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DISSERTATION

The Role of Chondrocyte-specific Endogenous Glucocorticoid Signalling in Age-related Cartilage Degeneration

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11β-HSD	11β-hydroxysteroid dehydrogenase
ACR	American College of Rheumatology
ACTH	adrenocorticotropic hormone
ADAMTS	a disintegrin and metalloproteinase with thrombospondin motifs
AES	aminopropyltriethoxysilane
AF	activation function
AGE	advanced glycation end-product
ALK	activin receptor-like kinases
AP	activator protein
BSA	bovine serum albumin
BV	bone volume
CC	calcified cartilage
cDNA	complementary deoxyribonucleic acid
CiOA	collagenase-induced osteoarthritis
Cre	cyclization recombinase
CRH	corticotropin-releasing hormone
DAB	diaminobenzidine
DBD	DNA-binding domain
DMM	destabilisation of medial meniscus
DMOAD	disease-modifying osteoarthritis drug
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
DPX	distyrene + plasticiser + xylene
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
ER	estrogen receptor
FOXO	forkhead-box class O
GAG	glycosaminoglycan
GC	glucocorticoid
GILZ	glucocorticoid-induced leucine zipper
GR	glucocorticoid receptor
GR KO	glucocorticoid receptor knockout
GR WT	glucocorticoid receptor wild-type

HC	hyaline cartilage (non-calcified cartilage)
H&E	hematoxylin and eosin
hGR	human glucocorticoid receptor
IASI	intra-articular steroid injection
IGF	insulin-like growth factor
IHC	immunohistochemistry
IL	interleukin
KO	knockout
LBD	ligand-binding domain
LoxP	locus of X-over P1
MC	mineralocorticoid
MMP	matrix metalloproteinase
MRI	magnetic resonance imaging
mRNA	messenger ribonucleic acid
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B-cells
OA	osteoarthritis
OARSI	Osteoarthritis Research Society International
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PFA	paraformaldehyde
PVN	paraventricular nucleus
RCT	randomized controlled trial
RNA	ribonucleic acid
RO	reverse osmosis
ROI	region of interest
ROS	reactive oxygen species
SASP	senescence-associated secretory phenotype
SBP	subchondral bone plate
TB	toluidine blue
TGF	transforming growth factor
TM	tamoxifen
TNF	tumor necrosis factor
TRAP	tartrate resistant acid phosphatase
TUNEL	terminal deoxynucleotidyl transferase dUTP nick end labelling
TV	tissue volume
WT	wild-type

1 SUMMARY

1.1 Abstract

Aim: The detrimental effects of exogenous glucocorticoids (GCs), in the sense of steroid-induced degradation of articular cartilage, are an acknowledged complication of repeated intra-articular GC administration in patients with knee joint osteoarthritis (OA). The impact of endogenous GCs on cartilage in the context of OA pathophysiology, in contrast, is rarely known. Therefore, the objective of this pilot-study was to investigate the role of chondrocyte-specific endogenous GC signalling in a murine model of age-related cartilage degeneration.

Methods: Chondrocyte-specific endogenous GC signalling was disrupted in C57BL/6 mice by tamoxifen-inducible deletion of the glucocorticoid receptor (GR) gene at 4 weeks of age. Male and female glucocorticoid receptor knockout (GR KO) mice and their wildtype (GR WT) littermates were housed under standard laboratory conditions with free access to a standard chow diet and water until the age of approximately one year (11.1 – 13.8 months). The knee joints of the mice were then harvested and histologically evaluated, with a focus on different OA-related changes in cartilage, subchondral bone and surrounding soft tissues. The results were statistically compared between GR WT and GR KO mice using the Mann-Whitney U test.

Results: The overwhelming majority of mice (42 out of 46 mice in total) developed structural cartilage degeneration, but predominantly to a mild extent. While cartilage erosion was significantly more pronounced in male GR WT mice compared to male GR KO mice ($p = 0.027$), no such difference was found between the genotypes of female mice ($p = 0.114$). Other cartilaginous parameters, including cartilage calcification and chondrocyte hypertrophy, did not confirm more severe degeneration in male GR WT as opposed to GR KO mice. Male GR WT mice developed a higher degree of cartilage erosion than their female counterparts ($p = 0.043$). Commensurate with the overall mild degree of cartilage damage, there was no evidence of subchondral bone sclerosis or osteophyte formation, except for one male GR WT outlier mouse. Synovitis was also absent in all investigated samples.

Conclusion: In male mice, chondrocytic endogenous GC signalling seems to influence structural cartilage degeneration, and results in more pronounced erosion in male GR WT mice. Hence, endogenous GCs probably exert negative effects on cartilage. Future studies on mice with manifest OA are necessary for conclusive elucidation of this observation.

Keywords: Osteoarthritis, endogenous glucocorticoids, cartilage, chondrocyte, aging

1.2 Kurzdarstellung

Zielsetzung: Schädigende Wirkungen exogener Glucocorticoide (GC) im Sinne einer steroid-induzierten Degradation des Gelenkknorpels sind eine anerkannte Komplikation wiederholter intraartikulärer Anwendungen von GC bei Patienten mit Kniegelenksarthrose. Im Gegensatz dazu sind die Effekte endogener GC auf den Knorpel im Kontext der pathophysiologischen Prozesse der Arthrose-Entstehung weitestgehend unbekannt. Deshalb bestand die Zielsetzung dieser Pilot-Studie in der Untersuchung der Bedeutung endogener GC-vermittelter Signaltransduktion spezifisch in Chondrozyten mit Hilfe eines Mausmodells zu altersabhängiger Knorpeldegeneration.
Methoden: In Mäusen mit C57BL/6 Hintergrund wurde im Alter von 4 Wochen mittels tamoxifen-abhängiger Ausschaltung des Glucocorticoidrezeptor (GR)-Gens eine Unterbrechung der GC-vermittelten Signaltransduktion exklusiv in Chondrozyten realisiert. Männliche und weibliche GR Knockout (GR KO) sowie Wildtyp (GR WT) Mäuse wurden für circa ein Jahr (11.1 – 13.8 Monate) unter freiem Zugang zu Wasser und einer Standard-Chow-Diät gehalten. Die Kniegelenke der Mäuse wurden daraufhin histologisch gesichert und in Bezug auf Arthrose-definierende Veränderungen des Gelenkknorpels, des subchondralen Knochens und der umliegenden Weichteilgewebe untersucht. Die gesammelten Messwerte dienten dem statistischen Vergleich zwischen GR WT und GR KO Mäusen mittels Mann-Whitney U Test.
Ergebnisse: Die überwiegende Mehrheit der Mäuse (42 von 46 Mäusen insgesamt) wies Zeichen struktureller Knorpeldegeneration auf, wenn auch in meist relativ milder Ausprägung. Während der Schweregrad der Knorpelerosion in männlichen GR WT Mäusen signifikant höher als in männlichen GR KO Mäusen war ($p = 0.027$), fand sich kein entsprechender Unterschied zwischen den Genotypen der weiblichen Mäuse ($p = 0.114$). Andere Parameter der Knorpelschädigung, darunter Knorpelkalzifikation und Hypertrophie der Chondrozyten, konnten einen schwerwiegenderen Verlauf der Degeneration in männlichen GR WT Mäusen gegenüber GR KO Mäusen nicht bestätigen. Männliche GR WT Mäuse hatten im Vergleich zu weiblichen GR WT Mäusen insgesamt höhergradige Knorpelerosion entwickelt ($p = 0.043$). In Übereinstimmung mit der milden Knorpeldegeneration existierten keine Anzeichen für eine Sklerosierung des subchondralen Knochens oder von osteophytären Anbauten mit Ausnahme eines Ausreißers unter den männlichen GR WT Mäusen. Außerdem war in keinem der untersuchten Schnitte eine Synovitis erkennbar.
Schlussfolgerung: In männlichen Mäusen scheinen endogene GC die Entwicklung struktureller Knorpeldegeneration zu beeinflussen, was in einer gesteigerten Anfälligkeit für erosive Prozesse in männlichen GR WT Mäusen ihren Ausdruck findet. Folglich vermitteln endogene GC wahrscheinlich negative Wirkungen auf den Gelenkknorpel. Zukünftige Studien in Mäusen mit manifester Arthrose sind zur abschließenden Klärung dieses Sachverhalts notwendig.
Stichwörter: Arthrose, endogene Glucocorticoide, Knorpel, Chondrozyt, Alterung

2 INTRODUCTION

2.1 Osteoarthritis

2.1.1 Definition and disease characteristics

Osteoarthritis (OA) is a chronic degenerative disease of the joints (1). In 2015, the Osteoarthritis Research Society International (OARSI) described OA as a "(...) *disorder involving movable joints characterized by cell stress and extracellular matrix degradation initiated by micro- and macro-injury that activates maladaptive repair responses including pro-inflammatory pathways of innate immunity. The disease manifests first as a molecular derangement (abnormal joint tissue metabolism) followed by anatomic, and/or physiologic derangements (characterized by cartilage degradation, bone remodeling, osteophyte formation, joint inflammation and loss of normal joint function), that can culminate in illness*." (2). In accordance with this definition, OA is nowadays considered to be a complex whole-joint disease, rather than a simple mechanical wear-and-tear issue of articular cartilage (3). Owing to the multifactorial aetiology, variability of main disease drivers and heterogeneous clinical presentation, there are most likely several pathogenetical subsets of OA, which are subsumed under the identical disease name as they have (at least in part) the above-mentioned phenotypical key features in common (1, 2). Aetiological subtypes of the condition can generally be divided into primary (or idiopathic) OA without specific underlying causes and secondary OA, when joint degeneration occurs as a result of anatomical, systemic or hereditary abnormalities such as musculoskeletal deformities (for instance valgus/varus alignment), traumatic injury, obesity or congenital chondrodysplasia (3, 4).

Regardless of regional or ethnic variability of disease prevalence, OA is considered to be the most frequent joint condition in the world (4). Aging is the most important risk factor for the development of primary OA, hence the disease prevalence tends to rise in the elderly (5). Approximately 10 % of people aged over 63 years suffer from symptomatic knee OA, while a third of them exhibit abnormal radiographic changes, as examined in the Framingham Osteoarthritis Study (4, 6). The percentage of people fulfilling the diagnostic criteria of radiographic knee OA increases to almost 50 % over the age of 75 years (7). While this epidemiological correlation is remarkable, it does not permit conceiving the phenomena of aging and OA-related pathologies as being synonymous, but should rather be interpreted in the sense that higher age predisposes towards abnormal joint degeneration (5). Female sex is another aspect which increases the likelihood of primary knee OA, both with regard to prevalence and severity, especially after menopause (8).

Clinically, OA is characterised by increasing joint pain, stiffness and/or intermittent swelling, which can result in progressive dysfunction, eventually diminishing quality of life and ability of physical activities (9). These subjective symptoms serve as the main basis for clinical diagnosis of OA, next to a reported age > 50 years and clinical examination findings like crepitus, bony enlargement or tenderness, as described by the American College of Rheumatology (ACR) (10). The subjective experience of joint degeneration and clinical findings, however, is not necessarily in line with radiographic changes on X-ray (7, 11), which has been the gold standard imaging technique for the radiological diagnosis of OA for a long time (12). The disease-defining radiographic features of OA are joint-space narrowing as a correlate of cartilage thinning alongside subchondral bone sclerosis (or cysts), osteophytosis and deformation of the articular surfaces (12). These items are the basis of common radiological OA severity classification systems, for instance the widely used system by Kellgren and Lawrence (13). Recently, magnetic resonance imaging (MRI) has become a valuable tool for the earlier and more precise evaluation of OA-related alterations as compared to a plain X-ray. Consequently, MRI is being used more often to support diagnostic and therapeutical decisions, especially for identifying soft-tissue pathologies or discreet changes in cartilage and bone. Other non-invasive imaging technologies like ultrasound can be used as an adjunct to X-rays and MRI to address specific diagnostic questions (3, 12).

2.1.2 Therapeutical options

Patients diagnosed with degenerative joint disease and their treating physician are confronted with a dilemma. While an abundance of physical programmes, questionable supplements and drugs are described in the literature, current therapeutical approaches for the treatment of OA are often only symptom-related, and thus limited in their efficacy. As a consequence, surgical procedures including total knee replacement are usually the last resort option in patients with otherwise unapproachable advanced OA. The choice of available non-pharmacologic, pharmacologic or surgical schemes should ideally depend on the stage of OA and the underlying aetiology (e.g. idiopathic, hereditary, instability-driven, inflammatory) (1). The ACR and European League Against Rheumatism (EULAR) published recommendations for the non-invasive, symptomatic treatment of knee OA (14, 15). Before drugs are considered, moderate aerobic and dynamic muscle strengthening exercises are recommended. These exercises can help to reduce pain, regain joint function and control overweight in obese patients (15). The next therapeutical step includes symptomatic pharmacological treatment. Prescription of paracetamol, oral or topical non-steroidal anti-inflammatory drugs (NSAIDs) and tramadol is "*conditionally recommended*" if the occasional use of over-the-counter analgesics is insufficient. The same restricted approval is given

with regard to the administration of intraarticular glucocorticoids (GC) like triamcinolone (14). Although these non-surgical "treatment" options are able to alleviate pain and inflammation temporarily, none of them has proven to prevent or delay the progression of OA. In fact, all therapies mentioned are purely symptomatic. Instead of preventing disease progress, certain drug prescriptions were identified to increase overall mortality in OA-affected patients (16). Safe and reliable approaches would naturally be preferable. For the time being, there is a lack of effective disease-modifying osteoarthritis drugs (DMOADs) (1). The earnest hope is to find agents which address the pathophysiology causally, thereby relieving OA-related symptoms and preventing or delaying further joint destruction at the same time. One essential prerequisite for the invention of targeted therapeutical solutions is more scientific insight into the pathogenesis of OA, in particular the age-induced changes in cartilage, as well as the associated effects on bone and surrounding soft-tissue, for which the current state of knowledge will be elaborated throughout this introduction. A couple of age-related cellular as well as extracellular alterations in articular cartilage have so far been identified, which are associated notably with the occurrence of idiopathic, age-related OA.

