pyridinoline cross-links during collagen synthesis, resulting in harder to degrade collagen. These pyridinoline cross-links are elevated in different fibrotic diseases. In previous studies in murine knee joints, we found a relationship between LH2b and TGF-β-induced irreversible synovial fibrosis. In this study, we examined whether the amount of pyridinoline cross-links per triple helix collagen was elevated in the synovium of murine knee joints during collagenase-induced OA-related fibrosis. In addition, to learn more about the mechanisms by which LH2b is induced and how to prevent this induction, we investigated whether the induction of LH2b was Smad2P or Smad3P depended.

Methods: We induced OA by intra articular injection of bacterial collagenase into the right knee joint of C57Bl/6 mice (collagenase model). Mice were sacrificed at day 7, 21, 28 and 42 after collagenase injection and the mRNA was isolated from the synovium for Q-PCR analysis. Paraffin sections of the murine joints were stained immunohistochemically for LH2 to determine the LH2 expression. The amount of pyridinoline cross-links per triple helix in synovium was determined biopsies with HPCL. All animal experiments were approved by the local animal ethics committee. Human fibroblasts (hSF) were isolated from synovial tissue of knee joints of OA patients undergoing arthroplasty. The hSF were stimulated with TGF-β with and without the Smad3P inhibitor SIS3 or the ALK5 kinase inhibitor SB-505124 (SB-5). RNA was isolated and the gene expression for LH2b and collagen type 1 (COL1A1) were analyzed with Q-PCR.

Results: LH2b mRNA expression in synovium of murine knee joints was significantly upregulated in the OA-affected joints compared to the healthy joints on al measured days. Histological sections of murine knee joints with collagenase-induced OA showed a mild increase in the thickness of the synovial membrane at day 7 whereas a large increase was seen on later days. Day 7 showed a strong increase in LH2 staining , at later time points there was still a clear increase but less intence than at day 7 (Figure 1). There was a significant increased in the amount of pyridinoline cross-links per triple helix after day 7 compared to control knee joints. TGF-β upregulated both LH2b and Col1A1 gene expression in hSF, however when SIS3 the Smad3P inhibitor was added Col1A1 was strongly (+/- 4 Ct cycles) down-regulated. In contrast to Col1A1, LH2b was still induced by TGF-β in the presence of SIS3. SB-5 blocked both TGF-β induced LH2b and Col1A1.

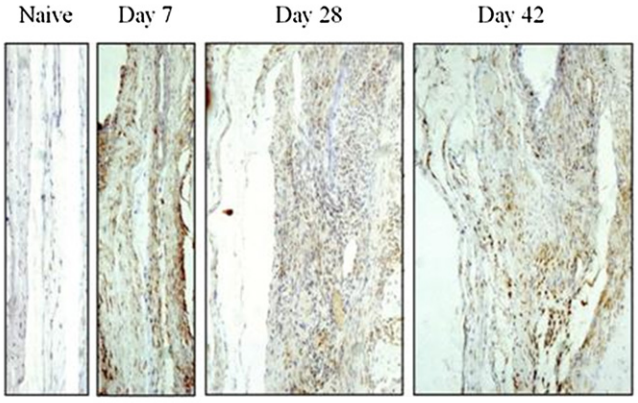

Figure 1. Synovium of murine knee joints with collagenase-induced OA stained immunohistochemically for LH2. At day 7 a mild increase in the thickness of the synovial membrane was seen whereas on day 28 and 42 a strong increase was observed. LH2 expression was strongly induced in the synovium at day 7 after collagenase injection on day 28 and 42 LH2 was still elevated but less intense compared to day 7.

Conclusions: We have published that TGF-β is crucial in irreversible synovial fibrosis in experimental OA. Now we show strong LH2b expression in synovium of murine knee joints with collagenase-induced OA and that pyridinoline cross-links were more than doubled per triple helix in the synovium of murine knee joints with collagenase-induced OA. Most likely, TGF-ß that is elevated during OA is the driving force of enhanced LH2b expression, which drives pyridinoline cross link formation. We showed previously that TGF-β induced LH2b relies exclusively on TGF-β ALK5 (Smad2/3) and not ALK1 (Smad1/5/8) signaling. Blocking both Smad2 and Smad3 signaling did prevent TGF-β induced LH2b, whereas LH2b was still induced by TGF-β when alone Smad3P was inhibited. This suggests that LH2b is mediated through ALK5/Smad2P. We propose that LH2b is responsible for the persistence of fibrosis during OA. Blocking LH2b, or the Smad2p route, in OA may therefore prevent the formation of persistent fibrosis.

