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„Biological activity of Galectin-8 in osteoarthritic chondrocytes and synoviocytes“

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Julia Kolb

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Abstract

Osteoarthritis (OA) is a degenerative joint disease which causes stiffness, pain and loss of joint function. Since many people suffer from OA and a successful therapeutic treatment does not exist, the interest in the pathophysiology of OA is increasing. Galectins are β-galactoside-binding animal lectins that bind to cell surfaces and extracellular matrix. They are present in different tissues and have influence on many biological reactions. Recent studies showed that some members of the family of galectins play an important role in the onset and progression of OA. To continue this work, the aim of the present thesis was to investigate the activity of the tandem-repeat Galectin-8 (Gal-8) in OA chondrocytes and synoviocytes to outline its role in OA progression. In immunohistochemical analyses we showed that severely degenerated cartilage areas exhibited a higher positivity for Gal-8 than moderately degenerated cartilage areas and that the presence of Gal-8 in OA chondrocytes significantly correlated with the level of cartilage degeneration ($p < 0.0001$, n=18 patients). Then, we focused on the impact of Gal-8 and a set of biochemical variants on OA chondrocytes and synoviocytes, whereby the results in OA chondrocytes were similar to that in OA synoviocytes. Dose-dependent experiments of Gal-8S showed a strong up-regulation of IL1B and CXCL8 mRNA levels, which suggests that Gal-8S might be an effector in joint inflammation. Specific aspects of related structure function relationships of Gal-8 could also be determined. As such, the linker length that differs between the isoforms Gal-8S and Gal-8L was shown to have no significant impact on the activity of Gal-8. In contrast, cleavage of the linker polypeptide resulted in a loss of activity, as shown using Gal-8C and Gal-8N. Moreover, experiments with a Gal-8 variant that contains the single nucleotide polymorphism F19Y showed a reduced up-regulation of IL1B and CXCL8 expression as compared to the native protein. This indicates that this mutation might play a yet to defined role in the induction of pro-inflammatory markers in OA chondrocytes and synoviocytes. In summary, the results of the current thesis suggest a correlation between OA cartilage degeneration and Gal-8 presence/activity. Furthermore, first insights into the structure function relationship of Gal-8 in OA tissues were revealed.

Zusammenfassung

Arthrose ist eine degenerative Gelenkserkrankung, bei der es zu Morgensteifigkeit der Gelenke, Schmerzen und im fortschreitenden Verlauf zu einer Beeinträchtigung der Gelenksfunktion kommt. Aufgrund der Häufigkeit dieser Erkrankung und des Mangels an geeigneter Pharmakotherapie, steigt das Interesse an den Vorgängen, die zur Entstehung der Arthrose beitragen. Galektine sind zuckerbindende Proteine, die an der Zelloberfläche und in der extrazellulären Matrix binden. Sie können in verschiedenen Gewebearten gefunden werden und sind an einer Vielzahl von zellulären Prozessen beteiligt. Neueste Studien konnten bereits nachweisen, dass einige Vertreter aus der Galektinfamilie eine wichtige Rolle während der Entstehung von Arthrose spielen. Aufgrund dieser Erkenntnisse wurde in der vorliegenden Studie der Einfluss des tandem-repeat Galectin-8 (Gal-8) auf arthrotische Chondrozyten und Synoviozyten untersucht, um seine Rolle im Krankheitsverlauf festzustellen. Immunhistologisch konnte gezeigt werden, dass es einen signifikanten Zusammenhang zwischen dem Auftreten von Gal-8 und dem Degenerationsgrad des Knorpels gibt ($p < 0.0001$, n=18 Patienten). Weiters wurde die Wirkung von Gal-8 und einigen biochemischen Varianten auf arthrotische Chondrozyten und Synoviozyten untersucht, wobei die Resultate der Chondrozyten in Synoviozyten reproduzierbar waren. Konzentrationsabhängige Versuche mit Gal-8S zeigten, dass die mRNA-Levels von IL1B und CXCL8 stark erhöht wurden. Dies deutet darauf hin, dass Gal-8S ein Mediator der Gelenksentzündung sein könnte. Darüber hinaus konnte ein Zusammenhang zwischen der Struktur und Funktion von Gal-8 festgestellt werden. So unterscheidet sich die Linkerlänge von Gal-8S und Gal-8L zwar, jedoch hatte dies keinen signifikanten Einfluss auf die Aktivität von Gal-8. Im Gegensatz dazu führte eine proteolytische Spaltung des Linkerpolypeptids zu einem Verlust der Aktivität. Schließlich untersuchten wir auch die Auswirkung der Punktmutation F19Y auf die IL1B und CXCL8 Genexpression. Die reduzierte Aktivität des Gal-8SF19Y deutet daraufhin, dass diese Mutation eine bis jetzt noch ungeklärte Rolle in der Auslösung von Entzündung in arthrotische Chondrozyten und Synoviozyten spielen könnte. Zusammengefasst zeigen diese Ergebnisse, dass es einen Zusammenhang zwischen Knorpeldegeneration und dem Auftreten und der Aktivität von Gal-8 gibt. Weiters konnten erste Einblicke in die Struktur-Funktionsbeziehung von Gal-8 gewonnen werden.

1 Introduction

1.1 The human knee joint

1.1.1 Anatomy and physiology

The knee joint is the largest joint in the human body. It is the most complicated one because on the one side it has to be flexible while walking but on the other side a great stability is needed while standing up. The knee joint consists of different bones. They are called tibia, femur and patella and connect the thigh with the lower leg. It is a diarthrodial joints, because it consists of two single joints, the kneecap joint (Articulatio femoropatellaris), which is located between femur and patella, and the femorotibial joint (Articulatio femorotibialis), which is composed of femur and tibia. The ends of these bones are covered with a hyaline articular cartilage which is important for a gliding and smooth surface. The two menisci, the meniscus medialis and lateralis act as shock absorbers between femur and tibia and disperse the weight of the body. The meniscus medialis is half-moon shaped and is grown together with the Lig. collaterale tibiae, which is important for the stability of the human knee joint. The meniscus lateralis is almost cricoid and more flexible than the meniscus medialis. Both exist of fibrocartilaginous tissue and enclosed chondroid cells. The menisci are often affected by injuries, especially the meniscus medialis because of its slight flexibility. The articular cavity of both joints is surrounded by an articular capsule, which exists of conjunctive tissue and is filled with synovial fluid. The joint capsule consists of two different tissues: The membrana fibrosa and the membrana synovialis which produces the synovial fluid. The synovial tissue has various important functions, such as shock absorption, reduction of friction or nutrition. The synovial fluid is composed of hyaluronic acid, lubricin, proteinases and collagenases. Lubricin and hyaluronic acid are lubricating components because they increase the viscosity of articular cartilage. A large number of bursae encompass the knee joint. The cruciate ligaments stabilize the whole knee joint but they also allow a huge range of motion. The Lig. cruciatum anterius is located in the front and the Lig.

cruciatum posterius is found at the back of the knee. Both secure the stability of the knee with the assistance of muscles and menisci.

Flexion (bending) and extension (straightening) are necessary for the movement of the human knee joint. There are also medial and lateral rotations possible. The flexion starts with the relaxation of different collateral ligaments, such as Lig. collaterale fibulare and Lig. collaterale tibiale. At the same time the cruciate ligaments are taut because they hinder the femur from sliding on the tibia. The menisci are drawn to the back of the knee. In the extended position the lateral, medial collateral ligaments and the Lig. cruciatum anterius are taut, also to prevent sliding. The condyles of the femur roll on the condyles of the tibia. So the human knee joint can be flexible and stable for different movements.

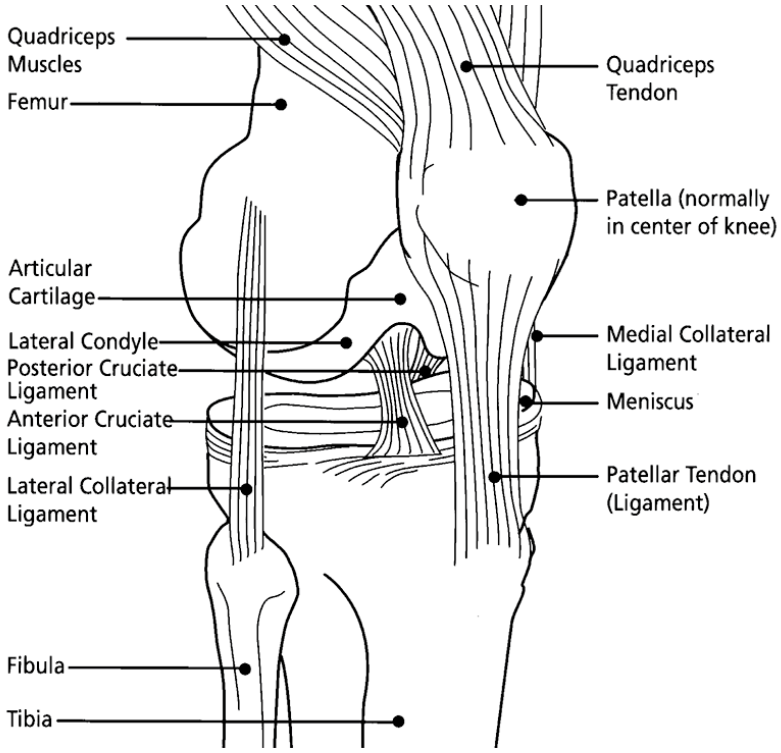

Figure 1.1: Anatomy of a human knee joint (Image taken from http://www.gustrength.com/injury:anatomy-of-torn-cartilage-and-other-knee-injuries, 12.09.2016)

1.1.2 Articular cartilage

Articular cartilage is the resilient and smooth elastic tissue of knee joints. Its important function is to provide a smooth, low friction surface for articulation and it minimizes pressure on the subchondral bone. Articular cartilage has no blood vessels or nerves, contrary to other tissue, like skin. It has a matchless mechanical behaviour and poor regenerative capacities. The hyaline articular cartilage consists of chondrocytes and extracellular matrix (ECM). Chondrocytes are the only cells, which are found in human cartilage. Because of their low metabolic activity, the repair of defects is a major challenge. The main components of the ECM are water, type II collagen and large proteoglycan aggregates. The ECM is viewed as a biphasic structure. On the one side there is a solid phase, which is composed of collagen and proteoglycan and on the other side there is a fluid phase. Aggrecan is the largest proteoglycan in articular cartilage and it is composed of a long protein core and many chrondroitin sulfate and keratan sulfate chains. The proteoglycan aggrecan molecules interact with hyaluronic acid via a link protein to form proteoglycan aggregates. Its main function is to bind water and the result is a hydrated gel structure, which is important for compressive resistance.

Cartilage can be classified into four zones, which are illustrated in the figure below:

- the superficial zone
- the middle zone
- the deep zone and
- the zone of calcified cartilage

Every zone is different in function and structure. The superficial zone or tangential zone is responsible for a smooth gliding surface and it is the thinnest part of the cartilage. The collagen fibres are oriented parallel to the articular surface and there is a high number of chondrocytes. This zone is affected first in osteoarthritis (OA). The middle zone makes up to 60% of the cartilage volume. The arrangement of the chondrocytes is completely different. They are spherical and the amount is low. The collagen is organized obliquely to the articular surface and the collagen fibrils are also thicker. The next zone, also known as deep zone, is made up from large collagen fibrils vertical to the articular surface. In this layer, the

chondrocytes are ordered as columns and parallel to the collagen fibres. It has the highest concentration of proteoglycans and because of that it is important for the resistance to compressive forces. The tide mark separates the deep zone from the calcified cartilage. The calcified layer builds a connection between cartilage and bone. (All details from Pearle et al., 2005)

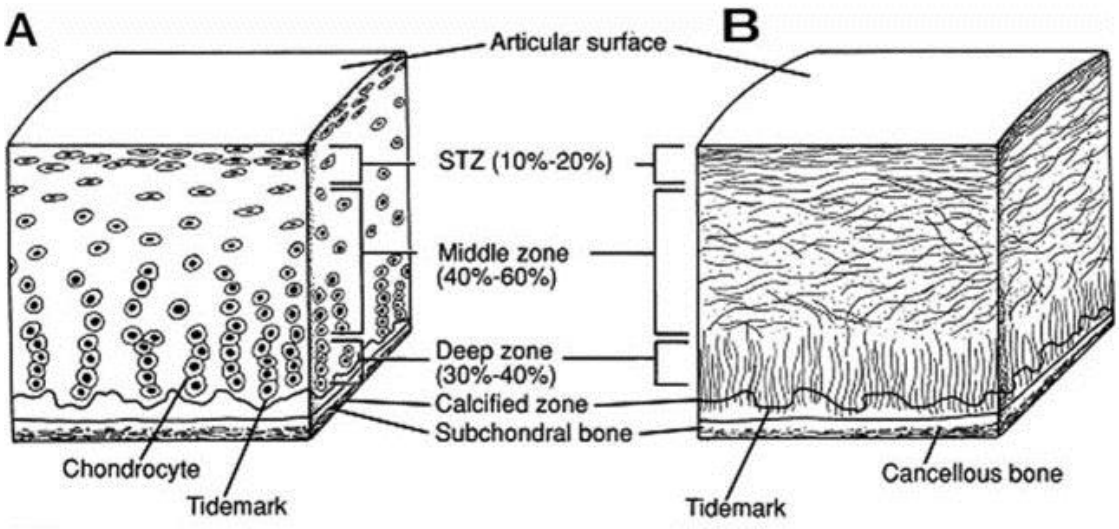

Figure 1.2: Structure of healthy articular cartilage. Picture A: cellular organisation, picture B: collagen fibrils architecture (Image taken from Buckwalter et al., 1994, Copyright American Academy of Orthopaedic Surgeons)

1.2 Osteoarthritis

OA is a degenerative joint disease and the most common form of arthritis worldwide. OA primarily affects weight-bearing joints, such as hips or knees. It affects the whole joint, including the subchondral bone, synovium, menisci and muscles. OA is the result of an imbalance between the breakdown and the repair of joint tissue. The cartilage gets thinner and as a result, the friction between the tibia and femur is higher. This causes knee pain, stiffness and loss of function. There are different risk factors, which can be divided in modifiable and non-modifiable. Age, genetic predisposition, bone density belongs to the non-modifiable risk factors, while obesity (Hunter et al., 2006), lack of exercise and joint injury (Wittenauer et al., 2013) rank to the modifiable risk factors. Gender also plays an important role because women are more often involved. It affects 10%

of men and 18% of women with 60 years of age (Woolf et al., 2003). There are also racial and ethnic differences. Black and Chinese people a have higher risk for developing OA. Other risk factors are vitamin D deficiency, smoking (Bennell et al., 2012), oestrogen levels and nutritional factors (Benito et al., 2005).

There are different strategies to diagnose OA but the widely used one is the physical examination, which can show crepitation or joint swelling. Additionally, OA is confirmed by radiography and the main characteristics to identify this degenerative joint disease are osteophytes, subchondral sclerosis and joint space narrowing (Hunter et al., 2011). Then there are other methods like arthroscopy to check whether it is not a kind of injury. MRI scan has also many benefits because it is more sensitive in discovering early structural changes (Guermazi et al., 2012)

Figure 1.3. illustrates a normal knee in comparison to a knee with OA. It clearly shows the effect of OA on the joint and the different tissues. An osteoarthritic knee has typical characteristics, such as inflammation of the synovial membrane or degeneration of articular cartilage.

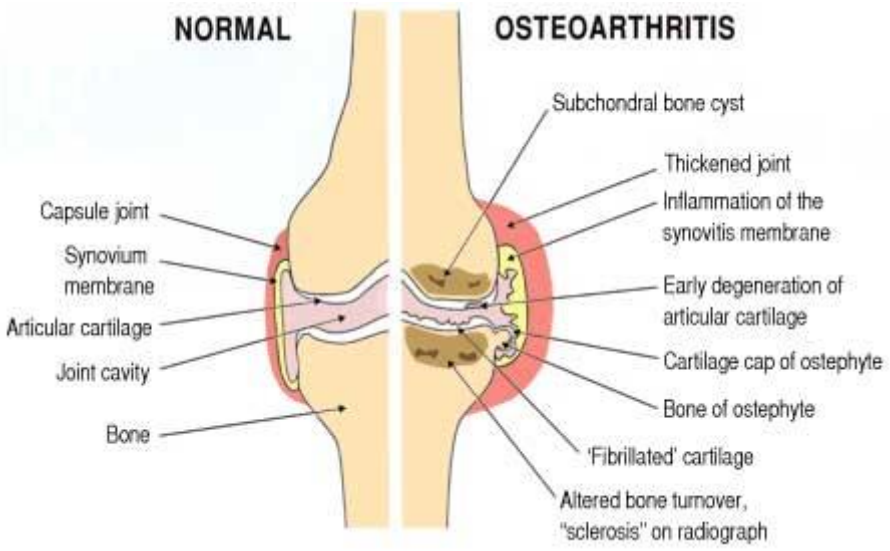

Figure 1.3: Anatomy normal knee vs. osteoarthritic knee (Image taken from https://chirobeans.wordpress.com/2012/05/03/what-are-osteoarthritis-oa-and-degenerative-joint-disease-djd, 12.09.2016)

1.2.1 Therapy of osteoarthritis

Since many risk factors for OA are modifiable, the first step is to change the lifestyle of obese or overweight patients. The aim is the loss of body weight. Because of the relief of weight- bearing joints, the symptoms get better and the risk of developing osteoarthritis will decrease. Weight loss and physical exercise also improve cardiovascular health and reduce mortality.

Nevertheless, there exists no cure of OA at the moment, only the symptoms can be controlled. Pharmaceutical drugs bring relief, because they control pain and the quality of life improves. At the beginning of pharmacological treatment acetaminophen (paracetamol) and NSAIDs are the first-line therapies. Acetaminophen is an oral analgesic drug with a dosage up to 4 g per day, but it is only a relief for mild to moderate pain (Hunter et al., 2006). Because of the risk of hepatotoxicity, the daily adult dose of 4 g should not be overdosed. Non-steroidal anti-inflammatory drugs (NSAIDs) show a stronger analgesic impact, although side effects like gastrointestinal bleeding occur. This effect is based on the inhibition of the cyclo-oxygenase 1 and 2. Due to the blockage of these enzymes the body is not able to produce prostaglandin, resulting in inhibition of pain and inflammation. COX-2 inhibitors like rofecoxib or etoricoxib have greater cardiovascular safety risks, so the usage should be well-considered. The idea for the development of COX-2 inhibitors was to reduce the side effects like GI bleeding, but later it turned out that the adverse drug reaction of COX-2 inhibitors was even greater. The topical use of NSAIDs or capsaicin is recommended for people over 75 years because the side effects are lower. Patients with chronic kidney disease stage IV or V are not allowed to take non- steroidal anti- inflammatory drugs, because the kidney capacity is restricted. Opioid analgesics can also be a choice, if NSAIDs are contraindicated or ineffective (Hunter et al, 2006). They belong to the second line treatment because NSAIDs and acetaminophen have better risk-benefit profiles. All these pharmaceutical drugs are very important in controlling symptoms.

