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Regulation of Lipocalin Prostaglandin-D Synthase expression by interleukin-1 β in Human chondrocytes

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Résumé

L'arthrose est une maladie multifactorielle complexe. Parmi les facteurs impliqués dans sa pathogénie, les certains prostaglandines exercent un rôle inflammatoire et d'autres un rôle protecteur. La prostaglandine D2 (PGD2) est bien connue comme une PG anti-inflammatoire, qui est régulée par l'enzyme «Lipocalin prostaglandine D-synthase». Avec l'inflammation de l'arthrose, les chondrocytes essaient de protéger le cartilage en activant certaines voies de récupération dont l'induction du gène L-PGDS. Dans cette étude, nous étudions la voie de signalisation impliquée dans la régulation de l'expression du (L-PGDS) sur les chondrocytes traités avec différents médiateurs inflammatoires.

Le but de projet: Nous souhaitons étudier la régulation de la L-PGDS dans le but de concevoir des approches thérapeutiques qui peuvent activer la voie intrinsèque anti-inflammatoire.

Méthode et conclusions: *In vivo*, l'arthrose a été suivie en fonction de l'âge chez la souris ou chirurgicalement suivant une intervention au niveau des genoux de souris. Nous avons confirmé les niveaux d'expression de L-PGDS histologiquement et par immunohistochimie. *In vitro*, dans les chondrocytes humains qui ont été traités avec différents médiateurs de l'inflammation, nous avons observé une augmentation de l'expression de la L-PGDS dose et temps dépendante. Nous avons montré, *in vivo* et *in vitro* que l'inflammation induit une sécrétion chondrocytaire de la L-PGDS dans le milieu extracellulaire. Enfin, nous avons observé la production de différentes isoformes de la L-PGDS en réponse à l'inflammation.

Mots clés: l'arthrose, Lipocalin prostaglandine synthase D, l'interleukine 1 bêta, la prostaglandine D2.

Abstract

Osteoarthritis is a complex multifactorial disease; many factors are involved in its pathogenesis, among those factors prostaglandins. Some prostaglandins have inflammatory role and some have anti-inflammatory role. Prostaglandin D₂ (PGD₂) is a well-established anti-inflammatory PG which is synthesized by the Lipocalin prostaglandin-D synthase (L-PGDS) enzyme. Upon the initiation of the inflammatory process in osteoarthritis, chondrocytes try to save themselves by activating salvage pathways, among these pathways is the induction of L-PGDS gene. In this study we are addressing the signaling pathways involved in the regulation of (L-PGDS) gene expression, in chondrocytes treated with different inflammatory mediator.

Rationale: understanding the regulation of L-PGDS will allow us to design therapeutic targets that can switch on the intrinsic anti-inflammatory pathway.

Method and findings: *In vivo*, osteoarthritis was induced in mice knees surgically or naturally following aging, the development of osteoarthritis was then confirmed histologically. The expression levels of L-PGDS were detected by Immunohistochemistry. *In vitro*, human chondrocytes were treated with different inflammatory mediators (interleukin-1 beta, interleukin-17, hydrogen peroxide and tert-Butyl hydroperoxide). Interestingly, in most cases chondrocytes increased expression of L-PGDS in a dose and time dependent manner. We discovered that chondrocytes release L-PGDS into the extracellular space in response to inflammation, *in vivo* and *in vitro*. Lastly we observed different isoforms of L-PGDS generated in response to inflammation.

Key words: osteoarthritis, Lipocalin prostaglandin D synthase, interleukin 1 beta, prostaglandin D₂.

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List of abbreviations:

- 4BHP: tert-butyl hydroperoxide.
- AA: arachidonic acid.
- ARE: antioxidant response element.
- c-AMP: cyclic adenosine monophosphate.
- COX: cyclooxygenase.
- COX-1: cyclooxygenase-1.
- COX-2: cyclooxygenase-2.
- CSF: cerebrospinal fluid.
- C-DNA: complementary deoxynucleic acid.
- BMDM: bone-marrow-derived macrophages.
- DMM: destabilization of medial meniscus.
- dNTP: deoxynucleotide triphosphate.
- H₂O₂: hydrogen peroxide.
- IL-1 β : interleukin 1 beta.
- IL-17: interleukin 17.
- IFN γ : interferon gamma.
- iNOS: inducible nitric oxide synthase.
- IP: immune precipitation.

- KD: kilodalton.
- L-PGDS: lipocalin prostaglandin D synthase.
- MAPK: mitogen-activated protein kinase.
- MMPs: matrix metalloproteinases.
- MMP-1 : matrix metalloproteinase-1.
- MMP-13: matrix metalloproteinase-13.
- MW : molecular weight.
- M-RNA: messenger ribonucleic acid.
- MgCl₂: magnesium chloride 2.
- NO: nitric oxide.
- NSAIDs: non-steroidal anti-inflammatory drugs.
- Nrf2: nuclear factor erythroid 2-related factor 2.
- OA: osteoarthritis.
- O/N: overnight.
- PCR: polymerase chain reaction.
- Pg: picogramme.
- PKA: phosphokinase A.
- PKC: phosphokinase C.
- PPAR- γ : peroxisome proliferator-activated receptor gamma.

- PG: prostaglandin.
- PGD2: prostaglandin D2.
- PGE2: prostaglandin E2.
- PGH2: prostaglandin H2.
- PGJ2: prostaglandin J2.
- RNA: ribonucleic acid.
- RPM: revolutions per minute.
- RT-enz: reverse transcriptase enzyme.
- RT-PCR: reverse transcriptase polymerase chain reaction.
- RT-qPCR: real-time quantitative polymerase chain reaction.
- ROS: reactive oxygen species.
- TNF- α : tumor necrosis factor alpha.
- WB: western Blot.

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Chapter 1: Introduction

A. Introduction

1.1 Osteoarthritis:

Osteoarthritis (OA) is a chronic inflammatory degenerative disease that affects joints cartilage, ligaments and underlying bone. It was long thought to be an inevitable process of aging. Further studies on OA revealed the interplay between many pathophysiological factors namely: weight, age, sex, genes, biomechanical, biochemical, environmental and local factors in the joint itself. In normal joints, there is a balance between the anabolic and the catabolic processes, at the level of articular cartilage, bones and synovial membranes. In OA, this balance is disrupted leading to the classic features of OA like degradation of articular cartilage (leading to narrowing of joint space), subchondral bone remodeling (osteosclerosis) and cyst formation, osteophytes formation and synovial membrane inflammation and thickening. Normal anatomy of the knee joint is detailed in **Figure 1**. **Figure 2** is a diagram showing the pathological changes in osteoarthritic knees versus normal knees.

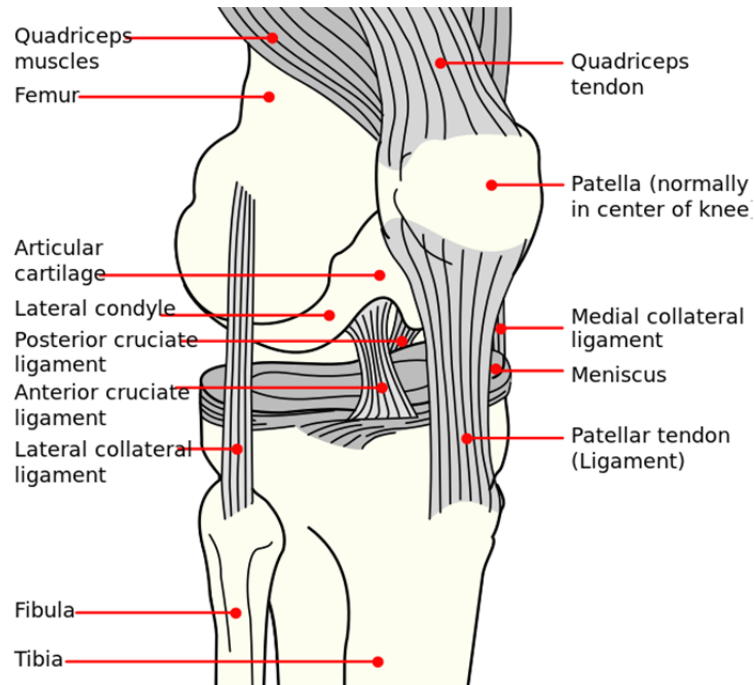


Figure 1: Normal anatomy and structure of knee joint.

Image source: http://commons.wikimedia.org/wiki/File:Knee_diagram.svg (open use).

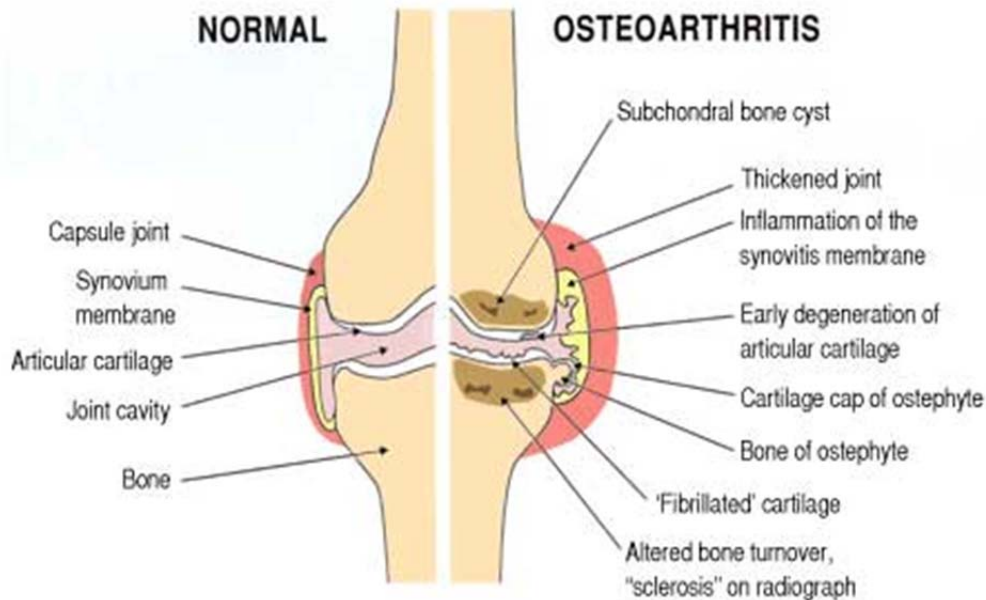


Figure 2: Diagram of normal Knee versus osteoarthritic one showing pathological features of OA.

Image source: <http://www.maurascollege.org/images/cervical-spondylosis-dizziness-treatment-information-disease-colorado-westminster-8933.jpg>

1.2 Epidemiology and risk factors:

OA is the most common joint disease in North America, with an incidence of 10% in males and 15% in females above age 60. It's expected that the total number of OA cases is going to grow with the aging of the population. There are many factors that affect the epidemiological distribution of OA cases, among these factors; the definition of what OA truly is, the method used to characterize it, the joints involved and the risk factors. For example, if we are going to check the prevalence among developed countries we may find obesity as a confounding factor raising the prevalence in this population. If we measure it among different age groups, the older the age group from which we take our samples the more prevalent the disease becomes. Most clinicians characterize OA radiologically according to the Kellgren and Lawrence scaling system where:

- Grade 0: no radiographic features of OA are present.
- Grade 1: doubtful joint space narrowing (JSN) and possible osteophytic lipping.
- Grade 2: definite osteophytes and possible JSN on the anteroposterior weight-bearing radiograph.
- Grade 3: multiple osteophytes, definite JSN, sclerosis, possible bone deformity.
- Grade 4: large osteophytes, marked JSN, severe sclerosis and definite bone deformity.

Regarding those patients radiologically characterized as OA, let us take the knee joint in people ≥ 40 years old as an example. In this population, the prevalence of knee OA was 19.2% according to the Framingham Study and 27.8% according to the Johnston County OA Project [1]. In the third National Health and Nutrition Examination Survey (NHANES III) researchers took a group of people >60 years old and characterized radiologically OA in their knees. They found that 37% of the study population had OA [1]. This suggests that different age groups have different prevalence. Conversely, if we take another factor such as which joint is involved, and we focus on hand OA for example, we will find the prevalence is 27.2% of the study subjects in the Framingham study. In addition, hip OA is less prevalent than knee OA or hand OA, with about 7% of women aged ≥ 65 years having a radiologically diagnosed OA. In Johnston County, the prevalence rate of hip OA reached 27% for individuals >45 years old [1]. Hence, in different age groups, the prevalence is totally different, as assessed by the Framingham study of hand OA (6.8%) and knee OA (4.9%) of the study subjects aged ≥ 26 year which is much different from the other study groups.

These differences in data reflect how different factors can affect the prevalence of OA, among those factors the definition of OA and the basis used to diagnose it i.e. clinically or radiologically, which joint is considered, and which age group is considered in the studies provided. OA is multifactorial in origin and factors such as age, gender, joint injury, body weight, bone density, collagen diseases (Ehlers-Danlos syndrome, Marfan syndrome) and muscle weakness around the joints are all important factors contributing to OA. Their modification determines the course of the disease. Genetic factors play a role also in the OA process [2, 3]. In a study to identify

the genetic loci that were associated with radiologically evident OA, researchers identified loci in 296 pedigrees on chromosomes 1, 7, 9, 13, and 19 particularly for OA of the hand [4]. OA of the hip also showed some genetic linkage to chromosome 11 [5]. Other chromosomes are also involved including 4 and 16 [6], 2 and 19 [7]. Chromosomes 2, 3 and 4 were shown to be involved in mutations in matrilin-3, a gene for non-collagenous matrix protein (MATN3). This mutation is found in 2% of hand OA patients in Iceland [8]. For patients with radiologically diagnosed OA of the knee single-nucleotide polymorphisms (SNPs) marker association within the gene for Leucine-Rich Repeats and Calponin Homology (LRCH1) on chromosome 13 was shown in studies from the UK and Canada [9]. In addition, the marker (rs91242a C/T) allele was detected more frequently in cases with significant knee OA versus control cases. Indeed, one out of 31 identified loci shows up more frequently in OA versus controls [9]. The function of the protein product of this gene is not fully known though it has a region similar to that of actin-binding protein that plays a role in cell shaping and intracellular signaling. Mutations which happen in structural components of articular cartilage and bone tissue potentiate the effect of other risk factors of OA and accelerate the pathology of the disease [3].

In conclusion the epidemiology and risk factors of OA are affected by the way you identify the cases, clinically versus radiologically, which joints are included, and which age group is targeted.

Age, race, acute and chronic repetitive trauma, are the main risk factors for OA, especially in the knee joint. To a lesser extent, they affect hip and hand OA. Age, obesity and gender, on the other hand, have a big role in the development of hip OA.

Bone mass in women, and whether they use hormonal replacement therapy or not, also play a role in the development of hip OA. Interestingly the higher the bone mass before and after menopause the higher the risk of developing OA of hip joints [10, 11].

Finally, obesity is the strongest modifiable risk factor for OA. Its strongest association is with the knee and hand OA while interestingly it's less associated with hip OA [12, 13]. Given this association, a reduction in weight has a noticeable protective role against knee OA [14].

1.3 Pathogenesis:

OA is a complex multifactorial disease and the mechanisms involved in its pathogenesis are not fully uncovered yet. There are different mechanisms that could initiate OA [15]. The most common initiating factor for OA is the mechanical damage, either as a single event macro-trauma or repeated microtraumas [16]. These mechanical injuries initiate the inflammatory process in chondrocytes causing them to release degenerative enzymes that degrade articular cartilage [16]. Another less common mechanism is the defective collagen type 1 and 2 formations in certain diseases leading to articular cartilage failure under normal mechanical loads [17]. Once the initial factor (for example trauma) starts the OA process, many other factors get involved. These factors include (on the molecular level) proteases, proteases inhibitors and cytokines, affecting the cartilage degeneration and repair balance. On the non-molecular level, other factors also play a role like age, weight, joint alignment, physical activity, and systemic hormones and mineral deposition. The biochemical cell response to mechanical stressors is termed mechanotransduction [18]. This

process has been studied in different cell types including chondrocytes [19]. Studies showed an increase in aggrecan messenger ribonucleic acid (mRNA) and a decrease metalloproteinase 3 mRNA by quantitative polymerase chain reaction (qPCR) in healthy human chondrocytes when cultures were subjected to repetitive loading versus those grown on solid support [20]. Osteoarthritic cartilages respond differently from healthy ones to the same mechanical loads, as they show more deformability and more loss of fluids under the same load [21]. These different biomechanical responses make normal every day loads considered pathological for osteoarthritic cartilage [22]. Biomechanical signaling pathways become now therapeutic targets to ameliorate the response of osteoarthritic cartilage to mechanical load. The difference in response between normal chondrocytes and osteoarthritic chondrocytes to the same mechanical load is what lead to the use of autologous chondrocytes implantation in OA. This therapeutic technique is aiming to improve the inflammatory process by restoring the normal signaling pathway by using healthy chondrocytes [23].

Trauma, even blunt ones make the cartilage susceptible to OA and degeneration. Tumor necrosis factor alpha (TNF- α) levels were observed to be high in synovial fluid early after trauma. TNF- α enhances neutrophils mediated proteoglycans degradation and has pro-apoptotic effect under certain enabling conditions [24]. Interestingly exposing chondrocytes *in vitro* to TNF- α did not increase cell death rates or levels of prostaglandin E2 (PGE2) or prostaglandin D2 (PGD2). Trauma, on the other hand, increased these mediators, independently of TNF- α [24]. TNF- α was found to induce the expression of matrix metalloproteinase-1 (MMP-1) regardless of trauma,

contributing to cartilage degradation. These mediators clarify to some extent the biomechanical pathways involving trauma and cartilage damage.

The role played by mechanical load is not fully understood in the pathogenesis of OA. It was thought to cause wear and tear to the cartilage, accelerating OA. Further clinical and research data revealed that mechanical load in the form of exercise (in normal weight subjects) can cause, accelerate, ameliorate and even reduce the risk of OA [25, 26]. In 2013, a systematic review of clinical trials on the effect of exercise on osteoarthritic weight-bearing joints (knee mainly) was done [27]. This review included 60 clinical trials with 12 different exercise intervention programs [27]. The review concluded to a statistically significant effect for exercise in reducing pain and improving functionality in osteoarthritic patients, particularly aquatic exercise [27]. Fewer similar studies were done for hip joint OA, where it was shown that physiotherapy and home-based exercise programs don't provide that much improvement in pain management and functionality [28]. Another study evaluated the effect of an intensive aerobic exercise program in patients with moderate to severe OA evidenced clinically and radiologically (grade III and IV). This study concluded that, there is no difference between exercise groups versus non-exercise groups regarding pain control and functionality [29]. In conclusion, exercise programs have to be tailored depending on many factors in each patient. We should always take into consideration the general medical condition of the patient particularly his cardiovascular capacity for exercise and whether his main complaint from OA is pain or limitation of movement. On the other hand, limitation of movement had put the osteoarthritic patients who are not following an exercise program at a higher risk for

cardiovascular diseases. This raises the question of screening sedentary osteoarthritic patient for cardiovascular diseases before assigning them to exercise programs. Excessive mechanical load induces biomechanical transformations in chondrocytes, for example cyclooxygenase 2 (COX-2) gene induction. These biomechanical transformations impair chondrocytes' ability to handle oxidative stress, which leads to chondrocyte toxicity and eventually OA [30]. This mechanical load on chondrocytes was also found to induce the lipocalin prostaglandin D synthase (L-PGDS) gene. L-PGDS through its end products PGD2 and 15d-PGJ2 is hypothesized to contribute to the different processes initiated by mechanical load [30]. **Figure 22** clarifies the biosynthesis of PGD2 and 15d-PGJ2.

1.4 Treatment:

Pharmacological and non-pharmacological management of OA mainly targets pain control, minimizing disability and improving the quality of life. General non-pharmacological management focuses on weight reduction and exercise programs.

Pharmacotherapy starts with acetaminophen for OA patients not showing inflammatory signs and symptoms [31]. Once there are symptoms of inflammation such as articular swelling, morning stiffness, night pain, joint effusion by examination, or synovitis on arthroscopic examination, non-steroidal anti-inflammatory drugs (NSAIDs) are indicated.

NSAIDs can be non-selective cyclooxygenase (COX) inhibitors including COX-1 and COX-2, or COX-2 selective inhibitors (coxib). NSAIDs should be started at the minimal dose that controls the inflammation to avoid the side effects of these drugs. NSAIDs and Capsaicin (topical analgesic) can be used topically in patients who can't tolerate NSAIDs orally. Patients with gastritis, peptic ulcer or renal diseases are intolerant to NSAIDs.

