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Biological activities of Galectins and biochemical Galectin-modifications in human arthritic Chondrocytes and Synoviocytes

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Abstract

Osteoarthritis (OA) is one of the most common joint diseases and very challenging to treat since there are no disease-modifying drugs available. In recent years, galectins were suggested to play a role in the pathophysiology of OA. Therefore, their effect and the elucidation of corresponding binding partners became of rising interest. In a previous study, it was proven that the glycan pattern in OA cartilage is altered, presumably providing altered binding sites for galectins *(Toegel et al., 2013)*. Another study proved that Gal-1, -3 and -8 were expressed in OA chondrocytes with an increasing tendency during progression of the disease *(Toegel et al., 2014)*. Considering these findings, the current thesis aimed to investigate the in vitro effects of various galectins and biochemically-modified galectin-variants in OA chondrocytes and synoviocytes.

In general, synoviocytes showed limited reactivity towards galectin treatment as compared to OA chondrocytes. The qPCR data in OA chondrocytes indicated an up-regulation of IL1B and MMP13 by all galectins, most effectively by Gal-3 and Gal-8. Moreover, Gal-1 induced a strong and consistent up-regulation of IL1B and MMP13 in OA chondrocytes. As shown in Western blot experiments, the activity of Gal-1 could at least be partially mediated by activation of NF-$_{K}$B signaling.

Further experiments focused on biochemical variants of Gal-1, Gal-3 and Gal-8. The insertion of a linker peptide into the homodimeric structure of Gal-1, as pursued in the Gal-1 variants, did not reveal an impact on the effect of Gal-1 regarding IL1B and MMP13 gene expression in OA chondrocytes. In case of Gal-3 and its variants, the data suggest that biochemical modification of the protein structure impacts on the activity of Gal-3 in OA chondrocytes. Gal-3_N induced a significantly stronger effect on IL1B and MMP13 up-regulation, whereas Gal-3tr revealed a reduced activity as compared to the full-length protein. The variants of Gal-8 – i.e., Gal-8_NF19Y and Gal-8_SF19Y – indicated a significantly lower effect on IL1B and MMP13 up-regulation in OA chondrocytes, suggesting that the naturally occurring single nucleotide polymorphism could impair the effect of this galectin.

The results obtained in current thesis suggest that glycobiology might play a role in OA and provide the first step towards further experiments to elucidate the role of galectins in the context of arthritic diseases.

Zusammenfassung

Arthrose zählt zu einer der häufigsten degenerativen Gelenkserkrankungen und hat, auch auf Grund des Mangels an geeigneter Pharmakotherapie, eine sehr schlechte Prognose. In den vergangenen Jahren stieg das Interesse an Galektinen und ihrem möglichen Beitrag zu Gelenkserkrankungen. Im Zuge dessen widmete sich eine rezente Studie der Identifizierung der Glykanmuster in arthritischen Knorpelzellen. Hierbei wurde gezeigt, dass sich das Glykanmuster in Knorpelzellen im Verlauf einer Arthrose verändert, wodurch veränderte Bindungsstellen für Galektine bereitgestellt werden können *(Toegel et al., 2013)*. Weiters wurde bewiesen, dass Knorpelzellen mit fortschreitender Degeneration vermehrt Gal-1, -3 und -8 exprimieren können *(Toegel et al., 2014)*. Diese Resultate richteten in Folge das Interesse auf die biologische Aktivität von Galektinen und modifizierten Galektin-Varianten in Knorpelzellen und Synovialzellen arthritischer Patienten. In der vorliegenden Arbeit wurden hierfür die Veränderung der Expressionsrate zweier Gene (IL1B und MMP13) herangezogen, die im Knorpelgewebe zu katabolen Prozessen beitragen.

Im Vergleich zu Knorpelzellen, haben Synovialzellen nur eine begrenzte Reaktivität auf eine Behandlung mit Galektinen gezeigt. In Knorpelzellen induzierten alle Galektine, vor allem Gal-1, -3 und -8, die Expression von IL1B und MMP13. In Western Blot Experimenten konnten wir zeigen, dass der starke Effekt von Gal-1 auf dessen Fähigkeit zur Aktivierung des NF-$_K$B Signalwegs zurückzuführen sein könnte. Ein weiterer Fokus wurde auf biochemisch modifizierte Varianten von Gal-1, -3 und -8 gelegt. Im Falle von Gal-1 zeigte die Insertion eines Linker-Peptids in das Homodimer keine Veränderung des Effekts auf IL1B und MMP13 in Knorpelzellen. Die Modifikation von Gal-3 hingegen rief veränderte Effekte in Knorpelzellen hervor. Gal-3_N induzierte eine signifikant höhere Regulierung von IL1B und MMP13, während Gal-3tr eine Reduktionn dieser Gene bewirkte. Die natürlich vorkommende Punktmutation in den Varianten von Gal-8 (Gal-8_NF19Y und Gal-8_SF19Y) führte zu einer Beeinträchtigung der Wirkung von Gal-8 in Knorpelzellen.

Diese Ergebnisse zeigen, dass die Glykobiologie eine Rolle in der Pathophysiologie der Arthrose spielen könnte. Weitere Forschungen sind notwendig, um die Effekte der Galektine im Zusammenhang mit Arthrose aufzuklären.

1. Introduction

1.1. Human articular cartilage

Cartilage is a non-innervated and avascular tissue, excluded from systemic regulation. Human articular cartilage consists of only one type of cells called chondrocytes. These cells are responsible for the maintenance of a stable extracellular matrix that is primarily composed of proteoglycans, water and collagen fibrils. In healthy individuals, cartilage carries important joint-protecting functions. It relieves joints due to distribution of the pressure on the subchondral bone, hinders interaction of bare bones and reduces attrition. Once articular cartilage is damaged, it cannot be renewed in its original form *(Pearle et al., 2005)*.

Human articular cartilage is organized in four zones that differ in structure and function (Fig. 1.1). The superficial or tangential zone constitutes about 10 to 20% of the cartilage thickness and is first affected by damage. This zone has the highest percentage of collagen fibrils, which are packed tightly and run parallel to the articular surface. The middle or transitional zone constitutes the largest amount of cartilage volume with about 40 to 60% and consists of thicker but loosely packed collagen fibrils. The next layer, known as deep or radial zone, includes the highest concentration of proteoglycans. Its collagen fibrils are thinner and longer, arranged vertically to the articular surface. It accounts for about 30 to 40% of the cartilage. The calcified zone builds the transition between cartilage and subchondral bone and interfaces directly with the bone. Deep zone and calcified zone are separated through the tidemark. The structure of human articular cartilage is depicted in the figure below (Fig. 1.1).

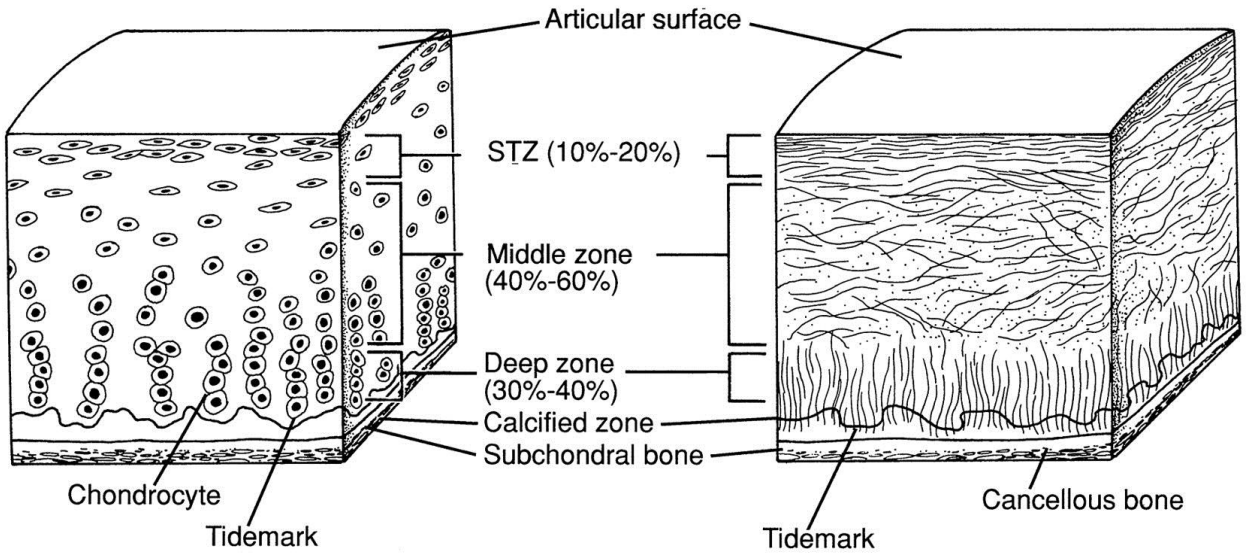

Fig. 1.1. Layered structure of articular cartilage. Left picture: cellular organization, **right picture:** architecture of collagen fibrils. (Taken from http://ajs.sagepub.com/content/26/2/309/F2.large.jpg, 18.05.2015)

Proteoglycans are most important for the conservation of a healthy cartilage. The main type in human articular cartilage is aggrecan, consisting of chondroitin and keratan sulfate. These glycosaminoglycans are due to their negative charge able to bind water and are responsible for compression resistance. Via link to a hyaluronate molecule they build a stable proteoglycan aggregate that is interacting with the collagen fibrils. The subchondral bone rests directly below the calcified zone. These are separated from each other by the so called cement line. It can be directly involved in the metabolism of cartilage and supplies chondrocytes with nutrients through canals extended into the calcified zone *(Madry et al., 2010)*. The following picture illustrates the anatomic structure of a healthy joint (Fig. 1.2.).

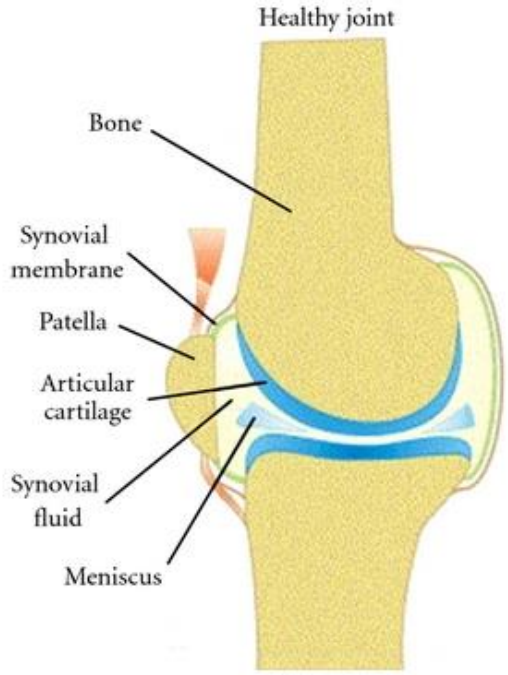

Fig. 1.2. Anatomy of a healthy joint.
(Taken from: http://www.hindawi.com/journals/jnm/2012/673968.fig.001.jpg, 18.05.2015).

1.1.1. Osteoarthritis

Osteoarthritis (OA) afflicts millions of people and is known as one of the most impairing diseases in the elderly population leading to disability. Patients often suffer from severe joint pain, stiffness, swelling and impaired movement. The disease affects the whole joint and comprises degeneration of articular cartilage, subchondral bone and also causes changes in synovial fluid and membrane. OA is usually associated with the family of degenerative diseases and is mainly characterized by a progressive cartilage damage and catabolism. The disease is primarily affecting weight-bearing joints, such as hips or knees. The prevalence of OA increases with age. However, younger people with genetically determined fragility of cartilage matrix, or regular stress on a particular joint, can also be affected from this disease. The pathogenesis of OA is still unexplained. In the end stage of this process, a total joint replacement is often inevitable due to functional impairment and a compromised life quality of the patients. OA is very challenging to treat, because once articular cartilage is damaged, its regenerative capacity is very poor due to a low metabolic activity of chondrocytes. The progression of OA is directly linked to an uncontrolled

proteolysis and change of the extracellular matrix, whereby cartilage loses its resilience to mechanical load and subjacent zones are thereby exposed to more pressure. Secondary to alterations in articular cartilage, a remodeling of the affected joint appears as a failed trial to improve the situation, which can result in osteophyte formation and/or bone thickening *(Heinegård et al., 2010)*. During the progression of OA, articular cartilage becomes thinner, changes its surface or can partially be completely degenerated. Furthermore, it comes to friction of the bare bones, joint space narrowing and reduction of synovial fluid that normally provides nutrients for the cartilage. Thickening of bone and capsule, inflammation of synovia, subchondral bone sclerosis and osteophyte formation are also features associated with OA. Osteophytes are bony extensions on the edge of a joint that are trying to enlarge its bearing area to reach joint relief. An osteoarthritic joint represents a thin cartilage layer with a thickened bone suggesting that micro-cracks in the subchondral bone are responsible for shifting up the tidemark due to increased reactivation and sclerosis formation *(Sharma et al., 2013)*. The following picture presents the anatomy of an osteoarthritic joint (Fig. 1.3.).

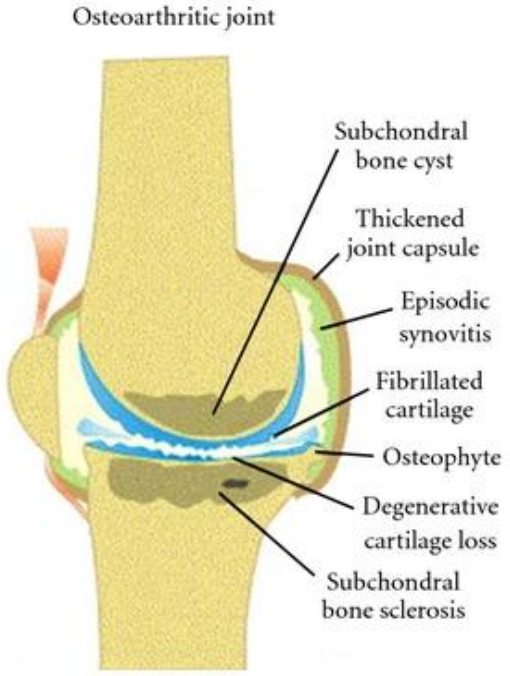

Fig. 1.3. Anatomy of an osteoarthritic joint.
(Taken from: http://www.hindawi.com/journals/jnm/2012/673968.fig.001.jpg, 18.05.2015)

It is still not possible to determine whether the process of the disease has its origin in the cartilage or in the underlying bone. A difficulty in dealing with this issue is that clinical manifestations of OA are recognized very late when alterations are already became too advanced *(Heinegård et al., 2010)*. The subchondral bone was revealed to carry a big role in load transmission and gives reason to consider OA not only as a cartilage disease. Furthermore, an interplay between both has been suggested due to formation of micro-cracks that may facilitate the crossover of molecules from one to the other *(Sharma et al., 2013)*.

1.1.2. Osteoarthritis as an inflammatory disease

For many years OA has been classified as a mechanically induced degenerative joint disease primarily characterized by cartilage damage. In recent years, however, an inflammatory theory became of interest since many studies discovered that cartilage breakdown may be partially induced through pro-inflammatory cytokines modulating the metabolism of chondrocytes to increase matrix metalloproteinase (MMP) synthesis *(Pearle et al., 2005; Martel-Pelletier, 2004)*. Especially MMP-13 was revealed a collagenases to be primarily involved in cartilage degradation *(Martel-Pelletier, 2004)* and shown to be synthetized by human chondrocytes *(Reboul et al., 2011)*. Among cytokines, IL-1β and TNF-α were revealed to be leading mediators of inflammation during OA *(Kobayashi et al., 2005)*, both able to increase MMP-13 levels and to activate catabolic pathways *(Fernandes et al., 2002)*. IL-1β is a cytokine protein encoded by the IL1B gene in humans. Furthermore, an imbalance between the synthesis of MMPs and its tissue inhibitors, directed towards MMPs in osteoarthritic chondrocytes, was already evidenced many years ago *(Dean et al., 1989)*. MMPs seem to play an important role in cartilage degradation. Moreover, inflammatory cytokines were found to be capable of activating the NF-$_{\kappa}$B pathway particularly in the superficial zones of OA cartilage *(Fan et al., 2007)*. This activated pathway plays an important role in inflammation and is in turn able to further increase IL-1β and TNF-α levels and to create a positive feedback loop (section 1.2.1.). Although the inflammation level in OA is not as high as in rheumatoid arthritis (RA), it has been repeatedly evidenced that inflammatory reactions occur during early and late OA and may contribute to the pathogenesis of this disease *(Benito et al., 2005)*.

1.1.3. The role of synovitis in osteoarthritis

The highly vascularized synovial membrane, consisting of synoviocytes, is physiologically important for the maintenance of a healthy cartilage. Via synovial fluid, the cartilage is supplied with nutrients and joint space is provided. Furthermore, it is responsible for the removal of products that occur during matrix degradation *(Sellam et al., 2010)*. As presented in Figure 1.3., during progression of OA, an inflammation of the synovial membrane resulting in synovitis may occur and can be evidenced by the existence of clinical symptoms, such as joint swelling, sudden pain increase and morning stiffness *(Krasnokutsky et al., 2008)*. In OA, angiogenesis in the synovial membrane was shown to be increased, which may support inflammation or vice versa *(Walsh et al., 2007)*. Furthermore, synovial hypertrophy and hyperplasia were additionally revealed exposed through an increase of synovial lining cells *(Myers et al., 1990)*. The existence of synovitis in patients with early OA could be already proved *(Benito et al., 2005)*. Still the question remains whether the changes in synovial membrane are primary or the consequence of cartilage degradation and joint inflammation. In OA patients, synovitis was only found at sites that are linked to degenerative cartilage, which may suggest that synovitis could be triggered through cartilage-breakdown products that immigrate into synovial fluid *(Ayral et al., 2005, Sellam et al., 2010)*. The role of synovitis in the pathogenesis of OA still needs to be elucidated in detail. An understanding of this context would provide new insights into therapy options.

1.1.4. Therapy of osteoarthritis

Although OA is one of the leading causes for disability and joint replacement, there is no disease-modifying therapy until now. The treatment is very challenging since the diagnosis of OA at an early stage constitutes a fundamental difficulty. The disease is often not clinically recognized until anatomic changes already reached an advanced stage. Efforts to find techniques for an earlier OA diagnosis also have to be pursued. To date, the pharmacological treatment of OA encompasses only a symptomatic pain relief. The table below shows commonly used pharmaceuticals in the therapy of OA including advantages and disadvantages of each (Tab. 1.1.).

Pharmaceuticals	Effect	Advantage	Disadvantage
Acetaminophen (Paracetamol)	Analgesic	No gastrointestinal (GI) bleeding	Hepatotoxicity, rarely anti-inflammatory effect
Non-steroidal anti-inflammatory Drugs (NSAIDs); e.g.: Naproxen, Ibuprofen	Cyclooxygenase (COX)-Inhibition (Analgesic, anti-inflammatory)	Stronger analgesic effect	GI bleeding
Selective NSAIDs; e.g.: Rofecoxib	COX-2-Inhibition (Analgesic, anti-inflammatory)	Thought to cause less GI bleeding	GI bleeding, cardiovascular concerns
Opiod analgesics, e.g.: Tramadol	Opiod receptor inhibition	Strong analgesic effect	Opioid addiction, elderly people
Intra-articular injection of steroids, e.g.: Cortisol	Glucocorticoid receptor inhibition (Anti-inflammatory, immunosuppressive, analgesic)	long lasting effect (2-4 weeks)	Disease progression, Osteoporosis

Tab. 1.1. Pharmaceutical drugs applied in OA.

Non-pharmaceutical interventions include a regular physical activity, loss of body weight and particularly body fat reduction to relief weight-bearing joints and to decrease the level of adipokines released by adipose tissue. Other possibilities encompass intra-articular injection of hyaluronic acid for an improved gliding and lubrication, or treatment with glucosamine and chondroitin sulfate products. For the last mentioned, a safe long-term use was reported but their efficacy remains controversial *(Zerkak et al., 2004)*. In most cases, a total joint replacement is inevitable to release patients from their suffering and increase their life quality. The development of disease-modifying drugs to provide symptom relief and delay OA progression is an important challenge in medical treatment. Therefore, key targets that are involved in catabolic mechanisms of OA are of interest. Experimental studies were already done towards MMP- and -NF-κB-inhibitors, as well as inhibitors of interleukin-converting enzyme (ICE). The ICE activates IL-1β that in turn is able to evocate a positive feedback loop through NF-κB activation. Furthermore, IL-1β and TNF-α receptor antagonists that are already successfully established drugs in RA treatment

(Mertens et al., 2009; Taylor et al., 2009) are suggested as disease modifiers in OA. It will take many more investigations on both, the pathogenesis and the treatment of OA to integrate successful drugs that may stop the progression and relief patients from severe pain.

1.2. Rheumatoid Arthritis

RA is classified as a rheumatic, inflammatory joint disease with participation of the autoimmune system. Contrary to OA, the synovium is the central area of the pathogenesis. The disease is characterized by inflammation of the synovial membrane, synovial hyperplasia, bone and cartilage degradation, as well as extra-synovial manifestations concerning the lungs, heart, eyes and causing nodular alterations of the skin. Patients suffer from severe joint pain, particularly in the morning from joint stiffness, swelling, overheating and reddening. Along with that, the inflammatory component can cause fever and general weakness, tiredness and anorexia. RA is affecting, contrary to OA, younger people and is one of the most common rheumatic diseases. The pain is initially first noticed in small joints of the hands and feet but in progression the disease affects more than just one joint. Typically, RA appears batch-wise in periods of high activity, in which the inflammation level increases abruptly. This flare can last for weeks or months until it decays. When the inflammation decreases again, a period of low disease activity starts, in which patients experience reduced pain and faded symptoms. This process makes RA challenging to treat. The pathogenesis is not fully explained but is suggested to be multifactorial including genetic and environmental factors. A leading role is attributed to an abnormal response of the autoimmune system and inflammation of the synovial membrane. The healthy synovial membrane adjacent to articular cartilage consists of an intimal lining layer and a sublining layer of very small thickness containing mainly synovial fibroblasts. The latter mentioned cells are the main secretor of hyaluronic acid into the joint place. Important functions of this membrane encompass cartilage lubrication and gliding, nutrition of chondrocytes and zoning of synovial fluid and its volume maintenance *(Smith, 2011)*. In RA, the intimal layer is thickened and becomes abnormally proliferative and infiltrated due to hyperplasia. Interactions with the immune system recruit T and B lymphocytes and macrophages that result in cytokine production in the synovial membrane. This processes lead to the generation of an inflamed synovial pannus tissue as a major source of cytokines, proteinases and cartilage damage. Synovial fibroblasts in RA were considered

to play the leading role in immune cell recruitment, increased vascularity, maintenance of pannus tissue and also MMP synthesis and secretion *(Abeles et al., 2006)*. The cellular interactions are complex, self-amplifying and trigger a vicious circle. Cytokines like TNF-α, IL-1β and other interleukins are produced by many cells during the RA process. Interleukin in turn amplifies inflammation, induces proteinases synthesis and activates chondrocytes, as well as precursors of bone resorbing osteoclasts. The tightly assigned processes during pathophysiology of RA are demonstrated in the next picture using the example of IL (Fig. 1.4.).

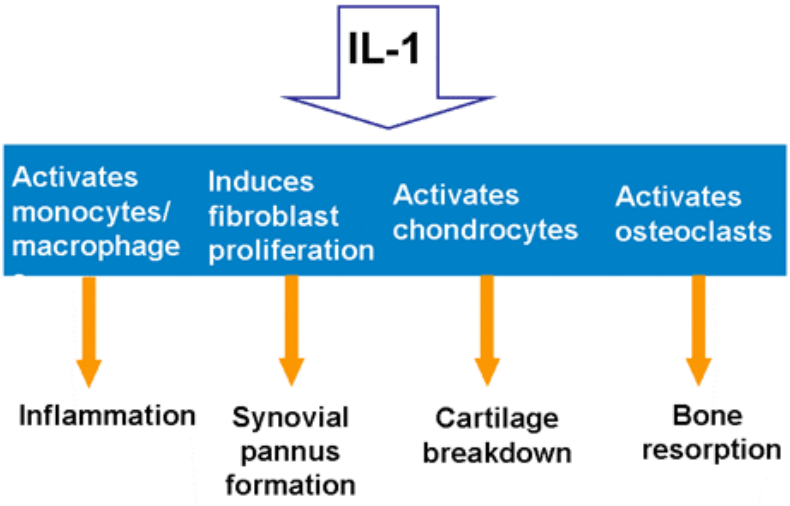

Fig. 1.4. Pro-inflammatory cytokine IL-1 entailing diverse effects in RA.
(Taken from: http://img.medscape.com/fullsize/migrated/editorial/casecme/2008/18719/markenson.fig3.gif, 20.05.2015)

Similarly to OA, the progression of RA leads to cartilage breakdown, bone damage, joint place narrowing and of course severe synovitis as hallmark. The complex cell interplay in RA is not clarified until date. Various cell types and processes are involved. The NF-$_{\kappa}$B signaling pathway is accepted to contribute to the pathogenesis of the disease. Therefore, the following section (1.2.1.) will provide an insight in its activation and effect.