Beside this, other pharmacological treatments, such as intra-articular injections, exist. They can be classified as either intra-articular steroids or intra-articular hyaluronan. Bellamy et al. (2005) found out, that joint injections of corticosteroids bring a short relief and reduce the pain better than a placebo. Hyaluronic acid is a

glycosaminoglycan and it decreases the friction between the tibia and femur. It was found out that the concentration of hyaluronic acid in osteoarthritic joints was much lower than in normal joints (Bastow et al., 2008). The impact relies on the improvement of the lubrication in the knee joint. However, the efficacy of hyaluronic joint injections is controversial. Other alternative treatments include glucosamine, but no clinically relevant benefit was proven. There is no great difference between glucosamine and placebo, because it shows a similar effect on pain. A total knee joint replacement is the last choice for patients, who suffer from daily pain and mobility limitations. The pharmacological treatment is ineffective or poorly tolerated and because of the severity of the symptoms, a surgery is inevitable. The following picture below shows the treatment scheme of patients with OA (Figure 1.4.).

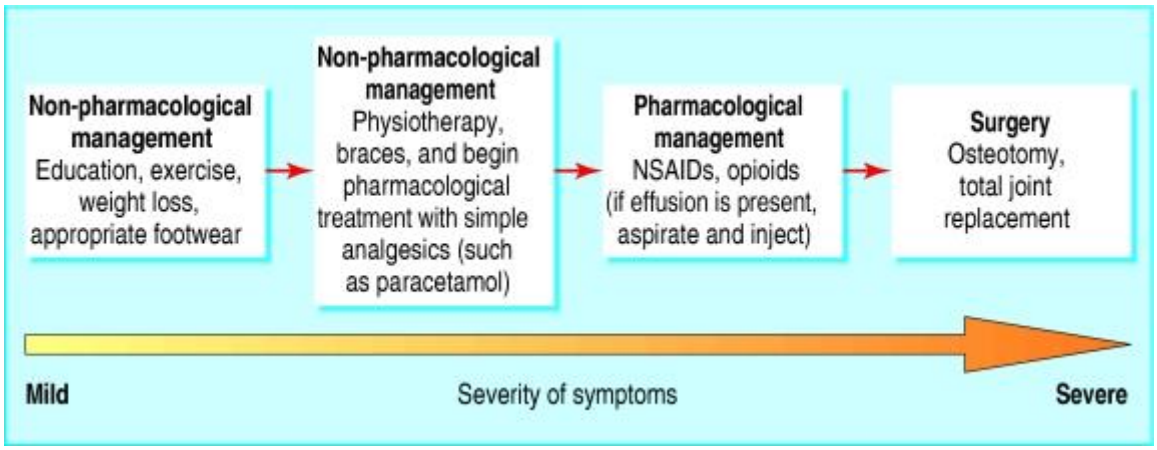

Figure 1.4: Treatment scheme of patients who suffer on OA (Image taken from Hunter et al., 2006).

1.2.2 The role of synovitis in osteoarthritis pathogenesis

The synovial membrane is a connective tissue, which contains highly active cells, the so called synoviocytes. There are two types of synoviocytes: fibroblastic and macrophage-like cells. Synoviocytes are important for nourishment of chondrocytes and for metabolic processes. The synovial fluid provides chondrocytes with nutrients. Its main function is to reduce the friction between the articulating joints. Other principal roles include shock absorption and waste transportation. The synoviocytes get rid of metabolites and products of matrix degradation. The inflammation of the synovial membrane is one of the characteristics of OA. Synovitis is the result of the inflammation and a key factor in OA because of the increased release of catabolic and proinflammatory mediators. It is responsible for different clinical symptoms, such as joint swelling or pain. Inflammatory OA pain includes night pain, morning stiffness or a sudden increase pain. There are various methods of detecting synovitis, but histological analyses are the best option, followed by arthroscopy and noninvasive imaging techniques, such as ultrasonography or MRI scan. Synovitis encompasses synovial hyperplasia, infiltration of macrophages and lymphocytes, neoangiogenesis and fibrosis and this variation has a massive influence on joint function. The presence of synovitis in OA correlates with higher rates of pain (Hill et al., 2007). Recent studies have also shown a relation between faster rates of cartilage loss and the occurrence of synovitis (Ayral et al., 2005). Cytokines, nitric oxide, prostaglandin E2 and neuropeptides belong to the catabolic and proinflammatory mediators, which are produced by the synovium. The cytokine Interleukin-1β (IL1B) promotes reactive oxygen species (ROS) generation and matrix metalloproteinases (MMP) synthesis, resulting in matrix proteolysis (Rousset et al., 2015). Interleukin-8 (CXCL8) is an inflammatory cytokine, which promotes the release of matrix metalloproteinase-13 (MMP-13), neutrophil accumulation and activation and leukocyte homing to the synovium (Olson et al., 2002; Borzi et al., 2004; Matsukawa et al., 1995). Taken together, IL1B and CXCL8 are strong pro-inflammatory markers. The result is an imbalance between repair and the breakdown of cartilage because these mediators have catabolic effects on chondrocytes. Thus, the first step for developing therapeutic targets designed to control symptoms and reduce structural joint damage is to understand the pathways that may promote synovitis in OA.

1.3 Galectins

Galectins are a group of lectins that have an affinity for ß-galactosides and share significant sequence similarity in their carbohydrate-recognition domains (CRDs) (Lobsanov et al., 1993). They are present in many organisms, including all animal kingdoms, plants and fungi (Leffler et al., 2004). Currently, there have been 15 numbered mammalian galectins identified. They have either one or two CRDs within a single polypeptide chain. One CRD contains of about 130 amino acids, which are responsible for carbohydrate binding (Yang et al., 2008). Based on the organisation of these CRDs, they can be classified in three subfamilies: proto-, chimera and tandem repeat type (Hirabayashi et al., 1993). The three subfamilies are illustrated in the following picture below (Figure 1.5.)

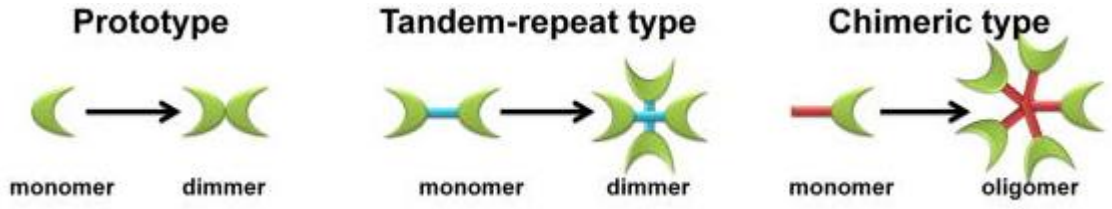

Figure 1.5: Galectin family members: Prototypical galectins have one CRD, which interacts with glycoproteins and glycolipids. The tandem repeat type galectins have two CRDs that are connected by a linker. Galectin -3, the only member of the chimeric type, consists of one CRD and non- lectin N- terminal domain. (Image modified from Li et al., 2013)

Prototypic galectins (galectin-1, -2, -5, -7, -10, -11, -13, -14, -15) consist of only one CRD and are either monomers or noncovalent homodimers depending on the ligand density. Tandem repeat galectins (galectin-4, -6, -8, -9, and -12) have two homologous CRDs joined by a linker polypeptide of up to 70 amino acids. Because of the two carbohydrate-binding sites, tandem repeat galectins are at least bivalent. Galectin-3 is the only member of the chimera type subfamily. It contains a single CRD and a non-lectin N-terminal region that promotes oligomerization and forms pentamers. Thereby, binding to multivalent glycoproteins or glycolipids is possible. In sum, each galectin has individual carbohydrate-binding prevalence (Hirabayashi et al., 2002).

The CRDs of all galectins have an affinity for the minimum saccharide ligand N-acetyllactosamine, which is found at many cellular glycoproteins, but at the same time the various galectins can also recognize different modifications to this minimum saccharide ligand. This demonstrates the specificity of galectins for tissue- or development-specific ligands (Ahmad et al., 2004).

Galectins can be found in numerous tissues and organs. Because of their different sugar-binding specificity and affinity, galectins have an important influence in multiple biological reactions, including mRNA splicing (Dagher et al., 1995), cell apoptosis (Hotta et al., 2001), cell cycle regulation (Kim et al., 1999), cell activation (Liu et al., 1995), cell adhesion and migration (Elola et al., 2007) and cell differentiation (Abedin et al., 2003). Galectins affect an amount of cellular reactions because of binding to cell-surface and extracellular matrix glycan. However, galectins have also typical characteristics of cytosolic proteins. They are synthesized on cytosolic ribosomes and from the cytosol they get to the nucleus or other subcellular sites. Secreting by non-classical pathways plays also an important role because it is a possible link between the intra- and extracellular activities. Non-classical secretion is very special for galectins because only a few lectins show this feature. So galectins are associated with a combination of intra- and extracellular activities (Ochieng et al., 2004 and Patterson et al., 2004). Based on these activities, galectins play a main role in various diseases, such as cancer, cardiovascular disease, liver fibrogenesis, asthma and RA (Klyosov et al., 2012). The different members of the galectin family can have positive and negative effects on immune responses and inflammatory reactions, depending both on the tissue and the disease context. Thus, the elucidation of their functions is necessary for any particular (patho)physiological situation. In the context of inflammatory joint diseases and joint tissue degradation, only a few members of the galectin family have been explored so far.

1.3.1 Galectin-1

Galectin-1 (Gal-1) is a carbohydrate-binding protein, which belongs to the family of lectins. It was the first member of the galectin family that was discovered. In humans, Gal-1 is encoded by the LGALS1 gene which is mapped to the q12 region of chromosome 22 (Mehrabian et al., 1993). The 0.6 kb transcript is the result of the splicing of four exons encoding a protein with 135 amino acids (Camby et al., 2006). Gal-1 belongs to the prototypic galectin subfamily and consists of a 14 kDa monomer and one CRD which can bind glycans as a monomer or homodimer. It is known that Gal-1 has different functions in- and outside of cells. While its extracellular activity is primary dependent on its lectin activities, the intracellular action is associated with carborbohydrate-independent interplay between Gal-1 and other proteins. Different effects were described for Gal-1 including activities in immune response, carcinogenesis, regeneration of the central nervous system and inflammation. The antiproliferative effect of Gal-1 is the result of the inhibition of the Ras-MEK-ERK pathway. The main functions of this pathway are cell proliferation, differentiation and survival. Aberrant regulation of the Ras-MEK-ERK pathway contributes to cancer and other disease. Gal-1 can have positive and negative effects on cell growth but the effect is dependent on cell type and cell activation status. The relative distribution of monomeric versus dimeric or intracellular versus extracellular forms might also play a role (Chamby et al., 2006). Gal-1 has also an impressive effect on OA-related mechanisms. There is a significant correlation between Gal-1 presence in chondrocytes and the level of cartilage degeneration. It was shown that Gal-1 upregulated the expression of enzymes involved in matrix degradation in vitro. At the same time the expression of matrix components was downregulated. Gal-1 promotes the proinflammatory process in osteoarthritic chondrocytes by stimulating NFκB pathway (Toegel et al., 2016). Taken together, Gal-1 might act as master regulator of the degeneration of osteoarthritic chondrocytes. This effect is contrary to the anti-inflammatory action of Gal-1 in other diseases, such as rheumatoid arthritis (RA). Besides its anti-inflammatory impact, Gal-1 can also be immunosuppressive. Treatments of T-cells with Gal-1 decrease pro-inflammatory cytokines such as TNFα or IL-1ß and increase anti-inflammatory cytokines such as IL-10 (Rabinovich et al., 1999, van der Leij et al., 2004). It is an important tool in the regulation of the immune response because

Gal-1 is highly expressed by immune-related cells, such as T-cells. The following picture shows the structure of Gal-1 (Figure 1.6.).

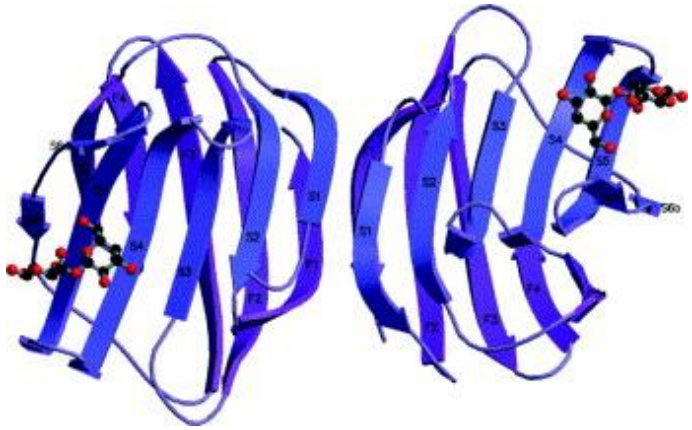

Figure 1.6: The structure of the human homodimeric Gal-1 (Image taken from Lopez-Lucendo et al., 2004).

1.3.2 Galectin-3

Galectin-3 (Gal-3) is a structurally unique member of the family of lectins because it is the only chimera type galectin. It is composed of one CRD which is linked to a proline, glycine and tyrosine rich N-terminal with multiple homologous repeats. The N-terminal region consists of tandem repeats of short amino acids segments. If multivalent carbohydrate ligands are present, Gal-3 self-assembles into higher order oligomers. As a result, oligosaccharides on the cell surface are crosslinked which might induce cell-signaling. Like Gal-1, Gal-3 is involved in many biological activities, such as cell adhesion, cell activation and immune regulation, cell growth and differentiation, cell cycle and apoptosis. Because of these biological functionalities Gal-3 plays important roles in different diseases, such as cancer, inflammation, heart diseases and stroke (Dumic et al., 2006). Gal-3 expression is necessary for cancer development because then, normal cells undergo a neoplastic transformation (Yoshi et al., 2001). Gal-3 has different roles in tumorgenesis-related processes such as transformation, cell-cycle regulation and tumor cell apoptosis. The transformation is caused by the interaction of Gal-3 with oncogenes, such as Ras and promotes the Ras-MEK-ERK pathway. Gal-3 has also influence on the cell cycle and can control tumor progression. So Gal-3 can regulate the levels of cell-cycle regulators and cell-cycle inhibitors. Cell cycle arrest can be the result

of this function. Gal-3 can have different effects in regulation apoptosis. This depends on the localization: cytoplasmic Gal-3 is anti-apoptotic, whereas nuclear Gal-3 is pro-apoptotic (Liu et al., 2005). Besides its role in malignant diseases, Gal-3 has also an impact on degenerative diseases, especially on OA. Cartilage lubrication accrues by cross-linking of Gal-3 with the glycoprotein lubricin (Reesink et al., 2016). In a mouse model intra-articular administered Gal-3 injections in knee joints led to OA-like lesions (Colnot et al., 2001). However, serum and synovial fluid of OA patients have no significant increase in their Gal-3 levels, contrary to tissues with RA (Oshima et al., 2003). Similarly, to Gal-1, Gal-3 seems to be a broad-spectrum upstream effector in OA because it induces pro-inflammatory cytokines and MMPs (Weinmann et al. 2016). Gal-3 also leads to an increase of IL-6 and CCL5 production/secretion from synovial fibroblasts of OA patients because of its upstream regulation of the NF-κB pathway (Filer et al., 2009). There is also a significant correlation between cellular positivity of Gal-3 and level of cartilage degeneration, which is also a similarity to Gal-1. A new study also shows a functional cooperation between Gal-1 and Gal-3 under conditions of progressing cartilage degeneration. Because of the overlapping of the activation profiles of Gal-1 and Gal-3, it is possible that a network of galectins acts as upstream master regulator in OA (Weinmann et al. 2016). The following picture shows the structure of Gal-3 (Figure 1.7.).

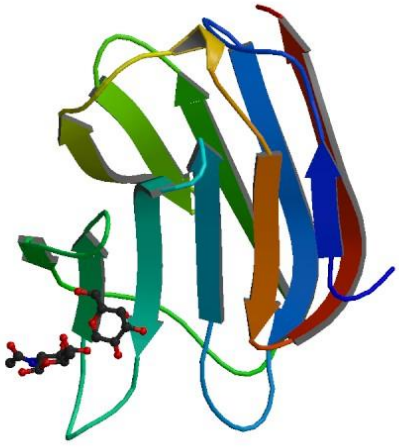

Figure 1.7.: The structure of the human monomeric Gal-3 (Image taken from RCSB Protein Data Bank; http://www.rcsb.org/pdb/explore.do?structureId=1a3k, 12.09.2016).

1.3.3 Galectin-8

This protein belongs to the tandem-repeat galectins, which contain two homologous CRDs separated by a linker of up to 70 amino acids in a single polypeptide chain. Galectin-8 (Gal-8) is a 34 kDa secreted protein, which is widely expressed (Levy et al., 2001). LGALS8 stands for the human Gal-8 gene, which encodes seven different isoforms, resulting from alternative splicing. Three of these isoforms belong to the tandem-repeat group and the main difference is the size of their hinge region. According to the length of their linker peptide, the isoforms are called as Gal-8S (small Gal-8 with a short linker region), Gal-8M (medium Gal-8, with an intermediate linker region) and Gal-8L (long Gal-8, with the longest linker peptide). Gal-8 isoforms, which belong to the prototype galectins and only have one CRD, are not found at the protein level (Bidon-Wagner et al., 2004). To determine the possible function of Gal-8 in endothelial cells, many studies were carried out. It has been shown that Gal-8 is localized in cytoplasmic and nuclear compartments of endothelial cells from human prostate and breast tissues (Delgado et al., 2011). Compared to human primary dermal vascular endothelial cells, human primary dermal lymphatic endothelial cells show higher Gal-8 mRNA and protein levels. Moreover, Gal-8 is mostly expressed in human primary dermal lymphatic endothelial cells. In summary, these findings led to the hypothesis that Gal-8 expression and localization control the biology of blood and lymphatic vessels. Gal-8 plays also an important role in vascular angiogenesis. It behaves similarly to VEGF by promoting angiogenesis in vivo in a dose-dependent manner. But contrary to Gal-1, Gal-8 showed no up-regulation by simultaneous addition of anti-VEGF treatment (Croci et al., 2012). Like all other galectins, Gal-8 can regulate cell adhesion positively or negatively, depending on the underlying carbohydrate-protein interactions. Gal-8 binds to the subunit of cell surface glycoproteins of the integrin family, but not all integrins are included. In human, Gal-8 interacts with α3, ß1 and α4 integrins. Besides, Gal-8 can recognize many other types of ligands, such as podoplanin, CD166 and CD44. All these interactions play a relevant role in angiogenesis, cell migration and cell adhesion (Troncoso et al., 2014). Moreover, Gal-8 promotes T-cell proliferation and so it has a tangible effect in autoimmune regulation (Tribulatti et al., 2009). Gal-8 leads also to apoptosis or growth arrest in immune and tumor cells. The level of Gal-8

expression may be related with the malignancy of some human cancers (Bidon-Wagner et al., 2004). The triggered apoptosis was shown in synovial fluid cells of RA patients (Eshkar Sebban et al., 2007). There is also a context between the cartilage degeneration in OA and the increase of Gal-8 presence in OA chondrocytes (Toegel et al., 2014). The following picture shows the structure of N- and C-terminus of Gal-8 (Figure 1.8.).