Intra-articular glucocorticoids injection is the next step when there is an inadequate response to non-pharmacological management plus acetaminophen or NSAIDs. Failure of the initial therapeutic lines necessitates a re-evaluation of the joint to detect the probability of another underlying pathology such as crystal disease and other inflammatory arthropathies. This could be done by arthrocentesis and synovial fluid

examination. Current data suggests an added value for the use of glucosamine and chondroitin together versus the use of each of them alone in treating OA.

Resistant cases of OA are treated by another spectrum of medications designated for those cases particularly. Those medications include opioid analgesics, intra-articular hyaluronans, a trial of glucosamine or chondroitin, and finally colchicine. These management guidelines are in accordance with the guidelines from American College of Rheumatology [31].

American college of rheumatology (ACR) stated guidelines for managing OA according to joints involved and according to modality of treatment as follow [36]:

Non-pharmacologic management of hand OA:

- 1) Determine how OA affects the patient's activities of daily living (ADLs).
- 2) Educate the patient about how to protect his joints.
- 3) Use supporting devices, like splints for patients with trapeziometacarpal OA.
- 4) Educate the patient about thermal techniques available for his condition.

Pharmacologic management of hand OA:

- 1) Topical capsaicin.
- 2) Topical NSAIDs.
- 3) Oral NSAIDs.
- 4) Tramadol.

ACR recommends against the use of intra-articular therapies and opioid analgesics. Generally persons who are ≥ 75 years old should use topical anti-inflammatory rather than oral ones.

Non-pharmacologic management of knee OA:

- 1) Aerobic exercise.
- 2) Aquatic exercise.
- 3) Weight loss.
- 4) Self-management training.
- 5) Physiotherapy plus exercise.
- 6) Psychosocial guidance and support.
- 7) Knee protective gears according to the internal lesion, for example medially directed patellar taping, and medially wedged insoles for those who have OA in lateral knee compartment or laterally wedged subtalar strapped insoles for medial knee compartment OA patients.
- 8) Advised about thermal and transcutaneous electrical stimulation modalities available.
- 9) Use supporting devices like walking aids as needed.
- 10) Tai chi programs.
- 11) Chinese acupuncture.

ACR didn't provide specific recommendations regarding balance exercises, strengthening exercises, laterally wedged insoles, physiotherapy alone, knee braces or laterally directed patellar taping.

Pharmacologic management of knee OA:

- 1) Acetaminophen.
- 2) Oral NSAIDs.
- 3) Topical NSAIDs.
- 4) Tramadol.
- 5) Intra-articular corticosteroid injections.
- 6) Chondroitin sulfate.
- 7) Glucosamine.
- 8) Topical capsaicin.

Also, ACR didn't have specific recommendations regarding the use of intra-articular hyaluronates, duloxetine, and opioid analgesics.

Non-pharmacologic management of hip OA:

- 1) Aerobic exercise.
- 2) Aquatic exercise.
- 3) Weight loss.

- 4) Self-management training.
- 5) Physiotherapy plus exercise.
- 6) Psychosocial guidance and support.
- 7) Advised about thermal modalities and walking aids, as needed.

ACR didn't have particular recommendations regarding balance exercises alone or with strengthening exercises, tai chi or physiotherapy alone.

Pharmacologic management of hip OA:

- 1) Acetaminophen.
- 2) Oral NSAIDs.
- 3) Tramadol.
- 4) Intra-articular corticosteroid injections.

ACR does not recommend the use of Chondroitin sulfate and Glucosamine and didn't have specific recommendations for topical NSAIDs, Intra-articular hyaluronate injections, Duloxetine or opioid analgesics.

Many studies had evaluated the role of NSAIDs in OA and the mechanisms by which they work. The initial idea was that NSAIDs work only by inhibiting COX enzymes, but recently it was discovered that they downregulate the expression of the COX gene as well [32]. **Figure 3** explains the synthesis of different PG's from AA by COX enzyme. NSAIDs affect the synthesis of all prostaglandins (PG's), the inflammatory as well as

the anti-inflammatory ones. This explains NSAIDs limited success in reversing or even stopping the progression of the disease. The moment we stop COX2 by NSAIDs we stop both the inflammatory and the salvation pathways. COX 1 and 2 activities are augmented by interleukin 1 beta (IL-1 β) and arachidonic acid (AA) to produce different types of PG's, as explained in **Figure 3**. The L-PGDS enzyme synthesizes PGD2 which contributes to the anti-inflammatory pathway through its end product 15-PGJ2. Via its inhibition of nitric oxide (NO) production PGD2 prevents cartilage degradation [33]. PGD2 even has a positive feedback effect on its own production through the stimulation of COX2; this is believed to be through its role in up-regulating other prostanoids such as PGF1 α , PGF2 α and thromboxane B2 (TXB2). PGE2 induces MMP1 and MMP13 which are inflammatory mediators in OA, and it inhibits proteoglycans promoting matrix degradation. PGD2, on the other hand, inhibits MMP1 & MMP13; this again suggests an anti-inflammatory role for PGD2. NSAIDs by blocking COX function and down-regulating it alter these entangled reactions, stopping the inflammatory and anti-inflammatory pathways. **Figure 3** explains the synthesis of different PG's from AA by COX enzyme.

Guidelines recommend the use of intra-articular glucocorticoids in cases of moderate to severe pain, especially after the failure of oral and topical management, or when they are contraindicated, like in cases of active peptic ulcer disease or renal failure [34-37]. This effect reaches its peak during the 1st two weeks after injection and then it wanes off. Even with the repeated injections, their efficacy is appreciated during the first year only [38]. No long-term benefits or harms had been proven for this modality of treatment [35]. The injected dose depends on the joint size, from 10mg for small,

20mg for medium sized and 40mg for large ones. All injection techniques should be done under complete aseptic conditions to minimize the risks of infection. Guidelines also recommend aspiration of any joint effusion before injection of steroids and sending the effusion fluid for analysis for better diagnosis and to exclude infection. Injection of steroids should be postponed until the culture results come back negative, especially if there is a high suspicion for infection like fever or rapidly accumulating joint effusion [39].

Studying the efficacy of this therapeutic modality showed a significant effect for intra-articular glucocorticoids injection versus placebo [34], even without a clinically evident joint inflammation [35]. Most studies were done on knee joint injections, then on the hip joint, and to a much lesser extent on carpometacarpal joints and other joints. Generally these injections cause pain relief rather than improvement of function or repair of joint damage.

Guidelines recommend the use of intra-articular hyaluronan injections in cases of OA (especially knee OA) which failed oral and topical therapeutic modalities, or in those patients who have a contraindication for the use of NSAIDs like active gastric ulcer or renal failure [31, 37]. Also, it's the next step in cases of failure of intra-articular glucocorticoids injection [34-37]. This modality improves mainly pain symptoms and to some extent functionality but doesn't prevent the progress of the disease or reverse it [40, 41]. Improvement of functionality in hyaluronan use is mainly through restoring the viscoelasticity of the synovial fluid that is usually lost in OA. These benefits were elaborated by many randomized trials and meta-analyses [42-46]. These injections

are usually given as one course per year (as one injection or five weekly injections according to the molecular weight (MW) of the drug). This is usually well tolerated at this rate with minimal to no side effects. Compared to glucocorticoids injections the hyaluronan compounds have similar efficacy with a slower onset of action, taking sometimes up to two months to reach its peak effect [47]. Studies compared also the effect of hyaluronan injections to that of NSAIDs and found almost similar effects [34, 42, 48, 49]. Hyaluronan showed some adverse reactions like flaring of the joint pain and swelling post injection in 1.5 to 5 percent of patients. These flares usually resemble septic arthritis with synovial fluid leukocytosis of 100,000 per mm³ [50]. These flares are related to injection techniques as well as to the MW of the injected drug, with higher rates in lateral approach versus medial one [51]. Also, higher rates of flares were found with the use of higher MW compounds compared to lower MW ones [52]. Generally no major side effects for these compounds were reported.

Surgical management is usually left as the last resort treatment of OA. There is a wide range of modalities available with different selection criteria for each modality. Arthroscopic intervention: it starts with joint irrigation [53] to improve visualization of the joint by removal of debris and blood. Following this, arthroscopy is used for debridement with or without arthroscopic synovectomy. Some hypotheses suggest that joint irrigation alone has a beneficial effect on the management of joint pain, by removal of tissue debris, crystalline materials and cartilage fragments, decreasing the burden on the joint and synovial membranes and slowing further destruction [54, 55]. Arthroscopic irrigation and lavage are an office procedure that can be done under local anesthesia and intravenous sedation.

Arthroscopic debridement is done mainly for meniscal tear usually with joint lavage. Some studies suggested that there is not much difference between arthroscopic debridement with lavage in addition to medical and physical therapy, compared to medical and physical therapy alone. This is regarding the pain level using WOMAC pain score [56]. Some randomized trials on patients with knee OA and meniscal tears showed no higher benefit with arthroscopic surgery, followed by physical therapy, compared to physical therapy alone [57-59].

Arthroscopic abrasion includes mainly drilling of sclerotic bone in affected joints. Randomized controlled trials did not show significant improvement with this technique compared to medical and physical therapy alone. Follow-up observations of patients who went through this procedure showed that 50 percent still needed a total knee replacement within 3 years [60].

No clinical trials were done to study the efficacy of arthroscopic synovectomy in patients with OA. This is usually reserved to those with severe inflammatory signs and symptoms unresponsive to initial NSAIDs, intra-articular glucocorticoid injections, arthroscopic irrigation, colchicine, and non-pharmacological measures.

Total joint replacement (arthroplasty) is another modality used as a final measure after the failure of other measures and in patients with significant limitation of activities [61]. Replacement surgery should be timed perfectly, ideally before significant joint deformity or instability, significant contractures or muscle atrophy. This aims to minimize the complications and to maximize outcomes [62, 63]. Preoperative medical management and postoperative rehabilitation affect greatly the surgical

outcomes [64, 65]. Total joint arthroplasty usually provides the best pain relief and functional improvement in patients with significant hip or knee OA [61, 66]. Improvement of functionality following surgery comes gradually, usually over a period of one year and lasts in average 5 years [67].

The joint resurfacing procedure is a treatment modality in which a metal cap or prosthesis replaces an arthritic femoral head; on the other side of the joint a metal acetabular component forms the articulation surface with the metal femoral head. This is known also as resurfacing arthroplasty, a common alternative for total hip replacement. This procedure shows some late complications, mainly femoral neck fracture and aseptic loosening. Other than these complications, this procedure is usually well tolerated and has excellent outcomes [68].

The chondrocyte grafting technique involves autologous chondrocytes implantation (ACI) into the defective areas of the cartilage aiming to fill these areas and restore the smooth surface as before. Chondrocytes are collected from the same patient from a non-weight bearing area. These are then allowed to grow in an *in vitro* culture media. Lastly, they are implanted into the cartilage defects under a cover of the periosteal membrane (1st generation, ACI-p); under a collagen type I/III membrane (2nd generation, ACI-c); or into a scaffold matrix (3rd generation, ACI-m). Usually, this technique is reserved to areas of confined and localized cartilage loss; this is usually seen in young patients who had joint trauma. Patients with advanced OA and wide areas of cartilage loss didn't show promising results with this technique [69]. Magnetic resonance imaging confirmed a better filling of small chondral defects when done by

a graft technique than by any other technique. This technique is also less likely to produce osteophytes [70].

Carpometacarpal or trapeziometacarpal OA is a unique situation necessitating different surgical techniques, usually including trapeziectomy with or without ligament reconstruction and tendon interposition [71]. Interpositional arthroplasty and joint replacement are other modalities used for this type of OA. Comparative studies for the outcomes of those different procedures didn't show any advantage for one technique over the others.

The demonstration of different treatment modalities, pharmacological and surgical ones, aims mainly to clarify the limitations that exist for current therapeutic modalities. Pharmacological modalities aren't specific enough to spare the anti-inflammatory pathways, and surgical ones aren't showing significant preference over the pharmacological ones. This justifies our search for more accurate understanding of this disease and for new therapeutic targets and accurate medications.

1.5 Prostaglandins:

PG's are a family of physiologically active lipid compounds that are involved in many physiological and pathological processes all over the body. One of their major roles is in inflammation. PG's can be both inflammatory and anti-inflammatory mediators. In this respect they are involved in the OA pathological process. The synthesis of PG's starts with the release of AA from the cell membrane, a reaction catalyzed by phospholipases. The next reaction is catalyzed by a COX that leads to the

intermediate PGH₂ from AA. Two isoforms of COX are known, COX-1 which is constantly expressed, and COX-2 which is induced by inflammatory mediators [72]. The unstable PGH₂ is then metabolized into more bioactive forms of PG's like PGE₂, PGD₂, PGF_{2α}, PGI₂, and thromboxane A₂ by different synthases [73]. **Figure 3** shows the pathways involved in the synthesis of different PG's from AA.

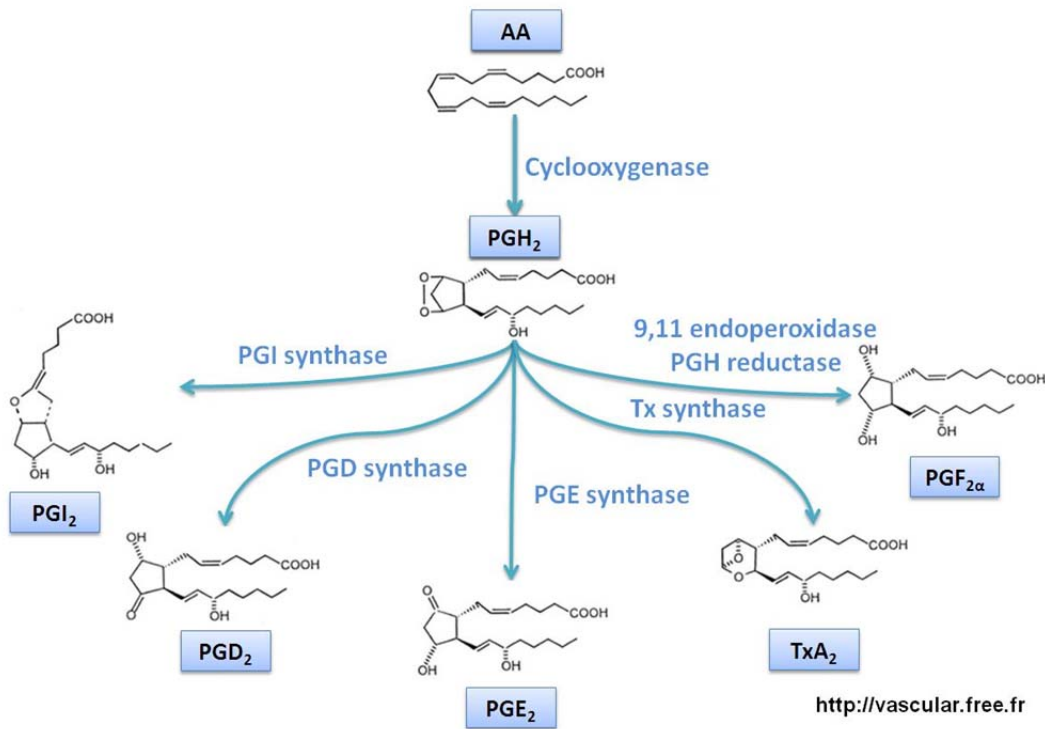


Figure 3: The synthesis of different PG's from AA acid by COX and different PG synthases.

Image source: <http://vascular.free.fr/cox-pathway.jpg>

PG's act via specific G protein-coupled receptors. Each prostaglandin acts via multiple different receptors subtypes and these receptors can cross react together. Via these multiple receptors PG's can multiply their coupling capacity to different signal transduction pathways leading to a more diverse range of downstream effects.

These effects can occur in functionally opposing directions within the same cell. PGE2 for example is widely expressed in the CNS. It has 4 receptors subtypes giving it a diverse functionality even inside the same cell. PGE2 can have neurodegenerative or neuroprotective effects depending on which receptor it binds to.

In articular cartilage, PGE2 plays a degenerative role. It inhibits proteoglycans synthesis, which are one of the main components of healthy cartilage. PGE2 also induces matrix metalloproteinase 13 (MMP13) and aggrecanase 5 (ADAMTS-5), which degrade cartilage proteins. The main effect of PGE2 on articular cartilage is mediated through a receptor known as EP4 [74].

Among different PG's, PGD2 is an interesting one. It's synthesized from PGH2 by PGD synthase and then gets enzymatically dehydrated into 15d-PGJ2. 15d-PGJ2 is the natural endogenous ligand of peroxisome proliferator-activated receptor gamma (PPAR- γ) nuclear receptor and transcription factor. PPAR- γ among other functions, is known to suppress inflammatory cytokines like COX-2, IL-1 β , TNF- α , inducible nitric oxide synthase (iNOS) MMP1, MMP13 [75]. This function was tested with synthetic ligands of PPAR- γ before discovering 15d-PGJ2 [75]. This makes 15d-PGJ2 (and thus PGD2) one of the most important natural anti-inflammatory PG's.

1.6 PGD2:

Many studies suggested a protective role for PGD2 in OA [73] [76]. Indeed, treating chondrocytes with PGD2 increases the production of collagen type 2 and aggrecan, which are involved in cartilage repair [77]. In addition, chondrocytes treated with PGD2 showed lower rates of cell death [76]. PGD2 has also a role in inhibiting the production of matrix metalloproteinase 1 and 13, which are involved in cartilage damage [78]. In light of these observations, it is noteworthy that studies noted increased levels of PGD2 during the resolution phase of the inflammation process. These high levels of PGD2 were lost by the use of COX-2 inhibitors suggesting a role for COX-2 in activating anti-inflammatory pathways [79]. Further exploring and confirming the role of PGD2 as anti-inflammatory, *in vivo* studies using PGD2 synthase knockout mice showed a failure of resolution of inflammation [80]. Another study confirmed the anti-inflammatory role of PGD2, using the delivery of PGD2 synthase into a murine air pouch model of monosodium urate monohydrate crystal-induced inflammation via a retroviral method [81]. PGD2 has two main receptors, D Prostanoid receptor (DP1) and chemoattractant-receptor-like molecule (CRTH2), also known as (DP2) [82]. Activation of the DP1 pathway is the one responsible for the down-regulation of MMP 1 & 13 in chondrocytes [78]. PGD2 exhibits its anti-inflammatory effect through its potent anti-inflammatory end product 15d-PGJ2 (15-deoxy-delta¹², 14-PGJ2) [83]. 15d-PGJ2 has a significant anti-arthritic effect through downregulation of inflammatory mediators of OA [83]. This includes IL-1 β , tumor necrosis factor-alpha (TNF-alpha), iNOS, and MMPs 1 and 13 [83]. 15d-PGJ2

induces its effect through nuclear PPAR- γ [83, 84] as well as other pathways independent of PPAR- γ [84].

PPAR- γ is a nuclear receptor protein that works as a transcription factor for different genes including anti-inflammatory genes. This receptor is noticeably downregulated in osteoarthritic cartilage *in vivo* and was downregulated also in chondrocytes treated with IL-1 β *in vitro* [85]. This down-regulation is interfering with the PGD2 protective function. PPAR- γ has a wide range of functions, besides being a potent anti-inflammatory effector through suppression of the previously mentioned mediators; it plays also a role in reversing insulin resistance. Studies found that PPAR- γ decreases insulin resistance and improves liver handling of glucose by upregulating insulin receptors [86], from which appeared the thiazolidinediones family of drugs as insulin sensitizers to treat type 2 diabetes. PPAR- γ also plays a key role in adipogenesis, therefore, PPAR- γ antagonists have been used to decrease steatosis and adipose tissue formation [87] [88].

1.7 L-PGDS:

PGD2 is formed from PGH2 by a PGD synthase. There are two isoforms of PGD synthase, lipocalin PGDS (L-PGDS) which is glutathione-independent and hematopoietic PGDS (H-PGDS) that is glutathione-dependent [89]. L-PGDS has a wide distribution all over the body especially within the central nervous system [90, 91], heart [92], retina [93], genital organs [94], and chondrocytes [95]. H-PGDS shows abundance mainly in hematopoietic tissues, mast cells [96], megakaryocytes [97],

and T-helper lymphocytes-2 [98]. **Figure 4** explains the distribution of L-PGDS and H-PGDS in different tissues.

