no such regional difference in type I collagen content in low-grade control specimens (p=0.181). Real-time PCR: COL2A1 expression was not significantly different in regions of damaged and preserved cartilage in AMG (p=0.156), low-grade control (p=0.625) or high-grade control specimens (p=0.813). COL1A1 expression in AMG specimens was significantly greater in the preserved cartilage than damaged cartilage (p=0.031). However, the regional difference in type I collagen expression was not seen in low-grade (p=0.625) or high-grade controls (p=0.219).

**Conclusions:** Type I collagen is abundant in fibro-cartilage but only found in very low levels in mature hyaline cartilage and is not usually found in articular cartilage. Macroscopically normal cartilage in AMG exhibits increased COL1A1 gene expression and a subsequent increase in type I collagen matrix content. Type I collagen is also increased in the equivalent region of knees with early histological features of OA.

These findings suggest that these cartilage matrix and chondrocyte phenotype changes may be an early feature of OA change in AMG.

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**A ROLE FOR TNF-LIKE WEAK INDUCER OF APOPTOSIS (TWEAK) DERIVED FROM SYNOVIAL TISSUES AND CARTILAGE IN THE PATHOGENESIS OF OSTEOARTHRITIS**

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**Purpose:** Previous studies have suggested that TWEAK may have an important role in stimulating bone and cartilage destruction in various arthritides. The aim of this study was to investigate TWEAK and TWEAK receptor (Fn14) expression in the cartilage and synovial tissues from osteoarthritic and normal (healthy) individuals. Protein expression of TWEAK and TWEAK receptors (Fn14) was determined in cartilage from samples of various grades of osteoarthritis (OA) and synovial tissue from patients with OA and normal controls. TWEAK and Fn14 gene expression was studied in various grades of OA cartilage. In addition, levels of soluble TWEAK present in OA synovial fluid were measured.

**Methods:** Synovial tissues from total of 10 OA and 10 normal subjects were studied for TWEAK and Fn14 using immunohistochemistry. Osteoarthritic cartilage was obtained from 21 patients undergoing primary hip replacement and stained for TWEAK and Fn14 expression. Staining was assessed using a standard semi-quantitative assessment carried out by two independent observers. In addition, mRNA levels of TWEAK and Fn14 were determined by real time RT PCR in the various grades of cartilage tissue. TWEAK levels were measured in 15 OA synovial fluid samples using a commercial ELISA kit.

**Results:** Significantly higher TWEAK (Figure 1A and B) and Fn14 was detected in OA synovial tissue compared to the normal synovial tissue (p<0.05) and expression was predominantly in the synovial lining. In OA cartilage, the majority (18/21) all grades of samples expressed TWEAK protein at low levels, whereas all samples (OA and normal) expressed high levels of Fn14 (Figure 1C and D). TWEAK and Fn14 mRNA was more abundant in OA patients (Figure 2) with grade 2 cartilage damage compared to those without any cartilage damage (p<0.05). TWEAK was present in all OA synovial fluids tested at a mean concentration of 713±134 pg/ml.

**Conclusions:** Previous studies have shown that TWEAK can induce metalloproteinase production in vitro by human chondrocytes and in murine intervertebral disc tissues. As yet no studies have directly investigated the presence of TWEAK and its receptor (FN14) in human OA tissues. The high expression of Fn14 by chondrocytes in OA cartilage indicates that these cells are able to respond to TWEAK. In OA tissues TWEAK was expressed by chondrocytes in cartilage and highly expressed in the adjacent synovial tissues. This is consistent with TWEAK protein in the OA synovial fluid. Overall the study indicates that TWEAK released from the synovial tissue and chondrocytes in OA has a role in cartilage degradation.

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**ELUCIDATION OF THE HUMAN CHONDROCYTE GLYCOME AND OF ALTERING GLYCANS UNDER CYTOKINE TREATMENT**

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**Purpose:** Recent evidence has indicated a relationship between osteoarthritic and altered glycosylation of cartilage and chondrocytes. Although the glycomics of cells is known to affect adhesion, receptor activity or apoptosis, little information exists on the N- or O-glycome of human chondrocytes and on their significance for the functionality of cartilage tissue. In part, this fact may result from the methodological challenges encountered with low glycosyltransferase transcription levels and linkage-type specific glycoprotein glycan analysis. This study aimed to elucidate the glycome of cultured human chondrocytes using a combination of RT-qPCR and structural glycan analysis (LC-ESI-MS) and to define alterations of cellular glycans upon treatment of chondrocytes with pro-inflammatory cytokines.