1.2.1. The role of NF-$_{\kappa}$B pathway in inflammation

Focusing on the canonical pathway, NF-$_{\kappa}$B is a ubiquitous transcriptional factor assembled of a heterodimer with a p65/Rel A and a p50 subunit. In its inactivated status the protein is localized in the cytosol and its transcriptional features are blocked by the inhibitor of NF-$_{\kappa}$B (I$_{\kappa}$B-α). The activation of the canonical NF-$_{\kappa}$B pathway can be triggered by many factors, such as cytokines, bacteria, viruses or stress. These are binding to receptors on its surface and initialize the phosphorylation of I$_{\kappa}$Bα by the inhibitor of NF-$_{\kappa}$B kinase (IKK). The inhibitor further dissociates from NF-$_{\kappa}$B and the activated heterodimers translocate into the nucleus and bind to specific responsive elements *(Marcu et al., 2010)*. This binding results in activation of specific target genes such as cytokines and inflammatory mediators, as well as matrix digesting enzymes. Due to a positive feedback loop, these products entail a further activation of the NF-$_{\kappa}$B signaling pathway. These processes demonstrate the role of NF-$_{\kappa}$B in inflammatory diseases and justifies the ambition to find therapeutic strategies that are aimed to block or modulate this signaling pathway. Beside RA, many other chronic inflammatory diseases are associated with NF-$_{\kappa}$B activation, such as asthma, chronic obstructive pulmonary disease or multiple sclerosis. Furthermore, this pathway is also assumed to play a role in OA. The following picture illustrates the activation of the canonical pathway and also shows the subunits of the IKK complex (Fig. 1.5.).

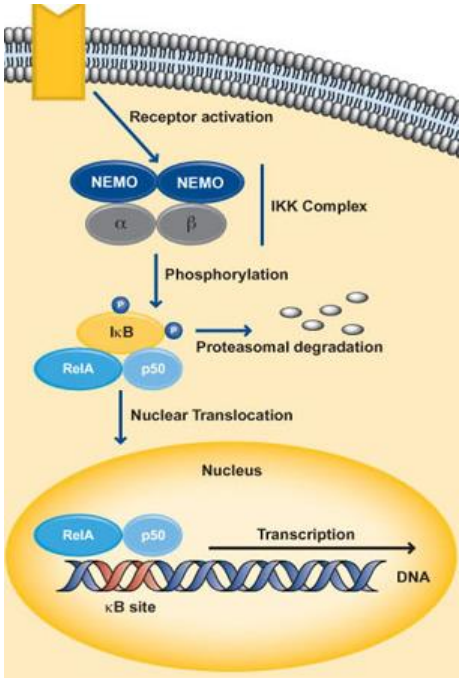

Fig. 1.5. NF-$_{\kappa}$B signaling pathway.
Receptor activation through cytokine binding leads to I$_{\kappa}$Bα phosphorylation and degradation by IKK. The activated heterodimers translocate into the nucleus and initialize transcription of target genes. (Taken from: http://a.static-abcam.com/CmsMedia/Media/abwire-diagram01---short.jpg, 20.05.2015)

1.2.2. Therapy of rheumatoid arthritis

In the last years, particular focus was set on the treatment of RA, although it affects less people than OA. Albeit there is still no cure provided for RA, the therapy includes not just pain relief, but also drugs that can delay the progression and limit damage development. The aim is to increase the life quality of patients and to extend terms of low disease activity. Beside non-pharmaceutical measures that include a regularly physical activity, healthy diet and loss of body weight, there are also different pharmaceutical drugs available that have to be adapted to the individual course of the disease in each patient. Medical treatment encompasses drugs of various classes including NSAIDs, disease-modifying anti-rheumatic drugs (DMARDs), glucocorticoids and biologicals. DMARDs and biologicals have immunosuppressive and progression slowing effects, while NSAIDs just bring pain relief. Biologicals are inhibitors of TNF-α or IL-1 that are meanwhile well-integrated in the therapy of severe rheumatoid arthritis. The table below lists common used drugs and some of their possible side effects (Tab. 1.2.).

Drug class	**Pharmaceuticals**	**Side effects**
NSAIDs	Diclofenac, Ibuprofen, Naproxen, acetylsalicylic acid	Gastrointestinal bleeding and ulceration
DMARDs	Methotrexate, Leflunomide, Azathioprine, Cyclosporine, Sulfasalazine, Hydroxychloroquine	Hematotoxicity, renal insufficiency, lung infections, fever, gastrointestinal complications
Biologicals	Infliximab, Adalimumab and Etanercept (TNFα) Anakinra (IL-1)	Reactivation of tuberculosis or hepatitis, incompatibility, hypertension
Glucocorticoids	Prednisolone, Dexamethasone, Methylprednisolone	Osteoporosis, disturbance of growth in children, manifestation of diabetes mellitus, hypertension

Tab. 1.2. Pharmaceuticals drugs applied in RA.

The treatment is complex and often requires a combined therapy to aim a desired effect. The following figure will demonstrate the treatment scheme pursued in RA (Fig. 1.6.).

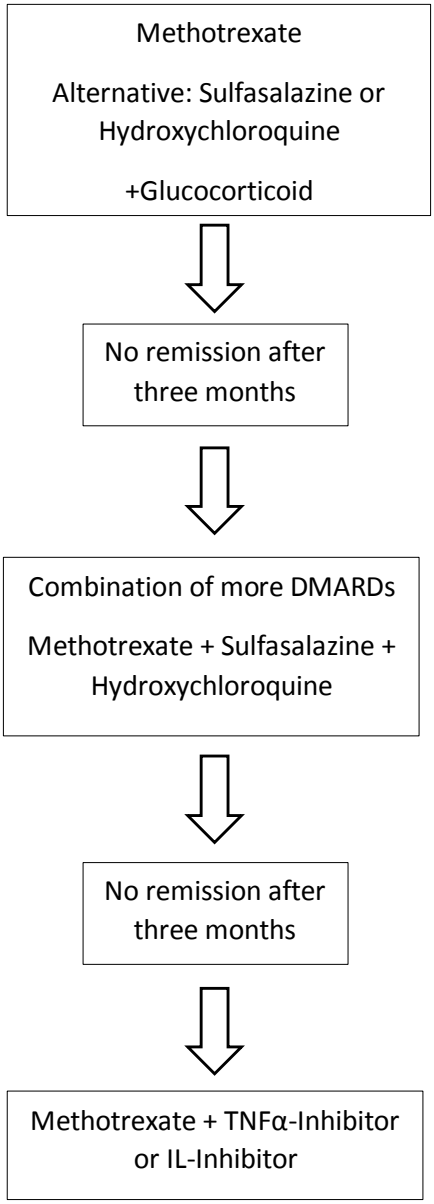

Fig. 1.6. Treatment scheme in RA. Initial therapy starts with methotrexate known as the gold standard in medical RA treatment and a glucocorticoid. In case of missing remission, combination of more DMARDs are necessary. Further absence of remission requires combination with a biological drug (cytokine inhibitor). If needed, an NSAID is prescribed additionally.

1.3. The family of galectins

Galectins, a subgroup of lectins, are a family of animal carbohydrate-binding proteins that all share a specifically binding affinity for β-galactoside carbohydrates and a conserved amino acid sequence in their binding site *(Barondes et al., 1994)*. Currently, 15 mammalian galectin members have been already described *(Liu et al., 2010)*, all containing a specific carbohydrate-recognition domain (CRD) that is responsible for their binding activity. This CRD encompasses a domain of about 130 amino acids *(Barondes et al., 1994)*. Galectins are classified into three subfamilies, based on the structure of the polypeptide chain: prototypical, tandem-repeat- and chimera-type galectins. The first group includes Gal-1, -2, -5, -7, -10, -11, -13, -14 and -15. These galectins have one single CRD and can build non-covalently homodimers through dimerization of the CRD. Tandem-repeat-type galectins are identified as Gal-4, -6, -8, -9 and -12 containing two homologous or different CRDs within one polypeptide chain that are connected by a linker consisting of about 70 amino acids. Gal-3 is the only member belonging to the chimera-type subfamily. It consists of one CRD linked to a non-lectin extended N-terminus of tandem-repeated segments encompassing about 120 to 130 amino acids. This terminus enables oligomerization of this lectin to pentamers. The following picture illustrates the three different subfamilies in which galectins are grouped (Fig. 1.7.).

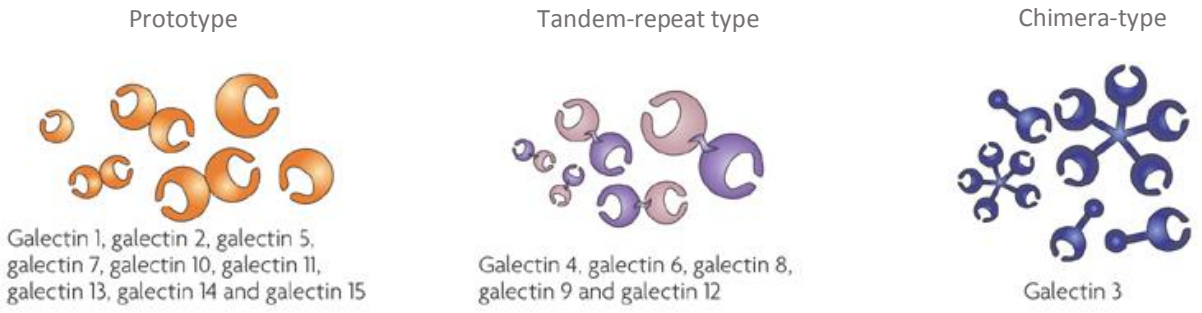

Fig. 1.7. Structure and classification of galectins into three subfamilies. Prototype galectins consisting of one CRD and building homodimers. Tandem-repeat-type galectins containing two CRDs connected via linker. Gal-3 having one CRD linked to an N-terminus forming pentamers.
(Taken from: http://www.nature.com/nri/journal/v9/n5/images/nri2536-f1.jpg, 21.05.2015)

Galectins can be bivalent or multivalent due to the presence of two CRDs or as a result of polymerization, respectively. Thus, they are able to form two- and three-dimensional cross-linked lattices with carbohydrates *(Brewer, 2002)*. The next picture presents lattice formation of galectins (Fig. 1.8.).

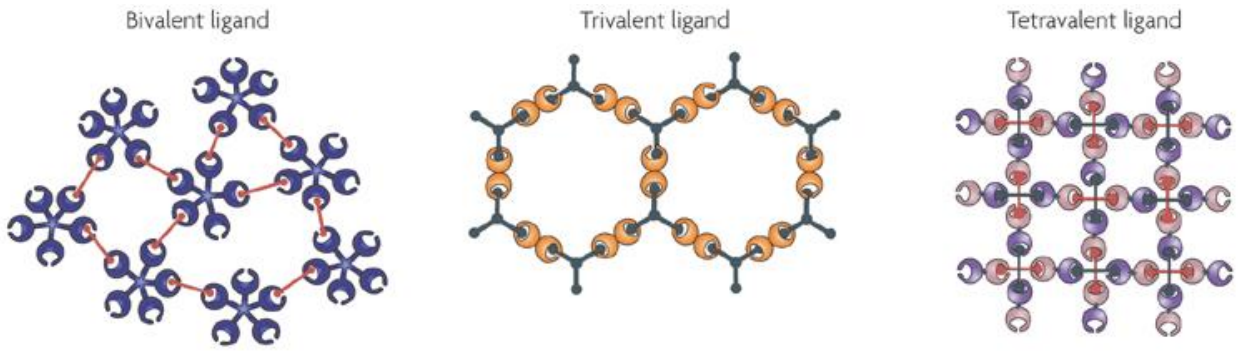

Fig. 1.8. Galectins forming lattices with glycans. Chimeric-type Galectin-3 binding to a bivalent ligand. Prototypical-homodimers forming a lattice with a trivalent ligand. Tandem-repeat galectins binded to tetravalent ligands. (Taken from: http://www.nature.com/nri/journal/v9/n5/images/nri2536-f1.jpg, 21.05.2015).

1.3.1. Galectins and their binding partners - small insight into the glycobiology

In the last years, galectins were shown to have various intracellular but also extracellular effects, which aroused the interest in characterizing their binding partners. Galectins are not able to be secreted through the usual pathway due to the absence of a signal sequence, but many galectins are found to be abundantly secreted into the extracellular place *(Hughes, 1999)* where their function is mainly dependent on their lectin feature. Thus, to understand mechanisms involving galectins, the science of glycobiology is inevitable, since galectins are known to bind to glycan chains, which are specific polysaccharide structures. Carbohydrates are ubiquitous and individual in every cell. The attachment of specific oligo- or polysaccharide chains to proteins, is called glycosylation and happens post-translationally as a protein modification due to the activity of specific glycosyltransferases. This process is very important, since it grants individuality in recognition, immunogenicity and biological activity. In previous studies, it was revealed that many factors, i.e. cytokines, are able to alter the cellular glycophenotype and thus provide altered surface receptors *(Brockhausen et al., 1998, Yang et al., 2007)*, suggesting that the glycan pattern might play an important role in the pathophysiology of many diseases. As mentioned above,

galectins were revealed to have multivalent binding properties and to form glycan-lattices *(Brewer, 2002)*. Thus, they were suggested to be involved in biological signal transduction in their interaction with multivalent glycoprotein-receptors. This was e.g. proven for Gal-3, whose pathogenetic extracellular role in joint tissues was just induced after forming multivalent lattices with surface glycoproteins, but not as a monomer *(Janelle-Montcalm et al., 2006)*. Since glycoproteins are known to be essential and abundant in the maintenance of extracellular cartilage matrix, the assumption that changes in the glycan pattern of human chondrocytes could play a role in OA became of growing interest. In a previous study, it was demonstrated that the glycophenotype of OA chondrocytes was actually altered, presumably providing binding sites for galectins during the progression of the disease *(Toegel et al., 2013)*. This small insight into the glycobiology should facilitate the demonstration of the relation between galectins, their carbohydrate binding partners and their role in OA pathophysiology before focus is set on particular galectin members.

1.3.2. Different galectins and their functions

Galectins have been implicated in numerous different functions including cell protection, disease pathogenesis, cell regulation, activation of inflammation and many more. Due to this, galectin-targeting became of interest as a possibility for new treatment options. Therefore, the unveiling of the various galectin functions is inevitable. The most extensive investigations were carried out on Gal-1 and Gal-3. Far less is known about the other family members.

1.3.2.1. Galectin-1

The first discovered prototypical Gal-1 consists of a 14 kDa monomer and a single CRD. Human Gal-1 occurs as a homodimer in solution but can also dissociate into a monomer. Its dimerization was shown to be not required for a high-affinity binding *(Salomonsson et al., 2010)*. The main cellular functions are not fully known, but intracellular and also extracellular activities were described for the protein. For the latter mentioned, its ability to from lattices with carbohydrates and in turn to affect intracellular signal trafficking plays an insistently role. In contrast, its intracellular activity is independent of the lectin feature. Many binding partners and effects were described for Gal-1 including regulation of immune responses in T-cells, inflammation and cancer

genesis. A relation between an overexpression/expression of Gal-1 in cancer cells and the malignant progression of tumors was reported in many studies *(Camby et al., 2006)*. These suggested that the intracellular apoptotic effect of Gal-1 in T-cells *(Pace et al., 1999)* might contribute to the progression of cancer diseases. In vivo, Gal-1 treatment in a mice model effected improvement of RA due to apoptosis of T-cells *(Rabinovich et al., 1999)*. This was the first link reported between RA and Gal-1. In further studies, down-regulated Gal-1 levels in RA synovium were correlated with a progression of the disease. Intracellular Gal-1 was shown to have an anti-inflammatory and immunosuppressive effect, while its extracellular effects were revealed to play an emerging role in inflammation processes *(Liu, 2000)*. Since it was also proven to be able to bind to components of extracellular matrix and inhibit the assembly of e.g. chondroitin sulfate *(Moiseeva et al., 2003)*, Gal-1 was also suggested to play a role in OA. This hypothesis was pursued and evidenced in following studies where the existence of Gal-1 and its induction of matrix degenerating enzymes could be revealed in porcine cartilage *(Marcon et al., 2005; Marsich et al., 2008)*. The expression of Gal-1 in human OA chondrocytes could further be evidenced *(Toegel et al., 2014).* Its role in joint tissues therefore became of big interest. The following picture shows the structure of the human Gal-1 protein (Fig.1.9.).

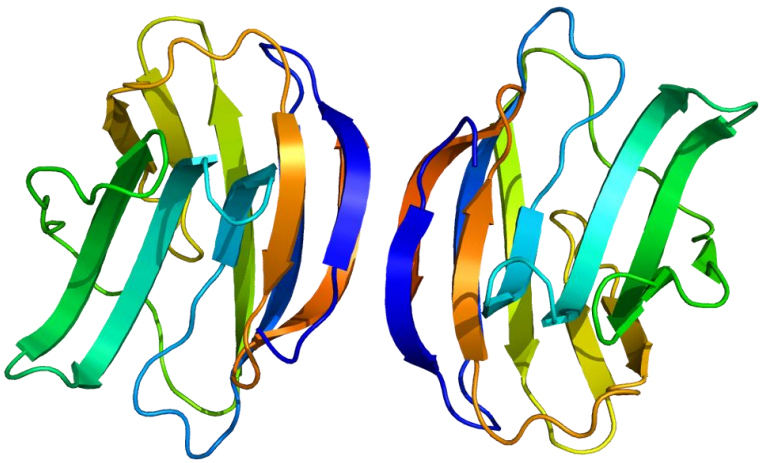

Fig. 1.9. Structure of a homodimeric human Gal-1.
(Taken from: http://upload.wikimedia.org/wikipedia/commons/a/a2/Protein_LGALS1_PDB_1gzw.png, 22.05.2015)

1.3.2.2. Galectin-3

This 30 kDa galectin is the only member belonging to the chimera-type subfamily of galectins. It is assembled of one CRD and an extended non-lectin N-terminus that consists of a proline-glycine-alanine-tyrosine repeat motif of about 100 amino acids and a short end of about 30 amino acids. In solution, Gal-3 occurs as a monomer, but via its N-terminus it is able to form pentamers in the presence of multivalent carbohydrate ligands. Due to its ability to crosslink glycans, it can initiate signal transduction on the cell surface. Similarly to Gal-1, its expression in many cancer tissues could be related to their malignancy *(Liu et al., 2005)*. In contrast to Gal-1, intracellular Gal-3 was shown to be anti-apoptotic *(Yang et al., 2003)*. Gal-3 was also revealed to play a role in joint tissues. It was proven to be markedly present in OA synovial tissue during the inflammatory phase and contribute to the activation of synovial fibroblasts *(Ohshima et al., 2003)*. Furthermore, it was shown to increase the secretion of cytokines and MMPs in synovial fibroblasts and promote inflammation in RA *(Filer et al., 2009)*. The role of extracellular Gal-3 in OA was also investigated. Levels of the protein were found to be increased in OA chondrocytes *(Guévremont et al., 2004)*. Additionally, exogenous multimerized Gal-3 was revealed to induce MMPs in human OA chondrocytes and induced swelling of mice's knee joints *(Janelle-Montcalm et al., 2006)*. An increasing Gal-3 level in chondrocytes during OA progression was also evidenced *(Toegel et al., 2014)*. In contrast to this and underscoring the various galectin functions, in an in vivo mouse model of OA, Gal-3 was found to play a key protective role *(Boileau et al., 2008)*. The following figure shows the structure of Gal-3 (Fig. 1.10.).

Fig. 1.10. Structure of a monomeric human Gal-3.
(Taken from: http://www.imperial.ac.uk/research/animallectins/ctld/images/galectin%203.gif, 23.05.2015)

1.3.2.3. Galectin-8

The tandem-repeat type Gal-8 consists of two carbohydrate-recognition domains that are connected to each other by a linker of about 70 amino acids. It can occur in six isoforms. Three of these have two CRDs and the other three have only one CRD. Gal-8 was revealed to have a high affinity to integrin receptors on the cell surface and therefore be able to induce cell growth or apoptosis *(Levy et al., 2001)*. In the context of joint tissues, Gal-8 was detected in synovial RA fluid at concentrations that can lead to apoptosis of synoviocytes *(Eshkar Sebban et al., 2007)*. The expression of Gal-8 in OA chondrocytes was revealed to be increased during the progression of the disease *(Toegel et al., 2014)*. Interestingly, an observed overexpression of Gal-8 in mouse models was correlated with an increased osteoclast activity and further loss of bone mass *(Vinik et al., 2015)*.

1.3.2.4. Galectin-2, -4, -7, -9

These galectins have been far less investigated. Gal-9 was recently suggested to play a suppressive role in RA since it was revealed to induce apoptosis of synovial fibroblasts (Seki et al., 2007). In the same study, it was shown that Gal-9 in the synovial tissue of RA patients was much higher expressed than in OA patients.

1.3.3. Variants of galectins

As mentioned above, the expression of galectins was proven to play a role in diverse inflammatory diseases, cancers and immune processes. This led to the assumption that targeting this proteins might result in the finding of promising treatments for many diseases, including also OA and RA. Efforts were pursued in characterizing the particular section within each full length protein that is actually contributing to mediation of a cellular response. Therefore, non-natural galectins were engineered to determine structure-function relationships.

1.3.3.1. Variants of Galectin-1

Variants included in the current thesis encompass Gal-1_GG, Gal-1_8S and Gal-1_8L. As already mentioned, Gal-1 consists only of one carbohydrate-recognition domain and can non-covalently homodimerize. The engineering of non-natural variants of this protein focused on turning the prototypical Gal-1 into covalently linked artificial tandem-repeat type products. This was performed by insertion of linker peptides with various lengths into the homodimeric structure of Gal-1. In case of Gal-1_GG, a minimal linker length consisting of two peptides was inserted. The artificial linker of Gal-1_8S encompasses a length of 33 peptides. In Gal-1_8L a linker consisting of 75 peptides was inserted *(Zhang et al., 2015)*. The following figure schematically presents the artificial products of tandem-repeat Gal-1 (Fig. 1.11.).

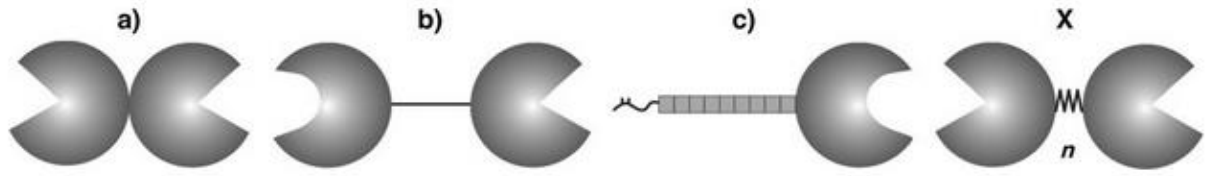

Fig. 1.11. a) Prototypical galectin, **b)** tandem-repeat type galectin, **c)** chimeric-type galectin. Gal-1 variants are engineered by turning the homodimeric structure of Gal-1 (a) into artificial covalently linked tandem-repeat products (**X**) with various lengths of linker peptides (n): n=2 (GG), n=33 (8S) and n=75 (8L).
(Taken from: Zhang et al., 2015)

1.3.3.2. Variants of Galectin-3

Variants of this protein include Gal-3_B1, Gal-3_C, Gal-3_CN, Gal-3_N and Gal-3tr. The full length protein is assembled of one CRD linked by a large tail of about 100 amino acids to an N-terminus. This tail consists of collagen-like repeats divided into nine sections (I-IX). Non-natural protein variants were produced by gradually dissecting the sections of this tail and engineering modified proteins with different truncation degrees of it *(Kopitz et al., 2014)*. The following picture presents the various variants of human Gal-3 (Fig.1.12.).

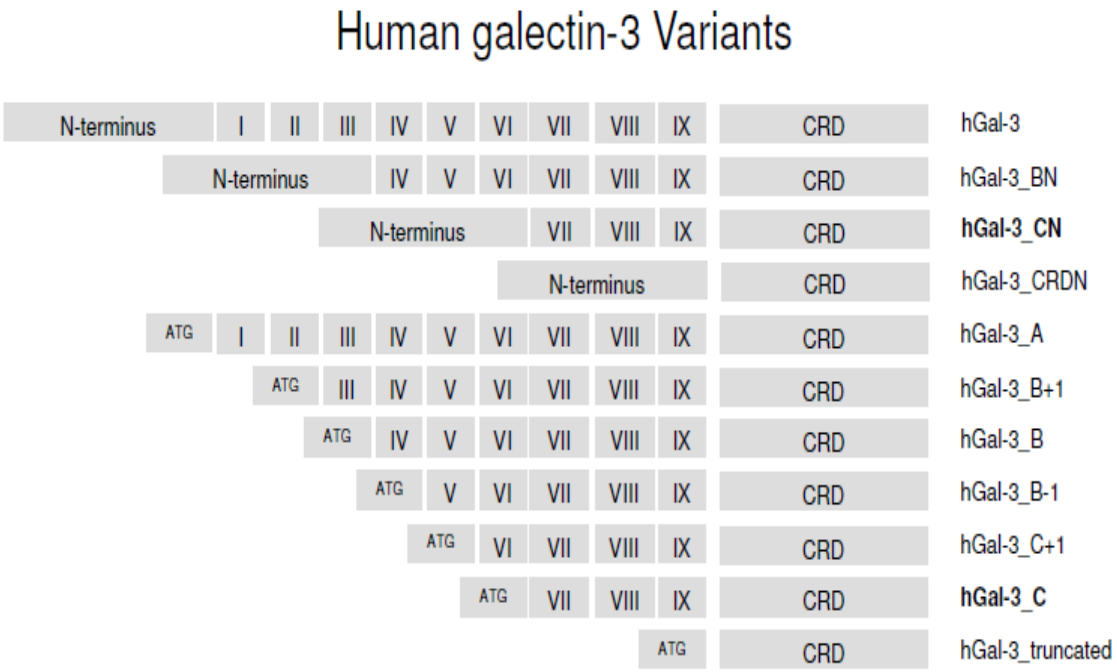

Fig. 1.12. Variants of human Gal-3 (hGal-3). The full length protein consists of 9 sections (I-IX), one CRD and an N-terminus peptide. A gradually truncation of the sections delivers the different variants. Not included in this thesis are hGal-3_BN, hGal-3_A, hGal-3_B+1, hGal-3_B and hGal3_C+1. Included in my thesis are hGal-3_CN (=**Gal-3_CN**), hGal-3_CRDN (=**Gal-3_N**), hGal-3_B-1 (=**Gal-3_B1**), hGal-3_C (=**Gal-3_C**) and hGal-3_truncated (=**Gal-3tr**).
(Image kindly provided by H. J. Gabius)

1.3.3.3. Variants of Galectin-8

Here, the variants Gal-8_SF19Y and Gal-8_NF19Y were included. The term "F19Y" describes a naturally occurring point mutation in the N-terminus of the human Gal-8. Thereby, it comes to a single nucleotide base change. Tyrosine (Y) is substituted for phenylalanine (F) at position 19. Gal-8_**S**F19Y is the human full length Gal-8 having the single nucleotide polymorphism. Gal-8_**N**F19Y is only the N-terminus of the galectin with its point mutation at position 19. *(Ruiz et al., 2014)*. The following pictures show a comparison of the wild-type N-domain of Gal-8 and the F19Y mutant (Fig. 1.13.). Figure A illustrates the unchanged wild type N-terminal. Figure B shows the mutant superposed to the respective structure of the wild-type of Gal-8.

