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Abstract

Osteoarthritis (OA) is a degenerative joint disease characterized by degradation of cartilage tissue, secondary bone lesion and inflammation of the joint capsule. Due to their high prevalence, degenerative joint diseases are of great socio-economic importance. However, no successful treatment has been found yet. Therefore, the importance to investigate the pathogenesis of OA has been recognized. Galectins are a family of proteins that specifically bind to β-galactoside sugars and have at least one carbohydrate recognition domain (CRD). They act as modulators of cell behaviour via regulation of signalling processes, inflammation and immune responses. Recent studies found that Galectin-1, -3 and -8 play a crucial role in the pathological mechanisms of OA. The aim of this thesis was to investigate the activity of Galectin-4 in OA chondrocytes. Immunohistochemical analysis of cartilage from 19 OA patients revealed that severely damaged cartilage shows a higher percentage of Galectin-4 positive cells than less damaged cartilage. A correlation between degeneration of cartilage and the increasing percentage of Galectin-4-positive chondrocytes was found. Lactose is known to bind galectins in vitro, therefore we tested the effects of lactose and Galectin-4 on OA chondrocytes and demonstrated its galectin inhibiting effects. Galectin-4 needs to bind to the cell surface to influence gene expression, since adding lactose inhibits this binding as demonstrated by confocal laser scanning microscopy. We also tested the impact of Galectin-4 and its biochemically altered variants on gene expression (IL1B and MMP13) in OA chondrocytes. Dose dependent experiments were conducted, and the results showed an upregulation of MMP13 and IL1B on mRNA levels, which backs the theory that Galectin-4 plays a role in joint inflammation. The altered variants with only one CRD showed increased effects on mRNA levels as compared to wild type Galectin-4. Shortening or omitting the linker had an impact on gene expression, which was comparable to that wild type Galectin-4. To test if Galectin-4 activates the NF-κB-pathway, experiments with Galectin-4 and specific inhibitors of NF-κB-effectors were conducted. The inhibitors attenuated the effect of Galectin-4 on gene expression of OA chondrocytes. Taking together, this thesis identified a correlation between OA cartilage degeneration and Galectin-4 positivity of chondrocytes, and described the effects of Galectin-4 on marker genes in isolated articular chondrocytes.

Zusammenfassung

Arthrose ist eine degenerative Gelenkerkrankung, die durch Abbau von Knorpelgewebe, sekundäre Knochenläsionen und Entzündungen der Gelenkkapsel gekennzeichnet ist. Degenerative Gelenkerkrankungen sind von großer sozioökonomischer Bedeutung. Aufgrund ihrer Häufigkeit nimmt die Behandlung von Arthrose einen wichtigen Stellenwert im Gesundheitssystem ein. Jedoch wurde noch keine erfolgreiche Behandlung gefunden, weshalb die Untersuchung der Pathogenese von Arthrose von hoher Bedeutung ist.

Galektine stellen eine Proteinfamilie dar, deren Vertreter spezifisch an β-Galaktosid-Zucker binden und mindestens eine Kohlenhydrat-Erkennungsdomäne (CRD) aufweisen. Sie wirken als Modulatoren des Zellverhaltens über die Regulation von Signalprozessen, Entzündungen und Immunantworten. Jüngste Studien ergaben, dass die Galektine 1, 3 und 8 eine entscheidende Rolle bei den pathologischen Mechanismen der Arthrose zu spielen scheinen. Ziel dieser Arbeit war es, die Aktivität von Galektin-4 in arthrotischen Chondrozyten zu untersuchen. Eine immunhistochemische Analyse des Knorpels von 19 Arthrose-Patienten ergab, dass der Anteil an Galektin-4-positiven Zellen bei stark geschädigtem Knorpel höher ist als bei weniger geschädigtem Knorpel. Es wurde eine Korrelation zwischen der Degeneration des Knorpels und dem zunehmenden Anteil an Galektin-4-positiven Chondrozyten gefunden. Es ist bekannt, dass Laktose die Wirkung von Galektinen beeinträchtigt. Daher haben wir die Wirkung von Laktose und Galektin-4 auf arthrotische Chondrozyten getestet und dessen galektin-hemmende Wirkung nachgewiesen. Galektin-4 muss an die Zelloberfläche binden, um die Genexpression zu beeinflussen. Die Zugabe von Laktose hemmt diese Bindung. Die Bindungshemmung ist mittels konfokaler Laserscanmikroskopie sichtbar gemacht worden. Wir haben auch den Einfluss von Galektin-4 und seiner biochemisch veränderten Varianten auf die Genexpression (IL1B und MMP13) in arthrotischen Chondrozyten getestet. Dosisabhängige Experimente wurden durchgeführt und die Ergebnisse zeigten eine verstärkte Expression der Gene MMP13 und IL1B auf mRNA-Ebene. Dies stützt die Theorie, dass Galektin-4 eine Rolle bei Gelenkentzündungen spielt.

Die Varianten mit nur einer CRD hatten einen noch stärkeren Einfluss auf die Genexpression als Wildtyp-Galektin-4. Das Verkürzen oder Weglassen des Linkers hatte jedoch einen

vergleichbaren Effekt. Um zu testen, ob Galektin-4 den NF-κB-Signalweg aktiviert, wurden Experimente mit Galektin-4 und bestimmten Inhibitoren von NF-κB-Effektoren durchgeführt. Die Inhibitoren schwächten die Wirkung von Galektin-4 auf die Genexpression von arthrotischen Chondrozyten ab. Zusammenfassend konnte diese Arbeit eine Korrelation zwischen der Degeneration des arthrotischen Knorpels und dem Auftreten von Galektin-4 in arthrotischen Chondrozyten zeigen, sowie den Effekt von Galektin-4 auf die Expression von Markergenen in vitro beschreiben.

1. Introduction

1.1. The human knee joint

1.1.1. Anatomy

The knee joint is the largest joint in the human body. It is a diathrodial joint, because it consists of two single joints, the kneecap joint (articulatio femoropatellaris), which is between femur and patella, and the femorotibial joint (articulatio femorotibialis), which lies between femur and caput tibiae. Articular surfaces are covered with hyaline articular cartilage which, makes certain that the joint surfaces can slide easily over each other. To resist pressure, the knee joint is supported by the medial and lateral meniscus (as seen in Figure 1.1). They consist of fibrous cartilage. The meniscus absorbs shocks between the femur and the tibia and evens out the uneven articular surfaces of femur and tibia. The meniscus medialis has a half-moon shape and is connected with the ligamentum collaterale tibiale. The meniscus lateralis has a nearly cricoid shape and is more flexible than the meniscus medialis, which is the reason why it is less affected from injuries. An articular capsule surrounds the articular cavities of both joints. It consists of two different tissues, the membrana synovialis, which is responsible for synovial fluid production, and the membrana fibrosa. The synovial tissue reduces friction in the joint, is responsible for nutrition and has a shock absorbing function. The synovial fluid consists of lubricin, proteinases, collagenases and hyaluronic acid. The knee joint is accompanied by a large number of bursae, which work like a cushion between the bones, tendons and muscles. The whole joint is stabilized by the anterior and posterior cruciate ligament, which limit certain movements, but also allow a big range of motion. The anterior cruciate ligament is found in the front part, the posterior in the back part of the knee. The stability of the knee is secured by these ligaments with the assistance of menisci and muscles. In the knee joint, flexion (bending) and extension (straightening) can be performed around approximately transversal axes. In flexed position a rotation around the lower leg axis is possible. When the knee is bent, the collateral ligaments (lateral and medial) are completely relaxed, at the same time the cruciate ligaments (anterior and posterior) are tensioned. When the knee is stretched, both collateral ligaments and the anterior cruciate ligament are stretched. During rotation, the

femur and the menisci move on the tibia, while on flexion and extension, the femur moves with rolling movements on the menisci.

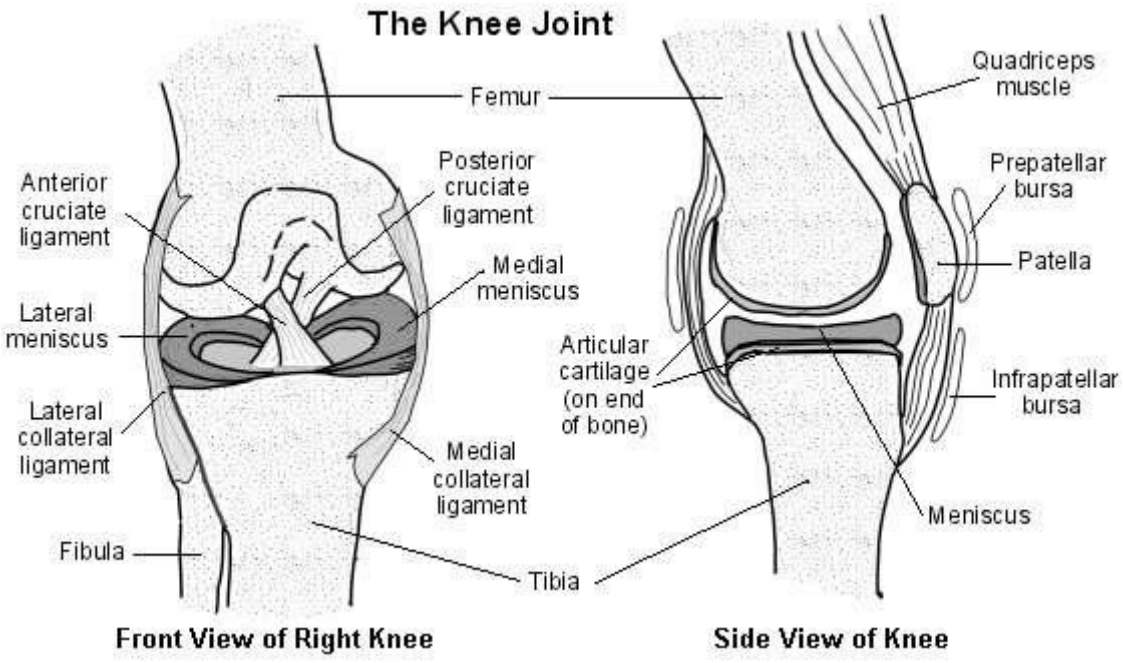

Figure 1.1: Anatomy of the knee joint. https://www.slideshare.net/ariffmahdzub/anatomy-of-knee-joint

1.1.2. Articular cartilage

Most of articular cartilage consists of hyaline cartilage, which is slightly bluish and milky and contains abundant collagenous. The collagenous fibrils always run in the direction of the greatest stress in the articular cartilage. The cells (chondrocytes) in the cartilage cavities are surrounded by a capsule, which is closed off against the rest of the intermediate tissue. The chondrocytes, which can be arranged more or less in rows or columns, form a chondron. The cells within a chondron are organized in complexes. The cells in those complexes are always multiple daughter cells created from one chondrocyte. Externally, the cartilage is covered with cartilaginous skin, the perichondrium, which more or less continuously passes into the cartilage. Chondrocytes are surrounded by extracellular matrix (ECM), which consists of large aggregates of proteoglycans, type II collagene and water. The ECM has two phases, a solid phase on the one side, which is composed of proteoglycans and collagens, and a fluid phase on the other side. Aggrecan, which is built by a large protein core and many chondroitin

sulfate and keratin sulfate chains, is the largest proteoglycan that can be found in hyaline cartilage. Hyaluronic acid and aggrecan interact via a link protein to form proteoglycan aggregates. Its main function is to bind water, which results in a hydrated gel structure, which is necessary for compressive resistance. Pressure-bearing hyaline cartilage (lower extremity joint surfaces) contains more glycosaminoglycans (chondroitin sulfate) than lesser burdened hyaline cartilage (e.g. articular surfaces at upper extremity). Due to the lack of vascularity of cartilage, degenerative processes can be favored in its interior. These are induced by "unmasking" of the collagenous fibers, which means that the collagen fibrils become visible in the microscope. As the content of water and chondroitin-sulphates decreases with age, the hyaline (joint) cartilage loses its load capacity.

As seen in Figure 1.2, cartilage can be classified into four zones:

- the superficial zone
- the mid zone
- the deep zone and
- the calcified cartilage zone

Each zone differs in structure and function. The thinnest part of the cartilage is the superficial zone. It is responsible for a surface that can glide smoothly. A high number of chondrocytes can be found in this area. Collagen fibers lie parallel to the articular surface. In Osteoarthritis (OA) the superficial zone is affected first. 60 % of the cartilage consists of the mid zone. Mid zone chondrocytes are differently arranged than the chondrocytes in the superficial zone. They are round and appear in much smaller numbers. The collagen fibrils are thicker and diagonally organized to the articular surface. In the deep zone, the collagen fibrils are organized orthogonal to the articular surface. The chondrocytes are ordered in rows parallel to the collagen fibers. In this zone the highest concentration of proteoglycans can be found, therefore it is important for resisting compressive forces. Beneath the deep zone, the tidemark can be found, which separates it from the calcified cartilage, which is the connection between cartilage and bone.

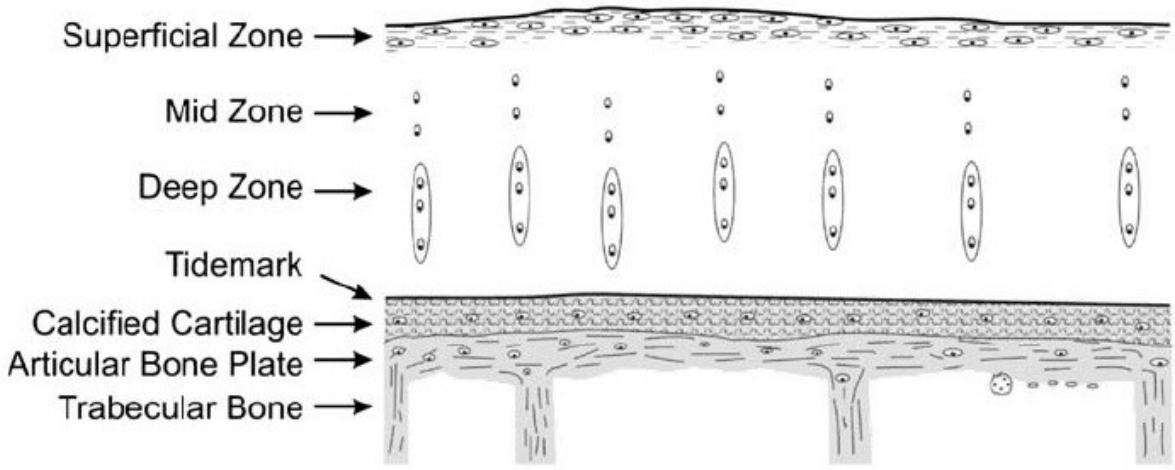

Figure 1.2: Articular cartilage (Osteoarthritis cartilage histopathology: grading and staging. Pritzker et al.; 2006)

1.2. Osteoarthritis

1.2.1. Definition

OA is a degenerative joint disease characterized by degradation of cartilage tissue, secondary bone lesion and inflammation of the joint capsule. The morphological changes are not always accompanied by corresponding clinical symptoms. Degenerative joint diseases are of great socio-economic importance. Due to its frequency, OA and its treatment occupy a central position. Every living human is able to develop OA, and in a lot of cases they do.
OA usually develops from a mismatch between load and load capacity of the joint. Immobilization also might promote the development of osteoarthritis through the reduction of joint metabolism. In case of primary OA there is an inferiority of the cartilaginous tissue, the cause of which is not clarified in detail. Secondary OA can develop on the basis of metabolic disorders, stress, trauma, inflammation or can be age-related.
The course of osteoarthritis is slowly progressive. At the beginning there is the loss of elasticity of the cartilage tissue with a change in the cartilage substance and unmasking of collagenous fibers.
First morphological changes (visible on X-rays) are the decrease in the level of cartilage tissue, the gap formation in the articular cartilage and the subchondral sclerosis of the articular surface. The cartilage damage causes shear forces on the edges of the articular surfaces, which lead to the formation of bulging bony prominences (Osteophytes). At this stage the deformation of the joint begins.
With progressive abrasion of the articular cartilage, the force-absorbing surface in the joint becomes smaller and smaller. Beneath the most stressed areas of the articular surface, the bone regresses. There are numerous small and large cysts. Reparative processes in the form of osteophyte formation and regressive processes in the form of bone abrasion and cyst formation can balance each other over many years, with less clinically conspicuous symptoms.
If the otherwise gradual course of the pathological-morphological changes accelerates, OA becomes clinically relevant. This may be due to an increased accumulation of cartilage abrasion products with reactive inflammation of the synovial membrane or else by concomitant subchondral bone necrosis (subchondral cysts). The pathogenesis of

degenerative changes is similar in all synovial joints.