**Methods:** Primary chondrocytes were isolated from human OA cartilage (n=8). Primary cells, SW1353 and C-28/I2 chondrocytes were cultured in DMEM with 10% FCS or 1% ITS. 10 ng/ml IL-1α or 40 ng/ml TNF-α were added as a pro-inflammatory stimulus. SYBR green based RT-qPCR assays for 19 glycosyltransferases and 5 galectins were established using GAPDH as reference. N- and O-glycan isolation was performed by PNGase (N-glycans) or sodium hydroxide (O-Glycan) digestion prior to structural identification by LC-ESI-MS using Porous Graphitized Carbon as stationary phase.

**Results:** About 100 different N- and O-glycans were detected and quantified in human chondrocytes in the presence or absence of FCS. Major N-glycans were found to be sialylated diantennary structures, oligomannosidic or hybrid-type glycans. The isoform of diantennary and oligomannosidic N-glycans were fully assigned. Some structures were sulfated, contained GalNAc or Lewis fucose residues, whereas tri and tetraantennary glycans were present only to a minor extent. O-glycosylation mainly constituted of core-1 and core-2 structures bearing one or two α2,3 linked sialic acids. Primary chondrocytes predominantly expressed α2,6-specific sialyltransferases (SiaT) and α2,6-linked sialic acid residues in glycoprotein N-glycans. In contrast, the preponderance of α2,3-linked sialyl residues and reduced levels of α2,6-specific SiaT were associated with the altered chondrocyte phenotype of C-28/I2 and SW1353 cells. Importantly, we found that both IL-1α and TNF-α increased overall sialylation of N- and O-glycans and induced a shift towards α2,3-linked sialic acid residues in primary chondrocyte glycoproteins. These results were supported by RT-qPCR showing increased expression of α2,3 SiaT in treated cells. Moreover, we found that both cytokines induced a considerable shift from oligomannosidic glycans towards complex-type N-glycans, whereas core α1,6-fucosylation was found to be reduced particularly by TNF-α.
Regarding galectin mRNA levels in human chondrocytes (n=8), we found expression in the order: LGALS1 (0.36±0.13) > LGALS3 (0.18±0.06) > LGALS8 (0.09±0.03) > LGALS9 (LGALS7 <0.00004).

**Conclusions:** Here, we have portrayed for the first time the complete glycome of human chondrocytes out of minute amounts of cultured cells (100,000 cells). IL-1 and TNF-α induce a range of specific alterations in chondrocyte glycoproteins supporting the hypothesis that the chondrocyte glyobiology might be of relevance for malfunctioning cell-matrix interactions or altered galectin function under disease conditions.

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**ROLE OF K(ATP) CHANNELS IN MODULATING GLUT-1 CONTENT IN NORMAL AND OSTEOARTHRITIC HUMAN CHONDROCYTES**

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**Purpose:** Our previous studies showed that human chondrocytes adjust their glucose transport capacity and facilitative glucose transporter (GLUT)-1 content in response to high and low extracellular glucose concentrations. OA chondrocytes, however, fail to downregulate GLUT-1 content and glucose transport when exposed to high extracellular glucose concentrations. The consequent intracellular glucose accumulation causes prolonged oxidative stress which can compromise chondrocyte functions. The mechanisms by which chondrocytes sense the extracellular glucose concentration and subsequently adjust GLUT-1 content are unknown. Functional ATP-sensitive potassium [K(ATP)] channels were demonstrated in equine chondrocytes and in other cells were shown to play an important role as metabolic sensors. In pancreatic β cells, high extracellular glucose causes these channels to close, increasing the intracellular calcium concentration and insulin secretion. Thus, we hypothesized that K(ATP) channels may function as metabolic sensors in chondrocytes, generating the signals that lead to adjustment of GLUT-1 content. To test this hypothesis, we evaluated the ability of specific blockers (glibenclamide) and openers (levcromakalin) of the K(ATP) channels to modulate GLUT-1 content in normal and OA human chondrocytes.

**Methods:** Normal (N=5, 31-59 years old, mean=44) and OA (N=6, 60–72 years old, mean=65) human chondrocytes were obtained from multi-organ donors or patients undergoing total knee replacement surgery, respectively, at the University Hospitals of Coimbra. Non-proliferating chondrocyte cultures were treated with glibenclamide or levcromakalin for 18h. GLUT-1 content and actin levels were evaluated in total cell extracts by western blot.