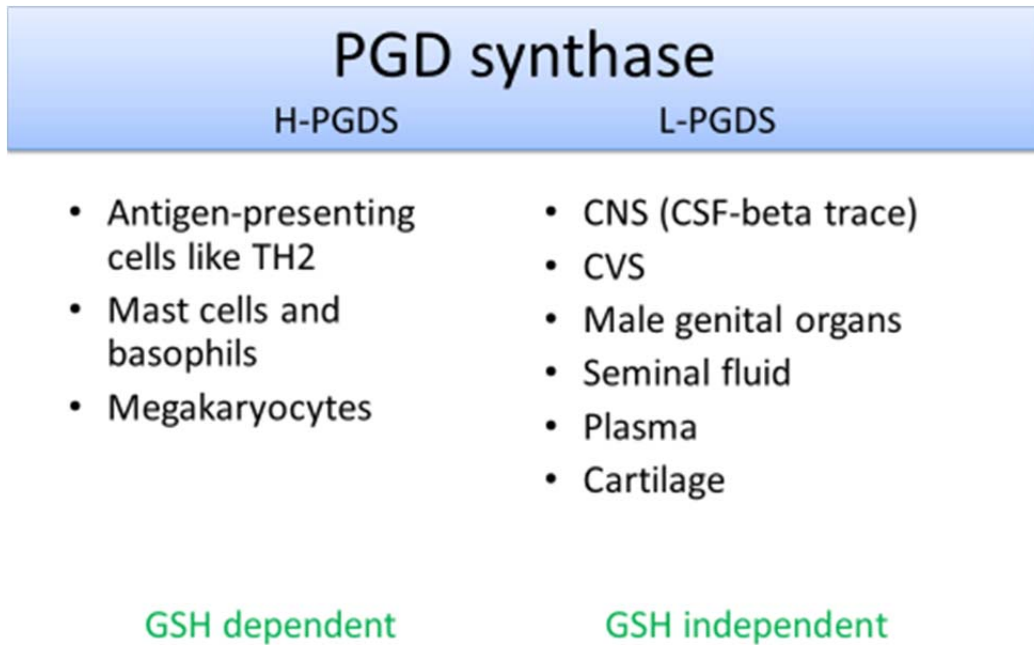


Figure 4: PGDS distribution in body tissues.

L-PGDS has a dual function; enzymatic as well as non-enzymatic. **Figure 5** describes its known functions. Enzymatically, it synthesizes PGD₂ from PGH₂. Non-enzymatically, it acts as a transmembrane transporter protein for small hydrophobic molecules including PGD₂, protecting it from metabolism and non-enzymatic dehydration into PGJ₂. Also, PGDS has lipophilic ligand-binding properties by which it binds retinoid, thyroid hormones, and bile pigments including bilirubin and biliverdin. PGDS increases in cerebrospinal fluid (CSF) after subarachnoid hemorrhage to act as a scavenger for bilirubin. It plays a role also in cell migration and morphological

changes mainly through retinoid. Lipophilic ligand binding properties of this protein contribute to its anti-cancer function, as many lipocalin-ligands (like retinoid) are known to be involved in cell cycle regulation and cell proliferation. The main mechanism of this function is not fully deciphered yet.

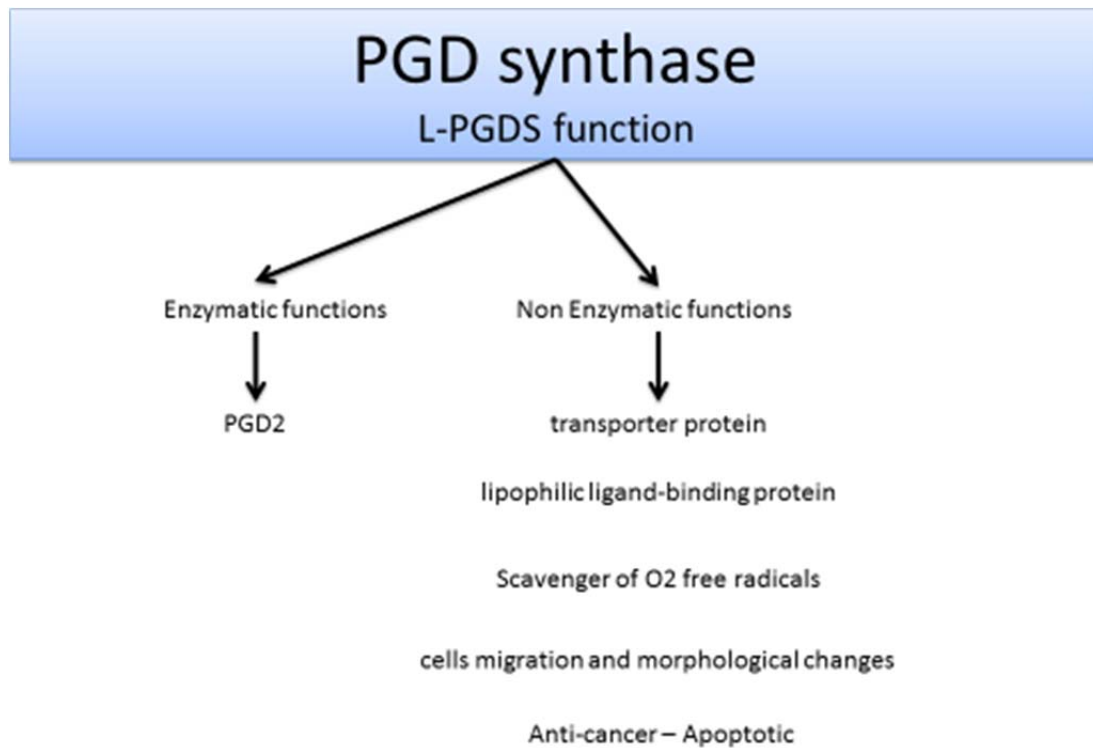


Figure 5: L-PGDS function.

The three-dimensional structure of L-PGDS is formed of 9 β -Strands and 3 α -helices. They are labeled A-I and 1–3 respectively in **Figure 6**. L-PGDS is formed of 190 AA giving it a calculated MW of 21 kilodalton (KD). Though, it actually appears at a MW range of 25 to 37Kd. This is because of the post-translational modification of the protein, as this protein has multiple glycosylation and phosphorylation sites giving it a

wide range of MW and functionality according to which site is phosphorylated and which is glycosylated [99]. It has an enzymatic active site, at cysteine 65 (Cys65), an important glycosylation site at asparagine 51 (Asn51), and an important phosphorylation site at serine 106 (Ser106) [99].

PGD synthase L-PGDS structure

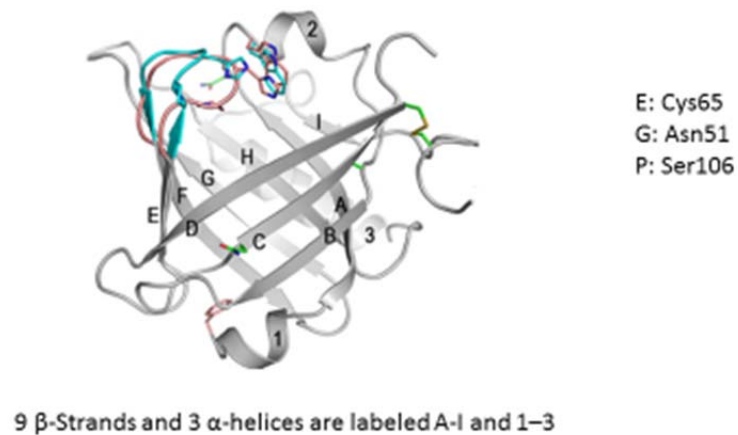


Figure 6: L-PGDS 3D structure.

Image source: <http://urade.wpi-iiis.tsukuba.ac.jp/research/analysis.php>

Depending on L-PGDS location inside the cell or in body fluids, different modified isoforms of L-PGDS appear. This is very important because it indicates that L-PGDS is modified in different ways to adapt for its multiple functions and in different locations also. For the purpose of using L-PGDS as a therapeutic target, we need to identify exactly the enzymatically active form to use as a medication avoiding the side

effects that may arise from other forms. These post translational modifications suggest different functions for different L-PGDS isoforms.

L-PGDS is the key regulatory enzyme for PGD₂ production from PGH₂ therefore; it is the key regulatory enzyme for 15d-PGJ₂ production also. This makes the regulation of L-PGDS production in chondrocytes the key for the regulation of natural anti-inflammatory pathways. The pathway we are working on in this study is described in **Figure 22** and explained in details in the discussion.

1.7.1 In the joint:

New studies started exploring the roles played by L-PGDS and H-PGDS as well as their regulation in cartilage. Higher abundance for L-PGDS mRNA and protein of about 20 fold was found in normal and osteoarthritic chondrocytes compared to H-PGDS [100]. L-PGDS is suggested to have a protective role in chondrocytes considering its end product, PGD₂. Pursuing this protective role, L-PGDS is expressed at higher levels in many pathological conditions. Among these conditions, multiple sclerosis [101], atherosclerosis and coronary artery diseases [92], diabetes mellitus [102], and hypertension [103]. An interesting study by Zayed *et al.* in 2008 showed a key role for IL-1 β in the induction of L-PGDS, both at the mRNA and protein levels, in chondrocytes, in a dose and time-dependent fashion. This eventually led to higher levels of PGD₂.

The increased levels of L-PGDS mRNA and protein were confirmed to be through gene induction. Different pathways were studied to identify those involved in L-PGDS gene induction. The successful blocking of IL-1 β induction of L-PGDS using

translation inhibitors like cycloheximide, C-jun N-terminal kinase (JNK), NF- κ B, p38 MAP kinase and Notch, confirmed the involvement of these pathways in L-PGDS gene induction by IL-1 β [100]. Interestingly, inhibition of the extracellular signal-regulated kinase/mitogen-activated protein kinase (ERK/MAPK) pathway didn't show any significant effect on blocking the induction of L-PGDS by IL-1 β [100]. The end product PGD₂ was found to have a negative feedback inhibitory effect on L-PGDS induction by IL-1 β in chondrocytes [100].

1.7.2 In the central nervous system:

L-PGDS is a major protein and it has many roles. It is used as a biomarker in patients with idiopathic normal pressure hydrocephalus (iNPH), a degenerative disease characterized by progressive dementia, gait problems and urinary incontinence. L-PGDS level is decreased in patient with iNPH, indicating the death of arachnoid cells responsible for its production. In the same disease, it plays a role as a scavenger of amyloid beta (A β) which has a neurotoxic effect leading to Alzheimer's disease, a major comorbid condition of iNPH [104]. Multiple sclerosis (MS) is an autoimmune disease leading to multifocal, recurrent, demyelination of the white matter of the central nervous system. L-PGDS generally plays a protective role in the CNS. In multiple sclerosis where there are plaques of degenerated myelin sheaths from the autoimmune process responsible for the pathology of the disease; L-PGDS is present at high levels in oligodendrocytes and astrocytes in the shadow plaques of MS. This is in contrast with other places within the white matter where there are no MS plaques, and at the same time of white matter from normal individuals. The observed

increased production of L-PGDS is a stress response in those cells and was not found to happen in parallel in the CSF itself [101]. L-PGDS also acts as a scavenger of Biliverdin in the CSF in cases of subarachnoid hemorrhage (SAH) [105]. Biliverdin is a metabolite of hemoglobin which is released from red cell corpuscles after their hemolysis in cases of subarachnoid hemorrhage. Hemolysis is due to low CSF osmolarity.

1.7.3 In the cardiovascular system:

In the cardiovascular system and in atherosclerosis particularly, L-PGDS plays a role as a marker for coronary artery diseases. This was based on particular findings linking degree of atherosclerosis with the levels of L-PGDS. In atherosclerosis, higher levels of PGE1 and its key enzyme “microsomal prostaglandin E synthase-1” (mPGES-1) and lower levels of PGD2 and its key enzyme (L-PGDS) were found in symptomatic plaques [77]. The opposite was found in asymptomatic plaques [77]. This suggests that a balance exists between those two types of PG’s upon which the pathology of the disease predominates [77]. This also suggests that the shift towards PGD2 may have a protective function in different tissues. Most recent studies used blood tests to detect L-PGDS levels in patients with different degrees of atherosclerosis and coronary artery disease. These studies were aiming to use L-PGDS serum levels as a diagnostic marker for the severity of atherosclerosis or a predictive tool of the probability and severity of coronary artery disease. Authors concluded that there were no significant differences in serum levels of L-PGDS

between different patients, suggesting a poor diagnostic value for L-PGDS level in atherosclerosis generally and coronary artery diseases specifically [106].

1.7.4 Reactive oxygen species (ROS):

Considering the fact that ROS activate Nuclear factor erythroid 2-related factor 2 (Nrf2) [107] and that Nrf2 induces L-PGDS by a recognized binding site for Nrf2 on the L-PGDS promoter region [108], it is reasonable to assume that ROS can induce L-PGDS in chondrocytes. This is part of a salvation pathway to protect chondrocytes against oxidative stress. Based on this hypothesis, ROS generators hydrogen peroxide (H_2O_2) and tert-butyl hydroperoxide (4BHP) were included in this study to determine their effect on L-PGDS expression levels. Fukuhara, *et al.* in 2012 [109] had shown an anti-apoptotic effect for L-PGDS in neuronal cells where L-PGDS was induced in neuroblastoma cells by oxidative stress using H_2O_2 (0, 50, 100 μ M) for 24H. L-PGDS levels were increased in a dose-dependent manner and cell viability assays showed decreased cell death with a direct correlation to the level of L-PGDS. They noticed that the Thiol group at Cys⁶⁵ of L-PGDS got oxidized into sulfonic acid after treating neuroblastoma cells with H_2O_2 . This suggested a ROS scavenger function for L-PGDS. These results were obtained by measuring the MW of L-PGDS before and after H_2O_2 treatment using MALDI-TOF (matrix-assisted laser desorption ionization - time of flight) mass spectrum. H_2O_2 -treated L-PGDS proteins showed a 32Da higher MS difference.

The structure of L-PGDS is composed of 9 stranded antiparallel β -sheet folded on itself to form a hydrogen bonded β -barrel and α -helix [110]. Human L-PGDS have two

thiol groups Cys⁶⁵ and Cys¹⁶⁷. Cys⁶⁵ is the active catalytic domain of the enzyme [111]. Thiol groups had been found to react with different reactive O₂ species and thus scavengers those harmful radicals, making those thiol groups key players in cell defense against free radicals [112, 113]. This suggests that the L-PGDS scavenger function also protects the cells against inflammation along with its role in the production of PGD₂. L-PGDS also functions as lipid transporter [114] which is not affected by H₂O₂ treatment, as its binding capacity to lipophilic molecules was the same before and after treatment [109].

1.7.5 Nrf2:

Kim *et al.* in their study in 2013 [108] showed a role for Nrf2 in L-PGDS induction by PGD₂. In bone marrow-derived macrophages, PGD₂ showed a positive feedback effect on L-PGDS gene induction, increasing its expression through activation of Nrf2, a well-known antioxidant transcription factor [108]. A specific binding site for Nrf2 was identified on the promoter region of L-PGDS gene. This paper concluded a positive feedback effect for PGD₂ on L-PGDS gene induction in macrophages.

Kim *et al.* didn't use a blocker of Nrf2 to confirm its involvement in PGD₂ mediated induction of L-PGDS gene but instead they used an Nrf2 Knockout (KO) mice approach to address this issue. This approach failed to induce L-PGDS gene in macrophages of KO mice treated with PGD₂. Further confirming this role, macrophages from KO mice were transfected with Nrf2 which restored the lost response [108]. Nrf2 is normally latent within the cells until it gets activated by one of its activators, usually oxidative stressors. At that point, Nrf2 migrates to the nucleus

and binds to promoter regions of specific genes known as the antioxidant response element (ARE). These genes are the master regulators of the whole antioxidant system in cells. Nrf2 is mainly activated through free radicals, other activators were found to be involved also, PGD2 for example [108]. Studies have shown a higher antioxidant protective effect by induction of intrinsic antioxidant pathway through Nrf2 compared to the use of exogenous antioxidants like vitamin C or E [115]. This makes Nrf2 and its agonist's potential therapeutic targets in OA, both for the Nrf2 role in L-PGDS induction and in activating intrinsic antioxidant pathways (ARE).

Infection and inflammation induce oxidative stress in cells and increase the production of ROS. Cells then activate their natural antioxidant system to maintain cell homeostasis. Nrf2 is a key transcription factor for the genes of ARE.

In normal conditions, Nrf2 is found bound to Kelch-like ECH-associated protein 1 (Keap1) which in turn is bound to actin in the cytosol. Keap1 promotes binding of Cul3-dependent E3 (Cul3) ubiquitin ligase complex to Nrf2 as a part of a post-translational modification of the protein. This leads to Nrf2 degradation under normal conditions making its half-life around 13-21 minutes [116, 117]. This keeps cellular Nrf2 levels relatively low. Keap1 is rich in the amino acid cysteine which makes it works as a sensor for the redox status of the cell.

Under oxidative stress, intracellular ROS and electrophiles increase leading to increasing oxidation of Keap1 cysteine residues (C¹⁵¹, C²⁷³, C²⁸⁸, C⁶¹³) that limits its activity as a bridge binding ubiquitin ligase complex to Nrf2 and thus limits its role in Nrf2 degradation. Here Nrf2 half-life extends to 100-200 minutes [117]. Once Nrf2 is

released from the Keap1 complex, and survives degradation, it migrates to the nucleus where it plays its role as a transcription factor for ARE genes [118]. Nrf2 binds to promoter regions of many cytoprotective genes including L-PGDS, as recently a Nrf2 binding site was identified on the promoter region of L-PGDS gene as explained before [108]. **Figure 7** explains the Nrf2 pathway and the role of ROS in its function.

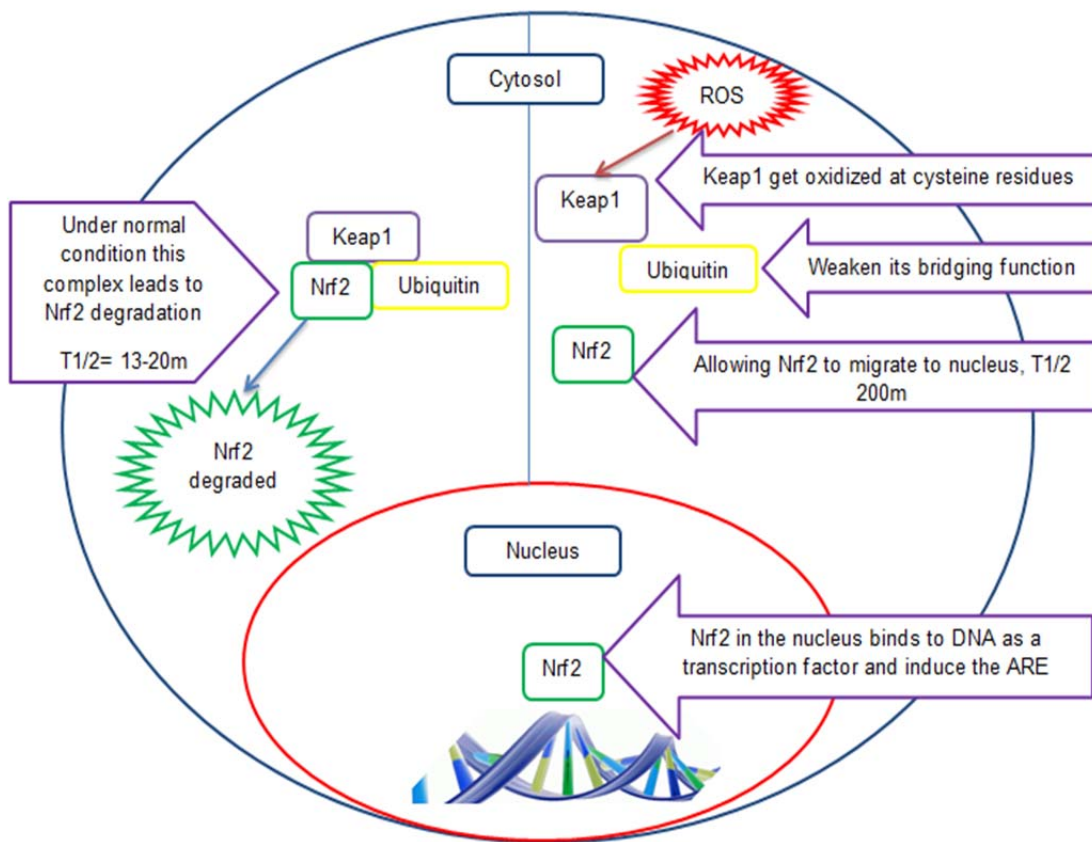


Figure 7: Nrf2 pathway and role of ROS in its activation.

ROS leads to oxidation of cysteine residues on Keap1 leading to loss of its function as a bridge for ubiquitin. This leads to longer t1/2 for Nrf2 which then migrates to the nucleus and binds to promoter regions of the ARE genes.

A recent study in 2013 used coffee alkaloid trigonelline (trig) as an inhibitor of Nrf2 pathway in pancreatic cancer cells to block its protective effect and its anti-apoptotic effect. They had promising results controlling cancer growth [119]. Estrogen-related receptor beta (ERR β) is a family of nuclear receptors that were found to block the Nrf2 pathway, particularly the (SFhERR β) member of this family [120]. Nrf2 siRNA can also be used to silence this transcription factor to test its involvement in IL1- β induced L-PGDS [121].