2.2 Age-related joint degeneration in the context of osteoarthritis

2.2.1 Age-related cartilage degeneration

Multiple age-induced changes of articular cartilage result in a pathological inclination towards cartilage breakdown. Once destructive processes prevail over regenerative efforts, and the load-bearing capacity of articular cartilage is exceeded, irreversible damage of the cartilaginous matrix and cellular decline are the ultimate consequence. The following indicated subdivision of the main degeneration-driving mechanisms - namely chondrocyte senescence, oxidative stress, terminal differentiation, changes of the extracellular matrix (ECM) and cell death - is adapted from the proposed classification by Rahmati et al. (17).

2.2.1.1 Chondrocytic cellular senescence

Cellular senescence was originally described as the limited quantity of proliferative cycles in the life of a cell, eventually resulting in irreversible growth arrest (18). This observation was later explained by the progressive shortening of DNA-protective telomeres, and at present is interpreted as a mechanism that on the one hand prevents uninterrupted proliferation of pre-cancerous cells, but on the other leads to accumulating cellular dysfunction towards the end of the cell's lifespan (19).

Therefore, cellular senescence is being discussed regarding its pathogenetical contribution to various age-related conditions like OA (19, 20). Decreasing telomere lengths and phenotypical features of cellular senescence in aged chondrocytes from human *in vivo* samples were reported by Martin and Buckwalter (21). Characteristic phenotypical hallmarks of senescent chondrocytes include reduced mitotic rates and raised levels of senescence-associated β-galactosidase (21), an enzyme commonly used as a biomarker of senescent cells in the context of increased age-related lysosomal activity (22). Another distinct feature of senescent osteoarthritic chondrocytes are altered concentrations of cell cycle regulators, especially the upregulation of tumour suppressor protein $p16^{INK4a}$, which was shown to contribute to a loss of chondrocytic repair potential through the inhibition of both cell renewal and matrix synthesis (23). Last but not least, age-related changes in the chondrocytic secretory profile, subsumed under the term "*senescence-associated secretory phenotype*" (SASP) (20), are a relevant attribute that may account for the predisposition to cartilaginous degeneration (24). Several pro-inflammatory cytokines and degradative proteases are released by senescent chondrocytes. For instance, cartilage samples from old but non-arthritic subjects and patients with beginning OA exhibit similar expression patterns of matrix metalloproteases (MMP) 1 and 13 (25). MMP-13 is considered to be one of the main drivers of enzymatic cartilage breakdown due to its high affinity to collagen type II, the principal collagen subtype of articular cartilage, and the ability to promote structural cartilage erosion like fibrillation or fissures (26). Increased levels of inflammatory cytokines like interleukin (IL) 1 or 6 and tumor necrosis factor (TNF) α have also been reported, both as a typical SASP feature (24) and as meaningful actors in the pathophysiology of OA, which facilitate, amongst other things, the pathological cross-talk between cartilage, subchondral bone and synovial tissue (1, 3). The presence of these mediators, either senescence-associated or along with degenerative processes, reveals striking parallels between hallmarks of aging and OA pathogenesis.

Concerning cellular senescence as a mechanism of age-related cartilage degeneration, it needs to be differentiated whether intrinsic or extrinsic mechanisms lead to the described senescent state of chondrocytes. Intrinsic (or replicative) senescence occurs inevitably when physiologic cells are approaching the maximum number of possible cell divisions (Hayflick limit) on account of telomere attrition. Extrinsic senescence, in contrast, is induced by factors which cause direct damage to telomeres, such as oxidative stress or inflammation (19, 24). Contrarily to chondrocytes in the epiphyseal growth plate, chondrocytes from articular cartilage do not typically proliferate unless they are exposed to heavy mechanical trauma (27). Therefore, the low proliferative rate of chondrocytes under physiological conditions may not be sufficient for the manifestation of classic replicative senescence (21). When reconsidering the directly age-dependent mechanisms of

cartilage senescence, extrinsic mechanisms may therefore appear more likely. There is accumulating evidence to support this theory, for instance an experiment performed by Dai et al. who were able to show that mediators of inflammatory (interleukin 1β) or oxidative stress (hydrogen peroxide) induce extrinsic senescence of chondrocytes (28). In particular, oxidative stress has received attention as a pivotal extrinsic senescence promoter since there is a natural increase of reactive oxygen species (ROS) generation during aging (29). Oxidative stress is a key component of cellular aging and drives age-related degeneration of cartilage in a manifold manner.

2.2.1.2 Oxidative stress

The relevance of oxidative stress in articular cartilage may seem counterintuitive at first sight considering the significantly lower oxygen levels in cartilage compared to vascularized tissues (30). In spite of the successful adaptation to hypoxia via anaerobic glycolysis (the Embden-Meyerhof-Parnas pathway), oxidative phosphorylation in mitochondria is still present in articular cartilage. Mitochondria, however, are considered to be the main producers of intracellular ROS (30, 31). Although oxidative phosphorylation is of minor importance with regard to energy production in cartilage, mitochondrial electron transport has recently been recognised as a precondition for maintaining normal cartilage metabolism via the supply of oxidants for the glycolytic pathway. Impaired mitochondrial electron transport could therefore critically affect the energy supply of chondrocytes and potentially disturb the physiological cartilage homeostasis as a consequence (32). In fact, mitochondrial dysfunction as a result of mitochondrial DNA damage and the occurrence of OA are associated with each other (31). Mutations in the mitochondrial genome are prone to accumulate over the course of life; emerging failure and insufficiency of the respiratory chain can result in enhanced ROS release, which can in turn lead to new oxidative damage of mitochondrial and nuclear DNA, forming a vicious circle (33). In addition, the anti-oxidative defence mechanisms are downregulated in aged articular cartilage. Therefore, age-related mitochondrial dysfunction is a crucial aspect of where excessive amounts of oxygen radicals in cartilage originate from (5).

Yudoh et al. investigated the effects of oxidative stress on telomere integrity and observed enhanced telomere attrition in cartilage exposed to oxygen radicals (34). But the impact of oxidative stress on chondrocytic cell functions extends beyond the induction of extrinsic senescence. Loeser et al. reported the presence of nitrotyrosine - a stable biochemical marker of reactions involving ROS - in human aged cartilage, and found a histological correlation between nitrotyrosine positivity and the intensity of OA-like degeneration (29). In addition, ROS-treated chondrocytes were characterised by diminished replicative capabilities and increased catabolism,

as defined by the decreasing capacity to synthesize glycosaminoglycans (GAG) of the ECM (34). Not only does age-related augmented oxidative stress lead to increased synthesis of cartilage-degrading MMPs by chondrocytes (35), but oxygen radicals were also described to cause active fragmentation of cartilaginous collagen (36) and proteoglycan (37). The equilibrium of anabolic and catabolic processes is further disturbed by weakened chondrocytic responsiveness to anabolic factors, namely insulin-like growth factor 1 (IGF-1) and osteogenic protein 1 (OP-1), following excessive ROS exposition (38). Lastly, there is evidence pointing towards an association between oxidative stress and subsequent changes of chondrocytic differentiation (39), as well as apoptosis of chondrocytes (40). These aspects of chondrocytic cell biology have particular relevance for the pathogenesis of age-related OA, so more light will be shed on them later on.

Apart from increased ROS generation on account of aging, certain environmental stimuli are able to boost the production of harmful radicals, thereby accelerating the above-mentioned processes. Coleman et al. discovered enhanced ROS levels via mitochondrial dysfunction following chronic mechanical overloading (41), and similar observations were made by Delco et al., who investigated the effect of acute mechanical injury (42). These studies provide a link between the exposition of physical force on cartilage and promoted oxidative stress, leading to consecutive cartilage degeneration. Pathological calcium deposition (43), inflammatory cytokines (44), and advanced glycation end products (45) are other confirmed triggers of nitric oxide generation, or similar radicals. The presence of these promoters of oxidative stress can themselves be related to cartilage aging or, fundamentally, the aging of the entire organism (24, 46, 47).

2.2.1.3 Terminal differentiation and age-related alterations of chondrocytic signalling

The quiescent, non-proliferative state of adult articular chondrocytes is not to be seen as the end point of chondrocytic differentiation, but rather as a pre-terminal stop in the course of chondrocytic lineage. Rather, terminal differentiation of chondrocytes leads to a hypertrophic phenotype, regularly observed in the growth plate of immature bones during the process of endochondral ossification, where hypertrophic chondrocytes are eventually replaced by bony tissue to enable longitudinal growth (48). As explained, terminal differentiation normally does not occur in hyaline articular cartilage, and the "*default route*" of chondroprogenitors towards bone is prevented by certain mechanisms (48). In the context of OA, however, processes similar to endochondral ossification can be initiated that induce a cellular phenotype resembling terminally differentiated chondrocytes. Afflicted chondrocytes start to form regional clusters, presumably reflecting an attempt to compensate for higher mechanical demands and potentially enable repair (27). This is accompanied by a phenotypic shift towards chondrocyte hypertrophy, characterised by the

occurrence of collagen type X. In addition, hypertrophy-like articular chondrocytes start to synthesize MMP-13 and ADAMTS-4/5 (a disintegrin and metalloproteinase with thrombospondin motifs), potent degrading enzymes of which the latter predominantly cleaves aggrecan (49). MMP-13 and ADAMTS-4/5 counteract and limit the hypothesized regenerative benefit of regional cluster formation through concomitant cartilage breakdown (27, 48). Unlike during endochondral ossification, mineralization does not inevitably follow chondrocyte hypertrophy in this case, but the phenomena can be associated with each other. Consequently, the proportion of calcified cartilage to non-calcified cartilage can increase in OA-affected cartilage (48).

Oxidative (39), mechanical (50) and inflammatory (51) stress acts as a potential trigger of the described cascade of phenotypical alterations. In line with this, markers of articular chondrocyte hypertrophy like collagen type X are elevated in cartilage from OA patients, especially in regions of structural cartilage damage (48). Under normal conditions, collagen type X is absent in the non-calcified hyaline cartilage layer, and its presence serves as an accepted marker of hypertrophic changes in early as well as late OA (48, 52). Interestingly, there is evidence that aging is an important modulator of articular chondrocytic differentiation and thus the occurrence of chondrocyte hypertrophy. Van der Kraan et al. thoroughly investigated the relation between age-dependent alterations of chondrocytic differentiation, predisposing to the occurrence of primary OA, and transforming growth factor β (TGF-β) signalling (53). TGF-β is a cytokine and ligand of the TGF-β superfamily which is involved in the regulation of cell growth, development and homeostasis. Signalling is transduced via TGF-β receptor type II and subsequent phosphorylation of different type I receptors (also called activin receptor-like kinases; ALK), which then facilitate further downstream signalling via various transcription factors called receptor-regulated SMADs (54). These downstream pathways of TGF-β signalling show differential activity in young vs. old articular cartilage (53). ALK5 transduces protective effects in young cartilage via SMAD 2 and 3, specifically by preventing terminal differentiation of articular chondrocytes (55) as their "*default route*" of cellular lineage (48). Furthermore, the SMAD 2/3 pathway suppresses actions of inflammatory cytokines and MMPs (53). In old cartilage, however, this shielding function of canonical ALK5 signalling seems to be diminished and is no longer sufficient to prevent cartilage degeneration. Instead, the alternative route of TGF-β signalling, namely activation of ALK1 which exerts effects via SMAD 1/5/8, continues to be stimulated and at some point prevails over ALK5 signalling (56). ALK1, in contrast to ALK5, is known to *induce* chondrocyte hypertrophy (with collagen type X expression) and is associated with *enhanced* MMP-13 production (57). Drawing a conclusion from these observations, the altered balance of TGF-β signalling via ALK5 or ALK1, with preferential activation of ALK1 routes in aged cartilage, serves as a profound argument for

age-related aberrant chondrocytic hypertrophy (collagen type X expression) plus secretory profile, increasing the probability of age-related cartilage degeneration as a consequence (53, 56).