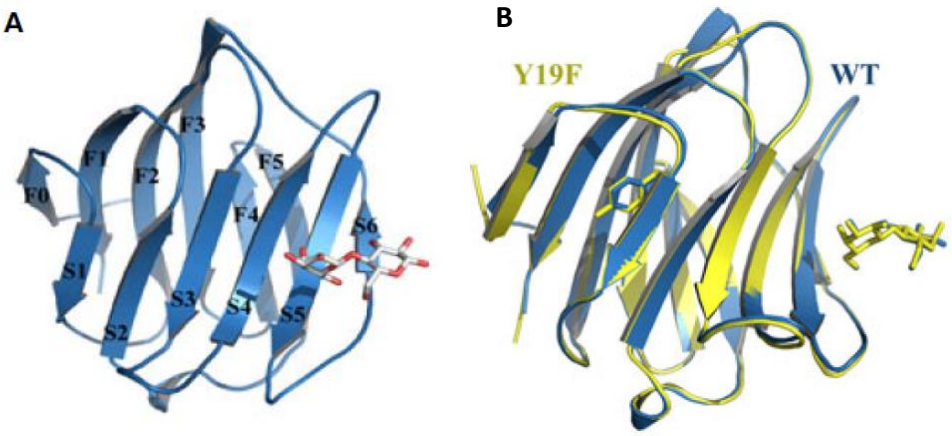

Fig. 1. 13. Comparison of the structures of the F19Y mutant and wild-type N-domain of human Gal-8.
A: structure of the wild-type (WT) N-terminus of Gal-8. **B:** Mutant F19Y (yellow) superposed to the respective structure of the wild-type (blue) protein. (Taken from: Ruiz et al., 2014)

1.4. Aim of the study

Recent studies suggested a potential role of glycobiology in the onset and progression of OA. Pro-inflammatory cytokines, such as IL-1β, were revealed to provoke alterations in the glycophenotype of human chondrocytes *(Pabst et al., 2010)*. Such changes could also be found in the glycan pattern of OA cartilage, presumably providing altered binding sites for galectins *(Toegel et al., 2013)*. These findings underscore the assumption that galectins may be involved in mechanisms that could lead to cartilage degeneration. Therefore, this study primarily aimed to investigate the in vitro effects of various galectins and also of modified galectin-variants in cultured human chondrocytes and synoviocytes of OA and RA patients regarding their ability to induce IL1B and MMP13 gene expression. In addition, the potential of Gal-1 to activate the inflammation-driving NF-$_K$B signaling pathway was addressed in this thesis.

2. Materials and Methods

2.1. Clinical specimens

Human osteoarthritic knee cartilage and synovial tissue were obtained from patients that underwent a 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.: 1065/2011). The resected material was received from the operating room and was kept in a small container with physiologic salt solution. Selected parts of the OA 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

Primary human OA chondrocytes were isolated from articular OA cartilage. The articular cartilage was cut off the bone, minced to little pieces and filled into a Falcon tube (Becton Dickinson Labware). Primary human OA synoviocytes were isolated from resected synovial tissue, which was also dissected into small parts. Then, these tissues were enzymatically digested with Collagenase B (Roche Diagnostics). Therefore, a solution of 80 mg lyophilized enzyme in 40 ml full medium (see section 2.2.3. Cell cultivation) was prepared (=2 mg Coll B/ml medium) and filtrated through a micropore filter to ensure its sterility. The solution was added to the pieces of tissue to digest their matrix. The Falcon tube was placed on a shaker for 24 hours at 37°C.

2.2.2. Counting of cells

After 24 hours, pieces of undigested tissue were removed by filtrating the solution through a cell strainer. Following centrifugation (unless otherwise indicated always at 1,000 rpm for 8 min), the resulting pellet was washed with 10 ml phosphate buffered saline (PBS) to remove cell components like erythrocytes and then centrifuged again. Finally, the pellet was resolved in 10 ml full medium. 10 µl of that cell solution were mixed with an equivalent portion of trypan blue to stain death cells and then given into a counting chamber (C-Chip) to determine the number of living cells under the microscope. The single use C-Chip counting chamber (Biochrom) consists of two independent injection areas A and B for one sample each (Fig. 2.1.). Its counting grid includes

four corner squares named a, b, c and d (Fig. 2.2.). For counting, 10 µl of the mixed cell solution were loaded into one of those injection areas using a pipette. Then, cells in all four corner squares were counted. The summed number was used to calculate the amount of cells in the whole solution (Fig. 2.3.).

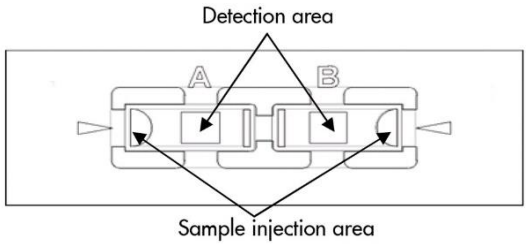

Fig. 2.1. Injection areas. (BIOCHROM)

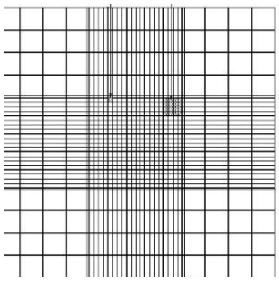

Fig. 2.2. Counting grid. (BIOCHROM)

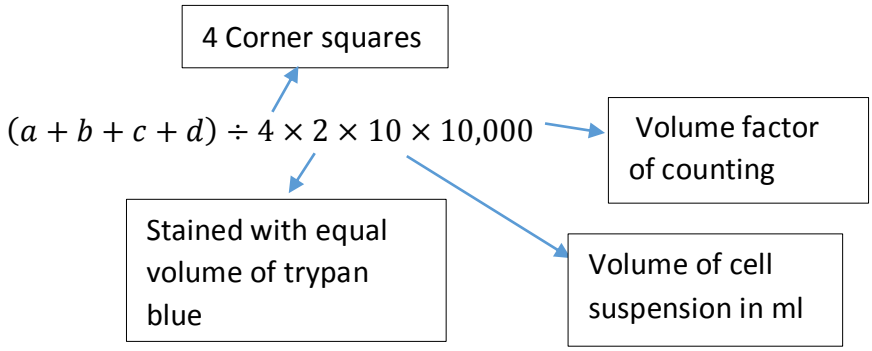

Fig. 2.3. Cell counting formula.

2.2.3. Cell cultivation

Isolated chondrocytes and synoviocytes were cultured in Dulbecco´s modified Eagles´s medium (DMEM), supplemented with 10% fetal bovine serum (FBS) and 1% gentamicin ("full medium") in a humidified atmosphere of 5% CO_2/95% air at 37°C. Chondrocytes were used at passage 0 because of dedifferentiation processes at higher passages. Synoviocytes were cultured in flasks and used between passage 4 and 8 to remove synovial macrophages. Between these passages, there are only the requested synovial fibroblasts left while at higher passages they are also able

to dedifferentiate. After reaching about 90% confluency, the cells were split into larger culture flasks. The following table shows the used cultivation consumables (Tab. 2.1.).

Culture flasks	Cultivation area	Capacity of medium
T25	25 cm²	5 ml
T75	75 cm²	15 ml
T150	150 cm²	30 ml
Culture plates		
6-well	9.5 cm²	2 ml/well
12-well	3.8 cm²	1 ml/well

Tab. 2.1. Cultivation consumables.

2.2.4. Passaging of synoviocytes

To passage synoviocytes, medium was discarded and cells were washed with PBS to avoid that two-valued ions in the medium inhibit the effect of trypsin. According to the size of the culture flask, a specified amount of trypsin, which was prewarmed to 37°C, was added to detach the cells. Therefore, the flasks remained in the incubator for the given time period (Tab. 2.2.).

Culture flask size	Trypsin	Incubation time
T25	1 ml	3 min
T75	2 ml	3.5 min
T150	4 ml	4 min

Tab. 2.2. Procedure of cell detachment with trypsin referring to the size of the culture flask.

After the indicated incubation time, the detachment of cells was checked under the microscope. The enzyme was then immediately inactivated by addition of full medium to a final volume of 10 ml. After that, the whole cell suspension was transferred into a Falcon tube and then centrifuged. The pellet was resuspended in full medium and cells were counted. The required amount of fresh full medium was added to seed the cells into a larger culture flask or in plates when cells were ready for treatment.

2.2.5. Changing of culture medium

To ensure that the cells are provided with enough nutrients for growing, the medium was changed regularly. Therefore, the old medium was replaced with fresh one. Freshly isolated chondrocytes and synoviocytes were additionally washed with PBS 24 hours after seeding to remove erythrocytes, other cell components or not adhered cells.

2.2.6. Freezing and thawing of synoviocytes

When cells successfully reached the requested passage but where not needed at that time, they were frozen for a later use. Therefore, 1 ml of the cell suspension was filled into a cryotube and 100 µl dimethylsulfoxide (DMSO) were added as a cryoprotectant to reduce ice formation and to prevent cell death during the freezing process. The cryotube was then placed into a Mr. Frosty Freezing Container to ensure a controlled freezing process. The container was then kept in the fridge at −80°C and on the next day the cryotubes were transferred into liquid nitrogen.
To thaw the cells, cryotubes were taken out of the nitrogen-tank and placed in a warm water bath for a fast thawing. The cell suspension was transferred into a Falcon tube containing 9 ml of full medium and centrifuged to remove the DMSO. Then, the cell pellet was resuspended with fresh full medium, the cells were counted and finally seeded depending on the requirements of the experiments.

2.2.7. Starvation and treatment of cells

Chondrocytes and synoviocytes that were ready for experimental use, first had to be starved by replacing full medium with starvation medium (=full medium without FBS). This was performed to make cells sensitive for the upcoming treatment. The cells were starved for different time periods before they were treated in fresh starvation medium and with various concentrations of galectins (see section 3. Results for precise protocols). For the treatment procedure, freshly isolated OA chondrocytes, as well as freshly isolated OA and thawn RA synoviocytes were used.

2.3. Cytotoxicity assay

To prove the viability of chondrocytes and synoviocytes under the applied treatment conditions, the EZ4U (Easy for You) non-radioactive cell proliferation and cytotoxicity assay (Biomedica) was used according to the manufacturer's instructions. In brief, the following steps were performed: 96-well microtiterplates were cultivated with 2,000 synoviocytes or 4,000 chondrocytes per well in full medium. Treatment was carried out in starvation medium for 24 h with five concentrations of each galectin (100, 50, 20, 10, 5 µg/ml).

The next day, reagents and samples were prepared. Therefore, one vial of lyophilized substrate (SUB) was dissolved in 2.5 ml activator (ACT) solution, which was prewarmed to 37°C prior to addition. This colored mixed substrate was immediately used by adding 20 µl to 200 µl of fresh starvation medium in each well. Depending on the metabolic capacity of the cells, the microtiterplates were incubated for 2 to 5 hours at 37°C until the yellow colored tetrazolium compound was convert to its red formazan derivative. The absorbance was then measured using a microtiterplate reader equipped with a 450 nm filter (Victor³ Perkin Elmer, Program Wallac 1420 workstation).

2.4. Isolation of RNA

For RNA purification from cultured cells, the NucleoSpin RNA protocol directions of Macherey-Nagel-02/2013, Rev. 15 were followed, whereby all mentioned reagents were supplied in the RNA-Kit.

Chondrocytes and synoviocytes were grown in 12-welland 6-well plates, respectively, and then used for extraction of total RNA. Therefore, cell culture supernatant was discarded and for lysis 350 µl Buffer RA1 together with 3.5 µl β-mercaptoethanol (β-ME) were added to the cell layer and thoroughly resuspended to reduce viscosity. The lysate was then filtrated through NucleoSpin Filter (violet ring) by centrifugation for 1 min at 11,000 rpm in a table centrifuge. Then, the filter was discarded and 350 µl of 70% ethanol were added to the lysate and mixed by pipetting 5 times up and down. To bind the RNA, this was then loaded to the NucleoSpin RNA Column (blue ring) placed in a Collection Tube and centrifuged for 30 s. From this step on, the NucleoSpin RNA Column was always placed into a new Collection Tube after centrifugation. Then, 350 µl of

Membrane Desalting Buffer (MDB) were added to the filter and again centrifuged for 1 min to dry the membrane. To digest the DNA, DNase reaction mixture was prepared in a sterile tube. For each isolation 10 µl reconstituted rDNase were added to 90 µl Reaction Buffer for rDNase and mixed by flicking the tube. 95 µl of this mixture were then applied onto the column and incubated at room temperature for 15 min. After the incubation time, the silica membrane of the filter was washed and dried in three steps. First, 200 µl wash Buffer RAW2 were added to the column to inactivate the rDNase and centrifuged for 30 s. The second wash was performed with 600 µl of Buffer RA3 and same centrifugation conditions. Finally, the column was washed with 250 µl of Buffer RA3 and completely dried by centrifugation for 2 min. After that, the column was placed into a nuclease-free (RNase-free) Collection Tube (1.5 ml) and RNA was eluted by pipetting 60 µl RNase-free H_2O directly onto the silica membrane of the column and centrifuging for 1 min. RNA was stored at −80°C.

2.4.1. Quantitation of RNA

The concentration of eluted RNA was then quantitated using the NanoDrop 2000 Micro Volume UV-Vis Spectrophotometer (Thermo scientific Peqlab Biotechnology GmbH) equipped with NanoDrop software according to the manufacturer's instructions. Before use, the upper and lower optical surfaces of the micro spectrophotometer were cleaned. Therefore, 2 µl of deionized water were pipetted onto the lower surface, lever arm was closed and then lift up to wipe the water off the surfaces with a soft laboratory wipe. Before sample measurement, a blank with water was performed by selecting the feature "blank". To prevent sample contamination, the pedestals were wiped off between all measurements and also after the blank. RNA samples were then measured by loading 2 µl onto the lower surface and closing the lever arm. All sample measurements were carried out without dilution and at a wavelength of 260 nm. RNA purity was checked by using 260/280 ratios.

2.5. Transcription of RNA into cDNA

For transcription into cDNA, quantitated RNA samples were diluted with RNase-free water, to ensure that the same amount of RNA in each sample was transcribed within each experiment. The cDNA samples were then used in quantitative polymerase chain reaction (section 2.6. qPCR). Transcription is a process in which single stranded RNA is transcribed into complementary DNA (cDNA) by using a special composition of reverse transcriptase (RT) enzyme, RT primer, dNTPs, RT buffer and nuclease-free H_2O to a total of 10 µl for each sample (Tab. 2.3.). All reagents 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
Nuclease-free H_2O	4.2 µl
Total per Reaction	10 µl

Tab. 2.3. Composition of reagents for transcription into cDNA.

10 µl of the reaction composition were pipetted to the previously diluted RNA samples and placed into a thermal cycler (Eppendorf Mastercycler Gradient) for transcription into cDNA. Transcription was performed in four steps, whereby the last one served for holding the samples at a constant low temperature (4°C) until they were taken out of the thermal cycler (Tab. 2.4.).

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

Tab. 2.4. Program of the thermal cycler.

2.5.1. Dilution of cDNA

Samples of cDNA were diluted with RNase-free H_2O in a ratio of 1:5. Therefore, 20 µl of nuclease-free water were pipetted into a 0.5 ml tube, and then 5 µl of the cDNA sample were added and resuspended. This dilution step was performed to achieve optimal performance of the following qPCR. Moreover, cDNA that had been prepared from galectin-treated non-OA chondrocytes was used.

2.6. qPCR analysis

Quantitative PCR 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 DNA synthesis: 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 DNA concentration. An advantage of SYBR-Green is that it does not bind to single strands. RT-qPCR was performed for quantification of three specific target genes: matrix metalloproteinase-13 (MMP13), interleukin-1B (IL1B) and succinate dehydrogenase A (SDHA). The last one serves as a so called "housekeeping gene", a constitutively active gene, whose expression is not influenced by galectin treatment. Because of the various sources of error that can occur during a PCR, 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 RT-qPCR is the so called threshold cycle (Ct). The selection of threshold setting is basically arbitrary, but with the requirement that it should be placed in 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.

2.6.1. Preparation of reagents

For each target gene, a composition of reagents, containing forward primer, reverse primer, SYBR-Green and nuclease-free H_2O was prepared to a total of 24 µl for each sample. In the following table the exact composition of reagents is listed (Tab. 2.5.).

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

Tab. 2.5. Composition of reagents for RT-qPCR.

Every gene was targeted by a specific set of primers (Tab. 2.6.).

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

Tab. 2.6. Sequences of forward and reverse primer of used target genes.

RT-qPCR was performed in a 96-well optical plate (MicroAmp, Applied Biosystems) that was 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. Non-template controls were performed for each target gene to which 1 µl nuclease-free H_2O were added to check for contamination of the reactions. The 96-well optical plate was covered with an optical adhesive film (MicroAmp, Applied Biosystems) before it was measured.

2.6.2. Computer and program setting

The measurement was carried out by the PC-controlled Applied Biosystems StepOnePlus Real-Time PCR System. Before starting the run, cycling conditions were defined by first selecting "advanced setup" and choosing the features 96-well, comparative Ct (ΔΔCt) for quantitation, SYBR®-Green and Standard run (2 h). Further, "plate setup" was done including two steps. First, targets were added and samples defined under the feature "define targets", then the pipetting scheme was determined under "assign targets and samples". Finally, the "run method" was chosen, which included the following cycle pattern with a total of 40 cycles. The following table presents the cycling processes (Tab. 2.7.).

Cycle	Length	Temperature
Holding Stage	10 min	95°C
Cycle Stage (40 cycles)	30 sec	95°C
	60 min	55°C
	60 min	72°C
Melt Curve Stage	15 min	95°C
	60 min	55°C
	15 min	95°C

Tab. 2.7. Run method of RT-qPCR.

After saving the file, the measurement started by selecting "start run" and stopped automatically when finished.

2.7. Western blot

Western blot was used to detect proteins in cell lysates, which had been separated according to their different molecular weight using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

2.7.1. Protein isolation for Western blot

Chondrocytes were cultivated (see section 2.2.3.) in 6-well plates or T25 cell culture flasks and treated with different concentrations of galectins (see section 3. Results). For cell lysis, radioimmunoprecipitation assay (RIPA) buffer (Thermo Scientific), supplemented with 2.5 µl of phenylmethylsulfonyl fluoride (PMSF), protease inhibitor (PI), and sodium orthovanadate was used. After treatment, starvation medium was discarded and cells were washed with PBS. Then, 64 µl of RIPA buffer were added to each 6-well, or 167 µl to T25 culture flasks. The plates or flasks were placed on ice for about 1 h. Cells were then harvested with a cell scraper and transferred into a 1.5 ml tube. Samples were then placed in an ice cold ultrasonic bath for 10 min, to ensure that all cells were lysed. Furthermore, the lysates were centrifuged at 5,000 rpm and 4°C for 10 min. The supernatants were then transferred into a new tube, while the pellets were discarded. These protein samples were used for the further Western blot experiment (see section 2.7.2.).

2.7.2. SDS-PAGE for Western blot

The following steps were performed:

1. Washing of glass and spacer plate: plates were washed with warm water and soap, then with 70% ethanol (EtOH). Finally, they were washed with deionized H_2O (dH_2O) and dried with science precision wipes (KIMTECH).
2. The protein and fat-free plates were then inserted into the Mini-PROTEAN Tetra cell casting frame and casting stand (BIORAD). Then separating and stacking gel were prepared, containing a special composition of Tris(hydroxymethyl)aminomethane (Tris, TRIZMA BASE, Sigma-Aldrich), Acrylamide/Bis (30%/2.67%, AA; Sigma-Aldrich), SDS, Tetramethylethylendiamin (TEMED), ammonium persulfate (APS) and dH_2O. The following table lists the components for an amount of two gels (Tab. 2.8.). APS was prepared freshly by solving 100 mg in 1,000 µl of dH_2O and added as last component immediately before using the gel, because it induces polymerization together with TEMED.

Separating gel 10%	Volume	Stacking gel 5%	Volume
dH_2O	4.17 ml	dH_2O	3.400 µl
1.5 M Tris	2.5 ml	1.5 M Tris	830 µl
30% AA	3.33 ml	30% AA	630 µl
10% SDS	100 µl	10% SDS	50 µl
TEMED	5 µl	TEMED	5µl
10% APS	100 µl	10% APS	50 µl

Tab. 2.8. Components of separating and stacking gel (amount for two gels).

3. After tightness testing with water, separating gel was filled three-quarter in between the glass plates and was then covered with a layer of dH_2O to prevent drying out of the gel and to remove air bubbles. After about 1 h, when the gel was sufficiently polymerized, dH_2O was poured off and the remaining rest was dried with filter paper without touching the gel. Stacking gel was then supplemented with APS and immediately pipetted onto the separating gel until the glass plates were filled. The slot combs were then inserted carefully and about 30 min were awaited. Glass plates were then wrapped in wet science precision wipes (KIMTECH) and stored in the fridge at 4-8°C for complete polymerization overnight.
4. On the next day, protein samples were prepared. Therefore, 8 µl of sample buffer (Laemmli Buffer) were added to 24 µl of each sample. For the composition of Laemmli Buffer see Tab 2.9.

Component	Volume
0,5 M Tris.HCl pH 6,8	4.4 ml
20% SDS	2.2 ml
Glycerol	4.4 ml
1% bromophenol blue	0.5 ml
β-mercaptoethanol	0.5 ml
dH_2O	2.8 ml

Tab. 2.9. Composition of Laemmli sample Buffer.

Samples were then heated for 5 min at 95°C and 300 rpm in the Thermomixer compact eppendorf and then again put on ice for a moment.

5. For loading sample, the gel cassette sandwich was given into the assembled Mini-PROTEAN Tetra Cell Electrophoresis Module (BIORAD). TANK and the area between the glasses plates were then filled with freshly prepared Running Buffer, composed of 100 ml 10x Tris-Glycin-Buffer (30.3 g Tris, 144 g Glycine, dH_2O to a total of 1,000 ml), 10 ml 10% SDS and 890 ml dH_2O. Slot combs were then pulled out carefully. Gel was finally loaded by pipetting 15 µl of each sample in one slot of a 15 slot comb or 3 µl of a marker (Precision Plus Protein Standards, All Blue, # 161-0373; BIORAD).
6. The area between the plates was completely filled with Running Buffer, the TANK was then closed and electrodes were connected. Running was carried out at 50 mA for about 90 min (until the blue front run out).
7. After the completed run, freshly prepared Transfer Buffer (100 ml 10x Tris-Glycin-Buffer, 700 ml dH2O, 200 ml Methanol) was filled into a plastic tub for blotting. Plates were taken out of the TANK, separated from each other carefully and stacking gel was removed with a scraper and discarded. The separating gel was peeled off the plate and transferred onto the filter paper of the gel sandwich (assembled of two fiber pads, two filter paper, the gel and the membrane in the middle). The membrane was then laid onto the gel followed by another layer of filter paper. Air bubbles were removed with a roller and the gel sandwich cassette was closed. The cassette application was then clamped between the electrode frames and a cool pack was added to the Blotting TANK. The TANK was filled with Transfer Buffer, closed and electrodes were connected. Blotting was performed at 250 mA for at least 120 min.
8. For blocking, a 5% blocking solution was prepared by resolving 5 g of powdered milk in 100 ml 1x PBS. Membrane was then overlaid with this solution and blocking was carried out overnight in the fridge at 4-8°C at a rocking platform.
9. On the next day, the membrane was taken out of the milk solution and washed for 10 min with PBS-Tween (PBS-T), composed of 100 ml 10x PBS, 900 ml dH2O and 1 ml Tween 20.
10. Primary antibody solution was prepared. Therefore, 7.5 ml of ODYSSEY blocking buffer were mixed with 7.5 ml PBS-T in a 15 ml Falcon tube and the antibodies were added in a

ratio of 1:1,000, means 15 µl of primary antibody: anti-phospho-NF-$_{\kappa}$B (Ser 536; 93H1; rabbit monoclonal antibody; cell signaling) and anti-α-tubulin (DM1A; mouse monoclonal antibody; cell signaling). The membrane was then probed with this primary antibody solution for 2 h at room temperature on the roll mixer.