To summarize, PGD₂ had a positive feedback on L-PGDS induction through Nrf2, so it may activate the intrinsic ARE as well as Nrf2. This suggests that PGD₂ can contribute to anti-inflammatory actions in two different ways, through 15d-PGJ2 or Nrf2 induced ARE. Nrf2 has a specific binding site on L-PGDS gene promoter region and induces it in macrophages. This opens the door for the hypothesis that the Nrf2 pathway may be involved in L-PGDS induction in chondrocytes also. IL-1 β has a known role as an inflammatory mediator in many tissues including chondrocytes. It induces many inflammatory pathways including those producing free radicals, the main activator of Nrf2. This may explain the mechanism by which IL-1 β induces L-PGDS, through Nrf2.

1.7.6 Objectives and rationale:

15d-PGJ2 is a major anti-inflammatory PG. It works through PPAR- γ to downregulate major inflammatory genes like MMP1, MMP13, iNOS, TNF- α , IL1 β and COX-2. 15d-PGJ2 is formed by a dehydration reaction from PGD₂. PGD₂ is formed by PGDS from PGH₂, the main precursor for all PG's. PGH₂ is in turn formed from AA by the

COX enzyme. This makes PGDS the rate-limiting step for 15d-PGJ2 synthesis and so for a major intrinsic anti-inflammatory pathway. **Figure 22** explains this pathway in details. Considering the abundance of L-PGDS versus H-PGDS in chondrocytes, both normal and OA, we are focusing in this study on L-PGDS. Therefore, the main objective of this study is to understand the regulation of L-PGDS expression in chondrocytes. We are aiming in this study to identify chondrocytes' response to different inflammatory conditions *in vivo* and *in vitro*, regarding L-PGDS. To do so, we are going to analyze the effect of IL-1 β and interleukin 17 (IL-17) (as inflammatory cytokines), as well as H₂O₂ and 4BHP (as ROS generators) on L-PGDS expression levels in human chondrocytes *in vitro*. ROS generators are used in this study for two main reasons, first, ROS induce inflammation and second, ROS increase the half-life of Nrf2 and so enable its translocation into the nucleus to express ARE genes (see **Figure 7**). Considering that Nrf2 has a binding site on L-PGDS gene promoter region and plays a role in its regulation as explained before, among our objectives is to study the effect of ROS generators on L-PGDS expression levels. *In vivo* also, we are going to analyze L-PGDS expression levels by chondrocytes in mice OA models, both surgically induced and naturally induced (by aging), using immunohistochemistry techniques.

Chapter 2: Materials and Methods

2.1 Destabilization of medial meniscus surgery:

The destabilization of medial meniscus (DMM) surgeries were done in 10-12 weeks old male mice, strain C57BL/6, to induce an OA model. These mice were sacrificed at 8 weeks of ages post-operatively; this time selection was based on observations from previous work done in our lab for the time needed to develop OA post DMM. Their knees were examined for OA changes histologically and for L-PGDS expression by immunohistochemistry.

The medial meniscus is fixed to the tibial plateau by the medial meniscotibial ligament (MMTL). A 3 mm longitudinal incision was made on the distal side of the patella with a # 15 blade exposing the joint capsule. The joint capsule was then opened by micro-iris scissors and a dissection was performed on the meniscotibial ligament. MMTL was dissected in a proximo-lateral direction with a micro-surgical knife. This led to the destabilization of the medial meniscus which eventually led to a medial displacement of the meniscus. The joint capsule was then closed by continuous sutures using 8-0 Vicryl®, and for the subcutaneous tissues and skin 7-0 Vicryl® was used. Sham surgeries were performed in animals following the same steps without dissecting the meniscotibial ligament.

2.2 Aging associated OA:

We followed the normal aging process in a large number of mice to determine the spontaneous appearance of OA compared to the DMM model. We sacrificed the mice and collected their knees at 3 or 18 months of age; this was also based on observations from previous work done in our lab for aging associated OA. Our aim

was to obtain knee joint samples at different age points to study the changes in the articular cartilages, bones, synovial membranes, genes and proteins at different time points.

2.3 Histology:

Histology blocks were made in collaboration with other labs at Notre Dame Hospital. Blocks were cut using Leica® rotary microtome into 5 micrometers slices. They were then put on histology glass slides, and kept in the incubator for 48h at 37°C.

For histological staining we used a protocol of different washing and staining steps, in which the slides were processed as follow:

First we washed the slides in Xylene for 5 minutes twice. The slides were then washed again in ethanol 100% for 3 minutes twice. Then, one more wash with double distilled water (ddH₂O) for 2 more minutes.

Slides were then stained with hematoxylin weigert 50% (Sigma-Aldrich®) for 4 minutes, then washed in ddH₂O for 2 minutes, incubated in ferrous chloride for 1 minute, and finally washed in ddH₂O for 2 more minutes.

Fast green staining (Sigma-Aldrich®) was done by incubating the slides in the dye for 30 seconds, then in acetic acid 1% for 5 seconds, followed by safranin-O (Sigma-Aldrich®) for 4 more minutes.

The final step was washing the slides with ethanol 100% for 1 minute, 3 times and then xylene for 2 minutes, two times. Slides were ready at that point and were covered with glass cover slips and glued.

2.4 Immunohistochemistry:

For immunohistochemistry, we washed the slides in Xylene for 5 minutes, 3 times and then in ethanol for 5 minutes, 3 more times (at concentrations of 100%, 95% and 75%). A final wash was performed in ddH₂O for 1 minute twice. The sections on the slides were then marked with histology PAP pen (Abcam®), incubated with one drop on each section of peroxidase (Dako®) for 10 minutes at room temperature on a humid rack. This last step was the 1st blocking step. Slides were then washed with ddH₂O for 1 minute, twice. They were then blocked again with 50µl of 1% BSA for 1h at room temperature. The 1st antibody was then incubated with the slides overnight (O/N) at 4°C in BSA 1% at different concentrations. For L-PGDS, the concentration was 1:100. Slides were then prepared for day 2 where they were washed in ddH₂O for 1 minute twice, and then 1 drop of biotinylated link antibody (Dako®) was added to each slide for 30 minutes at room temperature. Slides were then washed again in ddH₂O for 1 minute twice. Streptavidin HRP was added to each slide for 1 hour at room temperature. Another wash cycle was done. For the next step we prepared a diaminobenzidine (DAB) solution (Dako®), as 1 drop of DAB in 1ml of its buffer in a dark Eppendorf. The DAB solution forms a brown stain in peroxidase-based immunohistochemical reactions. We added 1 drop of the DAB preparation to each section and followed the change of the color under the microscopy. Once the tissues get the DAB brown stain we stopped the reaction by washing the slides in ddH₂O to avoid oversaturation of the signal. Lastly, the slides were counterstained with Eosin. Slides were washed in ddH₂O for 1 minute 4 times, then in 80% ethanol for 30 seconds, and then stained with Eosin for 2 seconds. Slides were then washed in

100% ethanol 3 times (5 minutes, 3 minutes and another 3 minutes). Lastly, they were washed in xylene for 2 minutes, after which we glued the slides with a glass cover. Each group of slides was treated under the same conditions and for the same periods so we can compare the results from different slides in the same group and within different groups. Expression levels of L-PGDS were quantified by counting cells stained with the DAB brown stain versus the total number of cells per high power microscopic field. This counting was done with at least 3 slides for each condition tested and the median of each condition was taken. The medians were compared in aged versus young mice, and in DMM versus the sham surgery group to quantify the increase in L-PGDS expression.

2.5 Cartilage collection & Chondrocyte culture:

Knee specimens were collected from patients undergoing joint replacement on the same day of operation or 1 day after. Normal knee specimens were collected from cadavers shortly after death. Synovial fluid samples were collected from patients with OA and normal subjects. All specimens were kept at 4°C until digestion to ensure the viability of chondrocytes.

Cartilage pieces were cut from the knees under aseptic conditions and kept them in cold sterile Phosphate buffered saline (PBS). The cartilage pieces were then washed twice with sterile PBS then kept in (penicillin/streptomycin) 5X in PBS for 1 hour at room temp. Cartilage pieces were then washed again twice with regular sterile PBS (without antibiotics), to remove any traces of the antibiotics. We started then digesting the cartilage pieces by incubating them in Pronase 1mg/ml in DMEM+ 10% FBS+ 1%

P/S Sigma-Aldrich® in a water bath at 37° for 1 hour with gentle agitation. Cartilage pieces were then washed with warm (37°C) sterile PBS twice. A collagenase digestion was then performed using 600units/ml or 2mg/ml of collagenase type I (Sigma-Aldrich) following its addition to the cartilage pieces for 5 hours incubated at 37°C in a water bath with gentle agitation. The fluid (containing chondrocytes) was then collected into 50ml falcon tubes and centrifuged at 1500 revolutions per minute (rpm) for 10 minutes. The supernatant was discarded, cells were re-suspended in DMEM 10% FBS 1% P/S, then seeded into a 175ml flask. The flasks were then incubated at 37°C in humidified 5% CO₂ incubator, for 2 weeks until they reach full confluence. The remaining cartilage pieces were then digested one more time by applying 50% diluted collagenase from the previous preparation, incubated at 37°C in a water bath with agitation O/N. The digestion fluid then went through the same previous steps to collect 2nd day chondrocytes and seed them into another 175ml flask. Both flasks were incubated for 2 weeks until they reached full confluence. Chondrocytes were then collected from the 175ml flask by trypsinization using 5ml trypsin 0.05% Multicell® per flask and kept in the incubator for 5 min. After the 5 minutes, the trypsinization reaction was stopped by adding 10ml DMEM media containing 10% FBS and 1% P/S. The fluid was then centrifuged at 1500rpm for 10 min, cells were re-suspended in different volumes of DMEM 10% FBS 1% P/S according to the final cell count needed per ml. After counting cells, they were seeded into 6 well plates at a density of 750,000 cells per well in 2ml media or into 12 well plates at a density of 250,000-300,000 cells per well in 1ml media. Cells were left 24h

to attach to the wells and then serum starved in a media containing 1% FBS for another 24h. At that point, chondrocytes were ready to be treated.

Chondrocytes were treated with:

- IL-1 β (an inflammatory cytokine) at different concentrations from 0-600pg/ml for 48h, or at 100pg/ml for time course assays.
- IL-17 (also an inflammatory cytokine) at different concentrations from 0-100ng/ml for 48h, or at 50ng/ml for time course assays.
- H₂O₂ (as a ROS generator) at different concentrations from 0-50 μ M/ml for 48h (re-added every 24h due to its instability), or at 20 μ M/ml for time course assays, (re-added also every 24h).
- 4BHP (also a ROS generator) at different concentrations from 0-50 μ M/ml for 24h and 48h, or 20 μ M/ml for time course assays.

For cell lysis, we removed the growth media from the plates from the previous step and washed the cells with cold sterile PBS 1ml/well twice. We then lysed the cells with a lysis buffer composed of Tris 20mM PH7.4, NaCl 150mM, EDTA 1mM PH 7.4, PMSF 1mM, protease inhibitors at concentration 1 μ g/ml and SDS 0.5% in double distilled water using 200 μ l/well for the 12 wells plates, or 400 μ l/well for the 6 well plates. The cells were incubated with the lysis buffer for 10min at room temperature with gentle shaking. Samples were then collected in Eppendorf tubes and sonicated for 10sec on ice. The lysate was then centrifuged at 13,000 rpm for 5min. Proteins

from the supernatant were then quantified by Pierce™ BCA Protein Assay Kit from Life technologies® according to the manufacturer's instructions.

2.6 Western Blotting (WB):

To detect L-PGDS produced by the chondrocytes, we used 20µg of the cell lysate protein per sample and added 5µl sample buffer (10X) to it; the total volumes were then equalized with lysis buffer to 50µl. The samples were heated for 2 minutes at 95°C, left to cool down, and then loaded into 12% acrylamide gel to get separated.

To detect L-PGDS released by the chondrocytes *in vitro*, we used 45µl per sample of the growth media of the treated chondrocytes. We added 5µl of the sample buffer (10X) to each; the samples were then processed as above.

To detect L-PGDS released by the chondrocytes *in vivo*, we used 20µl of the synovial fluid per sample plus 5µl of the sample buffer (5X) for each; and then processed as above.

Lastly, we used 1µl of the CSF as a positive control and we added 5µl of the sample buffer (10X) also; total volumes were then equalized to 50µl and processed as above. We used 12% sodium dodecyl sulfate SDS-page gel for better separation of this small MW protein (L-PGDS). Gels were prepared in our lab. Proteins were transferred to nitrocellulose membrane (Bio-Rad®) after migration on the gel.

Antibodies used:

- L-PGDS, (US-Biological®), at the concentration recommended by the manufacturer. For Immunohistochemistry 1:100, for WB 1:1000 with O/N incubation at 4°C.
- B-actin, (Santa Cruz biotechnology®) at a concentration of 1:200.
- iNOS and COX-2 were from Cayman chemicals®, both used at a concentration of 1:1000 for WB.
- The 2nd antibody was a goat anti-rabbit IgG coupled to horseradish peroxidase (HRP). This was used for all the primary antibodies and was bought from Cayman chemicals®. The 2nd antibody was used at a concentration of 1:10,000 for 1 hour at room temperature.

Nitrocellulose membranes were incubated O/N with the 1st antibody at 4°C then washed with TTBS wash buffer for 6 times, 10 minutes each. Then, they were incubated with the 2nd antibody at room temperature for 1h, and again washed 6 more times, 10 minutes each. Developing the membranes was done by the super-signal developing agent (Thermo scientific®) using the Bio-Rad® Chemidoc® imaging system.

Chapter 3: Results

All the experiments in this section were performed at least 3 times, and the results presented were consistent in at least 3 different experiments.

1. Histological changes in aging-associated and surgically-

induced OA:

To identify the pathological features of osteoarthritic cartilage either aging-associated or surgically induced, histological sections were compared from 3-month-old mice versus 18-month-old animals, and from 8-weeks post sham surgery mice versus 8-weeks post DMM animals, respectively. Upon confirming the development of OA histologically, samples were used to test for L-PGDS expression. In aging-associated OA (**Figure 8**), we identified areas of cartilage loss, damage and fibrillation (arrow). These changes represent the main pathological features of OA that can be histologically identified. **Figure 9** shows area of cartilage loss (arrow), using lost Safranin-O staining in surgically induced OA animals compared to the sham surgery group.

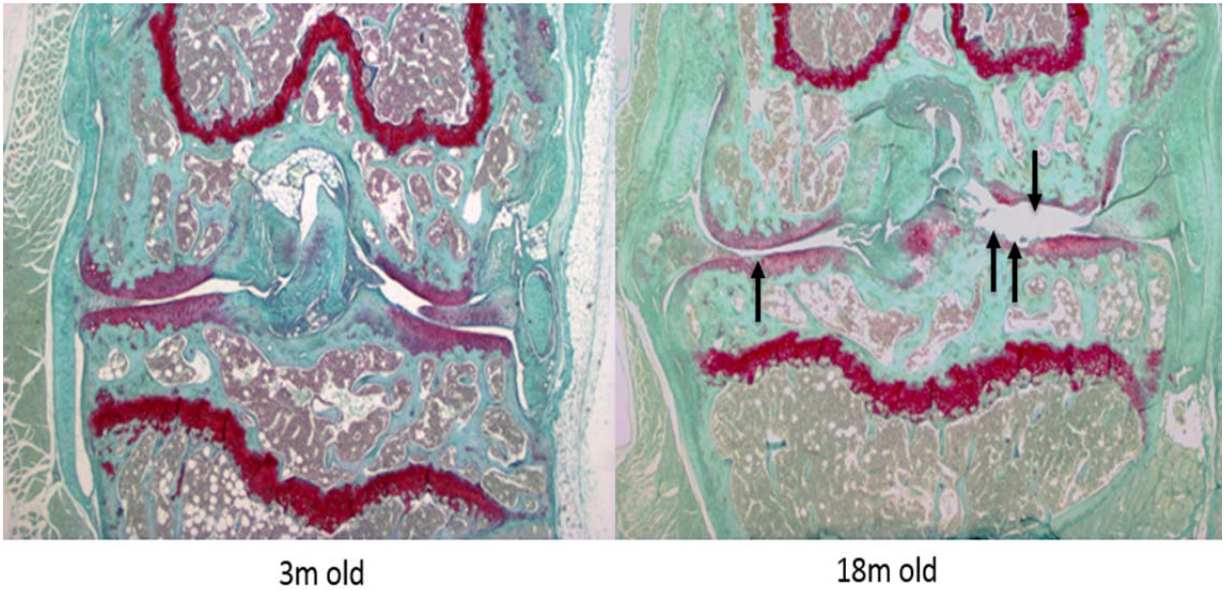


Figure 8: Histological changes in aging-associated osteoarthritis.

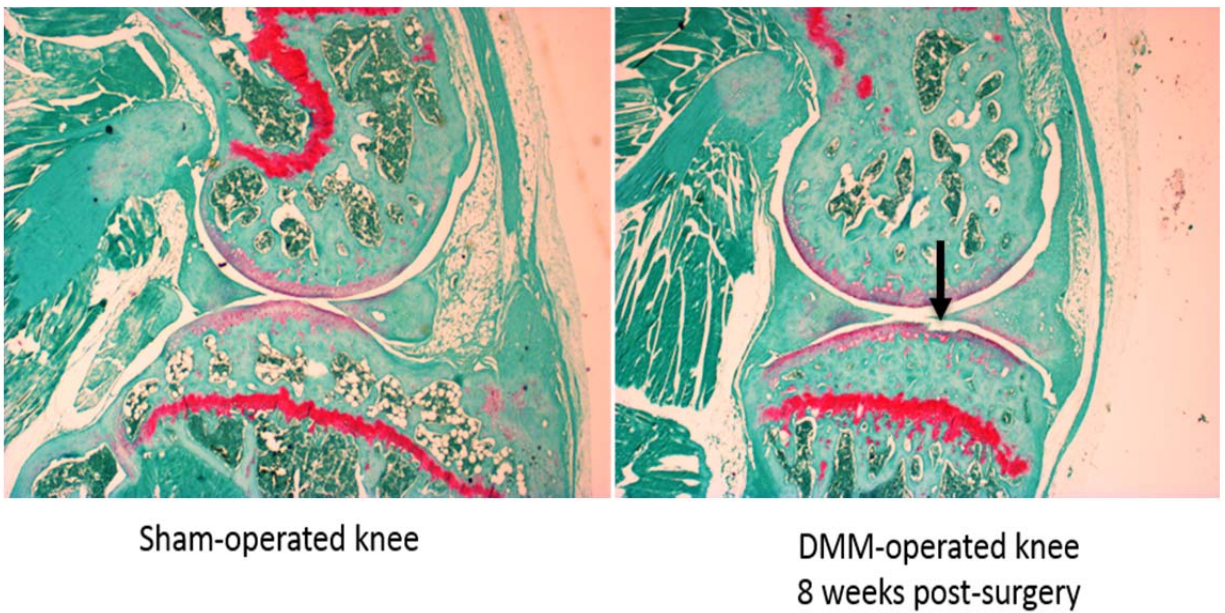


Figure 9 Histological changes in surgically induced OA.

Figures 8 and 9: Safranin-O was used to stain the proteoglycans in cartilage so we could visualize the articular cartilage versus the surrounding tissues which were

stained green by 'fast green stain'. The red coloration indicates proteoglycans in cartilage.

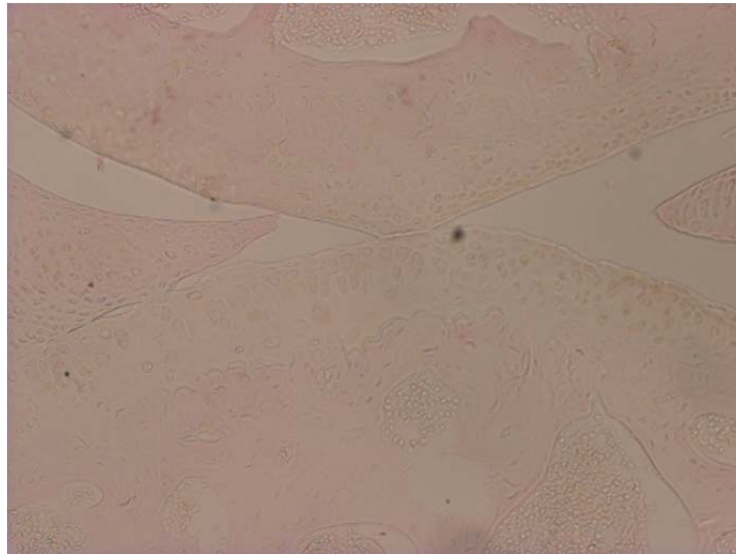
2. Expression of L-PGDS in aging-associated and surgically-

induced OA:

We investigated the expression levels of L-PGDS *in vivo* in aging-associated as well as in surgically-induced OA by immunohistochemistry. For aging-associated OA, L-PGDS expression levels were compared in 3-month-old mice versus 18-month-old animals in **Figure 10**, where we could see higher expression levels (2-3 folds) in the 18-month-old mice.