The transcription factors under control of TGF-β are not the only modulators of chondrocytic hypertrophy and homeostasis. Additionally, Wnt signalling has been recognised in its meaningfulness for cartilage development and maintenance, and also the pathogenesis of degenerative cartilage breakdown. Artificial augmentation of canonical Wnt signalling (via β-catenin) in chondrocytes was discovered to promote structural cartilage erosion, cell cloning, chondrocyte hypertrophy and osteophyte formation by Zhu et al. (58). OA-affected cartilage is characterised by enhanced β-catenin expression, commensurate with the previous study (59). Deletion of β-catenin signalling in chondrocytes, however, leads to accelerated apoptosis and concomitant cartilage degeneration (60), indicating an essential role of β-catenin for chondrocytic survival under physiological circumstances (58). Taniguchi et al. showed that there is an age-related decrease of β-catenin expression in the superficial zone of articular cartilage which could potentially make the articular surface more prone to degeneration (61).

Another promising discovery with regard to age-dependent differential activity of transcription factors was made by Akasaki et al., who studied the expression of forkhead-box class O (FOXO) protein in young, old and osteoarthritic cartilage (62). Three different members of this transcription factor family, that is FOXO1, 3 and 4, can be ubiquitously found in human cells, regulating essential functions such as the cell cycle, cell differentiation and stress response (63). Insulin- or growth factor-dependent phosphorylation of FOXO, via the phosphoinositide 3 kinase (PI3K)/ protein kinase B (Akt)/ serum and glucocorticoid inducible kinase (SGK) pathway, results in degradation of FOXO and inactivation of FOXO-dependent target genes. Generally speaking, translation of FOXO-dependent genes was identified to induce cell cycle arrest, support DNA repair and strengthen resistance to oxidative stress, but also to induce apoptosis, whereas inactivation of FOXO is associated with abnormal proliferation. The intricate balance of FOXO-mediated functions and their exact interplay, however, are presumably tissue- and cell-specific (63). Akasaki et al. were the first to investigate FOXO in articular cartilage. They reported the presence of FOXO1 and 3 in human as well as murine cartilage, which were significantly decreased in aged compared to young cartilage from both species. In OA-affected cartilage, FOXO was primarily observed in its phosphorylated, inactive isoform, especially in regions of hypertrophic chondrocyte clusters (62). A recent complementary study by Matsuzaki et al., using chondrocyte-specific murine knockout models of FOXO-encoding genes, revealed that loss of FOXO promotes the development of cartilage degeneration and OA-like changes. FOXO1 deletion caused inhibited synthesis of the lubricating substance lubricin by superficial

chondrocytes, while FOXO3 deletion primarily resulted in impaired antioxidative functions as well as decreased cartilage-protective autophagy (64). Drawing a conclusion from the studies by Akasaki et al. and Matsuzaki et al., the age-related decline of FOXO 1 and 3 (62) could predispose cartilage to degeneration via deficient lubrication (more mechanical friction), enhanced oxidative stress and accumulation of defective cell constituents (64).

2.2.1.4 Age-related alterations of the extracellular matrix

Since chondrocytes are the constituting cells of articular cartilage, the above-stated chondrocytic dysfunctions have direct implications on the stability of the surrounding extracellular matrix. Several age-related alterations of the ECM composition are known which may predispose or contribute to cartilage degeneration in the elderly. Accumulation of advanced glycation end-products (AGEs) like pentosidine is one of them and has received special attention, as increased AGE levels were shown to be a characteristic feature of aging tissues, including cartilage (46). AGEs originate from non-enzymatic linkage of reducing sugars to protein-rich substances via the Maillard reaction (65), a chemical process commonly known for the flavour-giving browning of food (66). Owing to the low turnover rate of ECM components, cartilage is predisposed to accumulation of glycated proteins (65). Apart from interfering with chondrocytic cell functions via AGE receptors and supporting catabolic pathways (67), AGEs exert direct negative effects on the ECM because they enhance loss of elasticity as a result of collagen cross-linking (68). Such age-related modifications of collagens are accompanied by changes of the proteoglycan content. For instance, the ratio of keratin sulphate to chondroitin sulphate (two important GAG proteins) was described as increasing, the core protein aggrecan shortens due to terminal cleavage, and the concentration of link protein declines, resulting in impaired aggregation and an overall destabilized ECM network (65). Once the polyanionic GAGs lose their ability for adequate H_2O binding, and tissue hydration is thus diminished, the concentration of ions like calcium exceeds the solubility capacity, which can result in age-related cartilage calcification (47). Morphologically, this is represented by a proceeding tidemark, the border between non-calcified (synonym: hyaline cartilage; HC) and calcified cartilage (CC) layers, and, consequently, a decreasing HC/CC ratio (69). Also, the progressive loss of hydrophilic ECM capacity ultimately leads to a loss of cartilage volume, which is a typical OA feature, but has also been identified in aged subjects free of clinical or (other) radiographic signs of cartilage disease (70). Finally, the concomitant loss of elasticity and mechanical resistance imposes more stress on subchondral bone, potentially initiating pathological changes that are not restricted to articular cartilage alone. Since the transition between exclusive age-related cartilage degeneration and the manifestation of whole-joint OA is actually fluid, characteristics of degenerative processes in adjacent joint parts will be addressed separately.

2.2.1.5 Autophagy and apoptosis

While the age-related changes of the cartilaginous matrix, chondrocytic gene activity, differentiation (hypertrophy-like) and metabolism are regarded to be the major principles of progressive cartilage degeneration, the role of chondrocytic cell death is less clear, and thus remains debatable. This is mainly due to the unresolved question of whether chondrocytic death is the cause or effect of cartilage breakdown, and to what extent dead cells are present in aged or OA-affected cartilage (17, 71). Three types of chondrocytic cell death are distinguished in the literature: autophagy, apoptosis and necrosis, of which the former two are examples of programmed cell death and have gained more attention in the pathophysiological research of primary, age-related OA, while necrosis might have more relevance in OA-subtypes following high-impact injury (17, 72).

Autophagy is a cell-inherent process which enables replacement of defective organelles. Principally, it is considered to be a cartilage-protective and homeostasis-ensuring cell function, unless the physiologic range of turnover of cellular components is exceeded (17, 72). During aging, the frequency of autophagy in cartilage was reported to decrease and this was originally interpreted to be a process which impairs cartilage integrity (73). FOXO3, as mentioned, seems to be an important age-dependent regulator of physiologic autophagy, and its age-related decline may explain the loss of cartilage-protective autophagy (64). The role of autophagy cannot be summarised in a simplified rule, but there is some agreement that autophagy may exert beneficial effects in early OA by eliminating defective organelles, whereas dysregulated autophagy promotes apoptosis and cartilage breakdown in late-stage OA (72).

Autophagy and apoptosis are morphologically and functionally distinct processes, although the mechanisms can run in parallel, maybe even synergistically, to each other. Again, the role of apoptosis in OA is not clearly defined, nor whether it is causal or consequential with regard to cartilage degeneration (17, 71, 72). Reported percentages of apoptotic cells in degraded cartilage are often diametrically opposed to each other (72). Aigner et al. firmly questioned the significant incidence and meaningfulness of apoptosis in OA-affected cartilage, reporting less than 1 % apoptotic chondrocytes (74). In contrast, a more recent study by McNulty et al. considered apoptosis an early indicator of cartilage deterioration prior to structural damage and detected increasing number of apoptotic cells with age (75). Other researchers also reported higher percentages of apoptotic cells than Aigner et al. (72). Regardless of the exact frequency of apoptosis in degenerated cartilage, several associations exist between the aforementioned signalling cascades and the induction or prevention of apoptotic programmes. Inhibition of TGF-β-mediated ALK5 signalling was shown to involve promoted chondrocyte apoptosis and

development of OA (76), hence the age-related downturn of ALK5 signalling may predispose to apoptosis. It is likely that age-related deficiency of canonical Wnt signalling in cartilage contributes to this predisposition, as explained above (60). Silencing of FOXO1 and 3 was shown to promote chondrocyte death following enhanced oxidative stress (77), so the age-related decline of FOXO1 and 3 signalling weakens cartilage resilience. Finally, the anabolic factor IGF-1 normally inhibits chondrocyte apoptosis (78), but responsiveness to IGF-1 declines with aging (38).

What becomes apparent, is that most of the hitherto reviewed mechanisms behind cartilage aging are closely related. Cellular actions and signals influence the extracellular environment, and vice versa. The common endpoint of all age-related cartilage alterations is cartilage degeneration in terms of cartilage loss, both structurally and functionally (3). This pathophysiological conception is illustrated below in Figure 1.

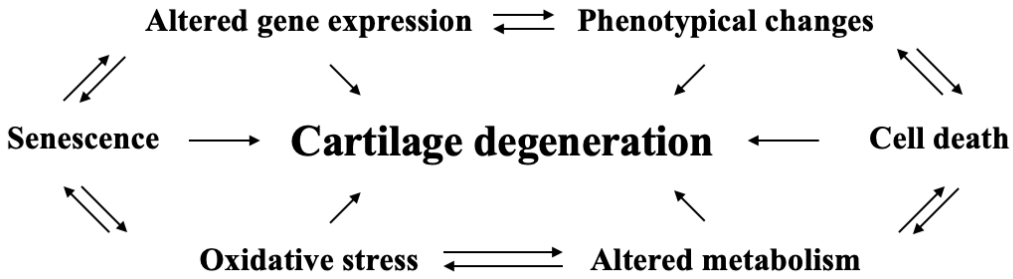


Figure 1: Several interrelated and age-dependent mechanisms contribute to the development of primary (idiopathic) OA. The mutual associations and relative impact of these factors are the subject of ongoing research, but their common endpoint is cartilage degeneration, as defined by progressive structural and functional cartilage loss. This is based on considerations by Aigner et al. (71) and Rahmati et al. (17).

2.2.2 Associated remodelling of subchondral bone

Idiopathic OA has primarily been considered an issue of articular cartilage against the background of progressive wear and tear. According to this theory, degeneration would take place from the outer surface of articular cartilage and proceed to the layers beneath, eventually reaching the level of subchondral bone in regions of completed cartilaginous denudation (1). Along with the accessibility of more advanced diagnostic technologies like MRI, this premise has been questioned. Not only are OA-affected joints characterised by the pathological features of subchondral bone and the adjacent soft tissues separately from cartilage damage, but visualisable

alterations like edema in these structures can occur prior to, or even in absence of, obvious cartilaginous erosion (79). The main features of OA and the processes contributing to their development are illustrated in Figure 2, originally published by Glyn-Jones et al. (3). Affected joint components as well as biochemical mediators are demonstrated under healthy (A) and diseased (B) circumstances.

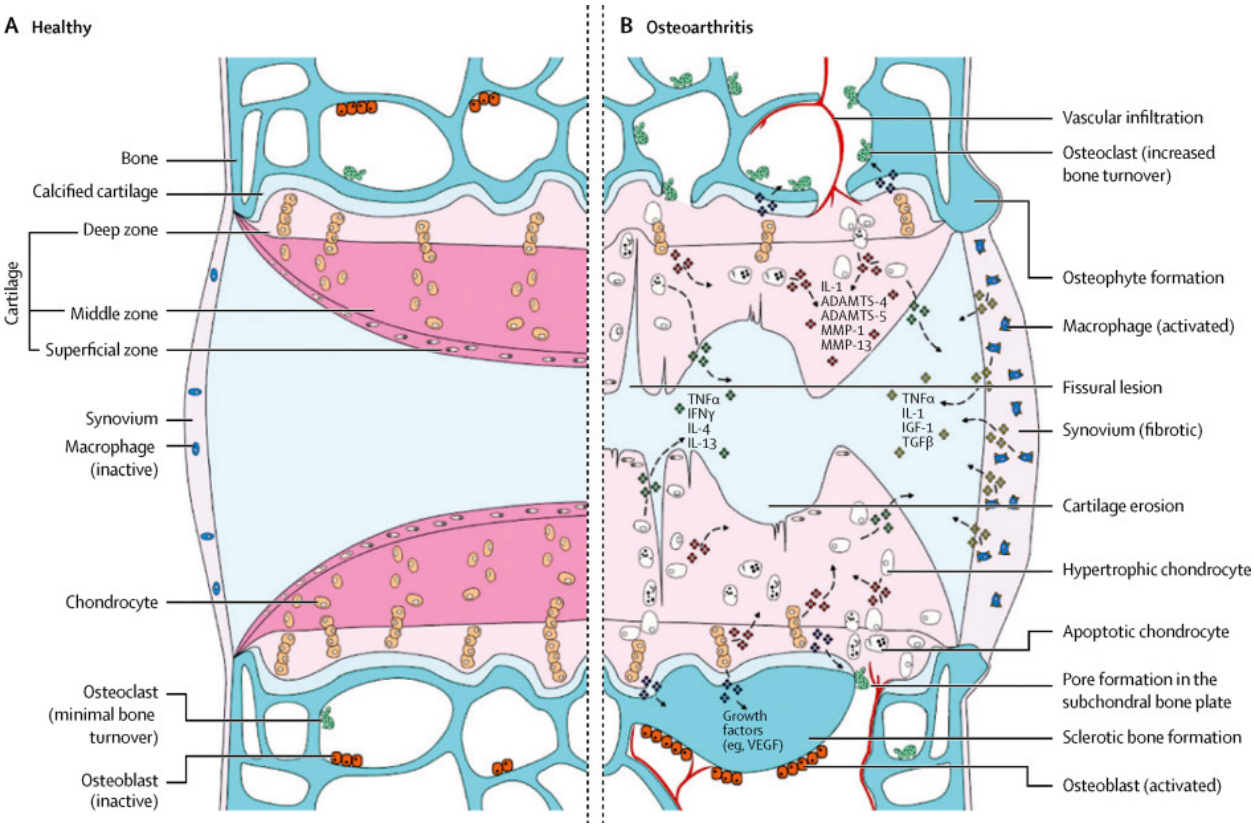


Figure 2: Scheme of a diarthrodial joint under healthy (A) and osteoarthritic (B) conditions. The involved anatomical joint structures and OA pathophysiology are displayed. Pay special attention to the disease's main features, namely cartilage erosion, subchondral bone remodelling, osteophyte formation and synovitis. Reprinted with permission from Glyn-Jones et al. 2015, *The Lancet* (3).