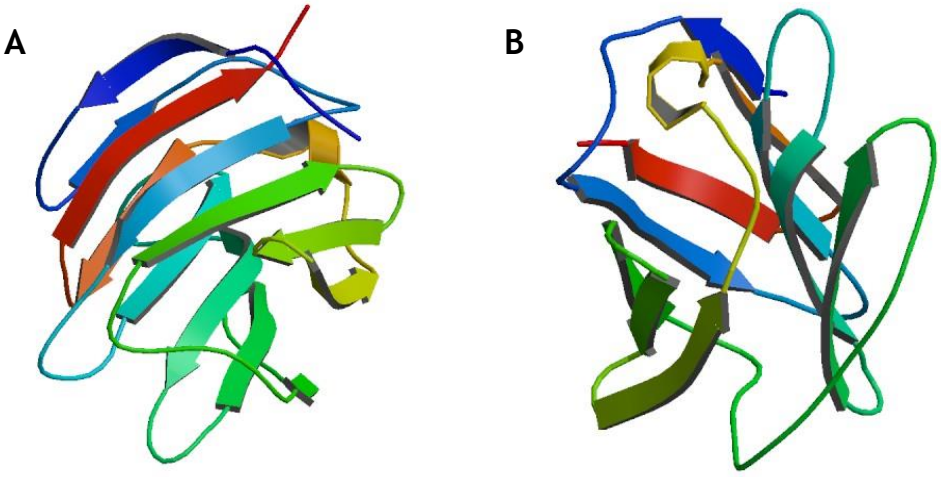

Figure 1.8: Components of the structure of human Gal-8
A: structure of the N-terminus (Image taken from Ideo et al., 2011), **B:** structure of C- terminus of human Gal-8 (Image taken from RCSB Protein Data Bank, http://www.rcsb.org/pdb/explore/explore.do?structureId=3OJB, 12.09.2016).

1.3.4 Variants of Galectin-8

Variants of Gal-8 including Gal-8S, Gal-8SF19Y, Gal-8N, Gal-8NF19Y, Gal-8L and Gal-8C have been tailored to address structure-function relationships for this galectin (Pal et al., 2012; Ruiz et al., 2014; Zhang et al., 2015). Gal-8 is a tandem-repeat galectin, which consists of N- and C-terminus and a linker peptide, which connects both regions. The linker is susceptible to proteolytic cleavage (Nishi et al., 2006) and because of that the two CRDs can be separated. The two free proteins, Gal-8N and Gal-8C, are the result of this separation. So Gal-8N only consists of the N- terminus, while Gal-8C is composed of only the C-terminus. Gal-8S (small Gal-8) and Gal-8L (long Gal-8) only differ in their size of the hinge region (Troncoso et al., 2014). While the linker peptide of Gal-8S consists of 33 amino acids, Gal-8L has a linker of 75 amino acids (Zhang et al., 2015). The term "F19Y" describes a single nucleotide polymorphism in the coding region of the LGALS8 gene. This missense mutation affects amino acid 19 in exon 1 and leads to substitution of phenylalanine (F) to tyrosine (Y) (Pal et al., 2012). This mutation naturally occurs in RA and showed a 50% lower capacity for agglutination, as compared to the wildtype protein (Zhang et al., 2015). Thus, Gal-8SF19Y describes Gal-8S with F19Y mutation, while Gal-8NF19Y is only the N-terminus with this mutation. The following picture below shows the structure of Gal-8 and some variants (Figure 1.9.).

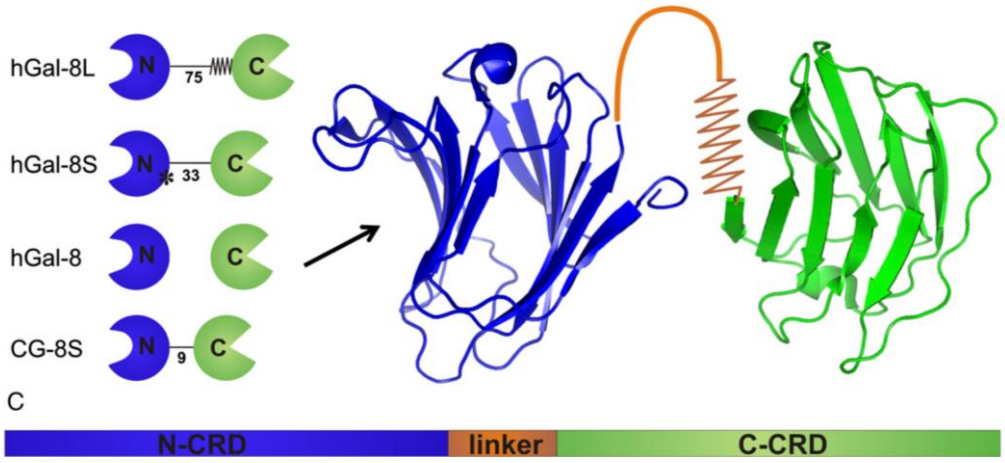

Figure 1.9.: Comparison of the different variants of Gal-8 (Image modified from Zhang et al., 2015).

1.4 Aim of the study

The aim of the study was to analyze the activity of Gal-8 in OA chondrocytes and synoviocytes to outline its role in OA progression. Recent studies showed that members of the galectin family are predominant factors in OA pathobiology. Gal-1 and Gal-3 seem to be upstream effectors because they induce pro-inflammatory cytokines and MMPs (Toegel et al., 2016; Weinmann et al., 2016). In the first part of the study, the presence of Gal-8 during cartilage degeneration in OA chondrocytes was analyzed histologically. Afterwards, a comparison between Gal-8 and a set of its biochemical variants was performed to show in vitro effects in cultured human OA chondrocytes and synoviocytes and to investigate specific aspects of related structure function relationships. Another aim of the study was to show the influence of a single nucleotide polymorphism, which naturally occurs in RA.

2 Materials and methods

2.1 Clinical specimens

Human articular cartilage and synovium was obtained from osteoarthritis patients, who underwent total knee replacement surgery with informed consent and in accordance with the terms in the ethics committee of the Medical University of Vienna (EK-no.: 1065/2011). The removed material was delivered from the operating room and was inserted in physiological salt solution. Useful parts of the OA joint were processed for cell culture, while the remaining parts were given for histological processing. Tissue specimens dedicated for cell culture were inserted overnight in physiological salt solution with 1% penicillin/streptomycin (=penstrep) (Gibco by Life Technologies) at 8°C prior to cell isolation on the next day.

2.2 Cell culture

2.2.1 Isolation of primary chondrocytes and synoviocytes

Primary human OA chondrocytes were isolated from OA knee joints under sterile conditions. The articular cartilage was washed with PBS (Gibco by Life Technologies), cut off the bone and dissected into small pieces with disposable scalpels (Heintel) on petri dishes (BD Falcon). Primary human OA synoviocytes were isolated from synovial tissue, which was also sliced in little pieces. Afterwards, the different tissues were filled into different Falcon tubes (BD Falcon) and digested by adding sterile-filtered medium (see 2.2.3) containing 300 U/ml lyophilized Collagenase type II (Gibco by Life Technologies). The Falcon tubes were shaken for 24 hours at 37°C.

2.2.2 Counting of cells

After 24 hours, the Falcons were taken off the shaker and the containing solution was filtered through a 40 µm nylon cell strainer (BD Falcon) to remove undigested pieces of tissue. The cell suspension was centrifuged at 1000 rpm for 8 minutes. Afterwards, the cell pellet was resuspended in PBS and counted in a Neubauer

hemocytometer (C-Chip, DHC-N01). Therefore, 10 µl cell suspension were mixed with 10 µl trypan blue for detection of death cells. 10 µl of the mixture were given into a cell counting chamber. Each of the four corner squares of the Neubauer hemocytometer was counted to calculate the amount of viable cells in the solution.

2.2.3 Cell cultivation

Primary human chondrocytes and isolated synoviocytes were cultured in Dulbecco´s Modified Eagle Medium (DMEM) containing 4.5 g/l glucose and supplemented with 10% fetal bovine serum (FBS). The used antibiotics were dependent on the cell type. For chondrocytes the usage of 1% penstrep was preferred, while 1% gentamycin (gibco by life technologies) was used for synoviocytes. So, the isolated cells were grown in "full medium" onto culture flasks or plates in an incubator with an atmosphere of 5% CO_2/ 95% air at 37°C.

2.2.4 Passaging of synoviocytes

If synoviocytes reached a confluency of 80%, they were split. After the removal of full medium, the cells were washed with PBS and a required amount (2 ml Trypsin for T75 and 4 ml for T150) of trypsin (Gibco by Life Technologies) was added for about 4 minutes. During this time period the flasks remained in the incubator. After that, the detachment was checked under the microscope and then the cell suspension was transferred into a Falcon tube with 10 ml full medium to stop the enzymatic reaction. Following centrifugation and removal of supernatant, the pellet was resuspended in full medium and cells were counted. The last step was the seeding of cells in larger flasks or in plates when cells were ready for treatment.

Chondrocytes for experiments were used in passage 0, while the synoviocytes were subcultured and used in passage 4 and 5.

2.2.5 Starvation and treatment of cells

The treatment of chondrocytes was performed in 12-well plates and 96-well plates, while synoviocytes were treated in 6-well plates. Because of different size of the cultivation area (Table 1.1.), the concentration of treatment per cm^2 also varied.

All cells, except of chondrocytes in 96-well plates for EZ4U, were starved with starvation medium (=full medium without FBS) before treatment to make cells more sensitive for the following treatment. The time period for starvation was dependent on cell type. Chondrocytes were starved overnight, followed by a 24 hours' treatment, while the starvation of synoviocytes lasted for about 6-8 hours and the treatment was overnight. The cells were treated in starvation medium and with various concentrations of different galectins. The different galectins were provided by Hans-Joachim Gabius´ Lab.

Culture plates	Cultivation area	Capacity of medium
6-well	9.5 cm^2	2 ml/well
12-well	3.8 cm^2	1 ml/well

Table 2.1: The difference of cultivation plates

2.3 Cell proliferation and cytotoxicity assay

EASY FOR YOU (EZ4U) assay (Biomedica) is a non-radioactive cell proliferation and cytotoxicity assay, which is performed in a 96-well plate. The aim of this assay was to test the viability of chondrocytes in presence of different concentrations and types of recombinant human Galectin-8. 96-well plates were seeded with 5000 chondrocytes per well in full medium. The treatment was performed in starvation medium for 24 hours with five concentrations of each galectin (5, 10, 20, 50, 100 µg/ml). One the next day, lyophilized substrate was dissolved in 2.5 ml activator. This solution was prewarmed to 37°C prior to addition. The culture medium was removed and 220 µl starvation medium containing 20 µl of mixed substrate. Given the metabolic capacity of the chondrocytes, the cells were incubated for 5 hours at 37°C. During this time the yellow coloured tetrazolium was reduced to its red formazan derivative. Then, the absorbance was measured at 450 nm using FLUOstar OPTIMA plate reader (BMG LABTECH).

2.4 Isolation of RNA

Total RNA was isolated from chondrocytes and synoviocytes, which were cultured in 12-well and 6-well plates respectively using innuPrep RNA Mini Kit (Analytic Jena). At first, cell culture supernatant was removed and the cell layer was washed with PBS to remove any culture medium residues. The next step was adding 350 µl Lysis Solution RL followed by repeated resuspending to lyse the cells. The lysate was then filtered through innuPrep Filter D, followed by centrifugation for 2 minutes at 12000 rpm in an Eppendorf Centrifuge 5415D. The filter was discarded to selectively remove gDNA. 350 µl of 70% ethanol were added to the filtrate and the solution was well mixed by pipetting up and down serval times. The mixture was filtered through innuPrep Filter R placed in a collection tube and centrifuged for 2 minutes. After that, the RNA was bound on the filter and the filtrate was discarded. In a next step, 500 µl washing solution HS was added and was followed by centrifugation for 1 minute at 12000 rpm. The next wash step was the adding of 750 µl washing solution LS prior to centrifugation for 1 minute. After every wash strep the innuPrep Filter R was placed into a new collection tube and at the end it was centrifuged for 2 minutes to dry the membrane. Then, the RNA was eluted by adding 40 µl of RNase-free water directly onto the membrane of the innuPrep Filter R. After incubation of 1 minute at room temperature, the sample was centrifuged for 1 minute at 8000 rpm. After the isolation of RNA, the sample was frozen at -80°C.

2.4.1 RNA quantification

The concentration and the purity (260/280 ratio) of isolated RNA were measured using the NanoDrop 2000 UV-Vis Spectrophotometer (Thermo Scientific Pequlab Biotechnologie GmbH). Before sample measurement, it was important to clean the upper arm and the lower surface of the micro-volume UV-Vis Spectrometer to avoid contamination. In the next step, a blank with water was performed, followed by sample measurement. Therefore, 2 µl of sample solution were loaded at the upper surface and the level arm was closed. Between every measurement the two surfaces were cleaned to prevent sample contamination and to make sure that the

results were correct. The RNA measurement was performed at a wavelength of 260 nm.

2.5 Transcription of RNA into cDNA

For quantitative polymerase chain reaction (PCR) the transcription of RNA to single-stranded complementary DNA (cDNA) is necessary. Therefore, the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) was used. In a first step, the samples were diluted with RNase-free water to have the same amount of RNA is in every sample. In every tube there was a total volume of 10 µl. To transcribe single-stranded RNA into cDNA, a 2x RT master mix composition containing reverse transcriptase enzyme (RT), RT buffer, dNTPs, RT primer and nuclease-free H_2O was used (Table 2.2.). To every sample, 10 µl of the reaction composition were added and mixed by pipetting up and down several times.

Component	Volume
10x RT Buffer	2.0 µl
25x dNTP Mix (100mM)	0.8 µl
10x RT Random Primers	2.0 µl
MultiScribe Reverse Transcriptase	1.0 µl
Nuclease-free H_2O	4.2 µl
Total per Reaction	10.0 µl

Table 2.2: Composition of 2x RT master mix

After that, the tubes were centrifuged and placed into a thermal cycler (Eppendorf Mastercycler Gradient). Transcription was performed in four steps (Table 2.3.). The completed samples were cooled down at 4°C until they were taken out of the thermal cycler. The final storage happened at -80°C.

Step	Length	Temperature
1	10 min	25°C
2	120 min	37°C
3	5 min	85°C
4	hold	4°C

Table 2.3: Program of the thermal cycler

2.6 RT-qPCR analysis

Quantitative real time polymerase chain reaction (RT-qPCR) is an important tool for the detection and quantification of mRNA levels in real time. It measures the accumulation of the PCR product during each cycle and that is the main advantage over conventional PCR. The detection by RT-qPCR is based on the emission of the fluorescence dye SYBR green, which binds to double-strand DNA but not on single-strand DNA. This binding emits fluorescence very brightly, which is measured during each cycle. So the increase of double-strand DNA leads to an increase of fluorescence. Three specific genes, succinate dehydrogenenase A (SDHA), IL1B and CXCL8, were quantified. SDHA is part of the citric acid cycle and is also known as "housekeeping gene". Its specific property is that its expression is not influenced by galectin treatment and because of that, it is used as stable reference to get reliable results.

In a first step, the thawed cDNA samples were diluted with RNase-free water. Therefore, 16 µl H_2O were pipetted into a small tube, followed by adding 4 µl of the cDNA sample. This was performed to achieve optimal performance for RT-qPCR analysis. For each reaction a master mix containing forward primer, reverse primer, SYBR green and nuclease-free water was prepared. The composition of reagents is shown in the following table (Table 2.4.).

Component	Volume
Forward Primer	0.5 µl
Reverse Primer	0.5 µl
SYBR-Green	12.5 µl
H_2O	10.5 µl
Total per Reaction	24 µl

Table 2.4: Composition of master mix for RT-qPCR

24 µl of master mix for each target gene were pipetted in a 96-well optical plate (MicroAmp Thin-Walled Reaction Tube, Applied Biosystems). Then, 1 µl cDNA samples was added to the master mix into the well. Each sample was prepared twice for target gene. Also, one negative control for every target gene was included to check the contamination of the reaction. After that, the plate was covered with an optical adhesive film and was placed in StepOnePlus Real-Time PCR System (Applied Biosystems). All working steps were carried out on ice.

For the targeting of the three specific genes different primer sets (Table 2.5.) were used.

	Forward Primer	Reverse Primer
SDHA	TGGGAACAGAGGGCATCTG	CCACCACTGCATCAAATTCATG
IL1B	CTTATTACAGTGGCAATGAGGATG	AGTGGTGGTCGGAGATTCG
CXCL8	CAAACTTTCAGAGACAGCAGAG	CAGTGAGATGGTTCCTTCCG

Table 2.5: Primer sequences of used target genes

During the thawing of the samples and kit components on ice, the setting of computer program was performed. The program “advanced setup” was adjusted, followed by selecting the setting 96-well, comparative C_T, SYBR-Green and Standard run. After that, the plate setup was done. Targets and samples were selected and were added to the pipetting scheme. Followed by choosing the “run method”, which determined the cycling process with a total of 40 cycles and saving the file, the measurement was ready for start. The RT-qPCR cycle program is shown in the following table (Table 2.6.)

Cycle	Step	Temperature	Length
Holding Stage	Step 1	95°C	10 min
Cycle Stage	Step 1	95°C	15 s
	Step2	55°C	1 min
	Step3	72°C	1 min
Melt Curve Stage	Step 1	95°C	15 s
	Step 2	55°C to 95°C	+1°C/min
	Step 3	95°C	15 s

Table 2.6: Cycling process of RT-qPCR

Every cycle consisted of three steps: denaturation at 95°C to separate the strands into single strands, primer annealing at 55°C and elongation at 72°C for DNA-polymerization.

For relative quantification of fluorescence, a threshold was used according to the $\Delta\Delta C_t$ method. The cycle threshold (C_T) is defined as the number of cycles at which the fluorescence crosses the threshold. The threshold should be placed in the region of exponential amplification across all samples with the same target gene. The difference between the C_T value of the treated samples and the C_T value of the control sample was calculated and related to the target gene SDHA, which was used as housekeeping gene.

