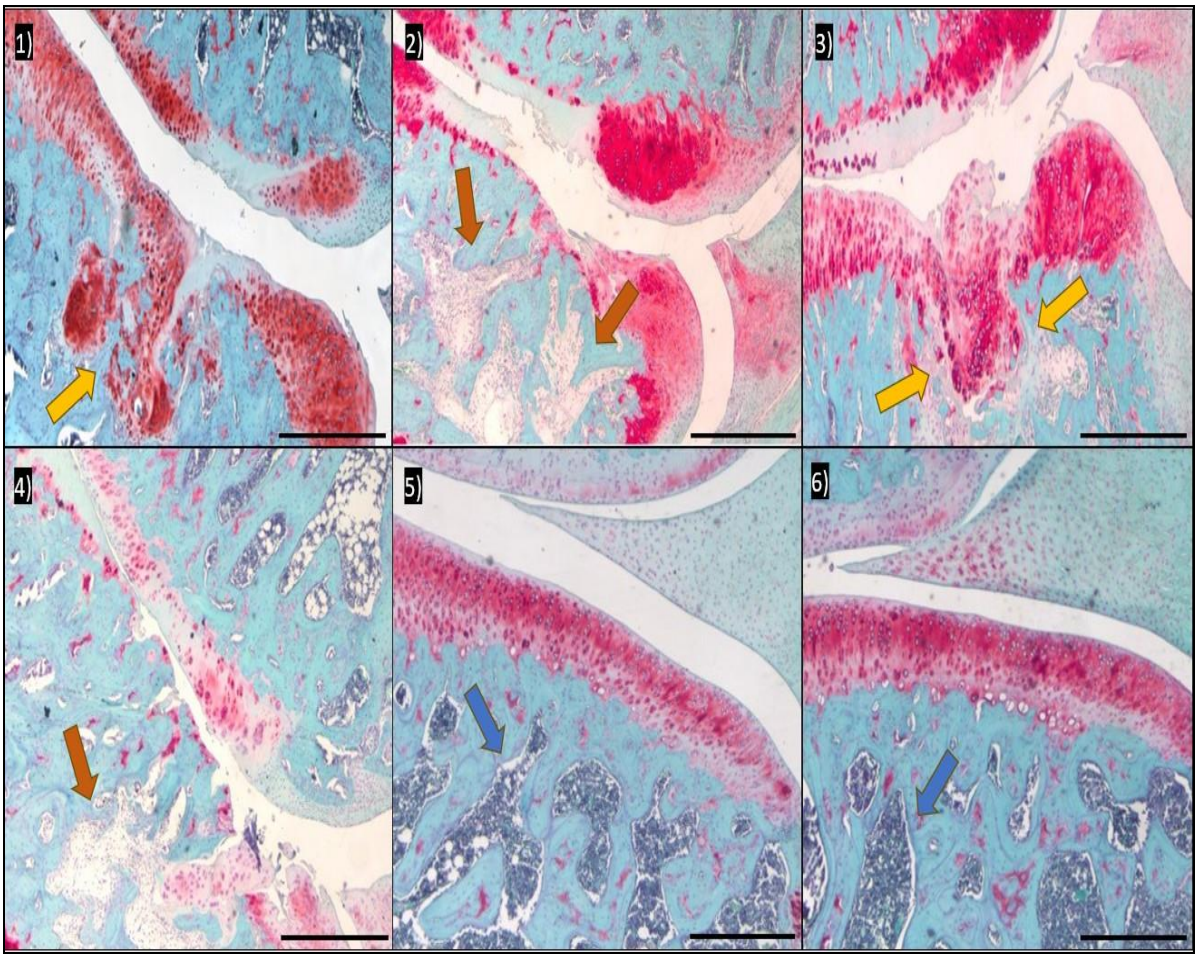


Fig. 3.7 Damage in Subchondral Bone is evident in different treatment groups. Image 1) Vehicle treatment (50% DMSO in deionized water) 2) AG1478 treated group 3) RS504393 treated group 4) AG1478+RS504393 treated group 5) Sham-operated group 6) Healthy naïve rats. Brown arrow shows the mesenchymal changes in the bone marrow (Score=3), while yellow arrows indicate the collapse of articular into subchondral bone indicating more severe form of damage. Blue arrows indicate the healthy bone marrow in sham-operated and healthy naïve rats. Images were taken from 10-week timepoint group. Scale bar-200µm.

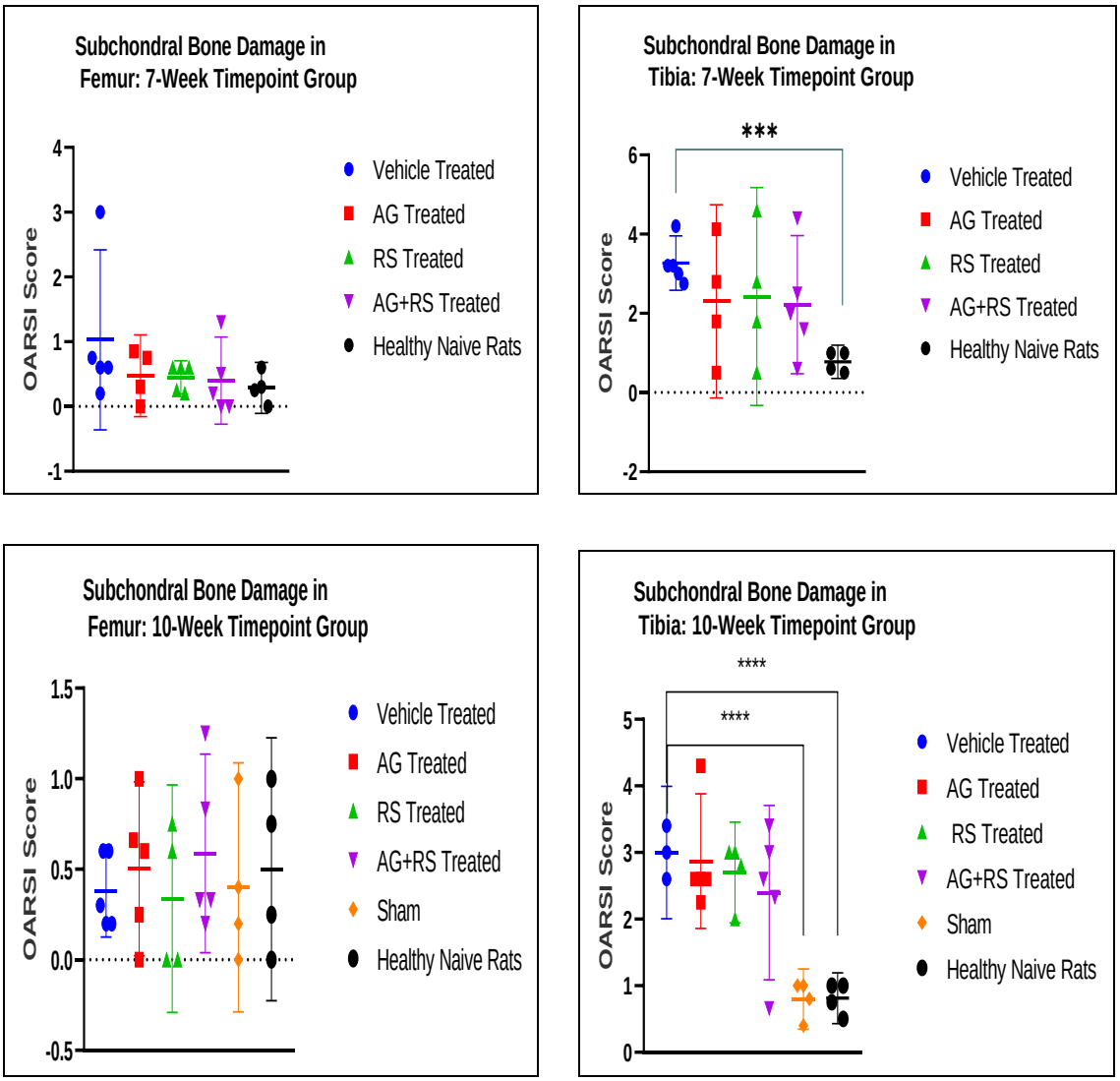


Figure 3.8 OARSI scores for the assessment of subchondral bone damage parameter in accordance with the recommended guidelines. One-Way ANOVA and Kruskal-Wallis test was performed along Dunnett's multiple comparison tests for both 7week and 10-week timeline groups. Scores were calculated with the help of ImageJ software. No significant differences were observed when vehicle group is compared with EGFR, CCR2 and combinational EGFR+CCR2 inhibited groups ($p>0.05$). N>3

3.2 Measurement of Cartilage area support OARSI scores

Measuring cartilage area is considered as a sensitive approach for evaluating changes into the cartilage. Samples were stained with Safranin O Fast green stain to measure the cartilage area with the help of OsteoMeasure. The bias value equals to 0.0002 was obtained when total cartilage area was plotted against the sum of unmineralized and mineralized cartilage suggesting good consistency while measuring the area (Figure 3.9). Since all the dots lie closer to the basal line, this indicates that we were very consistent while measuring cartilage area.

The datasets obtained from the measuring unmineralized and mineralized cartilage were analyzed separately for femur and tibial bone for both timeline groups (7-week & 10-week). RS504393 treated group in 7-week timeline rat groups show a significant difference (p-value $p \leq 0.05$) as compared to vehicle treated group on tibial bone cartilage area (Figure 3.10). However, this significant difference has not been seen in the 10-week timeline group. Also, we have not witnessed any significant differences between other treatments (Figure 3.10)

Healthy naïve rat groups showed considerably higher cartilage when compared to vehicle group ($p \leq 0.01$) which is the expected as it is non-OA group.

Also, the measurements of mineralized cartilage showed insignificant differences between vehicle, treatment groups and non-OA groups. This indicates that the matrix fibrillation and cartilage damage have mostly affected the unmineralized cartilage.

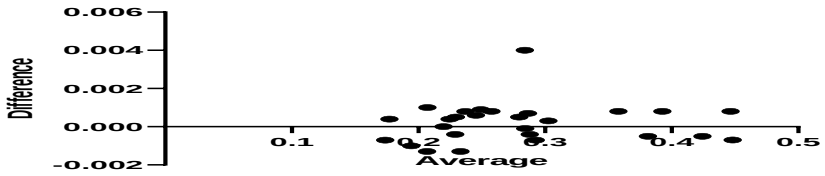
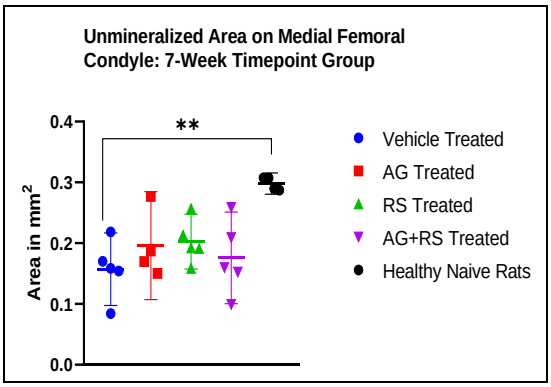
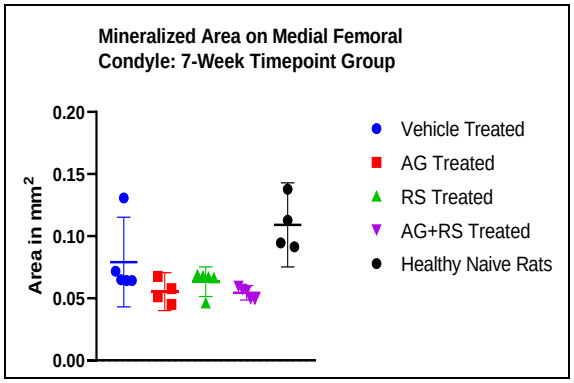
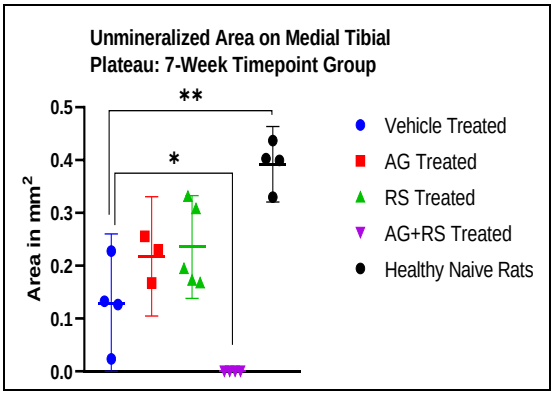
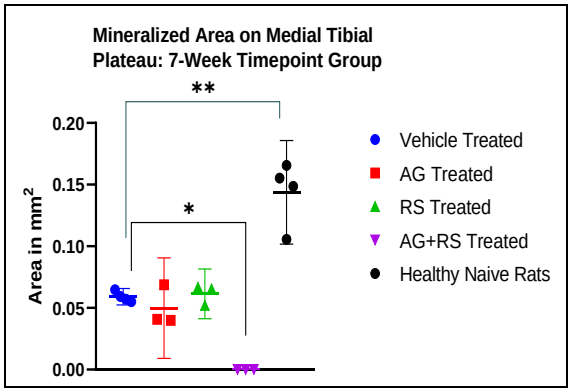


Figure 3.9 depicts Bland-Altman plot (difference vs average) of Total cartilage area vs Unmineralized + Mineralized Area. Almost all values lie closer to 0 indicate consistent measurements with bias value equals to 0.0002.