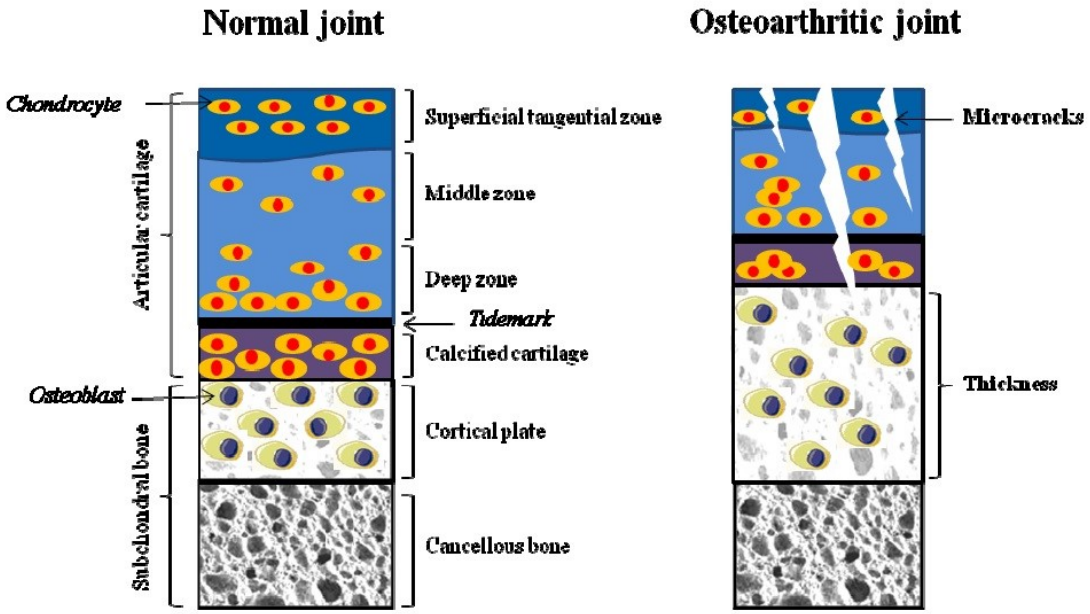

Figure1.3: Anatomy of normal and osteoarthritic cartilage (Quelle: Interplay between Cartilage and Subchondral Bone Contributing to Pathogenesis of Osteoarthritis, Sharma et al 2013)

1.2.2. Therapy

The prevention of degenerative joint disease plays an increasing role. Lack of exercise and obesity are two major risk factors for degenerative joint disease and must be considered as preventive measures. Important is the early detection of congenital or acquired deformities of the joints, which affect the joint functionality adversely. They are the only causes of OA that can usually be causally addressed by surgical measures. Otherwise, the treatment of osteoarthritis is symptomatic and tries to fight the main symptoms of pain, swelling and restricted mobility. What is important is an exact determination of the type and location of the ongoing degenerative changes. Thereafter, a treatment program may be established including mobilization, physical and physiotherapy, use of orthotics, non-steroidal and steroidal anti-inflammatory drug therapy, and possibly also surgical procedures.

In joint surgery, the body´s own tissue is used as an interpositum for destroyed joint surfaces through various surgical techniques. Of importance is, for example, the joint surgery in the hallux valgus and at the hip.

Joint-stiffening measures on the hip joint and knee joint have largely been pushed to the background by the success of endoprosthetic surgery. However, with painful OA in the area of the foot, the ankle, the wrist and finger joints and, above all, the spine, they are still of essential importance.

Joint replacement is used in almost all major limb joints - especially the hip and knee joint. In cases of primary OA, the indication for artificial joint replacement is usually given above the age of 60, but also in the case of secondary and advanced OA, as the durability of joint prostheses has improved considerably as a result of technological progress.

1.3. Glycobiology

Glycobiology covers the biosynthesis and function of saccharides (sugar chains or glycans). They induce a wide range of biological processes and are studied in a medical, biochemical and biotechnical context. Carbohydrates are divided into simple sugars (monosaccharides), double sugars (disaccharides), multiple sugars (oligosaccharides) and polysaccharides. Molecules composed of sugar structures and other components are called glycoconjugates. Proteins modified with sugar structures are referred to as glycoproteins (glycosylated proteins), fat molecules with attached sugar moieties are glycolipids. Glycoconjugates as well as molecules that recognize sugar building blocks and trigger biochemical reactions (eg. antibodies, enzymes, lectins) are of particular interest in glycobiology. Glycoconjugates are found mainly on the surface of the membrane and in cell secretion, they play an important role in cell-cell and cell-matrix interactions.

“Several human disease states are characterized by changes in glycan biosynthesis that can be of diagnostic and/or therapeutic significance.” (Varki et al., 2017).

1.4. Galectins

1.4.1. General aspects

“Lectins are proteins that bind to specific carbohydrate structures and can thus recognize particular glycoconjugates among the vast array expressed in animal tissues. Most animal lectins can be classified into four distinct families: C-type lectins (including the selectins), P-type lectins, pentraxins, and galectins, formerly known as S-type or S-Lac lectins.” (Barondes et al.; 1994)

Galectins are glycan-binding proteins that are characterized by their affinity for β-galactosides and the existence of at least one structurally conserved carbohydrate recognition domains (CRDs). Fifteen members of the galectin family can be found in vertebrates, they show diversity in ligand specificity and can be found inside and outside of the cell. It was shown that galectins work as modulators of cellular activity by influencing signaling processes, inflammation and immune responses. Therefore, galectins might be used as diagnostic markers or as targets for novel drugs in a number of human diseases. (Rustiguel et al.; 2016)

The nomenclature of the formerly known s-type lectins was reorganized in 1994. Afterwards, the first found galectin was renamed as Galectin-1 and all the other galectins, which were discovered later, were numbered consecutively.

Galectins are divided into 3 major groups, based on their organization and number of CRDs. The prototypical galectins (Galectin-1, -2, -7, -10, -11, -13, -14, and -15) have one CRD and are able to form homodimers. The chimera-type galectin (Galectin-3) also has one CRD but forms oligomers with its proline, tyrosine and glycine rich polypeptide tail.

The tandem-repeat galectins (Galectin-4, -8, -9, and -12) consist of 2 CRDs which are connected by a peptide linker, which can differ in its length. (Varki et al.; 2017) Those three types are shown in Figure 1.4.

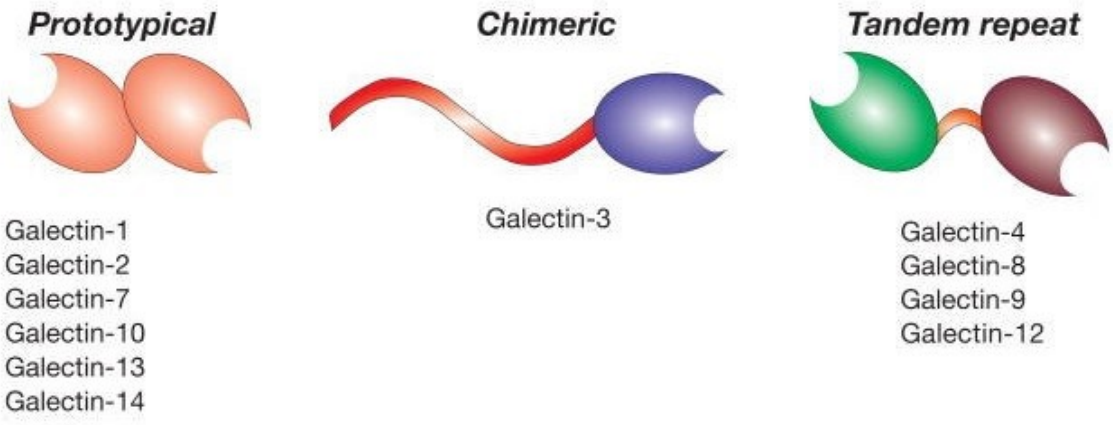

Figure 1.4: The three different types of galectins. (Varki et al.; 2017, chapter 36, figure 33.1)

Galectins essentially bind to glycans which contain galactose and galactose-derivatives. However, for significantly strong binding, they are likely to require N-acetyllactosamine or lactose. In general, if the saccharide chain is longer, the interaction with the galectin is stronger. Numerous factors determine the strength of the ligand binding: The variety of ligands and the galectins, the length of the carbohydrate and the mode of presentation of ligand to the CRD.

Depending on the tissue where they are expressed and their function, different galectins have different binding specificities for oligosaccharides, but galactose is always essential for binding.

Experiments have shown that binding between galectins and N-acetyllactosamine occur due to hydrogen bonds from the C4 and the C6 hydroxyl groups of galactose and C3 of N-acetylglucosamine to amino acid side chains of proteins. Binding to other sugars is not possible, because they wouldn´t fit inside the CRD. Galectins can bind internal or terminal sugars within a glycan, which makes bridging possible between two ligands on one cell or two ligands on different cells. (Varki et al.; 2017,chapter 9)

1.4.2. Galectin-1

Galectin-1 is a human protein that is 135 amino acids long and is encoded by the LGALS1 gene, which can be found on chromosome 22. It was the first discovered member of the galectin family. It belongs to the prototypical subfamily of galectins, consists of a monomer and a CRD, and is able to bind glycans as a monomer or a homodimer. Galectin-1 can be found inside and outside cells and has intracellular and extracellular effects.

The characteristics of Galectin-1 are typical for cytoplasmatic proteins. It has an acetylated N-terminus and lack of glycosylation and can be found in the cell nuclei, cytosols and on the intracellular surface of cell membranes. Even though Galectin-1 does not possess recognizable secretion signal sequences and does not go through the ER/Golgi pathway it still can be found on the extracellular side of cell membranes. It also was detected in the extracellular matrix of several normal and neoplastic tissue. Galectin-1 is important for neuronal cell differentiation and the persistence of the peripheral and the central nervous system. It also plays a vital role in the establishment and preservation of T-cell tolerance and homeostasis in vivo.

As Galectin-1 overexpression occurs in tumors and their surrounding tissue, it can be seen as a sign of their malignant progression, which is linked to metastasis and spreading of tumor cells in normal tissue. A poor prognosis and increased Galectin-1 expression are going hand in hand in large numbers of cancer patients.

As the overexpression of Galectin-1 can be found in some neurodegenerative pathologies, muscular dystrophies and various inflammation-related diseases, like Graft-versus-Host-Disease, arthritis, colitis, OA and nephritis, Galectin-1 itself could be a target for novel treatments. The inhibition of Galectin-1 overexpression could be an approach to fight the progression of cancer. (Camby et al.; 2006*)*

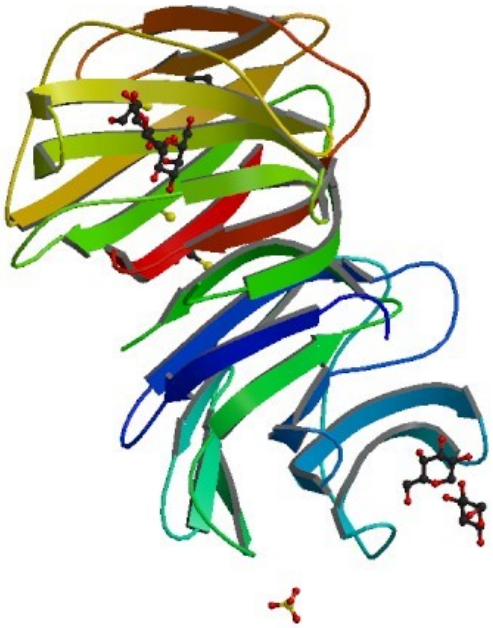

Figure 1.5: X-ray crystal structure of Galectin-1. (http://www.rcsb.org/structure/1GZW)

1.4.3. Galectin-3

Galectin-3 is a 29-35 kDa molecule of chimeric type. It consists of an N-terminal CRD with an attached glycine, proline and tyrosine rich peptide tail. “The repetitive domain is similar to ones found in certain other proteins (such as synexin and synaptophysin) where it is proposed to function in self-aggregation. Indeed, this seems to be the case for Galectin-3, which forms multimers and shows positive cooperativity in binding to immobilized ligand clusters.” (Cooper et al.; 2002) Only one gene, LGALS3, in the human genome codes Galectin—3. It is located on chromosome 14, locus q21-22. (Dumic et al.; 2006)
Galectin-3 will form oligomers, which occurs through its N-terminal domain, when it binds to multivalent carbohydrates. This means that oligosaccharides on the cell surface can be crosslinked, which can induce cell signaling.
Comparable to the other members of the galectin family, Galectin-3 is a soluble protein that can be found on the inside of cells, in the cell nucleus and the cytoplasm, or outside of the cell on the surface or in the matrix, depending on the type and differentiation stage of the cell. Galectin-3 does not carry a signal sequence and therefore is not secreted via the Golgi pathway. (Colnot et al.; 2001) "Its role outside the cell has been linked to modulation of cell adhesion in organogenesis, in the immune system, and during tumorigenesis. Inside the cell, Galectin-3 has also been implicated in splicing of mRNA and apoptosis of T-cells and tumor cells.” (Colnot et al.; 2001)
“Although it was found in many normal tissues, Galectin-3 expression in adults, similarly to its expression during embryogenesis, is mainly related to the epithelial cells and myeloid/amoeboid cells.” (Dumic et al.; 2006) It was found in cells of the small intestine epithelium, in limited areas of olfactory epithelium, also in epithelial cells of lung, kidney, thymus, prostate and breast. Galectin-3 was also found in ductal cells of salivary glands, pancreas, kidney, and eye and in intrahepatic bile ducts. It has been demonstrated, that Galectin-3 was expressed in uterine epithelia of pregnant animals shortly after the implantation, unlike in non-pregnant animals. (Dumic et al.; 2006)
Further, Galectin-3 was detected in chondrocytes, osteoblasts, fibroblasts, osteoclasts, Schwann cells, keratinocytes and gastric mucosa as well as in endothelial cells from numerous tissues and organs. Some experiments proved that Galectin-3 is expressed in cells which are involved in the immune response, like neutrophils, basophils, eosinophils,

Langerhans cells, mast cells dendritic cells, monocytes and macrophages. But there are also cells which usually do not express Galectin-3, but the expression can be induced by various stimuli. (Dumic et al.; 2006). It can also be found in different kinds of tumors.

“The expression intensity depends on the tumor progression, invasiveness and the metastatic potential. Galectin-3 was found to be involved in many biological processes, such as cell–cell and cell–extracellular matrix adhesion, cell growth and differentiation, cell-cycle, signalling, and apoptosis, as well as in angiogenesis. Consequently, Galectin-3 is involved in regulation of development, immune reactions, tumorigenesis, and tumor growth and metastasis.” (Dumic et al.; 2006).

Galectin-3 has different effect on apoptosis, depending on the localisation: cytoplasmic galectin-3 has anti-apoptotic and nuclear galectin-3 pro-apoptotic effects. Galectin-3 also plays a role in OA. It crosslinks lubricin which induces lubrication of the cartilage. (Reesink et al.; 2016) In a mouse model, Galectin-3 was injected to a knee joint which led to OA-like lesions. (Janelle-Montcalm et al.; 2007)

Interestingly, the serum and synovial fluid levels of galectin-3 comparable in and RA patients. Galectin-3 induces MMPs and pro-inflammatory cytokines, which makes it a broad-spectrum upstream effector of OA. A significant correlation between Galectin-3 cell positivity and the stage of cartilage degeneration was found. Galectin-1 and -3 have similar activation profiles and cooperate in cartilage degeneration in OA, which leads to the assumption that a network of lectins may work as a master regulator in OA. (Weinmann et al.; 2016)

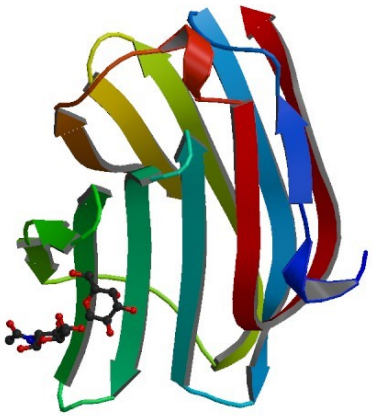

Figure 1.6: X-ray crystal structure of Galectin-3 (http://www.rcsb.org/structure/1A3K)

1.4.4. Galectin-8

Galectin-8, a tandem-repeat galectin, is a 34-kDa protein, which is expressed in a wide range of cells. It consists of two CRDs, connected by a short (26 amino acids) linking peptide. (Levy et al.; 2001) Galectin-8 is unique among the galectins, as many forms are encoded by the same gene, i.e. LGALS8. Six isoforms of the mature protein, harbouring either one or two CRDs, seem to be encoded by only one gene as a result of alternate splicing. (Bidon-Wagner et al.; 2002).