**Results:** No significant difference was found between basal GLUT-1 content in normal and OA chondrocytes. Treatment of normal chondrocytes with 10, 20 and 100 nM glibenclamide significantly decreased GLUT-1 content (61±8 ± 10.1%, 57±9 ± 11.3% and 61±2 ± 7.9%, respectively) while 20 μM (112±23.7%) had no effect, relatively to untreated cells. In the OA group, however, none of the glibenclamide concentrations tested significantly affected GLUT-1 content. Treatment with 0.5 μM levcromakalin had no significant effect on GLUT-1 content either in normal (96±24.8%) or OA (108±20.4%) chondrocytes.

**Conclusions:** These results suggest that closure of K(ATP) channels is involved in GLUT-1 downregulation in human chondrocytes, consequently modulating glucose transport. In OA chondrocytes, however, this process seems to be impaired and thus may compromise their ability to adjust glucose transport to metabolic needs.

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**HUMAN OSTEOARTHRITIC CHONDROCYTES EXPRESS THE CALCITONIN RECEPTOR**

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**Purpose:** A growing body of evidence suggests that calcitonin (CT) has a direct chondroprotective effect against degenerative joint disease in articular cartilage. The expression of the calcitonin receptor by chondrocytes is currently a matter of debate. In this study, we sought to test the hypothesis that human chondrocytes do express the calcitonin receptor.

**Methods:** We worked with fresh primary chondrocyte samples isolated from arthroplasty cartilage specimens dissected from osteoarthritic patients. We established a dedicated reverse transcription polymerase chain reaction (RT-PCR) protocol followed by a nested PCR assay aiming at amplifying the full coding region of the calcitonin receptor mRNA (CALCR). We optimized a western blotting protocol for characterizing calcitonin receptor protein (CTR) immunoreactivity in chondrocyte samples using human osteoclasts as positive controls. We validated an immunochromical procedure for localizing CTR in both primary and in situ chondrocytes.

**Results:** We amplified and sequenced four different allelic variants of CALCR in four patients. We detected a protein reacting against different CTR antibodies in four other patients. The molecular weight of the chondrocyte protein corresponds to that expressed by human osteoclasts. The plasma membrane of primary chondrocytes was intensely stained with one of the CTR antibodies validated by western blotting.

**Conclusions:** Human articular cartilage chondrocytes express both the mRNA and protein molecules of the calcitonin receptor. These findings signal the calcitonin receptor as a new pharmacological target in osteoarthritis treatment.

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**LEPTIN PRODUCED BY JOINT WHITE ADIPOSE TISSUE INDUCES CARTILAGE DEGRADATION BY UP-REGULATION AND ACTIVATION OF MATRIX METALLOPROTEINASES**

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**Purpose:** Investigate the effect of leptin on cartilage destruction in osteoarthritis.

**Methods:** The release of proteoglycan and collagen was assessed in bovine cartilage explant cultures, while collagenolytic activities and gelatinolytic activity in culture supernates were determined by bioassay and gelatin zymography. The expression and production of MMPs was analyzed by real-time reverse transcription polymerase chain reaction (RT-PCR). Signalling pathway activation was studied by immunoblotting. Levels of leptin in the supernatants from cultured osteoarthritic (OA) joint infrapatellar fat pad was measured by ELISA.

**Results:** Leptin significantly induced collagen release from bovine cartilage, by up-regulation of collagenolytic and gelatinolytic activity. When added in combination with IL-1, a marked synergistic and a dose-dependent increased cartilage collagen release and enzyme activity was observed. In cultured chondrocytes, leptin induced MMP-1 and MMP-13 expression with a concomitant activation STATs –1, –3, –5, MAPK (JNK, Erk, p38), Akt and NFκB pathways. Blockade of PI3K, P38, Erk and AKT pathway with selective inhibitors significantly reduced MMP-1 and MMP-13 expression in chondrocytes, and reduced collagen release induced by leptin or leptin + IL-1 in bovine cartilage. Furthermore, we found that supernatants from cultured OA joint infrapatellar fat pad contained leptin and can significant induce cartilage proteoglycan and collagen release from cartilage, and increase MMP-1 and MMP-13 expression chondrocytes. An anti-leptin antibody was able to partially suppress the effects of the fat conditioned media with respect to the induction of the collagenases by human chondrocytes.

**Conclusions:** This study demonstrates that leptin acts as a pro-inflammatory cytokine with a catabolic role on cartilage metabolism via the up-regulation and activation of proteolytic enzymes via PI3K, Akt, Erk and STATs signalling pathways. Moreover, our data suggests that the infrapatellar fat pad in arthritic joints is a local producer of leptin which may contribute to inflammatory and degenerative processes in cartilage metabolism, providing a mechanistic link between obesity and OA.