Unmineralized Area on Medial Femoral Condyle: 7-Week Timepoint Group
Area in mm2
0.4
0.3
0.2
0.1
0.0
**
Vehicle Treated
AG Treated
RS Treated
AG+RS Treated
Healthy Naive Rats


Mineralized Area on Medial Femoral Condyle: 7-Week Timepoint Group
Area in mm2
0.20
0.15
0.10
0.05
0.00
Vehicle Treated
AG Treated
RS Treated
AG+RS Treated
Healthy Naive Rats


Unmineralized Area on Medial Tibial Plateau: 7-Week Timepoint Group
Area in mm2
0.5
0.4
0.3
0.2
0.1
0.0
**
*
Vehicle Treated
AG Treated
RS Treated
AG+RS Treated
Healthy Naive Rats


Mineralized Area on Medial Tibial Plateau: 7-Week Timepoint Group
Area in mm2
0.20
0.15
0.10
0.05
0.00
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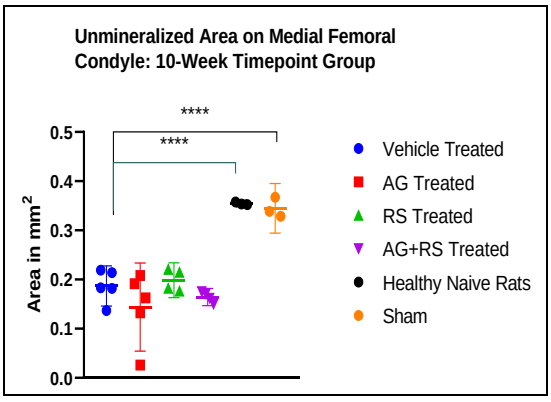
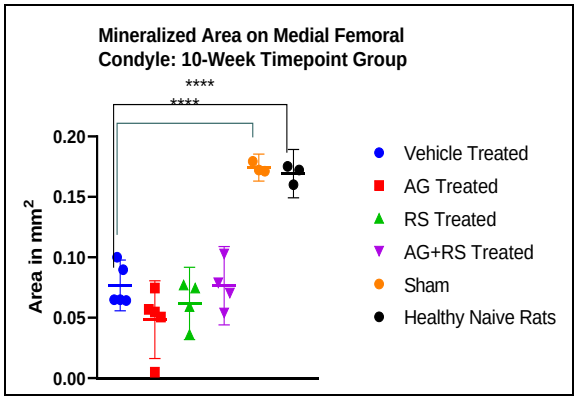
*
Vehicle Treated
AG Treated
RS Treated
AG+RS Treated
Healthy Naive Rats


Unmineralized Area on Medial Femoral Condyle: 10-Week Timepoint Group
Area in mm2
0.5
0.4
0.3
0.2
0.1
0.0
****
****
Vehicle Treated
AG Treated
RS Treated
AG+RS Treated
Healthy Naive Rats
Sham


Mineralized Area on Medial Femoral Condyle: 10-Week Timepoint Group
Area in mm2
0.20
0.15
0.10
0.05
0.00
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AG Treated
RS Treated
AG+RS Treated
Sham
Healthy Naive Rats

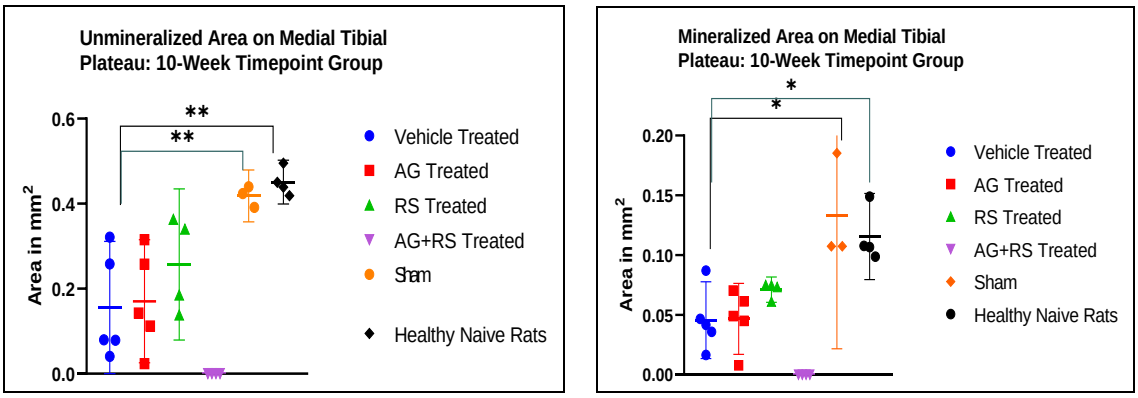


Figure 3.10 Measurement of cartilage area on Femoral & Tibia bone in 7-week and 10-week timepoints using Osteomeasure. To test the statistical significance, One-way ANOVA with Dunnett's multiple comparison tests was performed**.** No significant difference is seen between vehicle vs treated groups ($p>0.05$). The Cartilage Area (mm^2) is measured using OsteoMeasure with 10X objective lens. Mineralized femoral areas appear to be unaffected in all groups.

3.3 Synovitis Scoring

For assessing damage in synovial tissue, a semi-quantitative, 6-parametric scoring system was adopted.[108] Six distinct locations were considered for the scoring as indicated in Figure 3.11 to ensure that total knee joint is considered. The six compartments include lateral parapatellar compartment, medial parapatellar compartment, superior-lateral compartment, superior-medial compartment, inferior-lateral compartment, and inferior-medial compartment. The six parameters that were assessed include synovial hyperplasia, increased infiltration, fibrin deposition, increased vascularization, presence of fibrotic tissue, and presence of perivascular edema. All six parameters were scored individually for all six compartments in both 7-week & 10-week timeline groups.

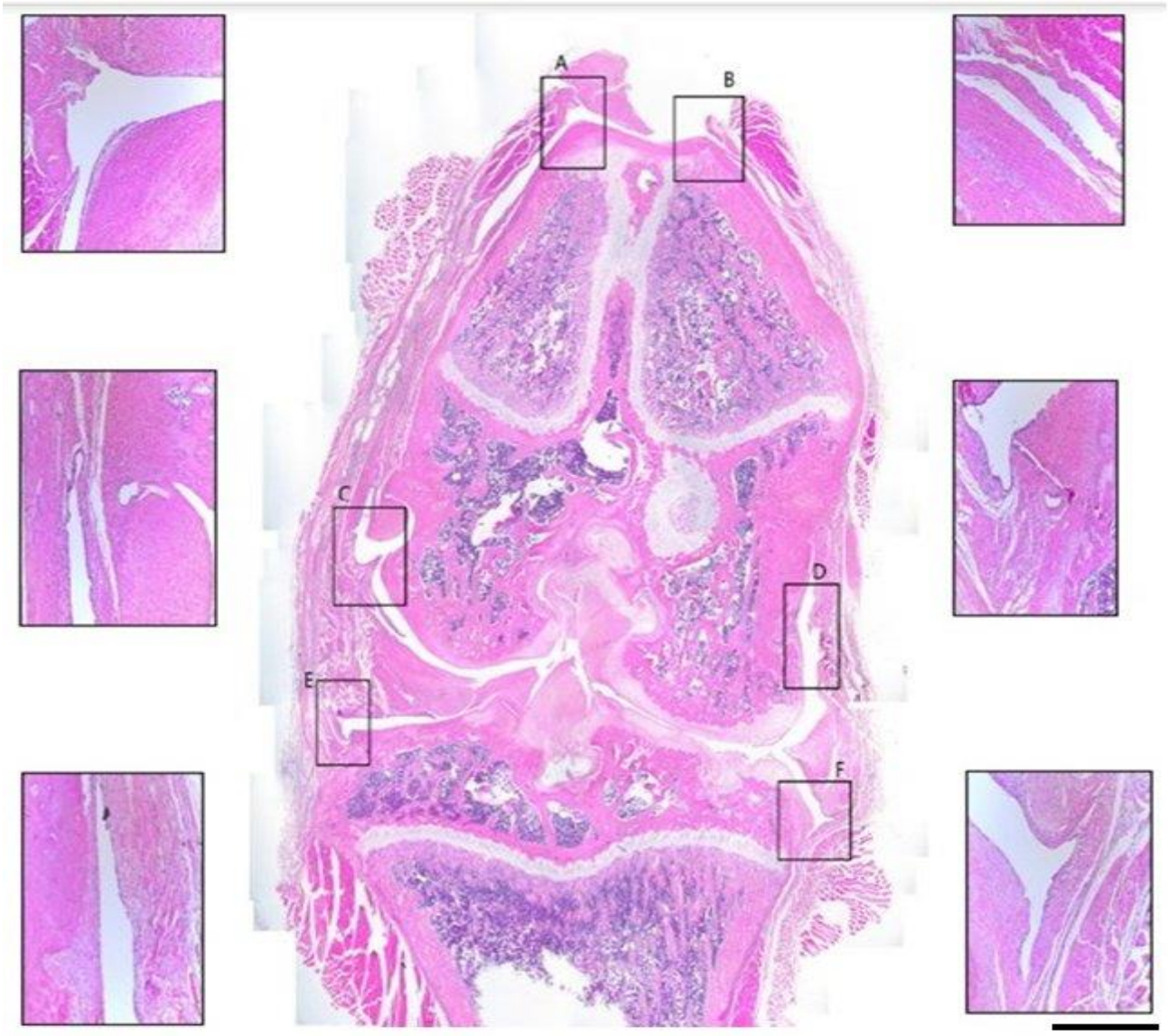


Figure 3.11 H&E-stained rat knee of 7week timeline group indicating distinct locations considered for Minten Scoring. Alphabetical letters indicate 6 compartments where all 6 parameters were scored. A) lateral parapatellar compartment B) medial parapatellar compartment C) superior lateral compartment D) superior medial compartment E) inferior lateral compartment F) inferior medial compartment

3.3.1 Significant thickening of intimal layer in all treatment groups

The first parameter is synovial hyperplasia where the intimal lining of synovium becomes thickened in response to inflammation (Figure 3.12) The data obtained from scoring this parameter indicates that no significant differences (p-value >0.05) were seen between the different treatment groups in any of the compartments on comparison to vehicle treated group which suggests that our agents were not able to provide any protection against the thickening of the intimal layer (Figure 3.13). However, data also suggested the presence of very low-grade synovial hyperplasia in just superior-lateral compartment of sham group in both 7 and 10-week timepoints while, in the other compartments, there was an insignificant difference between sham group and healthy naïve rat groups when compared with vehicle treated group (P>0.05).

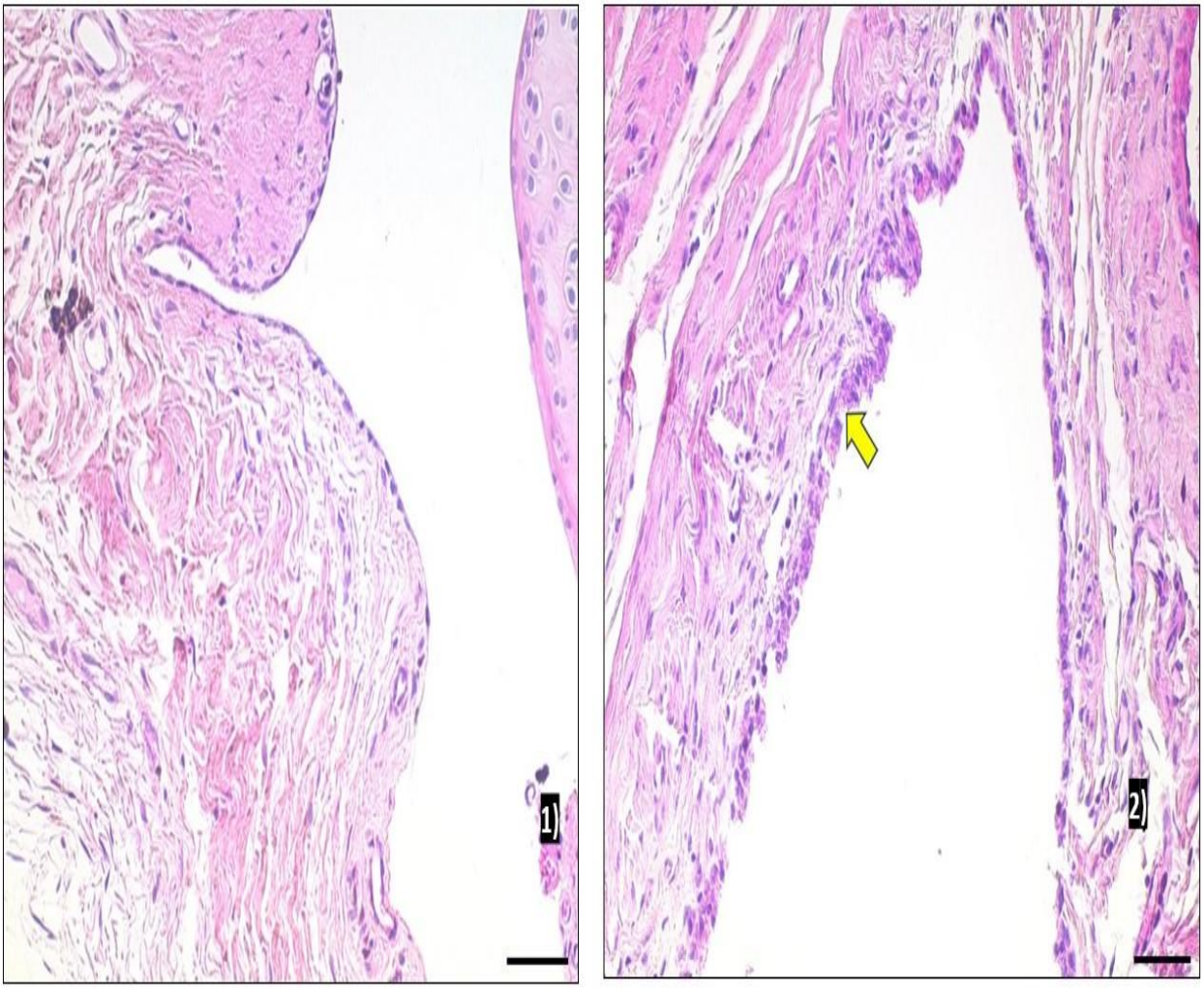
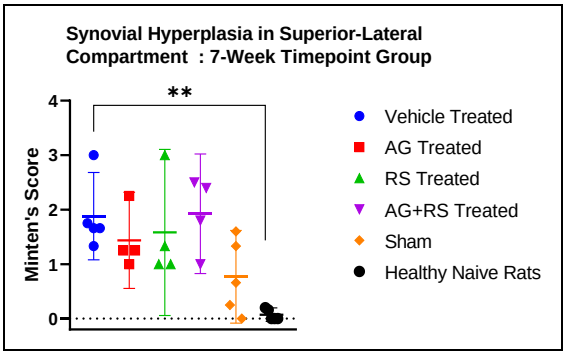
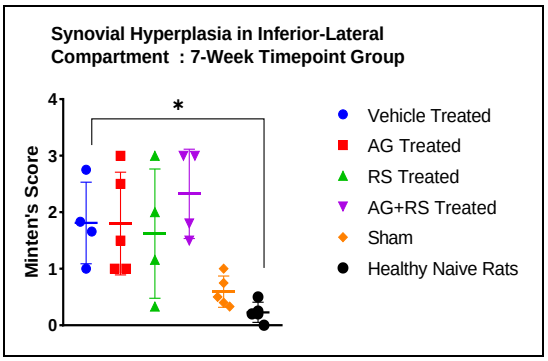
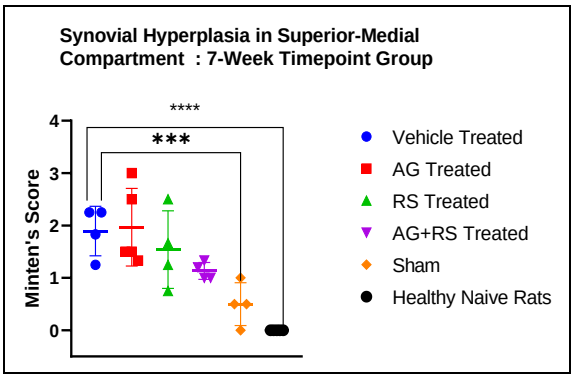
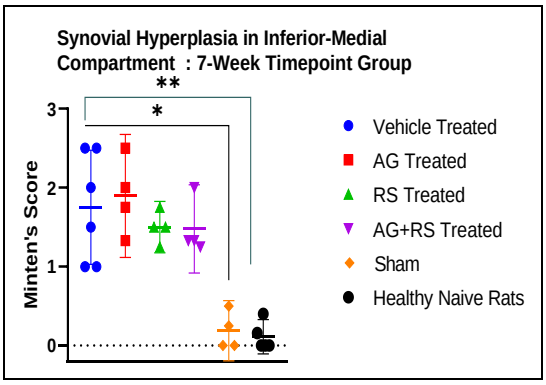


Figure 3.12 Hematoxylin & Eosin stained healthy vs inflammed synovial lining. 1) Image is taken from 10 week old healthy naïve rat in the inferior lateral compartment: Intimal lining is single-celled in thickness while 2) Image is obtained from 10 week timeline AG1478 treatment group in superior medial compartment. Yellow arrow marked the presence of more than 3-celled thick intimal lining which indicates significant synovial hyperplasia as a result of inflammation. Scale bar-100µm.