In tumor-associated cells of patients suffering from breast or prostate cancer, Galectin-8 was found in intracellular fluid and in the cell nucleus. Furthermore, a big amount of Galectin-8 is expressed in in the cytoplasm of human primary dermal lymphatic endothelial cells. (Troncoso et al., 2014)

"Galectin-8 mRNA and protein levels were significantly higher in lymphatic endothelial cells than in human primary dermal vascular endothelial cells. Collectively, these results suggest that Galectin-8 expression and localization control the biology of blood and lymphatic vessels. Galectin-8 promotes angiogenesis in vivo in a dose-dependent manner." (Troncoso et al., 2014) It behaves like an extracellular matrix protein which positively or negatively regulates cell adhesion, depending on the cell surface counterreceptors like integrins as well as on the extracellular context. (Levy et al.; 2001) Galectin-8 has a pro-apoptotic effect on several tumor cells.

The expression levels of Galectin-8 might correlate with the malignancy of some cancerous diseases. (Troncoso et al.; 2014) In OA patients, an association between Galectin-8 positive chondrocytes and cartilage degeneration was demonstrated. (Toegel et al.; 2014; Weinmann et al.; 2018).

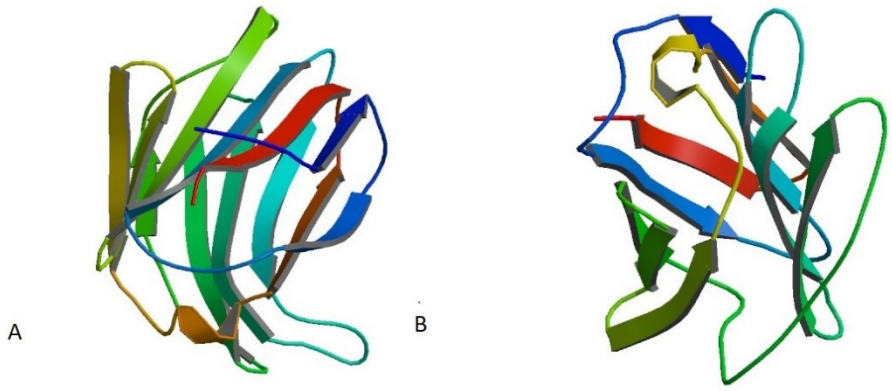

Figure 1.7: A: N-terminal domain of Galectin-8 (http://www.rcsb.org/structure/3VKN); B: C-terminal domain of Galecctin-8 (http://www.rcsb.org/structure/3OJB)

1.4.5. Galectin-4

Human Galectin-4, a tandem-repeat galectin, consists of 2 CRDs, one can be found N-terminal and the other one C-terminal, shown in Figure 1.7. Both CRDs are connected by a 42 amino acid long linker, which is rich in Proline and Glycine. The molecular weight of both CRDs is about 16-17 kDa. Due to its ability to link two distinct types of ligands, Galectin-4 might be a natural crosslinker. (Cao et al.; 2016)

“The carbohydrate binding specificities of the two CRDs are quite different and would be expected to show preference for different sets of ligands. The two CRDs in Galectin-4 bind lactose with similar affinity, but their preferences for other glycosphingolipids, oligosaccharides, and glycoprotein are distinctly different.” (Cao et al.; 2016)

Human LGALS4, which is located on chromosome 19 q13.1-13.3, gene codes Galectin-4. (Cao et al.; 2016)

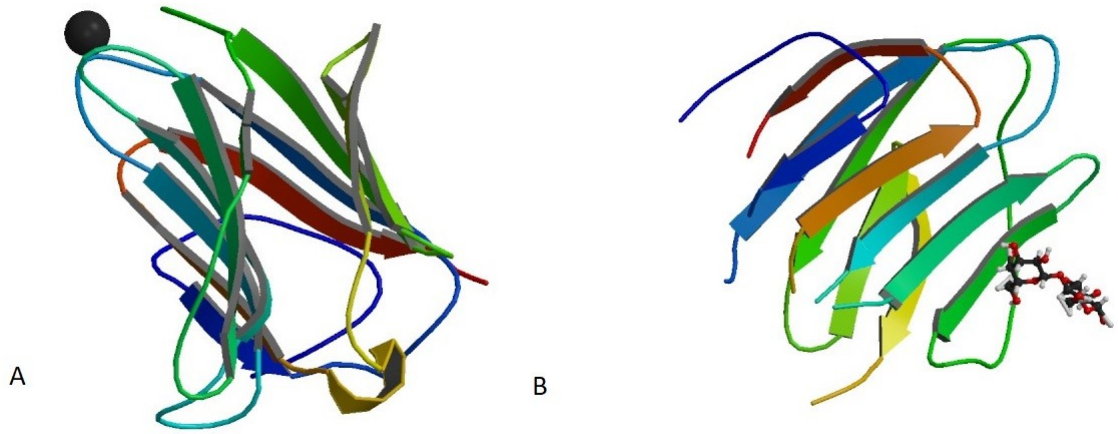

Figure 1.8: A: N-terminal domain of Galectin-4 (http://www.rcsb.org/structure/4XZP) B: C-terminal domain of Galectin-4 (http://www.rcsb.org/structure/5CBL)

Galectin-4 can be found on the cell surface, in circulation and intracellularly. In vitro experiments have shown, that Galectin-4 regulates cell proliferation, apoptosis and differentiation and mediates intercellular adhesion (Huflejt et al., 1997; Huflejt and Leffler, 2003). “Because of no signal sequence for endoplasmatic reticulum transport, the presence of galectin-4 on the cell surface is a consequence of secretion via nonclassical pathway.” (Cao et al.; 2016) In the small intestine Galectin-4 works as an organizer within the microvillar lipid rafts. It crosslinks glycoproteins and brush border proteins on the cell

surface and forms clusters and lattices, which protects them from being solubilized. (Danielsen et al.; 2008)

Galectin-4 possesses bactericidal activity against bacteria that express blood group antigens. The CRD2 can bin to blood group B carbohydrates which some bacteria express on the side chains of lipopolysaccharides. On the surface of E. coli O86 a human blood group B antigen can be found. E. coli O86 gets killed by Galectin-4 via destruction of the membrane integrity and bacterial motility.

In inflammatory bowel disease (IBD), Galectin-4 might also play an interesting role. While on the one hand it seems to lead to exacerbation in the T-cell-receptor mutated form of IBD, by stimulating the production of IL-6 in CD4+ T-cells (Hokama et al.; 2004) , on the other hand it ameliorates the wild type form of IBD by inducing apoptosis and reducing the secretion of inflammatory cytokines. (Paclik et al.: 2008)

“Galectin-4, which has been detected in many types of cancer, is associated with the development and progression of pancreatic carcinoma, hepatocellular carcinoma, colorectal cancer (CRC), breast carcinoma, gastric cancer, and lung cancer. However, it plays differing parts in different type of cancer cells. Furthermore, it has been detected in serum of some cancer patients. Among these cancer forms, only the role of Galectin-4 in CRC development has been revealed explicitly.” (Cao et al.; 2016)

Compared to normal colon tissue the expression level of Galectin-4 is decreased in CRC cells, which promotes tumor progression and metastasis through cell proliferation, migration and motility. However, Galectin-4 inhibits tumorigenesis via crosslinking Wnt signalling pathway proteins (APC, β-catenin and axin) which leads to degradation of β-catenin in cytoplasm. Therefore, the Wnt target genes in the nucleus will not be activated which results in an inhibition of cell proliferation, migration and motility. Additionally, IL-6 gets downregulated which inhibits NFKB and STAT3 in CRC. VEGF, COX-2 and other genes that are associated with tumorigenesis are downregulated leading to an inhibition of tumor progression. (Satelli et al.; 2011, Kim et al.; 2013)

Galectin-4 has similar effects in pancreatic cancer. Low expression levels also promote metastasis and cancer progression in hepatocellular cancer (Cai et al.; 2014) and in metastatic ileal carcinoids. Higher expression levels of Galectin-4 can be found in lung and gastric cancer, where they work as a predictor for metastasis.

In the serum of patients with colon, breast or hepatocellular cancer the level of free circulating Galectin-4 was significantly higher, compared to a healthy control group. (Barrow et al.; 2011)

That is why, circulating Galectin-4 could be a predictor for cancer patients, particularly those with metastasis. But, yet no significant correlation between cancer stages and the level of circulating Galectin-4 was found in the serum of breast and colorectal cancer patients. (Cao et al.; 2016)

1.4.6. Variants of Galectin-4

4 variants of Galectin-4 have been tailored to test if there is a relation between structure and function. Galectin-4 is a tandem repeat type galectin which consists of a N-terminal and a C-terminal domain with a 42 amino acid long linker peptide. (Kopitz et al.; 2012) Galectin-4N and Galectin-4C are simply the two domains separated from each other, as demonstrated in Figure 1.8.

In Galectin-4V, the linker-length was reduced to 17 amino acids (André et al.; 2013), whereas in Galectin-4P the linker peptide was completely removed, as shown in Figure 1.9.

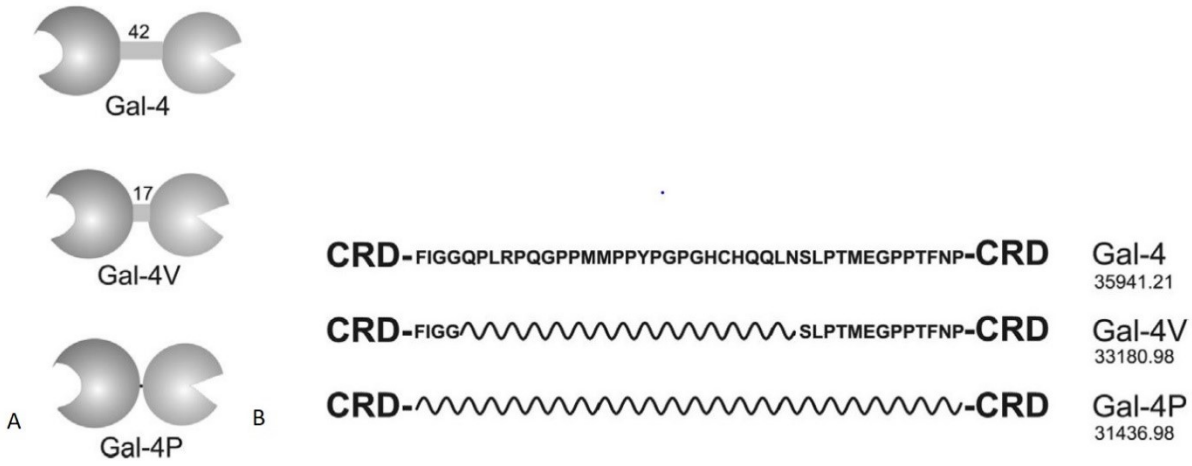

Figure 1.9: A: Illustration of the design of galectin-4 and its two shorter variants. B: Galectin-4 and its variants and the amino acid linker sequence, presenting the positions of deletions between N- and C-terminal domains.

1.5. Aim of the study

The first aim of this study was to histologically analyse, if the presence of Galectin-4 in OA chondrocytes correlates with the degeneration of cartilage. Earlier studies (Toegel et al.; 2013, 2014 and 2017; Weinmann et al.; 2016 and 2018) on other members of the galectin-family (Galectin-1, -3 and -8) revealed a correlation between galectin-positivity and cartilage degeneration.

A further aim of the study was to conduct in vitro-tests on the activity of Galectin-4 in OA chondrocytes as other members of the galectin family are known to induce the production of proinflammatory cytokines, such as IL1β (Toegel et al.; 2014 and 2017; Weinmann et al.; 2016 and 2018) and MMPs (Matrix metalloproteinases) which are playing important roles in pathological processes (Sneuk-van-Beurden et al.; 2005). Another aim of the study was to investigate whether the effects of Galectin-4 on OA chondrocytes are mediated by glycan-dependent binding to cells, and whether the gene regulatory effects of Galectin-4 are promoted by NF-κB signalling.

Furthermore, four biochemically altered variants of Galectin-4 were tested on cultured human OA chondrocytes, to evaluate if the length of the linker had an influence on the activity of Galectin-4.

2. Material and Methods

2.1. Clinical Specimen

Osteoarthritic knee cartilage was obtained from patients who underwent total knee replacement surgery with informed consent and in accordance with the terms of the ethics committee of the Medical University of Vienna (EK-Nr.: 1822/2017 and 1555/2019). The resected tissues were received from the operating room and were kept in in small sterile containers with physiological salt (NaCl) solution. Selected parts of the osteoarthritic joint were fixed with formalin for histological processing while the rest was processed for cell culture.

2.2. Cell Culture

2.2.1. Isolation of cells

The articular cartilage was cut off the bone and minced into small pieces, using two scalpels. Afterwards, the minced cartilage was transferred to Falcon tubes, washed with 30 ml of PBS (phosphate buffered saline) and afterwards enzymatically digested at 37° C for 24 hours using a Colllagenase II solution which was prepared from 30 ml of full medium and lyophilized Collagenase-II (Life Technologies) at a concentration of 300 Units/ml.

2.2.2. Counting

After 24 hours, pieces of undigested tissue were removed by filtration of the solution through a cell strainer, followed by centrifugation (1000 rpm for 8 minutes). The resulting pellet was washed with 10 mL PBS to remove cell components like erythrocytes and then centrifuged again. Finally, the pellet was resuspended in 5 ml PBS. 10 µl of the cell-solution (containing the chondrocytes) and 10 µL Trypan-Blue (to stain dead cells) were mixed.
10 µl of this solution were loaded into a counting chamber (C-Chip) to determine the number of living chondrocytes under the microscope. The single use C-Chip counting chamber consists of two independent injection areas (Figure 2.1, each for one 10 µl sample. Its counting grid includes four corner squares. Cells in all four corner squares were counted and the mean amount per square was determined.

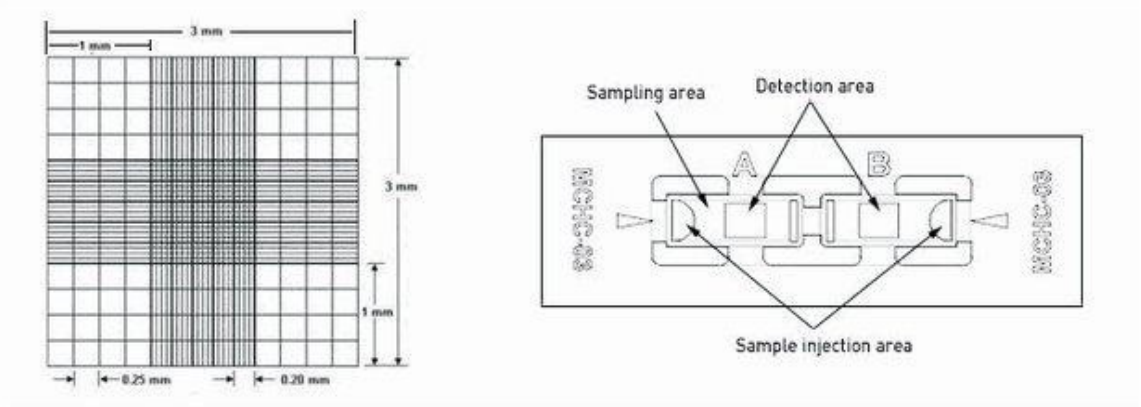

Figure 2.1: C-Chip counting chamber. The Counting grid (left) and the whole C-Chip (right).