A different perspective on the relation between pathological changes in cartilage and bone was proposed by Radin et al., who assumed reduced ability of shock-absorption due to subchondral bone stiffening as the first stage, and consecutive overstraining of articular cartilage by alternated force distribution as the second stage (80). A study by Nevitt et al. is in line with this mechanistic theory, since they reported a significant correlation between high bone mineral density and the risk for development of spontaneous knee OA (81). Such results based on sensitive imaging and measurement methods raise the question of whether subchondral bone changes are the

consequence or, in fact, the cause of other OA-related findings (27). For the time being, the sequence of pathologic mechanisms remains elusive and the pathogenesis of OA has to be discussed independently of a strict time-course, but with a focus, however, on interconnection between all involved parts of the joint.

OA-associated changes occur in both the cortical and trabecular elements of subchondral bone. In the early phase of OA, increased osteoclast activity was found to cause thinning of the subchondral bone plate (SBP) and to reduce trabecular bone volume. The loss of SBP material can result in focal pore formation, which allows vessels to penetrate the zone of calcified cartilage and supports mutual exchange of inflammatory cytokines, as well as other relevant OA mediators between cartilage and bone (82). Pathological vascularization is further enhanced by the release of vascular endothelial growth factor (VEGF) (3). During the later stages of OA, osteoblast-driven bone formation prevails over bone resorption and leads to the opposite phenomena, namely SBP thickening, subchondral bone sclerosis and osteophyte formation on the joint margins, but this is usually not followed by adequate mineralization. Therefore, biomechanical resistance of this adaptively formed tissue is impaired (82). It is thought that either stress-induced lesions or the influx of synovial fluid are responsible for the occasional development of pseudocysts within osteoarthritic subchondral bone (83). The changes in subchondral bone are associated with increased TGF-β levels (84), which demonstrates the relevance of this signalling pathway beyond mere cartilaginous degeneration.

Apart from these OA-related aspects of subchondral bone remodelling, one should keep mind that bone itself undergoes age-dependent alterations. Although not the primary focus of this thesis, aging clearly affects the physiologic functions of bone cells and bone tissue, not least on account of the possible coincidence with osteoporosis. In fact, the entire musculoskeletal and soft-tissue system can become deranged in the elderly organism, contributing to altered biomechanics and predisposition to trauma (17). The role of joint-surrounding soft tissue, especially inflammatory processes in synovium, is to date not absolutely clear, but there are certain attributes which can parallel OA-like degeneration.

2.2.3 Associated inflammatory processes in synovial tissue

Inflammation was classically not considered to be the essential determinant of OA, but accumulating evidence suggests that inflammatory processes take part in the overall disturbance during degenerative joint disease, both locally and systemically (1). On a microscopic level, inflammation is usually reflected by relatively mild to moderate histopathological signs: infiltration of leukocytes or macrophages, synovial lining cell hyperplasia, fibroconnective

remodelling or abnormal vascularization are possible OA-related findings in synovium and joint-surrounding soft tissue (85). On a macroscopic level, clinical examination or refined imaging by ultrasound or MRI is occasionally able to detect joint effusion and soft tissue edema/swelling in the sense of activated OA (79). A number of studies found evidence for synovitis prior to manifestation of radiographic OA, raising questions about the time course, starting point and, possibly, a causative role of synovial inflammation in OA pathogenesis (85).

The senescence-associated synthesis of inflammatory cytokines has been mentioned before (24), but release of IL-1, IL-6 and TNF-α can also occur as SASP independent. Damage-associated molecular patterns (DAMPs), such as collagen type II fragments or calcium crystals, are able to bind to toll-like receptors (TLRs), e.g. TLR-2 or -4. These receptors were shown to act as membrane-bound pattern recognition receptors (PRR) on chondrocytes, synovial fibroblasts and synovial macrophages, and to facilitate inflammatory responses, which can in turn promote further activation of innate immune cells (3, 43, 86). The cascade of released cytokines is able to accelerate cartilage degeneration by stimulating chondrocytic proteases secretion (85).

Accordingly, the idea of prescribing GCs to OA patients was originally based on anti-inflammatory properties and believed relief of symptoms (87). More recent studies suggest additional actions of GCs in the context of OA, which are not exclusively beneficial. Therefore, a differential understanding of the benefits and risks of GCs is essential.

2.3 Glucocorticoids and osteoarthritis

Intra-articular steroid injections (IASIs) are one of the most common treatment options in the outpatient care management of degenerative joint diseases. The locally targeted application of this potent drug family is routinely performed by many physicians dealing with orthopaedic patients (88). At the same time, there is ultimately no clear scientific evidence in favour of this regular practice. According to the American College of Rheumatology and American Academy of Orthopaedic Surgeons, IASIs are not supposed to be used as a first choice, but as an adjunct to physiotherapeutical measures and oral painkillers (14, 89). Awareness of the risk of steroid-induced arthropathy, in other words cartilaginous breakdown as a consequence of frequent GC injections, is one important reason why scientific experts and well-informed medical practitioners are reluctant to prescribe intra-articular GCs repeatedly (87, 88). Steroid-induced arthropathy is the central safety issue of IASIs, next to the general hazards of intra-articular administrations like infections or injury (90). "*Dosis sola facit venenum*" – a dose-risk relationship exists for any pharmacological agent, which raises the question whether there is a certain threshold beyond

which intra-articular GCs like triamcinolone can potentially become harmful and impair cartilage integrity. Moreover, it is not definite through which GC-dependent mechanisms desirable or undesirable effects are exerted (87). Despite the partly contradictory international studies on the harms and benefits of intra-articular GCs regarding OA cartilage, many reviewers declare agreement on dose-dependency and differences in short-term vs. long-term treatment when discussing their applicability (87, 91, 92). While short-term improvements in pain and joint functionality are more or less acknowledged, the implications in the long run are less well understood (92). As expected, higher cumulative GC doses also go along with a higher risk of steroid-induced side effects (91). Academic research has so far mainly focused on the effects of the above-mentioned exogenous GCs. What has often been neglected is the role of endogenous (i.e. naturally occurring) GCs during OA pathogenesis. In contrast to therapeutically administered steroids, the levels of natural GCs in synovial fluid are much lower (93). They are, however, potentially able to interfere with the mechanisms leading to age-related cartilage degeneration. Therefore, it might be worth enhancing the awareness of endogenous GCs in OA.

2.3.1 Physiology and functions of glucocorticoids

Glucocorticoids are steroid hormones which are physiologically produced in the *zona fasciculata* of the adrenal cortex. Together with mineralocorticoids (MCs), they are subsumed under the term "corticosteroids", which indicates their organic origin. Steroid hormones like GCs and MCs consist of a sterane skeleton as they derive from cholesterol chemically. That accounts for their lipophilic features and excellent tissue penetration properties (94, 95). Successfully used as pharmaceutical drugs for the first time in 1948 by Philip Showalter Hench, who was later awarded the Nobel Prize in Physiology or Medicine with co-laureates Kendall and Reichstein for their discovery, GCs have since then been the subject of wide-spread clinical and pharmaceutical research, and been modified to improve their therapeutically intended effects. Since they are supplied from the outside and intended for therapy, one can also call them "*exogenous GCs*", whereas the physiologically released GCs are referred to as "*endogenous GCs*" (96).

The average daily endogenous cortisol production of a healthy individual ranges from 5.7 to 7.4 mg per m^2 body surface or 9.5 – 9.9 mg in total (97). Synthesis of GCs is systemically regulated via the hypothalamic-pituitary-adrenal (HPA) axis, where corticotropin-releasing hormone (CRH) from the hypothalamic paraventricular nucleus (PVN) stimulates the secretion of adreno-corticotropic hormone (ACTH) from the anterior pituitary gland. ACTH then triggers the synthesis and release of cortisol, which in turn inhibits the release of CRH and ACTH, hence establishing self-regulatory control of the endocrinological axis by a negative feedback mechanism (94). As

the hypothalamic PVN receives signals from the neighbouring suprachiasmatic nucleus (SCN), the main chronobiologic control centre of the body, the levels of cortisol follow a circadian rhythm in which the concentration culminates in the morning hours with subsequent, gradual decline until approximately midnight (97). In addition to this systemic regulation, tissue-specific actions of GCs are locally modulated via 11β-hydroxysteroid dehydrogenase isoenzymes 1 and 2 (11β-HSD1/2). Under normal circumstances, 11β-HSD1 catalyses the reduction of biologically inactive cortisone (11-dehydrocorticosterone in rodents) to biologically active cortisol (corticosterone in rodents), and the counterpart enzyme 11β-HSD2 converts the two reactants in the opposite direction (98). As there is remarkable interaction between GCs and the MC receptor, which primarily serves in the constitution of electrolyte as well as fluid balance and belongs to the renin-angiotensin-aldosterone system (RAAS), 11β-HSD2 prevents exaggerated activation of the MC signalling pathway in MC-dependent organs like kidneys, despite much higher serum concentrations of cortisol than aldosterone (98, 99).

Other organs rely on adequate supply of the active form cortisol, since it is an essential stress hormone and indispensable for the maintenance of numerous body functions. The most important processes and organ systems under the influence of GCs include embryological development (sufficient organ maturation), metabolism (reinforced energy supply in situations of stress), the immune system (anti-inflammation and immunosuppression), the cardiovascular system (increased contractility and blood pressure) and the central nervous system (arousal and cognition) (87, 92, 100). The role of GCs in cartilage and OA will be illuminated separately.

2.3.2 Molecular mechanisms of glucocorticoid signalling

The molecular mechanisms of GC signalling can principally be divided into genomic and non-genomic actions. Genomic actions of GCs are based upon the interaction with the glucocorticoid receptor and are realised on the level of gene transcription. Encoded by a gene with 9 exons on chromosome 5 q31-q32, the GR protein resembles the typical structure of a nuclear receptor. A schematic illustration of the GR gene and protein organisation is provided in Figure 3, originally published by Nicolaides et al. (101).

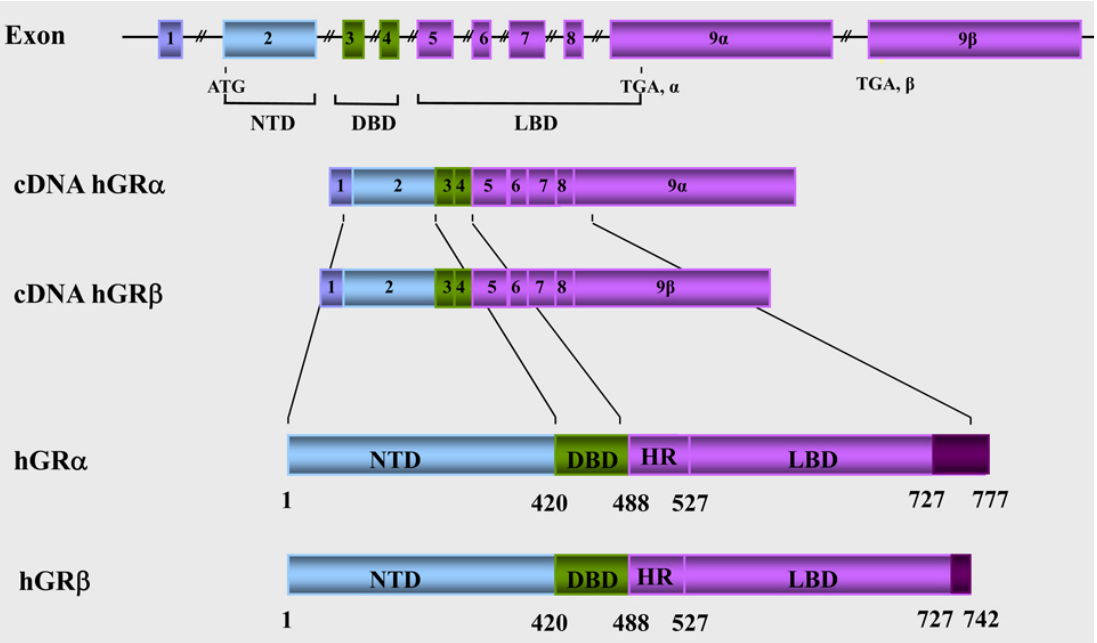


Figure 3: (Reverse) transcription and translation products of the human glucocorticoid receptor (hGR) gene. The major isoforms hGRα and hGRβ are depicted. The hGR gene consists of 9 exons, of which exon 2 is translated to the N-terminal domain (NTD), exons 3 and 4 to the DNA-binding domain (DBD) and exon 5-9 to the hinge region (HR) and ligand-binding domain (LBD) at the C-terminus of the hGR protein. Alternative splicing of exon 9 results in different cDNA/mRNA variants and hGR isoforms (α or β). Reprinted with permission from Nicolaides et al. (101).