For surgically induced OA, mice which underwent DMM surgeries were compared to those with sham surgeries. Eight weeks post DMM or sham surgeries, knees were collected and put in paraffin blocks and slides were made for immunohistochemistry. L-PGDS expression levels were 3 folds higher in DMM-operated mice compared to sham-treated animals (**Figure 11**).

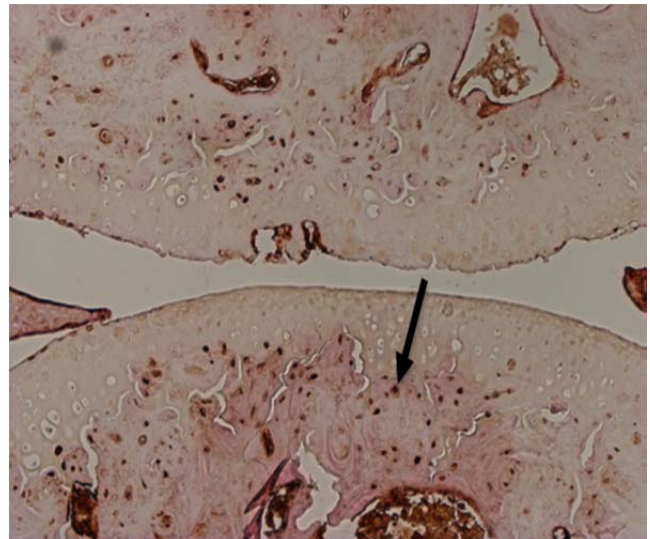
These results indicate that chondrocytes increase their expression of L-PGDS *in vivo* in response to either age-associated OA or following surgically-induced OA.



Negative control



3m old



18m old

Figure 10 L-PGDS levels in spontaneous age associated OA - immunohistochemistry.

Figure 10: Spontaneous age-associated OA. L-PGDS levels were higher in 18-months-old mice compared to 3-months-old animals. The arrow indicates the pigmentation visualized using the Dako® kit.

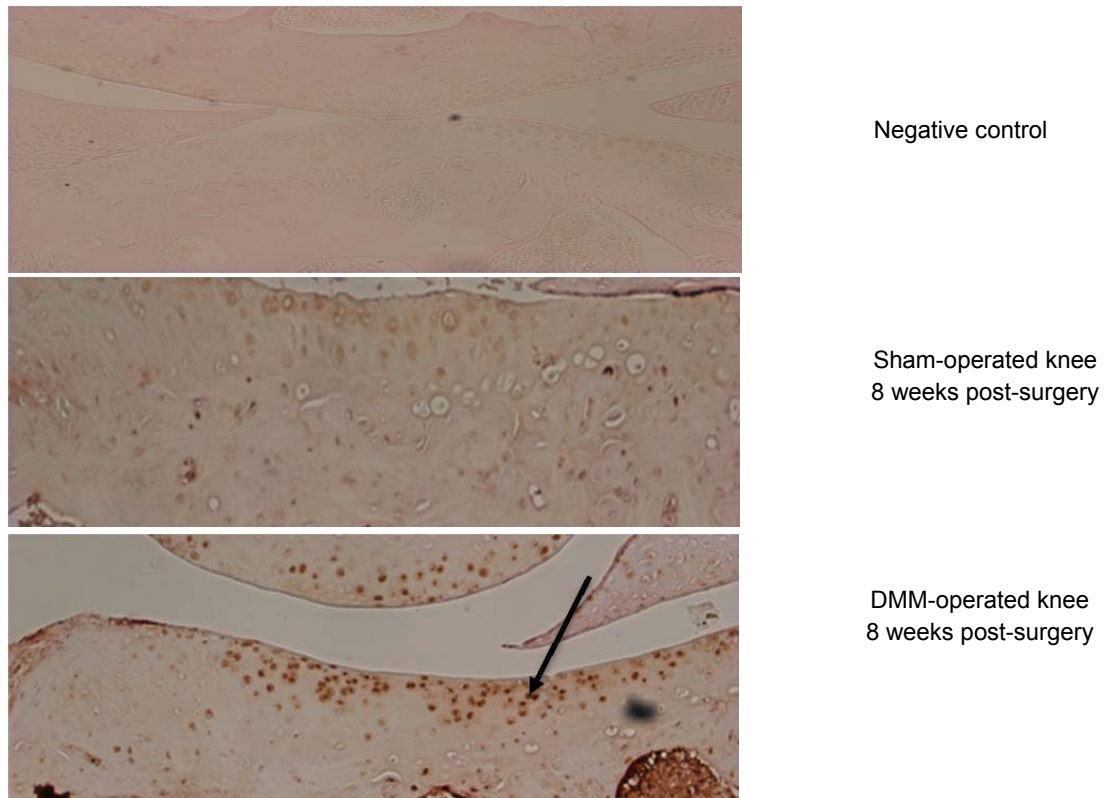


Figure 11 L-PGDS levels in surgically induced OA - immunohistochemistry.

Figure 11: DMM is a widely used surgery to induce OA models in knee joints. For the control mice, sham surgeries were done without cutting the knee ligaments or menisci. Knees were collected 8 weeks post-operatively from the 2 groups and expression levels of L-PGDS were detected by immunohistochemistry (IHC). In the negative control slides all the IHC steps were done without the use of the 1st antibody. In all experiments the concentration of the 1st antibody (L-PGDS) was 1:100 and the Dako® kit was used for the detection of the 2nd antibody. Pigmented cells were counted versus total number of cells in each slide to identify the percentage of stained cells. This was then used to compare the expression levels of L-PGDS in 3 month versus 18 months old mice and in DMM versus sham surgeries mice.

3. Expression of L-PGDS in chondrocytes treated with IL1 β ,

dose-dependent and time course:

- **Dose-dependent treatment:**

IL-1 β is a well-established inflammatory mediator [122] and widely used to induce inflammation *in vitro*. We previously showed that the levels of L-PGDS in chondrocytes treated with IL-1 β increase in a dose and time-dependent manner [95]. We used different doses and different durations of treatment to achieve the optimal results. The new finding here is that treating chondrocytes with different inflammatory cytokines (IL-1 β and IL-17) or ROS generators (H₂O₂ and 4BHP) not only increases L-PGDS expression but it also leads to the release of L-PGDS. The role of the released L-PGDS is not fully understood yet. Recent studies demonstrated that L-PGDS is a heavily glycosylated protein and each glycosyl group adds 3KD to the original protein MW.

We identified L-PGDS in IL-1 β treated chondrocytes cell lysate in a modified isoform of 37KD versus the original protein at 25KD, while we observed both the modified and non-modified isoforms in the growth media (**Figure 12**). We believe that different isoforms of L-PGDS do different functions in different locations, and also that they are produced in response to different stimuli.

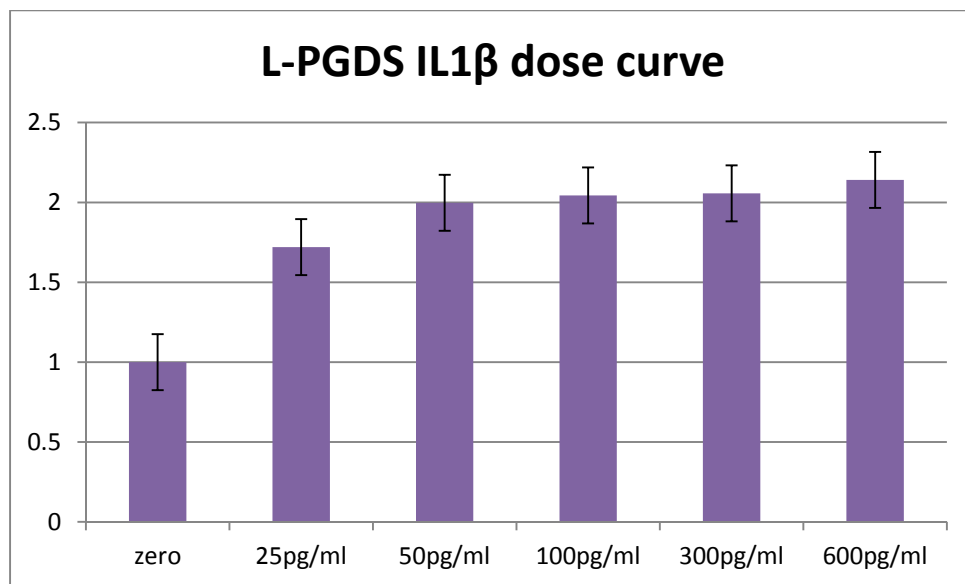
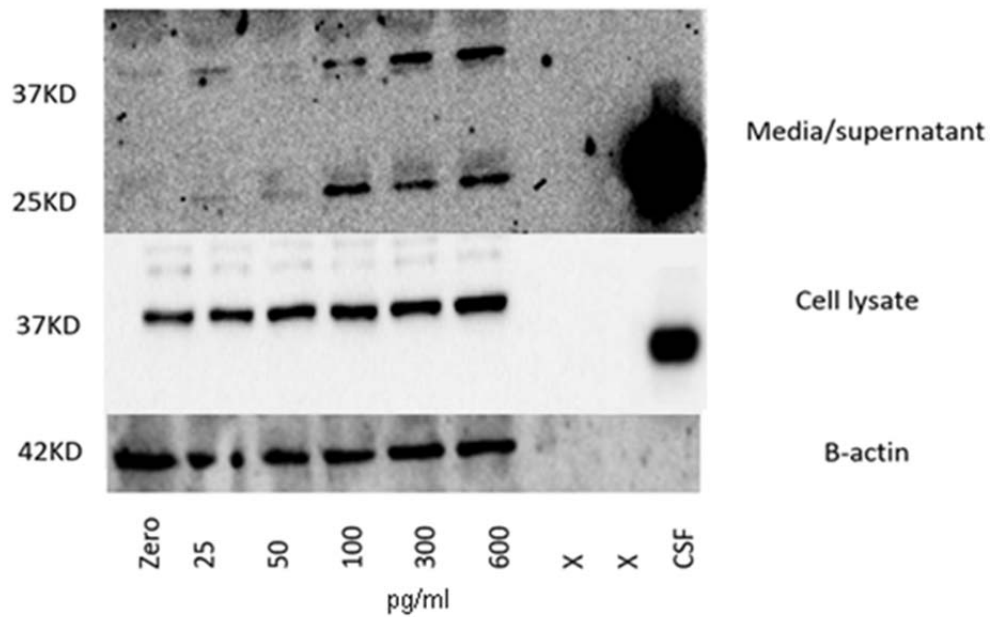


Figure 12: L-PGDS expression in IL1B treated chondrocytes dose curve.

Figure 12: Chondrocytes were treated with IL-1 β in at concentrations ranging from 25pg/ml to 600pg/ml for 48h. L-PGDS levels were detected in 20 μ g of the cell lysate and 40 μ l of the supernatant (growth media). CSF was used as a positive control in all

gels, as it is known to contain high levels of L-PGDS [123]. We used 0.5µl CSF per gel and L-PGDS was always detected at 25KD in all CSF samples. (X) Marks an empty well in all gels.

- **Time course treatment:**

To study the effect of prolonged exposure of chondrocytes to IL-1 β , we performed multiple time points and dose response curves to adjust for the best results. During these experiments, we observed an increase in L-PGDS expression levels in a time-dependent manner in the cell lysate as well as in the media. We further found that, in IL-1 β treated chondrocytes, L-PGDS levels in the cell lysate increased steadily with treatment periods. We also found that L-PGDS in the cell lysate was detected at a MW of 37KD (the modified isoform), (**Figure 13**). In the supernatant (growth media), the non-modified isoform of L-PGDS was the prominent form observed compared to the response from the dose curve treatment experiments. This may suggest a shorter half-life for the modified isoform of L-PGDS.

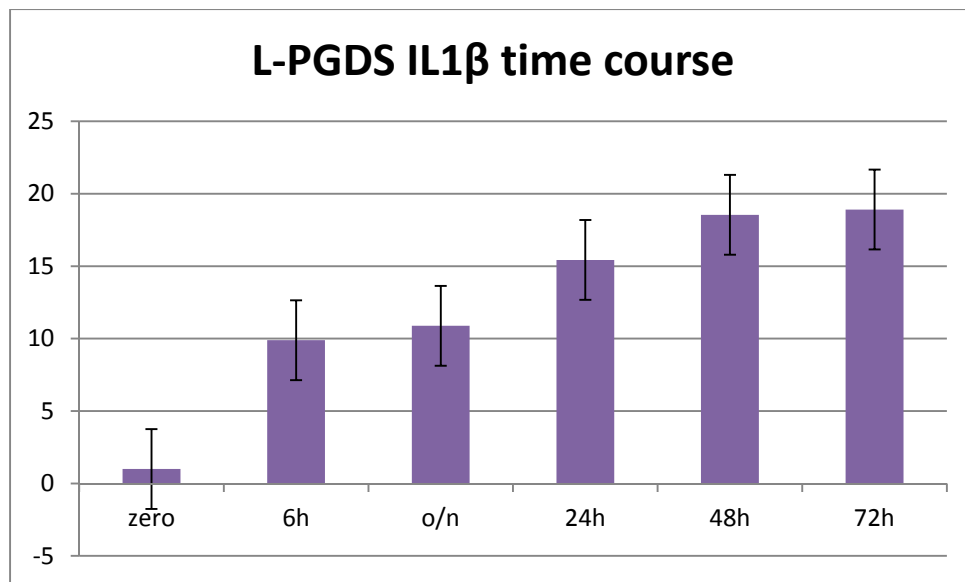
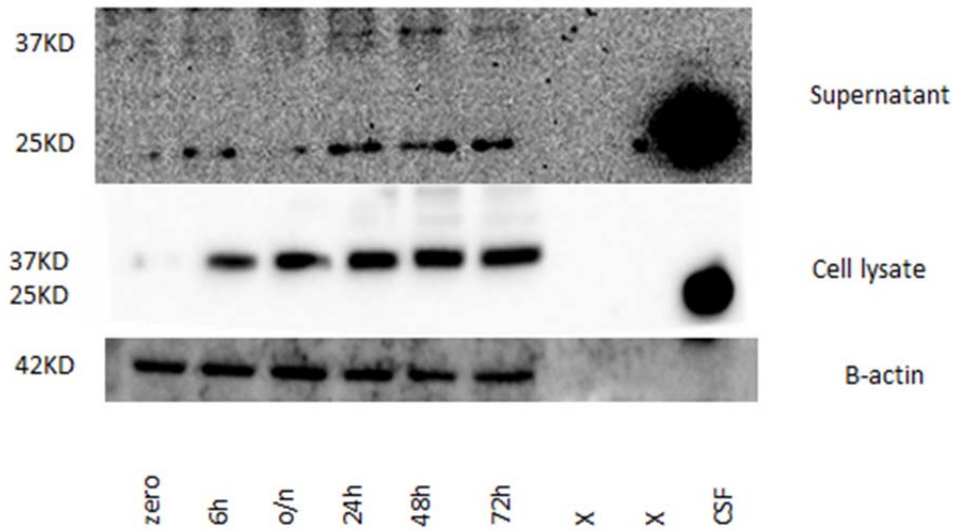


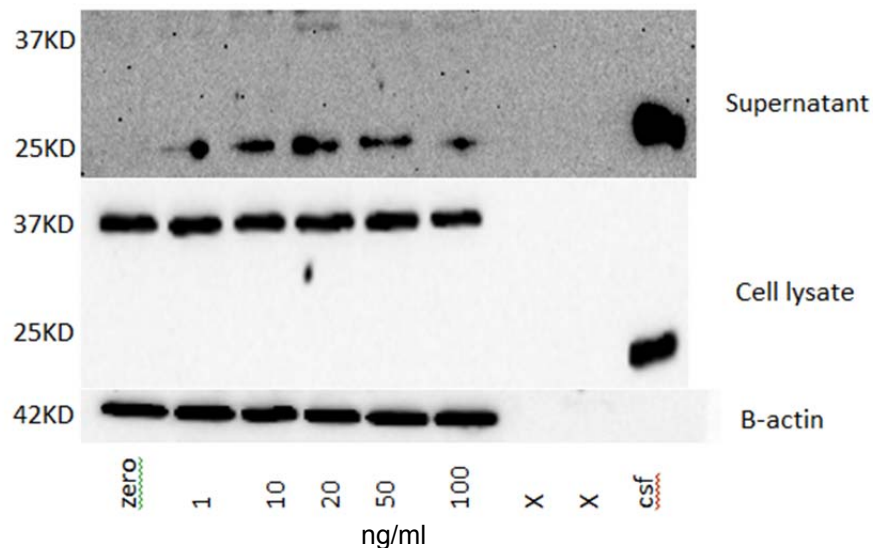
Figure 13: L-PGDS expression in IL-1 β treated chondrocytes time course.

Figure 13: Chondrocytes were treated with IL-1 β 100pg/ml for different time periods ranging from 6h to 72h. L-PGDS levels were detected in 20 μ g of the cell lysates and 40 μ l of the supernatants (media). CSF was used as positive control. (X) Marks an empty well.

4. Expression of L-PGDS in chondrocytes treated with IL-17, dose-dependent and time course:

- Dose-dependent treatment:

Our results suggest a weaker effect of IL-17 compared to IL-1 β on L-PGDS expression levels and its release into the media. Indeed we couldn't identify an increase in L-PGDS expression levels in chondrocytes cell lysates in response to IL-17 treatment. IL-17 treatment was still able to induce chondrocytes to release L-PGDS into the media, in the non-modified isoform also. An interesting finding here was that L-PGDS levels in the media increased in a bell-shaped curve pattern, where they peaked at 20ng/ml IL-17 and then went back down again (**Figure 14**).



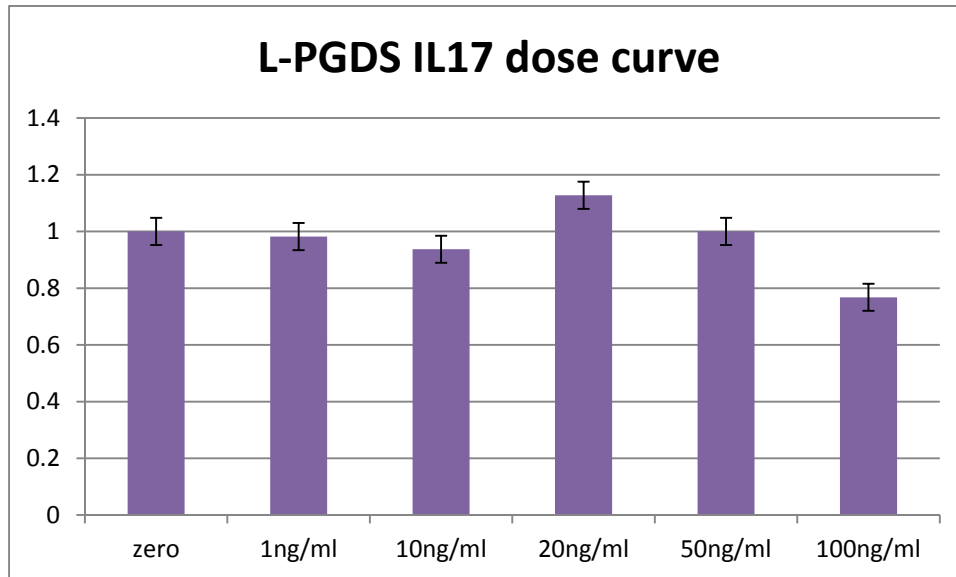


Figure 14: L-PGDS expression in IL-17 treated chondrocytes dose curve.