2.2.3. Cultivation of cells

Isolated chondrocytes were cultured in full medium in a humidified atmosphere of 5% CO2/ 95% air at 37° C. They were used at passage 0 due to dedifferentiation of cells at higher passages. Table 2.1 shows the different culture flasks and plates that were used to cultivate the chondrocytes. The medium was changed regularly to ensure that the growing cells were provided sufficiently with nutrients. If the cells were freshly isolated, they were washed with PBS 24 hours after seeding, to remove erythrocytes remaining tissue debris.

Culture flask	**Cultivation area**	**Capacity of medium**
T75	75cm²	15 mL
T150	150 cm²	30 mL
Culture plates		
6 Well	9.5 cm² / Well	2 mL / Well
12 Well	3.8 cm² / Well	1 mL / Well
96 Well	0.32 cm² / Well	0.2 mL / Well

Table 2.1: The culture flasks and plates and their differences.

2.2.4. Starvation of cells

When the cells were ready for experimental use, they were starved overnight. Therefore, the cells were first washed with PBS and the full medium was replaced by starvation-medium (Full-Medium without FBS), to make the cells sensitive for the upcoming treatment.

2.2.5. Treatment of cultured chondrocytes

The lyophilized galectins (provided by Prof. Hans-Joachim Gabius´ laboratory, institute of physiological chemistry at the Ludwig-Maximilian-University in Munich) were reconstituted with PBS to a yield 1 µg/µl concentration and mixed with starvation-medium to yield the final concentration for cell stimulation.

2.2.6. 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 galectins. 96-well plates were seeded with 5.000 chondrocytes per well in full medium. The treatment was performed in starvation medium for 24 hours with five concentrations of the galectins 1, 3, 4, 8 and 9 (5, 10, 20, 50, 100 µg/ml). On 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 were added. 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).

2.3. Isolation of RNA

After the treatment period, the supernatant of the treated cells was collected in labeled collection tubes and stored at -80° C and the layers of treated cells were washed with PBS. Total RNA was isolated using the innuPREP RNA mini Kit (by Analytik Jena) as described by the manufacturer. Briefly, 350 µl of lysis-buffer (lysis solution RL by Analytic Jena) were added to the cell layer and resuspended multiple times to lyse the cells. The lysate was also collected in labeled tubes. Those 350 µL were filtrated through a provided filter (Filter D) by centrifugation for 2 minutes at 12,000 rpm. 350 µL of ethanol (70%) were added and mixed by pipetting up and down several times. To bind the RNA, this solution was filtrated through Filter R by centrifugation for 2 minutes at 12,000 rpm. The RNA remained bound to filter R, while the filtrate was discarded. The RNA was washed with 500 µl washing solution HS and filtrated at 12,000 rpm for 1 minute. This washing-procedure was repeated with 750 µl of washing solution LS. To remove the ethanol the filter R was centrifuged at the maximum possible speed for 2 minutes. To elute the RNA, 40 µl of RNase-free water were pipetted directly onto the filter. The filter was placed in a 1.5 ml tube, incubated for 1 minute at room temperature and centrifuged at 8,000 rpm for 1 minute. Afterwards, the tubes were placed on ice.

2.3.1. Quantitation of RNA

To determine the concentration of the isolated RNA, a NanoDrop 2000 Micro Volume UV-Vis Spectrophotometer, equipped with NanoDrop software, was used according to the manufacturer´s instructions. Before the sample measurement, a blank measurement with water was performed. After every measurement the upper and lower surfaces of the micro spectrophotometer were wiped off to prevent sample contamination. The samples were measured by loading 2 µL onto the lower surface and closing the lever arm. All samples were measured without dilution at a wavelength of 260 nm. RNA purity was checked by determining the 260/280 nm ratio.

2.4. Transcription of RNA into cDNA

The quantitated RNA samples were diluted with RNase-free water to ensure that the same amount of RNA in each sample would be transcribed into cDNA. During transcription, the single stranded RNA gets transcribed into complementary DNA (cDNA) by using a

composition of reverse transcriptase (RT) enzyme, RT primer, dNTPs, RT buffer and RNase-free water. All reagents (listed in Table 2.2) were supplied in the High Capacity cDNA Transcription Kit (Applied Biosystems).

Component	Volume
10x RT Buffer	2.0 µl
25x dNTP Mix (100 mM)	0.8 µl
10x RT Random Primers	2.0 µl
MultiScribe Reverse Transcriptase	1.0 µl
RNase-free H2O	4.2 µl
Total per reaction	10 µl

Table 2.2: Composition of the RT Master Mix

10 µL of the diluted RNA and 10 µL of the reaction composition were mixed in a 0.5 mL tube and placed in a thermal cycler (Eppendorf Mastercycler Gradient) for transcription into cDNA. Transcription was performed in four steps at different temperatures, as shown in Table 2.3. At the last step the samples were hold at 4° C until they were taken out of the thermal cycler.

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

2.4.1. Dilution of cDNA

The cDNA samples were diluted with RNase-free water at a ratio of 1:5. 8 µL of RNase-free water were pipetted into a 0.5 mL tube then 2 µL of the sample cDNA were added and mixed. This dilution step was necessary to achieve optimal performance of the following qPCR.

2.5. Quantitative real-time polymerase chain reaction (qPCR)

qPCR is a method that enables the quantification of mRNA levels in real time, meaning "continuously during the process", while conventional PCR determines the product at the end of the reaction. The main procedure is similar to the conventional PCR, including three steps repeated in multiple cycles to synthesize DNA: denaturation at 95°C to create two single strands, primer annealing at 60°C and DNA-polymerization at 72°C. Detection of product is carried out by measuring the emission of the fluorescence dye SYBR-Green, whose fluorescence emission is dependent on the number of PCR cycles and the DNA concentration. SYBR-Green does not bind to single strands. RT-qPCR was performed for quantification of three specific target genes: matrix metalloproteinase (MMP13), interleukin-1β (IL1B) and succinate dehydrogenase A (SDHA), which was used as a so called "housekeeping gene", meaning that its expression is not influenced by galectin treatment. Because of the various sources of error, the "housekeeping gene" is important as an endogenous control to obtain reliable results.

For quantitation, the relative, comparative Ct method was used. Of central importance in the RT-qPCR is the threshold cycle (Ct). The selection of threshold setting is basically arbitrary, but with the requirement that it should be placed at the region of exponential amplification for all samples regarding the same target gene. The Ct value is the time expressed in cycles, when the amplification plot reaches the threshold. The Ct of every target gene is quantitated in relation to the Ct value of SDHA using the Pfaffl method (Pfaffl, 2001).

2.5.1. Preparation of the reagents

For each target gene, a composition of reagents containing forward primer, reverse primer, SYBR-Green and RNase-free water was prepared to a total of 24 µL for each sample. Components and used volumes are displayed in Table 2.4.

Component	Value
Forward Primer	0.5 µL
Reverse Primer	0.5 µL
SYBR-Green	12.5 µL
H2O	10.5 µL
Total per Reaction	24 µL

Table 2.4: Composition of master mix for RT-qPCR

Every gene was targeted by a specific set of primers, shown in table 2.5

	Forward Primer	Reverse Primer
MMP13	TCAGGAAACCAGGTCTGGAG	TGACGCGAACAATACGGTTA
IL1B	CTTATTACAGTGGCAATGAGGATG	AGTGGTGGTCGGAGATTCG
SDHA	TGGGAACAAGAGGGCATCTG	CCACCACTGCATCAAATTCATG

Table 2.5: Primer sequences of used target genes

RT-qPCR was performed in 96-well optical plates that were placed in a cooled rack during pipetting. First, 24 µL of the prepared mixture were pipetted in every required well. Then, 1 µL of the diluted cDNA sample was added into the well. “No template controls” were prepared for each target gene by replacing the diluted cDNA with RNase-free water to check for contaminations in the reactions. The 96-well optical plate was covered with an optical adhesive film and centrifuged for 1 minute at 1000 rpm at room temperature before measurement.

2.6. Confocal microscopy – Immunofluorescence staining of galectin binding sites

$2x10^5$ chondrocytes were kept in T75-culture flasks. The culture medium was changed regularly, and the condition of the cells was checked every day. Then a cell suspension was prepared by trypsinization and centrifugation of monolayer chondrocyte culture. Cells were incubated with 50 µL PBS containing 5 µg Alexa488-labeled Galectin-4 (and 10 ng DAPI) in the presence and absence of 0.1 M lactose for 10 minutes at 4 °C, to limit internalization of cell-bound galectins. Cells were thoroughly washed three times with PBS to remove unbound galectins. Cells were then immediately mounted for laser scanning microscopy without fixation. Confocal images of fluorescence-labeled cells were obtained using a Carl Zeiss LSM 700 Laser Scanning Microscope at 630x magnification and Zen software.

2.7. Immunohistochemistry

2.7.1. Tissue preparation and Safranin-O (SO) staining

For immunohistochemistry, cartilage specimens (n=19 patients) were selected macroscopically to include areas of mild and areas of severe degeneration. The tissues were fixed in formalin, followed by decalcification and embedding in paraffin. After that, the paraffin slides were incubated with SO (Sigma-Aldrich) and counter-stained using light-green Goldner III solution (Morphisto). SO staining correlates with the amount of proteoglycan in the cartilage. A deep red staining shows abundant proteoglycan presence, while the light-green counter staining visualizes the lack of proteoglycans. Consecutive sections were then processed for immunohistochemical staining. 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 using the “Mankin score”, which is illustrated in Table 2.6. The final grading ranges from 0 (most intact) to 14 (most degenerated). Areas of mild and severe cartilage degeneration were analyzed 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	
Slight reduction	1
Moderate reduction	2
Severe reduction	3
No dye noted	4
4. Tidemark Integrity	
Intact	0
Crossed by blood vessels	1

Table 2.6: Mankin score

2.7.3. Immunohistochemistry with anti-Galectin-4 antibody

After deparaffinization and blocking of the endogenous peroxidase activity, the slides were incubated overnight with anti-Galectin-4 antibodies, which were diluted to their final concentration with 2% BSA/PBS (Bovine Serum Albumin / Phosphate-Buffered Saline). The negative control was prepared by omission of primary antibodies. Later, slides were washed with PBS, followed by incubation with the secondary antibody. 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. Microscopic analysis

The immunohistochemical staining of Galectin-4 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 severe degeneration. Staining signals above the background levels were scored as positive. Afterwards, the percentage of positively stained cells was calculated.

2.8. Statistics

To analyze the correlation between Galectin-4 immunopositivity and the Mankin score, SPSS v24 was used. Scatterplots of “percentage of Galectin-4 positive cells” versus “Mankin scores” were prepared for each patient. The Pearson´s correlation coefficient was calculated for each of the 19 patients and Wilcoxon test was used to assess whether the distribution of correlation coefficients was significantly different from 0. The Wilcoxon signed-rank test was used to analyze the qPCR data. Shapiro Wilk test was performed to test the normality of distribution of the data. For the MTT results, the two-tailed t-test function of Microsoft Excel was used.

3. Results

3.1. Histological assessment of Galectin-4 in osteoarthritic cartilage

Cartilage specimens of 19 patients were analyzed to determine the immunopositivity of OA chondrocytes for Galectin-4.

Two to three samples, of a patient´s cartilage, were taken. The percentage of Galectin-4 positive chondrocytes was analyzed in three to four histological areas per sample and the grade of degeneration was determined by using the Mankin score (MS).

To determine the Mankin score (i.e. the grade of degeneration) SO stained slices of those areas were used. The Mankin score ranges from 0 to 14. The analyzed areas ranged from MS 2 (=mildly degenerated) to MS 13 (=severely degenerated).

The evaluated areas showed Galectin-4 cell positivity from 0 to 100%.

The more degenerated the area was the higher the cell positivity for Galectin-4 would be. Less degenerated areas would have less Galectin-4 positive cells. Figure 3.1 shows 3 areas: one mildly degenerated (MS 3), one medium degenerated (MS 8) and one severely degenerated (MS 11), with different Galectin-4 cell positivity.

A scatterplot was created, to show the correlation between degeneration and cell positivity. The percentage of Galectin-4 cell positivity was plotted against the Mankin score, with regression lines for each patient (Figure 3.2) and a regression line over all patients (Figure 3.3). The Pearson´s correlation coefficients for each patient were calculated and reached from 0.45 to 0.937 (mean=0.725±0.163). A Shapiro-Wilk-Test was performed showing that the data were normally distributed.

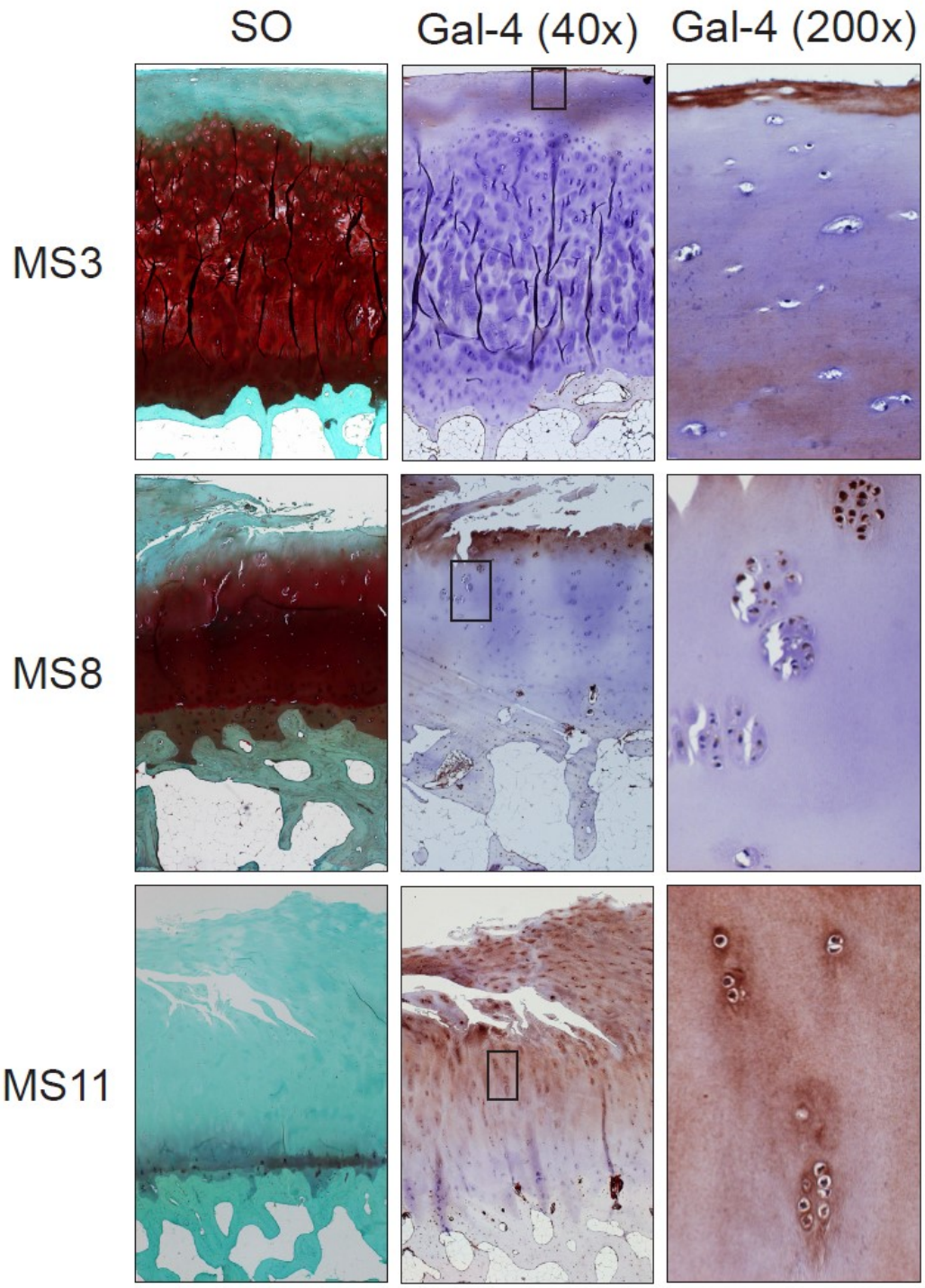

Figure 3.7: **Comparing different grades of degeneration and Galectin-4 positivity in OA cartilage.** Histological sections of OA cartilage and bone (n = 19 patients) were stained with Safranin O (SO). From each patient, 3–10 areas of interest were graded using the Mankin score (MS). Consecutive sections were processed immunohistochemically, and the percentages of Galectin-4-positive chondrocytes were assessed at 40 x and 200 x magnification.