The N-terminus is encoded by exon 2 (exon 1 remains untranslated) and serves as the ligand-independent domain of activation function 1 (AF-1). Messenger RNA from exon 3 and 4 is translated into the central, highly conserved DNA-binding domain (DBD), which represents a zinc finger motif. The hinge region, ligand-binding domain (LBD) with ligand-dependent activation function 2 (AF-2) and the C-terminus are encoded by exon 5-9. Owing to alternative splicing of exon 9, two major isoforms of the receptor exist, namely GRα and GRβ. While the α-subtype is by far the most frequent isoform and responsible for GC-mediated signalling, GRβ is considered to be the dominant negative inhibitor of GRα, or even to act independently of it. Interestingly, GRβ does not bind GCs as a ligand, but instead shows affinity to the artificial GR antagonist mifepristone. Besides GRα and GRβ as the main isoforms, other splicing variants (GRγ, GR-A, GR-P) have been described, mainly in the context of cancerous diseases. The GR heterogeneity is further enlarged by single nucleotide polymorphisms of the GR gene, by alternative translation (which causes length alterations of the N-terminus), and by post-translational modification of the completed protein (e. g. phosphorylation, acetylation, sumoylation); these mechanisms are able to influence transcriptional activity. (94, 101-103)

Without its ligand, the GR is located in the cytoplasm, protected by heat-shock proteins and immunophilins. Once GC molecules bind to the LBD of the GR, the attached multi-protein complex dissociates and allows conformational change of the receptor, followed by nuclear translocation via nuclear localization signals (87). Two pathways of genomic GR actions can be further distinguished: transactivation and transrepression. In case of transactivation, the homodimerized GR binds to glucocorticoid response elements (GREs) of GC-sensitive genes with its DBD. Supported by AF-1/2, which enables recruitment of coregulators in addition to general transcription factors (103), the preinitiation complex gets ready for action and promotes the expression of *anti-inflammatory* proteins like IL-10 or the inhibitor of NF-κB. Transrepression, in contrast, suppresses the expression of *pro-inflammatory* genes by direct interaction of an activated monomeric GR with the corresponding transcription factors of these genes, such as the previously mentioned NF-κB or AP-1 (87, 96, 102).

While the genomic actions of GC signalling naturally take time, specifically at least 30 minutes until changes at a biomolecular level are detectable, the non-genomic actions are transduced at much higher speed, which is the underlying principle and reason for GC application in several acute situations (96). Systematic classifications of non-genomic actions were provided by several researchers. The Mannheim classification from 2000 (104) was followed in 2008 by suggestions from Haller et al. (105) and eventually Stahn et Buttgereit (102), of which the latter involves three possible pathways of non-genomic GC actions: firstly, non-specific intercalation of GCs with cellular membranes that accounts for modified trans-membranous ion exchange; secondly, non-genomic effects mediated by the cytosolic GR, possibly counteracting inflammation via diminished release of arachidonic acid as a substrate of prostaglandin synthesis; and thirdly, specific interactions of GCs with membrane-bound GRs, which are probably another variant of GR gene expression and influence the immunological reactions of lymphocytes (102).

Structure-wise, the GR protein displays similarity to other steroid hormone receptors of the so-called nuclear receptor superfamily. The androgen receptor (AR) and estrogen receptor (ER) are also part of this superfamily (106). The close relationship between GR, AR and ER may account for potential interactions between GC signalling and sexual hormones, and there is some evidence suggesting sexually dimorphic actions of GCs in certain tissues. Recently, androgens were found to *increase* the sensitivity of adipose tissue to GC-regulated gene transcription (107). Conversely, estradiol was found to *repress* GC signalling in endometrial cells (108). This indicates a modulating role of sexual hormones with regard to GC signalling.

2.3.3 Effects of exogenous glucocorticoids on cartilage

The first theory which comes to mind when thinking about the target point of exogenous GCs in OA is related to their anti-inflammatory properties. Chondrocytes, osteocytes and synoviocytes are all capable of contributing to inflammatory processes (3). However, the status of inflammation in OA is disputable and most likely plays a subordinated role for the development of pain (109). Analyses by Maricar et al. in 2013 (110) and 2017 (111) aimed to identify predictors of successful response to intra-articular GC treatment in patients with manifest knee OA. Interestingly, pre-existent synovitis as an indicator of inflammation was not associated with increased GC efficacy in terms of pain reduction; one trial actually showed the opposite case, meaning that synovitis-affected joints paradoxically responded to a lower extent compared to non-inflamed joints (110-112). Commensurate with this, effusion as another sign of activated OA also did not serve as a predictor of response when assessed by sensitive MRI (111).

That is why other pharmacodynamic rather than anti-inflammatory properties of GCs may be relevant in OA-affected cartilage. An abundance of *in vitro* and animal *in vivo* studies were performed to understand the effects of artificial GCs on cartilage physiology better. Several experiments revealed beneficial outcomes of steroid treatment on cartilage metabolism. For instance, Lu et al. conducted an *in vitro* study of human and bovine cartilage explants in which the short-term application of dexamethasone in different constellations (before and after incubation with TNF-α; after mechanical injury, incubation with TNF-α plus IL-6 and soluble IL-6 receptor) decreased GAG loss and improved proteoglycan synthesis. The protective effects of GCs in this study were attributed to reduced nitric oxide generation and inhibition of aggrecanases (113). *In vivo* research by Pelletier et al., looking at canine OA models, showed ameliorated disease progression in terms of cartilage erosion and osteophyte formation after GC treatment (114, 115). The hypothesized role of GC-mediated metalloprotease suppression was proven by the reduction of chondrocytic stromelysin (MMP-3) expression in the latter study (115).

In contrast to these GC-approving results, other experiments reported negative impacts of steroids on cartilage health. Nakazawa et al. reported chondrocyte apoptosis in GC-rich milieu *in vivo* and *in vitro*, especially under higher GC concentrations (116). The scientists working with Nakazawa referred to GC-related cellular increase of pro-apoptotic Bax (Bcl-2-associated X protein) and reduction of anti-apoptotic Bcl-2 (B-cell lymphoma 2 protein) as a possible explanation (116). Another example of the potentially harmful side of GCs was provided by Richardson et Dodge (117), who confirmed suppression of metalloproteases expression (MMP-1/-3/-13) in equine chondrocyte cultures by various cortisol derivatives, but noticed a downturn of collagen type II and aggrecan synthesis at the same time. Interestingly, inhibition of MMP expression occurred at

markedly lower GC concentrations than the levels necessary to suppress proteins related to anabolic cartilage metabolism (117). Furthermore, exposition to dexamethasone was reported to impose more oxidative stress on cartilage via increased ROS levels (118). Since aged cartilage is characterised by diminished anti-oxidative FOXO3 activity (64), it might also be more susceptible to GC-induced oxidative stress. In addition, GC signalling counteracts TGF-β-mediated SMAD3 pathways (119). The protective SMAD3 functions, including anti-inflammatory and anti-proteolytic signalling, become impaired, which serves as another explanation for potential chondro-degenerative effects of GCs.

2.3.4 Effects of repeated intra-articular glucocorticoid injections in osteoarthritis

In vitro experiments and animal *in vivo* models provide valuable insight into the underlying mechanisms of exogenous GC actions, but frequently offer inconclusive or contradictory results. Although pre-clinical studies are indispensable, it is in the nature of things that their translation into actual clinics remains difficult. Since the GC signalling pathways are very complex, it is a challenge to predict both the effectiveness and safety of steroids administered to human OA patients. Moreover, methodologically reliable evidence from RCTs for the short- and mid-term (up to 6 months) usefulness of GCs is sparse, according to an extensive Cochrane review from 2015 (120). As far as we know, the availability of high-quality trials with a longer period of follow-up is even more limited. Therefore, a study by McAlindon et al. (121) from 2017 received wide attention. In their RCT, McAlindon and his team investigated the effects of intra-articular triamcinolone administration on cartilage volume and clinical response over a duration of 2 years. The idea and concept of this trial were analogous to an earlier study by Raynauld et al. (122) in 2003, but used more advanced imaging technology (MRI instead of X-ray). Furthermore, the RCT in 2017 was more effective due to more than twice as many participants. Since the parallels in the works by McAlindon and Raynauld are striking, except for the divergent results, the most important study characteristics and outcomes are displayed in Table 1 to allow quick comparison at a glance.

Although the study design, inclusion criteria and intervention of the two trials were very similar, the outcomes were incongruent. The conclusion of Raynauld et al., who did not observe significantly increased joint-space narrowing as the radiological correlate of cartilage thickness in patients treated with triamcinolone (as compared to the placebo group), but rather improved clinical parameters in the GC group, rehabilitated GCs at the time the paper was published as there was no proof of promoted cartilage loss by steroids (122). However, one swallow does not make a summer and part of the research world remained suspicious of IASIs.

Author Journal & year of publication	Raynauld et al.[122] *Arthritis & Rheumatism* 2003	McAlindon et al.[121] *JAMA* 2017
Study design	Double-blinded RCT	
n (treatment vs. control)	34 vs. 34	70 vs. 70
Inclusion criteria	OA knee joint (ACR definition[10]), Kellgren/Lawrence[13] 2 or 3	
	40 – 80 years old	≥ 45 y/o, WOMAC score 2 – 8 and sonographic synovitis
Intervention	40 mg triamcinolone acetonide vs. 0.9 % NaCl intra-articular administration in intervals of 3 months	
Duration of follow-up	2 years	
Surrogate endpoint (method)	Joint-space narrowing (X-ray)	Cartilage thickness (MRI)
Result	No between-groups difference in joint space width	Significantly decreased cartilage thickness in GC vs. control group
Clinical results	Area-under-curve night pain & stiffness improved in GC group	No between-groups differences in pain, stiffness or function

Table 1: Comparison of selected characteristics and outcomes of randomized controlled trials by Raynauld et al. (122) and McAlindon et al. (121), who investigated the long-term effects of repeated intra-articular GC injections. Abbreviations: WOMAC = Western Ontario and McMaster Universities Osteoarthritis Index.

The quasi repetition of the RCT with more patients and state-of-the-art radiology grants the newer study by McAlindon et al. more credibility in assessing the long-term risks. Neither relief of pain, nor improvement of stiffness and function were achieved by triamcinolone administrations, but MRI detected significantly reduced cartilage volume after two years of therapy (121). The observed decline in cartilage thickness did not correlate with a worsened clinical presentation, but was found to be a detrimental process which makes the necessity for joint replacement more likely (121, 123). The assessment of clinical values was perhaps distorted by the chosen 3-months interval of medical visits and the window of short-term improvement was thereby missed (121), but the frequency of injections (2 to 4 per year) is quite realistic (88). On a further note, only subjects with sonographic synovitis were included. The absent symptomatic recovery is consistent with the previously mentioned observations of Maricar et al. (110, 111), again reinforcing distrust in the anti-inflammatory functions of GCs for relief of pain and OA progression in general (121). To the best of my knowledge, no further RCTs of a comparable standard exist which inquire into

cartilaginous changes following IASI. Consequently, physicians should act with caution when including this treatment option in their therapy regimen. The aim should be to conduct additional clinical trials to see if the results are reproducible and if certain amendments (steroid type, dosage, frequency, restriction to particular patient subgroups or OA phenotype) can be implemented. Until then, the role of exogenous GCs in knee OA will not be conclusively clarified.

2.3.5 Effects of endogenous glucocorticoids on cartilage

During the pre-clinical phase of OA, cartilage degeneration usually takes place in the absence of artificial exogenous GCs, but in the presence of endogenous GC signalling. Since endogenous GCs are involved in the regulation of countless physiological body functions, it appears useful to know if the maintenance of healthy cartilage itself is inevitably GC-dependent. The hitherto conducted research about the role of endogenous GCs in chondrocytes created constellations of disrupted GC signalling and investigated the phenotypical alterations. What we know from these mouse studies, is that neither chondrocyte-specific GR knockout nor global GR knockout (GR^{null}) go together with changes in cartilage morphology, growth plate dimensions or long bone growth (124, 125). In contrast to absent endogenous GC signalling, higher levels of circulating cortisol (for instance stress-related during malnutrition) can inhibit longitudinal bone growth in adolescents by decreasing growth hormone receptor expression in the growth plate (92, 126).

Endogenous GCs might influence the inflammatory microenvironment in cartilage. At least this was hypothesized to be the reason why cartilaginous callous following fracture becomes less well mineralized and less stable in absence of endogenous GC signalling (124). This observation cannot be simply transferred to the complex processes of OA pathogenesis, but the mere fact that inflammation is involved in cartilage degeneration (85) – even if it is not the main driver – explains why the anti-inflammatory properties of GC signalling in OA cartilage need to be taken into consideration.