11. Membrane was then washed three times with PBS-T for 10 min. Primary antibody solution was retained.
12. Secondary antibody solution was also prepared in a 15 ml Falcon tube. Therefore, DyLight 800-conjugated goat anti-rabbit IgG (Thermo Scientific) and IRDye 680LT goat anti-mouse IgG (LI-COR Biosciences) were added 1: 15,000 to 15 ml of PBS-T, supplemented with 15 µl 20% SDS. These fluorescent conjugates had to be protected from light. The membrane was then probed with this secondary antibody solution for 1 h at room temperature on the roll mixer.
13. Membrane was again washed with PBS-T three times for 10 min. Secondary antibody solution was then also retained.
14. Scanning of Western blots were performed with Licor ODYSSEY Scanner and Image Studio Software.

2.8. Statistics

Statistical analyses were performed using the paired Student´s t-test. P-Values < 0.05 were considered statistically significant.

3. Results

3.1. Analysis of the metabolic cell activity

To analyze the impact of the applied treatment conditions on the metabolic cell activity of chondrocytes and synoviocytes, the EZ4U assay was performed. OA chondrocytes, RA synoviocytes, as well as OA synoviocytes of one patient (n=1) were treated in duplicate with 5 µg/ml, 10 µg/ml, 20 µg/ml, 50 µg/ml and 100 µg/ml of each galectin for 24 h. p-Values were considered statistically significant when $p < 0.05$ (t-test).

3.1.1. Effect of galectins on the cell activity of OA chondrocytes

Figure 1 (A-D) shows the effect of five concentrations of Gal-1 and its variants on the metabolic activity of OA-chondrocytes. Generally, Gal-1 and its variants tended to induce decreased metabolic activity at the applied concentrations. For yet undetermined reasons, Gal-1, Gal-1_GG and Gal-1_8S (Fig. 1A, B, D) showed a statistically significant decrease of metabolic activity at 5 µg/ml, which however were not considered biologically relevant. Analyses yielded statistically significant impairment of cellular activity at a concentration of 100 µg/ml for Gal-1_GG (Fig. 1B) and Gal-1_8L (Fig. 1C). Concentrations < 20 µg/ml appeared to be well tolerated by OA chondrocytes.

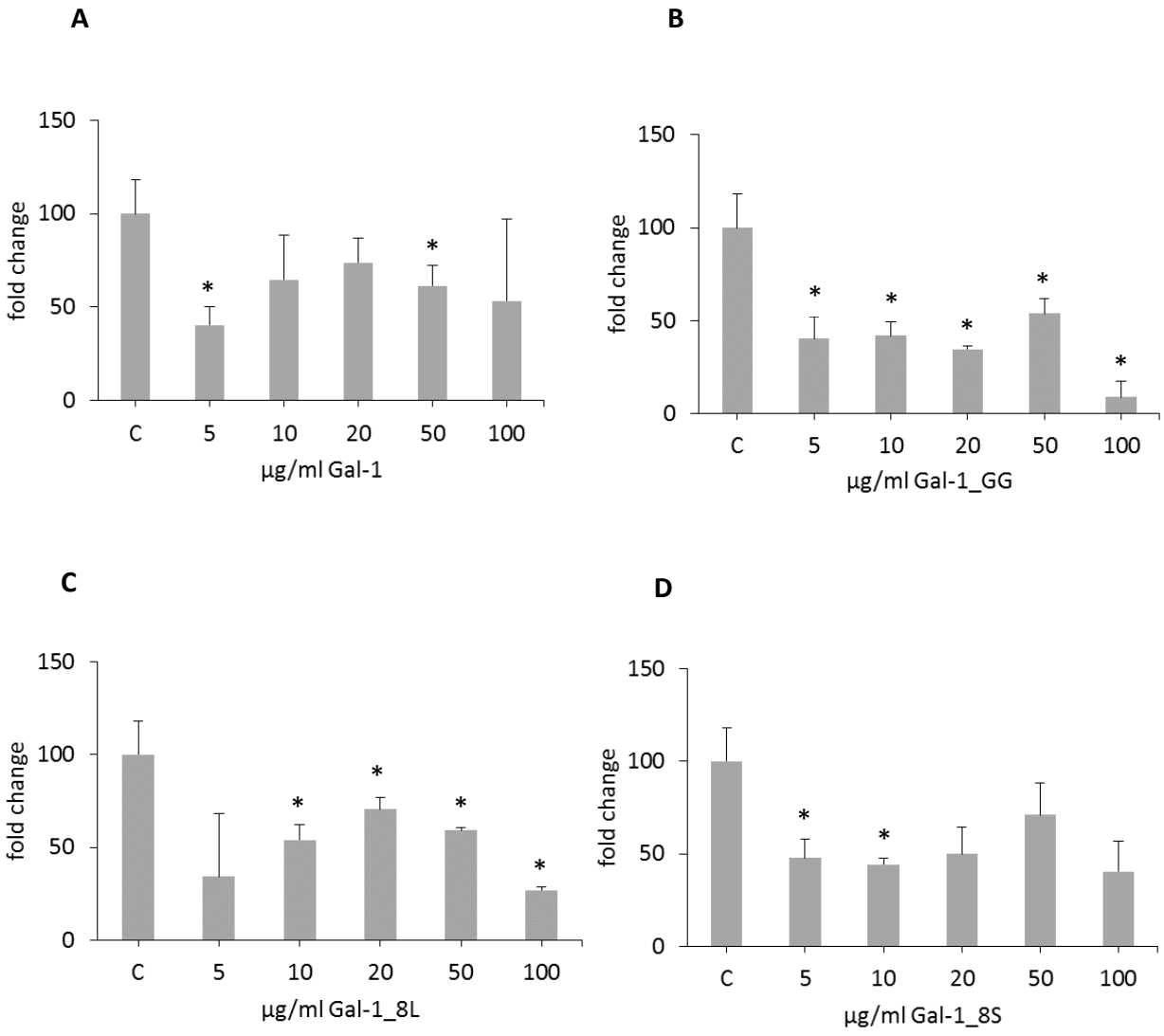

Fig. 1: OA chondrocytes (n=1) were treated for 24 h with 5, 10, 20, 50 and 100 µg/ml of Gal-1 and its variants to investigate their effects on metabolic cell activity.
A: Gal-1, *p< 0.05.
B: Gal-1_GG, *p< 0.05.
C: Gal-1_8L, *p< 0.05.
D: Gal-1_8S, *p< 0.05.

Figure 2 (A-F) presents the impact of Gal-3 and its variants at 5, 10, 20, 50 and 100 µg/ml on the metabolic cell proliferation of OA chondrocytes. Results revealed a statistically significant decrease of cellular activity at 100 µg/ml for all galectins (Fig. 1A-F). In general, concentrations between 5-50 µg/ml were tolerated well. For undetermined reason, Gal-3tr (Fig. 2F) promoted a statistically significant impairment of cell proliferation at 10-20 µg/ml, which however appeared to be caused by experimental errors rather than by a specific biological effect.

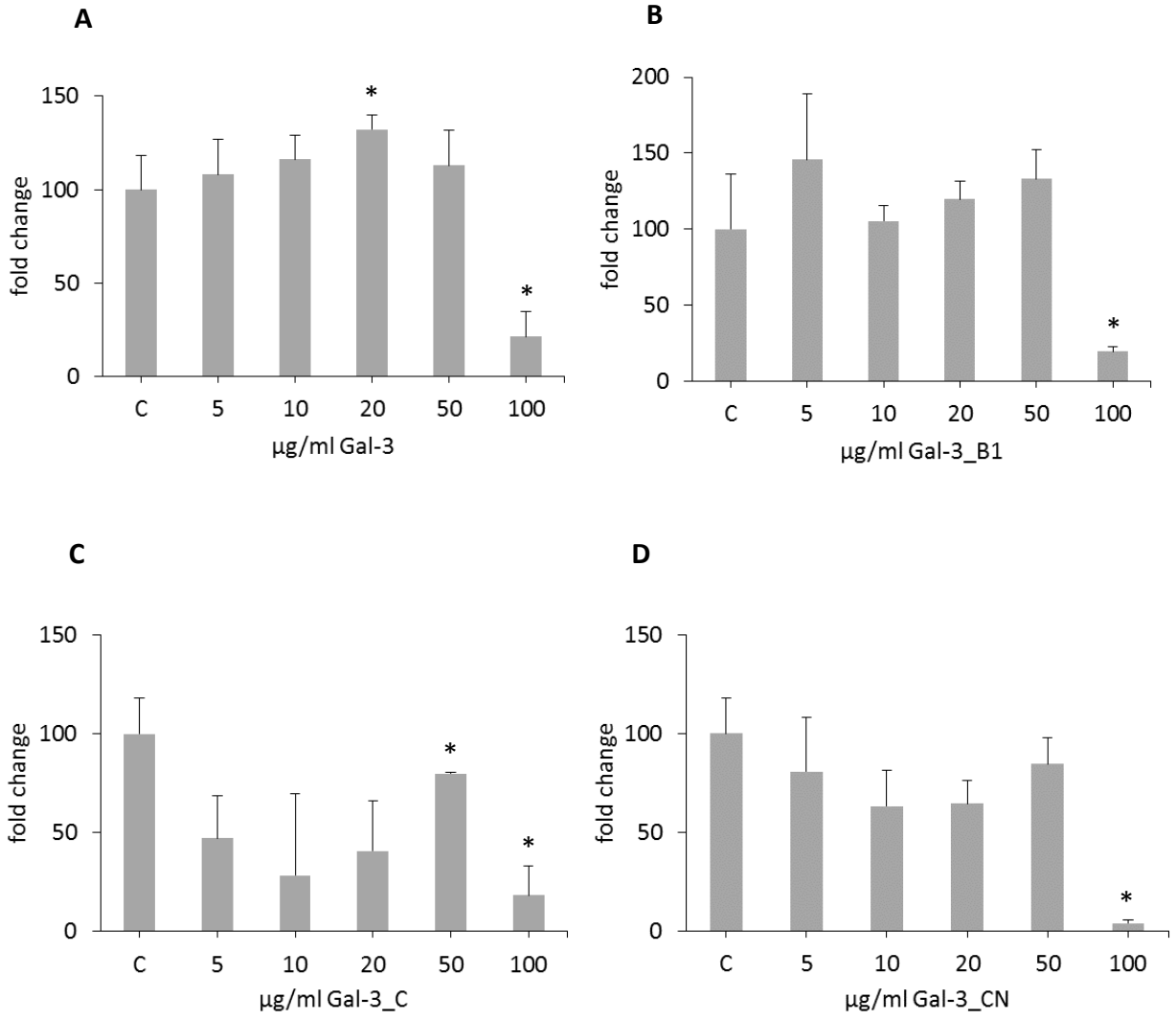

Fig. 2 (A-D): OA chondrocytes (n=1) were treated for 24 h with 5, 10, 20, 50 and 100 µg/ml of Gal-3 and its variants to investigate their effects on metabolic cell activity.
A: Gal-3, *p< 0.05, **B:** Gal-3_B1, *p< 0.05, **C:** Gal-3_C, *p< 0.05, **D:** Gal-3_CN, *p< 0.05
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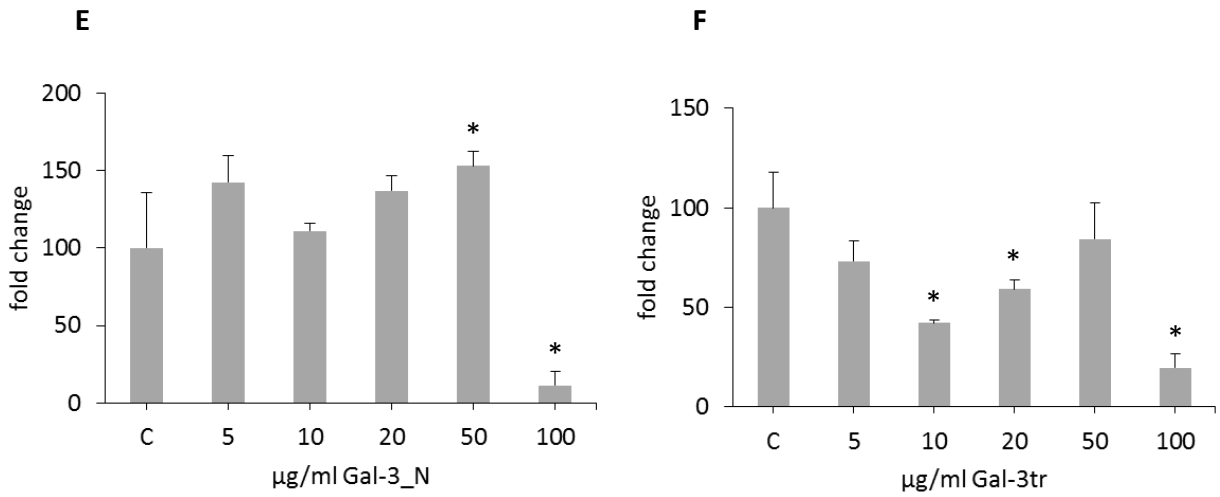


Fig. 2 (E-F): OA chondrocytes (n=1) were treated for 24 h with 5, 10, 20, 50 and 100 µg/ml of Gal-3 and its variants to investigate their effects on metabolic cell activity.
E: Gal-3_N, *p< 0.05.
F: Gal-3tr, *p< 0.05.

Figure 3 (A-C) depicts the concentration-dependent effects of Gal-8 and its variants on the metabolic cell activity of OA chondrocytes. Gal-8_NF19Y (Fig. 1B) showed a statistically significant decrease of metabolic activity at 100 µg/ml. Analyses further revealed no impairment of cellular activity at all other concentrations.

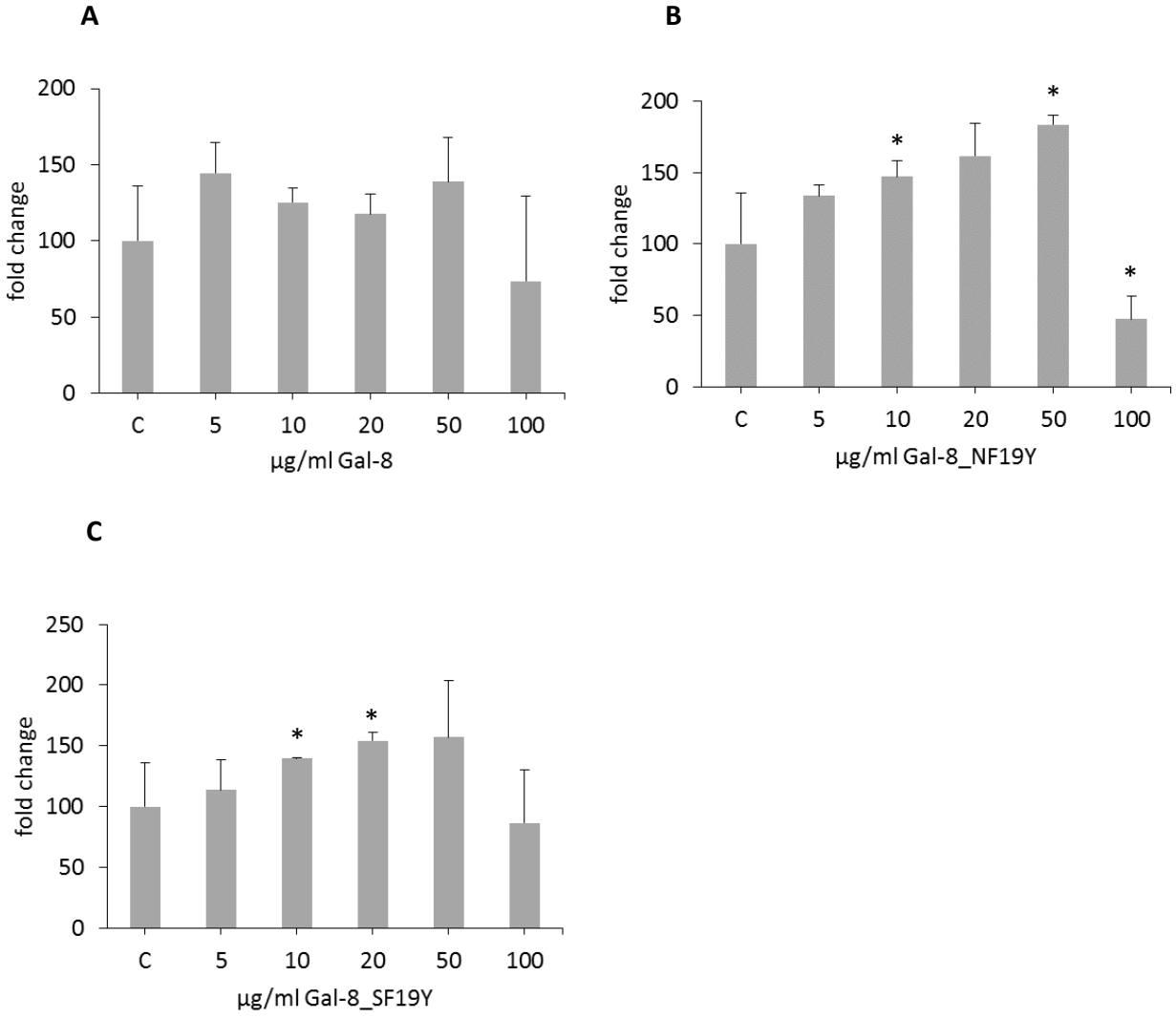


Fig. 3: OA chondrocytes (n=1) were treated for 24 h with 5, 10, 20, 50 and 100 µg/ml of Gal-8 and its variants to investigate their effects on metabolic cell activity.
A: Gal-8, **B:** Gal-8_NF19Y, *p< 0.05, **C:** Gal-8_SF19Y, *p< 0.05.

Figure 4 (A-D) illustrates the impact of Gal-2, Gal-4, Gal-7 and Gal-9 on the metabolic cell proliferation of OA chondrocytes. Gal-2, Gal-4 and Gal-7 (Fig. 1A-C) did not impair cellular activity at any applied concentration. Analyses revealed a statistically significant decrease of metabolic cell activity for Gal-9 (Fig. 4D) at concentrations > 50 µg/ml. At concentrations below 20 µg/ml, Gal-9 was well tolerated. In order to allow for a direct comparison of the biological effects induced by galectins, the following biological assays employing OA chondrocytes were carried out with galectins at a concentration of 10 µg/ml.

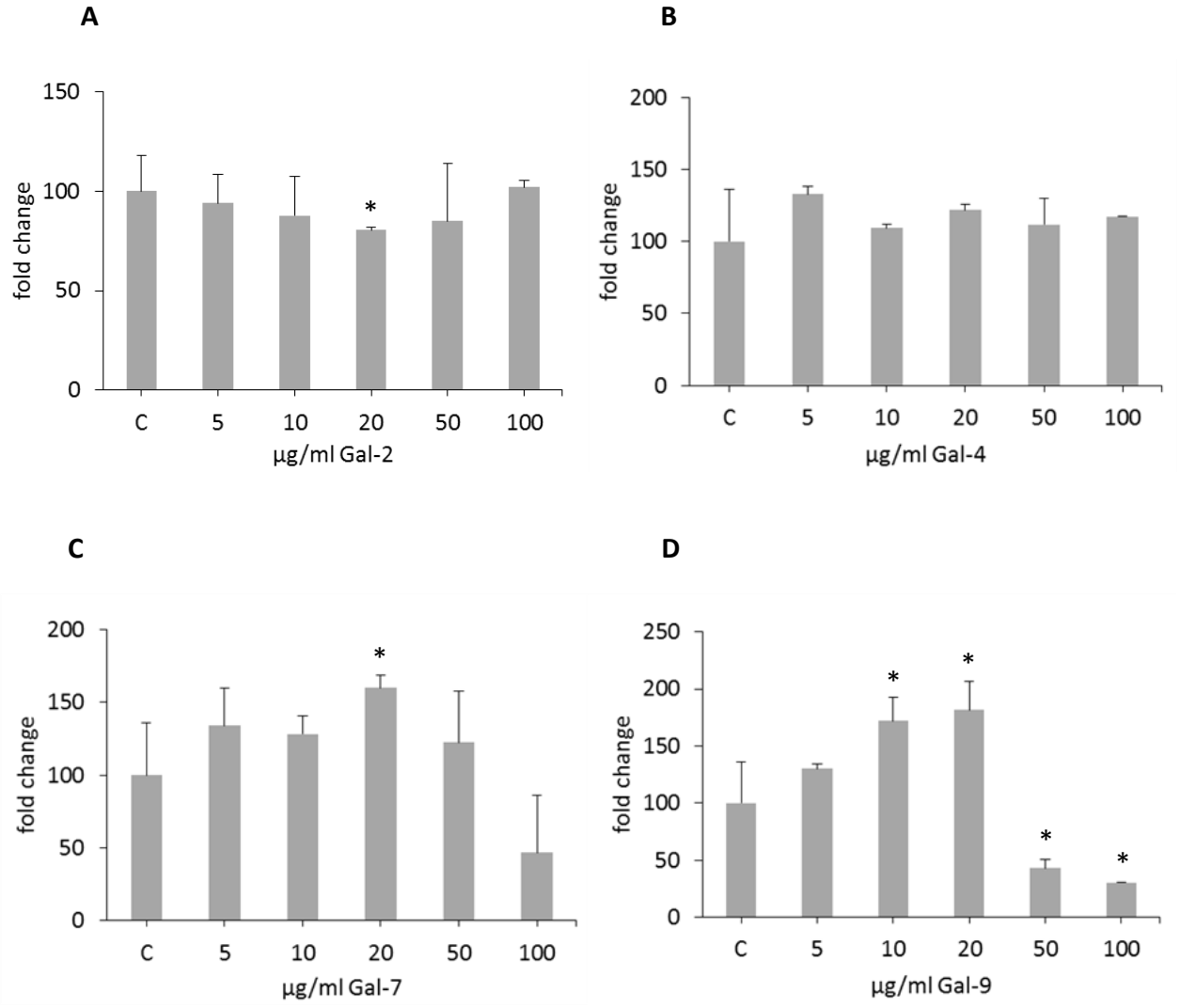


Fig. 4: OA chondrocytes (n=1) were treated for 24 h with 5, 10, 20, 50 and 100 µg/ml of Gal-2, -4, -7 and -9 to investigate their effects on metabolic cell activity.
A: Gal-2, *p< 0.05, **B:** Gal-4, **C:** Gal-7, *p< 0.05, **C:** Gal-9, *p< 0.05.

3.1.2. Effect of galectins on the cell activity of OA synoviocytes

Figure 5 (A-D) presents the impact of Gal-1 and its variants at 5, 10, 20, 50 and 100 µg/ml on the metabolic cell activity of OA synoviocytes. Gal-1_8S (Fig. 5D) induced a statistically significant decrease of metabolic activity at 100 µg/ml, but was well tolerated at concentrations < 50 µg/ml. All other galectins did not impair cellular activity at the applied concentrations.

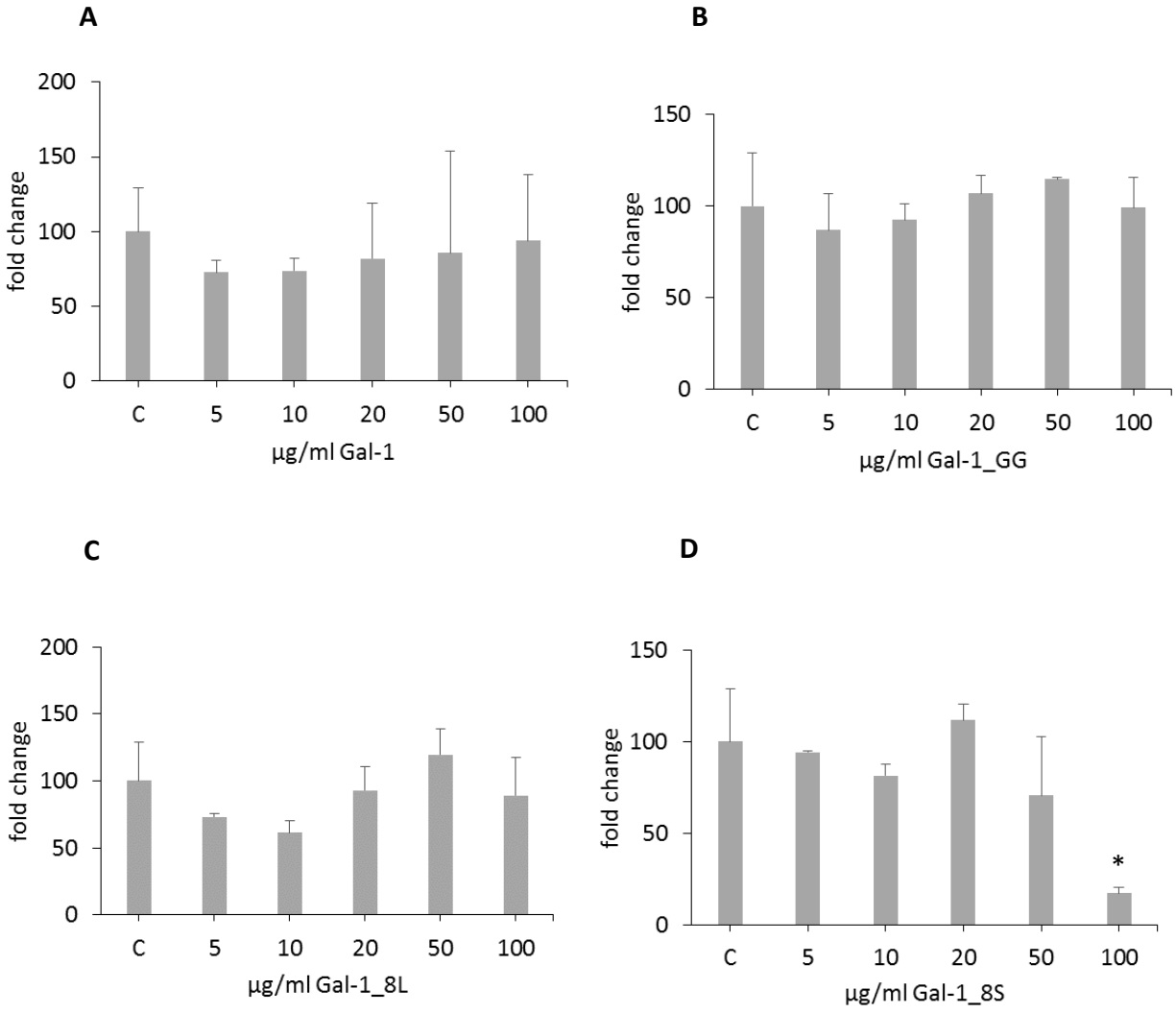


Fig. 5: OA synoviocytes (n=1) were treated for 24 h with 5, 10, 20, 50 and 100 µg/ml of Gal-1 and its variants to investigate their effects on metabolic cell activity.
A: Gal-1, **B:** Gal-1_GG, **C:** Gal-1_8L, **D:** Gal-1_8S, *p< 0.05.