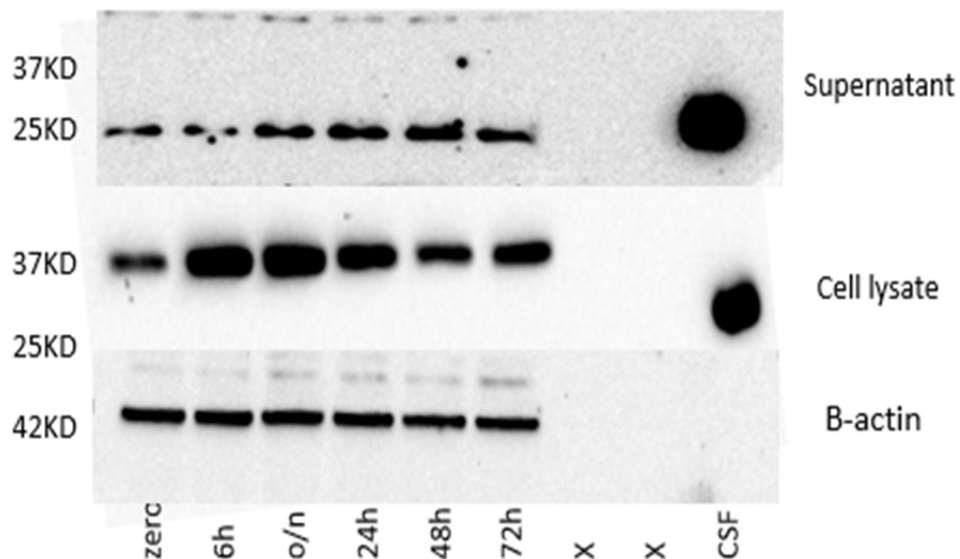
Figure 14: Chondrocytes were treated with IL-17 at concentrations ranging from 1ng/ml to 100ng/ml for 48h. L-PGDS levels were detected in 20µg of cell lysate and 40µl of supernatant (media). CSF was used as a positive control, (X) Marks an empty well.

The results from the two previous Figures imply that chondrocytes under inflammatory situations try to activate their salvage pathways among which the induction of L-PGDS to produce PGJ2, a major anti-inflammatory component [124].

- **Time course treatment:**

While treating chondrocytes with IL-17 for different periods of time, the response was challenging as we didn't initially have the expected results, especially that the dose curve was showing no increase in L-PGDS expression levels. At 50ng/ml, we started to have a response with the time course of IL-17 treatment. L-PGDS levels increased

in a bell-shaped curve also, peaking at 6h and O/N and then went back to almost basal levels at 48h. This actually partially explains why we didn't observe a response with the dose curve (in the cell lysate) where all plates were incubated for 48h. Thus, no matter what was the dose of IL-17 used, at 48h L-PGDS levels were back at almost baseline. This may suggest a short half-life of L-PGDS. This may also be due to the loss of IL-17 effect after a few hours. IL-17 is usually stable and doesn't need to be added every 24h like H₂O₂. So the expected effect of IL-17 is achievable mainly during the 1st few hours. L-PGDS was detected again in the modified isoform in the cell lysate. While, in the media we were able to observe a mild increase in L-PGDS expression levels (the non-modified isoform) that peaked at 48h and went back down to almost baseline at 72h (**Figure 15**).



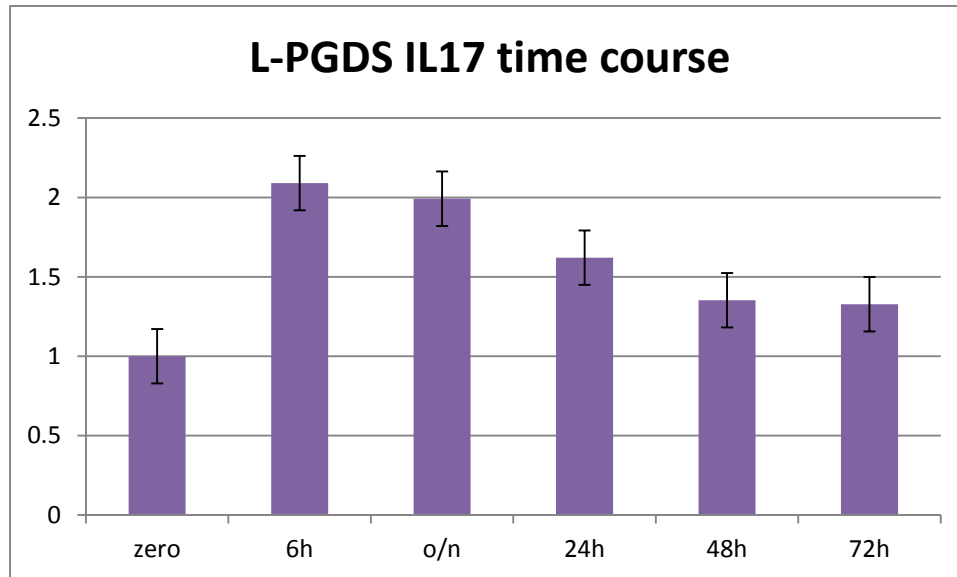


Figure 15: L-PGDS expression in IL-17 treated chondrocytes time course.

Figure 15: Chondrocytes were treated with IL-17 50ng/ml for different periods of time from 6h to 72h. L-PGDS levels were detected in 20µg of the cell lysate and 40µl of the supernatant (media). CSF was used as positive control. (X) Marks an empty well.

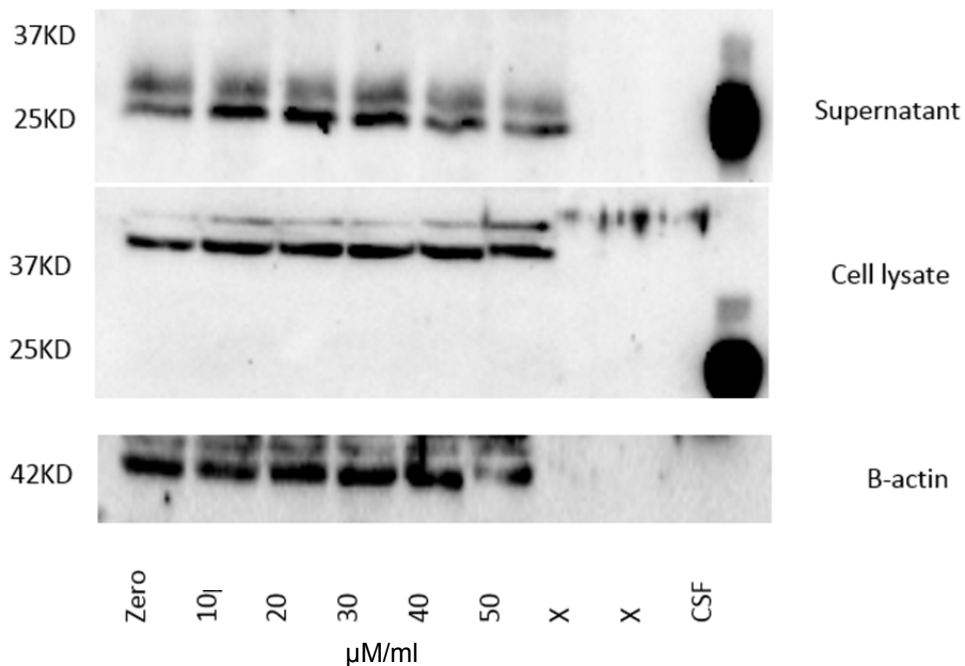
5. Expression of L-PGDS in chondrocytes treated with H₂O₂,

dose-dependent and time course:

- **Dose-dependent treatment:**

Chondrocytes were treated with H₂O₂ and tetra butyl hydroperoxide to produce ROS within the cells to study the effect of ROS on L-PGDS expression levels. ROS generators were used because according to our hypothesis Nrf2 is involved in L-PGDS gene regulation in response to ROS. We even hypothesized that this may be the same mechanism by which other inflammatory cytokines like IL-1β induce L-PGDS. This hypothesis is based on a study that identified a binding site for the Nrf2

transcription factor on the promoter region of L-PGDS gene in bone-marrow-derived macrophages (BMDM) [108]. Furthermore, researchers showed an increase in L-PGDS expression levels in BMDM when they were treated with PGD2 (positive feedback regulation) by a mechanism involving Nrf2 [108]. This induction effect was lost in Nrf2 KO mice and re-established by the re-introduction of Nrf2 using a vector. H₂O₂ treated chondrocytes showed no obvious increase in the expression of L-PGDS in the cell lysates. In contrast, chondrocytes released L-PGDS into the media in a dose-dependent manner in response to H₂O₂ treatment. Again here, L-PGDS in the media was in the non-modified isoform and its levels were showing a bell-shaped curve pattern (**Figure 16**), while in the cell lysate L-PGDS was in the modified isoform.



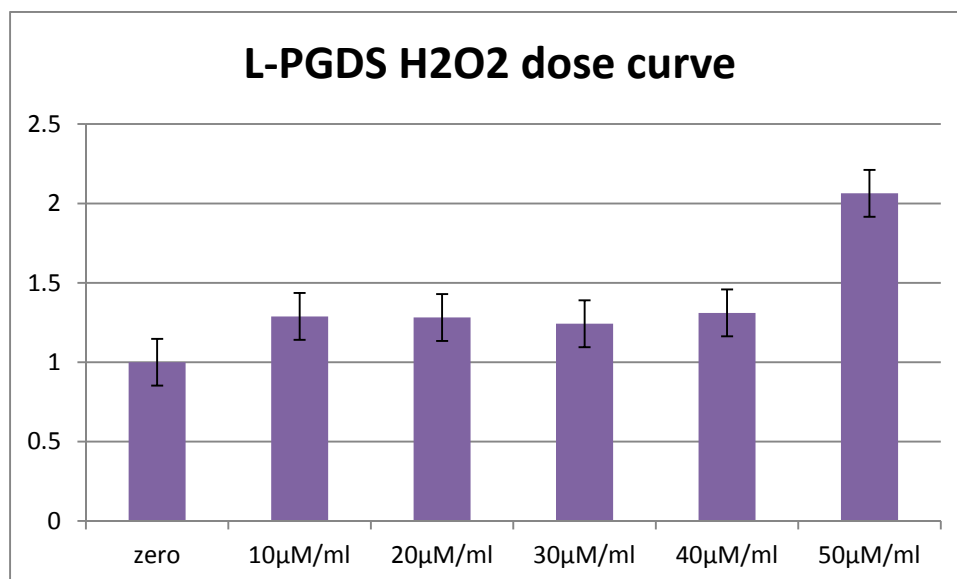


Figure 16: L-PGDS expression in H₂O₂ treated chondrocytes dose curve.

Figure 16: Chondrocytes were treated with H₂O₂ in a concentration ranging from 10µM/ml to 50µM/ml for 48h, and H₂O₂ was re-added every 24h due to its instability. L-PGDS levels were detected in 20µg of the cell lysate and 40µl of the supernatant (media). CSF was used as a positive control. (X) Marks an empty well.

- **Time course treatment:**

When chondrocytes were treated with H₂O₂ for different periods of time, we observed an increase in L-PGDS expression levels in treated cells versus control ones. This was also the case on the cell lysate level and for the modified isoform of L-PGDS. The expression of L-PGDS wasn't steadily rising; instead, it mildly rose at 6h then stayed at that level through the rest of the time points. L-PGDS wasn't detected in the media in all experimental time points with H₂O₂, (**Figure 17**).

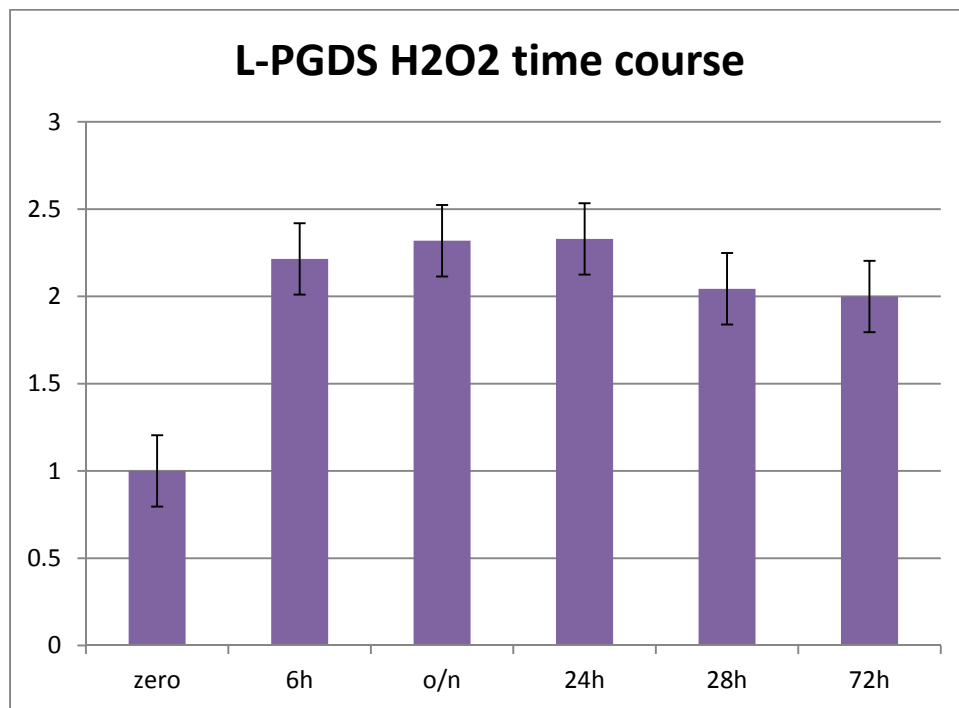
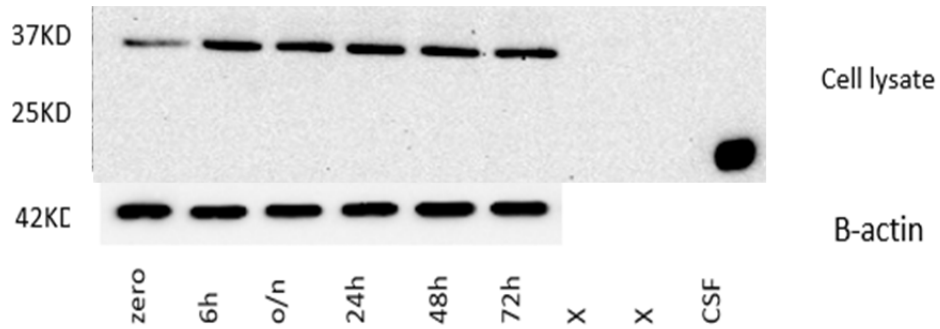


Figure 17: L-PGDS expression in H₂O₂ treated chondrocytes time course.

Figure 17: Chondrocytes were treated with 20 μ M/ml of H₂O₂ for periods of time ranging from 6h to 72h. Due to its instability, H₂O₂ was re-added every 24h to the media. L-PGDS levels were detected in 20 μ g of the cell lysate and 40 μ l of the supernatant (media). CSF was used as positive control. (X) Marks an empty well.

6. Expression of L-PGDS in chondrocytes treated with 4BHP,

dose-dependent and time course:

- **Dose-dependent treatment:**

We used 4BHP as a ROS generator to induce L-PGDS in chondrocytes. 4BHP is a more stable ROS generator compared to H₂O₂; it can also induce cell death if the concentration is high or the duration of treatment is long enough. Because of that, we used 4BHP in small doses and for 24h of treatment only. Treating chondrocytes with 4BHP caused an increase in L-PGDS expression levels in the cell lysates, as well as it led to the release of L-PGDS into the media in a dose-dependent fashion. The levels of L-PGDS in the media made a bell-shaped curve. L-PGDS was detected in the media mainly in the non-modified isoform while in the cell lysate it was in the modified isoform, (**Figure 18**). Interestingly, we observed a rise in the levels of the modified isoform of L-PGDS “in the media” concomitantly with the decrease of the same isoform in “the cell lysate” after 48h treatment with increasing doses of 4BHP (**Figure 19**). In these last experiments, cell death was observed in the form of detached and floating cells under the microscope. We didn't measure cell toxicity or apoptosis markers for the observed cells. Our observation of detached and floating cells may suggest that the modified isoform of L-PGDS is a potent oxygen free radicals scavenger, and it was the cells last line of defense against ROS before they died. It's one of the well-known functions of L-PGDS to act as an oxygen free radicals scavenger through a thiol group in Cyc65 at its active site [109, 125].

It may also suggest that the modified isoform of L-PGDS is the most enzymatically active isoform. The only other time when we noticed the modified isoform of L-PGDS in the media was when chondrocytes were treated with IL-1 β which can also generate ROS.

These contradictory opinions underline the need for a further understanding of this protein post-translational modification and the role each isoform plays. Doing so, we will be able to identify the protective isoform and further target it with medications. This is further analyzed in the discussion.

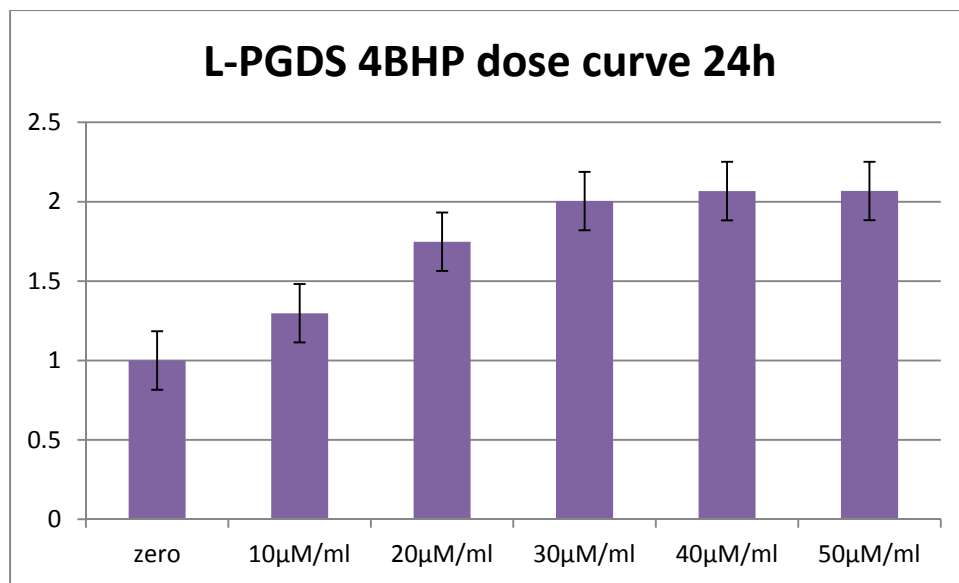
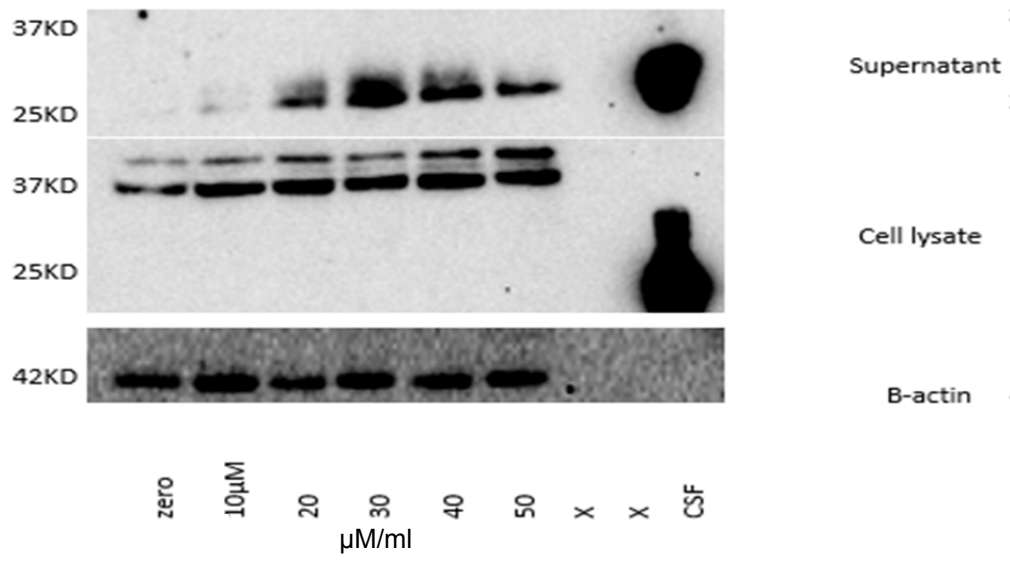


Figure 18: L-PGDS expression in 4BHP treated chondrocytes dose curve 24h.