Shown are representative areas from mildly (MS 3), moderately (MS 8) and severely (MS11) degenerated cartilage regions stained with SO (left) and using an antibody against Galectin-4 (middle and right).

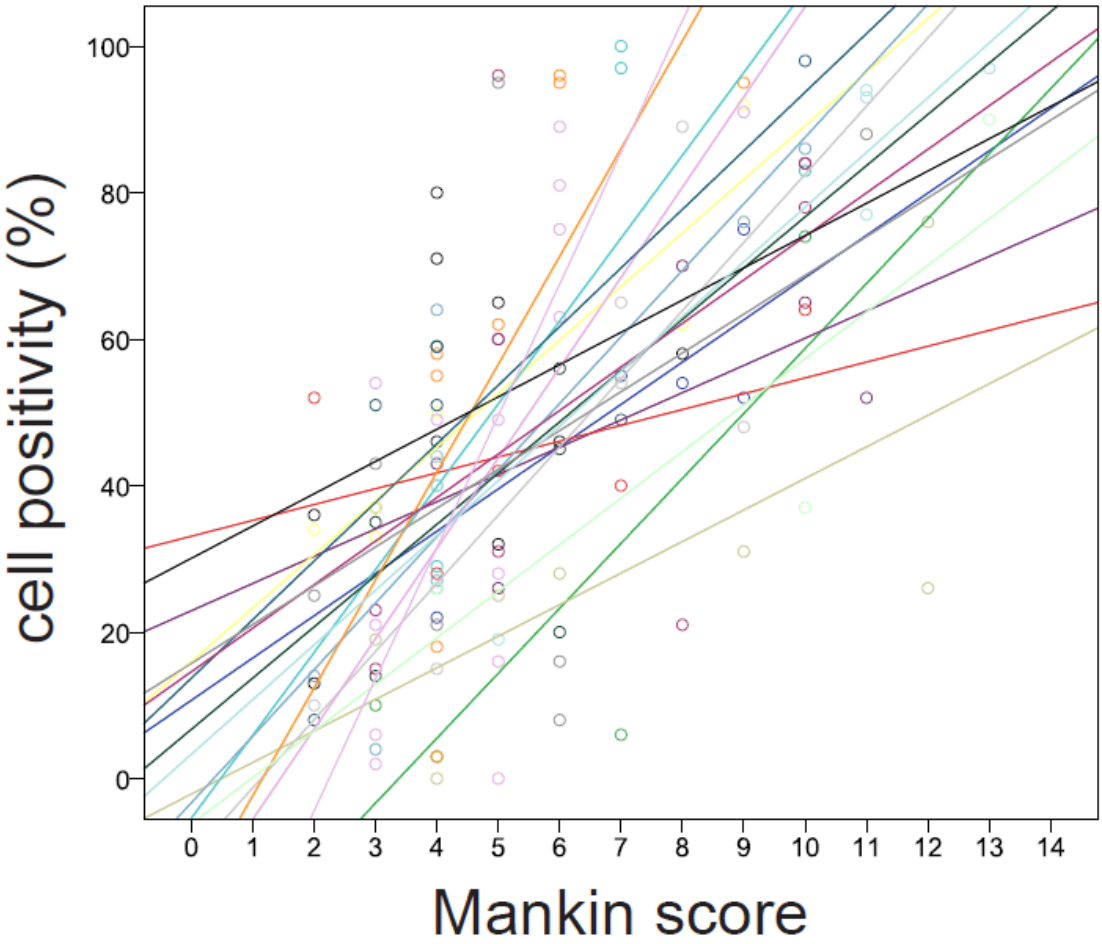

Figure 3.8: The presence of Galectin-4 in chondrocytes correlates with the degeneration of the cartilage. A scatterplot of Mankin scores versus the percentages of Galectin-4 positive cells in the cartilage of 19 patients, with regression lines for each patient, is shown. The determined Mankin scores ranged from 2 to 13 and the Galectin-4 positivity ranged from 0 to 100%. The Pearson´s correlation coefficients for each patient were calculated and reached from 0.45 to 0.937.

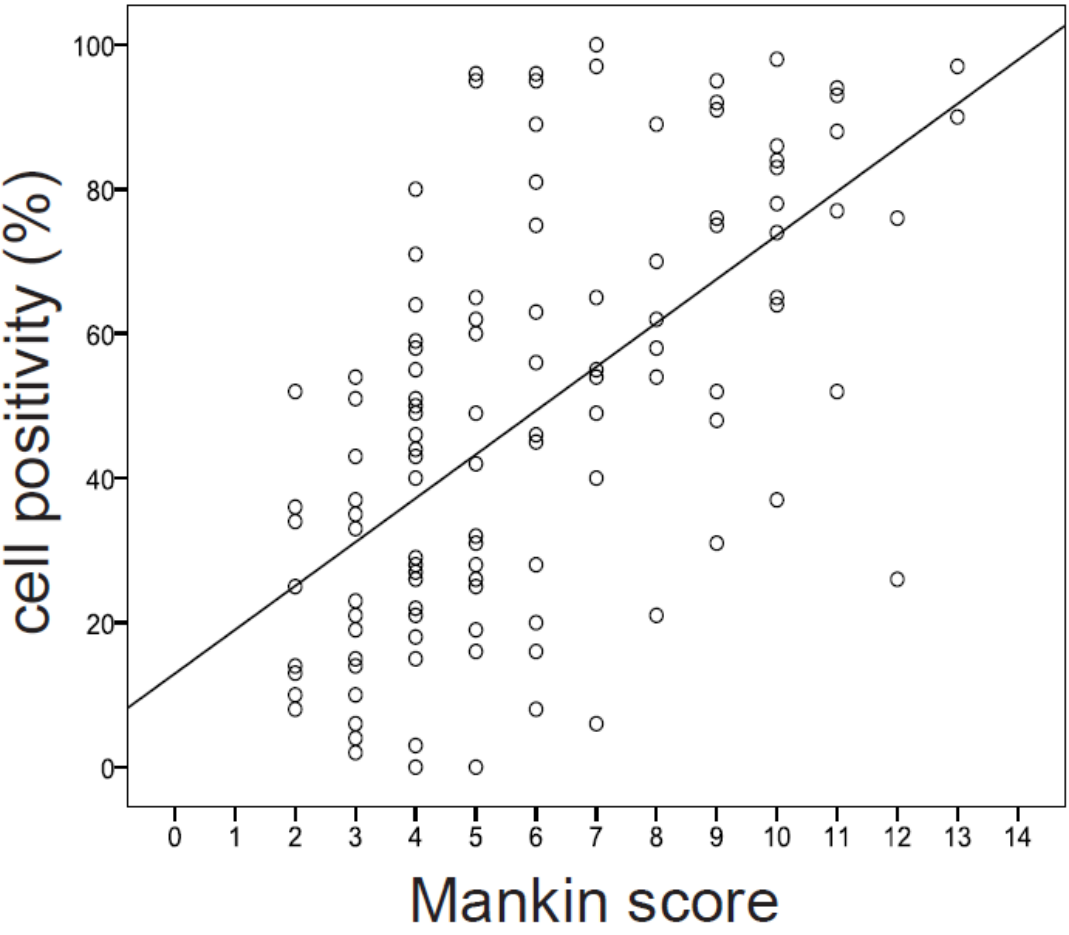

Figure 3.9: The presence of Galectin-4 in chondrocytes correlates with the degeneration of the cartilage. A scatterplot of Mankin scores versus the percentages of Galectin-4 positive cells in the cartilage of 19 patients, with an overall regression line for all patients, is shown. The determined Mankin scores ranged from 2 to 13 and the Galectin-4 positivity ranged from 0 to 100%. The Pearson´s correlation coefficients for each patient were calculated and reached from 0.45 to 0.937.

3.2. The binding of Galectin-4 to the surface of OA chondrocytes

Cultured OA chondrocytes were incubated with Galectin-4-FITC in absence or presence lactose. Afterwards, the cells were analyzed using a confocal laser scanning microscope. Figure 3.4 presents images of chondrocytes which were either untreated, treated with Galectin-4-FITC (without lactose), or treated with Galectin-4-FITC and 0.1 M lactose. Adding lactose precludes Galectin-4-FITC from binding to the surface of the chondrocytes.

The untreated cells on the left side are comparable to the cells treated with lactose and Galectin-4, as no fluorescence could be observed at cell surfaces. In the middle, cells with labelled Galectin-4 (green), which is bound to the surface, are presented. As seen on the right side, no fluorescence-labelled Galectin-4 was bound to the cell surfaces in presence of lactose. DAPI was used to stain the cell nucleus (blue).

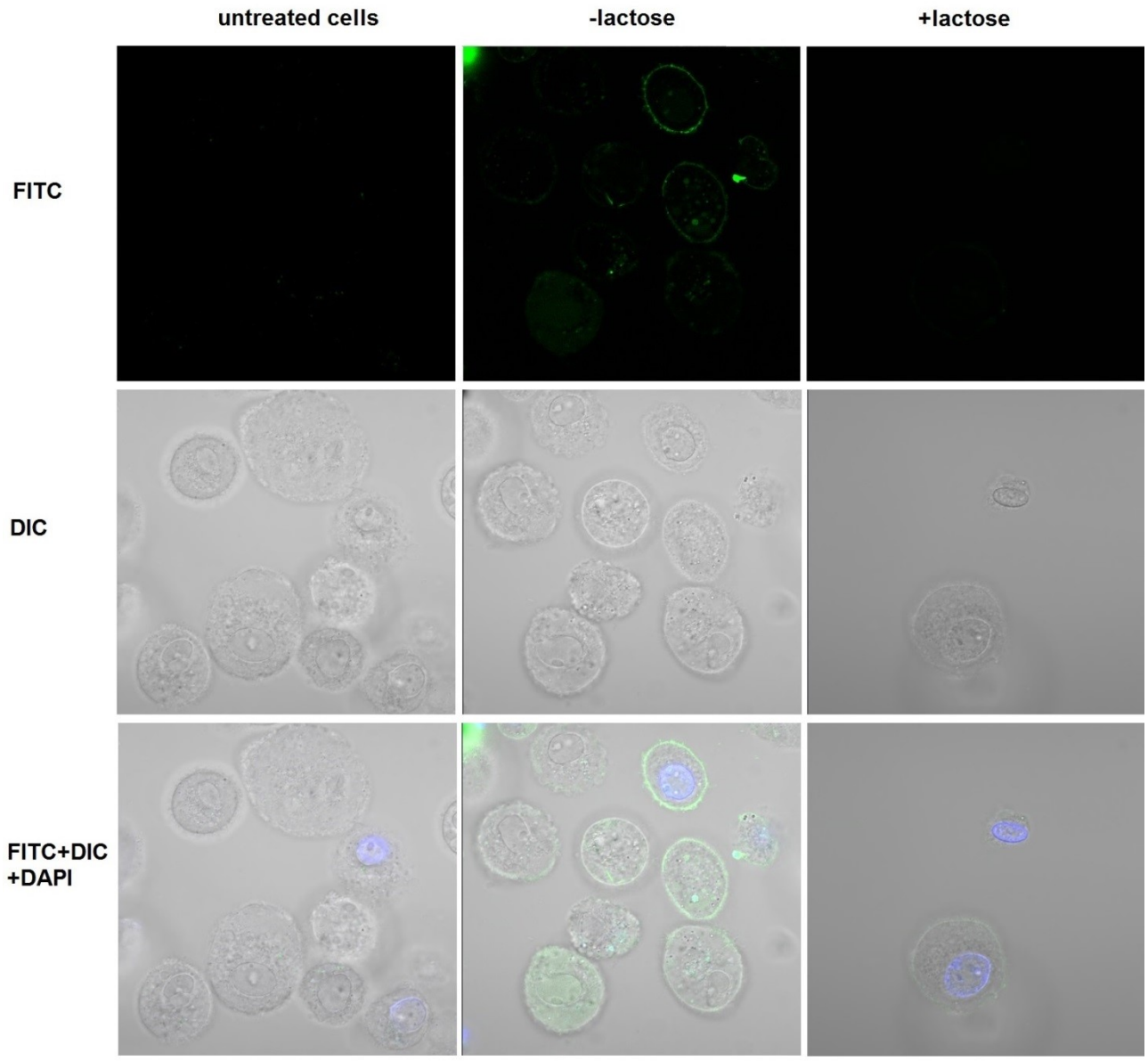

Figure 3.4: Confocal laser scanning microscopy images of OA chondrocytes, showing that lactose precludes Galectin-4 from binding to the cell surface. Untreated chondrocytes (left), chondrocytes treated with 100 µg/ml of Galectin-4-FITC (middle) and chondrocytes treated with 100 µg/ml of Galectin-4-FITC and 0.1M lactose (right) are presented in this figure. Cultured chondrocytes were trypsinized and resuspended prior to their treatment with FITC-labelled Galectin-4 in presence or absence of lactose for 10 minutes at 4°C. After washing, the cells were analyzed using a Carl Zeiss LSM 700 Laser Scanning Microscope at 630x magnification. Representative fluorescence images (top), corresponding differential interference contrast images (middle) and overlay images with DAPI (bottom) are presented.

3.3. Analysis of the metabolic cell activity

OA chondrocytes were treated with 5, 10, 20, 50 and 100 µg/mL of Galectin-4 for 24 hours. Afterwards, a cell toxicity and proliferation assay was performed to show the effect of the treatment. Control cells remained untreated and were cultured under the same conditions. Differences between Galectin-4 treated and untreated chondrocytes were considered significant if $p < 0.05$.

3.3.1. Effect of Galectin-4 on the metabolic cell activity of OA chondrocytes

Figure 3.4 shows the effect of Galectin-4 in 5 different concentrations (5, 10, 20, 50 and 100 µg/ml) on the metabolic activity of the OA chondrocytes, indicating that the applied concentrations did not have a negative impact on the cells or their metabolic activity.

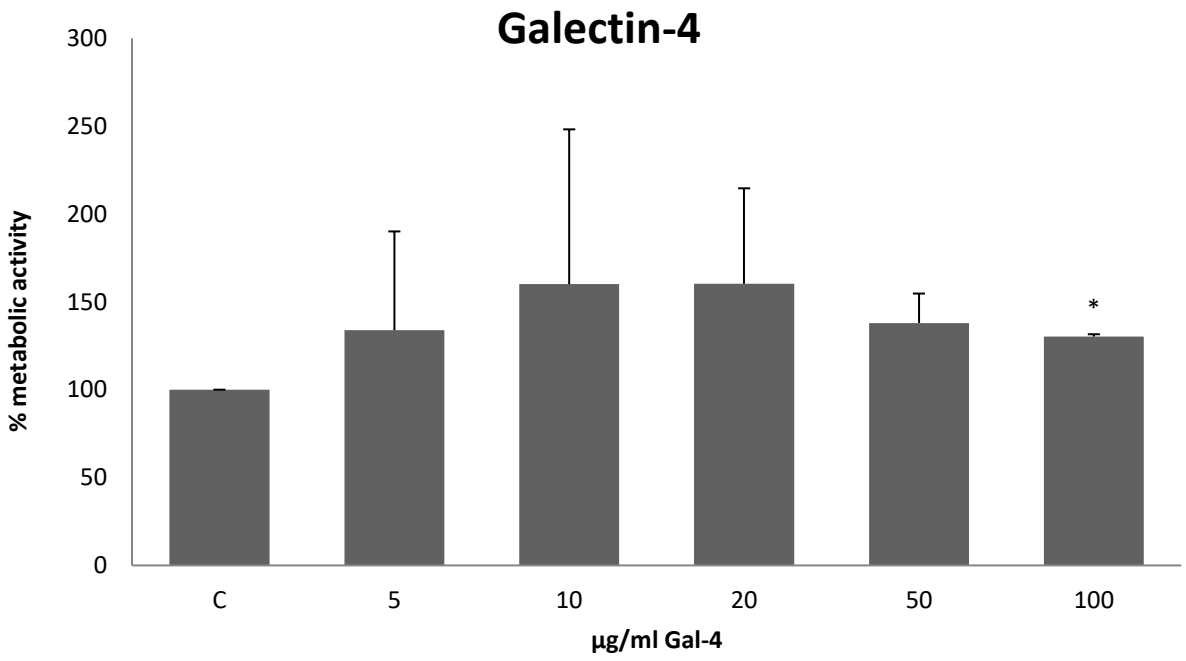

Figure 3.5: Effect of Galectin-4 on the metabolic cell activity of OA chondrocytes. OA chondrocytes (n=2 patients) were treated with five concentrations of Galectin-4 (5, 10, 20, 50, 100 µg/ml) for 24 hours. Control cells (C) remained untreated and were cultivated under the same conditions. The statistically significant ($p < 0.05$, t-test) effects, are marked with asterisks.