Figure 6 (A-F) shows the effect of Gal-3 and its variants on the cell activity of OA synoviocytes. The impact of all galectins appeared to be small, as indicated by the lack of dose-dependent effects. For undetermined reasons, Gal-3_C (Fig. 6C) decreased metabolic rate statistically significant at 5 and 10 µg/ml, which however was not considered biologically relevant. The other galectins were well tolerated at all applied concentrations, except for Gal-3tr (Fig. 6F), which induced a statistically significant decrease of cell activity at 100 µg/ml.

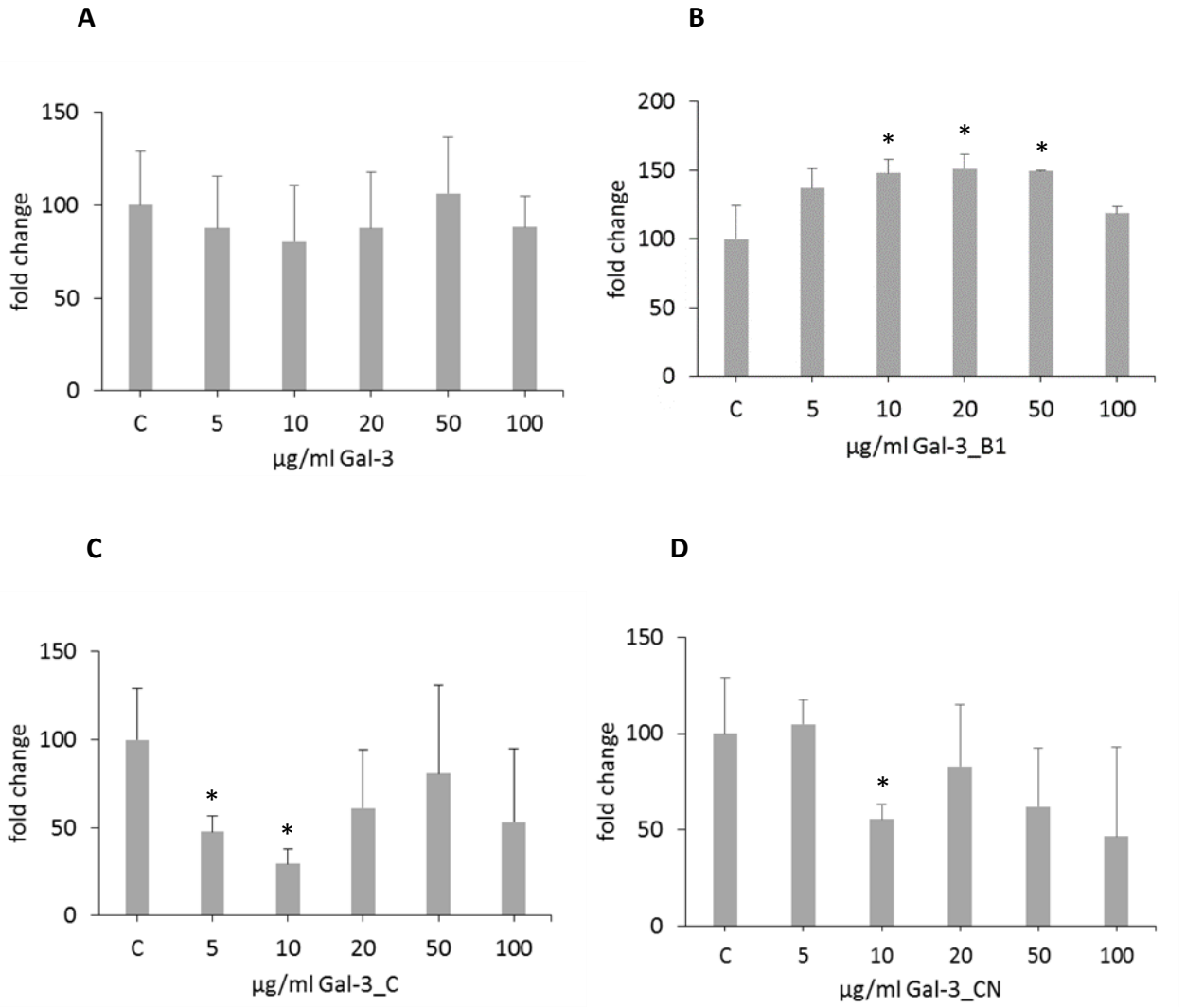


Fig. 6 (A-D): OA synoviocytes (n=1) were treated for 24 h with 5, 10, 20, 50 and 100 µg/ml of Gal-3 and its variants to investigate their effects on metabolic cell activity.
A: Gal-3, **B:** Gal-3_B1, *p< 0.05, **C:** Gal-3_C, *p< 0.05, **D:** Gal-3_CN, *p< 0.05.

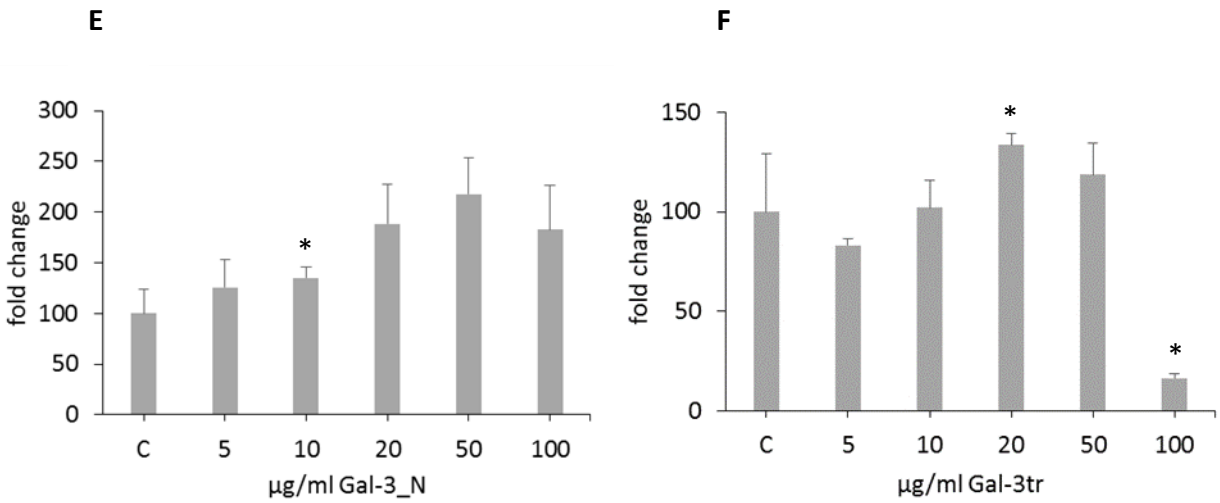


Fig. 6 (E-F): OA synoviocytes (n=1) were treated for 24 h with 5, 10, 20, 50 and 100 µg/ml of Gal-3 and its variants to investigate their effects on metabolic cell activity.
E: Gal-3_N, *p< 0.05.
F: Gal-3tr, *p< 0.05.

Figure 7 (A-C) shows the concentration-dependent effects of Gal-8 and its variants on the metabolic cell activity of OA synoviocytes. Analyses did not reveal a dose-dependent decrease of cell viability by any of these galectins.

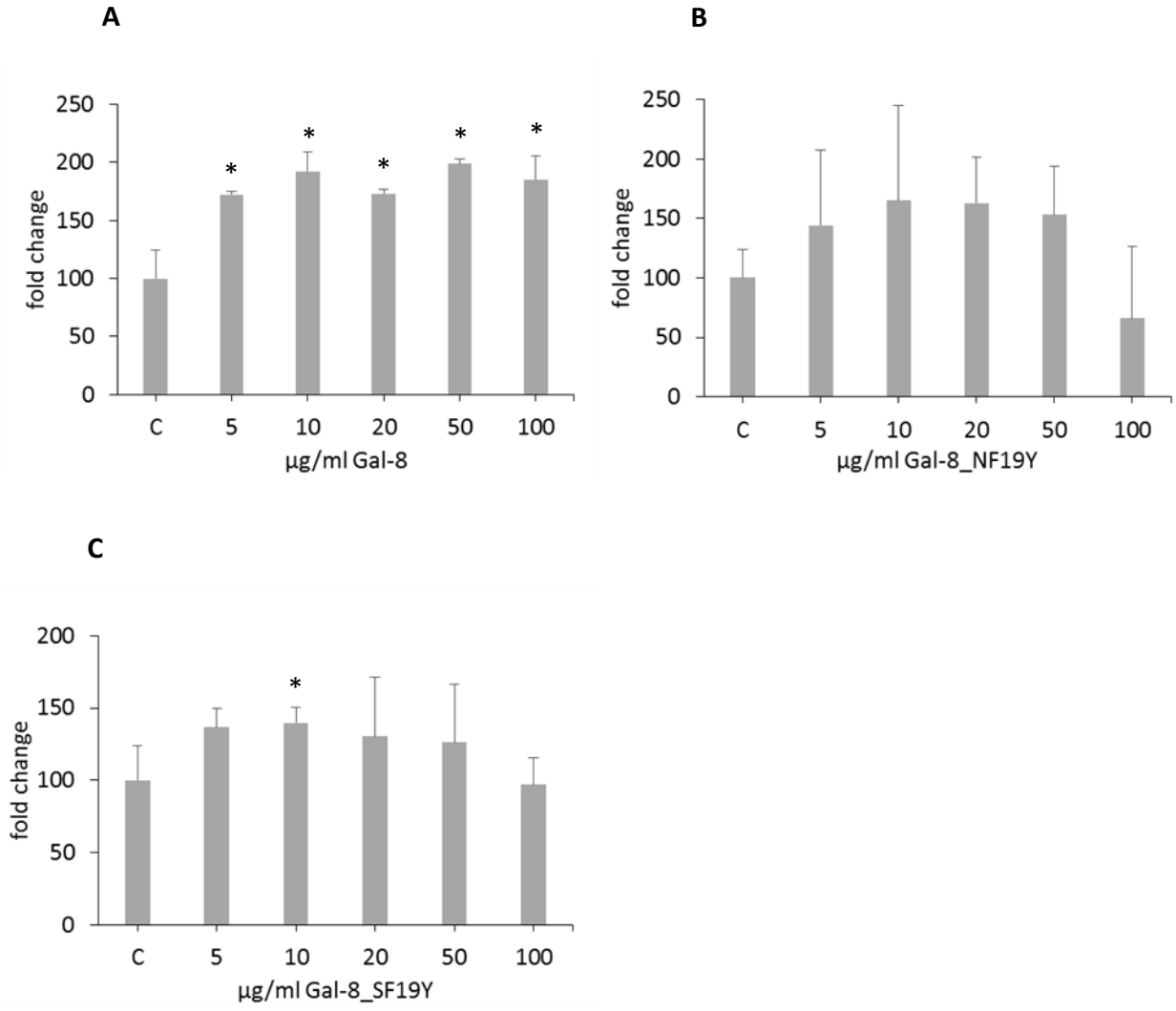


Fig. 7: OA synoviocytes (n=1) were treated for 24 h with 5, 10, 20, 50 and 100 µg/ml of Gal-8 and its variants to investigate their effects on metabolic cell activity.
A: Gal-8, *p< 0.05, **B:** Gal-8_NF19Y, **C:** Gal-8_SF19Y, *p< 0.05.

Figure 8 (A-D) presents the effect of Gal-2, Gal-4, Gal-7 and Gal-9 at five concentrations on the metabolic cell activity of OA synoviocytes. Analyses revealed no concentration-dependent effects of Gal-2, -4 and -7 on the cellular activity. Gal-9 (Fig. 8D) showed a statistically significant decrease at concentrations of 50 and almost statistically significant at 100 µg/ml. Concentrations below 20 µg/ml appeared to be well-tolerated. Based on these findings and preliminary experiments in 12-well plates (data not shown), further assays on galectin effects in OA synoviocytes were carried out at galectin concentration of 5 µg/ml.

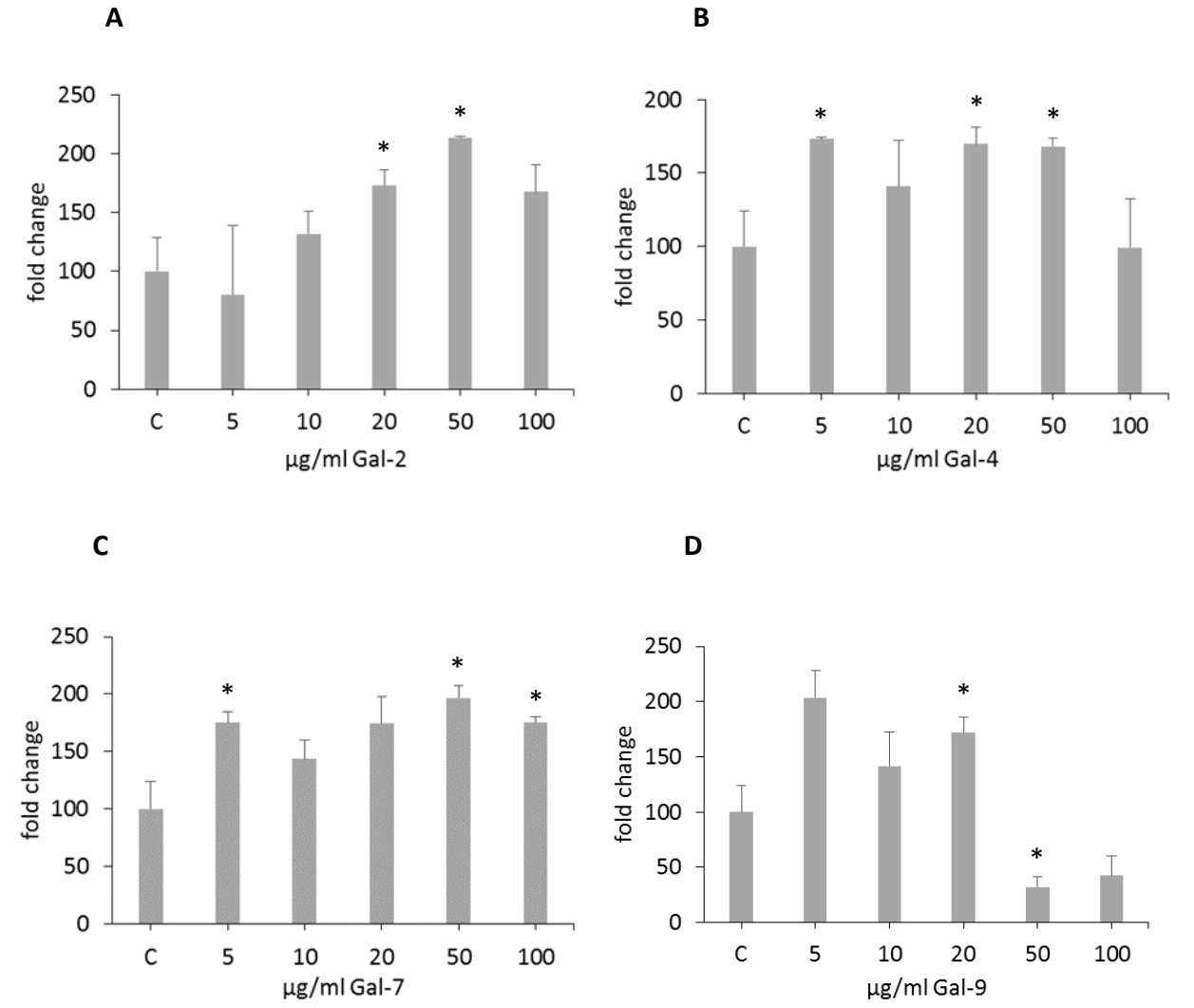


Fig. 8: OA synoviocytes (n=1) were treated for 24 h with 5, 10, 20, 50 and 100 µg/ml of Gal-2, -4, -7 and -9 to investigate their effects on metabolic cell activity.
A: Gal-2, *p< 0.05, **B:** Gal-4, *p< 0.05, **C:** Gal-7, *p< 0.05, **C:** Gal-9, *p< 0.05.

3.1.3. Effect of galectins on the cell activity of RA synoviocytes

Figure 9 (A-D) depicts the impact of Gal-1 and its variants at 5, 10, 20, 50 and 100 µg/ml on the metabolic cell activity of RA synoviocytes. Analyses revealed no concentration-dependent effect of Gal-1, Gal-1_GG and Gal-1_8L (Fig. 1A-C) on cellular activity. Gal-1_8L (Fig. 9C) decreased metabolic activity significantly at 100 µg/ml.

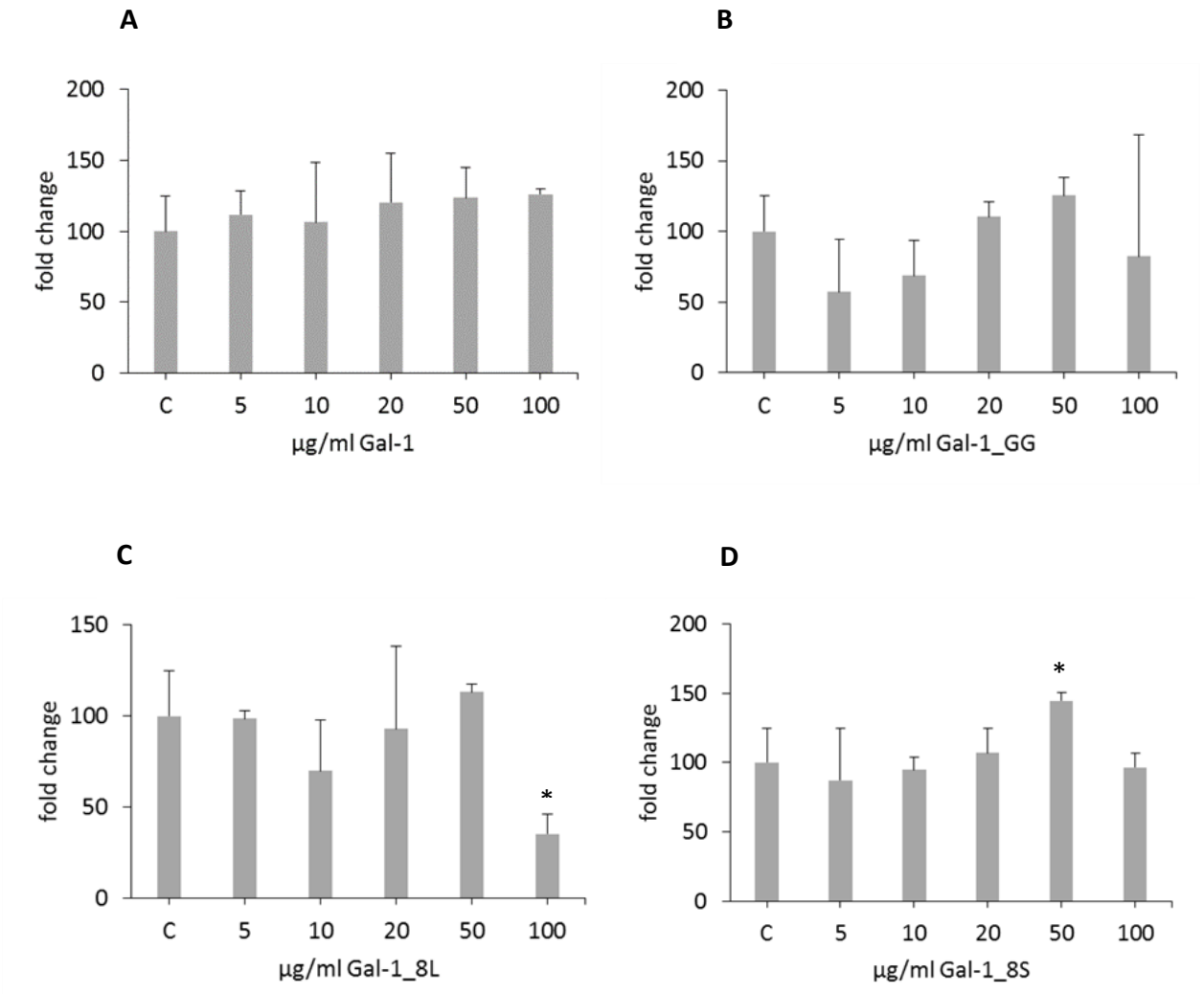


Fig. 9: RA synoviocytes (n=1) were treated for 24 h with 5, 10, 20, 50 and 100 µg/ml of Gal-1 and its variants to investigate their effects on metabolic cell activity.
A: Gal-1, **B:** Gal-1_GG, **C:** Gal-1_8L, *p< 0.05, **D:** Gal-1_8S, *p< 0.05.

Figure 10 (A-F) presents the concentration-dependent effects of Gal-3 and its variants on the metabolic cell rate of RA synoviocytes. Analyses again yielded no significant concentration-dependent impairment of cellular activity by these galectins. Gal-3 (Fig. 10A) decreased the metabolic activity statistically significant at 5 µg/ml, which however was not considered biologically relevant.

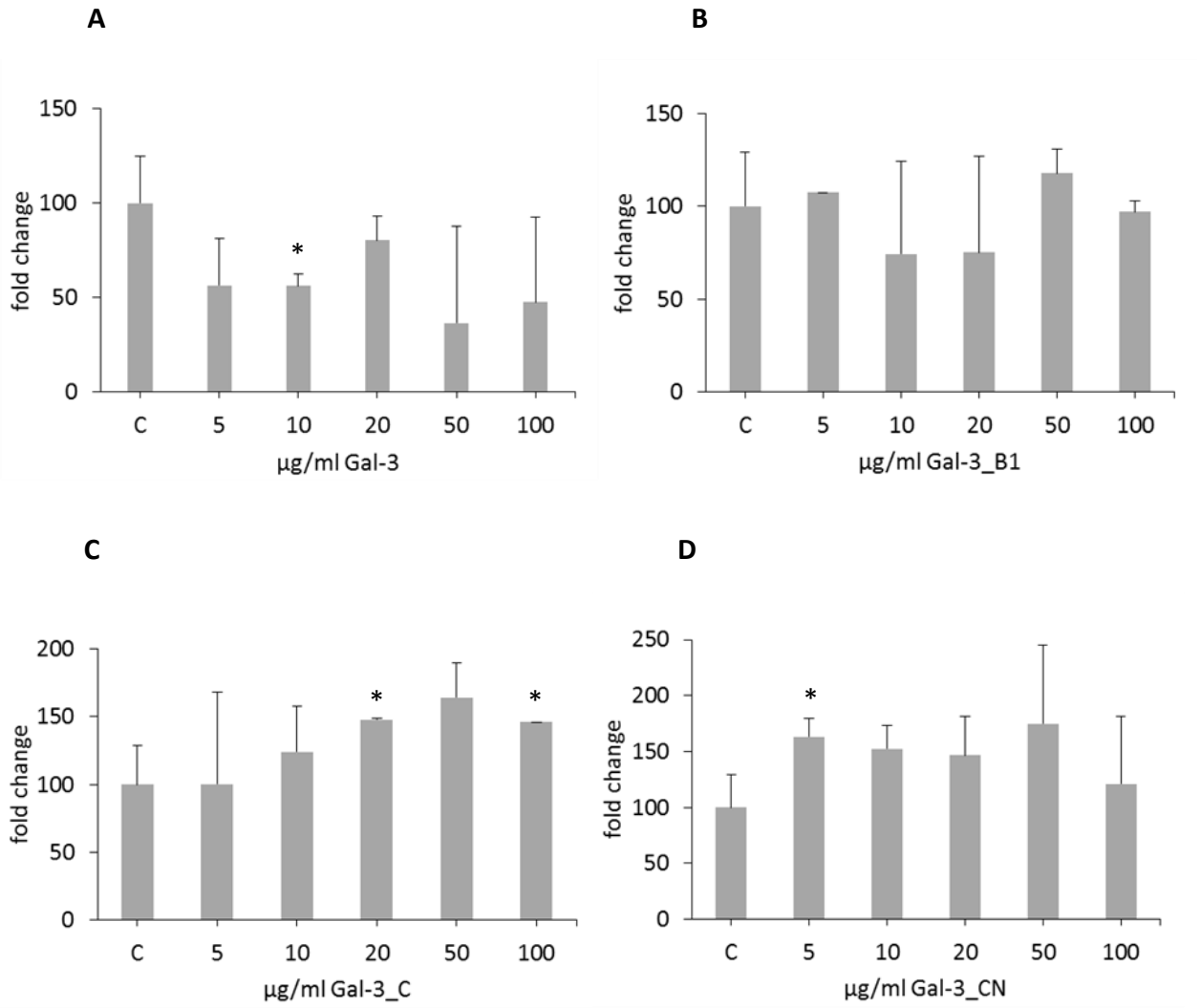


Fig. 10 (A-D): RA synoviocytes (n=1) were treated for 24 h with 5, 10, 20, 50 and 100 µg/ml of Gal-3 and its variants to investigate their effects on metabolic cell activity.
A: Gal-3, *p< 0.05, **B:** Gal-3_B1, **C:** Gal-3_C,*p< 0.05, **D:** Gal-3_CN, *p< 0.05.