2.7 Histology

2.7.1 Tissue preparation and Safranin-O staining

For immunohistochemistry, cartilage specimens (n=18 patients) were selected macroscopically to include areas of mild and areas of serve degeneration. According to standard procedures, the tissues were fixed in formalin, followed by decalcification and embedding in paraffin. After that, the paraffin slides were incubated in Safranin-O (Sigma-Aldrich) and counter-strained using light-green Goldner III solution (Morphisto). Safranin O staining correlates with the amount of proteoglycan in the cartilage. A deep red staining shows an intact proteoglycan existence, while the light-green counter staining visualizes the lack of proteoglycans. Consecutive sections were then processed for immunohistochemical staining (see 2.3.3.). All associated procedures were performed by the staff of the ´Karl Chiari Lab for Orthopaedic Biology´.

2.7.2 Histological and histochemical grading

The histological sections were graded according to histological and histochemical characteristics, which include cartilage structure, cellular abnormalities, matrix staining and tidemark integrity. The degree of cartilage degeneration was analysed with the so called “Mankin score”, which is illustrated in the following picture (Table 2.6.). The final grading ranges from 0 (most intact) to 14 (most degenerated). Areas of mild and serve cartilage degeneration were analysed from each patient.

Category	Grade
1. **Structure**	
Normal	0
Surface irregularities	1
Pannus and surface irregularities	2
Clefts to transitional zone	3
Clefts to radial zone	4
Clefts to calcified zone	5
Complete disorganisation	6
2. **Cells**	
Normal	0
Diffuse hypercellularity	1
Cloning	2
Hypocellularity	3
3. **Safranin O staining**	
Normal	0
Slight reduction	1
Moderate reduction	2
Serve reduction	3
No dye noted	4
4. **Tidemark integrity**	
Intact	0
Crossed by blood vessels	1

Table 2.7: Mankin score

2.7.3 Immunohistochemistry with anti-Galectin-8 antibody

After deparaffinization and blocking of the endogenous peroxidase activity, the slides were incubated overnight with the anti-Gal-8 antibody, which was diluted with 2% PBS. The negative control was treated by omission of primary antibodies. Later, slides were washed with PBS, followed by incubation with the secondary antibody for a short time. In a next step, the slides were again washed with PBS and were incubated with VECTASTAIN Elite ABC Kit (Vector Labs). After repeated washing, the horseradish peroxidase-mediated reaction converted 3,3´-

diaminobenzidine tetrahydrochloride hydrate (DAB, FLUKA) to an insoluble brown reaction product. All associated procedures were performed by the staff of the ´Karl Chiari Lab for Orthopaedic Biology´.

2.7.4 Morphometric analyses

The immunohistochemical staining of Gal-8 presence in OA cartilage was evaluated using an Olympus Vanox AHBT3 microscope and microphotographs were processed using cell D software (Olympus). The fraction of stained cells was evaluated under the miscroscope (200 x magnifications) on the basis of 100 chondrocytes per area of mild and serves degeneration. Staining signals above the background levels were scored as positive. Afterwards, the percentage of positive stained cells was calculated.

All used overview images were photomerged by using Adobe Photoshop from single photographs.

2.7.5 Statistical analyses

Statistical analysis of a correlation between Galectin-8 immunpositivity and the Mankin Score was performed using SPSS 20.0. Scatterplots of "percentage of Gal-8 positive cells" versus "Mankin scores" were prepared for each patient. For each of the 18 patients the Spearman's and Pearson's correlation coefficients were calculated. At the beginning of the statistical analysis it was important to determine if the data were normally distributed or not. The Shapiro Wilk test was used for this detection. If the p-value was smaller than 0.05 ($p< 0.05$), the data was not normally distributed. Then, the Wilcoxon signed-rank test was performed to test whether the median Pearson´s or Spearman´s correlation coefficients were different from 0.

qPCR data was also analysed using the paired Student´s t-test or the Wilcoxon signed-rank test. At first, the distribution of the data was performed using the

Shapiro Wilk test. Normally distributed data was analysed using paired t-tests, while the significance of not normally distributed data was delineated using Wilcoxon signed-rank test.

3 Results

3.1 Histological assessment of Gal-8 in cartilage

The immunopositivity of chondrocytes for Gal-8 was detected in cartilage specimens of 18 patients using the anti-Gal-8 antibody. Nine to ten different areas per patient were analysed to determine the percentage of Gal-8 positive chondrocytes and the degeneration grade using the Mankin score. Safranin O stained slices were used to determine the Mankin score. The scores of the different cartilage areas ranged from 1 (mildly degenerated) to 13 (severely degenerated). The evaluated areas showed Gal-8 cell positivity from 0% to 98%. Mildly degenerated areas (Figure 3.1) seemed to have a lower percentage of Gal-8 positivity, while in severely degenerated areas (Figure 3.3) the percentage of Gal-8 positive cells was higher. Figure 3.2 shows cartilage areas with a moderate degeneration and an intermediate Gal-8 positivity.

To show the relation between Gal-8 cell positivity and the Mankin score a scatterplot was performed. So the percentage of Gal-8 positive cells was plotted against the Mankin score with the regression lines for each patient (Figure 3.4) and with the regression line over all patients (Figure 3.5). For statistical analysis, Spearman's and Pearson's correlation coefficients were calculated for each patient and the Wilcoxon signed rank test showed a significant ($p < 0.05$) correlation between Mankin scores and the percentage of Gal-8 positive chondrocytes.

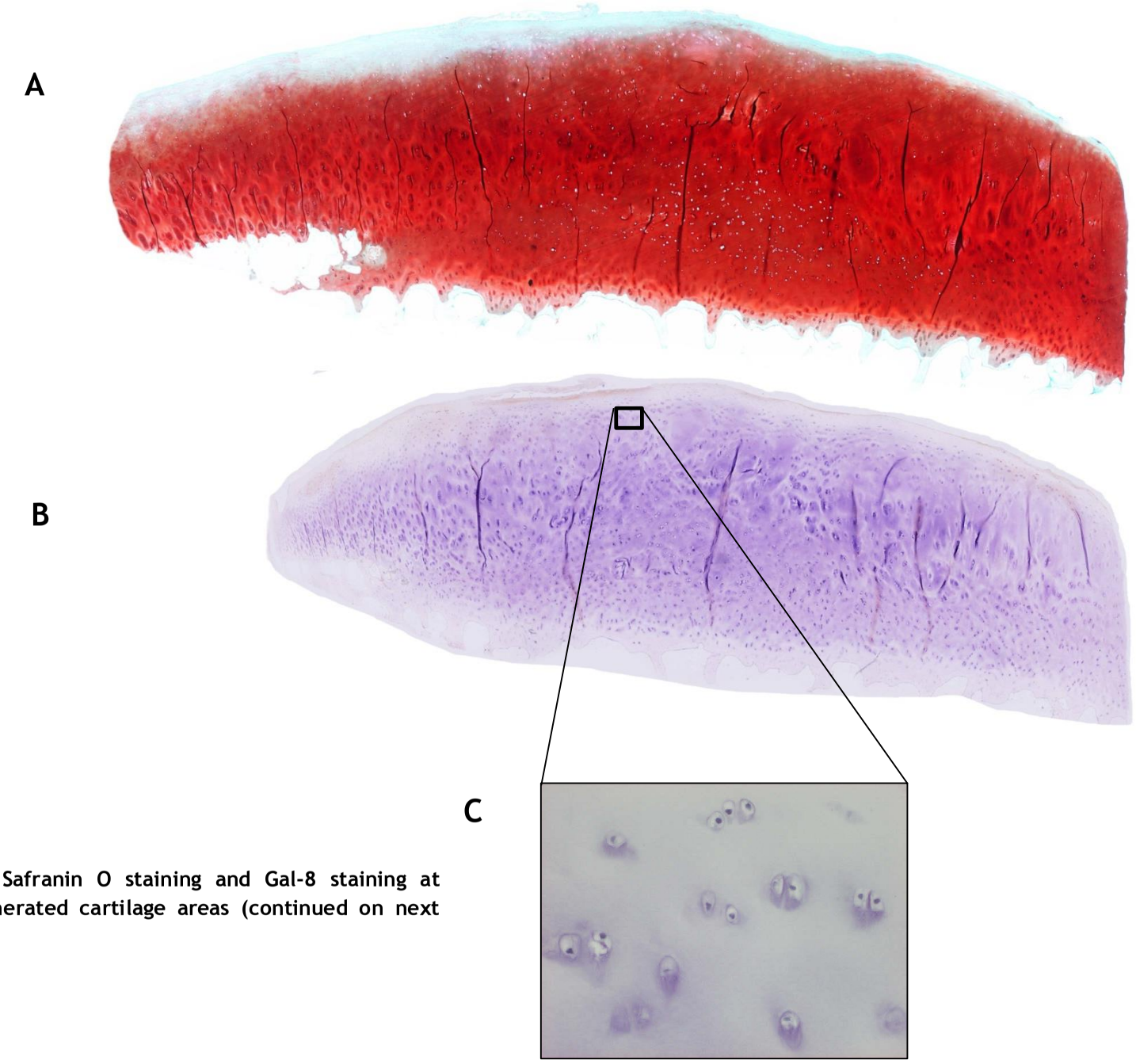

Figure 3.1: Safranin O staining and Gal-8 staining at mildly degenerated cartilage areas (continued on next page)

Figure 3.1 (continued): Safranin O staining and Gal-8 staining at mildly degenerated cartilage areas

Representative images of cartilage areas with Mankin score 1-3 (mild degeneration) are shown. Safranin O staining at 40x magnification (A) was used to analyse the degree of cartilage degeneration. The anti-Gal-8 antibody stained slice at 40x magnification (B) and at 400x magnification (C) were used to determine the percentage of Gal-8 positive stained cells. In this case, Mankin score 1-3 comes together with 13%-17%Gal-8 positive cells (B).

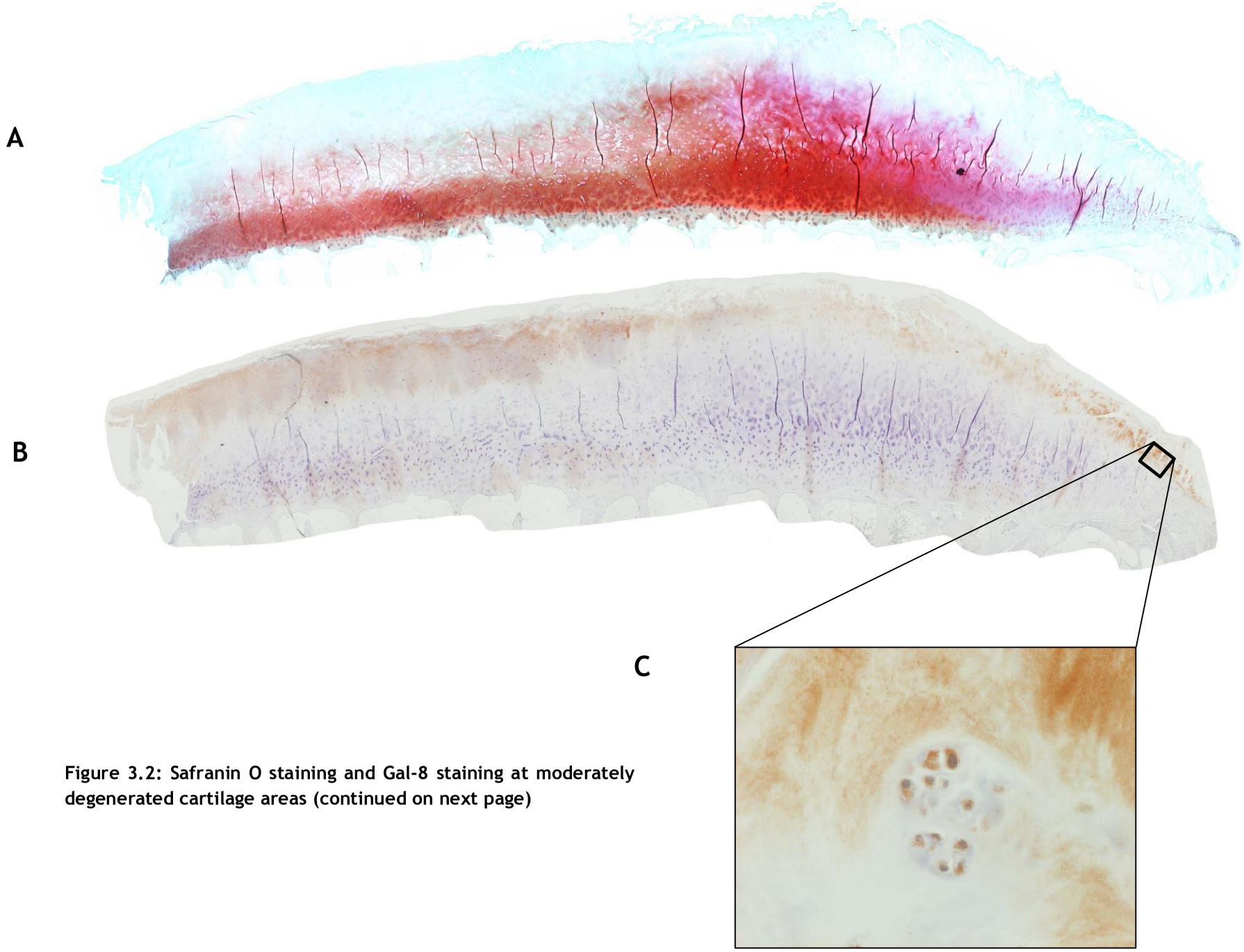

Figure 3.2: Safranin O staining and Gal-8 staining at moderately degenerated cartilage areas (continued on next page)

Figure 3.2 (continued): Safranin O staining and Gal-8 staining at moderately degenerated cartilage areas

Representative images of cartilage areas with Mankin score 5-6 (moderate degeneration) are shown. Safranin O staining at 40x magnification (A) was used for to analyse the degree of cartilage degeneration. The anti-Gal-8 antibody stained slice at 40x magnification (B) and at 400x magnification (C) were used to determine the percentage of Gal-8 positive stained cells. In this case, Mankin score 5-6 comes together with 28%-39%Gal-8 positive cells (B).

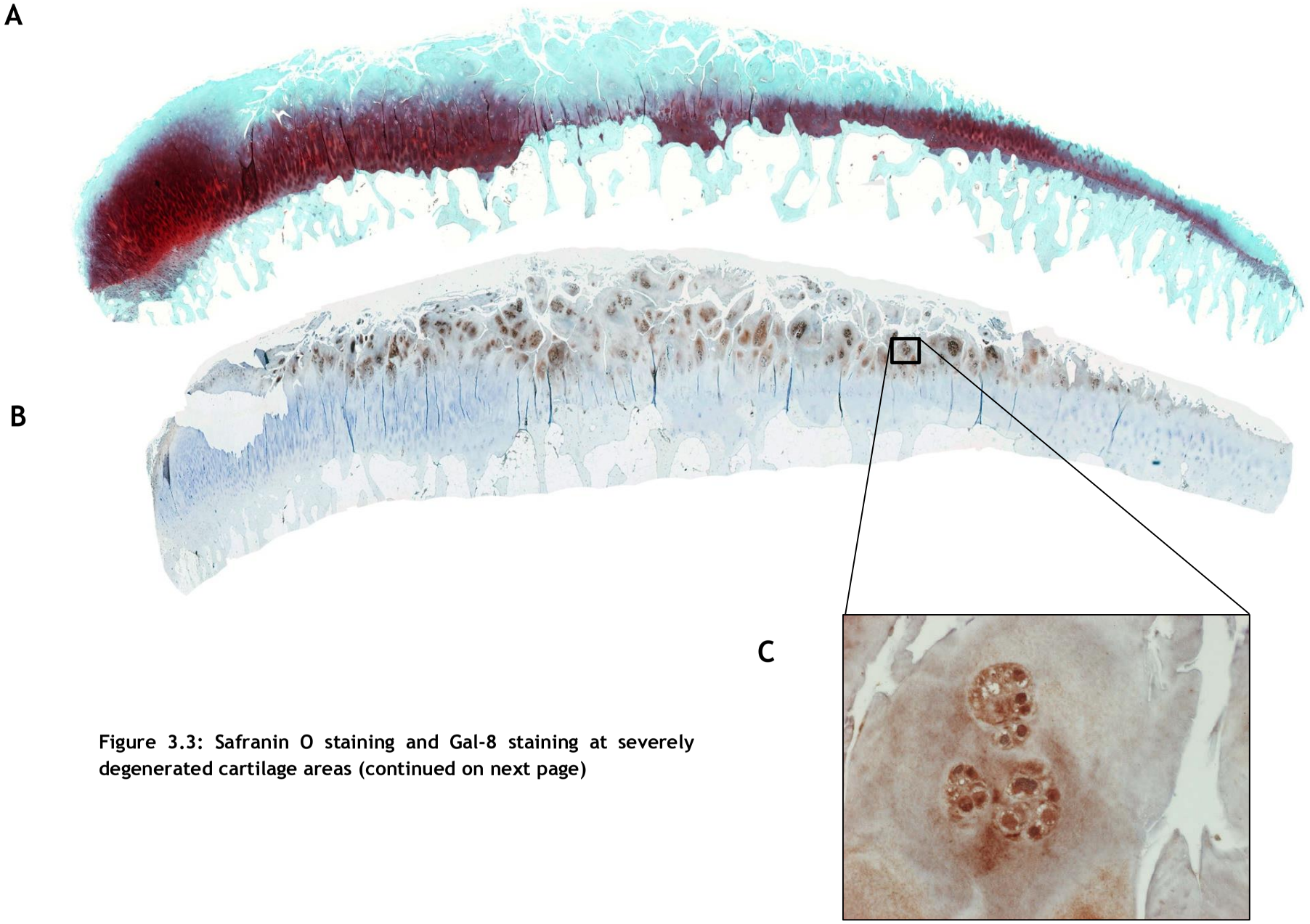

Figure 3.3: Safranin O staining and Gal-8 staining at severely degenerated cartilage areas (continued on next page)

Figure 3.3 (continued): Safranin O staining and Gal-8 staining at severely degenerated cartilage areas

Representative images of cartilage areas with Mankin score 8-10 (severely degeneration) are shown. Safranin O staining at 40x magnification (A) was used to analyse the degree of cartilage degeneration. The anti-Gal-8 antibody stained slice at 40x magnification (B) and at 400x magnification (C) were used to determine the percentage of Gal-8 positive stained cells. In this case, Mankin score 8-10 comes together with 20%-94%Gal-8 positive cells (B).