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THE POTEINTIAL ROLE OF PROSTAGLANDIN-F2ALPHA IN REGULATING INFRAPATELLAR FAT PAD INDUCED FIBROTIC PROCESSES IN CULTURED SYNOVIOCYTES

Y.M. Bastiaansen-Jenniskens [1], W. Wei [1], R. Stoop [2], C. Feijt [1], R. Hanemaaijer [2], G. Van Osch [1]. [1] *Erasmus MC, Univ. Med. Ctr., Rotterdam, Netherlands;* [2] *TNO, Metabolic Hlth.Res., Leiden, Netherlands*

Purpose: Stiffening of the joint is one of the features of knee osteoarthritis, which can be caused by fibrosis of several tissues within the joint, such as the synovium. Fibrosis is characterized by high collagen deposition and increased collagen cross-linking. A large fat pad is present in the knee joint (infrapatellar fat pad, IPFP) and adipose tissue is known to be capable of excreting inflammatory and angiogenic factors. We recently found that the IPFP of end-stage OA patients is able to inhibit catabolic processes in cartilage. The goal of the present study was to investigate whether IPFP and its secreted factors influence fibrotic processes in synovial fibroblasts and to determine the role of TGFβ and $PGF_{2\alpha}$, two potent fibrotic inducers, in the relation between IPFP and synovial fibrosis.

Methods: Fat conditioned medium (FCM) was made by culturing small pieces of IPFP, obtained from osteoarthritic knees after total knee replacement, in serum-free medium for 24 hours. 13 different batches of FCM were made, representing 13 different IPFP donors. Human synoviocytes were isolated from synovium obtained after total knee replacement, expanded for two passages, seeded at 50.000 cells per cm^2, and pre-cultured in 10% fetal calf serum. After pre-culture and washing with saline, cells were cultured in FCM for 4 days for gene expression analysis of PLOD2 (a cross-linking enzyme) and α-smooth muscle actin (αSMA), and for collagen analysis. In parallel, proliferation of the synoviocytes was analyzed at 1, 4 and 5 days and migration (scratch-wound assay) during 19 hours. To test involvement of two potent fibrotic inducers, inhibitors of the TGFβ receptor/ALK5 (SB505124, 1 μM) or $PGF_{2\alpha}$ (AL8810, 10 μM) were used together with the FCM.

Results: Fat Conditioned Medium induced synoviocytes to produce significantly more collagen and express more PLOD2 than control medium. No clear effects were seen on αSMA gene expression. In addition, synoviocytes cultured in FCM had a higher proliferation rate and migrated faster. The FCM-induced increase in PLOD2 gene expression

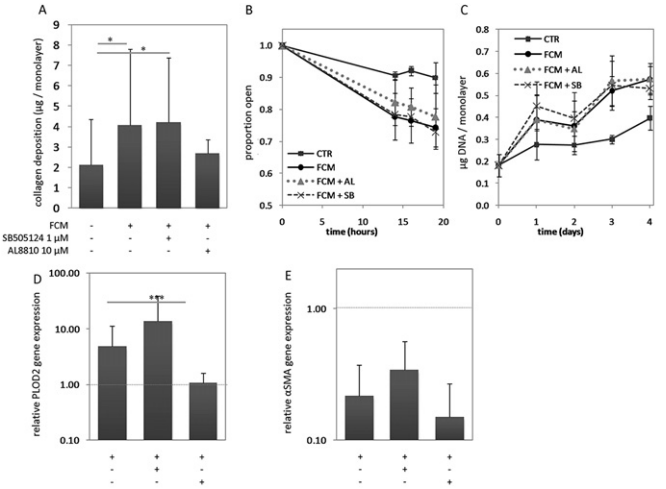

Figure: The effect of the TGFβ/ALK5 (SB505124) or PGF2α signaling (AL8810) inhibition on the effect of FCM in synoviocyte cultures on A) collagen production, B) migration and C) proliferation and gene expression of D) PLOD2 and E) αSMA relative to the control without these additions (set at 1, dotted line). Experiment was performed with 8 different FCM batches. * indicates $p<0.05$, *** indicates $p<0.005$

returned to control levels when synovial fibroblasts were co-incubated with $PGF_{2\alpha}$ inhibitor AL8810 whereas inhibition of TGFβ signaling with SB505124 had no effect. αSMA gene expression and collagen production were unaffected by inhibition of TGFβ or $PGF_{2\alpha}$ signaling. The increased synovial fibroblast migration in response to FCM could not be counteracted by SB505124, but was partially counteracted by AL8810. Synovial fibroblast proliferation in response to FCM was unaffected either by SB505142 or AL8810.