Synovial Hyperplasia in Superior-Lateral Compartment : 7-Week Timepoint Group
Minten's Score
**
Vehicle Treated
AG Treated
RS Treated
AG+RS Treated
Sham
Healthy Naive Rats


Synovial Hyperplasia in Inferior-Lateral Compartment : 7-Week Timepoint Group
Minten's Score
*
Vehicle Treated
AG Treated
RS Treated
AG+RS Treated
Sham
Healthy Naive Rats


Synovial Hyperplasia in Superior-Medial Compartment : 7-Week Timepoint Group
Minten's Score
****
***
Vehicle Treated
AG Treated
RS Treated
AG+RS Treated
Sham
Healthy Naive Rats


Synovial Hyperplasia in Inferior-Medial Compartment : 7-Week Timepoint Group
Minten's Score
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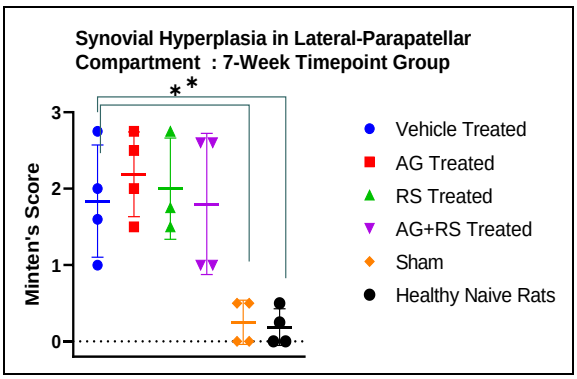
Vehicle Treated
AG Treated
RS Treated
AG+RS Treated
Sham
Healthy Naive Rats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Minten's Score
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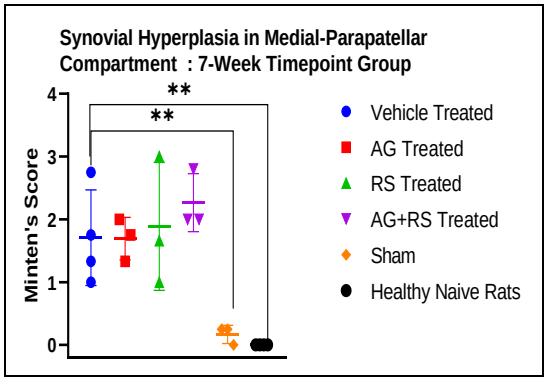
AG Treated
RS Treated
AG+RS Treated
Sham
Healthy Naive Rats


Synovial Hyperplasia in Medial-Parapatellar Compartment : 7-Week Timepoint Group
Minten's Score
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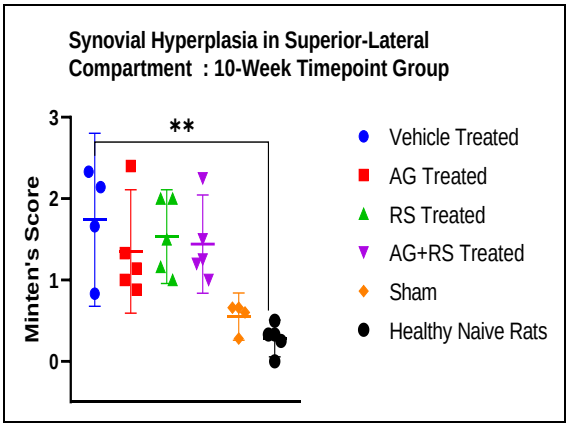
AG Treated
RS Treated
AG+RS Treated
Sham
Healthy Naive Rats


Synovial Hyperplasia in Superior-Lateral Compartment : 10-Week Timepoint Group
Minten's Score
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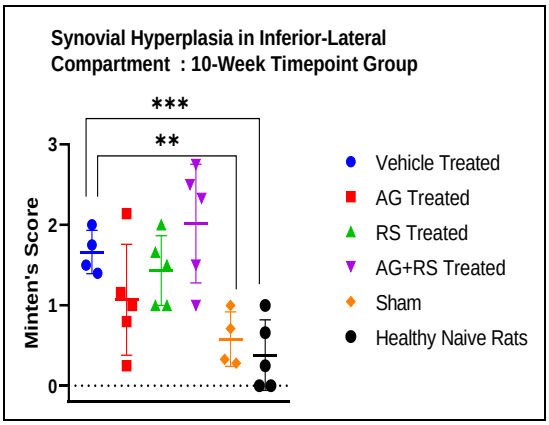
Vehicle Treated
AG Treated
RS Treated
AG+RS Treated
Sham
Healthy Naive Rats


Synovial Hyperplasia in Inferior-Lateral Compartment : 10-Week Timepoint Group
Minten's Score
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Vehicle Treated
AG Treated
RS Treated
AG+RS Treated
Sham
Healthy Naive Rats

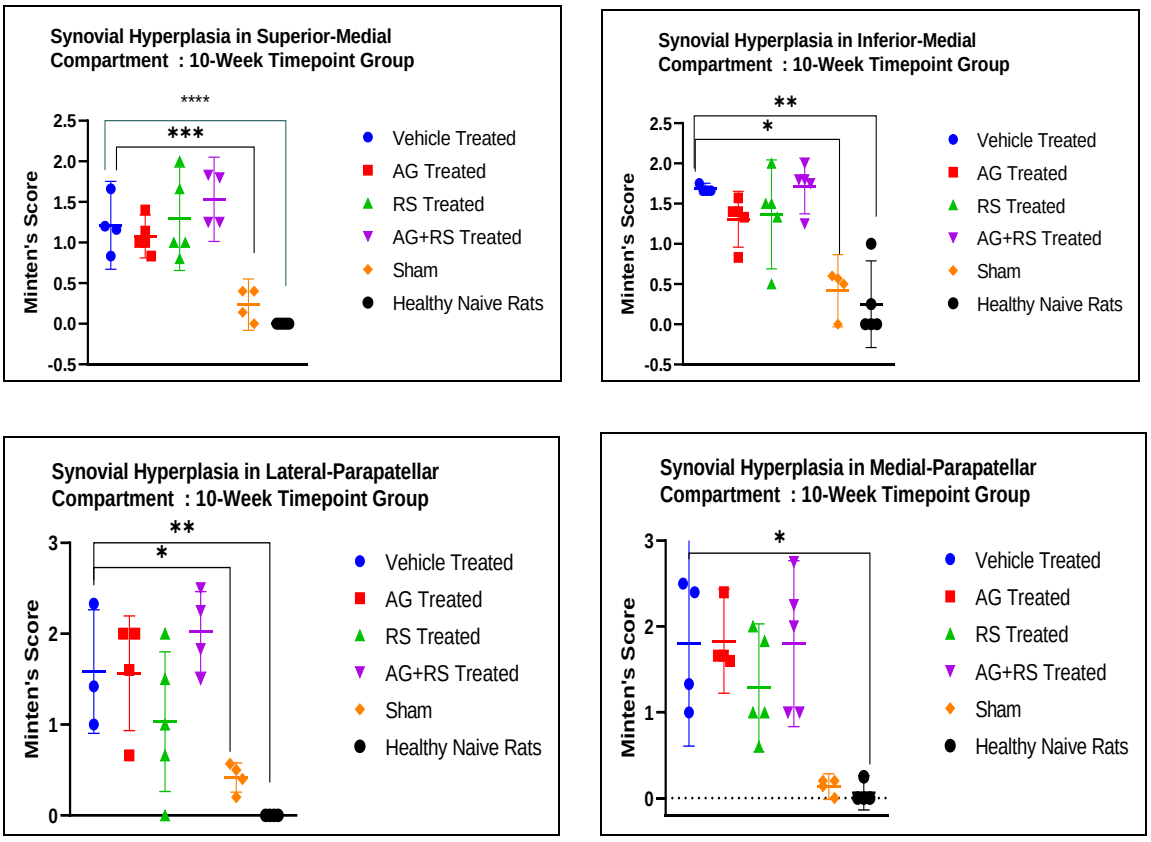


Fig 3.13 Histological scoring of Synovial Hyperplasia according to Minten's Scoring System. One-way ANOVA and Kruskal-Wallis test with Dunnett's and Dunn's multiple comparison test was done to test the statistical significance for all six compartments at 7 & 10w timepoints. OA and non-OA groups were compared with vehicle treatment separately. Blocking EGFR with AG1478, CCR2 with RS504393, and combinational inhibition of EGFR and CCR2 does not safeguard the synovial lining from undergoing hyperplasia ($p>0.05$) in both 7-week & 10-week timepoints. N>4

3.3.2 Increased Infiltration is observed in every treated group

The second parameter assessed histologically was increased infiltration of monocytes in response to inflammation (Figure3.14). Our results indicate that AG1478/RS504393 does not provide any protection against this parameter. In all compartments, no significant differences were observed between vehicle and other treatments ($p>0.05$). We have witnessed insignificant differences between vehicle & sham group in superior-lateral &

medial compartment, inferior-medial compartment, lateral-parapatellar compartment, suggesting the presence of low-grade inflammation in fewer animals of the sham group (Figure 3.15).

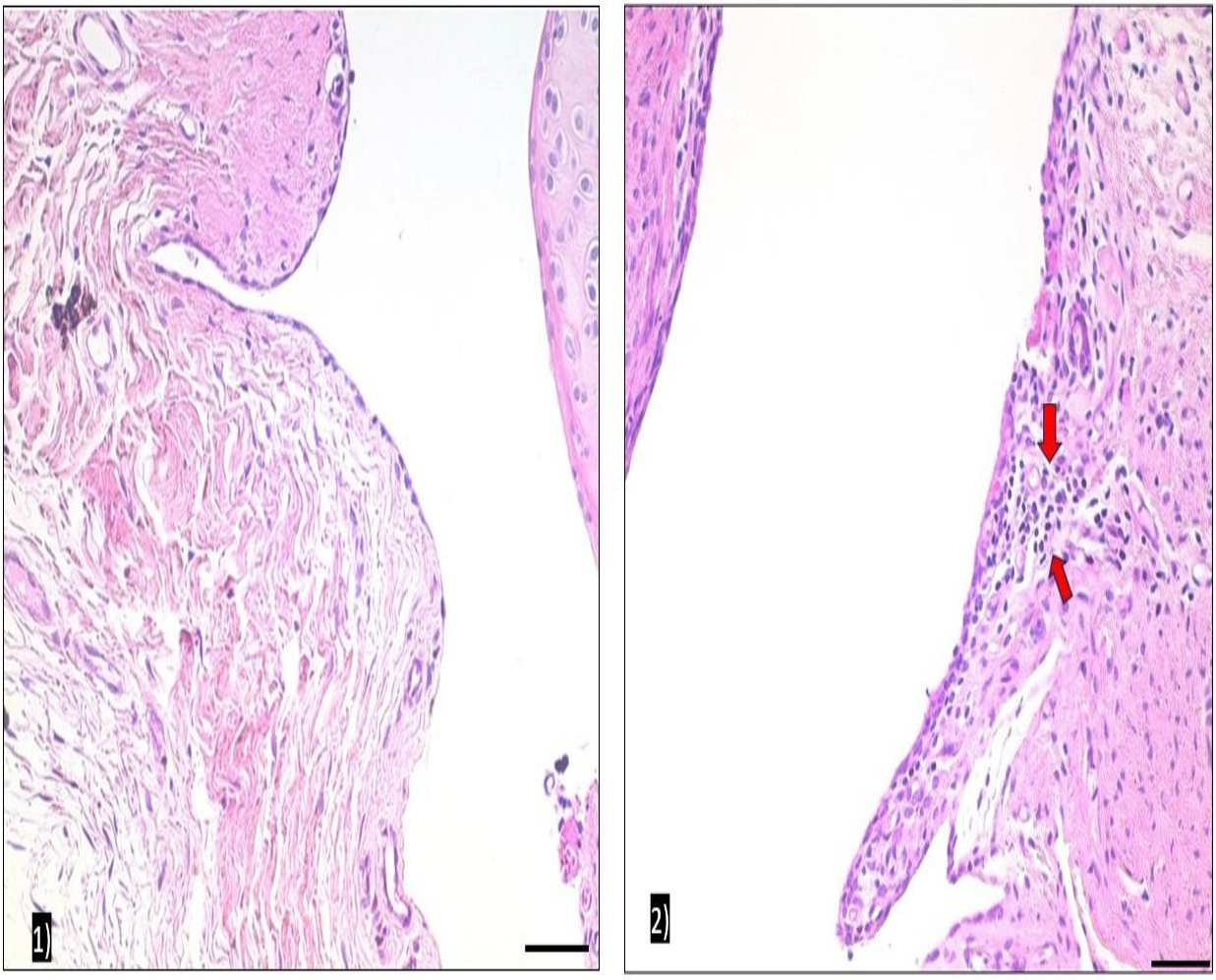
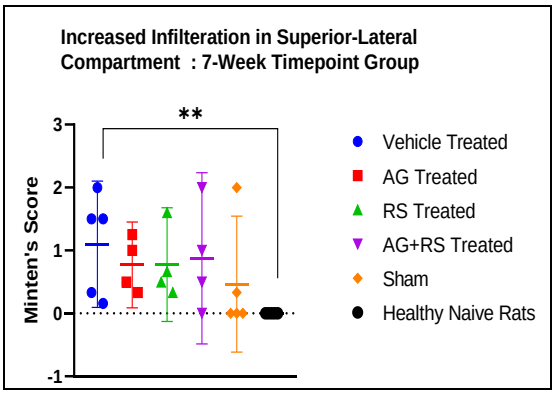
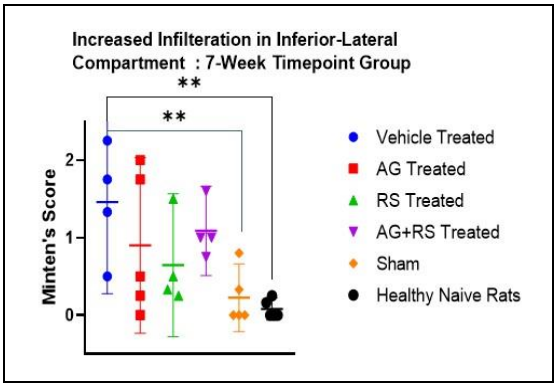
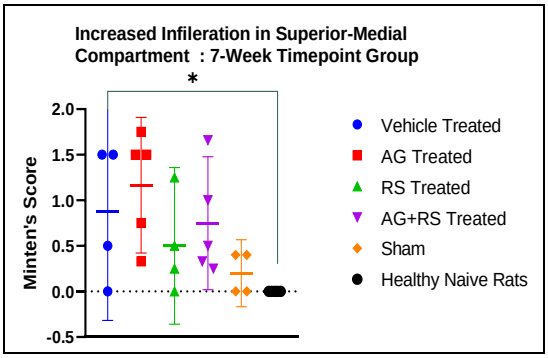
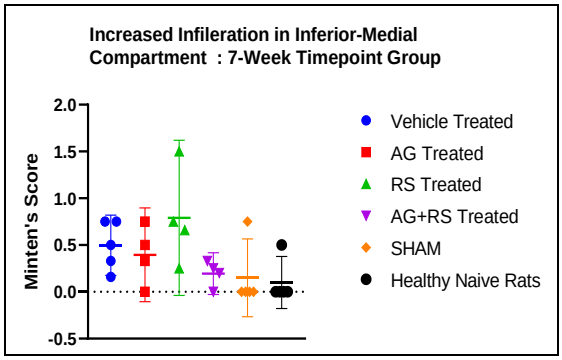