The serum concentrations of endogenous GCs rise as a result of aging, both in humans and mice (127). Furthermore, the local activation of endogenous GCs in bone shows age-related increase due to enhanced 11β-HSD1 expression in bone. This circumstance was recently identified to be a potential promoter of post-traumatic OA in aged mice, since mice with disruption of endogenous GC signalling (via transgenic overexpression of 11β-HSD2, the GC-inactivating enzyme) presented mitigated disease progression following destabilization of the medial meniscus (DMM) (128). Although the 11β-HSD2 transgenic model exclusively targets cells of the osteoblast lineage, disease prevention was not restricted to subchondral bone, but also affected articular cartilage erosion. The authors concluded that osteoblast-specific endogenous GC signalling may indirectly

also influence degenerative processes within cartilage (128). While the exact causal pathways have not yet been discovered, this experiment indicates a significant role of endogenous GC signalling for OA pathogenesis during aging. It has not been investigated whether endogenous GC signalling specifically and exclusively in chondrocytes has the same relevance for the development of age-related OA. Little is known about the effects of endogenous GCs on aged cartilage in general. The mechanisms by which physiological GCs influence cartilage integrity are even less well studied than the effects of therapeutical GCs. Therefore, chondrocyte-specific disruption of endogenous GC signalling seems to be a promising tool for conducting research in murine OA models.

2.4 Hypothesis and aim

Aging is the central risk factor for the development of idiopathic knee OA (5), characterised by different pathological alterations of cellular and extracellular functions of articular cartilage (17). To date, incomplete understanding of these pathogenetical mechanisms is the main reason accounting for the lack of effective disease-modifying OA drugs (1). The clinical routine of repeated intra-articular steroid injections was recently revealed to cause promoted cartilage volume loss in patients with symptomatic knee OA, indicating a harmful impact of exogenous GCs on disease progress (121). Conversely to therapeutically administered GCs, the role of naturally occurring, endogenous GCs on age-related cartilage degeneration has so far not been studied. Endogenous GC levels are elevated in the elderly organism (127), and endogenous GC signalling, mediated by osteoblasts, was recently shown to enhance post-traumatic OA disease severity in mice of higher age, both with regard to bony and cartilaginous degeneration parameters (128). Based on these observations and considerations, I hypothesized that endogenous GC signalling promotes age-related cartilage degeneration and, eventually, OA. My aim was to study the role of chondrocyte-specific endogenous GC signalling in a model of spontaneously developed, age-induced OA in one-year-old mice. For this purpose, I made use of the established chondrocyte-specific glucocorticoid receptor knockout mouse line (124). In this way, the aim was to investigate the direct effects of endogenous GC signalling on age-related cartilage degeneration. The nature of this pilot-study was explorative and observational, but I also hoped for insights into underlying pathophysiological mechanisms, and for general contributions to the characterisation of murine models in the OA research field. In the long run, a better understanding of GC effects could eventually support therapeutical decision-making in patients with chronic degenerative joint disease.

3 MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemical reagents

Agarose	Astral Scientific Pty Ltd., Taren Point, NSW, AU
3-Aminopropyl-triethoxysilane (AES)	Sigma-Aldrich Co., St. Louis, MO, USA
Bovine serum albumin (BSA), lyophilized	Sigma-Aldrich Co., St. Louis, MO, USA
3,3′-Diaminobenzidine (DAB) substrate kit	Vector Laboratories Inc., Burlingame, CA, USA
Deoxyribonucleoside triphosphates	Invitrogen Corp., Carlsbad, CA, USA
Distyrene + plasticiser + xylene (DPX)	Sigma-Aldrich Co., St. Louis, MO, USA
Eosin	Fronine Laboratory Suppl., Taren Point, NSW, AU
Ethanol	Labtech Service & Supplies, Windsor, NSW, AU
Ethylenediaminetetraacetic acid (EDTA)	Merck Pty Ltd., Bayswater, VIC, AU
Fast red violet LB salt	Sigma-Aldrich Co., St. Louis, MO, USA
Glacial acetic acid	Fronine Laboratory Suppl., Taren Point, NSW, AU
Gill's hematoxylin	Fronine Laboratory Suppl., Taren Point, NSW, AU
Goat serum	Vector Laboratories Inc., Burlingame, CA, USA
Harris' hematoxylin	POCD Healthcare, Artarmon, NSW, AU
Hydrogen peroxide 3 %	Sigma-Aldrich Co., St. Louis, MO, USA
Ketamine	Cenvet Pty Ltd., Kings Park, NSW, AU
Magnesium chloride 20 mM solution	Ajax Finechem Pty Ltd., Taren Point, NSW, AU
Magnesium chloride 50 mM solution	Bioline Pty Ltd., Alexandria, NSW, AU
Mango Taq DNA polymerase	Bioline Pty Ltd., Alexandria, NSW, AU
Milli-Q water	Millipore Pty Ltd., North Ryde, NSW, AU
Naphthol	Sigma-Aldrich Co., St. Louis, MO, USA
N, N – Dimethylformamide	Sigma-Aldrich Co., St. Louis, MO, USA
Paraffin	Merck KGaA, Darmstadt, Germany
Paraformaldehyde (PFA)	Merck KGaA, Darmstadt, Germany
PCR buffer	Fisher Biotec Pty Ltd., Wembley, WA, AU
Phosphate buffered saline (PBS)	Medicago AB, Uppsala, Sweden
Potassium sodium tartrate	Sigma-Aldrich Co., St. Louis, MO, USA
Proteinase K	Roche Applied Sciences, Castle Hill, Australia
Reverse osmosis (RO) water	Millipore Pty Ltd., North Ryde, NSW, AU
Sodium acetate anhydrous	Sigma-Aldrich Co., St. Louis, MO, USA
Sodium citrate trisodium salt dehydrate	Sigma-Aldrich Co., St. Louis, MO, USA

SYBR Safe DNA gel stain	Invitrogen Corp., Carlsbad, CA, USA
Tamoxifen	Sigma-Aldrich Co., St. Louis, MO, USA
Toluidine blue O	Sigma-Aldrich Co., St. Louis, MO, USA
Tris-borate-EDTA buffer	Amresco LLC, Solon, OH, USA
Triton X100	Sigma-Aldrich Co., St. Louis, MO, USA
Vectastain® ABC kit	Vector Laboratories Inc., Burlingame, CA, USA
Xylazine	Troy Laboratories Pty Ltd., Ringwood, VIC, AU
Xylene	Labtech Service & Supplies, Windsor, NSW, AU

3.1.2 Antibodies

Primary antibodies				
Specificity	Catalogue Nr.	Species	Type	Company
anti-Collagen X	abcam-58632	rabbit	polyclonal, IgG	Abcam PLC, Cambridge, GB
anti-GR (M-20)	sc-1004	rabbit	polyclonal, IgG	Santa Cruz Biotechnology Inc., Dallas, TX, USA
Secondary antibodies				
Specificity	Catalogue Nr.	Species	Type	Company
anti-rabbit IgG (H+L chains)	BA-1000	goat	biotinylated, IgG	Vector Laboratories Inc., Burlingame, CA, USA

Table 2: Overview of antibodies used in the present research project.

3.1.3 Laboratory equipment and software

Embedding workstation: Histostar®	Thermo Fisher Scientific, Waltham, MA, USA
Microtome: Leica RM 2125 RT	Leica Microsystems GmbH, Wetzlar, Germany
Microscope: Olympus BX60 F-3	Olympus Optical Co., Ltd., Japan
PCR machine: Eppendorf Mastercycler EP	Eppendorf AG, Hamburg, Germany
Scales: Mettler-Toledo, Model ML 104	Mettler-Toledo Inc., Columbus, OH, USA
Tissue processing machine: Excelsior ES	Thermo Fisher Scientific, Taren Point, NSW, AU
Histomorphometry software:	
OsteoMeasure XP v3.3.0.2	OsteoMetrics Inc., Decatur, GA, USA
Imaging software (histology):	
cellSens Entry, v1.4.1	Olympus Optical Co., Ltd., Japan
Imaging software (PCR): Image Lab, v6.0	Bio-Rad Laboratories Inc., Hercules, CA, USA
Statistics software: GraphPad Prism, v5.02	GraphPad Software Inc., La Jolla, CA, USA

3.2 Chondrocyte-specific glucocorticoid receptor knockout mouse line

Disruption of chondrocyte-specific endogenous GC signalling in mice was first established and delineated by Tu et al. (124). GR knockout by means of the Cre/LoxP system is characterised by the possibility of a) tissue-specific targeting due to selected promoters and b) time-controlled activation, as the external application of tamoxifen is necessary for inducing the genetic knockout (129). As described by Tu et al., these two important characteristics were proven by the help of Rosa26R reporter mice prior to application of the GR KO mouse line (124). A visualization of the Cre/LoxP system and the principle behind spatially and temporally controlled Cre activation is provided in Figure 4, originally published by Greco et Guo (129).

The method for creating GR KO mice started with the generation of a fusion protein consisting of Cre recombinase and a modified version of the murine estrogen receptor (ER), including three point mutations in the ligand-binding domain (LBD). This fusion protein, abbreviated Cre-ER^{T2}, only responds to the selective ER modulator tamoxifen - it doesn't respond to physiologically circulating estrogens (124, 129). Cre-ER^{T2} was then linked to a tissue-specific promoter (Col2a1 or collagen type II, alpha 1) for it to be selectively expressed in chondrocytes of articular cartilage, the growth plate and the intervertebral disc (124), resulting in Col2a1-CreER^{T2} mice. For the GR gene to be the target of Cre, the corresponding genetic site needed to be flanked by loxP (locus of X-over P1), which serves as a recognition sequence for Cre to turn into action. This was achieved by cross-breeding of Col2a1-CreER^{T2} mice (provided by Prof. Di Chen, University of Rochester, New York, USA) with $GR^{flox/flox}$ mice (provided by Prof. Jan Tuckermann, University of Ulm, Ulm, Germany) that had the loxP sequences integrated upstream and downstream of exon 3 (124), which encodes the DNA-binding domain of the GR protein (101). Thereby created heterozygous Col2a1-$Cre^{+/-}ER^{T2}$;$GR^{flox/flox}$ offspring were defined as GR KO and homozygous $Cre^{-/-}$;$GR^{flox/flox}$ mice were defined as WT (124). After injection of tamoxifen, only the modified LBD allowed Cre-ER^{T2} to translocate from the cytosolic to the nuclear compartment and to exert its recombinase function. In this way, exon 3 was excised from the genome and a stop codon (TAG) was generated which impaired further GR translation, thereby disabling any GR-mediated GC actions in chondrocytes of Col2a1-$Cre^{+/-}ER^{T2}$;$GR^{flox/flox}$ mice (= GR KO mice) (124).

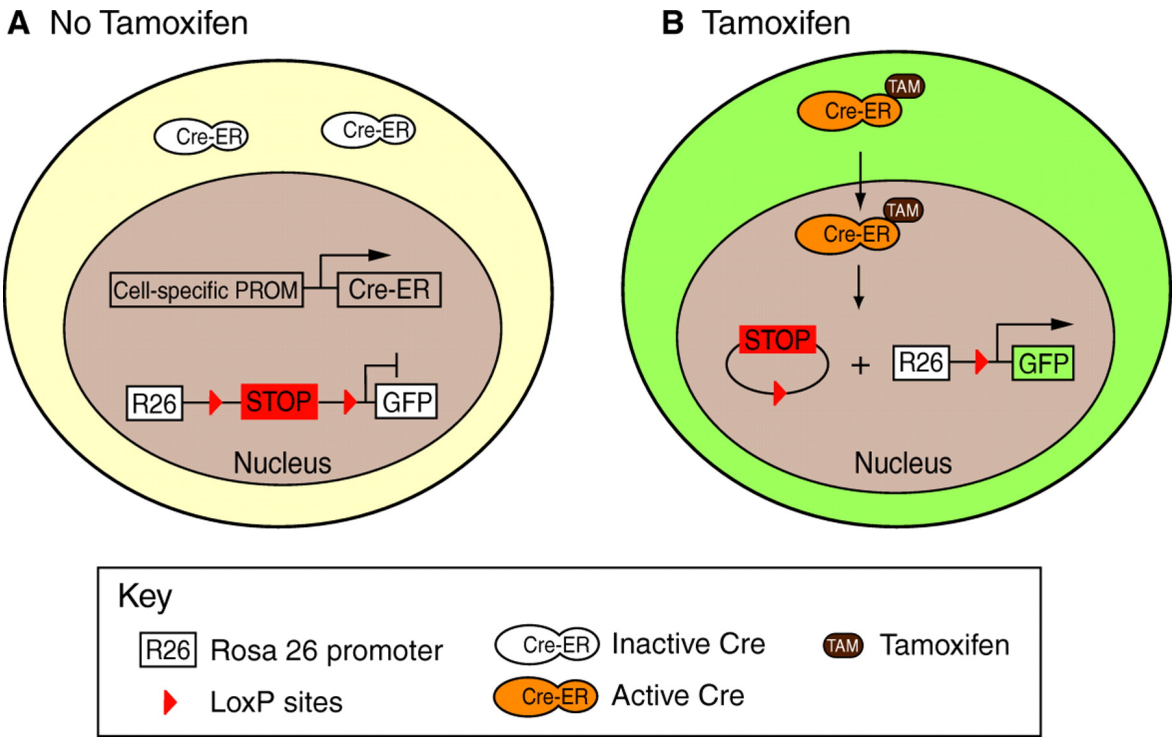


Figure 4: Scheme of the Cre/LoxP system for the generation of GR KO mice with chondrocyte-specific knockout of the glucocorticoid receptor (GR). In absence of tamoxifen (A), a cell-specific Col2a1-promoter drives the cartilage-targeted expression of Cre recombinase, which was fused to a modified estrogen receptor only responsive to tamoxifen (Cre-ER^{T2}). Upon presence of tamoxifen (B), Cre-ER^{T2} translocates to the nucleus, recognizes LoxP sequences and leads to the excision of genes flanked by LoxP. The image above illustrates how the spatial and temporal specificity of Cre expression was checked with the help of Rosa26R reporter mice, enabling marking of Cre-positive cells by certain reporter genes (for instance GFP = green fluorescent protein, or X-Gal, as used in our GR KO mouse line (124)). Reprinted with permission from Greco et Guo (129).