Conclusions: These results indicate that infrapatellar fat can contribute for the development of synovial fibrosis by increasing collagen production, PLOD2 gene expression, cell proliferation and cell migration; all characteristics of a fibrotic process. Based on our results, not TGFβ but the more recently discovered pro-fibrotic factor $PGF_{2\alpha}$ seems partly responsible for the observed effects.

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EFFECT OF CHONDROITIN SULFATE ON THE FACTORS INVOLVED IN SYNOVIAL INFLAMMATION

J. Martel-Pelletier, Sr. [1], F. Mineau, Sr. [1], E. Montell, Sr. [2], J. Vergés, Sr. [2], J.-P. Pelletier, Jr. [1]. [1] *Osteoarthritis Res. Unit, Univ. of Montreal Hosp. Res. Ctr. (CRCHUM), Notre-Dame Hosp., Montreal, QC, Canada;* [2] *Pharmacological Res. Unit, Scientific Med. Dept., Bioibérica, Barcelona, Spain*

Purpose: Chondroitin sulfate (CS), an interesting candidate for the therapeutic treatment of osteoarthritis (OA), has been shown in clinical trials to reduce swelling and effusion in knee OA. We thus aimed to further investigate the effect of CS alone or in combination with glucosamine sulfate (GS), an anti-COX-2 (celecoxib), or acetaminophen on some synovial membrane anti-angiogenic and inflammatory factors.

Methods: Treatment with CS (200 μg/ml; CS*b*Bio-*Active*®, Bioibérica, Spain), GS (5 mM), celecoxib or acetaminophen alone, and CS in combination with the other mentioned products was investigated on human OA synovial fibroblasts in the presence or absence of IL-1β at 10 and 100 ng/ml. To determine whether CS has an additional effect with celecoxib or acetaminophen, preliminary experiments were performed to find concentrations of these products that induced about a 50% reduction in prostaglandin E_2 production under IL-1β at 100 ng/ml. A concentration of 10 nM was found for celecoxib and 25 μM for acetaminophen. The expression levels (real time PCR) and/or protein production of vascular endothelial growth inhibitor (VEGI), thrombospondin-1 (TSP-1), hyaluronic acid (HA), secreted phospholipase 2 (sPLA2), and cytosolic phospholipase 2 (cPLA2) were determined using specific primers (expression) and ELISAs (protein).

Results: On the anti-angiogenic factors VEGI and TSP-1, IL-1β dose-dependently decreased their levels. On cells under basal conditions or treated with IL-1β, CS significantly induced the levels of VEGI expression and TSP-1 production. All the other products tested (GS, celecoxib, and acetaminophen) alone had no effect or decreased these anti-angiogenic factors, but in conjunction with CS their levels were significantly increased. HA production was slightly but significantly increased by IL-1β at 10 ng/ml and a heightened induction was found at 100 ng/ml. CS significantly increased HA under basal conditions and in the presence of IL-1β at 10 ng/ml. GS had no effect and celecoxib and acetaminophen significantly decreased it. Concomitant incubation of CS with GS, celecoxib, or acetaminophen significantly increased HA production. Interestingly, sPLA2, which is considered an anti-inflammatory (resolving) factor, demonstrated a significant decrease under IL-1β. Incubation with CS alone and in conjunction with celecoxib or acetaminophen significantly increased its level. The inflammatory factor cPLA2 showed significantly decreased expression levels by CS, GS, and the combination of CS and GS. IL-1β markedly and significantly increased it at both concentrations and under IL-1β, CS alone had no effect, but in combination with GS, its level was significantly reduced.

Conclusions: The anti-inflammatory effect of CS appears to occur through a number of mechanisms including the inhibition of the anti-angiogenic factors TSP-1 and VEGI as well as sPLA2, a factor associated with an anti-inflammatory effect, and through increasing HA production and decreasing cPLA2. Importantly, these effects of CS occurred in the presence of other products, even though those products alone had no or a reverse effect.