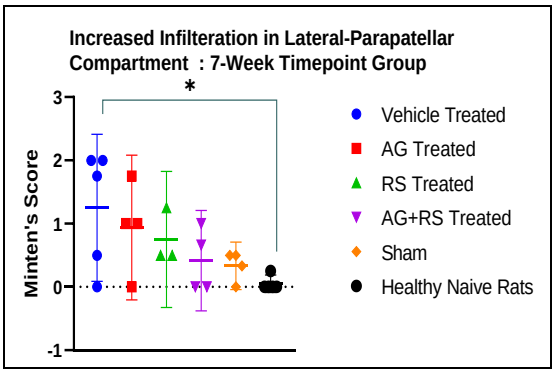
Fig 3.14 Healthy synovium (Image 1) vs synovial membrane with increased infiltration (Image 2) in response to inflammation. Red arrow indicates increased influx of monocytes and other cells at the site of inflammation. Both images are taken from the 10-week timeline group from inferior lateral compartment. Scale bar-100µm.

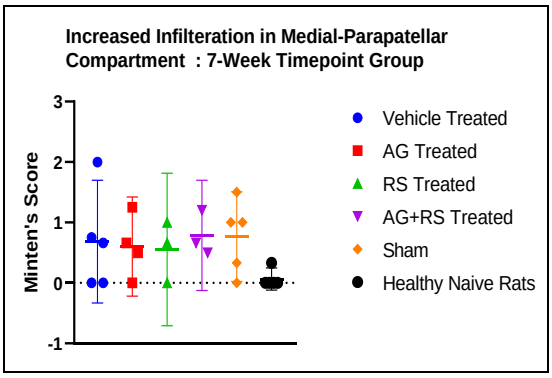

Increased Infilteration in Superior-Lateral Compartment : 7-Week Timepoint Group
Minten's Score
**
Vehicle Treated
AG Treated
RS Treated
AG+RS Treated
Sham
Healthy Naive Rats

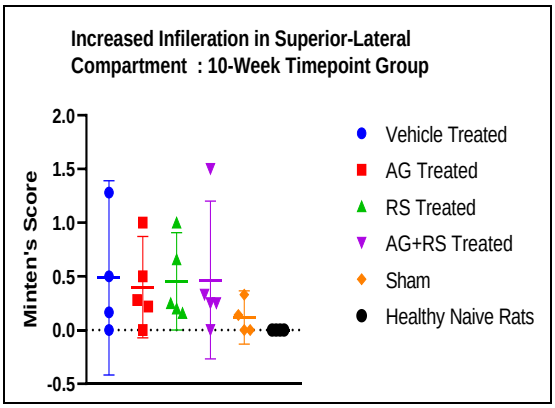

Increased Infilteration in Inferior-Lateral Compartment : 7-Week Timepoint Group
Minten's Score
**
**
Vehicle Treated
AG Treated
RS Treated
AG+RS Treated
Sham
Healthy Naive Rats

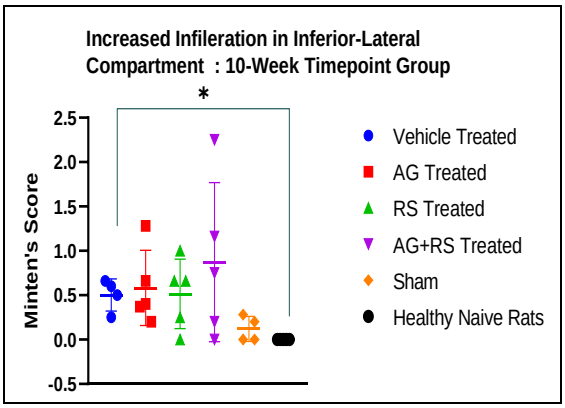

Increased Infileration in Superior-Medial Compartment : 7-Week Timepoint Group
Minten's Score
*
Vehicle Treated
AG Treated
RS Treated
AG+RS Treated
Sham
Healthy Naive Rats


Increased Infileration in Inferior-Medial Compartment : 7-Week Timepoint Group
Minten's Score
Vehicle Treated
AG Treated
RS Treated
AG+RS Treated
SHAM
Healthy Naive Rats


Increased Infilteration in Lateral-Parapatellar Compartment : 7-Week Timepoint Group
Minten's Score
*
Vehicle Treated
AG Treated
RS Treated
AG+RS Treated
Sham
Healthy Naive Rats


Increased Infilteration in Medial-Parapatellar Compartment : 7-Week Timepoint Group
Minten's Score
Vehicle Treated
AG Treated
RS Treated
AG+RS Treated
Sham
Healthy Naive Rats


Increased Infileration in Superior-Lateral Compartment : 10-Week Timepoint Group
Minten's Score
Vehicle Treated
AG Treated
RS Treated
AG+RS Treated
Sham
Healthy Naive Rats


Increased Infileration in Inferior-Lateral Compartment : 10-Week Timepoint Group
Minten's Score
*
Vehicle Treated
AG Treated
RS Treated
AG+RS Treated
Sham
Healthy Naive Rats

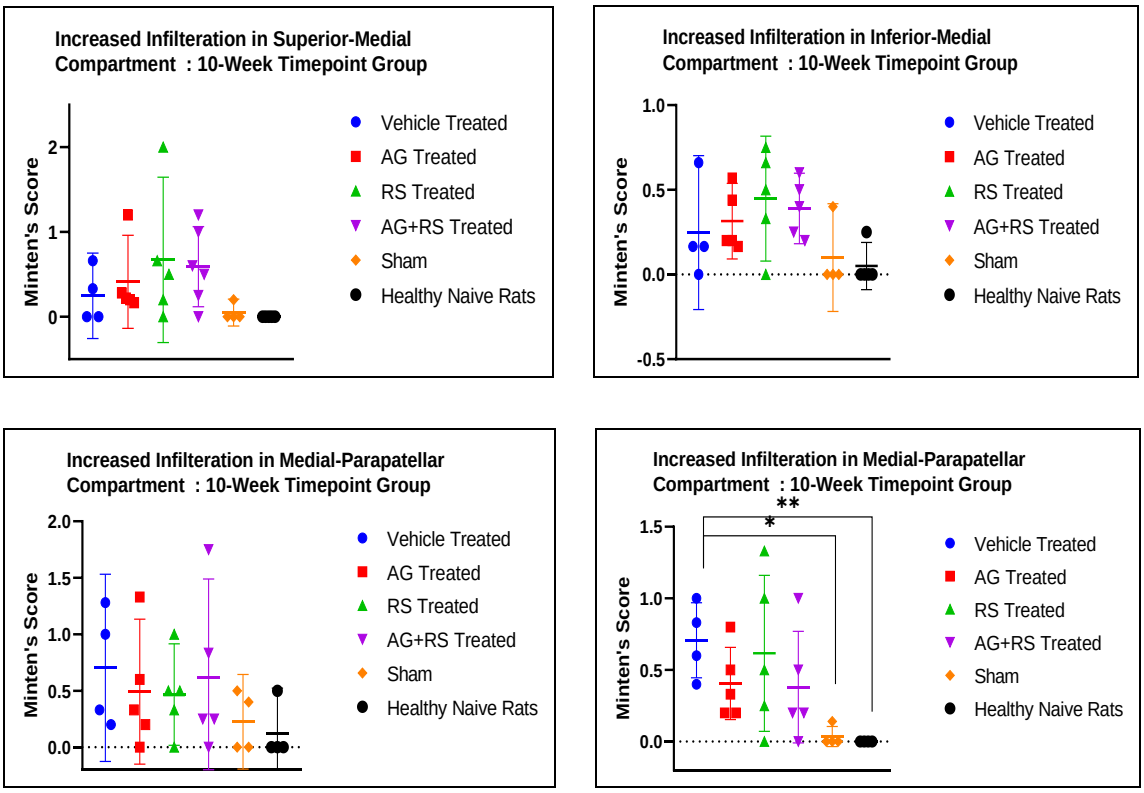


Figure 3.15 Minten's scores representing increased infiltration of monocytes and macrophages in response to inflammation in different compartments within the knee joint. One-way ANOVA and Kruskal-Wallis test along with Dunnett's and Dunn's multiple comparison test was performed in both 7-week and 10-week time points in different treatment groups. No statistically significant differences were seen on comparing vehicle group with other treatment groups ($p>0.05$), indicating the presence of inflammation in synovial lining. N>4

3.3.3 None of the treatment groups were successful in preventing the Fibrin deposition

The third parameter for scoring synovitis is the deposition of fibrin. The data obtained from scoring this parameter indicated that no significant difference is present between the vehicle & AG1478, RS504393 and both AG1478+RS504393 treated groups at both 7-week & 10-week timepoints (Figure 3.17). Comparison of sham-operated group with vehicle also showed insignificant differences in most of the compartments.

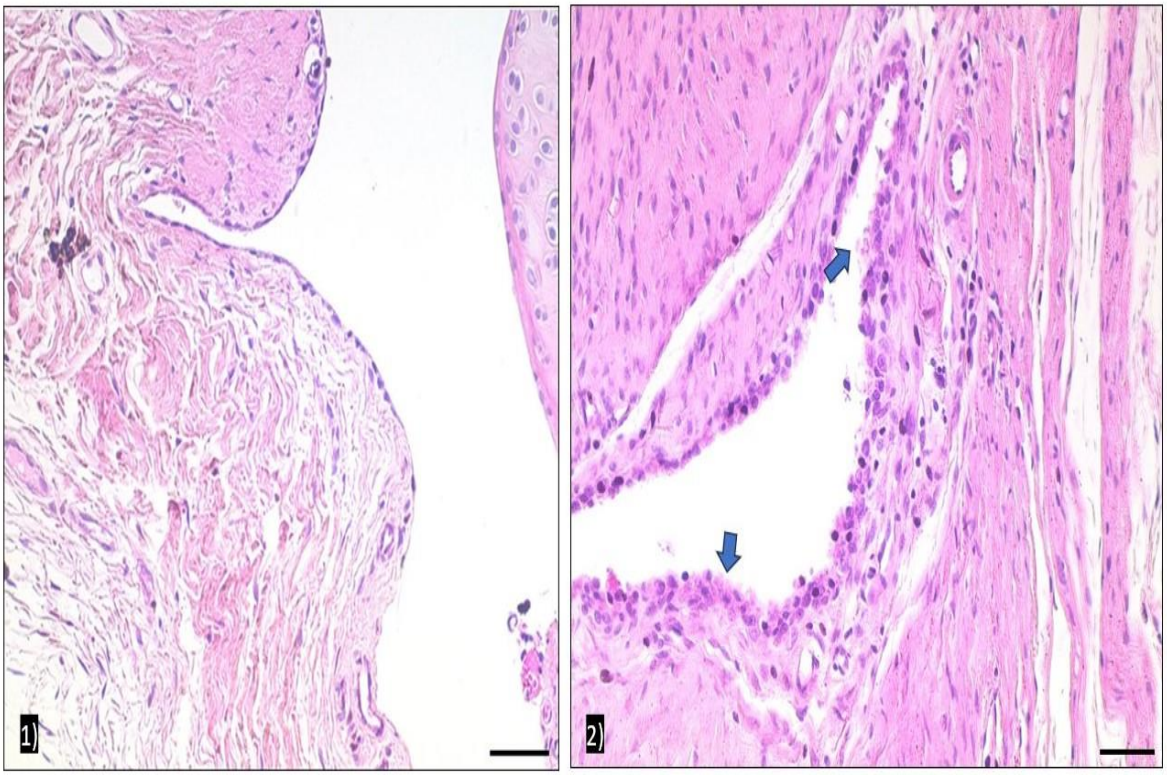
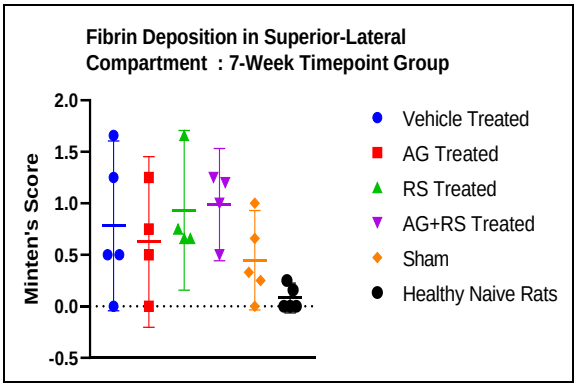
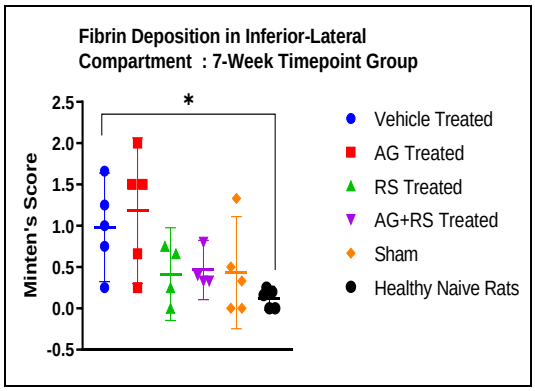
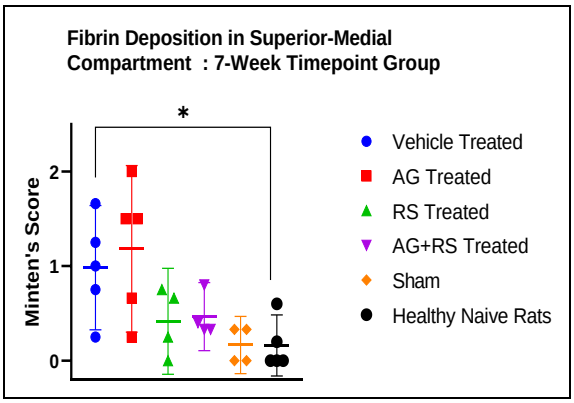
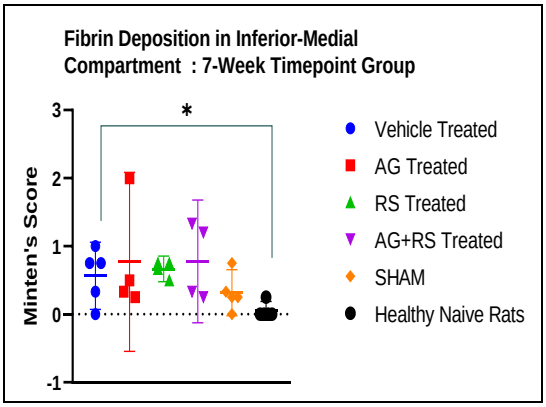