3.3 Experimental design

The main focus of this research project concerned spontaneous, age-related cartilage degeneration and related pathologies in the context of OA. Mice of advanced age were used for this study, which tried to contrast potential differences based on the chondrocytic glucocorticoid receptor status (GR WT vs. GR KO mice of both sexes).

Both GR WT and GR KO mice used for the primary age-related OA project had been injected with tamoxifen (TM, 1 mg/ 10 g body weight) intraperitoneally for 5 consecutive days at the age of 4 weeks (124). Afterwards, mice were kept in the molecular physiology unit of the ANZAC Research Institute (Sydney, Australia) until approximately 12 months of age (exact range: 11.1 – 13.8 months). Between 2 to 4 same-sex littermates were kept together in cages with a base area of

roughly 340 cm^2 (length 15.5 cm x width 22 cm). They were given unlimited access to food and water and an alternating rhythm of 12 hours light or darkness was applied. Spontaneous movement was enabled, but not supported by any means. Furthermore, animals underwent no other kind of intervention. The final distribution of experimental group sizes of 1-year-old mice was as follows:

Genotype \ Sex	♂	♀
WT	13	12
GR KO	14	7

Table 3: Size of experimental groups (n =) for the study of spontaneous, age-related OA.

In addition, 11 young male WT mice from a previous publication (124) were included to identify age-related changes in cartilage calcification. Those younger mice had been injected with TM at the age of roughly 4 weeks and kept until the age of roughly 12 weeks. They were exposed to the same living and housing conditions (meaning food, water, cage size, light-dark-rhythm) as explained above. All animal experiments were conducted in accordance with the regulations and guidelines of the Animal Care and Ethics Committee of the Government of New South Wales (Australia) by officially licensed and trained researchers under approval number 2015/015.
Conceptualization of the project and its experimental design, breeding, induction of chondrocyte-specific GR knockout, genotyping, weighing and animal euthanisation were planned and performed by my supervisors Dr. Jinwen Tu, Prof. Hong Zhou and Prof. Markus Seibel, kindly supported by members of the Bone Biology Unit of ANZAC Research Institute (Sydney, Australia). Strategies included have previously been published by Tu et al. (124) and are recapitulated here briefly for the sake of completeness. My own contributions to this project commenced with the preparation of tissue for histology and were followed by histological staining, histopathological assessment, statistical analysis and data interpretation.

3.4 Genotyping

Mice toe clips served as materials for genotype analysis. To extract DNA from the toe clips, each toe clip was put into 250 µl of lysis buffer, containing 25 µl magnesium chloride (20 mM), 25 µl PCR buffer, 1.7 µl proteinase K and 198.3 µl autoclaved Milli-Q water, and was incubated at 55 °C for 2 hrs. After that, samples were incubated at 98 °C for 15 min to deactivate proteinase K.

Extracted DNA was the substrate for subsequent PCR analysis (Eppendorf Mastercycler EP). 5 µl of the extracted DNA lysate were added to the PCR reaction mix, consisting of 6.8 µl Milli-Q water, 5 µl 5x buffer, 1 µl magnesium chloride (50 mM), 5 µl dNTPs (1 mM), 1 µl forward primer, 1 µl reverse primer and 0.2 µl mango taq DNA polymerase (5 U/ µl). The CreF (F, 5' – ATC CGA AAA GAA AAC GTT GA - 3') and CreR (R, 5' – ATC CAG GTT ACG GAT ATA GT - 3') primers were used to detect the Cre expression, and the GR1 (F, 5′ - GGC ATG CAC ATT ACT GGC CTT CT - 3′) and GR8 (R, 5'-CCT TCT CAT TCC ATG TCA GCA TGT - 3) primers were used for GR flox detection. Confirmation of successful GR knockout after tamoxifen injection, i.e. detection of excised exon 3, was conducted with the GR1 and GR4 (R, 5′ - GTG TAG CAG CCA GCT TAC AGG A - 3′) primers. After an initial cycle at 94 °C for 5 min, 35 cycles of denaturation (94 °C for 30 sec), annealing (60 °C for 30 sec) and elongation (72 °C for 45 sec) followed, and PCR finished with one cycle at 72 °C for 5 min. Then, 1.5 weight % agarose was dissolved in tris-borate-EDTA buffer (0.089 M tris, 0.089 M borate, 0.002 M EDTA) with 10 µl SYBR Safe DNA gel stain (10,000x concentrated) per 100 ml agarose gel, for intercalation with the amplified DNA. Electrophoresis was done for 25 min with 120 V and the gel imaged afterwards.

3.5 Histology

3.5.1 Tissue harvesting and preparation for histology

Animal euthanasia was always conducted under adequate anaesthesia and analgesia. A ketamine/xylazine mixture was administered intraperitoneally for this purpose. A dosage of 75 mg ketamine/kg body weight and 10 mg xylazine/kg body weight was given to each mouse. Cardiac exsanguination with a 25 G needle was performed prior to cervical dislocation. The legs were surgically removed and the knee joints along with parts of the adjacent bones were carefully excised.
After harvesting, tissues were fixed in freshly made 4% paraformaldehyde (PFA) buffer for 48h at 4 °C. PFA powder was dissolved in phosphate buffered saline solution (PBS) consisting of 0.14 M NaCl, 0.0027 M KCl, 0.01 M phosphate buffer (pH = 7.4) at 60 °C, followed by cooling down to 4 °C. After fixation, the right mouse legs were decalcified in 10% ethylenediaminetetraacetic acid solution (EDTA, pH = 7.5) for 2-3 weeks at 4°C on a shaker with a solution change every 2 days. After decalcification, the soft tissue around the tibia and femur was carefully removed, while tissue directly surrounding the knee joints was maintained. The samples then underwent an automated dehydration/clearing/waxing process in a tissue processing machine (Excelsior ES, Thermo Fisher Scientific). The dehydration step included washing in a series of increasing concentrations of

ethanol (70% for 1h x 2, 75% 2h, 85% 2h, 95% 2h, 100% 4h x 3). Clearing was done by washing three times with 100% xylene for 2h. The samples were then transferred in paraffin in three intervals for 2, 2 and 6 hours at 62 °C.

Finally, the samples were embedded in paraffin (using the Histostar® embedding machine) with the knee joint's medial side oriented towards the surface of the latter paraffin block.

Serial longitudinal 5 μm-thick sections were obtained using a Leica microtome (Leica RM 2125 RT) and mounted onto AES coated slides (see section 3.5.2 below). Cutting was performed from the medial to the lateral joint compartment in the sagittal plane. Three sections were placed next to each other on one slide. After the slides were kept at 37 °C overnight to allow sections to attach to the slide surface properly, they were ready for staining.

3.5.2 Coating slides with aminopropyltriethoxysilane

The slides were coated with aminopropyltriethoxysilane (AES) to enhance adhesion. The slides were first soaked in hot water containing detergent for 2 hours and then rinsed with tap water until clean, followed by soaking in reverse osmosis (RO) water (5 min x 2). After that, they were immersed in 80% ethanol for 2 hours before drying at 37 °C overnight. Dried slides were then coated with AES by 4 processes, starting with soaking in acetone with 2 % AES for 30s, followed by 3 washes with a few gentle dips in two racks with pure acetone and one with RO water. The slides were then dried at 37 °C overnight again before they were ready to use.

3.5.3 Histological staining

Prior to every histological staining procedure, the obtained sections needed to be cleansed of paraffin. Sections were first heated up on a 60 °C hot plate for 30 seconds to melt the paraffin, and then put in three changes of 100 % xylene for 5 min each for dewaxing. The slides were then put in three changes of 100 % ethanol for 5 min each for rehydration before the slides were rinsed in tap water for 3 min to get ready for the subsequent different specific stainings.

3.5.3.1 Hematoxylin and eosin staining

Harris' hematoxylin and eosin solutions were prepared following a standard laboratory protocol. As a first step, the slides were immersed in 1:10 Harris' hematoxylin solution for 7 min. Afterwards they were gently rinsed with tap water for 10 min and stained with eosin for 10 min. For dehydration, the slides were then washed in three changes of 100 % ethanol (2 min each) and finally cleared in three changes of xylene (3 min each), before cover slipping with DPX mounting medium.

3.5.3.2 Toluidine blue staining

A 0.1% toluidine blue (TB) solution at pH 3.7 was prepared according to a standard laboratory protocol. TB is a cationic dye which enables staining of anionic proteoglycans and GAGs in cartilage (130). Also, TB allows distinction between calcified and non-calcified layers as a precondition for histological assessment (131) and is an accepted staining dye for murine OA studies (132). The slides were put into the TB staining solution for 5 min and then gently rinsed with tap water until the water turned clear. After that they were dried at 37 °C for overnight. The dried slides were immersed in 100 % xylene to enable cover slipping with DPX mounting medium.

3.5.3.3 Tartrate-resistant acid phosphatase staining

Tartrate-resistant acid phosphatase (TRAP) staining is a specific histological staining method which allows to assess the activity of osteoclasts in bony tissue and correlates with increased bone turnover (133). The buffer used for TRAP staining consisted of sodium acetate anhydrous (50 mM) and potassium sodium tartrate (40 mM) dissolved in RO water, being adjusted to a pH of 5.0 with glacial acetic acid. To prepare the staining solution, 5-10 mg Naphthol were dissolved in 0.5 ml N, N – dimethylformamide and mixed with 50 ml of TRAP buffer. Then 20-30 mg of Fast red violet LB salt were added.

To perform TRAP staining, dewaxing and rehydration were executed as stated above, but after washing with three changes of 100 % ethanol slides underwent treatment with decreasing concentrations of ethanol, i.e. 95 % ethanol (3 min) and 70 % ethanol (2 min), before they were rinsed in tap water. TRAP solution was carefully pipetted upon the sections so that they were entirely covered. The reaction took place at room temperature and was attentively checked under the microscope until osteoclasts gained correct colour intensity, which took approximately 8 min. At this point, the reaction was stopped by rinsing the slides in tap water. After 3 minutes, counterstaining was done with 1:10 Gill's hematoxylin for 30 seconds. The slides were rinsed in tap water again, dried at 37 °C overnight and coverslipped with DPX mounting medium.

3.5.4 Immunohistochemistry

A variety of solutions and buffers were necessary to perform immunohistochemistry (IHC). Sodium citrate trisodium salt dehydrate (0.01 M) served as antigen retrieval buffer at an adjusted pH of 6.0. PBS/BSA/Triton X100 buffer with 0.015 % BSA and 0.01 % Triton X100 was used as washing buffer. 5 % goat serum in PBS/BSA/Triton X100 served as a blocking solution and all antibodies were made up in 2 % goat serum in PBS/BSA. ABC reagent (avidin DH and

biotinylated horseradish peroxidase H) was used as described in the Vectastain® ABC kit. DAB solution was made according to the DAB substrate kit.

The initial procedure of dewaxing and rehydration was slightly modified for IHC, since three additional washing steps with 80 % ethanol (3 min each) were included before the slides were immersed in RO water for 5 min.

After the citrate buffer had been boiled and allowed to cool down to 75 °C, the slides were gently placed into the buffer to prevent sections from falling off. Incubation in citrate buffer took 90 min until the temperature of the buffer dropped to room temperature in order to achieve optimal antigen retrieval. The slides were washed in RO water for 5 min afterwards and then incubated in 3 % hydrogen peroxide for 20 min to block intrinsic peroxidase activity. They were then washed in RO water for 5 min before carefully placing them in an IHC sequenza rack. As a next step, the slides were incubated with 5 % goat serum for 30 min to avoid non-specific staining, before the primary antibodies, anti-GR (diluted 1:100) or anti-collagen X (diluted 1:400), were added. After overnight incubation at 4 °C, the slides were washed with washing buffer for three times (10 min per wash). Then the secondary anti-rabbit antibody, diluted 1:200 in 2 % goat serum in PBS/BSA, was added for 1-hour incubation at room temperature, followed by washing three times in washing buffer (10 min per washing). The slides were then incubated with ABC reagent for 30 min and washed again three times before DAB staining. DAB solution was added onto the slides (100 µl/section) and observed under the microscope, to be stopped immediately after the positive staining was distinct by washing in RO water. For counterstaining, the slides were immersed in Harris' hematoxylin for 1 min. After tap water washing until the colour was clear, washing three times in 100 % ethanol and washing three times in xylene (3 min for each step) followed. The slides were ready for mounting with DPX mounting medium afterwards.