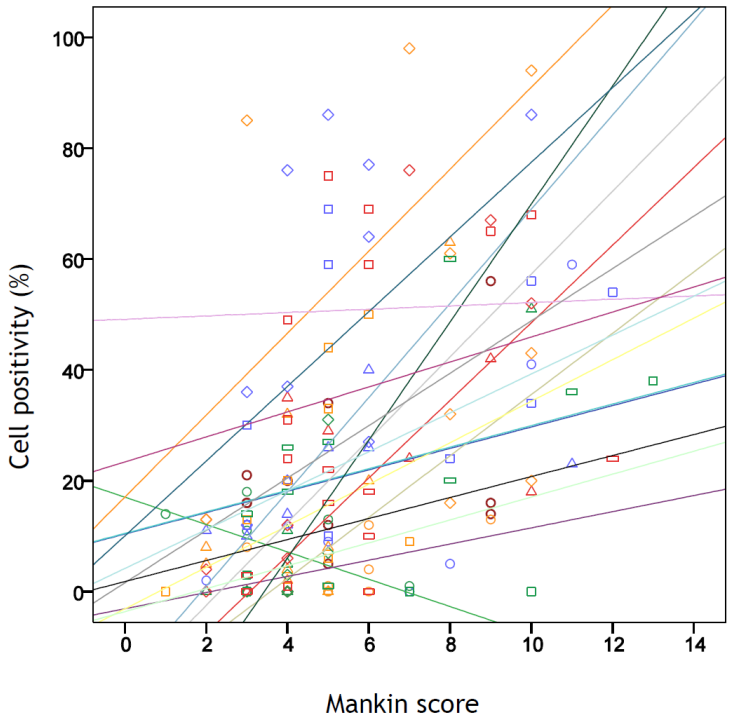

Figure 3.4: Scatterplot of Mankin score vs. the percentage of Gal-8 positive cells with regression lines for each patient

The scatterplot shows the relation between the Mankin score and the percentage of Gal-8 positive cells in cartilage areas of 18 patients. The Mankin scores ranged from 1 to 13, while the areas showed Gal-8 cell positivity from 0% to 98%. Statistical analysis was performed using Wilcoxon signed rank test, after Pearson´s and Spearman´s correlation coefficients were calculated for each patient. A strong correlation between Galectin-8 levels and degeneration severity ($p < 0.05$) was found.

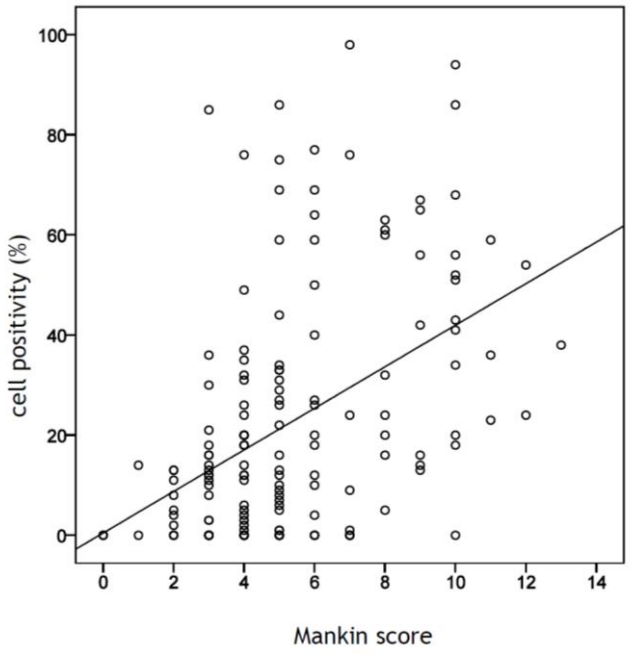

Figure 3.5: Scatterplot of Mankin score vs. the percentage of Gal-8 positive cells with regression lines over all patients

The scatterplot shows the relation between the Mankin score and the percentage of Gal-8 positive cell in cartilage areas of 18 patients. The Mankin scores ranged from 1 to 13, while the areas showed Gal-8 cell positivity from 0% to 98%. Statistical analysis was performed using Wilcoxon signed rank test, after Pearson´s and Spearman´s correlation coefficients were calculated for each patient. A strong correlation between Galectin-8 levels and degeneration severity ($p < 0.05$) was found.

3.2 Analysis of the metabolic cell activity

A cell proliferation and cytotoxicity assay was performed to show the impact of the applied treatment on OA chondrocytes (n=3 patients). The cells were treated with 5 μg/ml, 10 μg/ml, 20 μg/ml, 50 μg/ml and 100 μg/ml of Gal-8S and its variants for 24 hours. The untreated control cells were cultured under the same conditions. Differences between study group and control were considered statistically significant when $p < 0.05$.

3.2.1 Effect of galectins on the metabolic cell activity of OA chondrocytes

Figure 3.6 (A-F) indicates the impact of five different concentrations of Gal-8S and its variants on the metabolic activity of OA chondrocytes. In general, all applied concentrations were well tolerated and showed no considerable impairment of cellular activity. Only Gal-8N induced a statistically significant decrease to 82% of metabolic activity at a concentration of 100 μg/ml.

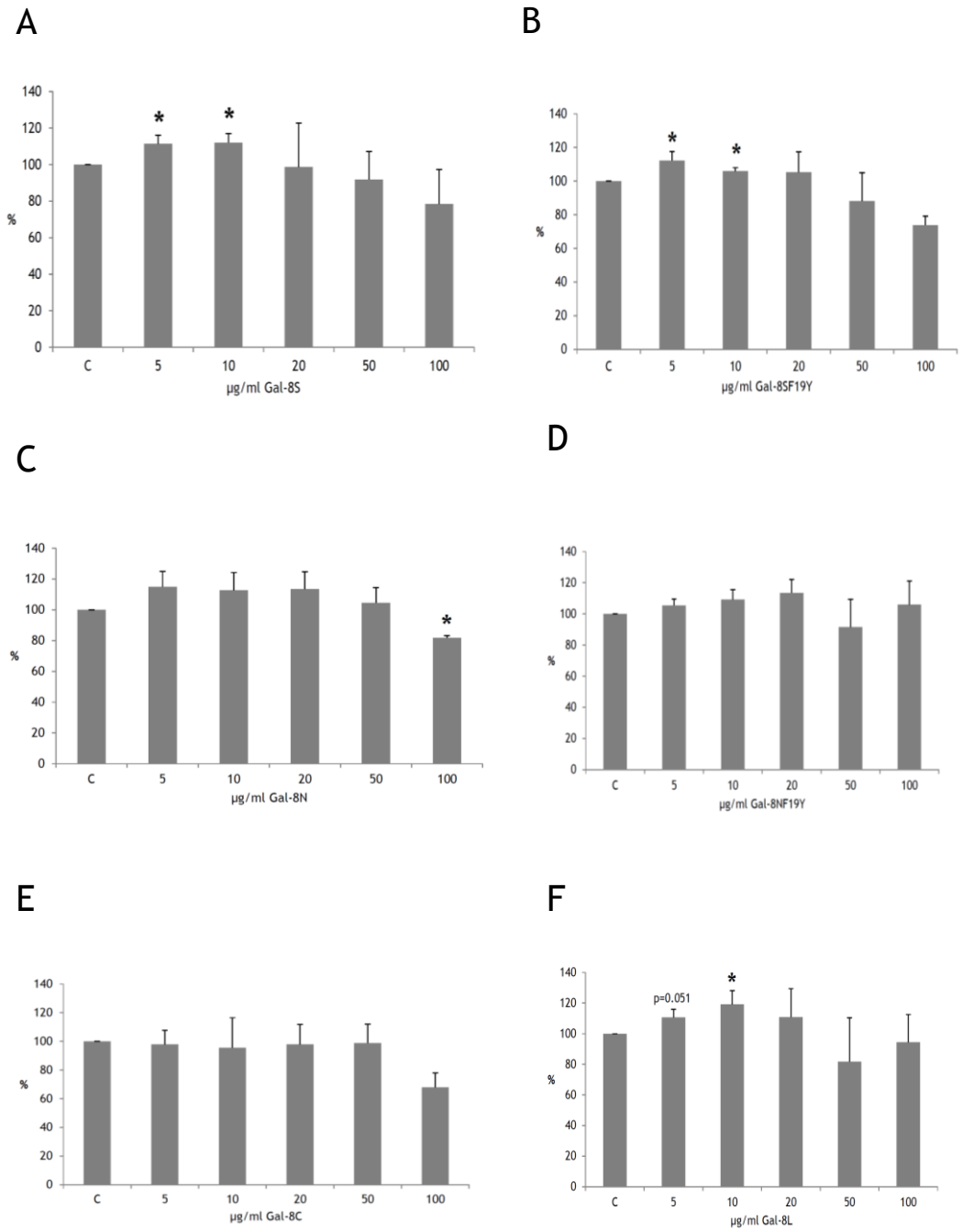

Figure 3.6: Effects of galectins on the metabolic cell activity of OA chondrocytes

OA chondrocytes (n=3 patients) were treated for 24 hours with different concentrations of Gal-8S and its variants (5, 10, 20, 50, 100 µg/ml). Untreated control cells (C) were cultured under the same conditions. Asterisks indicate significant differences compared to the control which was set to 100% ($p < 0.05$).

A: Gal-8S, **B:** Gal-8SF19Y, **C:** Gal-8N, **D:** Gal-8NF19Y, **E:** Gal-8C, **F:** Gal-8L

3.3 Effect of galectins on mRNA levels in galectin-treated cells

To analyze the impact of different galectins on IL1B and CXCL8 gene expression in chondrocytes and synoviocytes, RT-qPCR was performed. Three different experiments were designed. First, concentration-dependent treatments of chondrocytes were carried out with Gal-8S with concentrations from 1 µl/ml to 100 µl/ml, whereas in synoviocytes this experiment was performed with Gal-8S concentrations from 1 µl/ml to 50 µl/ml. In a second experiment, a comparison between Gal-1, Gal-3 and Gal-8S was carried out to evaluate differences in their activity on mRNA levels of chondrocytes. Third, biochemical variants of Gak-8S were compared regarding their impact on marker gene expression in chondrocytes and synoviocytes. Compensating for the different molecular weights of the protein variants, the used concentrations were adapted for each variant to allow direct comparison. While the treatment of chondrocytes was accomplished at two concentration levels (based on 10 µl/ml or 50 µl/ml Gal-8S), synoviocytes were only treated with concentrations of around 10 µl/ml. The work processes for RNA isolation, RNA quantification and cDNA synthesis of chondrocytes and synoviocytes were kept constant. The quantification of mRNA levels was analyzed using RT-qPCR.

3.3.1 Effect of galectins in OA chondrocytes

Figure 3.7 presents a concentration-dependent experiment in OA chondrocytes and the ability of Gal-8S to induce IL1B mRNA levels. The up-regulation of IL1B, as compared to the untreated control, was statistically significant for all concentrations of Gal-8S. This figure indicates a steady increase in IL1B mRNA levels up to 20 μg/ml, then, concentrations of 50 μg/ml and 100 μg/ml seemed to remain on the same level of up-regulation. At 20 μg/ml, Gal-8S significantly induced a 29917-fold (±39233, p=0.028) up-regulation, while Gal-8S with the highest concentration of 100 μg/ml significantly modulated the mRNA level of IL1B 2188-fold (±20829, p=0.028), compared to the untreated control.

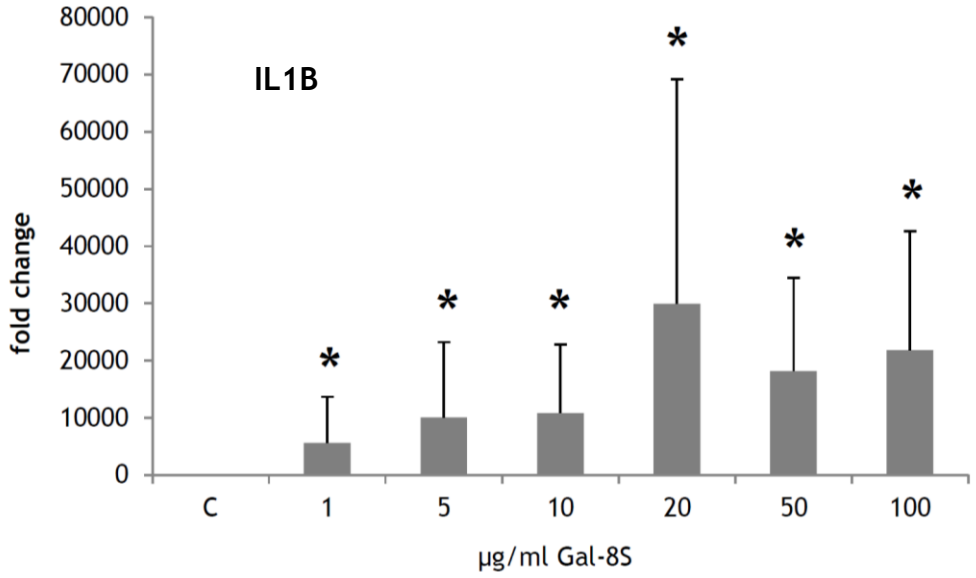

Figure 3.7: Concentration-dependent effect of Gal-8S on IL1B mRNA levels in chondrocytes
Cells (n=6 patients) were treated for 24 hours with different concentrations of Gal-8S (5, 10, 20, 50, 100 μg/ml). As reference, untreated OA chondrocytes (C) were cultured under the same conditions. After incubation, the mRNA levels of IL1B were quantified using RT-qPCR with SDHA as reference gene. The asterisks mark statistical up-regulations of IL1B expression levels compared to the control ($p < 0.05$).

Figure 3.8 illustrates the concentration-dependent effect of Gal-8S in its efficacy to increase CXCL8 levels in OA chondrocytes. As compared to the control, the up-regulation of CXCL8 was statistically significant at all applied concentrations. Gal-8S induced a 3907-fold (±5190, p=0.028) at the lowest concentration of 1 µg/ml and an 8117-fold (±6049, p=0.028) up-regulation of CXCL8 at the highest concentration of 100 µg/ml. Again, there was a slightly tendency towards peak at 20 µg/ml.

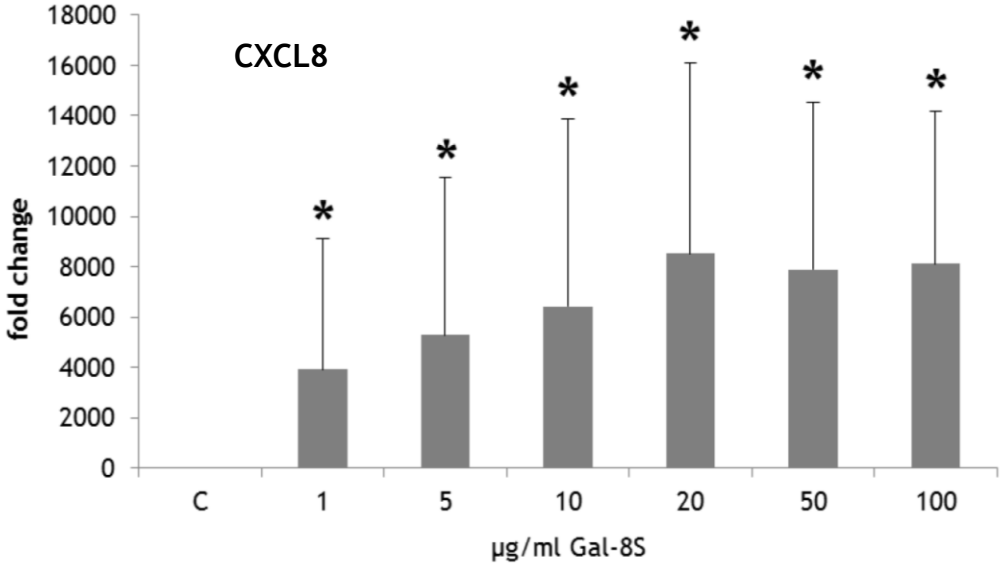

Figure 3.8: Concentration-dependent effect of Gal-8S on CXCL8 mRNA levels in chondrocytes
Cells (n=6 patients) were treated for 24 hours with different concentrations of Gal-8S (5, 10, 20, 50, 100 µg/ml). As reference, untreated OA chondrocytes (C) were cultured under the same conditions. After incubation, the mRNA levels of CXCL8 were quantified using RT-qPCR with SDHA as reference gene. The asterisks mark statistical up-regulations of CXCL8 expression levels compared to the control ($p < 0.05$).

Figure 3.9 shows Gal-8S in comparison to Gal-1 and Gal-3 in their potential to induce IL1B mRNA levels in OA chondrocytes. This figure presents a statistical significant up-regulation of IL1B by all galectins in comparison to the untreated control. Gal-1 induced a 13349-fold (±9574, p=0.025), Gal-3 a 10014-fold (±7236, p=0.025) and Gal-8S a 21896-fold (±11684, p=0.010) increase of IL1B mRNA levels. Although, there was no significant difference between Gal-1 and Gal-3, this could be found in the comparison of Gal-1 to Gal-8S.

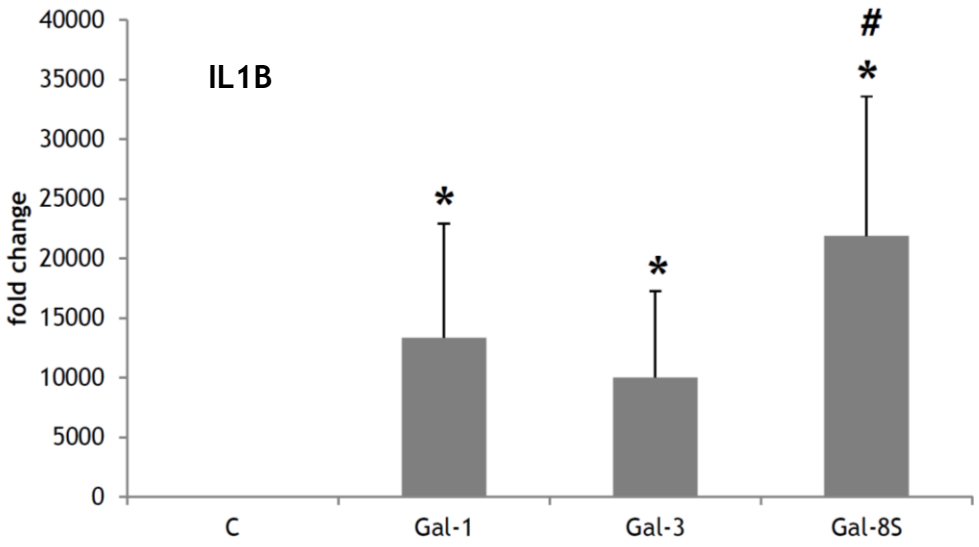

Figure 3.9: IL1B mRNA levels of chondrocytes after treatment with Gal-1, Gal-3 or Gal-8S
Cells (n=5 patients) were treated for 24 hours with 30 µg/ml Gal-1, 50 µg/ml Gal-3 or 70 µg/ml Gal-8S. As reference, untreated OA chondrocytes (C) were cultured under the same conditions. After incubation, the mRNA levels of IL1B were quantified using RT-qPCR with SDHA as reference gene. The asterisks mark statistical differences of IL1B expression levels compared to the control. The rhombus indicates the statistical difference of IL1B expression levels in comparison to Gal-1.