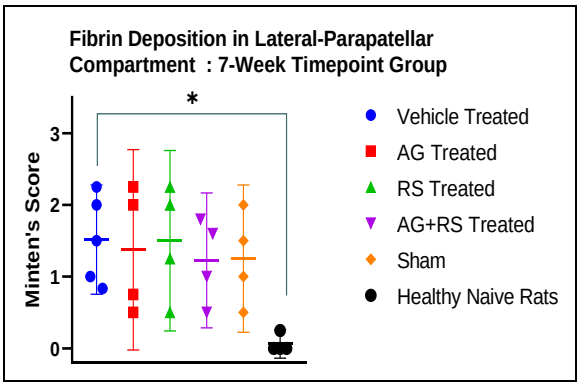
Fig 3.16 Healthy synovial lining vs inflamed membrane with the presence of Fibrin. Blue arrow indicates the presence of fibrin in the affected area. Images were taken from a 10-week timeline group with the help of Leica Application Suite Version 4.13 using objective lens with magnification power of 10X. Scale bar-100µm.

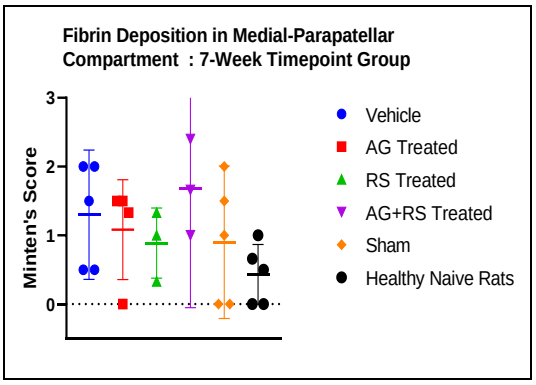

Fibrin Deposition in Superior-Lateral Compartment : 7-Week Timepoint Group
Minten's Score
2.0
1.5
1.0
0.5
0.0
-0.5
Vehicle Treated
AG Treated
RS Treated
AG+RS Treated
Sham
Healthy Naive Rats


Fibrin Deposition in Inferior-Lateral Compartment : 7-Week Timepoint Group
*
Minten's Score
2.5
2.0
1.5
1.0
0.5
0.0
-0.5
Vehicle Treated
AG Treated
RS Treated
AG+RS Treated
Sham
Healthy Naive Rats


Fibrin Deposition in Superior-Medial Compartment : 7-Week Timepoint Group
*
Minten's Score
2
1
0
Vehicle Treated
AG Treated
RS Treated
AG+RS Treated
Sham
Healthy Naive Rats


Fibrin Deposition in Inferior-Medial Compartment : 7-Week Timepoint Group
*
Minten's Score
3
2
1
0
-1
Vehicle Treated
AG Treated
RS Treated
AG+RS Treated
SHAM
Healthy Naive Rats


Fibrin Deposition in Lateral-Parapatellar Compartment : 7-Week Timepoint Group
*
Minten's Score
3
2
1
0
Vehicle Treated
AG Treated
RS Treated
AG+RS Treated
Sham
Healthy Naive Rats


Fibrin Deposition in Medial-Parapatellar Compartment : 7-Week Timepoint Group
Minten's Score
3
2
1
0
Vehicle
AG Treated
RS Treated
AG+RS Treated
Sham
Healthy Naive Rats

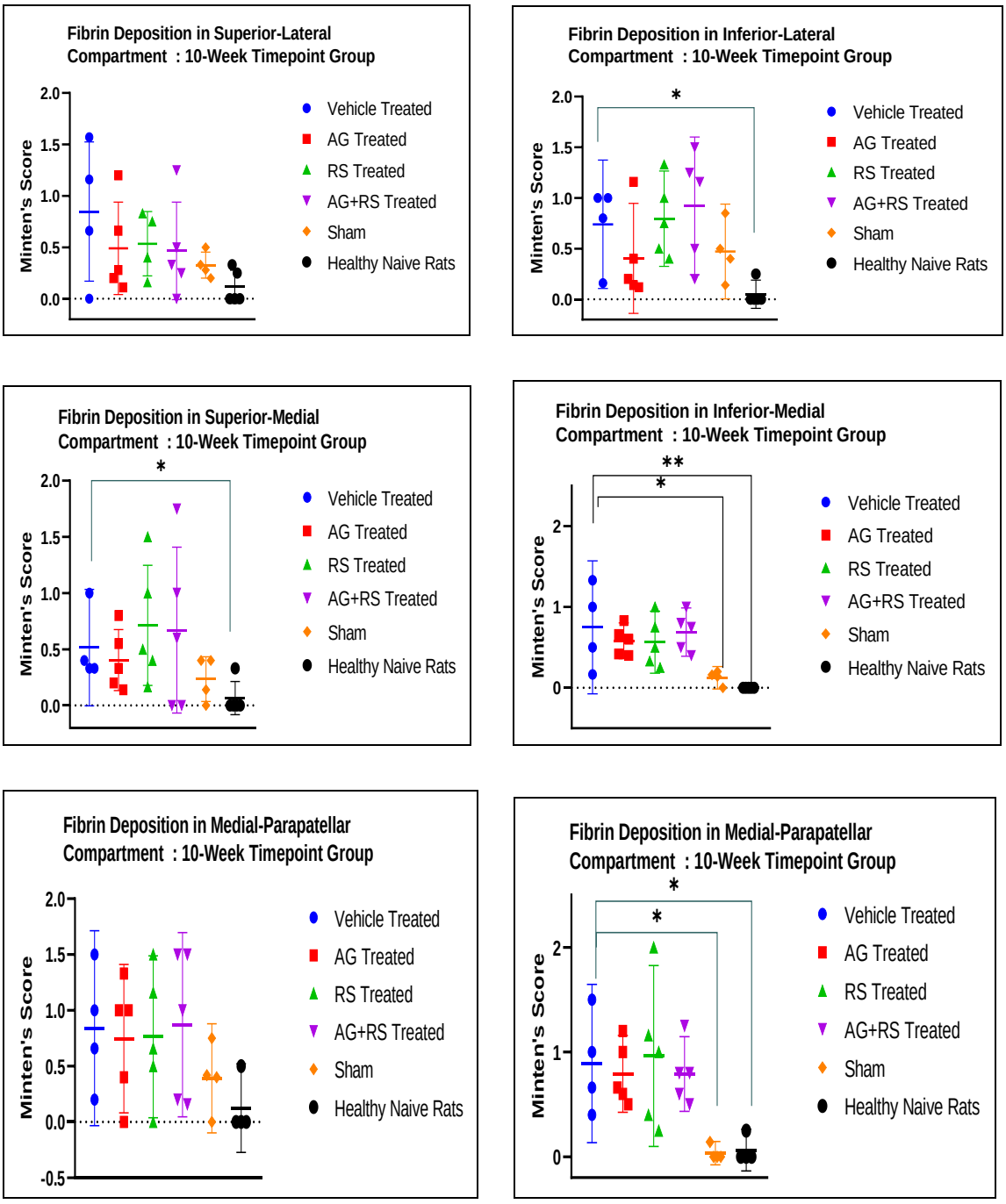


Figure 3.17 Histologically assessed scores for Fibrin Deposition. One-way ANOVA and Kruskal-Wallis test with Dunnett's and Dunn's multiple comparison test was carried out for statistical significance in all 6 compartments at 7w and 10W timepoints between different treatment groups. However, insignificant differences were seen between vehicle and other treated groups in most of the compartments ($p>0.05$) at both timepoints. N>4

3.3.4 AG1478 treatment group reduced vascularization in superior-lateral compartment

The fourth parameter is increased vascularization near intimal lining as a result of inflammation. In this parameter, newly formed blood vessels near the intimal layer were considered. After scoring, the data confirmed that vehicle and other treatments groups have insignificant differences in most of the compartments, while the superior lateral compartment has shown significantly less vascularization in AG1478 treated group ($p \leq 0.01$) and RS504393 treatment group ($p \leq 0.05$) as compared to vehicle treated group.

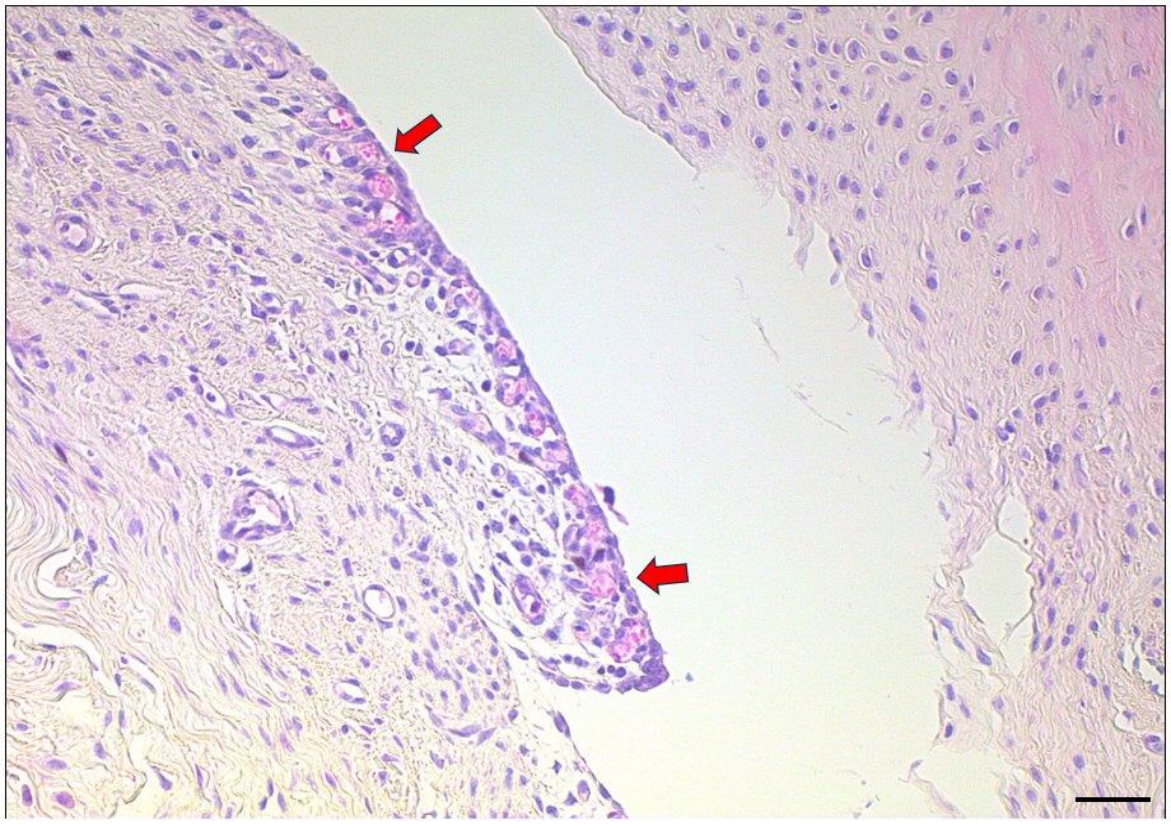
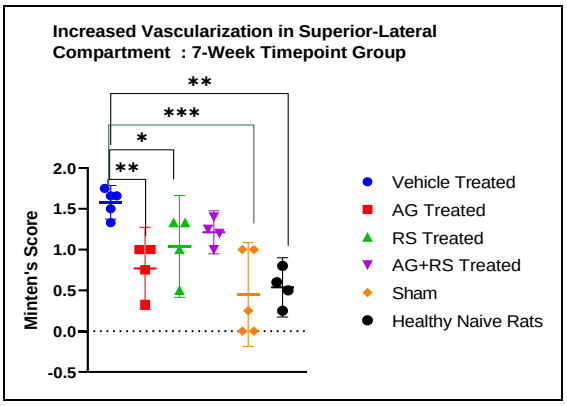
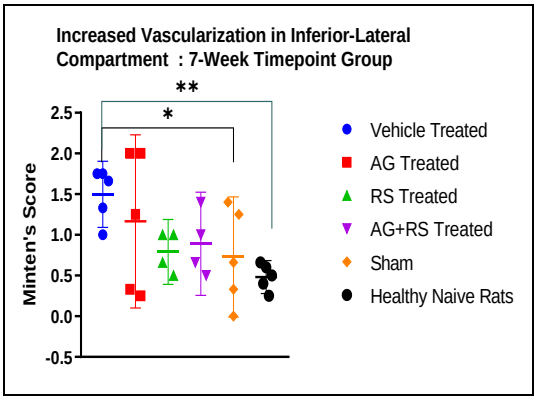
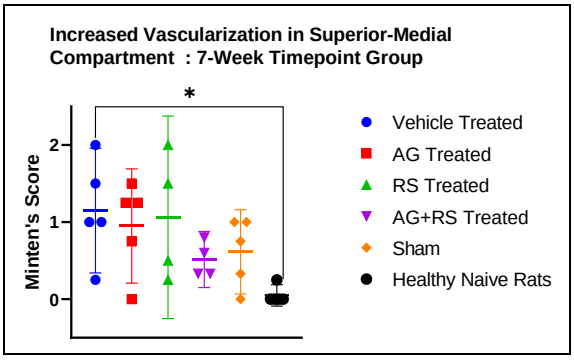
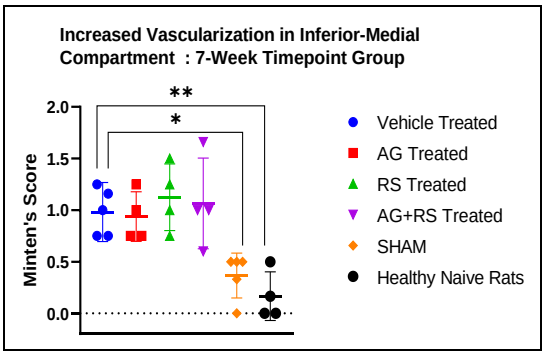


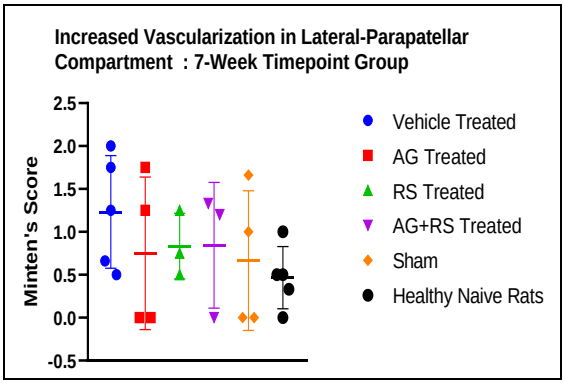
Fig 3.18 Increased vascularization in RS504393 treated group (CCR2 antagonist) from 7week timeline rats. Red arrows indicate the presence of newly formed blood vessels near intimal layer of synovial membrane in response to inflammation. Scale bar-100µm.