3.3.2. Effect of Galectin-4 on metabolic cell activity compared to the activity of Galectin-1, -3 and -8.

Galectin-4 treated chondrocytes showed similar metabolic cell activity to cells treated with the Galectins-1, -3 and -8 in the sense that the metabolic cell activity was not impaired by the treatment with galectins.

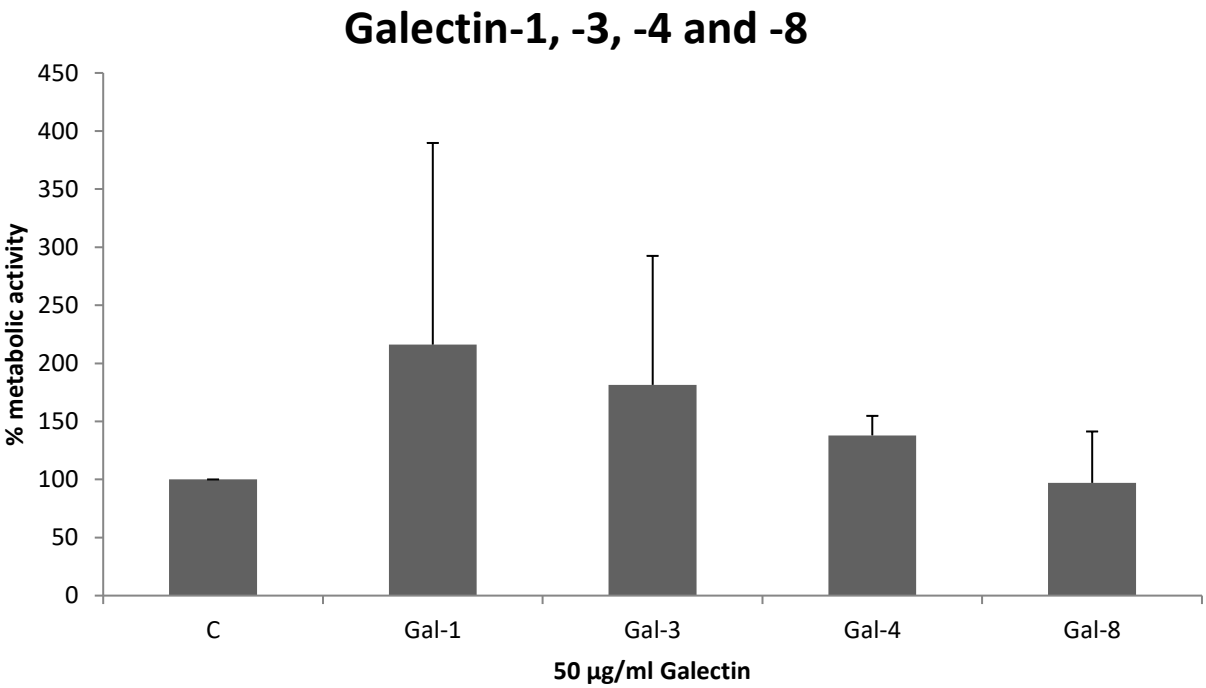

Figure 3.6: Effect of Galectin-1, -3, -4 and -8 on the metabolic cell activity of OA chondrocytes. OA chondrocytes (n=2 patients) were treated with 50 µg/ml of galectin 1, 3, 4 or 8. All four galectins did not impair the metabolic cell activity significantly.

3.4. Effect of Galectin-4 on mRNA levels in OA chondrocytes

RT-qPCR was performed to measure the impact of galectin treatment on the gene expression of IL1B and MMP13 in OA chondrocytes. Five different experiments were conducted. First, OA chondrocytes were treated with Galectin-4 at different concentrations (1, 5, 10, 20, 50, 100 µg/ml). The second experiment compared the effects on mRNA levels of 4 different galectins (i.e. Galectin-1, -3, -4 and -8). The third experiment analyzed if the presence of lactose influenced the activity of Galectin-4. Fourth, four biochemically altered variants of Galectin-4 were compared regarding their impact on gene expression in OA chondrocytes. The fifth experiment was conducted to analyze if inhibition of the NF-κB-pathway reduces the effect of Galectin-4 on gene expression in OA chondrocytes. The work processes for RNA isolation, RNA quantification and cDNA synthesis of chondrocytes were kept constant.

3.4.1. Concentration-dependent effects of Galectin-4 on the gene expression of IL1B and MMP13 in OA chondrocytes

The mRNA levels of IL1B in OA chondrocytes after the treatment with different concentrations of Galectin-4 (1, 5, 10, 20, 50, 100 µg/ml) are shown in Figure 3.6. The upregulation of the gene was statistically significant ($p < 0.05$) for the concentrations from 5 to 100 µg/ml, as compared to the untreated control.

As shown, the expression of IL1B mRNA increased with rising concentrations of Galectin-4.

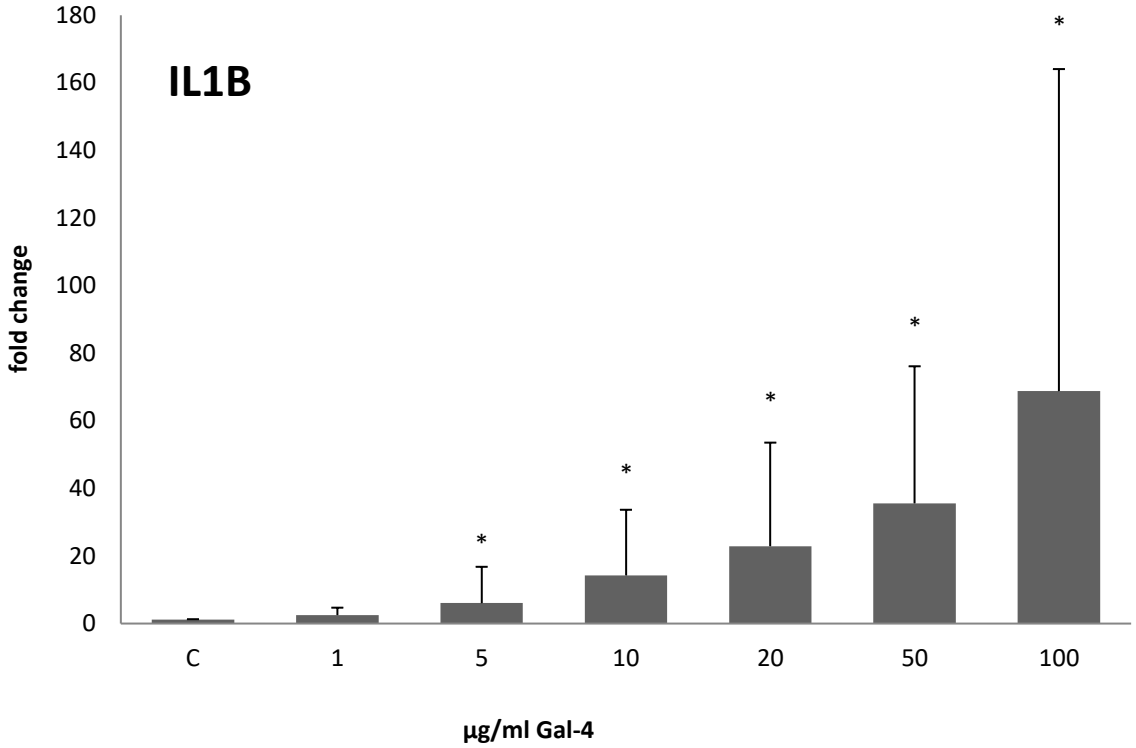

Figure 3.7: Concentration-dependent effect of Galectin-4 on IL1B mRNA levels in OA chondrocytes. OA chondrocytes (n=8 patients) were treated with different concentrations (1, 5, 10, 20, 50, 100 µg/ml) of Galectin-4 for 24 hours. Untreated chondrocytes, which were cultured under the same conditions, were used as a control (C). After incubation, the mRNA levels of IL1B were quantified using RT-qPCR with SDHA as reference gene. The statistically significant ($p < 0.05$, Wilcoxon-test) up-regulations compared to the control, are marked with asterisks.

Figure 3.7 shows that the expression of MMP13 mRNA increased with rising concentrations of Galectin-4 in treated OA chondrocytes. mRNA levels of the gene were significantly up-regulated ($p < 0.05$, Wilcoxon-test) for the concentrations from 5 to 100 µg/ml, as compared to the untreated control.

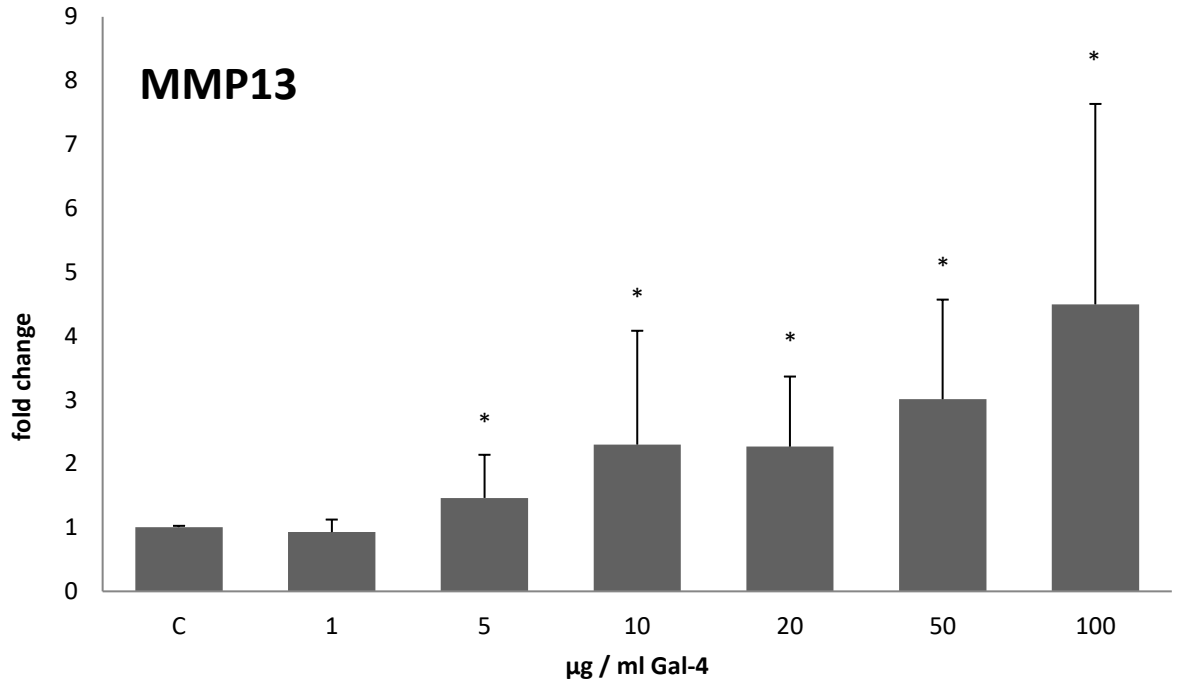

Figure 3.8: Concentration-dependent effect of Galectin-4 on MMP13 mRNA levels in OA chondrocytes. OA chondrocytes (n=8 patients) were treated with different concentrations (1, 5, 10, 20, 50, 100 µg/ml) of Galectin-4 for 24 hours. Untreated chondrocytes, which were cultured under the same conditions, were used as a control (C). After incubation, the mRNA levels of MMP13 were quantified using RT-qPCR with SDHA as reference gene. The significant ($p < 0.05$, Wilcoxon-test) up-regulations, compared to the control, are marked with asterisks.

3.4.2. Comparison of Galectin-1, -3, -4 or -8 regarding their effects on mRNA levels in OA chondrocytes

Due to the fact, that the different galectins have different molecular weights, equimolecular concentrations were used to allow direct comparison. The Galectin-4-mediated up-regulation of IL1B-gene expression was compared to the effects of Galectin-1, -3 and -8. Figure 3.9 shows that mRNA levels of IL1B are significantly higher in the galectin-treated groups in comparison to the untreated control groups. However, the Galectin-4-mediated up-regulation was significantly lower than that mediated by Galectin-1 or Galectin-3.

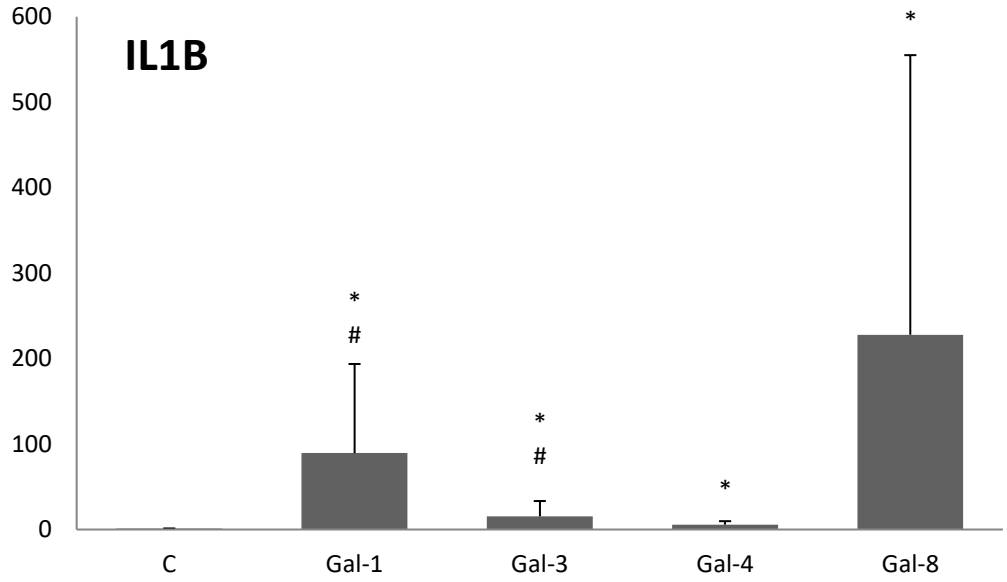

Figure 3.9: IL1B mRNA levels after treatment with Galectin-1,-3, -4 or -8. OA chondrocytes (n=6 patients) were treated for 24 hours with 10 µg/ml Galectin-1, 18 µg/ml Galectin-3, 24 µg/ml Galectin-4 or 24 µg/ml Galectin-8. Untreated OA chondrocytes were cultivated under the same conditions and used as control (C). After incubation, the mRNA levels of IL1B were quantified using RT-qPCR with SDHA as reference gene. Statistical differences of IL1B expression levels compared to the control (C) are marked with asterisks ($p<0.05$, Wilcoxon-test). IL1B expression levels of Galectin-1 and Galectin-3 treated groups were significantly higher than in the Galectin-4 treated group. Those statistical differences are marked with number signs ($p<0.05$, Wilcoxon-test).

The Galectin-4-induced up-regulation of the MMP13-gene was also compared to the effects of Galectin-1, -3 and -8. As seen in Figure 3.10, the upregulation of the gene mediated by each galectin is significantly higher than the untreated control. However, the Galectin-4-mediated up-regulation was significantly lower than the Galectin-1 and Galectin-3-mediated up-regulation.