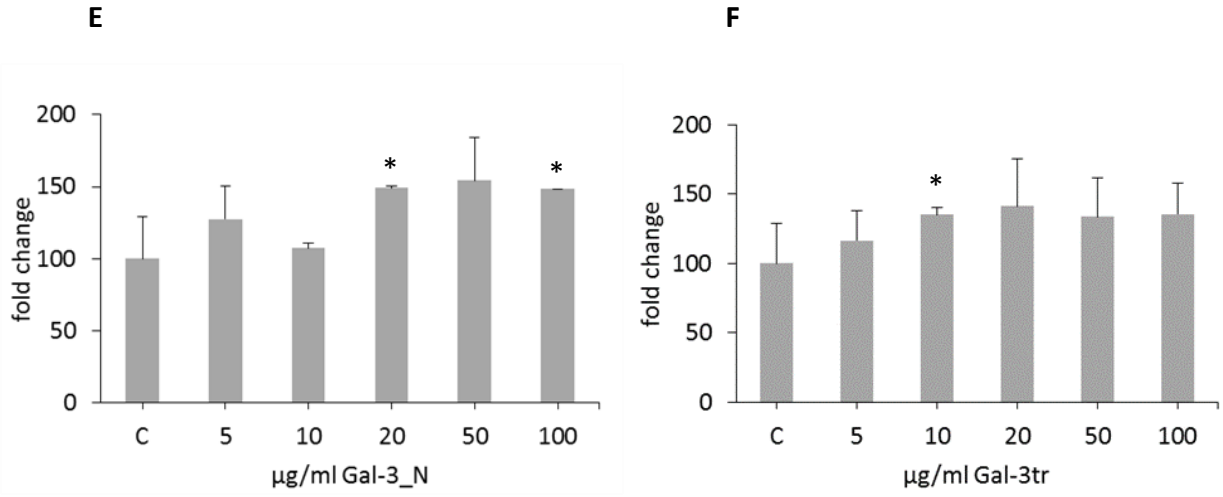


Fig. 10 (E-F): RA synoviocytes (n=1) were treated for 24 h with 5, 10, 20, 50 and 100 µg/ml of Gal-3 and its variants to investigate their effects on metabolic cell activity.
E: Gal-3_N, *p< 0.05.
F: Gal-3tr, *p< 0.05.

Figure 11 (A-C) shows the impact of Gal-8 and its variants at 5, 10, 20, 50 and 100 µg/ml on the metabolic cell activity of RA synoviocytes. These galectins again showed no concentration-dependent impairment of cell activity. Gal-8 (Fig. 11A) decreased cellular activity significantly at 5 µg/ml, which however appeared to be caused by experimental errors rather than by a specific biological effect.

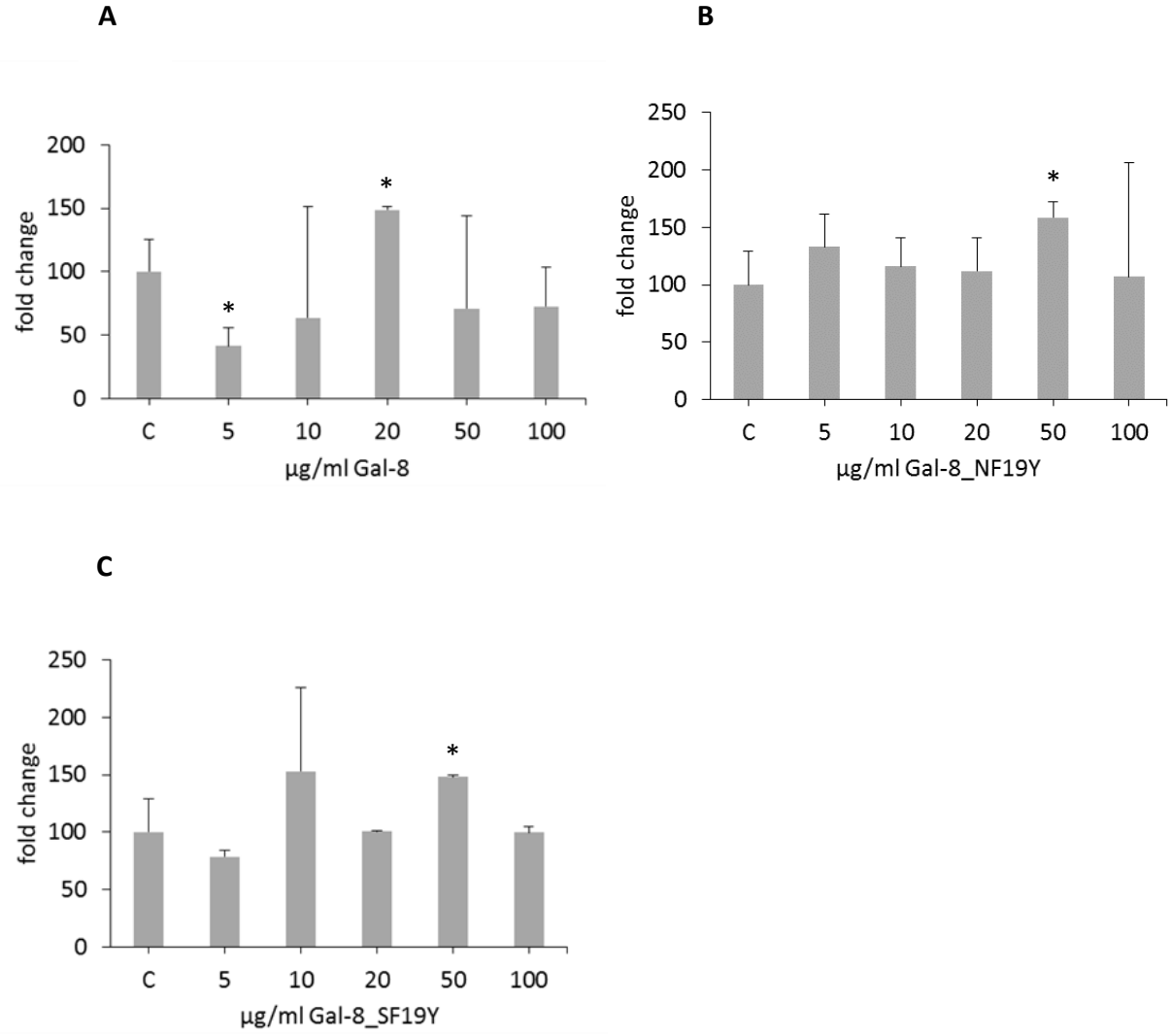


Fig. 11: RA synoviocytes (n=1) were treated for 24 h with 5, 10, 20, 50 and 100 µg/ml of Gal-8 and its variants to investigate their effects on metabolic cell activity.
A: Gal-8,*p< 0.05, **B:** Gal-8_NF19Y, *p< 0.05, **C:** Gal-8_SF19Y, *p< 0.05.

Figure 12 (A-D) depicts the effect of the full length proteins Gal-2, Gal-4, Gal-7 and Gal-9 on the metabolic cell activity of RA synoviocytes. Analyses revealed no concentration-dependent impairment of cellular activity for Gal-2, -4 and -7 (Fig. 12A-C). Gal-9 (Fig. 12D) strongly affected a decrease of metabolic activity at 100 µg/ml. Due to the analyses revealed for RA synoviocytes and preliminary experiments (data not shown), the following biological assays were carried out at a concentration of 5 µg/ml.

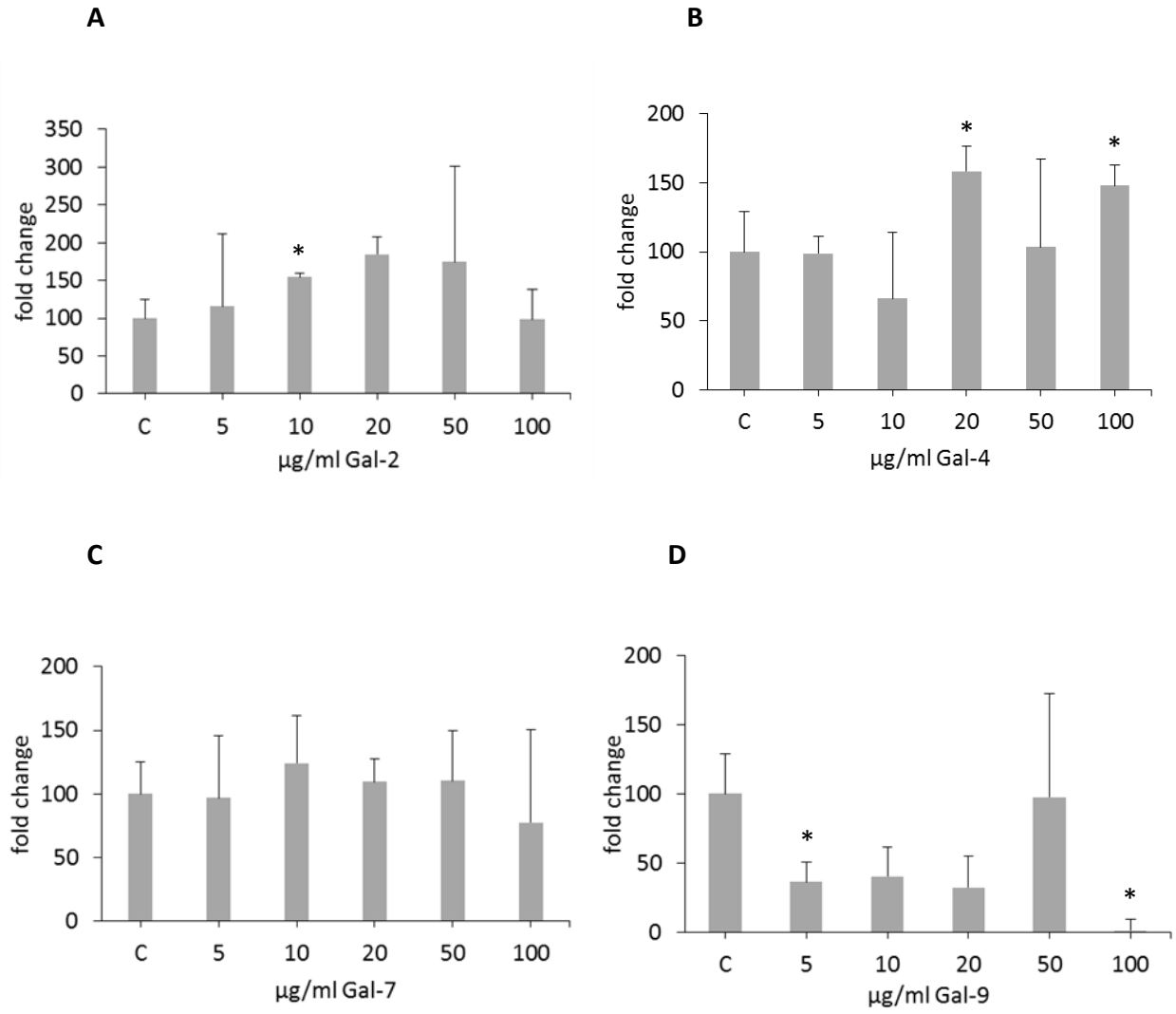


Fig. 12: RA synoviocytes (n=1) were treated for 24 h with 5, 10, 20, 50 and 100 µg/ml of Gal-2, -4, -7 and -9 to investigate their effects on metabolic cell activity.
A: Gal-2, *p< 0.05, **B:** Gal-4, *p< 0.05, **C:** Gal-7, **C:** Gal-9, *p< 0.05.

3.2. Quantification of mRNA levels in galectin-treated cells

The effect of various galectins in cultured chondrocytes and synoviocytes was investigated using qPCR for determining their impact on MMP13 and IL1B gene expression. Chondrocytes were usually stimulated with 10 µg/ml of each galectin for 24 h after "overnight" starvation. Concentration-dependent experiments were carried out with Gal-3 and Gal-3tr including concentrations of 5, 10 and 20 µg/ml for cell stimulation.

Synoviocytes were starved for 5 h and treated for 12 h. Stimulation of cells was usually performed with a concentration of 5 µg/ml and included only Gal-3 and its variants. Concentration-dependent experiments were only performed for Gal-3 and Gal-3tr at 2.5, 5 and 10 µg/ml.

Total RNA was extracted and equal amounts were used for cDNA synthesis, prior to analysis of mRNA expression levels using qPCR. p-Values < 0.05 were considered statistically significant (t-test).

3.2.1. Activity of galectins in OA chondrocytes

Figure 13A shows a direct comparison between Gal-1, Gal-2, Gal-3, Gal-4, Gal-7, Gal-8 and Gal-9 regarding their ability to induce MMP13 levels in OA chondrocytes. An up-modulation was induced by all galectins, including differences among each other. The range encompassed a minimum of 4.39-fold up-regulation (± 3.69) by Gal-4, to a maximum of 22.95-fold (± 8.69) by Gal-9. Although not statistically significant, a difference between Gal-1 (up-regulation 17.52-fold ± 4.59) and Gal-3 (7.25-fold ± 0.94) is apparent. As compared to the control, statistical significance was given for Gal-3 ($p=0.03$) and Gal-8 ($p=0.048$). There were no statistical significances among each galectins.

The difference between Gal-1 and Gal-3 regarding their potential to increase MMP13 gene expression was repeated for non-OA chondrocytes (Fig. 13B). Gal-1 induced a 12.79-fold up-regulation (± 1.93) with a statistical significance ($p=0.004$). Gal-3 elevated MMP13 expression 2.59-fold (± 3.68), with no statistical significance as compared to the control. The effect of Gal-1 compared to Gal-3 reached statistical significance ($p=0.04$).

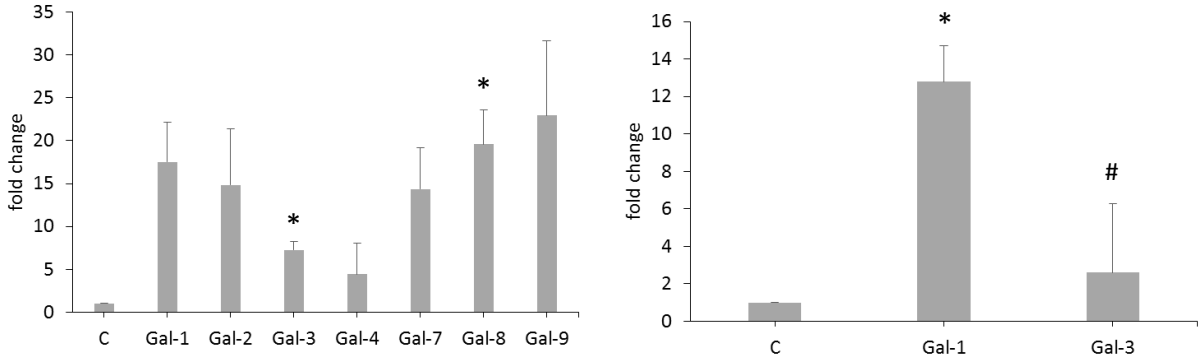


Fig. 13A (left): **Effect of Gal-1, -2, -3, -4, -7, -8 and -9 at 10 µg/ml on MMP13 gene expression in OA chondrocytes (n=2) incubated for 24 h**, *p<0.05.
Fig. 13B (right): **Effect of Gal-1 and Gal-3 at 10 µg/ml on MMP13 gene expression in non-OA chondrocytes (n=3) incubated for 24 h**, *p<0.05, #p<0.05 Gal-3 vs. Gal-1.

Figure 14 depicts the effects of Gal-1, Gal-2, Gal-3, Gal-4, Gal-7, Gal-8 and Gal-9 on IL1B gene expression in OA chondrocytes. In comparison to the control, all galectins tended to induce an up-regulation of IL1B. Although statistically not significant, Gal-9 induced a 34074.51-fold (± 48161.79) and Gal-2 a 9660.31-fold (± 13642.03) increase of IL1B. Lowest alteration of IL1B expression was mediated by Gal-3 (40.53-fold ± 48.21). Because of the limited number of donor samples (n=2) and high interindividual variability, there was no statistical significance for each galectin, although the up-regulation of IL1B was partially remarkable regarding the values on the y-axis. Gal-1 modulated the mRNA-levels of this gene 1061.51-fold (±1495.51) and Gal-8 up to 2820.69-fold (±3978.29).

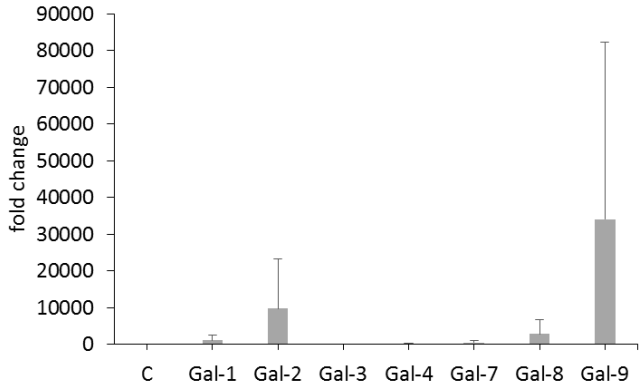


Fig. 14: **Effect of Gal-1, -2, -3, -4, -7, -8 and -9 at 10 µg/ml on MMP13 gene expression in OA chondrocytes (n=2) incubated for 24 h**.

Figure 15A and Figure 15B present a comparison between Gal-1 and its variants in their potential to induce MMP13 and IL1B gene expression in OA chondrocytes. Fig. 15A suggests an up-regulation of MMP13 by all galectins, despite the lack of statistical significance. The numerical differences ranged from a 15.39-fold (± 6.52) increase by Gal-1_GG to a 20.18-fold by Gal-1_8L (± 8.12). There was no statistical significance among the galectins. Similarly, there was no significant difference between the full length protein Gal-1 (17.52 ± 4.59) and its variants.

Albeit statistically not significant, Figure 15B suggests an up-regulation of IL1B by all galectins as compared to the control. The fold change range numerically went from a 1061.51-fold up-modulation (± 1495.51) by Gal-1 to a 3448.24-fold (± 4868.57) up-modulation by Gal-1_8L. Although statistical significance across all patients was compromised by high interindividual differences, a numerically high up-regulation of IL1B was observed for all galectins, as compared to the control. In comparison to the full length protein, its variants did not show significant differences in their effect.

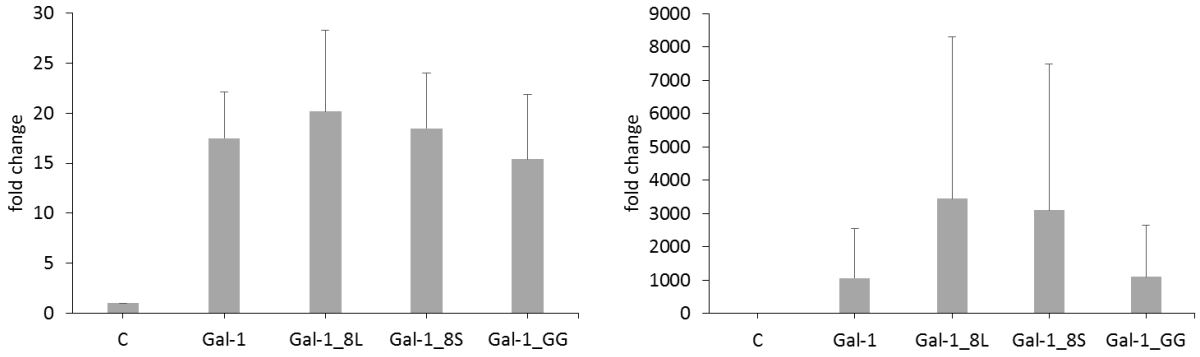


Fig. 15A (left): Effect of Gal-1 and its variants at 10 µg/ml on MMP13 gene expression in OA chondrocytes (n=2) incubated for 24 h.
Fig. 15B (right): Effect of Gal-1 and its variants at 10 µg/ml on IL1B gene expression in OA chondrocytes (n=2) incubated for 24 h.

Figure 16A and Figure 16B illustrate Gal-3 in comparison to its variants in their ability to increase mRNA-levels of MMP13 and IL1B in OA chondrocytes. Fig. 16A shows a statistical significance (p=0.001-0.1) for all galectins in the up-regulation of MMP13. As compared to the control, Gal-3 significantly induced a 8.04-fold (± 3.31, p=0.004) up-regulation and Gal-3tr a 4.08-fold (± 1.73, p=0.008) up-regulation. Gal-3_N evoked the highest increase up to a 12.26-fold (± 4.31, p=0.002) as compared to the control. The potential of Gal-3_C to induce MMP13 was also statistically significant (9.32-fold ± 5.42, p=0.01) and similar to the significant up-regulation evoked by Gal-3_CN (9.62-fold ± 2.60, p=0.001). As compared to the effect of the full length protein (8.04 ± 3.31), there was a statistical significance for Gal-3_CN (p=0.04), Gal-3_N (p=0.01), and Gal-3tr (p=0.005).

Despite the lack of statistical significance, Figure 16B suggests an up-regulation of IL1B gene expression by all galectins. As compared to the control, Gal-3 showed a 66.43-fold (± 83.12) tendency towards up-regulation of IL1B. Gal-3tr exhibited the lowest modulating effects on IL1B gene expression (10.41-fold ± 13.27). Albeit statistical significance across all patients was compromised due to the high level of interindividual variability, Gal-3_N was suggested to induce a remarkably high up-regulation of IL1B (548.78-fold ± 1054.06) as compared to the control. Gal-3_C (84.24 ± 103.22) and Gal-3_CN (89.45 ± 101.22) modulated IL1B mRNA-levels numerically similarly. As compared to the full length protein of Gal-3, none of its variants reached statistical significance.

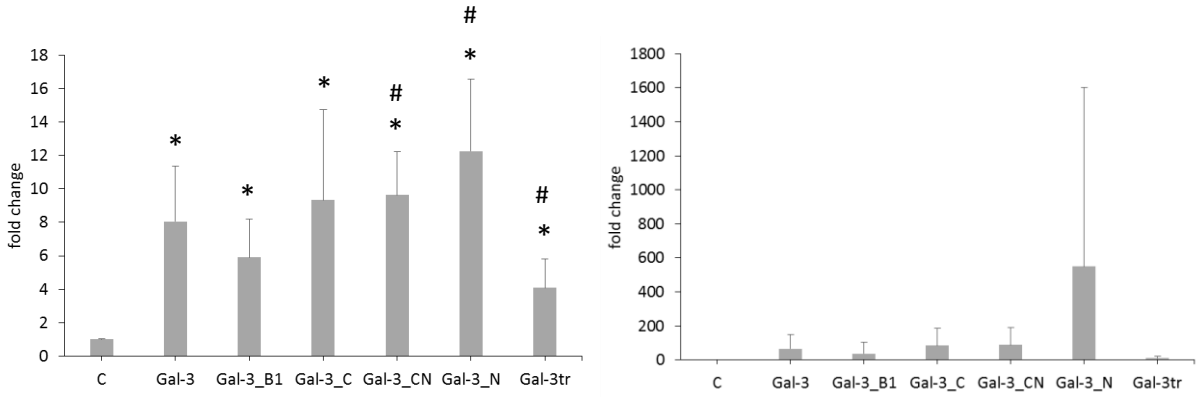
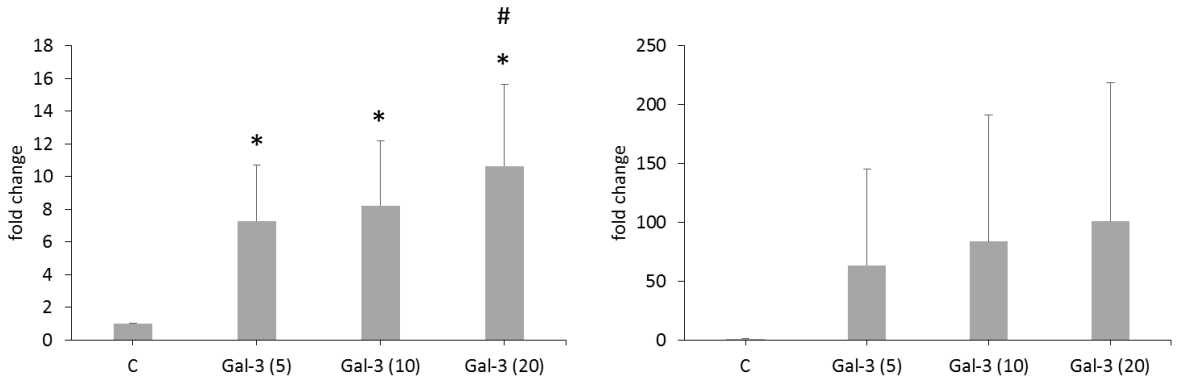


Fig. 16A (left): Effect of Gal-3 and its variants at 10 µg/ml on MMP13 gene expression in OA chondrocytes (n=5) incubated for 24 h, *p<0.05, #p<0.05 all vs. Gal-3.
Fig. 16B (right): Effect of Gal-3 and its variants at 10 µg/ml on IL1B gene expression in OA chondrocytes (n=5) incubated for 24 h.