Figure 3.10 demonstrates Gal-8S in comparison with Gal-1 and Gal-3 in their ability to increase CXCL8 mRNA levels of chondrocytes. As compared to the untreated control, all galectins induced a statistical significant up-regulation of CXCL8 gene expression. Gal-3 with a 5304-fold (±4603, p=0.028) up-regulation showed the lowest modulating effects on CXCL8 mRNA levels, while Gal-8S induced the highest up-regulation (6851-fold ±4603, p=0.028). The gene expression level of Gal-1 with a 6578-fold (±5172, p=0.028) up-regulation was similar to Gal-8S. Statistically significant was also the difference of CXCL8 expression levels between Gal-3 and Gal-1.

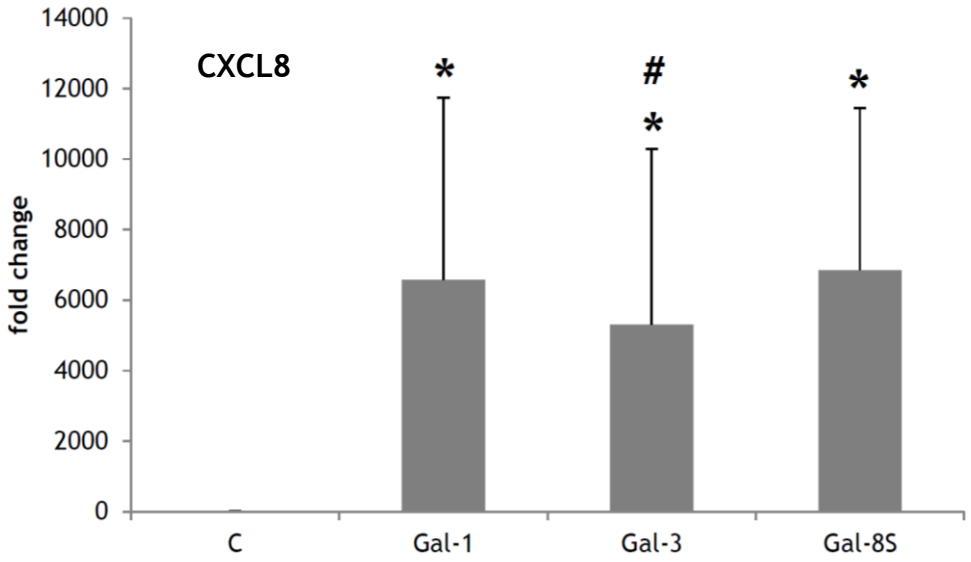

Figure 3.10: CXCL8 mRNA levels of chondrocytes after treatment with Gal-1, Gal-3 or Gal-8S
Cells (n=5 patients) were treated for 24 hours with 30 µg/ml Gal-1, 50 µg/ml Gal-3 or 70 µg/ml Gal-8S. As reference, untreated OA chondrocytes (C) were cultured under the same conditions. After incubation, the mRNA levels of CXCL8 were quantified using RT-qPCR with SDHA as reference gene. The asterisks mark statistical differences of CXCL8 expression levels compared to the control. The rhombus indicates the statistical difference of CXCL8 expression levels in comparison to Gal-1.

Figure 3.11 depicts the comparison of Gal-8S and its variants regarding their capacity to increase IL1B gene expression in OA chondrocytes. Each galectin showed a modulation of this gene with different ranges of fold changes. Gal-8N demonstrated the lowest up-regulation (28±35-fold) and did not reach statistical significance with regard to the untreated control. All other galectins had significant effects compared to the control. Gal-8S induced a 10855-fold (±11961, p=0.028) and Gal-8L a 6782-fold (±7593, p=0.028) increase of IL1B mRNA levels. As compared to the small length protein Gal-8S, Gal-8SF19Y (2581±4650-fold), Gal-8N and Gal-8C (590±728-fold) showed statistically significant differences of IL1B expression levels. Also, a statistically significant difference between Gal-8NF19Y and Gal-8N treated cells was found.

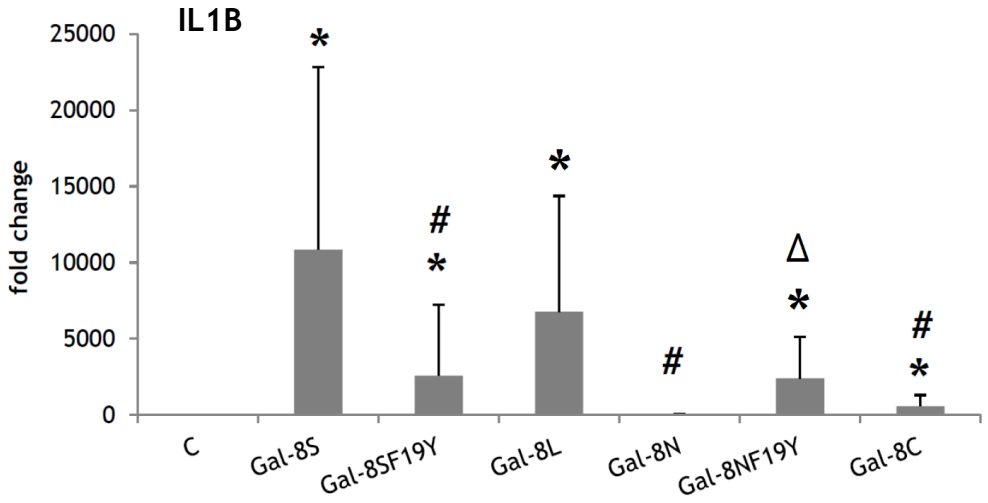

Figure 3.11: IL1B mRNA levels of chondrocytes after treatment with Gal-8S and its variants
Cells (n=6 patients) were treated for 24 hours with 10 µg/ml Gal-8S, 10 µg/ml Gal-8SF19Y, 11 µg/ml Gal-8L, 5 µg/ml Gal-8N, 5 µg/ml Gal-8NF19Y and 4 µg/ml Gal-8C. As reference, untreated OA chondrocytes (C) were cultured under the same conditions. After incubation, the mRNA levels of IL1B were quantified using RT-qPCR with SDHA as reference gene. The asterisks mark statistical differences of IL1B expression levels compared to the control. The rhombus indicates the statistical difference of IL1B expression levels in comparison to Gal-8S. The triangle marks the statistical significance of IL1B expression levels between Gal-8NF19Y and Gal-8N-treated cells.

Figure 3.12 presents the ability of Gal-8S and its variants to increase CXCL8 gene expression in OA chondrocytes. All applied galectins showed a significant up-regulation of gene expression compared to the control. The activities ranged from a minimum of a 4-fold (±3, p=0.028) up-regulation by Gal-8N to a maximum of a 6416-fold (±7476, p=0.028) up-regulation by Gal-8S. Gal-8L induced a 6238-fold (±4439, p=0.028) up-regulation of CXCL8 mRNA levels. Gal-8SF19Y (1456±1729), Gal-8N and Gal-8C (358±387) were found to be statistically significant in comparison to the small length protein Gal-8S, while Gal-8L reached no statistical difference of gene expression levels compared to Gal-8S. A statistical significance of CXCL8 expression levels between Gal-8NF19Y and Gal-8N was also revealed.

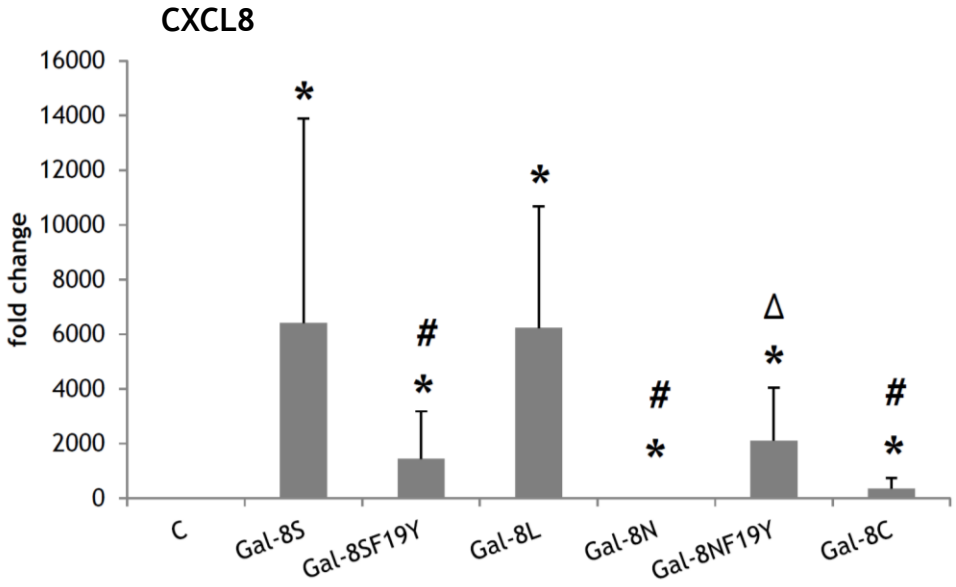

Figure 3.12: CXCL8 mRNA levels of chondrocytes after treatment with Gal-8S and its variants
Cells (n=6 patients) were treated for 24 hours with 10 µg/ml Gal-8S, 10 µg/ml Gal-8SF19Y, 11 µg/ml Gal-8L, 5 µg/ml Gal-8N, 5 µg/ml Gal-8NF19Y and 4 µg/ml Gal-8C. As reference, untreated OA chondrocytes (C) were cultured under the same conditions. After incubation, the mRNA levels of CXCL8 were quantified using RT-qPCR with SDHA as reference gene. The asterisks mark statistical differences of CXCL8 expression levels compared to the control. The rhombus indicates the statistical difference of CXCL8 expression levels in comparison to Gal-8S. The triangle marks the statistical significance of CXCL8 expression levels between Gal-8NF19Y and Gal-8N-treated cells.

Figure 3.13 shows Gal-8S and its variants in their efficacy to increase Il1B mRNA levels in chondrocytes. Compared to the untreated control, each galectin significantly enhanced mRNA expression up-regulation of IL1B. As such, Gal-8L modulated the mRNA levels of IL1B 19100-fold (±12301, p=0.018) and had the highest modulating effect, followed by Gal-8S with an 18189-fold (±16241, p=0.025) up-regulation of IL1B gene expression. Again, Gal-8N (71±45) showed the lowest modulating effects. Gal-8SF9Y (3513±2462), Gal-8N and Gal-8C (5336±3743) reached the statistical significance compared to Gal-8S. Repeatedly, this did not apply for Gal-8L. Gal-8NF19Y (9865±7842) was found to be statistically significant compared to Gal-8N.

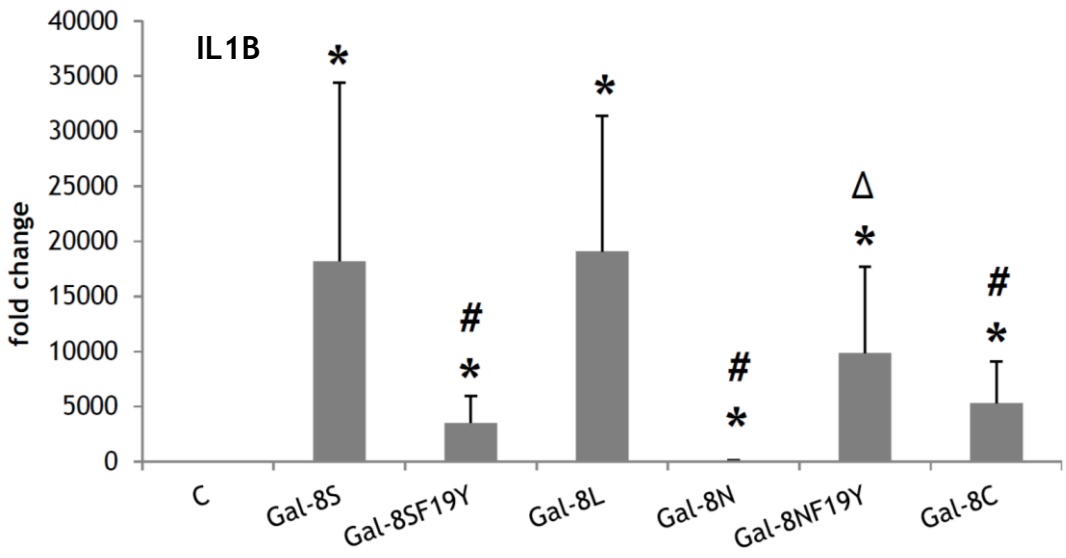

Figure 3.13: IL1B mRNA levels of chondrocytes after treatment with Gal-8S and its variants
Cells (n=5 patients) were treated for 24 hours with 50 µg/ml Gal-8S, 50 µg/ml Gal-8SF19Y, 55 µg/ml Gal-8L, 25 µg/ml Gal-8N, 25 µg/ml Gal-8NF19Y and 20 µg/ml Gal-8C. As reference, untreated OA chondrocytes (C) were cultured under the same conditions. After incubation, the mRNA levels of IL1B were quantified using RT-qPCR with SDHA as reference gene. The asterisks mark statistical differences of IL1B expression levels compared to the control. The rhombus indicates the statistical difference of IL1B expression levels in comparison to Gal-8S. The triangle marks the statistical significance of IL1B expression levels between Gal-8NF19Y and Gal-8N-treated cells.

Figure 3.14 demonstrates the ability of Gal-8S and its variants to induce CXCL8 mRNA levels in OA chondrocytes. Each galectin showed a statistically significant increase of CXCL8 gene expression, as compared to the untreated control. The activities ranged from a minimum of a 44-fold (±34, p=0.028) up-regulation by Gal-8N to a maximum of a 7893-fold (±6633, p=0.028) up-regulation by Gal-8S. As compared to the small length protein Gal-8S, Gal-8SF19Y (2748±2154), Gal-8N and Gal-8C (3481±2788) statistically enhanced CXCL8 expression levels. Again, a statistically significant difference between Gal-8NF19Y (5919±5274) and Gal-8N treated cells was detected.

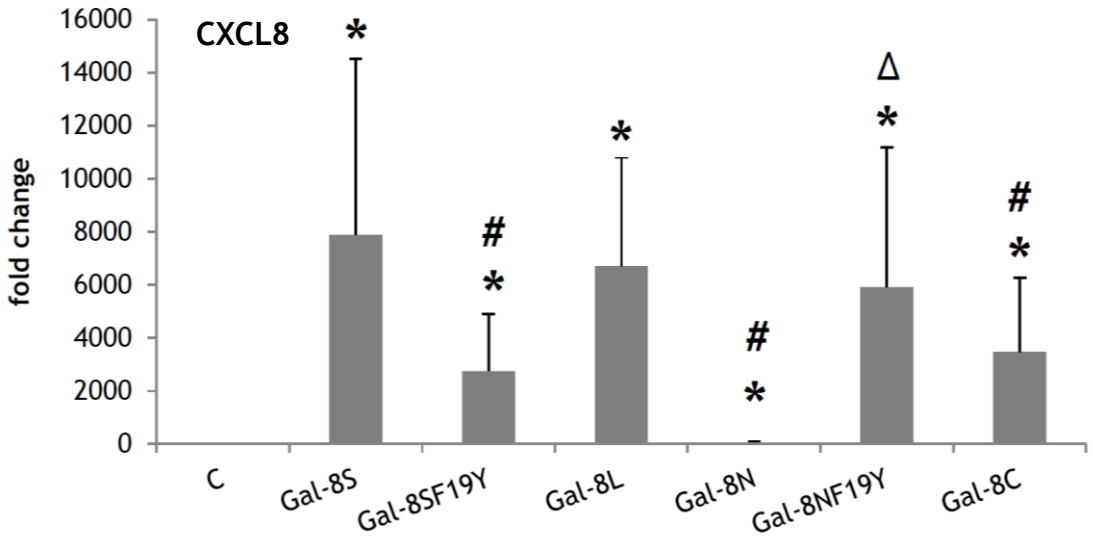

Figure 3.14: CXCL8 mRNA levels of chondrocytes after treatment with Gal-8S and its variants
Cells (n=5 patients) were treated for 24 hours with 50 µg/ml Gal-8S, 50 µg/ml Gal-8SF19Y, 55 µg/ml Gal-8L, 25 µg/ml Gal-8N, 25 µg/ml Gal-8NF19Y and 20 µg/ml Gal-8C. As reference, untreated OA chondrocytes (C) were cultured under the same conditions. After incubation, the mRNA levels of CXCL8 were quantified using RT-qPCR with SDHA as reference gene. The asterisks mark statistical differences of CXCL8 expression levels compared to the control. The rhombus indicates the statistical difference of CXCL8 expression levels in comparison to Gal-8S. The triangle marks the statistical significance of CXCL8 expression levels between Gal-8NF19Y and Gal-8N-treated cells.

3.3.2 Effect of galectins in OA synoviocytes

Figure 3.15 illustrates the results of a concentration-dependent experiment, which was designed to show the ability of different Gal-8S concentrations to increase IL1B mRNA levels in OA synoviocytes. Although statistical significance was not reached at any concentration, a clear tendency towards up-regulated IL1B levels was revealed. As compared to the untreated control, 1 µl/ml Gal-8S induced a 40-fold (±40, p=0.149) and 50 µl/ml Gal-8S a 1631-fold (±1325, p=0.112) increase of IL1B mRNA levels. The high level of inter-individual variability among patients and the low number of repetitions (n=3 patients) might have contributed to the lack of statistically significance.