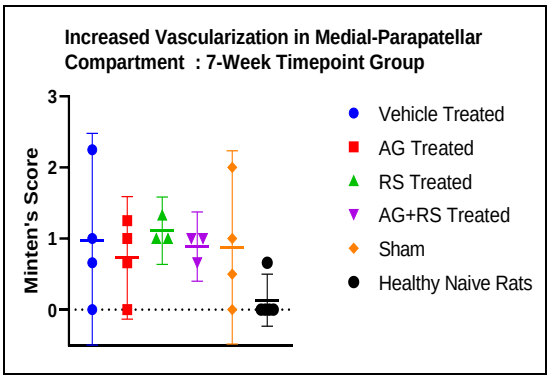

Increased Vascularization in Superior-Lateral Compartment : 7-Week Timepoint Group
Minten's Score
Vehicle Treated
AG Treated
RS Treated
AG+RS Treated
Sham
Healthy Naive Rats

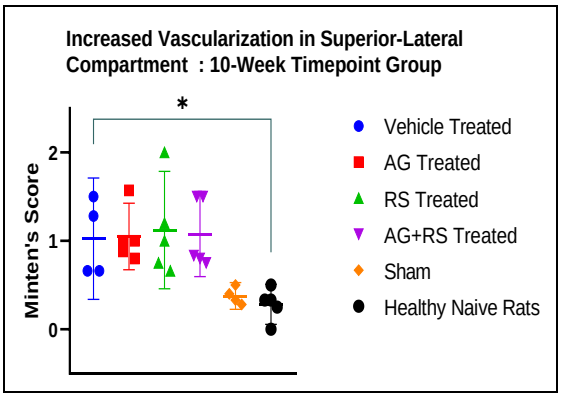

Increased Vascularization in Inferior-Lateral Compartment : 7-Week Timepoint Group
Minten's Score
Vehicle Treated
AG Treated
RS Treated
AG+RS Treated
Sham
Healthy Naive Rats

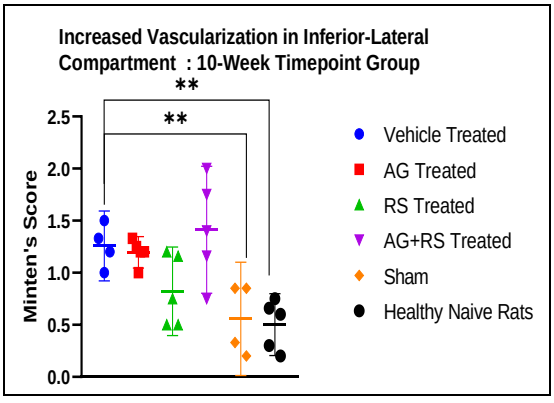

Increased Vascularization in Superior-Medial Compartment : 7-Week Timepoint Group
Minten's Score
Vehicle Treated
AG Treated
RS Treated
AG+RS Treated
Sham
Healthy Naive Rats


Increased Vascularization in Inferior-Medial Compartment : 7-Week Timepoint Group
Minten's Score
Vehicle Treated
AG Treated
RS Treated
AG+RS Treated
SHAM
Healthy Naive Rats


Increased Vascularization in Lateral-Parapatellar Compartment : 7-Week Timepoint Group
Minten's Score
Vehicle Treated
AG Treated
RS Treated
AG+RS Treated
Sham
Healthy Naive Rats


Increased Vascularization in Medial-Parapatellar Compartment : 7-Week Timepoint Group
Minten's Score
Vehicle Treated
AG Treated
RS Treated
AG+RS Treated
Sham
Healthy Naive Rats


Increased Vascularization in Superior-Lateral Compartment : 10-Week Timepoint Group
Minten's Score
Vehicle Treated
AG Treated
RS Treated
AG+RS Treated
Sham
Healthy Naive Rats


Increased Vascularization in Inferior-Lateral Compartment : 10-Week Timepoint Group
Minten's Score
Vehicle Treated
AG Treated
RS Treated
AG+RS Treated
Sham
Healthy Naive Rats

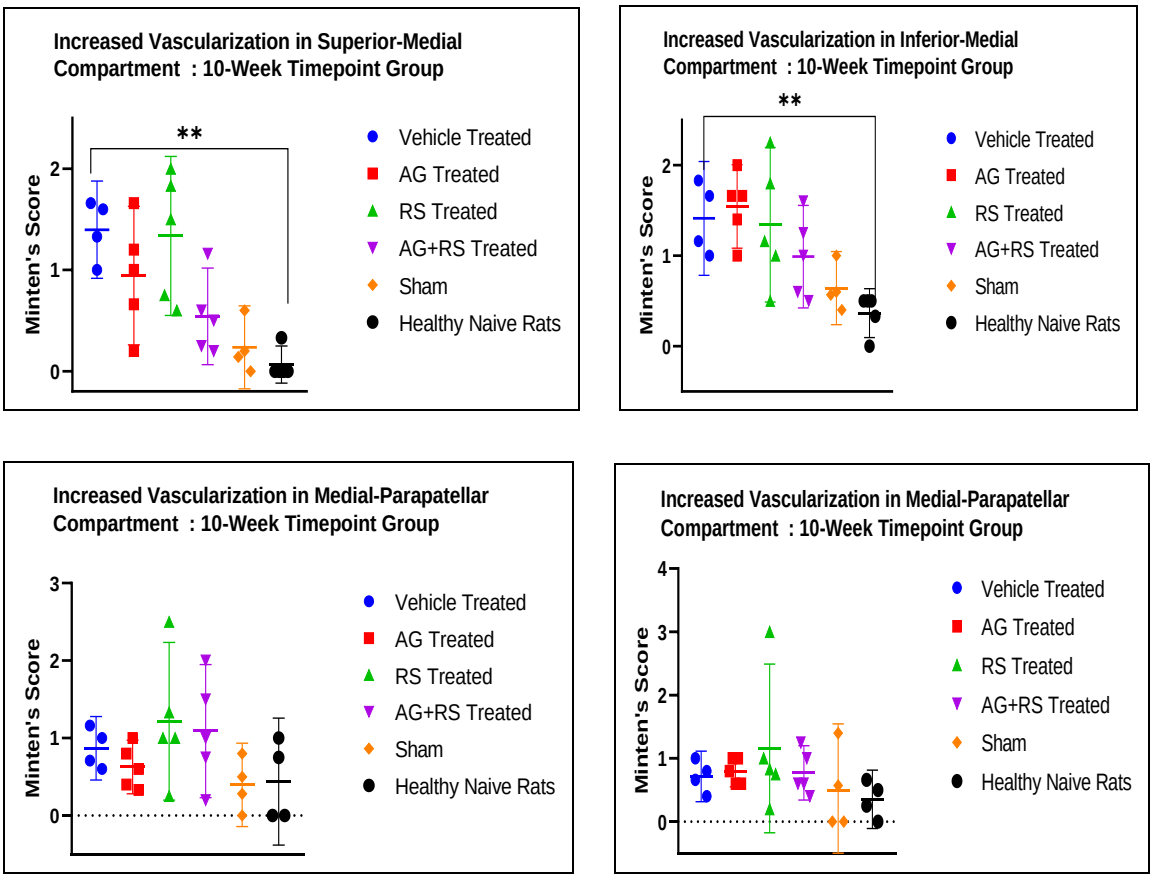


Figure 3.19 Minten's scores for increased vascularization parameter at both 7-week and 10-week timepoints. Kruskal-Wallis test was performed along Dunn's multiple comparison test to analyze the statistical significance between different treatment groups. EGFR inhibited (AG1478 treated) group shows significantly less vascularization in superior-lateral compartment as compared to vehicle ($p \leq 0.01$) and other treatment groups at 7-week timepoint group. CCR2 inhibited group also shows reduced vascularization in the same compartment ($p \leq 0.01$) at 7-week timepoint. No significant differences were seen between vehicle and other treatment groups at 10-week timepoint. N>4

3.3.5 No substantial difference is seen between vehicle and other treatment groups

The presence of fibrotic tissue marked the presence of advanced form of inflammation and is the fifth parameter scored. Only a fewer number of animals showed the presence of fibrotic tissue (Figure 3.20, Figure 3.21) in the combinational treatment group, although statistically insignificant when compared with vehicle treatment group.

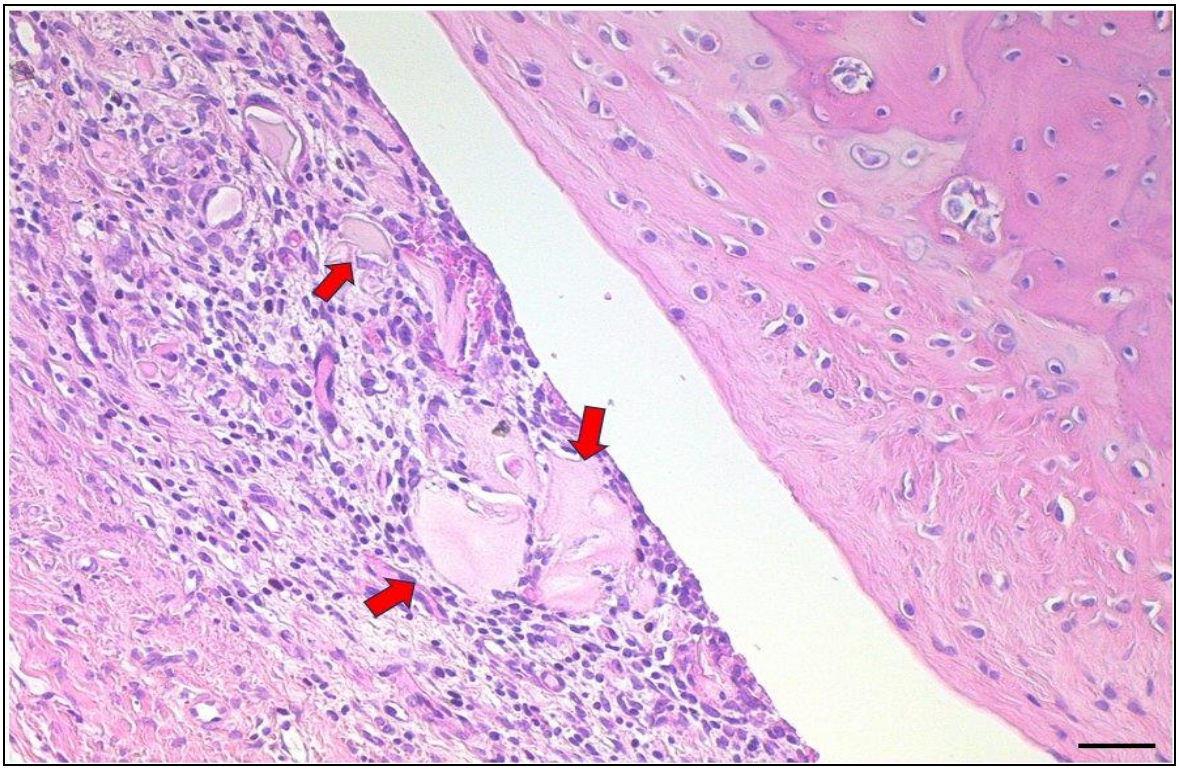
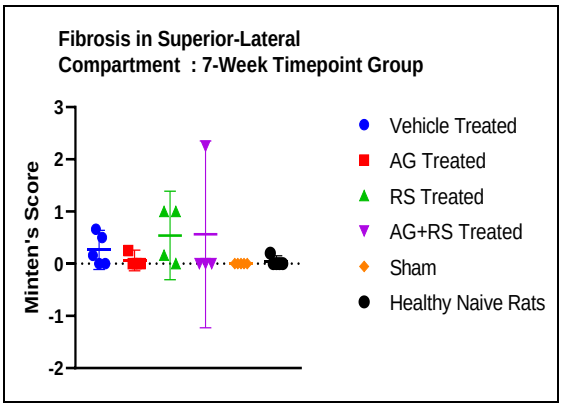
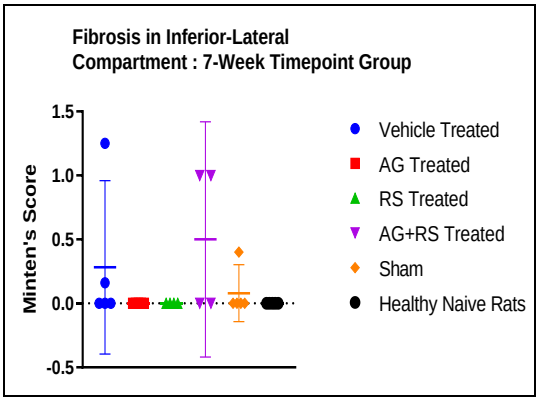
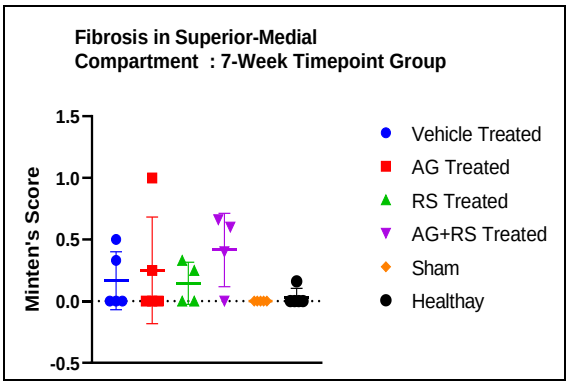
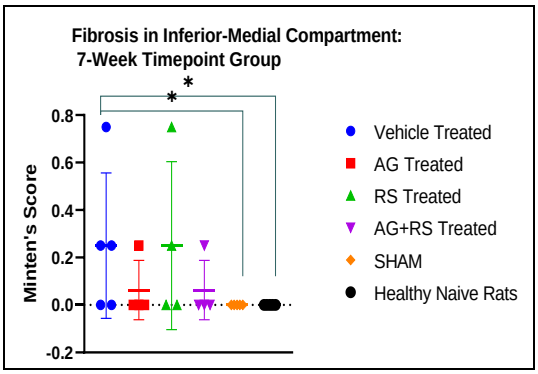


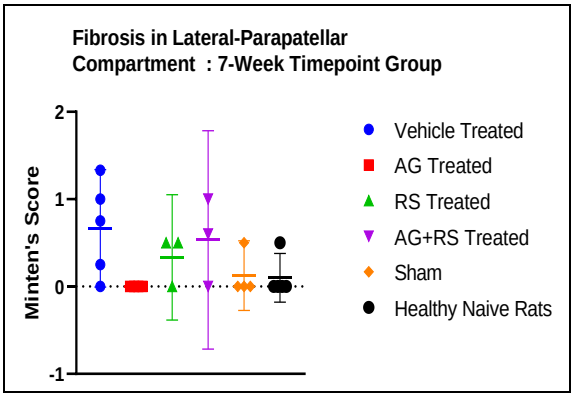
Fig 3.20 Presence of fibrotic tissue in an inflamed synovial membrane (indicated by red arrows) of 10-week timeline group in AG1478+RS504393 (combinational inhibition of EGFR & CCR2) treatment group. Scale bar-100µm.