With particular focus placed on Gal-3 and Gal-3tr, Figure 17A and 17B illustrate concentration-dependent experiments in OA chondrocytes. As shown in Figure 17A, the up-regulation of MMP13 was significant for all concentrations of Gal-3 in comparison to the control. Relative quantity of mRNA level was slightly increasing from the lowest to the highest concentration. Gal-3 significantly induced a 7.29- fold (± 3.41, p=0.04) and a 10.65-fold (± 5.02, p=0.04) increase of MMP13 at 5 µg/ml and 20 µg/ml, respectively. At 10 µg/ml, Gal-3 significantly promoted a 8.22-fold (± 3.97, p=0.04) up-regulation. As compared to the standard concentration (10 µg/ml), Gal-3 at 20 µg/ml induced a significantly (p=0.03) higher increase of MMP13.

Although statistically not significant, Figure 17B suggests an up-regulation of IL1B at all applied concentrations of Gal-3. As compared to the control, Gal-3 induced a 63.37-fold (± 81.75) and a 83.71-fold (± 107.41) increase of IL1B at 5 µg/ml and 10 µg/ml, respectively. At 20 µg/ml, the modulation of IL1B expression was numerically the highest (100.99-fold ± 117.74). Despite the lack of statistical significance, the tendency to up-regulate IL1B was prominent. In comparison to the standard concentration (10 µg/ml), the other concentrations did not induce statistically significant differences.



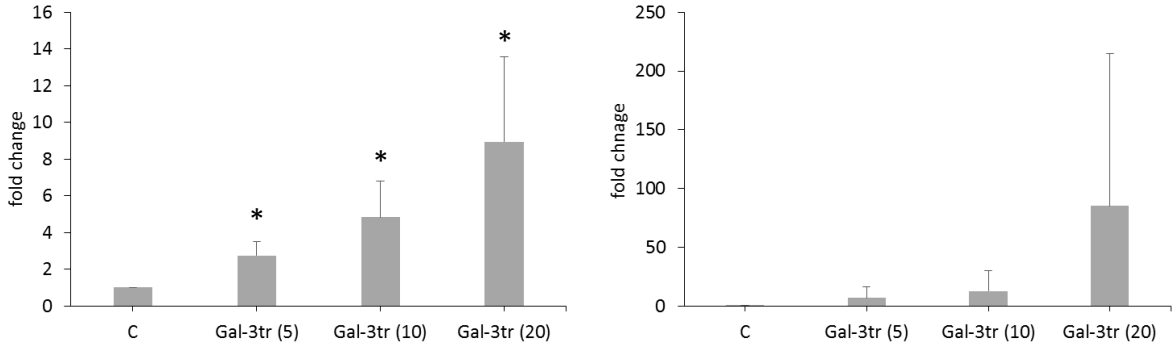
Concentration-dependent experiments.

Fig. 17A (left): Effect of Gal-3 at 5, 10 and 20 µg/ml on MMP13 gene expression in OA chondrocytes (n=3) incubated for 24 h, *p<0.05, #p<0.05 all concentrations vs. Gal-3 (10).

Fig. 17B (right): Effect of Gal-3 at 5, 10 and 20 µg/ml on IL1B gene expression in OA chondrocytes (n=3) incubated for 24 h.

Figure 18A and 18B demonstrate the concentration-dependent ability of Gal-3tr to induce MMP13 and IL1B mRNA-levels in OA chondrocytes. Fig. 18A shows a significantly up-regulation of MMP13 at all applied concentrations. As compared to the control, Gal-3tr significantly induced a 2.75-fold (± 0.75, p=0.03) and a 8.94-fold (± 4.64, p=0.049) up-regulation of MMP13 at 5 µg/ml and 20 µg/ml, respectively. Gal-3tr at the standard concentration of 10 µg/ml significantly promoted a 4.84-fold (± 1.95, p=0.04) up-regulation of MMP13. As compared to this standard concentration, the others induced no significantly different effects.

Figure 18B presents the same experiment for IL1B gene expression. Despite the lack of statistical significance, an up-regulation of IL1B was suggested for all concentrations. As compared to the control, Gal-3tr modulated the mRNA-level of IL1B 6.94-fold (± 9.62) and 12.81-fold (± 17.23) at 5 µg/ml and 10 µg/ml, respectively. Although statistically not significant, Gal-3tr at 20 µg/ml induced the numerically highest IL1B up-regulation (85.15-fold ± 129.62). In comparison to the standard concentration of 10 µg/ml, there was no statistical significance for the other concentrations.



Concentration-dependent experiments.

Fig. 18A (left): Effect of Gal-3tr at 5, 10 and 20 µg/ml on MMP13 gene expression in OA chondrocytes (n=3) incubated for 24 h, *p<0.05.

Fig. 18B (right): Effect of Gal-3tr at 5, 10 and 20 µg/ml on IL1B gene expression in OA chondrocytes (n=3) incubated for 24 h.

Figure 19A and 19B depict a comparison of Gal-8 and its variants in their efficacy to increase MMP13 and IL1B gene expression in OA chondrocytes. Fig. 19A shows a statistically significant 19.58-fold (± 3.99, p=0.048) up-regulation of MMP13 by Gal-8, as compared to the control. Although statistically not of significance, Gal-8_SF19Y induced a 7.60-fold (± 2.11) and Gal-8_NF19Y a 11.89-fold (± 5.53) up-regulation of MMP13. As compared to the full length protein, the effects of Gal-8_NF19Y (p=0.045) and Gal-8_SF19Y (p=0.035) were significantly lower.

Figure 19B illustrates the effects of Gal-8 and its variants on IL1B gene expression. Although the up-regulation of IL1B was highly modulated by Gal-8 (2820.69-fold ± 3978.29), it did not reach statistical significance across all patients, as compared to the control. Despite the lack of statistical significance due to high interindividual differences, Gal-8_SF19Y (50.04-fold ± 65.09) and Gal-8_NF19Y (425.23-fold ± 594.70) also suggested an up-regulation of IL1B. There was no statistical significance for the variants in comparison to Gal-8, albeit a striking difference is apparent in Figure 19B, which might be explained by the limited number of patients in this experiment (n=2).

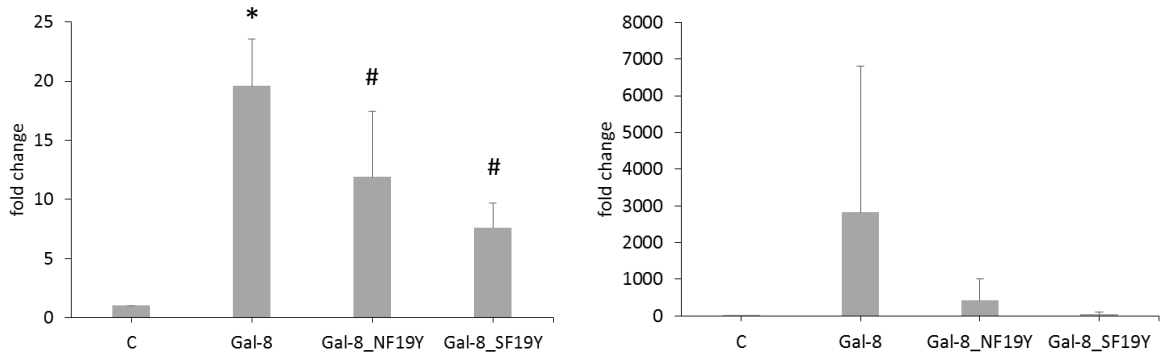


Fig. 19A (left): Effect of Gal-8 and its variants at 10 µg/ml on MMP13 gene expression in OA chondrocytes (n=2) incubated for 24 h, *p<0.05, #p<0.05 variants vs. Gal-8.
Fig. 19B (right): Effect of Gal-8 and its variants at 10 µg/ml on IL1B gene expression in OA chondrocytes (n=2) incubated for 24 h.

3.2.2. Activity of galectins in OA synoviocytes

Figure 20A and 20B present a direct comparison of Gal-3 and its variants in their effect on MMP13 and IL1B gene expression in OA synoviocytes. Figure 20A shows a low modulation of MMP13 by all galectins with only minor differences. These differences numerically ranged from a 2.09-fold (± 1.77) up-regulation by Gal-3_C to a 7.57-fold (±10.57) up-regulation by Gal-3_N. As compared to the control, none of the galectins reached statistical significance, although Gal-3_N suggested the numerically highest up-regulation of MMP13 (7.57-fold ± 10.57). In comparison to the modulating effects of the full length protein Gal-3 (2.45-fold ± 1.69), the variants did not induce statistically significant differences.

Figure 20B depicts the effect of Gal-3 and its variants on IL1B expression in OA synoviocytes. The modulation of this gene by the various galectins numerically ranged from a minimum of 1.58-fold (± 0.24) by Gal-3_B1 to a maximum of a 33.66-fold (± 32.01) by Gal-3_N. The full length protein Gal-3 showed a low modulation of IL1B (4.59-fold ± 4.90). As compared to the control, none of the galectins reached statistically significant effects. Despite the lack of statistical significance, Gal-3_N suggested a high up-regulation of IL1B as compared to Gal-3. There were no significantly differences in the effects of the variants, as compared to Gal-3.

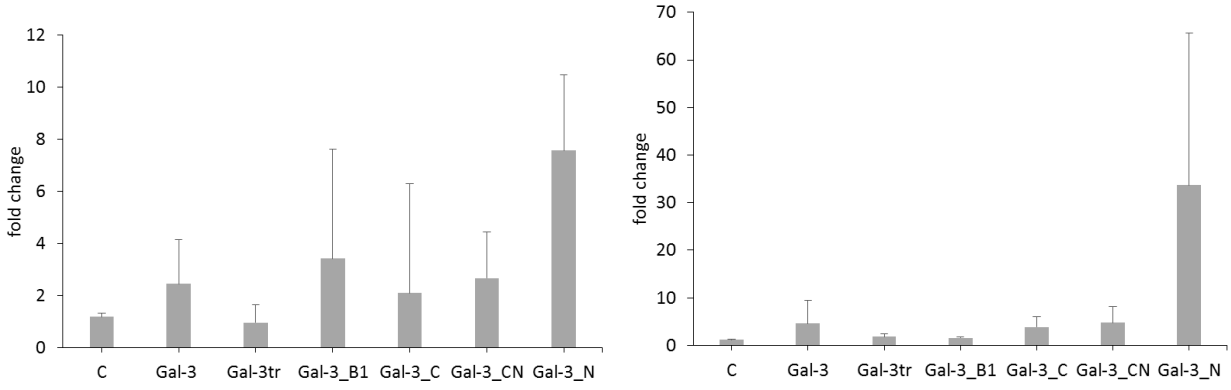
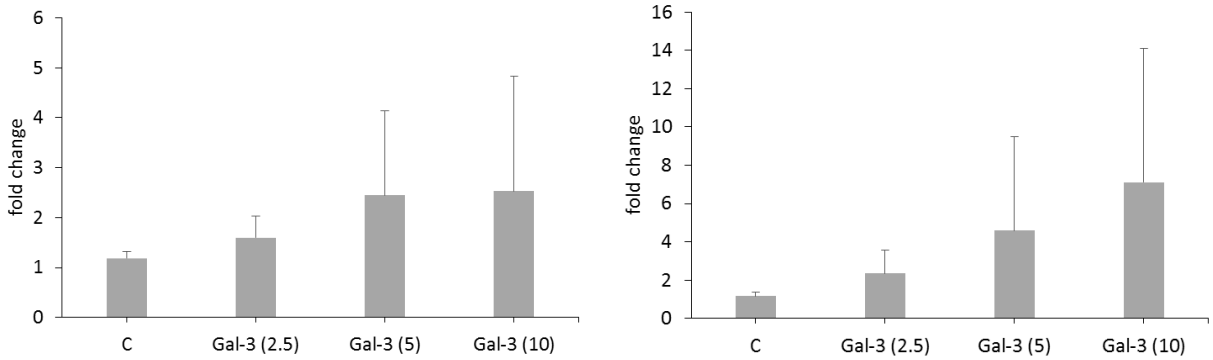


Fig. 20A (left): Effect of Gal-3 and its variants at 5 µg/ml on MMP13 gene expression in OA synoviocytes (n=3) incubated for 12 h.
Fig. 20B (right): Effect of Gal-3 and its variants at 5 µg/ml on IL1B gene expression in OA synoviocytes (n=3) incubated for 12 h.

Figure 21A and 21B illustrate concentration-dependent effects of Gal-3 in OA synoviocytes. Fig. 21A shows no significant concentration-dependent effect of Gal-3 on MMP13 gene expression. As compared to the control, Gal-3 numerically induced a 1.59-fold (± 0.43), 2.45-fold (± 1.69) and a 2.53-fold (± 2.31) increase of MMP13 at 2.5, 5 and 10 µg/ml, respectively. In comparison to the control, Gal-3 revealed no significant differences at applied concentrations regarding its ability to increase MMP13 gene expression. As compared to the effect of Gal-3 at a standard concentration of 5 µg/ml, the other concentrations did not induce statistically significant differences.

Figure 21B presents the concentration-dependent effect of Gal-3 on the IL1B gene regulation. The low modulation of IL1B by Gal-3 numerically ranged from a 2.35- fold (± 1.21) at 2.5 µg/ml to a 7.11-fold (± 7.00) at 10 µg/ml. Analyses revealed no statistically significant concentration-dependent effect of Gal-3 as compared to the control. In comparison to the effect of Gal-3 at a standard concentration of 5 µg/ml (4.59-fold ± 4.90), this galectin did not induce significantly different effects at the other concentrations.



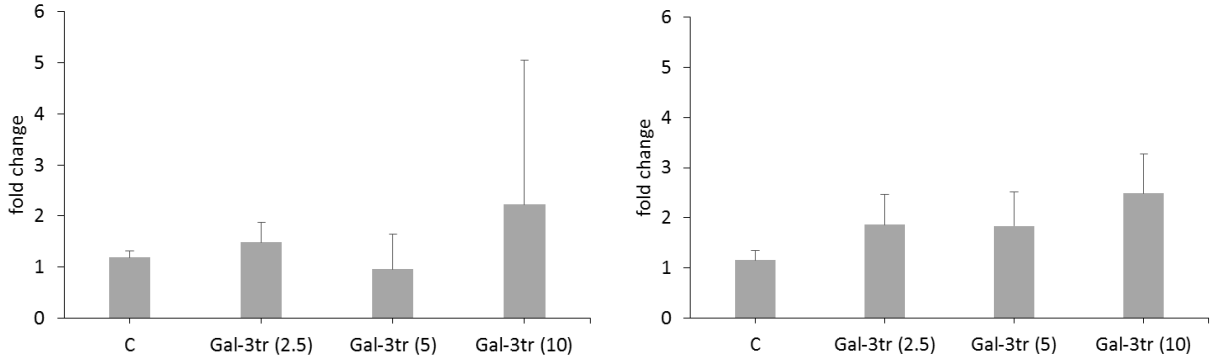
Concentration-dependent experiments.

Fig. 21A (left): Effect of Gal-3 at 2.5, 5 and 10 µg/ml on MMP13 gene expression in OA synoviocytes (n=3) incubated for 12 h.

Fig. 21B (right): Effect of Gal-3 at 2.5, 5 and 10 µg/ml on IL1B gene expression in OA synoviocytes (n=3) incubated for 12 h.

Figure 22A and 22B present the concentration-dependent effect of Gal-3tr on MMP13 and IL1B gene expression in OA synoviocytes. Figure 22A shows a very low alteration of MMP13 by Gal-3tr at 2.5 µg/ml (1.48 ± 0.39), 5 µg/ml (0.95 ± 0.70) and at 10 µg/ml (2.22 ± 2.82). However, the differences were statistically not significant as compared to the control. Also in comparison to the standard concentration of 5 µg/ml, the other concentrations did not induce statistically significant differences in their effect on MMP13 gene expression.

Figure 22B illustrates the concentration-dependent effect of Gal-3tr on IL1B gene regulation. Modulation of IL1B by Gal-3tr was very low at all concentrations as compared to the control. The alteration of IL1B mRNA levels numerically ranged from a 1.86- fold (± 0.60) up-regulation at 2.5 µg/ml, to a 2.49-fold (± 0.79) up-regulation at 10 µg/ml. There was no significant difference revealed between the single concentrations regarding their effect on IL1B gene expression. Thus also, Gal-3tr seemed to evoke hardly any effects in OA synoviocytes.



Concentration-dependent experiments.

Fig. 22A (left): Effect of Gal-3tr at 2.5, 5 and 10 µg/ml on MMP13 gene expression in OA synoviocytes (n=3) incubated for 12 h.

Fig. 22B (right): Effect of Gal-3tr at 2.5, 5 and 10 µg/ml on IL1B gene expression in OA synoviocytes (n=3) incubated for 12 h.

3.2.3. Activity of galectins in RA synoviocytes

Figure 23A and 23B present the effect of Gal-3 and its variants on MMP13 and IL1B gene expression in RA synoviocytes. In Figure 23A, a modulation of MMP13 by all galectins is shown as compared to the control, including a fold change range from 1.59-fold (± 0.58, induced by Gal-3tr) to 11.96-fold (± 15.16, induced by Gal-3_N). Gal-3 provided a 2.39-fold (± 1.77) up-regulation of MMP13. Although statistical significance across all patients was compromised by high interindividual differences, Gal-3_N induced a numerically high up-regulation of MMP13. In comparison to the control, there was a lack of statistical significance for all galectins. As compared to the full length protein, the variants did not induce significant differences in their effect on RA synoviocytes.

Figure 23B illustrates the effect of Gal-3 and its variants on the regulation of IL1B in RA synoviocytes. As compared to the control, the increase of IL1B was found to be significant in case of Gal-3_N with a 13.31-fold (± 1.57, p=0.03) up-regulation. Although Gal-3_CN did not reach statistical significance, a 7.00-fold (± 4.78) up-regulation as compared to the control is suggested. Gal-3 and Gal-3tr numerically modulated the IL1B gene expression 1.73-fold (± 0.60) and 0.75-fold (± 0.18), respectively. Except for Gal-3_N, there was no statistical significance for any galectin, as compared to the control. There were also no statistically significant differences revealed for the variants, as compared to the full length protein.

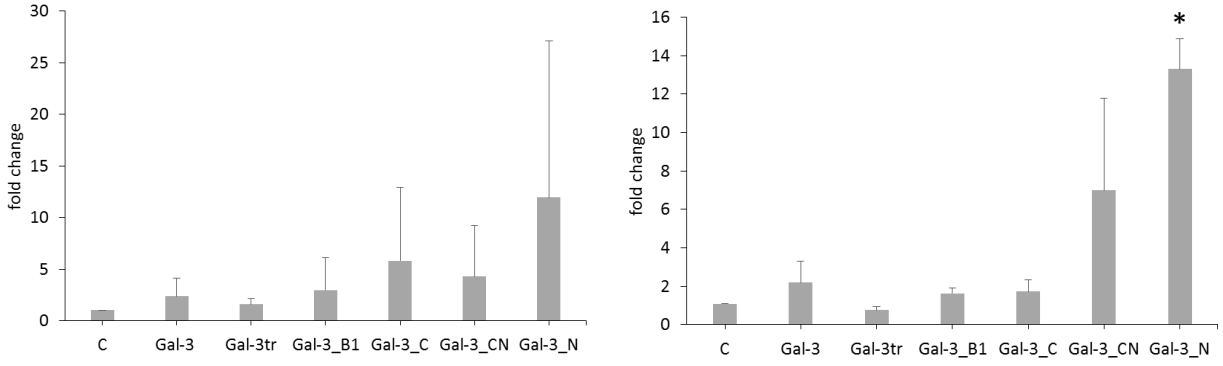
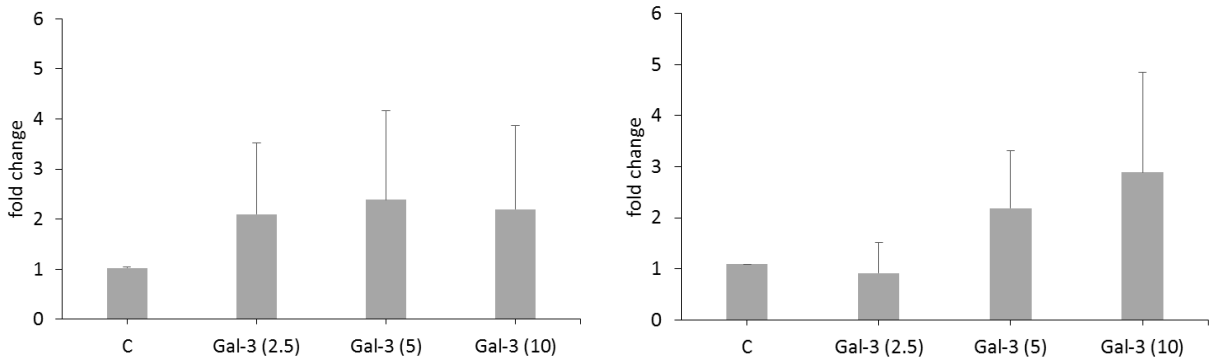


Fig. 23A (left): Effect of Gal-3 and its variants at 5 µg/ml on MMP13 gene expression in RA synoviocytes (n=2) incubated for 12 h.
Fig. 23B (right): Effect of Gal-3 and its variants at 5 µg/ml on IL1B gene expression in RA synoviocytes (n=2) incubated for 12 h, *p<0.05.

Figure 24A and 24B depict concentration-dependent effects of Gal-3 on MMP13 and IL1B gene regulation in RA synoviocytes. Figure 24A reveals no significant concentration-dependency of Gal-3 regarding its ability to induce MMP13 gene expression. A similarly low modulation of MMP13 was suggested by Gal-3 at 2.5 µg/ml (2.09-fold ± 1.43), 5 µg/ml (2.39-fold ± 1.77) and at 10 µg/ml (2.20-fold ±1.67). However, none of the differences reached statistical significance as compared to the control or to the standard concentration of 5 µg/ml.

Figure 24B shows the concentration-dependent effects of Gal-3 on the regulation of IL1B. In comparison to the control, there was very low modulation of this gene, including a fold change range from 0.92-fold (± 0.60, induced at 2.5 µg/ml) to 2.89-fold (± 1.96, induced at 10 µg/ml). The effect of Gal-3 in RA synoviocytes regarding the ability to induce IL1B mRNA-levels, showed no statistical significance at any of the applied concentrations.



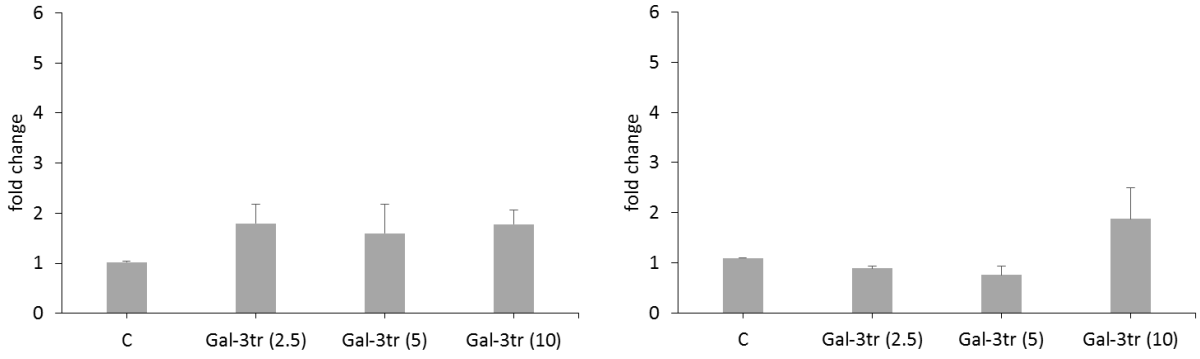
Concentration-dependent experiments.

Fig. 24A (left): Effect of Gal-3 at 2.5, 5 and 10 µg/ml on MMP13 gene expression in RA synoviocytes (n=2) incubated for 12 h.

Fig. 24B (right): Effect of Gal-3 at 2.5, 5 and 10 µg/ml on IL1B gene expression in RA synoviocytes (n=2) incubated for 12 h.

Figure 25A and 25B present concentration-dependent effects of Gal-3tr on mRNA-levels of MMP13 and IL1B in RA synoviocytes. In Figure 25A, a very low modulation of MMP13 is shown at all applied concentrations as compared to the control. Gal-3tr numerically induced a 1.79-fold (± 0.38) and a 1.78-fold (± 0.27) increase at 2.5 µg/ml and 10 µg/ml, respectively. At 5 µg/ml, Gal-3tr modulated MMP13 1.59-fold (± 0.58). There was no statistical significance observed at any concentration as compared to the control. In addition, there was no significant difference between the effects of various concentrations of Gal-3tr.