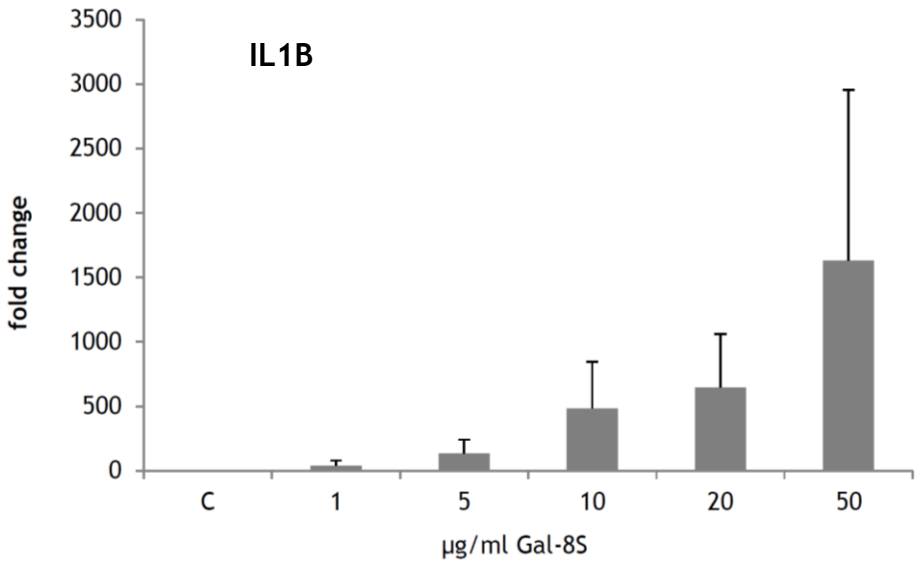

Figure 3.15: Concentration-dependent effect of Gal-8S on IL1B mRNA levels in OA synoviocytes
Cells (n=3 patients) were treated for 24 hours with different concentrations of Gal-8S (5, 10, 20, 50 µg/ml). As reference, untreated OA synoviocytes (C) were cultured under the same conditions. After incubation, the mRNA levels of IL1B were quantified using RT-qPCR with SDHA as reference gene. Statistical analysis of the data revealed no statistical significance ($p > 0.05$, paired t-test).

Figure 3.16 depicts the concentration-dependent effect of Gal-8S to induce CXCL8 mRNA levels in OA synoviocytes. Although statistical significance across all patients was not found, a tendency towards up-regulated CXCL gene expression levels was observed. As compared to the control, 5 µl/ml Gal-8S induced a 58-fold (±32, p=0.064) up-regulation and 50 µl/ml Gal-8S a 1309-fold (±647, p=0.052) up-regulation of CXCL8 mRNA levels.

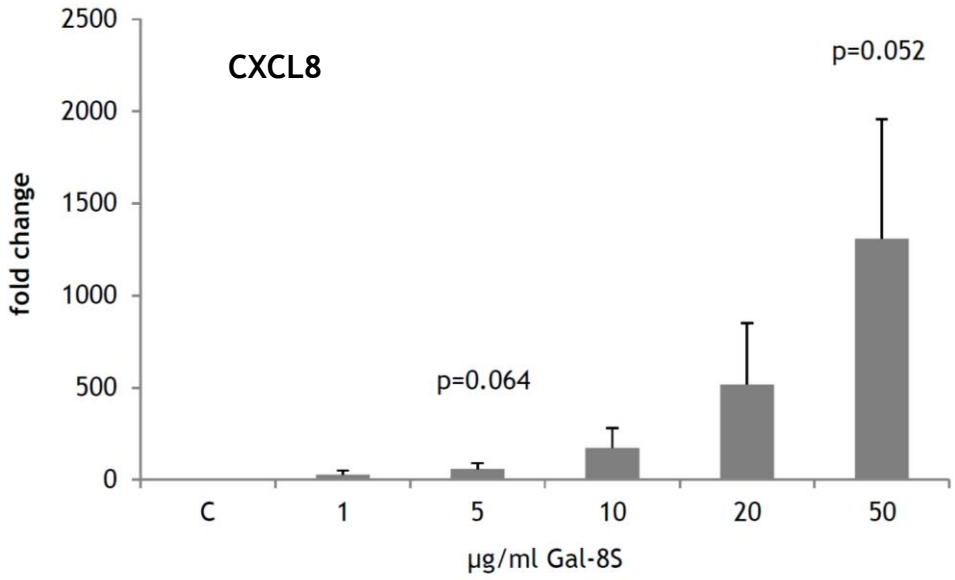

Figure 3.16: Concentration dependent effect of Gal-8S on CXCL8 mRNA levels in OA synoviocytes

Cells (n=3 patients) were treated for 24 hours with different concentrations of Gal-8S (5, 10, 20, 50 µg/ml). As reference, untreated OA synoviocytes (C) were cultured under the same conditions. After incubation, the mRNA levels of CXCL8 were quantified using RT-qPCR with SDHA as reference gene. Statistical analysis of the data revealed no statistical significance ($p > 0.05$, paired t-test). In certain cases, where the evaluation almost reached statistical significance, exact p-values are given.

Figure 3.17 presents the efficacy of Gal-8S and its variants to increase IL1B mRNA levels in OA synoviocytes. As compared to the untreated control, the increase of IL1B was statistically significant in case of 11 µl/ml Gal-8L with a 691-fold (±281, p=0.037) up-regulation. None of the other galectins reached statistical significance compared to the control. Despite the lack of statistically significant differences, a clear tendency towards up-regulated IL1B levels and marked differences between study groups was observed. As compared to the control, Gal-8S had the highest modulating effect with a 792-fold (±440, p=0.063) up-regulation.

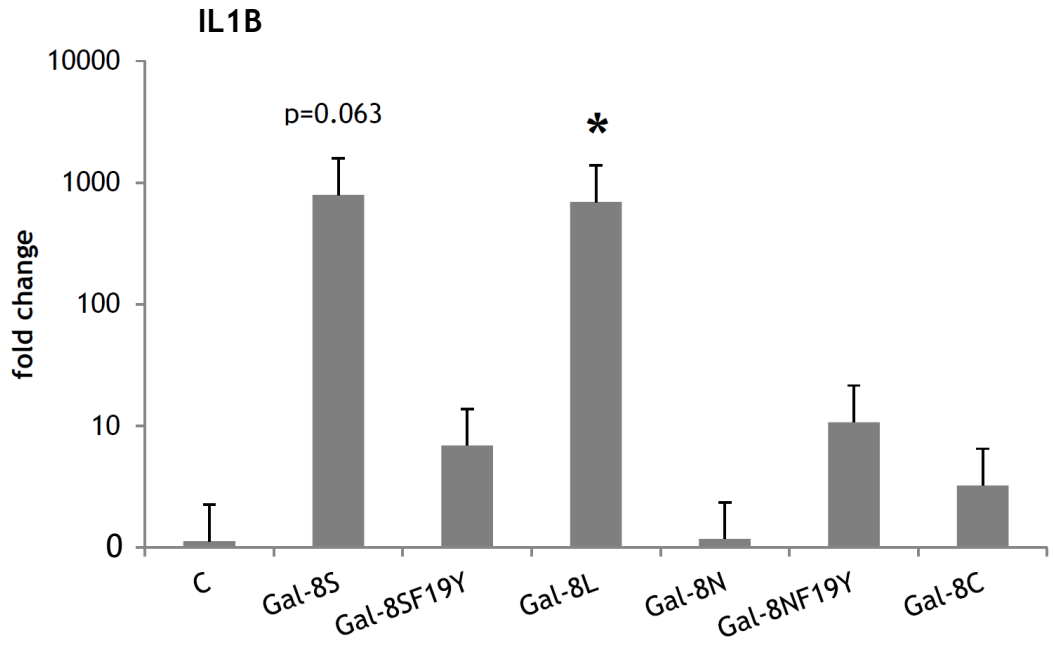

Figure 3.17: IL1B mRNA levels in synoviocytes after treatment with Gal-8S and its variants
Cells (n=3 patients) were treated for 24 hours with 10 µg/ml Gal-8S, 10 µg/ml Gal-8SF19Y, 11 µg/ml Gal-8L, 5 µg/ml Gal-8N, 5 µg/ml Gal-8NF19Y and 4 µg/ml Gal-8C. As reference, untreated OA synoviocytes (C) were cultured under the same conditions. After incubation, the mRNA levels of IL1B were quantified using RT-qPCR with SDHA as reference gene. To improve readability, these data are presented on a logarithmic scale. The asterisks mark statistical differences of IL1B expression levels compared to the control. In certain cases, where the evaluation almost reached statistical significance, exact p-values are given.

Figure 3.18 shows Gal-8S and its variants in their ability to induce CXCL8 mRNA levels in OA synoviocytes. The activities ranged from a minimum of a 1.4-fold (±0.1, p=0.020) up-regulation by Gal-8N to a maximum of a 1431-fold (±1320, p=0.133) up-regulation by Gal-8S. Only Gal-8N reached a statistical significance compared to the untreated control. Despite the lack of statistically significance in most cases, a clear tendency towards up-regulated CXCL8 mRNA levels and marked differences between study groups was observed. The p-values of Gal-8SF19Y (12±6.5) and Gal-8C (7±12.9) almost reached statistical significance.

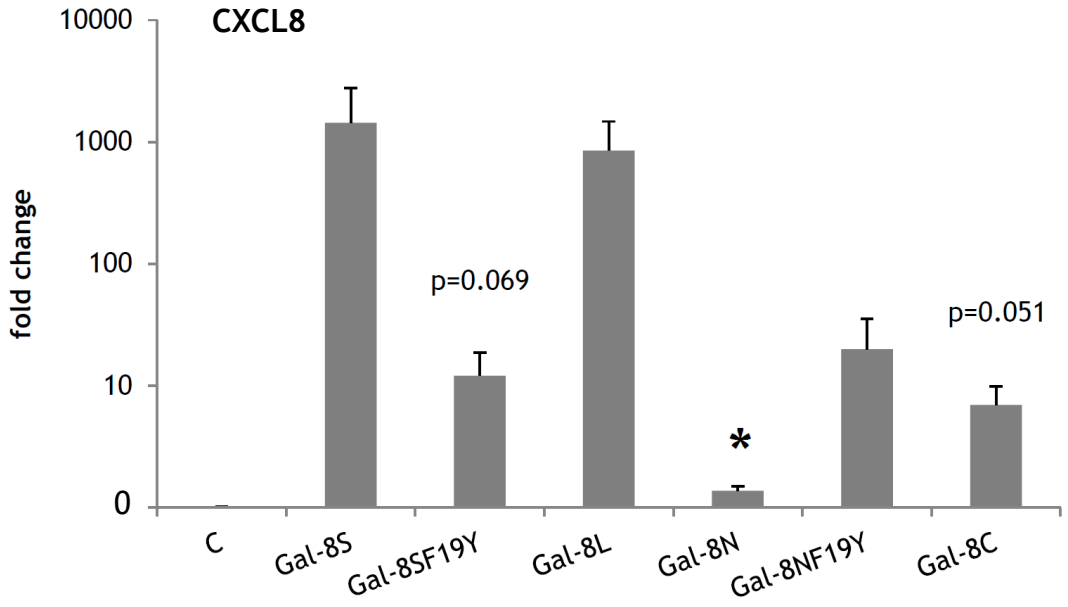

Figure 3.18: CXCL8 mRNA levels in synoviocytes after treatment with Gal-8S and its variants
Cells (n=3 patients) were treated for 24 hours with 10 µg/ml Gal-8S, 10 µg/ml Gal-8SF19Y, 11 µg/ml Gal-8L, 5 µg/ml Gal-8N, 5 µg/ml Gal-8NF19Y and 4 µg/ml Gal-8C. As reference, untreated OA synoviocytes (C) were cultured under the same conditions. After incubation, the mRNA levels of CXCL8 were quantified using RT-qPCR with SDHA as reference gene. The asterisks mark statistical differences of CXCL8 expression levels compared to the control. In certain cases, where the evaluation almost reached statistical significance, exact p-values are given.

4 Discussion

This study was performed to determine the presence of Gal-8 in OA cartilage and to evaluate the effect of Gal-8 on IL1B and CXCL8 gene expression in OA chondrocytes and synoviocytes. Recent studies showed that Gal-1 (Toegel et al., 2016) and Gal-3 (Weinmann et al., 2016) play an important role in the pathophysiology of OA. These presented data gave reason for further work on the galectin network. Thus, the next step was testing cellular effects of other galectins. Gal-8 was selected, since in a previous study it showed high cell positivity in OA cartilage in immunohistochemical detection. There, the Quick score of Gal-8 was calculated and showed a statistically significant difference between mildly and severely degraded cartilage regions (Toegel et al., 2014).

To evaluate the occurrence of Gal-8 in OA cartilage, immunohistochemical analyses of 18 OA patients were performed. We found that Gal-8 presence in OA chondrocytes significantly correlated with the level of cartilage degeneration ($p < 0.0001$). Cartilage areas with moderate degeneration showed low levels of Gal-8 cell positivity, while severely degenerated areas exhibited higher positivity for Gal-8. This might lead to the hypothesis that Gal-8 is an effector in cartilage degeneration. However, compared to a previous study (Toegel et al., 2016), the positivity of OA chondrocytes for Gal-8 was clearly lower than that for Gal-1. However, in comparison, the presence of Gal-8 in OA cartilage was higher than that of Gal-3 (Weinmann et al., 2016).

To verify that Gal-8 and its variants have no cytotoxic effects on chondrocytes at the applied concentrations, the metabolic cell activity was tested following treatment with 5, 10, 20, 50 and 100 µg/ml of the proteins. In summary, all concentrations were well tolerated and showed no significant impairment of metabolic activity, except Gal-8N at a concentration of 100 µg/ml. These data were important to determine the concentration used for RT-qPCR experiments. Therefore, and talking the molecular masses of the galectins into account, the highest used concentrations for ensuring RT-qPCR experiments were set to 50 µg/ml for Gal-8SF19Y, 55 µg/ml for Gal-8L, 25 µg/ml for Gal-8N and Gal-8NF19Y, and 20 µg/ml for Gal-8C. The concentration-dependent experiment of Gal-8S was performed with concentrations up to 100 µg/ml. In OA chondrocytes, a

concentration-dependent treatment with Gal-8S was performed and revealed the highest effect at a concentration 20 µg/ml. This may lead to the hypothesis that at this concentration of Gal-8S the effect is at its peak in OA chondrocytes and that a higher up-regulation of IL1B and CXCL8 at even higher concentrations is not possible. This result is also important for the study design of future co-treatments using mixtures of different galectins, because the concentrations should be applied at a sub-saturating level to enable the detection of differences between the individual and combined galectins. In addition, further analyses such as microarrays might be interesting to identify Gal-8-induced gene expression and associated signalling pathways. Since the maximal effect of Gal-8S occurred at a concentration of 20 µg/ml, a concentration of 50 µg/ml, as recently applied for Gal-1 and Gal-3 (Toegel et al., 2016; Weinmann et al., 2016), might not be useful. In comparison, the dose-dependent experiments in OA synoviocytes showed other findings. The used concentration of Gal-8S ranged from 1 to 50 µg/ml. Although the effect of Gal-8S on the gene expression reached no statistical significance at any concentration, a clear dose-dependent up-regulation was shown as compared to the control. The highest increase of IL1B and CXCL8 was at a concentration of 50 µg/ml Gal-8S and this differed from the effect of Gal-8S in OA chondrocytes, where the maximal effect was at a concentration of 20 µg/ml. Taken together, the suggested pro-inflammatory activity of Gal-8S in OA synoviocytes is of particular interest since Gal-8 was previously found in synovial fluid cells of RA patients and the concentration was high enough to induce apoptosis (Eshkar Sebban et al., 2007).

The impact of Gal-8S in comparison to that of Gal-1 and Gal-3 was shown in further RT-qPCR experiments. Figure 3.9 suggests that Gal-8S up-regulates IL1B mRNA levels in OA chondrocytes to a significantly higher extent than Gal-1 or Gal-3. Figure 3.10 showed the same effect for CXCL8 mRNA levels but the difference was not so clear. Therefore, this finding provides the experimental evidence that Gal-8S up-regulates IL1B and CXCL8 mRNA levels in OA chondrocytes in a numerically higher dimension than Gal-1 or Gal-3.

Further analyses in this diploma thesis were focused on the effect and the comparison of the biochemical variants of Gal-8. OA chondrocytes were treated with equal quantities of Gal-8S, Gal-8SF19Y, Gal-8L, Gal-8N, Gal-8NF19Y and Gal-

8C. The first step was the treatment with concentrations adapted to 10 µg/ml Gal-8S, and in a second experiment the concentrations of the variants were set to correspond to 50 µg/ml Gal-8S. The resulting data, however, were similar in these two experiment approaches.

The variants Gal-8SF19Y, Gal-8N and Gal-8C showed a significant decrease of IL1B and CXCL8 gene expression levels at both concentrations, as compared to Gal-8S. Although Gal-8L and Gal-8NF19Y reached no statistical significance in comparison to Gal-8S, a reduced induction of IL1B and CXCL8 was shown.

Regarding the linker length, it was shown in previous studies that the linker length had an effect on the cross-linking activity of Gal-8. Moreover, it was reported that Gal-8L can sense ligand presence at low concentrations (Zhang et al., 2015). In the present study, the activity of Gal-8L was not statistically significant compared to Gal-8S. This might lead to the hypothesis that the linker length has no significant influence on the effect of Gal-8 in OA chondrocytes and synoviocytes.

In addition, the results of Gal-8N and Gal-8C are of interest. Both separated CRDs are the result of proteolytic cleavage of Gal-8. Gal-8C only consists of the C-terminus and Gal-8N is composed of the N-terminus. Both galectin fragments showed no marked up-regulation of IL1B and CXCL8 gene expression as compared to untreated controls. This suggests that proteolytic cleavage leads to the inactivation of Gal-8, at least regarding its impact on IL1B and CXCL8 transcription. It appears conceivable that this mechanism constitutes a physiologic switch-off for the activity of Gal-8. Future studies are needed to define the physiologic role of this process in the tissue or disease context, and to elaborate any possible consequences for therapeutic applications.

Further analyses focused on the influence of the naturally occurring single nucleotide polymorphism F19Y, compared to the effect of Gal-8S and Gal-8N. This mutation leads to replacement of phenylalanine (F) to tyrosine (Y) at amino acid 19 in exon 1. This single nucleotide polymorphism naturally occurs in RA (Pal et al., 2012) and causes a 50% lower agglutination capacity of the galectin in comparison to the wildtype protein Gal-8S (Zhang et al., 2015). In our study, Gal-8SF19Y showed a significantly reduced induction of IL1B and CXCL8 gene expression levels, as compared to Gal-8S. This data is in agreement with the previous study by Zhang

et al. In comparison with Gal-8N, however, Gal-8NF19Y induced a significant increase of mRNA levels. This means that Gal-8NF19Y shows a higher up-regulation of IL1B and CXCL8 expression, as compared to Gal-8N, while Gal-8SF19Y shows a reduced induction of mRNA levels in comparison to Gal-8S. Gal-8NF19Y even induced a higher up-regulation of mRNA levels of IL1B and CXCL8 than Gal-8SF19Y. Previous studies showed that the human Gal-8 variant with the single nucleotide polymorphism was more active as growth regulator in different types of cancer (Ruiz et al., 2014). Until now, experiments with single nucleotide polymorphism in human Gal-8 were not performed in OA chondrocytes and OA synoviocytes. Thus, further studies are needed to elaborate the relevance of this single nucleotide polymorphism in the context of OA.