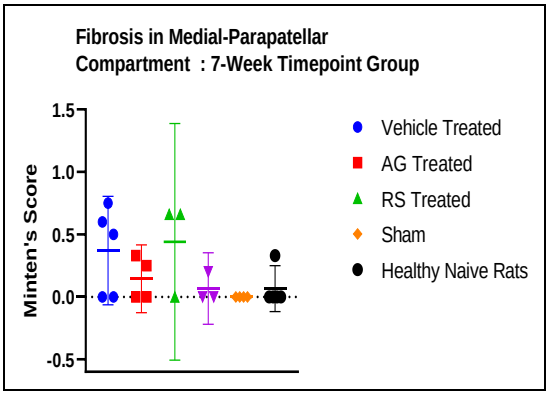

Fibrosis in Superior-Lateral
Compartment : 7-Week Timepoint Group
Minten's Score
3
2
1
0
-1
-2
Vehicle Treated
AG Treated
RS Treated
AG+RS Treated
Sham
Healthy Naive Rats

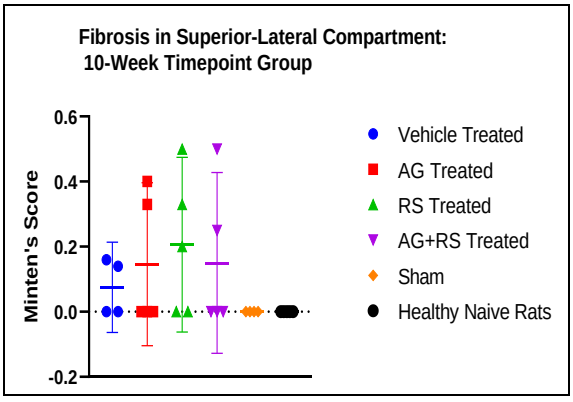

Fibrosis in Inferior-Lateral
Compartment : 7-Week Timepoint Group
Minten's Score
1.5
1.0
0.5
0.0
-0.5
Vehicle Treated
AG Treated
RS Treated
AG+RS Treated
Sham
Healthy Naive Rats

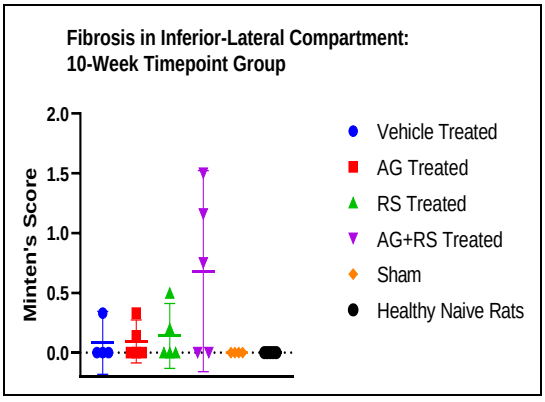

Fibrosis in Superior-Medial
Compartment : 7-Week Timepoint Group
Minten's Score
1.5
1.0
0.5
0.0
-0.5
Vehicle Treated
AG Treated
RS Treated
AG+RS Treated
Sham
Healthay


Fibrosis in Inferior-Medial Compartment:
7-Week Timepoint Group
*
*
Minten's Score
0.8
0.6
0.4
0.2
0.0
-0.2
Vehicle Treated
AG Treated
RS Treated
AG+RS Treated
SHAM
Healthy Naive Rats


Fibrosis in Lateral-Parapatellar
Compartment : 7-Week Timepoint Group
Minten's Score
2
1
0
-1
Vehicle Treated
AG Treated
RS Treated
AG+RS Treated
Sham
Healthy Naive Rats


Fibrosis in Medial-Parapatellar
Compartment : 7-Week Timepoint Group
Minten's Score
1.5
1.0
0.5
0.0
-0.5
Vehicle Treated
AG Treated
RS Treated
Sham
Healthy Naive Rats


Fibrosis in Superior-Lateral Compartment:
10-Week Timepoint Group
Minten's Score
0.6
0.4
0.2
0.0
-0.2
Vehicle Treated
AG Treated
RS Treated
AG+RS Treated
Sham
Healthy Naive Rats


Fibrosis in Inferior-Lateral Compartment:
10-Week Timepoint Group
Minten's Score
2.0
1.5
1.0
0.5
0.0
Vehicle Treated
AG Treated
RS Treated
AG+RS Treated
Sham
Healthy Naive Rats

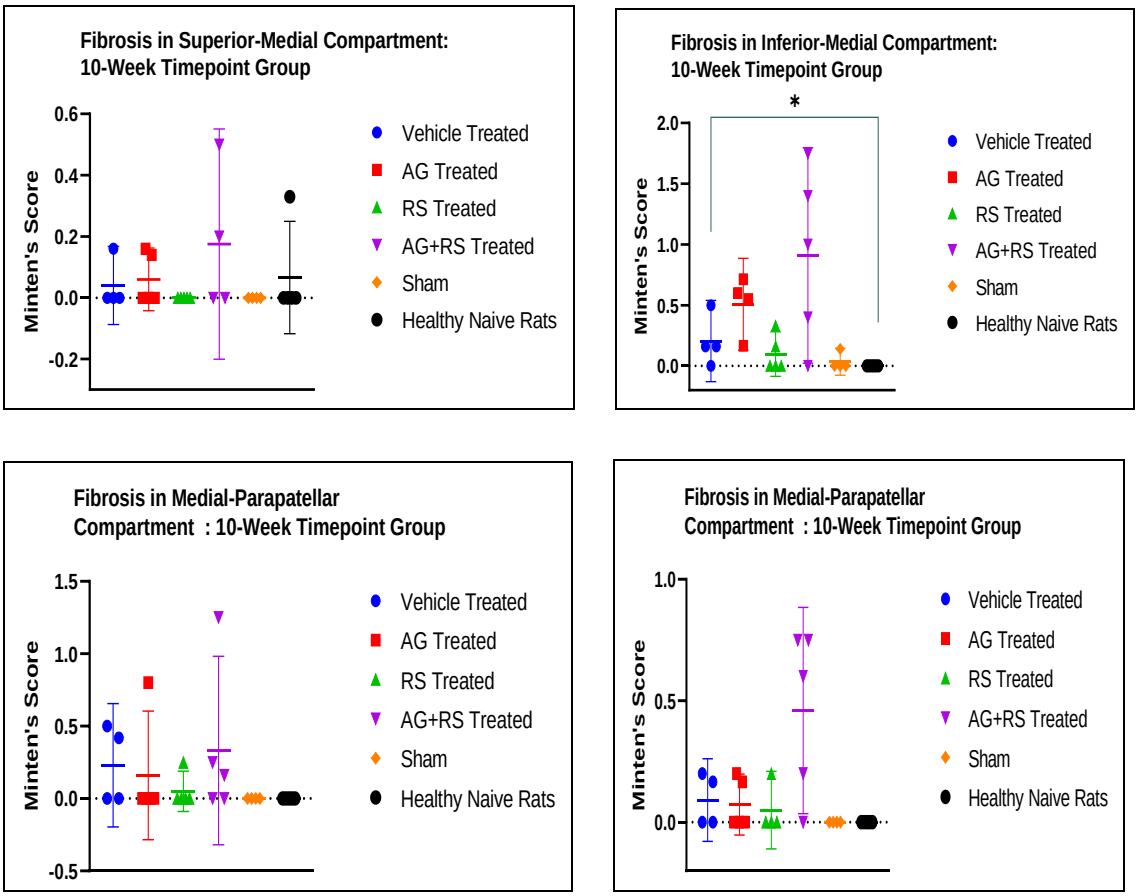
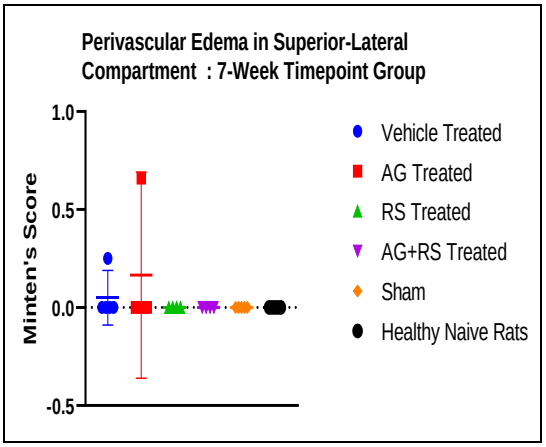


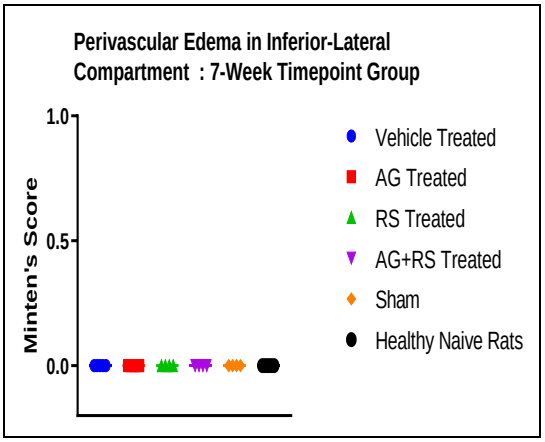
Figure 3.21 Presence of fibrotic tissue was seen in treated groups on histological assessment of H&E-Stained samples. To test for statistical significance, One-Way ANOVA and Kruskal-Wallis test was carried out along Dunnett's and Dunn's multiple comparison test for both 7- and 10-week timepoints. No significant difference is observed in AG1478 or RS504393 treated groups vs vehicle group. N>4

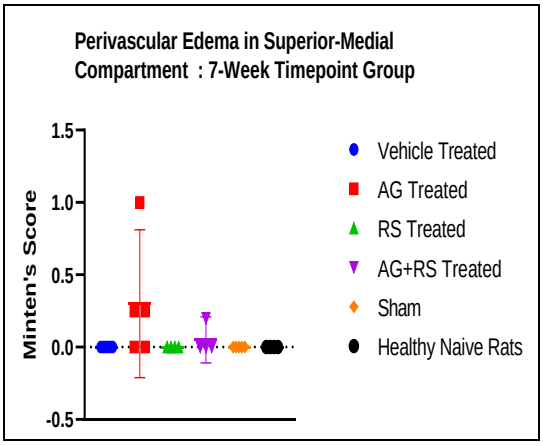
3.3.6 Perivascular edema is not evident in any treatment group

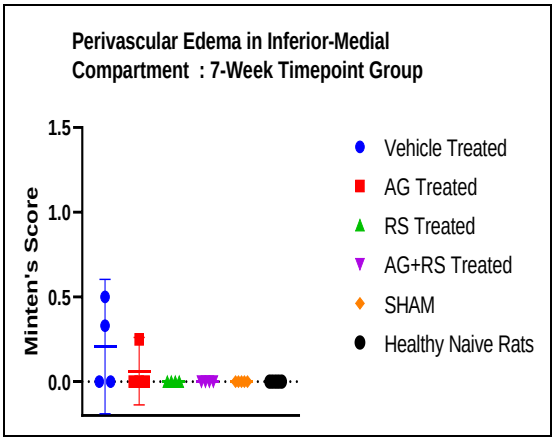
The sixth parameter considered in synovitis scoring was the presence of perivascular edema. In our rat groups, we have not witnessed the significant presence of perivascular edema in any of the groups. On comparison with vehicle group, the p-value was >0.05 for all treated groups and non-OA healthy groups (Figure 3.22).

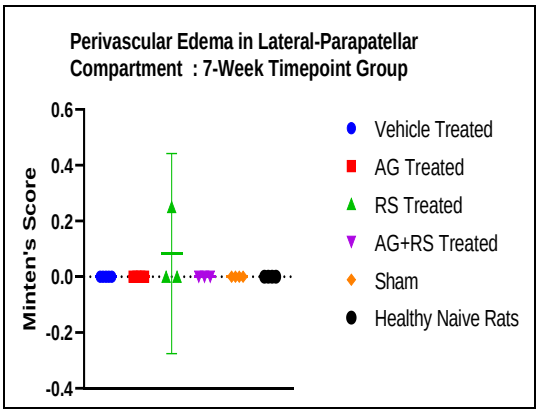
Thus, our results from synovitis scoring indicate that EGFR, CCR2 or combinational blockade do not protect the synovial membrane from inflammation.