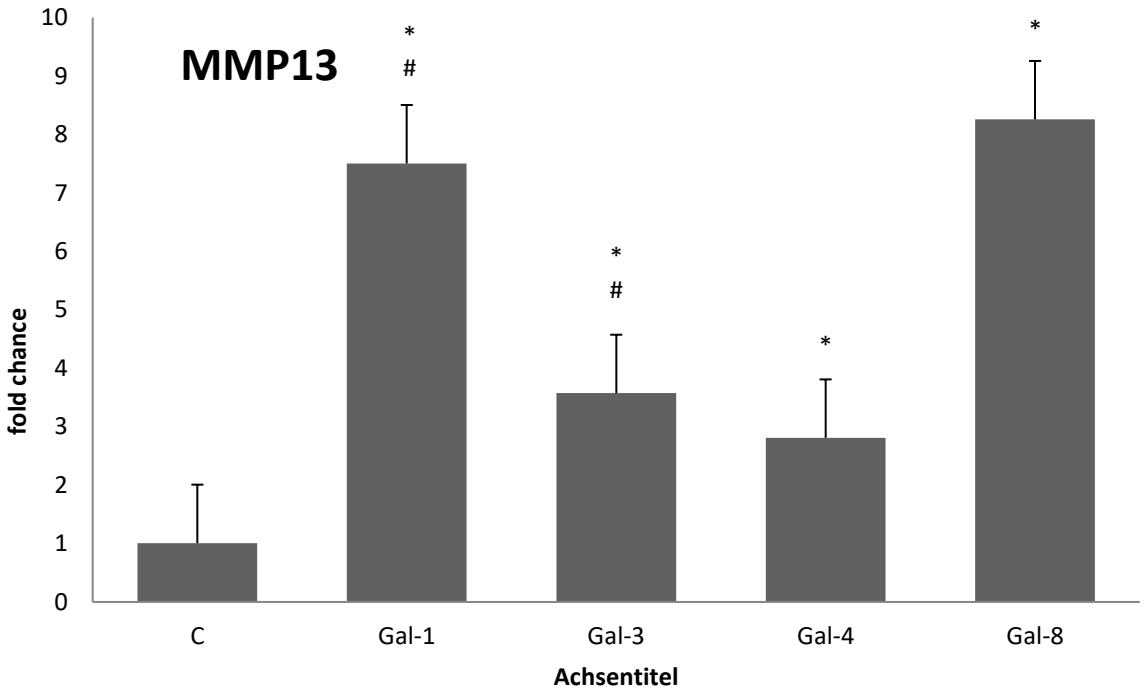

Figure 3.10: MMP13 levels after treatment with Galectin-1, -3, -4 or -8. OA chondrocytes (n=6 patients) were treated for 24 hours with 10 µg/ml Galectin-1, 18 µg/ml Galectin-3, 24 µg/ml Galectin-4 or 24 µg/ml Galectin-8. Untreated OA chondrocytes were cultivated under the same conditions and used as control (C). After incubation, the mRNA levels of MMP13 were quantified using RT-qPCR with SDHA as reference gene. Statistical differences of MMP13 expression levels compared to the control are marked with asterisks ($p<0,05$, Wilcoxon-test). MMP13 expression levels of Galectin-1 and Galectin-3 treated groups were significantly higher than in Galectin-4 treated groups. Those statistical differences are marked with number signs ($p<0.05$, Wilcoxon-test).

3.4.3. Inhibition of Galectin-4 activity by lactose

The effects of Galectin-4 on mRNA levels in OA chondrocytes were attenuated in presence of lactose. As seen in Figure 3.11, the expression of IL1B was significantly lower if a solution of 0.2M lactose was added to the cultivated cells before they were treated with Galectin-4.

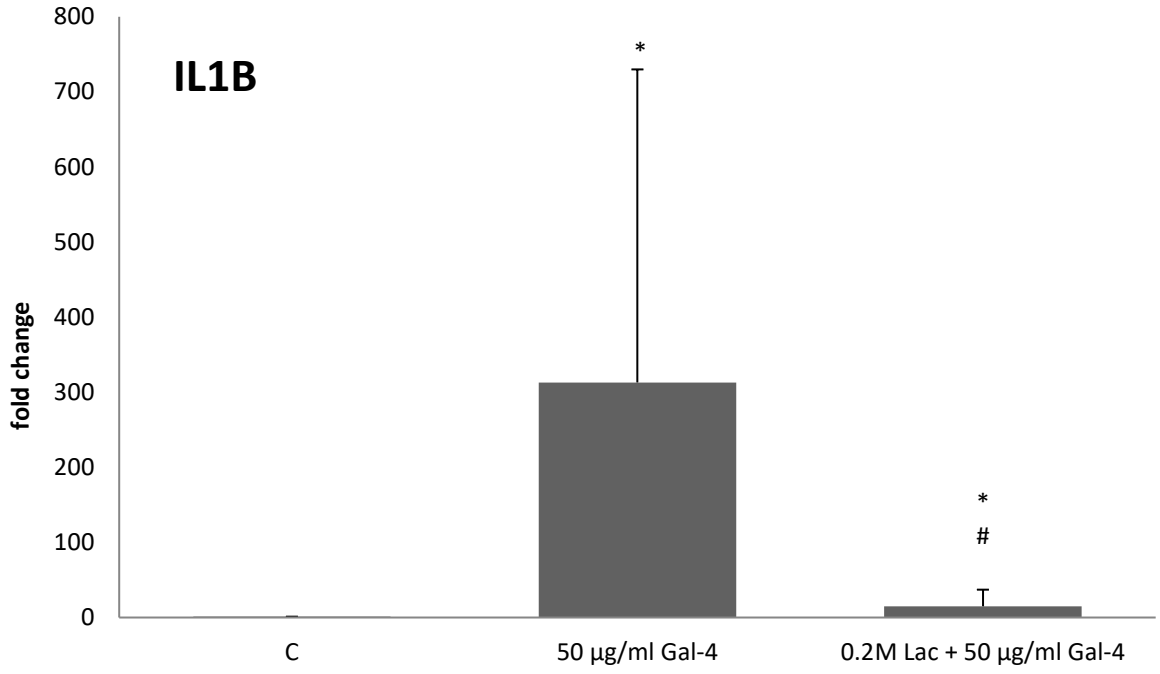

Figure 3.11: IL1B mRNA levels after treatment with Galectin-4 in absence and presence of lactose. OA chondrocytes (n=6 patients) were treated for 24 hours with 50 µg/ml of galectin-4. One test-group underwent a co-treatment with a 0.2 M lactose-solution and Galectin-4. Untreated OA chondrocytes were cultivated under the same conditions and used as control (C). After incubation, the mRNA levels of IL1B were quantified using RT-qPCR with SDHA as reference gene. Statistical differences of IL1B expression levels compared to the control are marked with asterisks ($p<0.05$, Wilcoxon-test). The statistically significant difference between galectin-treated cells and those cells treated with both galectin and lactose is marked by the number sign ($p<0.05$, Wilcoxon-test).

The effect of lactose was also observed in case of MMP13 mRNA-levels. Figure 3.12 shows that MMP13 levels were significantly reduced when OA chondrocytes were co-treated with lactose as compared to cells that were treated with Galectin-4 only.

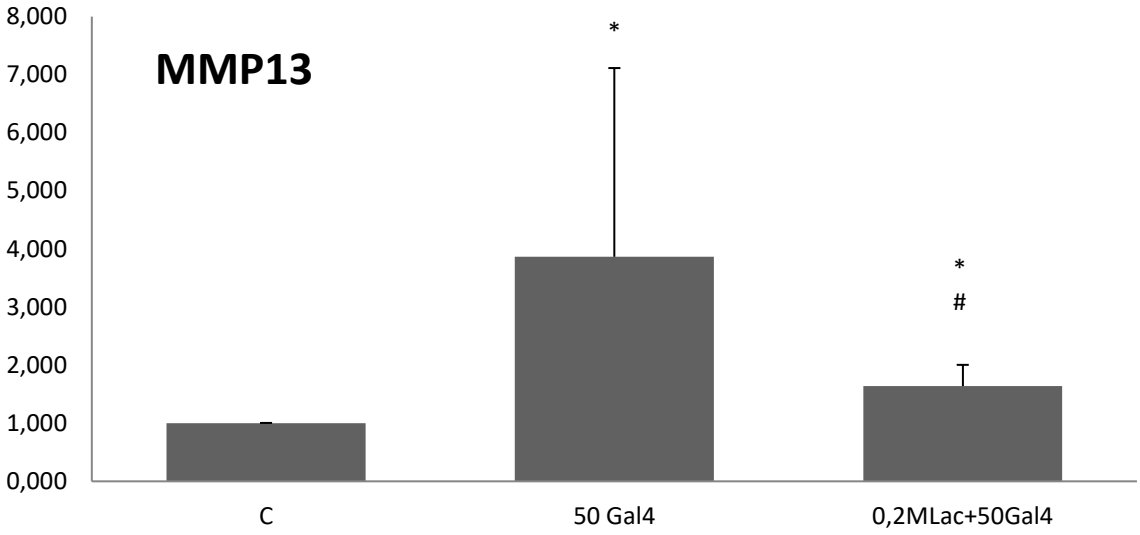

Figure 3.12: MMP13 mRNA levels after treatment with Galectin-4 in absence and presence of lactose. OA chondrocytes (n=6 patients) were treated for 24 hours with 50 µg/ml of Galectin-4. One test-group underwent a co-treatment with a 0.2 M lactose-solution and Galectin-4. Untreated OA chondrocytes were cultivated under the same conditions and used as control (C). After incubation, the mRNA levels of MMP13 were quantified using RT-qPCR with SDHA as reference gene. Statistical differences of MMP13 expression levels, compared to the control, are marked with asterisks ($p<0.05$, Wilcoxon-test). The statistically significant difference between galectin-treated cells and those cells treated with both galectin and lactose is marked by the number sign ($p<0.05$, Wilcoxon-test).

3.4.4. Effect of Galectin-4 and its variants on mRNA levels in OA chondrocytes

Cultured OA chondrocytes were treated with Galectin-4 and four biochemically altered variants of Galectin-4. Figure 3.13 demonstrates that the expression of IL1B is significantly upregulated after the treatment of chondrocytes with Galectin-4 or its variants (N, C, V or P). The activity of Galectin-4N or Galectin-4C was even significantly higher than that of Galectin-4. Galectin-4V and Galectin-4P showed a similar level of activity compared to Galectin-4, but were less active than Galectin-4N and C. To compensate the different molecular weights of the Galectin-4 variants, the used concentrations were adjusted for each variant.

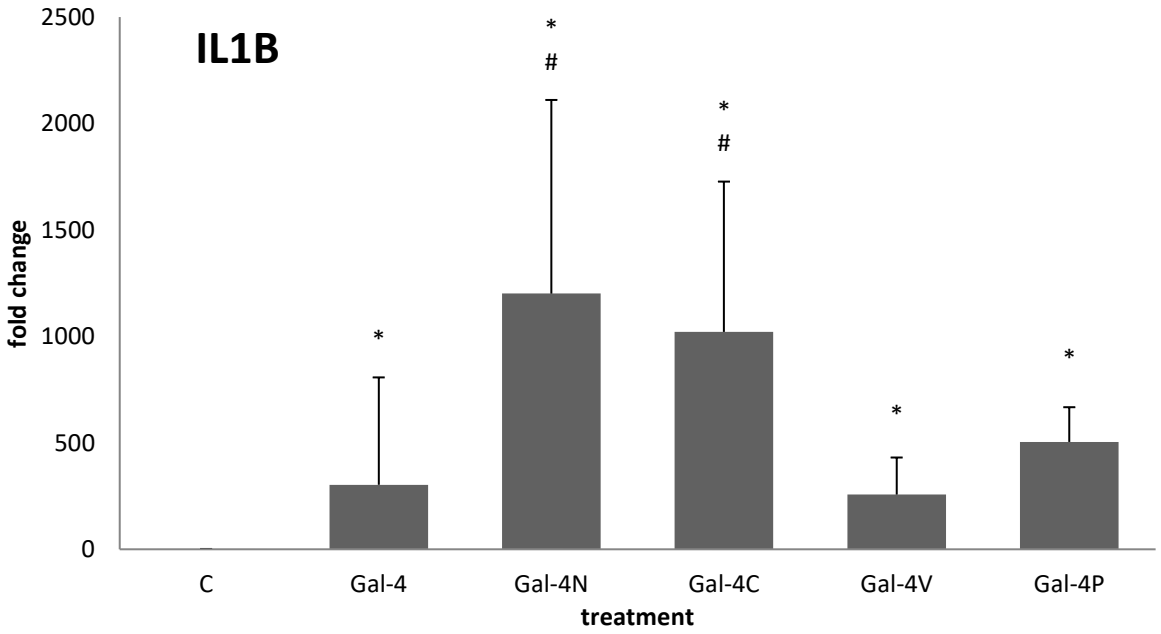

Figure 3.13: IL1B mRNA levels in OA chondrocytes after treatment with Galectin-4 and its biochemical variants (Galectin-4-N, -C, -V and -P). OA chondrocytes (n=7 patients) were treated for 24 hours with 50 µg/ml Galectin-4, 107 µg/ml Galectin-4N, 121 µg/ml Galectin-4C, 54 µg/ml Galectin-4V and 57 µg/ml Galectin-4P to compensate for differences in molecular weights. Untreated OA chondrocytes were cultivated under the same conditions and used as control (C). After treatment, the mRNA levels of IL1B were quantified using RT-qPCR with SDHA as reference gene. Statistically significant differences between galectins and the control are marked with asterisks ($p<0.05$, Wilcoxon-test). Statistical differences between Galectin-4-treated cells and cells treated with Galectin-4 variants are marked with the number sign ($p<0.05$, Wilcoxon-test).

Figure 3.14 presents the ability of Galectin-4 and its variants to increase MMP13 expression in OA chondrocytes. The applied galectins induced a significant up-regulation of the gene, compared to the untreated control. The activity of Galectin-4N or Galectin-4C was even significantly higher than that of Galectin-4, whereas the activity of Galectin-4V and Galectin-4P was also higher, but not significantly.

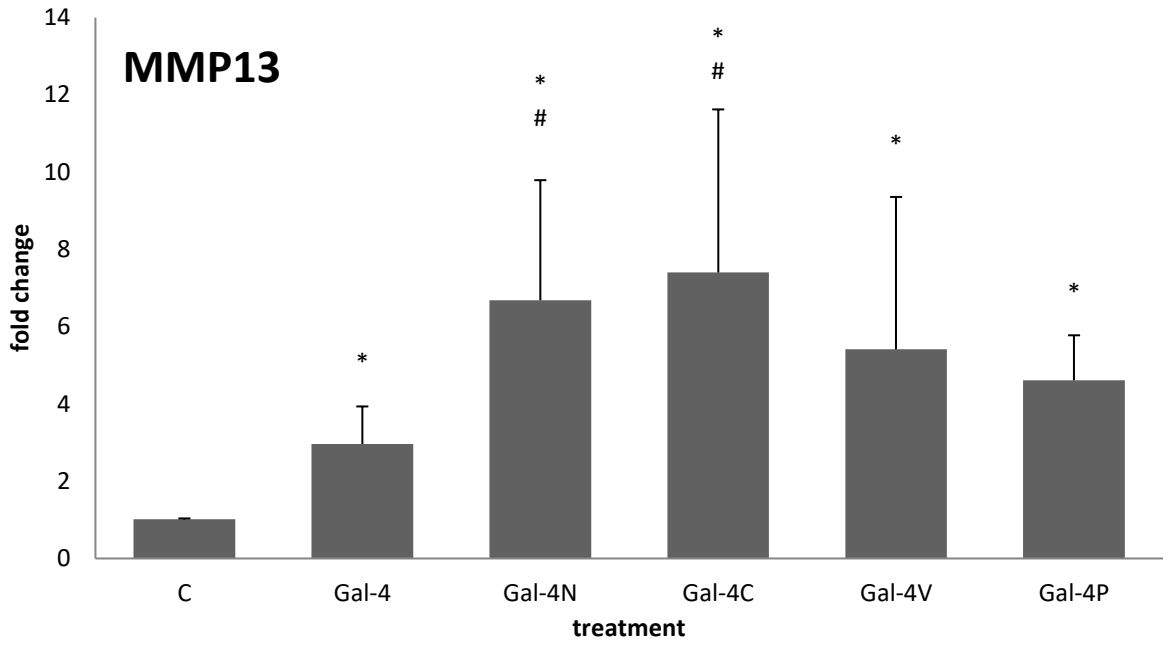

Figure 3.14: MMP13 mRNA levels in OA chondrocytes after treatment with Galectin-4 and its biochemical variants (Galectin-4-N, -C, -V and -P). OA chondrocytes (n=7 patients) were treated for 24 hours with 50 µg/ml Galectin-4, 107 µg/ml Galectin-4N, 121 µg/ml Galectin-4C, 54 µg/ml Galectin-4V and 57 µg/ml Galectin-4P to compensate for differences in molecular weights. Untreated OA chondrocytes were cultivated under the same conditions and used as control (C). After incubation, the mRNA levels of MMP13 were quantified using RT-qPCR with SDHA as reference gene. Statistically significant differences between galectins and the control are marked with asterisks ($p<0.05$, Wilcoxon-test). Statistical differences between Galectin-4 treated cells and cells treated with Galectin-4 variants are marked with the number sign ($p<0.05$, Wilcoxon-test).