Interestingly, the effect of Gal-8 variants in OA chondrocytes was similar to that in OA synoviocytes. Although only a few galectins reached statistical significance, a clear tendency towards up-regulated IL1B and CXCL8 levels and marked differences between study groups were observed. Taken together, the results of Gal-8 variants in OA chondrocytes were reproducible in OA synoviocytes.

The results in this diploma thesis provide the experimental evidence that Gal-8 is an up-regulator of IL1B and CXCL8 mRNA levels in OA chondrocytes and OA synoviocytes. Compared to previous studies (Toegel et al., 2016), Gal-8 seemed to have an even greater influence than Gal-1. Therefore, further studies are justified to obtain more information about the effect of Gal-8. The first step for developing therapeutic targets is to understand the pathways that may promote OA. Therefore, galectin antagonists could be developed as pharmacological treatment in the future. Furthermore, other target genes including other inflammatory cytokines should be analysed. It is also important to know more about the secretion of Gal-8 by OA chondrocytes and to determine the molecular response to carbohydrate-dependent cell surface binding of Gal-8. In this context, the counterreceptors of Gal-8 are yet to be defined in OA chondrocytes and synoviocytes. More experiments, such as microarray and bioinformatic investigations, are needed to identify the Gal-8-induced gene expression and signalling pathways. Regarding OA synoviocytes, further experiments with an increased number of patients are needed to reach statistical significance of the data.

In summary, this diploma thesis established the innovative concept that Gal-8 might be a novel player in inflammatory processes during the progression of OA.

5 Conclusion

The results of this diploma thesis suggest that Gal-8 might have an influence on cartilage degeneration during the progression of OA. First, immunohistochemical experiments revealed that the presence of Gal-8 in OA chondrocytes was significantly correlated with OA severity, suggesting a functional link between Gal-8 and disease manifestation. Furthermore, dose-dependent experiments of Gal-8S showed a high up-regulation of pro-inflammatory markers in OA chondrocytes and synoviocytes, indicating that Gal-8S might be an effector in inflammation and matrix degeneration. In addition, experiments with Gal-8 with two different linker lengths revealed comparable activity towards IL1B and CXCL8 gene expression. This might suggest that the two physiologic Gal-8 isoforms (Gal-8S and Gal-8L) equally contribute to inflammatory processes in OA. In contrast, cleavage of the linker polypeptide resulted in a loss of activity, as shown by the reduced activity of the fragments Gal-8N and Gal-8C. Furthermore, experiments with the single nucleotide polymorphism F19Y that naturally occurs in RA showed a reduced up-regulation of pro-inflammatory markers as compared to the native protein. This suggests that this mutation might have an influence on the expression of pro-inflammatory markers in OA chondrocytes and synoviocytes. Taken together, the results of this study suggest a correlation between OA cartilage degeneration and Gal-8 presence/activity. Moreover, the evaluation of biochemical variants of Gal-8 revealed first insights into the structure function relationship of Gal-8 in OA tissues.

6 References

6.1 Books

Taschenatlas Anatomie, Band 1: Bewegungsapparat, 11. Auflage; Georg Thieme Verlag 2013. Werner Platzer

Prometheus: Lernatlas der Anatomie, Allgemeine Anatomie und Bewegungssystem, 4. Auflage, Georg Thieme Verlag 2014. Schünke M, Schulte E, Schumacher U, Voll M, Wesker K.

6.2 Papers

Abedin MJ, Kashio Y, Seki M, Nakamura K, Hirashima M. Potential roles of galectins in myeloid differentiation into three different lineages. J Leukoc Biol. 2003; 73(5):650-6.

Ahmad N, Gabius HJ, Sabesan S, Oscarson S, Brewer CF. Thermodynamic binding studies of bivalent oligosaccharides to galectin-1, galectin-3, and the carbohydrate recognition domain of galectin-3. Glycobiology. 2004; 14(9):817-25.

Ayral X, Pickering EH, Woodworth TG, Mackillop N, Dougados M. Synovitis: a potential predictive factor of structural progression of medial tibiofemoral knee osteoarthritis– results of a 1-year longitudinal arthroscopic study in 422 patients. Osteoarthritis Cartilage. 2005; 13(5):361-7.

Bellamy N, Campbell J, Robinson V, Gee T, Bourne R, Wells G. Intraarticular corticosteroid for treatment of osteoarthritis of the knee. Cochrane Database Syst. Rev 2005; (2):CD005328.

Bastow ER, Byers S, Golub SB, Clarkin CE, Pitsillides AA, Fosang AJ. Hyaluronan synthesis and degradation in cartilage and bone. Cell Mol Life Sci. 2008; 65(3):395-413.

Benito MJ, Veale DJ, FitzGerald O, Van den Berg WB, Bresnihan B. Synovial tissue inflammation in early and late osteoarthritis. Ann Rheum Dis 2005; 64:1263-1267.

Bennell KL, Hunter DJ, Hinman RS. Mangement of osteoarthritis of the knee. BMJ 2012; 345:e4934.

Bidon-Wagner N, Le Pennec JP. Human galectin-8 isoforms and cancer. Glycoconj J. 2004; 19(7-9):557-63.

Borzì RM, Mazzetti I, Marcu KB, Facchini A. Chemokines in cartilage degradation. Clin Orthop Relat Res. 2004; (427 Suppl):S53-61.

Buckwalter JA, Mow VC, Ratcliffe A. Restoration of injured or degenerated articular cartilage. J Am Acad Orthop Surg. 1994; 2:192-201.

Camby I, Le Mercier M, Lefranc F, Kiss R. Galectin-1: a small protein with major functions. Glycobiology. 2006; 16(11):137R-157R.

Colnot C, Sidhu SS, Balmain N, Poirier F. Uncoupling of chondrocyte death and vascular invasion in mouse galectin 3 null mutant bones. Dev Biol. 2001; 229(1):203-14.

Croci DO, Cerliani JP, Dalotto-Moreno T, Méndez-Huergo SP, Mascanfroni ID, Dergan-Dylon S, Toscano MA, Caramelo JJ, García-Vallejo JJ, Ouyang J, Mesri EA, Junttila MR, Bais C, Shipp MA, Salatino M, Rabinovich GA. Glycosylation-dependent lectin-receptor interactions preserve angiogenesis in anti-VEGF refractory tumors. Cell. 2014; 156(4):744-58.

Dagher SF, Wang JL, Patterson RJ. Identification of galectin-3 as a factor in pre-mRNA splicing. Proc Natl Acad Sci U S A. 1995; 92(4):1213-7.

Delgado VM, Nugnes LG, Colombo LL, Troncoso MF, Fernández MM, Malchiodi EL, Frahm I, Croci DO, Compagno D, Rabinovich GA, Wolfenstein-Todel C, Elola MT. Modulation of endothelial cell migration and angiogenesis: a novel function for the "tandem-repeat" lectin galectin-8. FASEB J. 2011; 25(1):242-54.

Dumic J, Dabelic S, Flögel M. Galectin-3: an open-ended story. Biochim Biophys Acta. 2006; 1760(4):616-35.

Elola MT, Wolfenstein-Todel C, Troncoso MF, Vasta GR, Rabinovich GA. Galectins: matricellular glycan-binding proteins linking cell adhesion, migration, and survival. Cell Mol Life Sci. 2007; 64(13):1679-700.

Eshkar Sebban L, Ronen D, Levartovsky D, Elkayam O, Caspi D, Aamar S, Amital H, Rubinow A, Golan I, Naor D, Zick Y, Golan I. The involvement of CD44 and its novel ligand galectin-8 in apoptotic regulation of autoimmune inflammation. J Immunol. 2007; 179(2):1225-35.

Filer A, Bik M, Parsonage GN, Fitton J, Trebilcock E, Howlett K, Cook M, Raza K, Simmons DL, Thomas AM, Salmon M, Scheel-Toellner D, Lord JM, Rabinovich GA, Buckley CD. Galectin 3 induces a distinctive pattern of cytokine and chemokine production in rheumatoid synovial fibroblasts via selective signaling pathways. Arthritis Rheum. 2009; 60(6):1604-14.

Guermazi A, Niu J, Hayashi D, Roemer FW, Englund M, Neogi T, Aliabadi P, McLennan CE, Felson DT. Prevalence of abnormalities in knees detected by MRI in adults without knee osteoarthritis: population based observational study (Framingham Osteoarthritis Study). BMJ 2012; 345:e5339.

Hill CL, Hunter DJ, Niu J, Clancy M, Guermazi A, Genant H, Gale D, Grainger A, Conaghan P, Felson DT.Synovitis detected on magnetic resonance imaging and ist relation to pain and cartilage loss in knee osteoarthritis. Ann Rheum Dis. 2007; 66(12):1599-603

Hotta K, Funahashi T, Matsukawa Y, Takahashi M, Nishizawa H, Kishida K, Matsuda M, Kuriyama H, Kihara S, Nakamura T, Tochino Y, Bodkin NL, Hansen BC, Matsuzawa Y. Galectin-12, an Adipose-expressed Galectin-like Molecule Possessing Apoptosis-inducing Activity. J Biol Chem. 2001; 276(36):34089-97.

Hirabayashi J, Kasai K. The family of metazoan metal-independent beat-galactoside-binding lectins: Structure, function and molecular evolution. Glycobiology. 1993; 3(4):297-304.

Hirabayashi J, Hashidate T, Arata Y, Nishi N, Nakamura T, Hirashima M, Urashima T, Oka T, Futai M, Muller WE, Yagi F, Kasai K. Oligosaccharide specificity of galectins: a search by frontal affinity chromatography. Biochim Biophys Acta. 2002; 1572(2-3):232-54.

Hunter DJ, Felson DT. Osteoarthrits. BMJ 2006; 332(7542):639-42.

Hunter DJ. Osteoarthrits. Best Pract Res Clin Rheumatol. 2011; 25(6):801-14.

Ideo H, Matsuzaka T, Nonaka T, Seko A, Yamashita K. Galectin-8-N-domain recognition mechanism for sialylated and sulfated glycans. J Biol Chem. 2011; 286(13):11346-55.

Kim HR, Lin HM, Biliran H, Raz A. Cell cycle arrest and inhibition of anoikis by galectin-3 in human breast epithelial cells. Cancer Res. 1999; 59(16):4148-54.

Klyosov AA, Traber PG. Galectins and disease implications for targeted therapeutics. Oxford University Press, USA 2012.

Leffler H, Carlsson S, Hedlund M, Qian Y, Poirier F. Introduction to galectins. Glycoconj J. 2004; 19(7-9):433-40.

Levy Y, Arbel-Goren R, Hadari YR, Eshhar S, Ronen D, Elhanany E, Geiger B, Zick Y. Galectin-8 functions as a matricellular modulator of cell adhesion. J Biol Chem. 2001; 276(33):31285-95

Li S, Yu Y, Koehn CD, Zhang Z, Su K. Galectins in the Pathogenesis of Rheumatoid Arthritis. J Clin Cell Immunol. 2013; 4(5).

Liu FT, Hsu DK, Zuberi RI, Kuwabara I, Chi EY, Henderson WR Jr. Expression and function of galectin-3, a beta-galactoside-binding lectin, in human monocytes and macrophages. Am J Pathol. 1995; 147(4):1016-28.

Liu FT, Rabinovich GA. Galectins as modulators of tumour progression. Nat Rev Cancer. 2005; 5(1):29-41.

Lobsanov YD, Gitt MA, Leffler H, Barondes SH, Rini JM. X-ray crystal structure of the human dimeric S-Lac lectin, L-14-II, in complex with lactose at 2.9-A resolution. J Biol Chem. 1993; 268(36):27034-8.

López-Lucendo MF, Solís D, André S, Hirabayashi J, Kasai K, Kaltner H, Gabius HJ, Romero A. Growth-regulatory human galectin-1: crystallographic characterisation of the structural changes induced by single-site mutations and their impact on the thermodynamics of ligand binding. J Mol Biol. 2004; 343(4):957-70.

Matsukawa A, Yoshimura T, Maeda T, Ohkawara S, Takagi K, Yoshinaga M. Neutrophil accumulation and activation by homologous IL-8 in rabbits. IL-8 induces destruction of cartilage and production of IL-1 and IL-1 receptor antagonist in vivo. J Immunol. 1995; 154(10):5418-25.

Mehrabian M, Gitt MA, Sparkes RS, Leffler H, Barondes SH, Lusis AJ. Two members of the S-lac lectin gene family, LGALS1 and LGALS2, reside in close proximity on human chromosome 22q12-q13. Genomics. 1993; 15(2):418-20.

Nishi N, Itoh A, Shoji H, Miyanaka H, Nakamura T. Galectin-8 and galectin-9 are novel substrates for thrombin. Glycobiology. 2006; 16(11):15C-20C.

Ochieng J, Furtak V, Lukyanov P. Extracellular functions of galectin-3. Glycoconj J. 2004; 19(7-9):527-35.

Ohshima S, Kuchen S, Seemayer CA, Kyburz D, Hirt A, Klinzing S, Michel BA, Gay RE, Liu FT, Gay S, Neidhart M. Galectin 3 and its binding protein in rheumatoid arthritis. Arthritis Rheum. 2003; 48(10):2788-95.

Olson TS, Ley K. Chemokines and chemokine receptors in leukocyte trafficking. Am J Physiol Regul Integr Comp Physiol. 2002; 283(1):R7-28.

Pál Z, Antal P, Srivastava SK, Hullám G, Semsei AF, Gál J, Svébis M, Soós G, Szalai C, André S, Gordeeva E, Nagy G, Kaltner H, Bovin NV, Molnár MJ, Falus A, Gabius HJ, Buzás EI. Non-synonymous single nucleotide polymorphisms in genes for immunoregulatory galectins: association of galectin-8 (F19Y) occurrence with autoimmune diseases in a Caucasian population. Biochim Biophys Acta. 2012; 1820(10):1512-8.

Patterson RJ, Wang W, Wang JL. Understanding the biochemical activities of galectin-1 and galectin-3 in the nucleus. Glycoconj J. 2004; 19(7-9):499-506

Pearle AD, Warren RF, Rodeo SA. Basic science of articular cartilage and osteoarthritis. Clin Sports Med 2005; 24(1):1-12.

Rabinovich GA, Ariel A, Hershkoviz R, Hirabayashi J, Kasai KI, Lider O. Specific inhibition of T-cell adhesion to extracellular matrix and proinflammatory cytokine secretion by human recombinant galectin-1. Immunology. 1999; 97(1):100-6.

Reesink HL, Bonnevie ED, Liu S, Shurer CR, Hollander MJ, Bonassar LJ, Nixon AJ. Galectin-3 Binds to Lubricin and Reinforces the Lubricating Boundary Layer of Articular Cartilage. Sci Rep. 2016; 6:25463.

Rousset F, Hazane-Puch F, Pinosa C, Nguyen MV, Grange L, Soldini A, Rubens-Duval B, Dupuy C, Morel F, Lardy B. IL-1beta mediates MMP secretion and IL-1beta neosynthesis via upregulation of p22(phox) and NOX4 activity in human articular chondrocytes. Osteoarthritis Cartilage. 2015;23(11):1972-80.

Toegel S, Bieder D, André S, Kayser K, Walzer SM, Hobusch G, Windhager R, Gabius HJ. Human osteoarthritic knee cartilage: fingerprinting of adhesion/growth-regulatory galectins in vitro and in situ indicates differential upregulation in severe degeneration. Histochem Cell Biol. 2014; 142(4):373-88.

Toegel S, Weinmann D, André S, Walzer SM, Bilban M, Schmidt S, Chiari C, Windhager R, Krall C, Bennani-Baiti IM, Gabius HJ. Galectin-1 Couples Glycobiology to Inflammation in Osteoarthritis through the Activation of an NF-κB-Regulated Gene Network. J Immunol. 2016; 196(4):1910-21.

Tribulatti MV, Cattaneo V, Hellman U, Mucci J, Campetella O. Galectin-8 provides costimulatory and proliferative signals to T lymphocytes. J Leukoc Biol. 2009; 86(2):371-80.

Troncoso MF, Ferragut F, Bacigalupo ML, Cárdenas Delgado VM, Nugnes LG, Gentilini L, Laderach D, Wolfenstein-Todel C, Compagno D, Rabinovich GA, Elola MT. Galectin-8: a matricellular lectin with key roles in angiogenesis. Glycobiology. 2014; 24(10):907-14.

van der Leij J, van den Berg A, Blokzijl T, Harms G, van Goor H, Zwiers P, van Weeghel R, Poppema S, Visser L. Dimeric galectin-1 induces IL-10 production in T-lymphocytes: an important tool in the regulation of the immune response. J Pathol. 2004; 204(5):511-8.

Weinmann D, Schlangen K, André S, Schmidt S, Walzer SM, Kubista B, Windhager R, Toegel S, Gabius HJ. Galectin-3 induces a pro-degradative/inflammatory gene signature in human chondrocytes, teaming up with galectin-1 in osteoarthritis pathogenesis. Sci. Rep. 2016; 6:391122016,

Wittenauer R, Smith L, Aden K. Priority medicines for Europe and the world "A public health approach to innovation" (Backround paper 6.12 osteoarthritis). WHO Department, 2013. Available at: http://www.who.int/medicines/areas/priority_medicines/BP6_12Osteo.pdf

Woolf AD, Pfleger B. Burden of major musculoskeletal conditions. Bull World Health Organ 2003; 81(9):646-56.

Yang RY, Rabinovich GA, Liu FT. Galectins: structure, function and therapeutic potential. Expert Rev Mol Med. 2008; 10:e17.

Yoshii T, Inohara H, Takenaka Y, Honjo Y, Akahani S, Nomura T, Raz A, Kubo T. Galectin-3 maintains the transformed phenotype of thyroid papillary carcinoma cells. Int J Oncol. 2001; 18(4):787-92.

Zhang S, Moussodia RO, Vértesy S, André S, Klein ML, Gabius HJ, Percec V. Unraveling functional significance of natural variations of a human galectin by glycodendrimersomes with programmable glycan surface. Proc Natl Acad Sci U S A. 2015; 112(18):5585-90.

7 Appendix

7.1 Abbreviations

C	control
CRD	carbohydrate recognition domain
CXCL8	interleukin-8 gene
DMEM	Dulbecco´s modified eagle medium
ECM	extracellular matrix
EZ4U	easy for you
FBS	fetal bovine serum
Gal-1	Galectin-1
Gal-3	Galectin-3
Gal-8	Galectin-8
IL1B	interleukin-1 beta gene
kDa	kilo Dalton
MMP	matrix metalloproteinase
NFκB	nuclear factor-κB
NSAID	non-steroidal anti-inflammatory drug
OA	osteoarthritis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
Penstrep	penicillin/streptomycin
RA	rheumatoid arthritis

RT	reverse transcription; real time
RT-qPCR	quantitative real time PCR
SDHA	succinate dehydrogenase A
TNF	tumor necrosis factor

7.2 List of figures

7.3 List of tables