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EXPRESSION OF SPECIFIC PATHWAYS IN THE INFLAMED SYNOVIAL MEMBRANE OF OSTEOARTHRITIS PATIENT: IDENTIFICATION OF NEW POTENTIAL KEY INTERMEDIATES

C. Lambert [1], J.-E. Dubuc [2], B. Hennuy [3], E. Montell [4], J. Vergés [4], Y. Henrotin [1]. [1] *Bone and Cartilage Res. Unit, Univ. of Liège, Liège, Belgium;* [2] *Orthopaedic Dept., Cliniques Univ. Saint Luc, Brussels, Belgium;* [3] *GIGA Genomics Core Facility, Univ. of Liege, Liège, Belgium;* [4] *Pharmacological Res. Unit, Scientific Med. Dept., Bioibérica, S.A, Barcelona, Spain*

Purpose: Synovitis is a key factor in osteoarthritis (OA) pathophysiology, contributing to both patient symptoms and disease progression. In this study, using an original methodology comparing normal/reactive (N/R) and inflammatory (I) synovial membranes zones, we investigated the gene expression profiles of synovial cells from these areas and identified differentially regulated pathways.

Methods: Synovial cells (SC) were isolated from OA synovial specimens obtained from 12 patients undergoing knee replacement. The inflammatory status of the synovial membrane was characterized by the surgeon according to macroscopic criteria including the synovial vascularization, the villi formation and the hypertrophic aspect of the tissue. At the surgery time, the synovial membrane was dissected and biopsies from N/R and I areas cultured separately for a period of 7 days. Total RNA was extracted using the RNeasy Mini Kit. RNA purity and quality were evaluated using the Experion RNA StdSens Analysis kit (Bio-rad Laboratories). Gene expression profiling between N/R and I areas was performed using Illumina's multi-sample format Human HT-12 BeadChip (Illumina Inc.). Differential analysis was performed with the BRB array tools software. Class Comparison test between N/R and I areas was based on paired t-test where N/R and I were paired for each patient. The biological relevance of up- and down-regulated genes was analyses with Ingenuity Pathways Analysis (Ingenuity® Systems). Western blot was performed to confirm certain intermediate expression.

Results: From among 47000 probes, 17500 were filtered out. Probes with a p-value below than 0.005 were chosen and classified as up- or down-regulated ones. By this way, 896 differentially expressed genes between N/R and I zones were identified. Among these, 576 genes were upregulated (I/NR > 1.5) and 320 downregulated (I/NR < 0.75). With Ingenuity Pathways Analysis, a significant number of the top ranking differentially expressed genes were identified as inflammatory, Wnt and angiogenic pathways. Interleukin (IL)-6 and -8, chemokines (CXCL1, CXCL2, CXCL5, CXCL6, CXCL16) and arachidonate 5-lipoxygenase (ALOX5) were identified as the most upregulated in I zones in the inflammatory pathway. Interestingly, the alarmin S100A9 was found strongly upregulated in this pathway. Wnt5A and LRP (Low density lipoprotein receptor-related protein) 5 were upregulated whereas FZD (Frizzled homolog) 2 and DKK (dickkopf homolog) 3 were downregulated in the Wnt signaling pathway. Finally, stanniocalcin (STC)-1, an intermediate in angiogenesis was identified as the most upregulated gene in I zones compared to N/R zones. This difference of expression was confirmed at the protein level.

Conclusions: Using a unique culture system, this study is the first to identify different expression pattern between two areas of synovial membrane from the same OA patient. These differences concern several key pathways involved in OA pathogenesis, i.e. inflammation, Wnt and angiogenesis. This analysis also provided interesting information regarding new potent intermediates as S100A9 and STC-1. They could be potential targets for chondroitin sulfate, one of the most used molecules in the management of OA. New experiments are being perfomed at the moment to elucidate the potential effect of this molecule on these specific differentially expressed genes in the same culture system.

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SYNOVIAL INFLAMMATION CORRELATES WITH MENISCAL PATHOLOGY IN A COHORT OF PATIENTS UNDERGOING ACL RECONSTRUCTION FOR TRAUMATIC ACL RUPTURE

M. Favero [1,2], J.L. Scott [1], C. Dragomir [1], R.F. Warren [1], M.K. Crow [1], E. Attia [1], K. Bohnert [1], E.F. DiCarlo [1], S.R. Goldring [1], M.B. Goldring [1], H.A. Jo [1], S.A. Rodeo [1]. [1] *The Hosp. for Special Surgery, Weill Cornell Med. Coll.,*