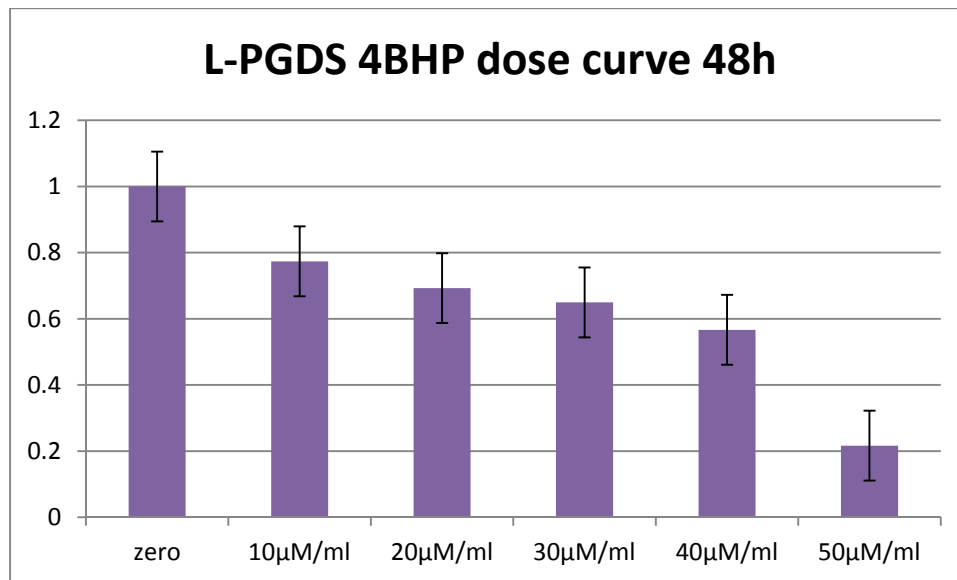
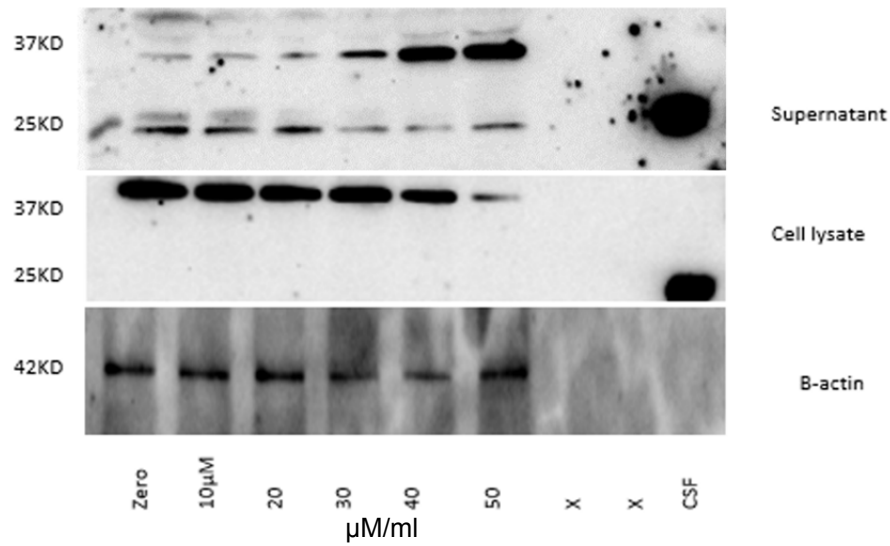


Figure 19: L-PGDS expression in 4BHP treated chondrocytes dose curve 48h.

Figures 18 and 19: Chondrocytes were treated with 4BHP in a concentration ranging from 10µM/ml to 50µM/ml for 24h in **Figure 18** and 48h in **Figure 19**. L-PGDS levels were detected in 20µg of the cell lysates and 40µl of the supernatants (media). CSF was used as a positive control. (X) Marks an empty well.

- **Time course treatment:**

Treating chondrocytes with 20µM/ml of 4BHP for different durations lead to inconsistent results (data not shown). This was mainly because of the cell death that was observed at many points during the experiments. This was observed while increasing the dose or the duration of 4BHP treatment. Cell death made the preliminary results inconclusive for anything other than the rising of the levels of the modified isoform of L-PGDS in the media concomitantly with the decrease of these levels in the cell lysate. These findings were demonstrated in **Figure 19**. Reducing the dose of 4BHP to allow longer exposure of chondrocytes didn't give any specific signal. Cell death was not measured here and no apoptosis markers were investigated. Cell death was just observed as detached and floating cells under the microscope. These observations were used only to adjust the dose of 4BHP and the duration of treatment, to study its effects without inducing cell toxicity or cell death.

7. Expression of L-PGDS in synovial fluid:

As we showed in previous results, chondrocytes release L-PGDS into the media for an unknown function (*in vitro*), in response to different inflammatory triggers. Aiming to detect the presence of this process *in vivo*, we examined synovial fluid samples for L-PGDS from different patients with or without OA. We found that L-PGDS is released by chondrocytes to the synovial fluid *in vivo* also. Even more, both the modified and the non-modified isoforms of L-PGDS were detected in some cases (**Figure 20**). This particular result needs further investigation and a large numbers of samples to identify which conditions are associated with L-PGDS release in the synovial fluid and which

aren't. Also to identify which isoform of L-PGDS is linked to which condition. This can be one of the future steps of this project.

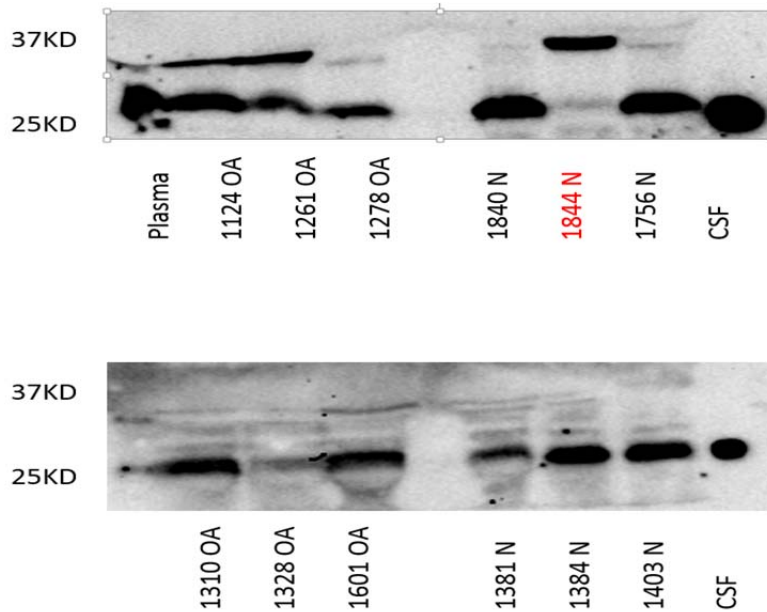


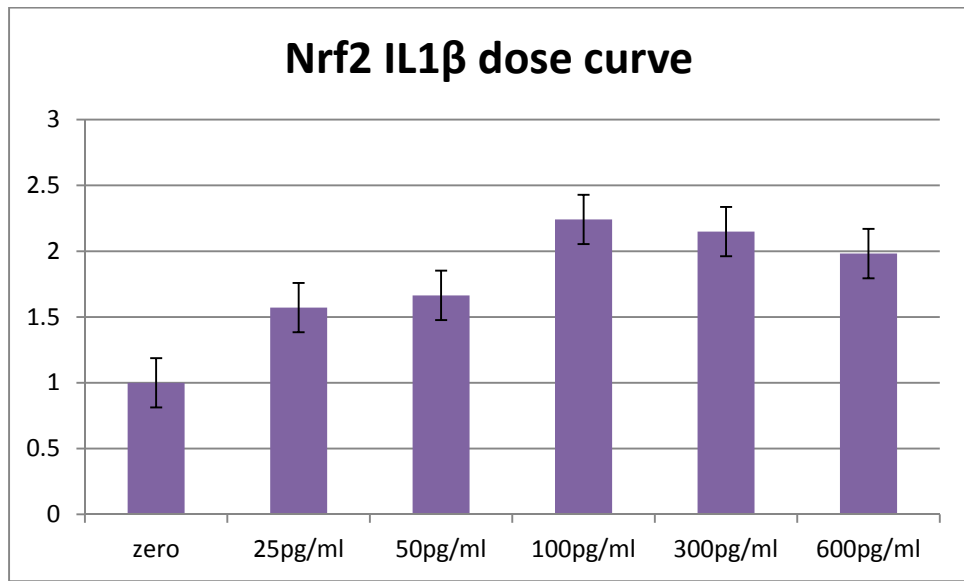
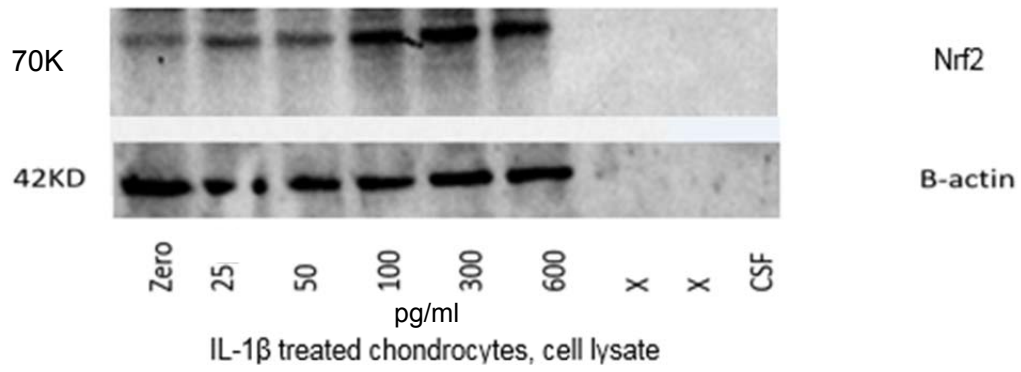
Figure 20: L-PGDS expression in synovial fluid samples

In further experiments synovial fluids were treated with Hyaluronidase to enhance the detection of L-PGDS. 20µl of Hyaluronidase treated synovial fluid samples were used. CSF and plasma were used as a positive control. Samples were labeled with the patients code numbers, (OA) indicates a patient diagnosed with osteoarthritis while (N) indicates a non-osteoarthritic one. The modified isoform of L-PGDS was detected mainly in OA patients, while in non OA individuals L-PGDS in the synovial fluid was mainly in the non-modified isoform, except for one N patient with the modified form of L-PGDS (1844 N). Due to limited access to the medical data of these patients we weren't able to exclude other inflammatory joints diseases, so we consider these

results preliminary and just to demonstrate that chondrocytes release L-PGDS to the extracellular space for an unknown function, to be explored. This goes in line with the results we found *in vitro* when chondrocytes were treated with IL-1 β or 4BHP in which both induced significant inflammation, and consequently the modified isoform of L-PGDS started to appear in the media.

8. Expression of Nrf2:

In our hypothesis, Nrf2 transcription factor plays an important role in upregulating L-PGDS gene expression in osteoarthritic chondrocytes. A recent paper showed a direct role for Nrf2 in L-PGDS gene expression in bone marrow-derived macrophages [108]. Furthermore, a binding site for Nrf2 was identified on the promoter region of L-PGDS gene. To study Nrf2 response to different inflammatory mediators, we treated chondrocytes with IL-1 β , IL-17, H₂O₂ and 4BHP, and checked for the level of expression of Nrf2 (total Nrf2). In IL-1 β treated chondrocytes, the expression levels of Nrf2 were directly increasing with increasing IL-1 β doses. Similar results were obtained from 4BHP treated chondrocytes (**Figure 21**). 4BHP as a ROS generator is expected to increase the Nrf2 half-life to 200 minutes (see **Figure 7** for Nrf2 pathway), which leads to accumulation of Nrf2 in the cytosol and thus its translocation into the nucleus to transcribe ARE genes. We believe that by the same mechanism Nrf2 plays a role in L-PGDS gene induction in chondrocytes treated with 4BHP [108, 126, 127]. The mechanism by which IL-1 β contributes to increasing Nrf2 level is not well known yet though it may also be through the production of ROS. We plan in future work to confirm the involvement of Nrf2 in L-PGDS gene regulation, by using Nrf2 agonists like Protandim® and check for the different isoforms of L-PGDS. We plan also to check for the loss of L-PGDS induction effect when Nrf2 pathway is disrupted by siRNA.



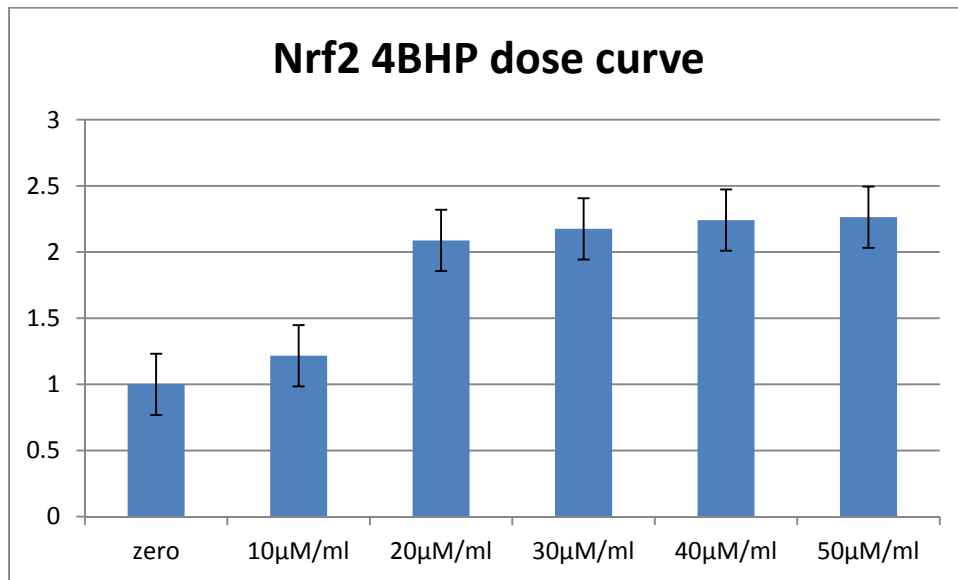
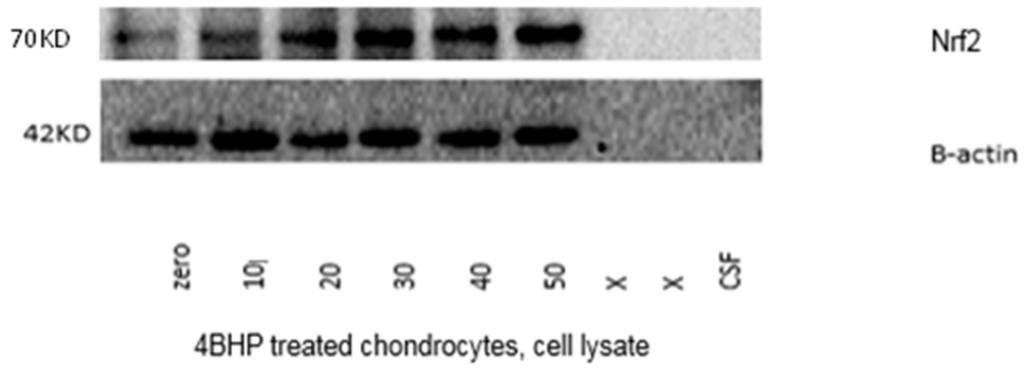


Figure 21: Nrf2 expression levels in chondrocytes treated with IL1 β and 4BHP.

Figure 21: Chondrocytes were treated with IL1 β in a concentration ranging from 25pg/ml to 600pg/ml for 48h, or 4BHP in a concentration ranging from 10 μ M/ml to 50 μ M/ml for 24h. Nrf2 levels were detected in 20 μ g of the cell lysates. (X) Marks an empty well in all gels. These experiments were done with different samples and different treatments. The results presented here were from IL1 β and 4BHP dose curves stripped membranes, where they were re-incubated with Nrf2 and further developed. We didn't notice any increase in Nrf2 levels with IL-17 nor H₂O₂ (results not shown).

Chapter 4: Discussion

Osteoarthritis:

Osteoarthritis is a degenerative inflammatory disease where eicosanoids play a key role in its pathology. This disease was long thought to be a natural aging process, and so inevitable. With deciphering the role of PG's in OA pathophysiology, research made the 1st real step towards a definitive cure for it. Until now most of the therapeutics available for OA were managing pain and to some extent joint stiffness providing symptomatic treatment only. Hence, these treatments do not slow down or reverse joint degeneration. Some experimental techniques are out there to replace the damaged cartilage and fill the cartilage gaps using stem cells directed to differentiate into new chondrocytes. These techniques are promising but until now they do not work perfectly, probably due to the tenacious blood supply of the articular cartilage which made the grafts fail.

OA treatment starts with NSAIDs. Non-selective COX inhibitors were the 1st line used, yet they had a lot of side effects from blocking COX-1 enzyme as it plays a vital role in the synthesis of constitutive PG's. COX-2, the inducible form of COX, is the enzyme mainly involved in inflammation while COX-1 is mainly involved in normal physiological functions. COX-1 through its production of PG's plays a role in many physiological functions in the body among them platelet function and hemostasis, the formation of the mucous lining of the stomach and the control of glomerular filtration rate and renal function. Hence, the side effects of general NSAIDs necessitated the development of a new generation of NSAIDs, the selective COX-2 inhibitors. These had fewer side effects, but still they were not 100% COX-2 selective. Moreover, all

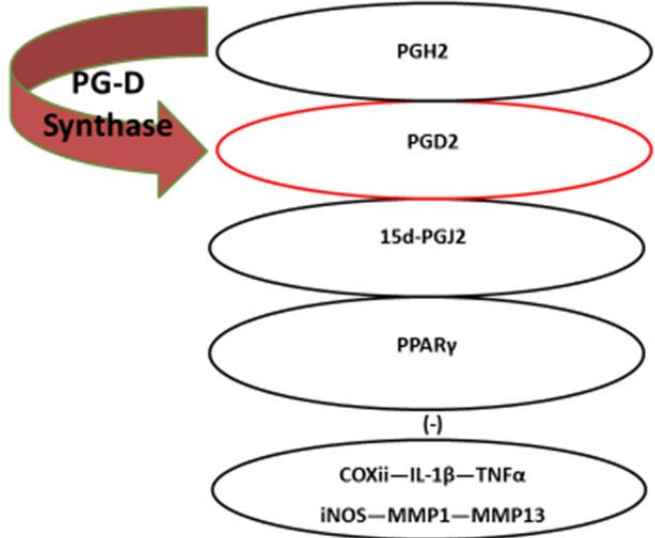
COX inhibitors suppress PG's non-selectively as they block the synthesis of degenerative and regenerative PGs. This fact raised the concern for a more selective approach in treating OA, as patients are probably going to take these medications for life.

From this point of view, we are aiming for an in-depth analysis of which PG plays a regenerative role, and how we can up-regulate it or deliver it with minimal side effects. Due to the wide range of distribution of systemic drugs including NSAIDs and so the wide range of side effects from it, we are focusing on delivering the medication locally. Steroids and hyaluronidase injections are widely used nowadays and they show equal efficacy in relieving pain and improving functionality [48]. They are more efficient and work for a longer duration than oral anti-inflammatory drugs. In this context, we are aiming for a product that can be locally injected to antagonize inflammation by activating natural anti-inflammatory pathways. Based on what we are showing in our results, L-PGDS is present in the synovial fluid under different conditions and since it can synthesize "good PGs", this makes L-PGDS optimum for local injection with minimal side effects expected. We showed also from previous results that L-PGDS is particularly abundant in chondrocytes of both osteoarthritic patients and normal individuals. Moreover, its levels are elevated in chondrocytes under different inflammatory conditions. This is assumed to be one of the main defense mechanisms of chondrocytes against OA. Based on that, L-PGDS became our therapeutic target, especially that the 15d-PGJ₂ receptor (PPAR- γ) [124] was found to be downregulated in OA, both *in vivo* and *in vitro* [85]. We used herein the same plan established to treat diabetes type 2, where the problem is decreased

insulin production plus downregulation of insulin receptors. The treatment in that case focuses on increasing insulin supply from outside as well as increasing the expression of insulin receptors. In OA, we can up-regulate PPAR- γ receptors and increase its natural ligand (15d-PGJ₂) to achieve a higher anti-inflammation effect. What we focused on here is increasing 15d-PGJ₂ levels through increasing L-PGDS expression in articular cartilage. The axis we are working on is detailed in **Figure 22**, where PGH₂ the precursor of all PG's is catalyzed by PGDS to produce PGD₂ which is then enzymatically dehydrated into 15d-PGJ₂. This later triggers PPAR- γ to downregulate the expression of inflammatory gene mediators such as COX2, MMP1&13, IL-1 β , TNF- α as well as iNOS.

Another interesting function of PGDS is the protection of PGD₂ from the non-enzymatic dehydration into 15d-PGJ₂ while transporting it to the site where it is supposed to work. This can also explain why chondrocytes release L-PGDS into the media and the synovial fluid in cases of inflammation, probably to deliver anti-inflammatory products or as a paracrine message.