Figure 25B illustrates the effect of Gal-3tr at various concentrations regarding the IL1B gene expression in RA synoviocytes. As compared to the control, none of the applied concentrations reached statistical significance. A very low alteration was induced by Gal-3tr at 10 mg/ml (1.87-fold ± 0.62). At 2.5 µg/ml and 5 µg/ml, Gal-3tr caused a slight decrease of IL1B gene expression of 0.89-fold (± 0.04) and 0.75-fold (± 0.18), respectively. There was no concentration-dependent effect of Gal-3tr on the IL1B regulation observed.



Concentration-dependent experiments.

Fig. 25A (left): Effect of Gal-3tr at 2.5, 5 and 10 µg/ml on MMP13 gene expression in RA synoviocytes (n=2) incubated for 12 h.

Fig. 25B (right): Effect of Gal-3tr at 2.5, 5 and 10 µg/ml on IL1B gene expression in RA synoviocytes (n=2) incubated for 12 h.

3.3. Activation of the NF-$_K$B pathway by Gal-1 in OA chondrocytes

Primary OA chondrocytes were treated for 1, 5, 15, 30 and 60 min with 50 µg/ml Gal-1. Western blots were performed to assess the phosphorylation of the NF-$_K$B p65 protein subunit. α-tubulin was used as a reference protein. This experiment was repeated with cells obtained from three donors (n=3). The blot of one representative experiment is shown below. The NF-$_K$B pathway was revealed to be time-dependently activated under the influence of Gal-1 (Fig. 26).

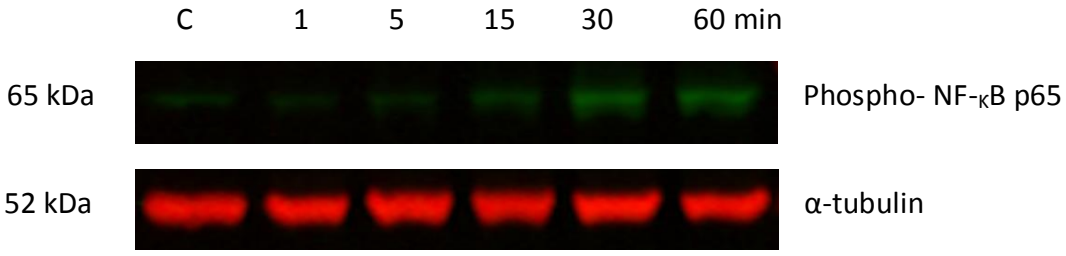


Fig. 26: Time-dependent activation of NF-$_K$B p65. Primary OA chondrocytes (n=3) were left untreated (C) or treated for 1, 5, 15, 30 and 60 min with 50 µg/ml Gal-1. Western blots were performed with α-tubulin serving as a reference protein. The blot of one representative experiment is shown.

4. Discussion

This diploma thesis aimed to characterize and compare the effects of a broad range of galectins regarding their contribution to cartilage breakdown through the regulation of genes that are assumed to be important modulators in the progression of OA. Therefore, mRNA-levels of MMP13 and IL1B were monitored by qPCR. In a previous study, it was already proven that the glycan pattern in OA cartilage is altered during the progression of the disease, presumably providing altered binding sites for galectins *(Toegel et al., 2013)*. This was pursued in another study with the central aim to define which galectins were present in OA cartilage and how their expression pattern changes with OA progression *(Toegel et al., 2014)*. Briefly, this previous study demonstrated that Gal-1, -3 and -8 were the most prominent galectins in OA cartilage. These findings gave reason to determine the biological activity of galectins in OA chondrocytes and synoviocytes, also including galectin-variants to determine which section of the full length protein contributes to mediating a cellular response.

To ensure that the galectins did not induce apoptosis in cultured chondrocytes and synoviocytes, their effect on metabolic cell activity was investigated at concentrations of 5, 10, 20, 50 and 100 µg/ml as a first step. In OA chondrocytes, analyses revealed a significantly impairment of the cellular activity by all galectins at 100 µg/ml, most effectively by Gal-9 already at 50 µg/ml. Concentrations between 5-20 µg/ml were observed to be well tolerated. Therefore, concentrations for qPCR experiments were established at 10 µg/ml. In OA and RA synoviocytes, data did not reveal a dose-dependent impairment of the metabolic cell rate for any galectin, except for Gal-9, which significantly reduced cellular activity at concentrations above 20 µg/ml. Since concentrations < 20 µg/ml appeared to be well tolerated and based on results of preliminary experiments (data not shown), further qPCR experiments employing OA and RA synoviocytes were performed at 5 µg/ml.

The presented qPCR data in OA chondrocytes indicated a significant up-regulation of MMP13 following treatment with Gal-8 and Gal-3 as compared to the control. Generally, all full-length proteins modulated an up-regulation of MMP13, including differences among each other, as shown in Figure 13A. Without reaching significance, Gal-9 and Gal-1 numerically caused the highest up-regulation of MMP13. This pattern was repeated for IL1B gene expression in a very

high tendency towards up-regulation, but without reaching significance for any galectin. The apparent impact of Gal-1 and Gal-3 on MMP13 was verified also in non-OA chondrocytes, where the difference between these two galectins turned out to be significant. This finding underscores the important point that the expression and the effect of these galectins in articular chondrocytes is irrespective of the underlying OA disease. In previous studies, it was shown that Gal-1 and Gal-3 are not expressed in normal articular cartilage *(Boeuf at al., 2010; Toegel et al., 2014)*. In the latter mentioned study, it was additionally shown that within OA cartilage, the presence of Gal-1 and Gal-3 was increased in areas of severely degraded cartilage as compared to almost intact areas. Summing up, these findings hypothetically suggest that galectins which are produced by OA chondrocytes may be able to affect healthy cartilage areas and impact on non-OA chondrocytes as a trigger of disease progression.

Further analyses in this thesis focused on the comparison of the full-length proteins of Gal-1, Gal-3 and Gal-8 with their respective variants. Although statistical significance was not reached, the strong and consistent up-regulation of MMP13 and IL1B in OA chondrocytes by Gal-1 was considered biologically relevant. In the context of joint tissues and OA, the presence of this galectin was first evidenced in porcine chondrocytes *(Marcon et al., 2005)*. This finding led to another study that investigated the mechanisms involving Gal-1 and finally proved its catabolic effect in porcine OA cartilage through up-regulation of MMP13 *(Marsich et al., 2008)*. In agreement, this diploma thesis provides the first experimental evidence that Gal-1 is able to promote mRNA-levels of MMP13 in human OA chondrocytes. In comparison to Gal-1, its variants did not reveal significant differences in their impact on MMP13 and IL1B. The modulation of these genes was observed to be similar for all variants, again IL1B was modulated in a numerically higher dimension. This finding may be the first evidence that the insertion of a linker peptide into the non-covalent homodimeric structure of Gal-1, as pursued in the tailored Gal-1 variants, may not crucially impact on the effect of Gal-1 in OA chondrocytes.

Gal-8 showed a statistically significant up-regulation of MMP13 in OA chondrocytes. Although its effect on the IL1B gene expression reached no statistical significance, the up-modulation was numerically remarkable as compared to the control. This observation might be of interest, since it was shown that Gal-8 and corresponding binding sites are highly expressed in OA cartilage *(Toegel et al., 2014)*. In comparison to Gal-8, its variants Gal-8_NF19Y and Gal-8_SF19Y showed a

significantly reduced induction of MMP13. This trend, although not statistically significant, was repeated in case of IL1B induction. These findings may suggest that the single nucleotide polymorphism in human Gal-8 reduces its affinity to bind to corresponding binding sites in OA chondrocytes. The single base change is located in the N-terminus of the protein, this domain might contribute to some extent to the binding and biological effectivity of Gal-8.

Gal-3 induced a significant increase of MMP13 gene expression in OA chondrocytes. The up-modulating effect on IL1B, however, did not reach statistical significance. Considering the findings of previous studies in which the levels of Gal-3 were revealed to be increased in OA chondrocytes *(Guévremont et al., 2004)*, this result might be of importance. All variants of Gal-3 induced a statistically significant up-regulation of MMP13. For the IL1B up-regulation there was no statistical significance reached. Compared to the full-length protein, Gal-3_N and Gal-3_CN promoted a significantly higher up-regulation of MMP13. In contrast, Gal-3tr promoted a significantly lower up-regulation (Fig. 16A) suggesting that the N-terminus of the protein might play a role for the binding activity. Interestingly, in another study the truncation of Gal-3 was revealed to impair its potency upon cell surface binding, suggesting that the N-terminus could be an important structure for modulatory functions of Gal-3 *(Kopitz et al., 2001)*.

The statistically significant differences between Gal-3 and Gal-3tr, aroused the interest to investigate the effects of these galectins also at rising concentrations. Both, Gal-3 and Gal-3tr induced a significant concentration-dependent increase of MMP13 in OA chondrocytes, whereby the effect of Gal-3tr at 5 and 10 µg/ml was always lower than that of Gal-3. This difference is well presented in Figure 17B and 18B. Although numerically striking, the dose-dependent up-regulation of IL1B did not reach statistical significance, neither for Gal-3 nor for Gal-3tr. These results may lead to the hypothesis that a higher concentration of these galectins seem to induce a higher gene up-regulation in OA chondrocytes. In light of the results of a previous study, suggesting that Gal-3 and corresponding binding sites increased with OA progression *(Toegel at al., 2013)*, the presented data suggest that with progression of the disease cartilage may be able to bind more galectins that in turn trigger a higher expression of genes that may lead to cartilage degeneration.

Although statistical significance across all patients was sometimes compromised by high interindividual differences and the limited number of donor samples in the current thesis, the

quantitative data always revealed a numerically higher impact of galectins on the IL1B up-regulation than on MMP13. This suggests that galectins may primarily induce pro-inflammatory cytokines that in turn could modulate the expression of other genes through activation of signaling pathways. This hypothesis would be in agreement with a previous study that revealed that cartilage degeneration is partially induced through pro-inflammatory cytokines modulating the metabolism of chondrocytes to increase MMP13 synthesis *(Martel-Pelletier, 2004)*. Also, numerous other studies could already show a relation between OA and inflammation *(Sharif et al., 1997; Benito et al., 2005)*. In recent years, this led to the assumption of OA being a mechanically driven but chemically mediated process (*Pearle et al., 2005).* To our knowledge, this study provides first evidence that galectins are able to increase gene expression of inflammatory cytokines and matrix metalloproteinase in human OA chondrocytes.

Since the role of synovitis in OA is still controversial, we further explored the activity of galectins in human synoviocytes. The experiments in RA and OA synoviocytes focused on Gal-3 and its variants and again on concentration-dependent effects of Gal-3 and Gal-3tr. In general, synoviocytes showed limited reactivity towards galectin treatment as compared to OA chondrocytes. Gal-3 did not induce statistically significant increase of MMP13 and IL1B in OA or RA synoviocytes. Among the variants, Gal-3_N showed the numerically highest effect on MMP13 and IL1B gene expression. In RA synoviocytes, the IL1B up-regulation by Gal-3_N reached statistical significance. Gal-3tr induced the numerically lowest modulation of MMP13 and IL1B in OA and RA synoviocytes. The impact of Gal-3 and Gal-3tr did not show a concentration dependency neither in OA nor in RA synoviocytes. These findings are very interesting considering that many studies could already prove inflammation in synovial tissue of patients with OA *(Benito et al., 2005)*. This lack of reactivity to Gal-3 in synoviocytes might be explained by a possible lack of required binding sites for Gal-3 that might transduce respective signaling. However, an intracellular effect of galectins during RA or OA might still persist. In contrast, galectins demonstrated strong effects in OA chondrocytes regarding the up-regulation of MMP13 and IL1B gene expression. In a previous study, the expression of Gal-3 in synovial tissue could be demonstrated *(Ohshima et al., 2003)*. In light of these previous findings, the presented data might suggest that synoviocytes could produce Gal-3 that in turn induces an effect on the cartilage adjacent to the synovial membrane.

The NF-$_K$B signaling pathway captures a central role in the regulation of inflammatory processes. In the context of joint tissues and OA, a previous study could prove its contribution to the progression of this disease, since an inhibition of this pathway resulted in a reduced degeneration of cartilage *(Marcu et al., 2010)*. In the current thesis, a time-dependent activation of NF-$_K$B by Gal-1 could be demonstrated using Western blotting. This result might suggest that the data obtained by qPCR analyses, especially the up-regulation of IL1B gene expression, could at least be partially mediated by NF-$_K$B signaling.

The results obtained in this thesis suggest that glycobiology might be a relevant factor in the progression of OA. Therefore, further studies should determine the corresponding binding partners of galectins in OA chondrocytes. More experiments are needed and the range of galectins has to be extended on synoviocytes, including other family members than Gal-3. The limited number of donor samples in this diploma thesis often led to high overall standard deviations that might cover the actual significance of some results. Further experiments have to be performed to increase the number of patients to allow for more powerful statistical analyses. Also, further target genes including other inflammatory cytokines that may be able to activate pathological pathways should be assessed in the future. For instance, TNF-α should be included in further experiments, since this cytokine was revealed to be - together with IL1B - a leading mediator of inflammation *(Kobayashi et al., 2005)*. The key question of OA being not just a degenerative disease, but also accompanied or even triggered by inflammatory processes requires many more investigations. Regarding the lack of treatment options in OA, galectins could represent a potential target to broaden the therapeutic options. Since some MMP inhibitors were already tested, but failed in clinical studies, galectin variants could be developed as partial antagonists in the future. Another therapeutic target might be the NF-$_K$B pathway.

Finally, the current thesis suggests that it is justified to draw attention to galectins and also their variants to find modified proteins as eventual inhibitors in the treatment of OA. Many more investigations have to be performed in future to unveil the multifaceted properties and applications of the various galectins.

5. Conclusion

The results obtained in the current thesis suggest that glycobiology might be a relevant factor in the progression of OA. The impact of galectins on the up-regulation of MMP13 and IL1B gene expression was tested in OA chondrocytes as well as in RA and OA synoviocytes at non-toxic concentrations.

In general, synoviocytes showed limited reactivity towards galectin treatment as compared to OA chondrocytes. The qPCR data in OA chondrocytes indicated an up-regulation of IL1B and MMP13 by all galectins, including differences among each other. A significant up-regulation was revealed for Gal-3 and Gal-8. The effect of Gal-1 on IL1B and MMP13 up-regulation was shown to be strong and consistent in OA chondrocytes. As shown in this thesis, the activity of Gal-1 could at least be partially mediated by the activation of NF-$_K$B signaling.

Further experiments focused on biochemical variants of Gal-1, Gal-3 and Gal-8. The insertion of a linker peptide into the homodimeric structure of Gal-1, as pursued in the Gal-1 variants, did not reveal an impact on the effect of Gal-1 regarding IL1B and MMP13 gene expression in OA chondrocytes. In case of Gal-3 and its variants, the data suggest that biochemical modification of the protein structure impacts on activity of Gal-3 in OA chondrocytes. Gal-3_N revealed a stronger effect on IL1B and MMP13 up-regulation, whereas Gal-3tr entailed a reduced activity.

The natural occurrence of the single nucleotide polymorphism in the variants of Gal-8 impaired the effect of this galectin on IL1B and MMP13 up-regulation in OA chondrocytes.

Taken together, this study provides the first step towards further experiments elucidating the role of galectins in the context of arthritic diseases.

6. References

Abeles AM, Pillinger MH. The role of the synovial fibroblast in rheumatoid arthritis: cartilage destruction and the regulation of matrix metalloproteinases. Bull NYU Hosp Jt Dis 2006; 64:20-4.

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. Osteoarthr Cartilage 2005; 13:361-7.

Barondes SH, Cooper DN, Gitt MA, Leffler H. Galectins: Structure and function of a large family of animal lectins. J Biol Chem 1994; 269:20807-10.

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: 1263-7.

Boeuf S, Bovée JV, Lehner B, Hogendoorn PC, Richter W. Correlation of hypoxic signalling to histological grade and outcome in cartilage tumours. Histopathol 2010; 56:641-51.

Boileau C, Poirier F, Pelletier JP, Guévremont M, Duval N, Martel-Pelletier J, Reboul P. Intracellular localisation of galectin-3 has a protective role in chondrocyte survival. Ann Rheum Dis 2008; 67:175-81.

Brewer FC. Binding and cross-linking properties of galectins. Biochim Biophys Acta 2002; 1572:255-62.

Brockhausen I, Schutzbach J, Kuhns W. Glycoproteins and their relationship to human disease. Acta Anat (Basel) 1998; 161:36-78.

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

Dean DD, Martel-Pelletier J, Pelletier JP, Howell DS, Woessner JF Jr. Evidence for metalloproteinase and metalloproteinase inhibitor imbalance in human osteoarthritic cartilage. J Clin Invest 1989; 84:678-85.

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; 179:1225-35.

Fan Z, Söder S, Oehler S, Fundel K, Aigner T. Activation of interleukin-1 signaling cascades in normal and osteoarthritic articular cartilage. Am J Pathol 2007; 171:938-46.

Fernandes JC, Martel-Pelletier J, Pelletier JP. The role of cytokines in osteoarthritis pathophysiology. Biorheol 2002; 39:237-46.

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; 60:1604-14.

Guévremont M, Martel-Pelletier J, Boileau C, Liu FT, Richard M, Fernandes JC, Pelletier JP, Reboul P. Galectin-3 surface expression on human adult chondrocytes: a potential substrate for collagenase-3. Ann Rheum Dis 2004; 63:636-43.

Heinegård D, Saxne T. The role of the cartilage matrix in osteoarthritis. Nat Rev Rheumatol 2011; 7:50-6.

Hughes RC. Secretion of the galectin family of mammalian carbohydrate-binding proteins. Biochim Biophys Acta 1999; 1473:172-85.

Janelle-Montcalm A, Boileau C, Poirier F, Pelletier JP, Guévremont M, Duval N, Martel-Pelletier J, Reboul P. Extracellular localization of galectin-3 has a deleterious role in joint tissues. Arthritis Res Ther 2007; 9:R20.

Kobayashi M, Squires GR, Mousa A, Tanzer M, Zukor DJ, Antoniou J, Feige U, Poole AR. Role of interleukin-1 and tumor necrosis factor alpha in matrix degradation of human osteoarthritic cartilage. Arthritis Rheum 2005; 52:128-35.

Kopitz J, von Reitzenstein C, André S, Kaltner H, Uhl J, Ehemann V, Cantz M, Gabius HJ. Negative regulation of neuroblastoma cell growth by carbohydrate-dependent surface binding of galectin-1 and functional divergence from galectin-3. J Biol Chem 2001; 276:35917-23.

Kopitz J, Vértesy S, André S, Fiedler S, Schnölzer M, Gabius HJ. Human chimera-type galectin-3: defining the critical tail length for high-affinity glycoprotein/cell surface binding and functional competition with galectin-1 in neuroblastoma cell growth regulation. Biochim 2014; 104:90-9.

Krasnokutsky S, Attur M, Palmer G, Samuels J, Abramson SB. Current concepts in the pathogenesis of osteoarthritis. Osteoarthr Cartilage 2008; 16 Suppl 3:S1-3.

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; 276:31285-95.

Liu FT. Galectins: a new family of regulators of inflammation. Clin Immunol 2000; 97:79-88.

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

Liu FT, Rabinovich GA. Galectins: regulators of acute and chronic inflammation. Ann N Y Acad Sci 2010; 1183:158-82.

Madry H, van Dijk CN, Mueller-Gerbl M. The basic science of the subchondral bone. Knee Surg Sports Traumatol Arthrosc 2010; 18:419-33.

Marcon P, Marsich E, Vetere A, Mozetic P, Campa C, Donati I, Vittur F, Gamini A, Paoletti S. The role of Galectin-1 in the interaction between chondrocytes and a lactose-modified chitosan. Biomaterials 2005; 26:4975-84.

Marcu KB, Otero M, Olivotto E, Borzi RM, Goldring MB. NF-kappaB signaling: multiple angles to target OA. Curr Drug Targets 2010; 11:599-613.

Marsich E, Mozetic P, Ortolani F, Contin M, Marchini M, Vetere A, Pacor S, Semeraro S, Vittur F, Paoletti S. Galectin-1 in cartilage: expression, influence on chondrocyte growth and interaction with ECM components. Matrix Biol 2008; 27:513-25.

Martel-Pelletier J. Pathophysiology of osteoarthritis. Osteoarthr Cartilage 2004; 12 Suppl A:S31-3.

Mertens M, Singh JA. Anakinra for rheumatoid arthritis: a systematic review. J Rheumatol 2009; 36:1118-25.

Moiseeva EP, Williams B, Samani NJ. Galectin 1 inhibits incorporation of vitronectin and chondroitin sulfate B into the extracellular matrix of human vascular smooth muscle cells. Biochim Biophys Acta 2003; 1619:125-32.

Myers SL, Brandt KD, Ehlich JW, Braunstein EM, Shelbourne KD, Heck DA, Kalasinski LA. Synovial inflammation in patients with early osteoarthritis of the knee. J Rheumatol 1990; 17:1662-9.

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; 48:2788-95.

Pabst M, Wu SQ, Grass J, Kolb A, Chiari C, Viernstein H, Unger FM, Altmann F, Toegel S. IL-1beta and TNF-alpha alter the glycophenotype of primary human chondrocytes in vitro. Carbohydr Res 2010; 345:1389-93.

Pace KE, Lee C, Stewart PL, Baum LG. Restricted receptor segregation into membrane microdomains occurs on human T cells during apoptosis induced by galectin-1. J Immunol 1999; 163:3801-11.

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

Rabinovich GA, Daly G, Dreja H, Tailor H, Riera CM, Hirabayashi J, Chernajovsky Y. Recombinant galectin-1 and its genetic delivery suppress collagen-induced arthritis via T cell apoptosis. J Exp Med 1999; 190:385-98.

Reboul P, Pelletier JP, Tardif G, Cloutier JM, Martel-Pelletier J. The new collagenase, collagenase-3, is expressed and synthesized by human chondrocytes but not by synoviocytes. A role in osteoarthritis. J Clin Invest 1996; 97:2011-9.

Ruiz FM, Scholz BA, Buzamet E, Kopitz J, André S, Menéndez M, Romero A, Solís D, Gabius HJ. Natural single amino acid polymorphism (F19Y) in human galectin-8: detection of structural alterations and increased growth-regulatory activity on tumor cells. FEBS J 2014; 281:1446-64.

Salomonsson E, Larumbe A, Tejler J, Tullberg E, Rydberg H, Sundin A, Khabut A, Frejd T, Lobsanov YD, Rini JM, Nilsson UJ, Leffler H. Monovalent interactions of galectin-1. Biochem 2010; 49:9518-32.

Seki M, Sakata KM, Oomizu S, Arikawa T, Sakata A, Ueno M, Nobumoto A, Niki T, Saita N, Ito K, Dai SY, Katoh S, Nishi N, Tsukano M, Ishikawa K, Yamauchi A, Kuchroo V, Hirashima M. Beneficial effect of galectin 9 on rheumatoid arthritis by induction of apoptosis of synovial fibroblasts. Arthritis Rheum 2007; 56:3968-76.

Sellam J, Berenbaum F. The role of synovitis in pathophysiology and clinical symptoms of osteoarthritis. Nat Rev Rheumatol 2010; 6:625-35.

Sharif M, Elson CJ, Dieppe PA, Kirwan JR. Elevated serum C-reactive protein levels in osteoarthritis. Br J Rheumatol 1997; 36:140-1.

Sharma AR, Jagga S, Lee SS, Nam JS. Interplay between cartilage and subchondral bone contributing to pathogenesis of osteoarthritis. Int J Mol Sci 2013; 14:19805-30.

Smith MD. The normal synovium. Open J Rheumatol 2011; 5:100-6.

Taylor PC, Feldmann M. Anti-TNF biologic agents: still the therapy of choice for rheumatoid arthritis. Nat Rev Rheumatol 2009; 5:578-82.

Toegel S, Bieder D, André S, Altmann F, Walzer SM, Kaltner H, Hofstaetter JG, Windhager R, Gabius HJ. Glycophenotyping of osteoarthritic cartilage and chondrocytes by RT-qPCR, mass spectrometry, histochemistry with plant/human lectins and lectin localization with a glycoprotein. Arthritis Res Ther 2013; 5:R147.

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; 142:373-88.

Vinik Y, Shatz-Azoulay H, Vivanti A, Hever N, Levy Y, Karmona R, Brumfeld V, Baraghithy S, Attar-Lamdar M, Boura-Halfon S, Bab I, Zick Y. The mammalian lectin galectin-8 induces RANKL expression, osteoclastogenesis, and bone mass reduction in mice. Elife 2015; doi: 10.7554/eLife.05914.

Walsh DA, Bonnet CS, Turner EL, Wilson D, Situ M, McWilliams DF. Angiogenesis in the synovium and at the osteochondral junction in osteoarthritis. Osteoarthr Cartilage 2007; 15:743-51.

Yang RY, Liu FT. Galectins in cell growth and apoptosis. Cell Mol Life Sci 2003; 60:267-76.

Yang X, Yip J, Anastassiades T, Harrison M, Brockhausen I. The action of TNFalpha and TGFbeta include specific alterations of the glycosylation of bovine and human chondrocytes. Biochim Biophys Acta 2007; 1773:264-72.

Zerkak D, Dougados M. The use of glucosamine therapy in osteoarthritis. Curr Pain Headache Rep 2004; 8:507-11.

Zhang S, Moussodia RO, Murzeau C, Sun HJ, Klein ML, Vértesy S, André S, Roy R, Gabius HJ, Percec V. Dissecting molecular aspects of cell interactions using glycodendrimersomes with programmable glycan presentation and engineered human lectins. Angew Chem Int Ed Engl 2015; 54:4036-40.

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