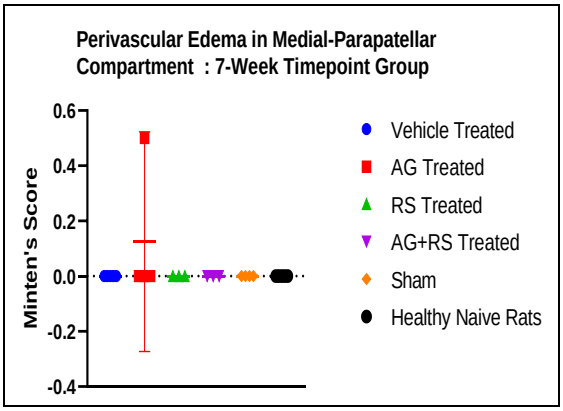












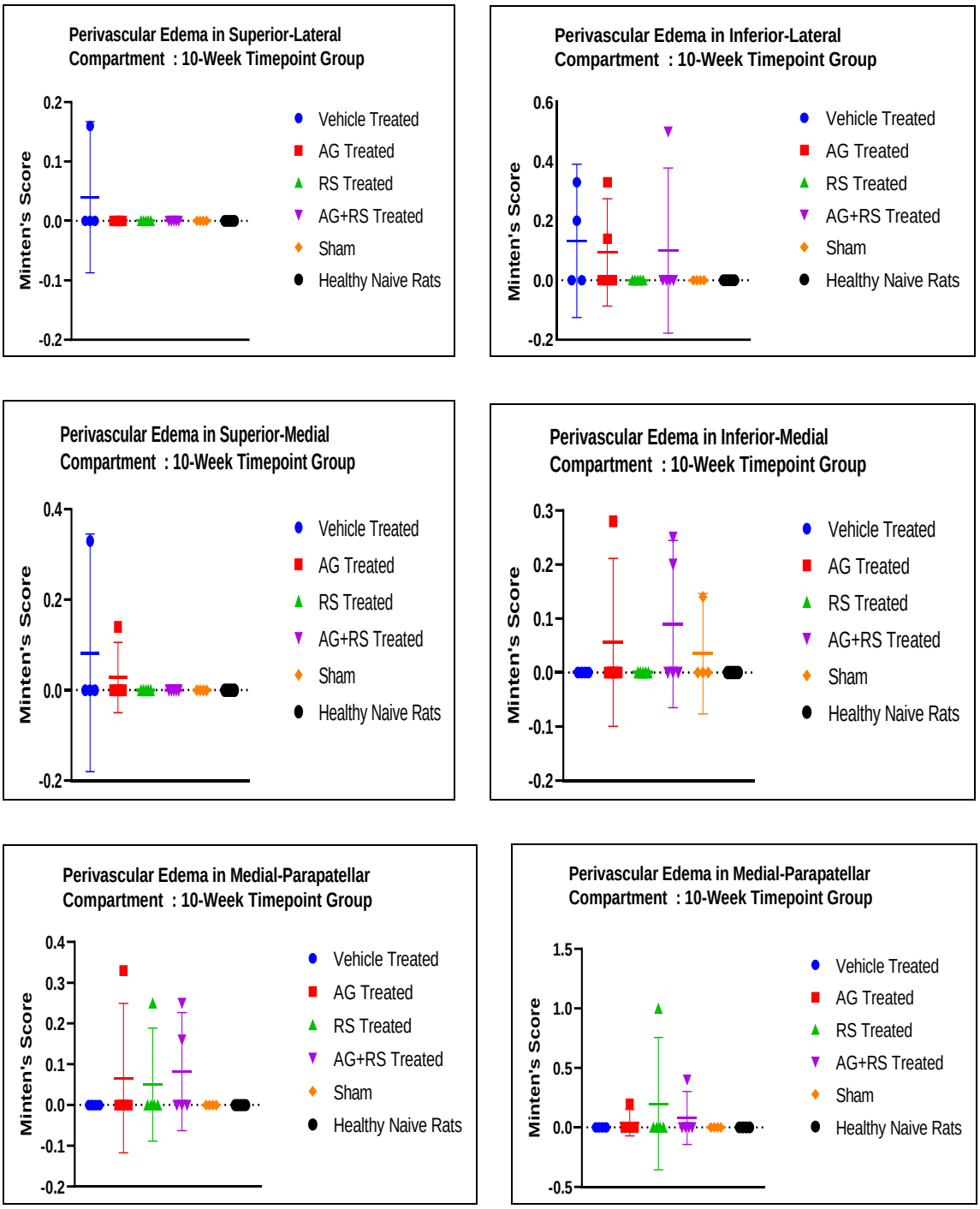


Fig 3.22 Minten's scores for the presence of Perivascular Edema on H&E-Stained samples. No significant differences were observed when vehicle group was compared with other treatment groups (n>0.05) when tested for statistical significance using One-Way ANOVA and Kruskal-Wallis test based on normal distribution curve along with Dunnett's and Dunn's multiple comparison testing. N>4

4.0 DISCUSSION

4.1 Discussion

Investigation into EGFR and CCR2 signaling has provided insights into the crucial role of these receptors in the pathophysiology of OA. Based on the research conducted by Appleton et.al 2015, targeting EGFR and CCR2 axis has provided some protection against the development of OA when treatment starts just after surgery.[101] Thus, we decided to target the same axis, but initiation of treatment administration starts one-month post-surgery, as it is more translatable situation to humans. Based on this, we hypothesized that inhibition of EGFR and CCR2 will reduce the severity of PTOA. We decided to use AG1478 and RS504393 to block EGFR and CCR2, respectively, along with combinational treatment of both AG1478 and RS504393 to target both receptors together. We set two timepoints, at 7-weeks and 10-weeks, to determine the effects of EGFR and CCR2 inhibitors.

Our first aim set out to analyze the damage in the cartilage. To achieve this, we performed histological assessment of cartilage based on the guidelines provided by OARSI.[41] Our results from cartilage scoring, however, indicate that the inhibition of EGFR, CCR2 or the combinational inhibition of EGFR and CCR2 have not imparted any protection against cartilage damage (Fig 3.3, 3.4), or against subchondral bone damage (Fig. 3.8). However, we have noticed a mild decrease in osteophyte formation in EGFR inhibited group on tibial bone at 7-week as well as 10-week time points (Figure 3.6).

We further performed the measurement of cartilage area on femur and tibia using Osteomeasure. Our results have not shown statistically significant differences in cartilage area when the treated groups compared with vehicle group at both timepoints (3.10).

Our next aim was to analyze the damage in the synovium. To achieve this, histological evaluation was performed on H&E stained samples using Minten's scoring system for synovitis.[108] The result of synovial scoring implies that inhibition of EGFR/CCR2 axis has not offered protection against synovial inflammation. All three treatments have

shown significant synovial hyperplasia, fibrin deposition, escalated infiltration of monocytes and macrophages and even milder formation of fibrotic tissue. Although, we witnessed less vascularization in the EGFR inhibited group (AG1478 treatment), this occured just in the superior-lateral compartment at the 7-week timepoint. This limited protection does not necessarily indicate overall efficacy of AG1478 in reducing vascularization as it was still evident in all other compartments (Figure 3.19). Also, this reduction is not seen in 10-week timepoint group within same compartment (Figure 3.19).

EGFR is known for its context specific role in maintaining joint homeostasis.[83] We know that EGFR has both catabolic and anabolic activities in cartilage but the driving force that initiates catabolic activities including EGFR mediated suppression of SOX-9 (a key transcription factor for the synthesis of collagen-II, aggrecan and matrix proteins) is still unresolved.

While scoring for cartilage damage, we have witnessed reduction in osteophyte size in EGFR-inhibited group at both timepoints (Figure 3.6). Osteophytes are the bone spurs that are capped with fibrocartilage and develop in response to damage in articular cartilage.[110] However, it is not yet clear whether the formation of osteophyte is a functional adaptation in response to damage. Mesenchymal stem cells (MSCs) from the synovium and periosteal cells covering the bone surface are considered as precursor cells in the process of chondrogenesis and formation of osteophytes.[110] During this process, some growth factors are expressed such as IGF-2, TGF-beta, leptin etc. that are believed to be the driving force for the differentiation of MSCs.[111] TGF-beta has the capacity to transactivate EGFR which could provide a possible explanation for the reduction in osteophyte size in the EGFR inhibited group.[110] But researchers have also found that EGFR downregulates TGF-beta signaling and thus the cross-talks between EGFR and TGF-beta in this context is not yet clear.[110][111]

Based on our results and research conducted by previous lab members and other researchers, the context-specific role of EGFR could be influenced by dose of EGFR

inhibitor, type and stage of OA, and time at which drug administration starts.[71][101][112][113]

Zhang X et.al (2014) have given Gefitinib, FDA-approved EGFR inhibitor, 100mg/kg every other day in 3 month old male mice (DMM model) for 12 weeks and witnessed accelerated OA.[112] Similar experiment was conducted by Heng Sun (2018) in 2 month old mice, but with much lesser dose of Gefitinib.[113] They administered 25mg/kg/day for up to 8 weeks and found out reduction in OA severity.[113] This suggests that moderate suppression of EGFR appears to yield protective anabolic effects while excessive inhibition in OA has deleterious effects.

Apart from this, it also depends on the type of OA. Usmani et.al 2016 conducted an experiment on 10-week old TGF-α knockout mice after DMM surgery and found out that these TGF-α knock-out mice were protected from development of OA but this protection was not seen in 6-month old TGF-α null mice that showed significant cartilage damage and development of OA.[71] This suggests that inhibition of EGFR in PTOA seems to have protective effects, but only in young adult mice.

The third important factor is the time of initiation of treatment. Appleton attempted to block EGFR with AG1478 in male rats at the time of injury and found less severe OA at 4 and 7 weeks.[101] However, he witnessed no reduction at 10-week timeline group. In our experiment, we initiated drug administration one-month post-surgery, and we did not witness any protection or delay in the advancement of OA in 7-week or 10-week timepoint rats. This all implies that these factors likely play a significant role in shaping the impact of EGFR. It is also to bear in mind that EGF receptors belong to the class of erbB4s receptors that show structural and functional similarity with the other three subtypes of erbB receptors (erbB1, erbB2, erbB3).[83] Their ability to form heterodimers and transactivate one another adds another line of complexity in understanding EGFR signaling.[72][75]

In this thesis, we also witnessed that inhibition of CCL2 has neither provided any protection against the cartilage loss or chondrocyte cell death nor proven any role in alleviating inflammation in the synovial lining. However, many studies have shown that

CCL2 and its receptor are heavily regulated in post-traumatic inflammation and OA by recruiting monocytes and macrophages, increasing collagenase activity and inhibiting proteoglycan synthesis in response to injury. Zhang, Y et.al (2023) have shown in both in-vitro model and in mice that CCL2 increases VCAM-1 expression, induces MMP-3 secretion, inhibits proteoglycan synthesis, increases collagenase activity, all factors that damages cartilage and contribute to OA.[91] They have also shown that CCL2 is responsible for cellular chemotaxis and plays key role in attracting monocytes and macrophages to the OA joint. [91]

One possible explanation that CCL2 inhibition has not provided protection in our experiment, would be the lack of direct association of CCL2 with its receptor CCR2. D. T. Graves et.al mentioned in his research that CCL2 is not only a ligand to CCR2 but it can also bind to CCR4 receptor.[114] Jablonski in his research, also showed that the macrophages showed variance in number and expression post full cartilage defect injuries in CCL2, CCR2 and CCL2/CCR2 deficient mice.[103] They also found that CCL2 performed defensive role and is essential for proliferation of mesenchymal stem cells for cartilage renewal while CCR2 have prevented the differentiation of these cells post-injury which otherwise are important for cartilage regeneration. [103] They also published that CCL2 favors MMP3 and MMP13 expression in chondrocytes. Harris et.al also published supportive results pointing towards the inhibition of MSCs by CCR2.[102]

A comparable study to our research was conducted by L.Longobardi et.al (2017) but used a different surgical model.[105] They gave RS404393 as CCR2 inhibitor in male mice with DMM surgery and found out that blockade of CCR2 axis has not provided any protection against OA in the later stages (8 to 12-week after surgery), while during earlier stages (4-weeks after surgery) CCR2 inhibition resulted in decreased cartilage and subchondral bone damage along with reduction in osteophyte size.[105]

Appleton et.al have shown a positive effect when CCR2 blockers were given at the time of injury at earlier timepoints.[101] This indicates that treatment works prophylactically when used at the time of trauma. While, in this thesis, we administered the drug 1 month post trauma and have not witnessed any protection against cartilage damage.

This signifies that treatment initiation time, and duration of therapy, holds central importance in targeting EGFR/CCR2 downstream signaling as a potential therapy for the treatment of OA. Also, there is a need for further investigation to fully understand the specific molecular mechanism through which CCR2/CCL2 and EGFR contribute to cartilage damage and eventually progression of OA.

4.2 Limitations

We understand that it is important to outline the limitations associated with the research so that future studies can be done more comprehensively. Our research too involved a few limitations due to completion of animal work long before the onset of this thesis.

Our first limitation is related to the artifacts that occur during sectioning of samples. We were not able to include lateral side of the rat knee while scoring for cartilage damage or subchondral bone damage as the depth was too deep or shallow on the lateral side. Thus, cartilage scoring was only conducted on the medial side of the joint. However, it is important to consider both sides due to differences in the joint biomechanics in weight-bearing joints and care should be taken to avoid this in future.

Secondly, our study has not included any behavioral assessment of the animals such as pain responses. This would be important as pain is directly associated with the disease progression. Moreover, the samples were too old to use antibodies on them, hence, we were not able to perform any studies involving immunohistochemistry (IHC) such as immunohistochemical studies involving MMPs. This could have provided better understandings of associated involvement of CCL2/CCR2 or EGFR with MMPs.

Lastly, our research was limited to male rats and sex-related variations have not been considered in this thesis. Epidemiological evidence suggests that difference in the severity of OA in male and female are due to the different sex hormones.[115] Also, post-menopausal women are at greater risk for the development of OA due to decrease in

estrogen levels in the body. Thus, it is important to consider female sex into research design.

4.3 Future Directions

Despite the fact that our results have not aligned with our hypothesis, but our thesis has demonstrated the necessity of continued research for further investigation of the role of the EGFR and CCR2 axis in OA**.** Certainly, EGFR and CCL2 have a crucial role in the pathophysiology of osteoarthritis. Since researchers have witnessed variation in the number and location of macrophages attracted by CCR2 and CCL2, it is important to understand the molecular mechanism of chemokines and their receptors. From our thesis and research carried out by other researchers, it is important to consider the concentration of drug, time of drug administration after injury while targeting C-C axis. It would be equally intriguing to conduct a study that would demonstrate the interaction between EGFR and the other members of sub-family of erbB receptors.

Also, CCR2 inhibition has been proven beneficial during initial stages. However, in our thesis, blockade of CCR2 receptor has not provided any protection against OA over the longer period of time. It would be worthwhile to carry out research that would demonstrate the possibility of CCR2 antagonist tolerance over time. To date, no studies have tested the effects of increasing doses of CCR2 inhibitor over time or tested the administration of CCR2 blocker intermittently with intervals instead of continuous drug delivery. Also, it would be interesting to test the possibility of non-specific binding of CCL2 with other CC-receptors needs to be considered and targeted for developing more novel approaches.

In spite of the multifaceted and intricate nature of these receptors, focusing on EGFR and CCR2 axis can be the potential strategy for the treatment of osteoarthritis in earlier stages.

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Poster Presentations

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