Prostaglandins



Prostaglandins

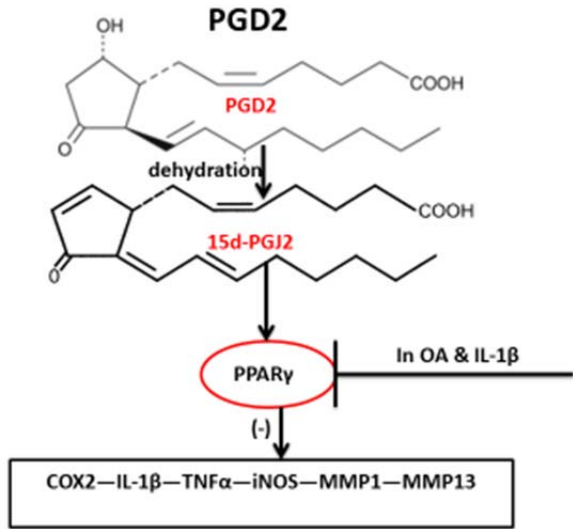


Figure 22: PGD2 Anti-inflammatory pathway.

The Role of Prostaglandins

To further understand the roles played by PG's in OA and to identify potential therapeutic targets we went through the literature about this topic. Among degenerative PG's in articular cartilage, PGE₂ inhibits proteoglycans formation, one of the main components of healthy cartilage. We showed the effect of age-associated OA and surgically induced OA, on proteoglycans levels in articular cartilage versus normal cartilage in **Figure 8** and **9** respectively. PGE₂ also increases collagen degradation through induction of MMP13 and aggrecanase 5 (ADAMTS-5) which degrade cartilage proteins. IL1 is also known to induce MMP13 and ADAMTS-5. Furthermore it augments the PGE₂ induction of these enzymes and so stimulates degradation. These effects are usually blocked by COX-2 selective blockers (like celecoxib®), from which comes their therapeutic value as anti-inflammatory in OA. The PGE₂ effect is mediated through EP4 receptors and these receptors antagonists are able to reverse the effect of PGE₂, opening the door for new therapeutic targets [74]. Oxidative stress has a direct role in inflammation in OA mainly via activation of COX-2 and this is reversible by antioxidants like N-acetylcysteine and hyaluronic acid [128]. Inhibiting COX-2 leads to suppression of pro-inflammatory PG's as well as anti-inflammatory ones. This raises the need for a new strategy in treating OA other than the most commonly used one by NSAIDs.

The Role of PGDS

The enzyme Prostaglandin-D synthase is a protein with a wide range of functions that are not fully explored yet. However, its enzymatic activity is well understood and it is the rate limiting step for PGD₂ synthesis from PGH₂ and for 15d-PGJ₂. PGD₂ is a well-known anti-inflammatory PG that works through different receptors among which DP₁ and DP₂, these receptors activate cyclic adenosine monophosphate (c-AMP) and so increase protein kinase-A (PKA). This leads to a downstream anti-inflammatory effect [129]. PPAR-γ is a receptor of 15d-PGJ₂ that acts as a transcription factor that downregulates important inflammatory mediators and cytokines including COX-2, IL-1β, TNF-α, iNOS, MMP1, MMP13 [124]. From this comes our focus on regulating the Prostaglandin-D synthase enzyme for its potential use as a therapeutic target. This aims to induce the intrinsic salvation pathways used by chondrocytes to protect themselves against stress. Hence, we studied the role of Prostaglandin-D synthase in OA, including its regulation and downstream effects. We focused here on its regulation, as its downstream end product 15d-PGJ₂ is a well-studied anti-inflammatory PG. Prostaglandin-D synthase is present in two isoforms, the hematopoietic and the lipocalin form. The tissue distribution of these isoforms is detailed in **Figure 4**. Depending on which isoform is present, the function of Prostaglandin-D synthase varies; a general overview of the different functions is presented in **Figure 5**. From a previous work in our lab, we showed a specific abundance for the lipocalin isoform of Prostaglandin-D synthase (L-PGDS) in chondrocytes. Furthermore, we previously showed a particular increase in L-PGDS expression compared to H-PGDS in osteoarthritic chondrocytes versus normal [95].

These results directed us to a more focused attention on the regulation of L-PGDS. As explained before in the Introduction, L-PGDS is formed of 190 amino acids giving it a calculated MW of 21KD and it is post-translationally modified by at least two glycosylation sites and many phosphorylation sites [99]. This gives it a wide range of MW depending on which modifications take place; each glycosyl group adds 3KD to the MW. During our work, we showed that chondrocytes produce L-PGDS in the modified form mainly inside the cells and in the non-modified form extracellularly.

From what we observed in our results, we developed the hypothesis that during inflammation chondrocytes release L-PGDS in the non-modified form to suppress inflammation. Moreover, since the released isoform of L-PGDS is the non-modified form this may suggest a paracrine function for this specific form. While with prolonged stress like exposure to 4BHP or IL-1 β , chondrocytes release more of the modified form of L-PGDS (most likely glycosylated, according to the MW) which may contribute to cell death after failure to reduce inflammation. The fact that L-PGDS acts as an oxygen free radical scavenger through thiol group in Cys65 at its active site can be another explanation for the over production of the glycosylated isoform. This may suggest that this form is the most active form as a scavenger. The different functions of L-PGDS and the different modifications modulating these functions necessitate an accurate identification of which isoform of L-PGDS is doing which function exactly. Doing so, we would be able to use the right isoform therapeutically, while avoiding potential side effects that may arise from other isoforms.

The Role of Nrf2

Kim *et al.* in 2013 showed an interesting role for the Nrf2 transcription factor in the induction of L-PGDS in murine bone marrow-derived macrophages. Nrf2 is known for its protective role against inflammation [130-132]. The mechanism by which it suppresses inflammation is variable and still new mechanisms are being discovered. Among these mechanisms, Nrf2 plays a role in transcribing ARE genes and Phase 2 detoxification enzymes. One mechanism presented by Kim *et al.* shows that Nrf2 uses also L-PGDS to suppress inflammation in mice. They used murine bone marrow derived macrophages to induce L-PGDS by PGD₂ (positive feedback mechanism). They studied the role of Nrf2 in this process by studying the response in Nrf2 knockout mice. This effect was lost. Then, they used Nrf2 agonists in one experiment and they overexpressed Nrf2 in another experiment to induce L-PGDS. Interestingly L-PGDS levels increased with such treatments. Furthermore, they did a sequence analysis of the murine L-PGDS gene and identified a binding site for Nrf2 in its promoter region [108], showing a role for Nrf2 in L-PGDS gene regulation. Next, they observed in mice lung tissues a decreased infiltration of inflammatory mediators and neutrophils in response to PGD₂-induced Nrf2 mediated L-PGDS induction [108]. In chondrocytes, PGD₂ has a negative feedback effect on L-PGDS gene expression.

This was shown in our lab by treating chondrocytes with IL-1 β , with or without PGD₂, which decreased IL-1 β induced expression of L-PGDS in PGD₂-treated chondrocytes [133]. We believe that the same mechanisms shown in murine macrophages and lung cells are present in human chondrocytes and that inflammatory mediators induce L-

PGDS by different mechanisms including the Nrf2 induction pathway. We chose IL-1 β and IL-17 as inflammatory cytokines because of their well-known role in the induction of inflammation. H₂O₂ and 4BHP were also used because they generate ROS, which induce inflammation and contribute to increasing the half-life of Nrf2. This increases Nrf2 levels in the cytosol and so stimulates its shift to the nucleus where it expresses ARE and L-PGDS genes playing an antioxidant and anti-inflammatory role. The role of ROS in Nrf2 pathway activation is detailed in **Figure 7**. In our results, we could detect increasing levels of Nrf2 in chondrocytes treated with IL-1 β or 4BHP in a dose-dependent manner. In IL-17 and H₂O₂ treated chondrocytes there wasn't any increase in Nrf2 levels (results aren't shown). IL-1 β may have increased the half-life of Nrf2 by oxygen free radicals production also, the same mechanism by which 4BHP works. Further addressing the role of Nrf2 in L-PGDS induction, future experiments should test the use of Nrf2-siRNA to block this pathway and detect to which extent this will affect L-PGDS production in response to different inflammatory stimuli.

The Role of 4BHP

Treating chondrocytes with 4BHP generates ROS which induce inflammation, and when they reach a certain limit they induce cell toxicity and death. What we observed during our work was that at higher doses of 4BHP or with prolonged exposure, expression levels of L-PGDS decreased in the cell lysate. Concomitantly, the modified form of L-PGDS appeared in the cell supernatant increasing with raising 4BHP dose or duration of treatment. At the same time, cell death, as evaluated by detached and floating cells, was noted microscopically with higher concentrations of

4BHP as well as with prolonged treatment. 4BHP as a ROS generator has a well-known role in apoptosis [134]. These results may suggest a role for the modified form of L-PGDS in 4BHP-induced cell toxicity and possibly cell death when they were treated for 48h. This goes in line with many papers that proposed a role for L-PGDS in apoptosis in Alzheimer disease and different cancer cell lines [135-138]. Furthermore, a paper suggested that the apoptotic effect was related to the glycosylated form of L-PGDS in Alzheimer disease [135]. Another interesting paper suggested that the mechanism by which L-PGDS contributes to apoptosis is through its transporter function for retinoic acid [137]. The post-translational modification of L-PGDS has also been linked with its potential apoptotic activity, particularly the glycosylation and phosphorylation [139]. The detection of apoptosis markers in chondrocytes, such as caspase 3, or cell viability measurements, would give us an explanation for the results we had with prolonged 4BHP treatment (**Figure 19**). This hypothesis is currently tested in the laboratory. A further step in understanding those modified forms of L-PGDS would be through IP L-PGDS in the cell lysate and in the cell supernatant and identifying which modifications are observed upon which treatment. In the IP, we would try to detect the glycosylation and phosphorylation changes in response to different treatments. This would help us to identify which modification is responsible for which function, and eventually find the correct isoform that plays an anti-inflammatory role. Hence, we could use the correct isoform as a medication avoiding at the same time any potential side effects that may arise from other isoforms.

Our main contribution

In this study, we showed that chondrocytes produce L-PGDS in response to different stimuli and that different forms of L-PGDS are detectable in the cells and secreted by these cells. The function of L-PGDS can be affected by which isoform is produced and which post-translational modification happens to the protein. Furthermore, its location (intracellular and extracellular) in different isoforms suggests different functions. Many different studies discussed post-translational modification of L-PGDS by glycosylation and/or phosphorylation, as explained in the introduction. Some studies also pointed out specific function for different isoforms, we did not directly study the function of the different isoforms functions in this study. Instead we used the literature to better understand the results we obtained.

To study arthritis, we induced inflammation in human chondrocytes *in vitro*. We used IL-1 β and IL-17 as well-known pro-inflammatory cytokines. Upon treating chondrocytes with IL-1 β , we observed an increase in L-PGDS expression levels both intracellularly and extracellularly in a dose and time-dependent fashion. The isoform produced intracellularly (in cell lysate) was the modified one (37KD), while extracellularly (in the media), chondrocytes released both modified and non-modified isoforms of L-PGS.

Compared to IL-1 β , IL-17 is a weaker pro-inflammatory cytokine; this may partially explain why we did not observe an increase in L-PGDS expression levels in dose-dependent treatment in the cell lysate. While in the time course treatment, a bell-shaped curve rise in L-PGDS levels was detected in the cell lysate. In all cell lysates,

chondrocytes produced the modified form of L-PGDS (37KD). In contrast, we detected a bell-shaped curve rise of the levels of the non-modified form (25KD) of L-PGDS in a dose and time-dependent manner in the cell media, hence representing the secreted form of L-PGDS.

We also used 4BHP and H₂O₂ as ROS generators to induce inflammation and also increase Nrf2 half-life allowing it to translocate into the nucleus and transcribe ARE and L-PGDS. We hypothesized this based on different studies showing a role for Nrf2 in L-PGDS induction, as detailed in the Introduction.

Treating chondrocytes with 4BHP for 24h showed an increase in expression levels of L-PGDS in a dose-dependent manner. This was observed both intracellularly and extracellularly. In addition, L-PGDS was detected in the cell lysate in the modified isoform and in the media in the non-modified isoform. When we tried treating chondrocytes with 4BHP for longer periods (>24h) we observed signs of cell toxicity and death. A big percentage of cells were detached and floating in the media, suggesting cell death or at least toxicity. However we did not perform quantification of cell death or toxicity nor apoptosis markers here, we only used this observation to adjust the 4BHP dose and time of exposure in further experiments. Interestingly, during the prolonged treatment (48h), L-PGDS levels, in its modified form, were decreasing in the cell lysate with increasing doses of 4BHP. Though, L-PGDS levels continued to rise in the media albeit in the modified isoform, for the 1st time for all 4BHP experiments. We observed also in the media decreasing levels of the non-modified isoform of L-PGDS with increasing 4BHP doses. This would suggest that

there is a shift from the non-modified isoform to the modified isoform with increasing 4BHP dose (at 48h). These results are summarized in **Figure 19**.

The observed detached and floating chondrocytes suggests a toxic effect for 4BHP at 48h of exposure and a possible relation between this toxicity and the modified form of L-PGDS. This observation drove us to adjust the dose and time of exposure of chondrocytes to 4BHP to 24h only. This also limited our capacity for testing a time course effect of 4BHP on chondrocytes as we had inconsistent results, hence they were not included in this study.

H₂O₂ is a weaker ROS generator due to its instability at 37°C. Treating chondrocytes with increasing H₂O₂ doses for 24h did not show an increase in L-PGDS expression levels in the cell lysate. In the media however, we observed increasing levels of the non-modified isoform of L-PGDS in a bell-shaped curve with increasing H₂O₂ dose.

In the time course experiments, a small increase in L-PGDS levels in cell lysates was observed at 6 hours; this level was maintained with longer exposure. L-PGDS was detected in the cell lysates in the modified isoform also. This suggests a saturated induction of L-PGDS by H₂O₂. In the supernatants, L-PGDS could not be detected mainly due to the weak effect of H₂O₂ on L-PGDS induction and due to the instability of H₂O₂ overtime. These findings, along with the bell-shaped curves in previous results may suggest that L-PGDS is not stable and that there is a degradation mechanism to clear it when the inflammation process is stopped (for example when H₂O₂ is degraded).

The appearance of the modified isoform of L-PGDS in the cell lysates and in the media of 4BHP and IL-1 β treated chondrocytes can be explained by L-PGDS function as an oxygen free radical scavenger as detailed in the Introduction. This L-PGDS function is achieved mainly through the thiol group at Cys65. Why chondrocytes release L-PGDS either in the non-modified isoform or the modified isoform is not fully understood yet. This may be part of a paracrine system to the surrounding cells to induce anti-inflammatory effect in the adjacent cells as well as in chondrocytes.

The different functions of L-PGDS and the different modifications modulating these functions necessitate accurate identification of which isoform of L-PGDS is the one working as anti-inflammatory, so we can use the right isoform therapeutically to avoid possible side effects from other isoforms.

In 4BHP and IL-1 β dose curves experiments that showed increased expression of L-PGDS in the cell lysates, the nitrocellulose membranes were stripped and re-incubated, we tested the presence of Nrf2. Interestingly, both treatments (4BHP and IL-1 β) showed increased Nrf2 expression levels as well. The mechanism by which 4BHP increases Nrf2 by generating ROS was explained in details in the introduction, but the IL-1 β involvement in Nrf2 expression levels is not fully understood yet. This could be due to ROS production by IL-1 β in chondrocytes. The increase in Nrf2 and L-PGDS levels in chondrocytes treated with IL-1 β and 4BHP goes in line with the studies that discussed a direct role for Nrf2 in L-PGDS induction, as detailed in the Introduction. This role is important because we can use a Nrf2 agonist therapeutically to induce L-PGDS in articular cartilage by local injection, to suppress inflammation.

Aiming to study the release of L-PGDS by chondrocytes *in vivo*, we tried detecting L-PGDS in different synovial fluid samples. Interestingly, L-PGDS was found in many different synovial fluid samples from OA patients and normal individuals both in the modified isoform and the non-modified isoform. Due to limited access to these individuals' medical records we couldn't exclude other joint diseases in the non OA patients, hence we have limited possibilities to explain the presence of both isoforms in the "normal" samples. This needs further studying to identify in which conditions L-PGDS is released into the synovial fluid and in which isoform. The results from the synovial fluids confirmed only our *in vitro* findings that chondrocytes release L-PGDS.

Aiming to study the production of L-PGDS inside the chondrocytes *in vivo*, we detected L-PGDS expression levels by immunohistochemistry in OA mice models versus normal mice. OA was induced via two different routes, either surgically by DMM or naturally by aging. Sham surgery mice models were used as a control for DMM surgery, and young mice were used as a control for the age-induced OA models. There was a 3-5 fold increase in L-PGDS expression levels in OA models, either surgically-induced or age-induced. All the OA models were first confirmed histologically, by staining some slides with safranin-O and fast green to identify the characteristic pathological lesions of OA histologically. Hence, these data confirmed our *in vitro* studies of the potential role of L-PGDS in OA pathology.

Limitations:

One of the important limitations we had during this study was the toxic effect of 4BHP on human chondrocytes with prolonged exposure. Therefore, we couldn't determine L-PGDS expression patterns in 4BHP time course treatments. Alternatively we may try using lower doses of 4BHP or higher doses of H₂O₂ with a more frequent application (6h) to generate enough ROS without inducing cell toxicity. H₂O₂ instability also was a limitation. *In vivo* studies weren't enough, and so we may try silencing L-PGDS gene by siRNA or knockout model and observe the inflammatory effect of IL-1 β on the induction of COX-2, iNOS or MMP1, 13 for example. Comparing those results in knockout mice versus normal mice can reflect L-PGDS anti-inflammatory effect if COX-2 and iNOS levels are higher in the knockout. We may also use siRNA or knockout mice for Nrf2 to confirm its role in L-PGDS induction in response to different stimuli. We can also use a Nrf2 vector to overexpress the gene in chondrocytes and observe its effect on L-PGDS levels.

We couldn't collect synovial fluid samples from mice to detect L-PGDS under different conditions, so we may collect dogs' samples or humans' synovial fluid samples from different OA patients as well as normal volunteers to detect L-PGDS levels and its different isoforms.

Future studies:

This study opens the door for many other questions to answer. In future projects, we should try to identify which isoform of L-PGDS is produced in the media, synovial fluid and inside the chondrocytes themselves in response to different other inflammatory stimuli. This can be done by immunoprecipitation techniques where we can pull-down L-PGDS and check for glycosylation or pull-down glycosylated proteins and check for L-PGDS among them. We can also use glycosylation inhibitors and observe the loss of protein bands or change in L-PGDS band location in WB. The same techniques can be used for detection of the phosphorylated form of L-PGDS.

Another interesting question would be to assess the effect of Nrf2 agonists on L-PGDS expression levels and different isoforms. This can be tested by using Nrf2 agonist or deliver Nrf2 by a vector inside the chondrocytes to overexpress it, and then we could observe the changes of L-PGDS levels and isoforms.

Further studying L-PGDS anti-inflammatory effect, we could treat chondrocytes with L-PGDS and then measure their response to inflammatory mediators (for example IL-1 β), by detecting other inflammatory gene expression levels (like COX-2, iNOS, MMP1 and 13). Another way to do it would be by silencing L-PGDS gene with siRNA and see if this will lead to a more inflammatory response in chondrocytes when treated with IL-1 β for example.

Chapter 5: Conclusion

From our results, we conclude that L-PGDS is produced by human chondrocytes *in vitro* in response to different inflammatory stimuli. This happens in response to inflammatory cytokines and ROS generators, aiming to ameliorate inflammation. The same process happens *in vivo*, for example in mice articular cartilage in response to mechanical stimuli generating OA. L-PGDS induction is dose and time dependently expressed in response to the inflammatory stimuli. Chondrocytes release L-PGDS into the media *in vitro* and the synovial fluid *in vivo* in response to different inflammatory stimuli. This is probably a paracrine function to suppress inflammation even in the surrounding tissues. The fact that L-PGDS is present in the synovial fluid is interesting when we think about using it locally as a medication, avoiding possible systemic side effects. There are different isoforms of L-PGDS depending on which post-translational modification happened to the protein. This modulates its function in response to different stimuli and in different locations. Consistent with what we found in the literature review, our results suggest that some isoforms of L-PGDS (probably the glycosylated isoform) may play a role in cell toxicity in human chondrocytes. The fact that there are different isoforms of L-PGDS, and that some may contribute to cell death, raises the concern for identifying which isoform is enzymatically active as anti-inflammatory and which isoform is contributing to cell death so we can work on the right isoform as a therapeutic target. Nrf2 is induced in human chondrocytes by different inflammatory mediators, either cytokines or ROS generators. Nrf2 is most probably one of the pathways by which L-PGDS gene is induced in response to inflammation. This opens the door for another potential therapeutic target using Nrf2 direct ligands to induce L-PGDS.

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