3.4.5. Inhibition of NF-κB signaling blocks Galectin-4 activity in OA chondrocytes

Cultured OA chondrocytes were treated with 50 µg/ml of Galectin-4 in presence or absence of NF-κB inhibitors for 24 hours prior to RT-qPCR analyses of IL1B and MMP13 mRNA levels. Figure 3.15 shows the expression of the IL1B gene after the treatment with Galectin-4 and the inhibitors BAY 11-7082, IKK inhibitor VII or CAPE. Despite the lack of statistical significance, a clear tendency of inhibiting the effects of Galectin-4 could be observed.

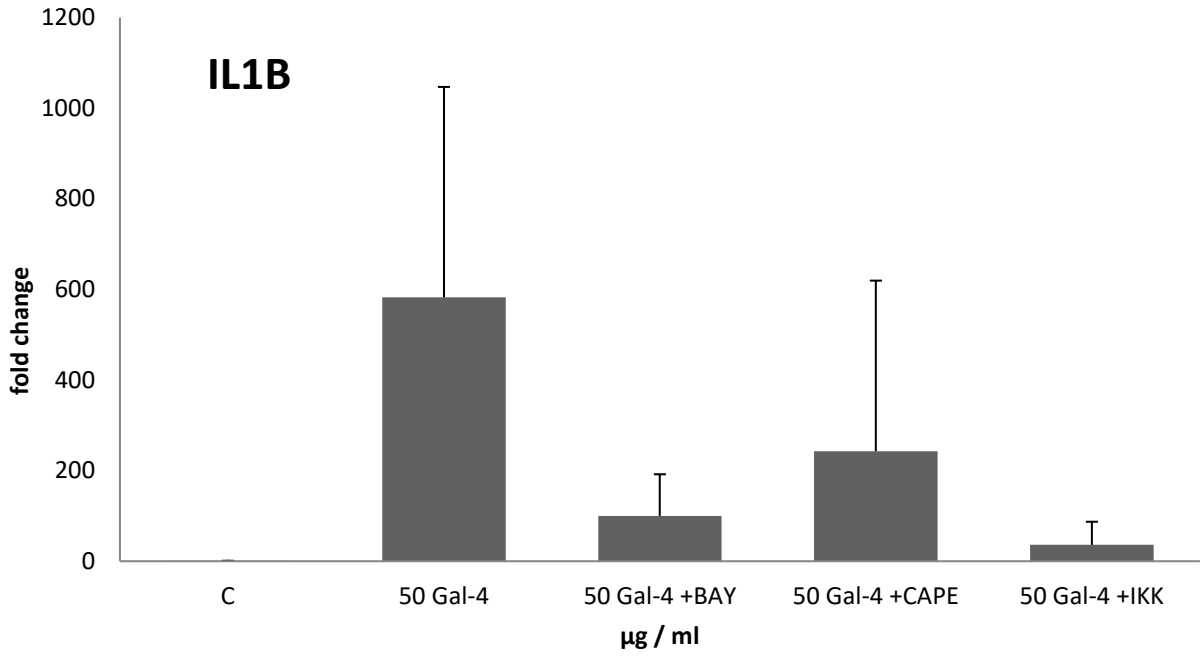

Figure 3.15: Reduction of Galectin-4-induced IL1B mRNA levels by NF-κB inhibitors (BAY 11-7082, IKK inhibitor VII and CAPE). OA chondrocytes (n=3 patients) were treated for 24 hours with 50 µg/ml of Galectin-4, combined with either 4 µM BAY-11-7082, 40µM CAPE or 4µM IKK inhibitor VII. Untreated OA chondrocytes were cultivated under the same conditions and used as control (C). After incubation, the mRNA levels of IL1B were quantified using RT-qPCR with SDHA as reference gene. No statistically significant difference ($p<0.05$) was found using the Wilcoxon-test.

As seen in Figure 3.7, treatment with Galectin-4 increases the expression of MMP13 in OA chondrocytes. Figure 3.16 shows that the effects of Galectin-4 on MMP13 levels were reduced when the cells were additionally treated with NF-κB inhibitors (BAY 11-7082, IKK inhibitor VII or CAPE). A tendency can be seen, although the results were not statistically significant.

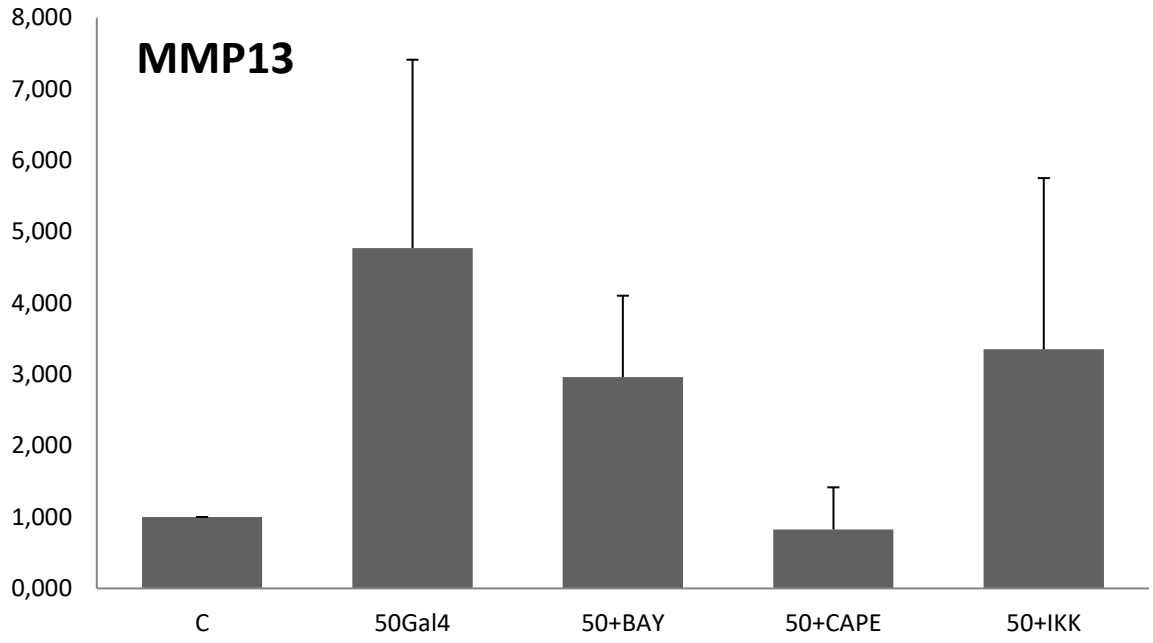

Figure 3.16: Reduction of Galectin-4-mediated MMP13 mRNA levels by NF-κB inhibitors (BAY 11-7082, IKK inhibitor VII and CAPE). OA chondrocytes (n=3 patients) were treated for 24 hours with 50 µg/ml of Galectin-4, combined with either 4 µM BAY-11-7082, 40µM CAPE or 4µM IKK inhibitor VII. Untreated OA chondrocytes were cultivated under the same conditions and used as control (C). After incubation, the mRNA levels of MMP13 were quantified using RT-qPCR with SDHA as reference gene. No statistically significant difference ($p<0.05$) was found using the Wilcoxon-test.

4. Discussion

This study was carried out to determine if Galectin-4 is present in OA cartilage and if it influences the gene expression of IL1B and MMP13 in osteoarthritic chondrocytes. Previous studies have shown that Galectin-1 (Toegel et al., 2016), Galectin-3 (Weinmann et al., 2016) and Galectin-8 (Weinmann et al., 2018) play significant roles in the pathophysiology of OA. The data of those three studies suggested the presence and role of a network of galectins in OA cartilage. As a further member of the galectin family of proteins, Galectin-4 was chosen based on a study (Toegel et al., 2014) that showed that it was one of four galectins (Galectin-1, -3, -8 and -4) that showed a statistically significant difference between mildly and severely damaged cartilage, after a quick score of immunohistochemical detection was performed.

First, articular cartilage specimens of 19 OA patients were immunohistochemically analysed, to evaluate the occurrence of Galectin-4 in OA chondrocytes. The data showed that the percentage of Galectin-4-positive chondrocytes increased with the Mankin score of the affected region. As such, the calculated Pearson´s coefficients showed a positive correlation between degeneration and cell positivity. This would lead to the assumption that Galectin-4 plays a role in degenerative processes of cartilage, similar to those of Galectin-1, -3 and -8 (Toegel et al., 2016; Weinmann et al. 2016 & 2018).
Other immunohistochemical studies (Huflejt et al., 2004; Nagy et al., 2003) showed that Galectin-4 could be used as a biomarker in cancer diagnostics or even plays a cancer inducing role.

To test the cytotoxicity of Galectin-4 on chondrocytes, a metabolic cell activity test was performed after the treatment of cells with 5, 10, 20, 50 and 100 µg/ml of Galectin-4. The treatments did not show negative effects on the metabolic activity, which was important for upcoming RT-qPCR experiments, where those concentrations of Galectin-4 were used. The results show that the higher the concentration of applied Galectin-4 was, the higher were the levels of the expressed IL1B or MMP13 genes. Only at 1 µg/ml there were no statistically significant differences to the control group, in both cases. This could be, because Galectin-4 seems to be less active than the Galectins-1, -3 and -8, as shown by experiments that directly compared the different galectins. In both cases (IL1B and MMP13), Galectin-4 was the least

active substance. Galectin-1 and Galectin-3 were both significantly more active than Galectin-4. Galectin-8 was more active, but not at a statistically significant level.

Further experiments tested the effects of biochemically altered variants of Galectin-4 on gene expression in OA chondrocytes after, always in comparison to a treatment with wild-type Galectin-4. Equal quantities of Galectin-4, Galectin-4-N, Galectin-4-C, Galectin-4-V and Galectin-4-P were used in those treatment. Based on a concentration of 50 µg/ml Galectin-4, the variants were adapted taking their molecular weights in account. Galectin-4 N and -4-C are the N and C carbohydrate recognition domains (CRD) of the tandem-repeat type Galectin-4, respectively. Galectin-4-V has a shorter linker between the 2 CRDs, whereas Galectin-4-P has no linker at all, mimicking a dimer of the prototype group. The experiments suggested that the variants have a stronger effect on the upregulation of IL1B and MMP13 genes than the wild-type Galectin-4. As such, the upregulation of marker genes after the treatment of cells with all variants was significantly higher than in the untreated control groups. However, the upregulation after the treatments with the variants C and N was even significantly higher than after Galectin-4 treatment. This is an interesting observation, because Galectin-4 was still active after losing its bivalent character. Former studies (Toegel et al., 2016) suggested that the ability to crosslink glycans on a cell surface may trigger an outside-in signalling, which might modulate gene expression in OA chondrocytes. A previous study (Stowell et al., 2008) suggests that the single-CRD variants of galectins might form dimers which might restore their ability for crosslinking.

Former studies on Galectins-1, -3 and -8 (Toegel et al., 2016; Weinman et al.,2016; Weinmann et al., 2018) showed that lactose inhibits their binding to the cell surface and therefore alters their gene regulatory activity.

Before OA chondrocytes were treated with 50 µg/ml of Galectin-4, the galectin was mixed with 0.2 M lactose. Lactose would bind to the Galectin-4 molecules and impair galectin binding to the cell surface. This hypothesis was confirmed by 2 experimental approaches. The first experiment showed that the upregulation of the IL1B and MMP13 mRNA levels was reduced to basal levels after mixing galectin with lactose.

In the second experiment, the inhibitory effect of lactose was visualized using FITC-conjugated Galectin-4 and confocal microscopy. The experiment shows that the binding

of Galectin-4 to the OA chondrocyte surface was impaired in presence of lactose. Compared to the Galectins-1, -3 and -8 (Toegel et al., 2016; Weinman et al.,2016; Weinmann et al., 2018), Galectin-4 seems to bind to the cell surfaces to a lesser extent, which could be the reason why it shows less effects than the others. Future studies will need to identify the glycoprotein targets at chondrocyte surfaces that act as binding partners for galectins and trigger cell signalling.

Recent studies on other galectins (Toegel et al., 2016; Weinman et al.,2016; Weinmann et al., 2018) have shown that they activate the NF-κB-pathway which leads to inflammation via upregulation of IL1B gene expression. To test if this is also the case with Galectin-4, OA chondrocytes were treated with Galectin-4 and three inhibitors that block three different parts of the NF-κB-pathway. The experiments showed that after treatment with those inhibitors, the expressions of IL1B and MMP13 genes were attenuated. This leads to the assumption, that Galectin-4 also activates the NF-κB-pathway in OA chondrocytesIn contrast, recent studies (Kim et al., 2013; Maftouh et al., 2014) have shown that
Galectin-4 inhibits the activity of the NF-κB-pathway and therefore alleviates tumorigenesis via downregulation of IL-6 in patients suffering from CRC or pancreatic cancer. It seems that Galectin-4 plays a different role in inflammatory diseases, such as OA, than in tumour diseases. Thus, further experiments need to be conducted to elaborate this assumption of a disease-specific activity of Galectin-4.

5. Conclusion

The results of this thesis suggest that Galectin-4, plays a role in the pathogenesis of osteoarthritis, which is similar to - but less pronounced than - that of Galectin-1, -3 and -8. The immunohistochemical experiments showed, that Galectin-4 is present in OA cartilage and that its presence in chondrocytes correlates with the severity of cartilage degeneration. As inflammation plays a well described role in OA, the dose-dependent upregulation of pro-inflammatory markers after treatment with Galectin-4 indicated that Galectin-4 might have effects on inflammation and matrix degeneration. As inflammation in OA is linked to the activation of the NF-κB pathway, blocking specific mediators of this pathway attenuated the inflammatory effects of Galectin-4, which leads to the assumption that Galectin-4 activates the NF-κB-pathway.
The biochemically altered Galectin-4 variants investigated in this thesis allowed us to test which parts of Galectin-4´s molecular structure are crucial for the observed effects in OA chondrocytes. The single CRDs of the tandem-repeat-type Galectin-4 were even more active than the wild type Galectin-4 with both CRDs, while shortening of the linker length between the CRDs had less impact on the activity. This might indicate that linker length plays a minor role in the activity of Galectin-4 and that the single-CRD variants might form dimers, comparable to the situation described for Galectin-8N (Stowell et al., 2008). However, further experiments need to be conducted to further elaborate these structure-function-relationships.

Taking together, Galectin-4 might be a useful target for the treatment or diagnosis of osteoarthritis in the future.

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7. Appendix

7.1. Abbrevations

C	Control
CRC	colorectal cancer
CRD	Carbohydrate Recognition Domain
DAPI	4’,6-Diamidin-2-phenylindol
EZ4U	easy for you
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
Gal-1	Galectin-1
Gal-3	Galectin-3
Gal-4	Galectin-4
Gal-8	Galectin-8
IL1B	interleukin-1 beta gene
MMP	matrix metalloproteinase
MS	Mankin score
OA	osteoarthritis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
RT	reverse transcription; real time
RT-qPCR	quantitative real time PCR
SDHA	succinate dehydrogenase A
SO	Safranin-O
TNF	tumor necrosis factor

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