In each IHC staining procedure, negative controls were performed by omitting the primary antibody to check for unspecific binding of the secondary antibody.

3.6 Histopathological assessment

3.6.1 Scoring of cartilage erosion

In order to evaluate signs of cartilage erosion, an established semi-quantitative scoring system by Kamekura et al. was applied, which differentiates lesion depth in terms of affected cartilage layers (131). Table 4 (on page 42) summarises the descriptive criteria according to which cartilage damage was quantified (131). Representative images and corresponding grades are shown in Figure 5 (page 42). Femur and tibia were always graded separately.

Morphologic feature	Grade
Smooth cartilage surface and intact cartilage tissue	0
Superficial lesions to cartilage	1
Vertical clefts limited above tidemark	2
Vertical clefts extending to or beyond tidemark	3
Complete erosion extending to subchondral bone	4

Table 4: Semi-quantitative scoring system for histological grading of cartilage erosion. Adapted from Kamekura et al. (131).

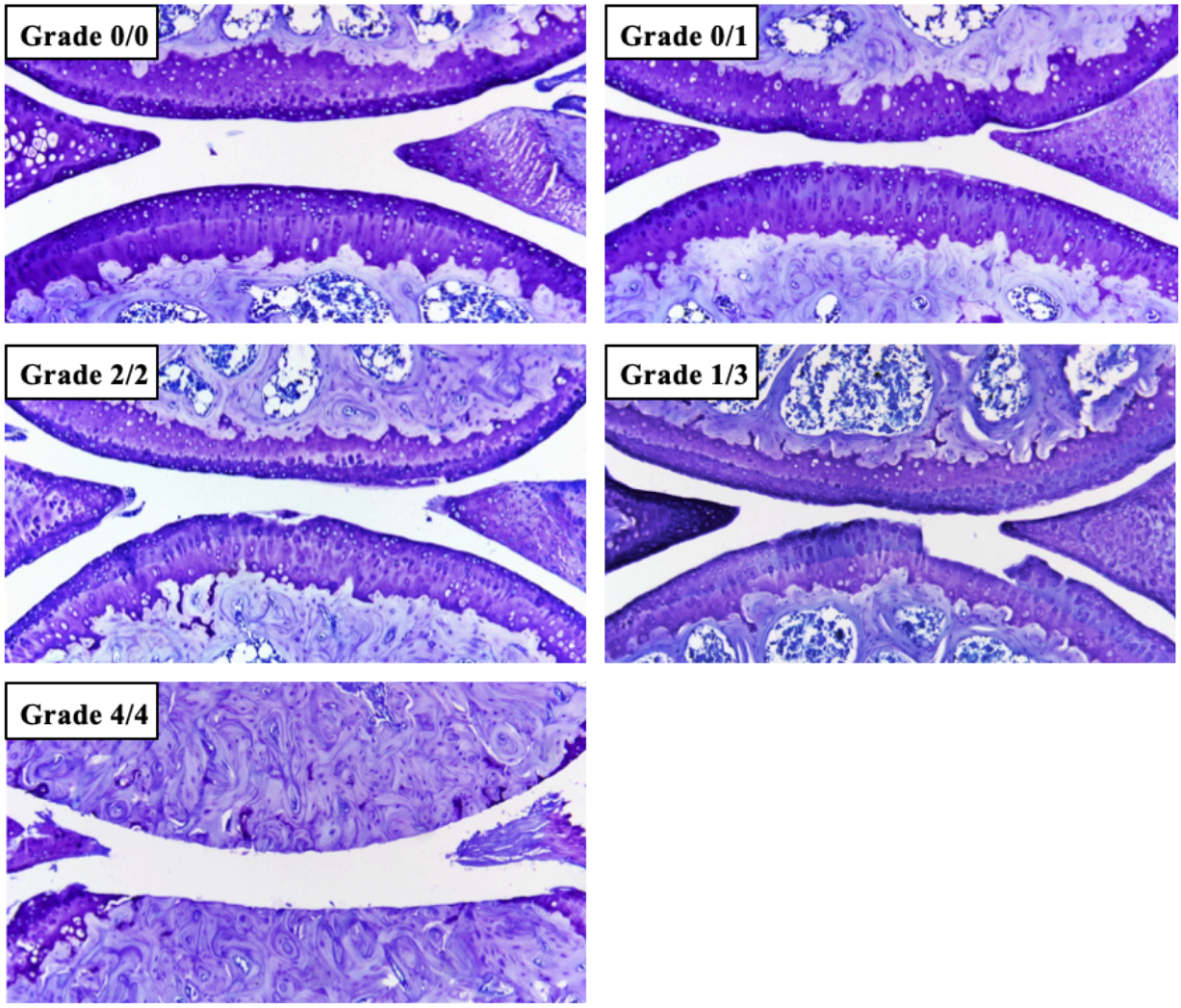


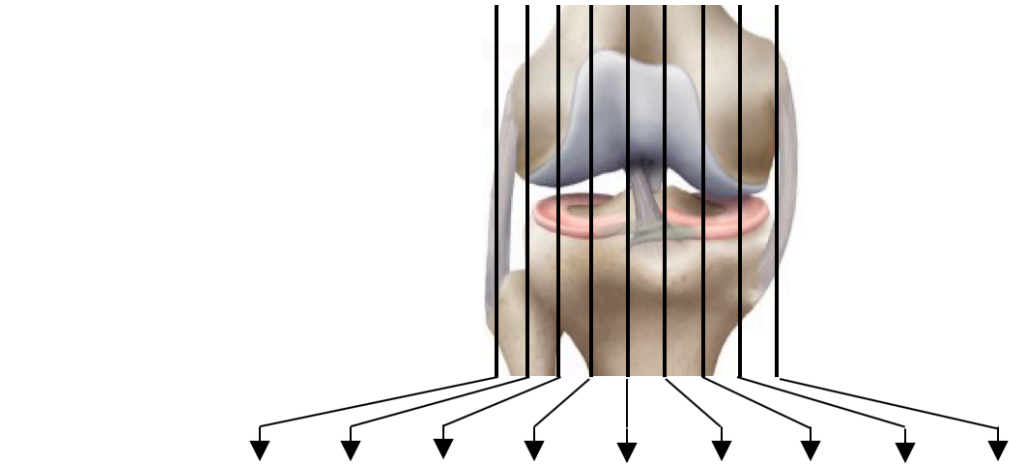
Figure 5: Representative images of cartilage erosion as a reference for scoring. The first grade refers to the femoral (upper), the second grade to the tibial (lower) side. Toluidine blue staining. Magnification 10x.

As shown, toluidine blue stained sections were used for cartilage erosion scoring. Images were taken with the help of an Olympus BX60 F-3 microscope and cellSens Entry imaging software. Afterwards, the images were assessed by two independent, blinded observers, namely my supervisor Dr. Jinwen Tu and me. The entire right knee joint of each mouse had been cut in serial, sagittal sections of 5 µm thickness and every seventh slide (with three sections on it) was stained with toluidine blue dye. In this way, every 90 µm of the joint were evaluated histologically. In case of inter-observer discrepancy, the particular section was re-evaluated and one consensus score was given. The cartilaginous tidemark is the neuralgic point where splitting of the cartilaginous layers is predestined to occur, as described by Fawns and Landells (134). Therefore, sharp separations in this area were not considered to be artefacts, but as damage that was present *in vivo*; consequently, scoring grade "3" was assigned in this case. If any lesion seemed to be of questionable genuineness, the neighbouring sections on the same slide were used for clarification. Sections from the cruciate ligament zone (without cartilaginous surface) were excluded from analysis. Approximately 8 levels per joint were assessed. Owing to individual anatomical and sex-related differences in joint size and width, the exact number of assessable sections per joint differed slightly.

Calculations were based on modifications from the OARSI histopathology initiative and their "*recommendations for histological assessments of osteoarthritis in the mouse*", as described by Glasson et al. (135). Accordingly, a maximum score and an average score for the right joint of each mouse was calculated and these values were used for the comparison of experimental groups. The maximum score of each joint represents the arithmetic mean of the worst score noted for the femoral side and the worst score noted for the tibial side. In this way, the maximum score differentiates between joints with one-sided (only tibial or femoral) or two-sided damage (both tibial and femoral) and allows more accurate distinction of joint degeneration.

The average score is calculated from the sum of all single scores (femoral and tibial) divided by the number of all given scores (femoral and tibial). Whereas the maximum score correlates with the deepest observed damage of a joint only, the average score represents levels from the entire joint and thus also gives an idea of the affected area and overall extent of damage.

Figure 6 on the next page illustrates how single grades from a joint were principally recorded and how an overall maximum and average score was calculated from the original values.



Joint level	1	2	3	4	5	6	7	...	n
Score Femur	f_1	f_2	f_3	f_4	f_5	f_6	f_7	...	f_n
Score Tibia	t_1	t_2	t_3	t_4	t_5	t_6	t_7	...	t_n

Calculations (based on OARSI recommendations[135])

Maximum score $= \frac{\text{worst femoral score } (f_1 \ldots f_n) + \text{worst tibial score } (t_1 \ldots t_n)}{2}$

Average score $= \frac{\text{sum of all femoral scores } (f_1+f_2+\cdots+f_n)+\text{sum of all tibial scores } (t_1+t_2+\cdots+t_n)}{\text{number of femoral scores (n)}+\text{number of tibial scores (n)}}$

Figure 6: Diagram of cartilage erosion scoring. Femoral and tibial scores from on average 8 joint levels were used to calculate a maximum and an average score for each knee joint, based on modified OARSI recommendations by Glasson et al. (135). Further description in the text above. Knee image adapted and reprinted with permission from Rodríguez Hidalgo under the Creative Commons Attribution-Share Alike 4.0 International license (136).

3.6.2 Measurement of the ratio of non-calcified to calcified cartilage

Since calcification of articular cartilage is subject to osteoarthritic or age-related processes (47), the ratio of non-calcified cartilage (synonym, hyaline cartilage; abbreviation, HC) to calcified cartilage (CC) can vary. Therefore, these two histological layers were evaluated by histomorphometric analysis. A representative section of the central medial compartment of the tibial articular cartilage was chosen for measurement. Instead of width measurement which only allows acquisition of HC/CC ratios at a certain location, the central area of each layer of articular cartilage was measured at 10 times magnification (Figure 7). A virtual pen was used to trace the respective areas on a computer display (OsteoMasure XP software). Based on those measured values, the HC/CC ratio was calculated and interpreted with regard to potential meaningfulness in OA or aging.

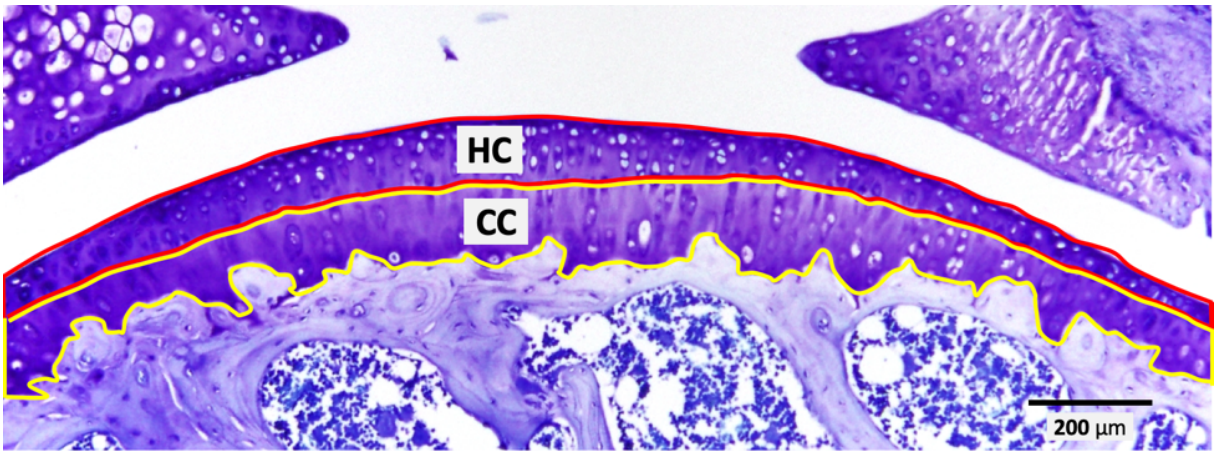


Figure 7: Diagram of histomorphometric analysis of cartilage layers by tracing the respective areas. HC = hyaline cartilage, CC = calcified cartilage. TB staining. Magnification 10x.

3.6.3 Chondrocyte hypertrophy

The occurrence of collagen type X (marker of chondrocyte hypertrophy) was investigated using IHC. As chondrocyte hypertrophy can potentially be accompanied by synthesis of degradative enzymes like MMPs (48), structurally affected and unaffected sections from male WT and KO mice were differentiated to possibly relate chondrocyte hypertrophy to concomitant cartilage erosion. Accordingly, one structurally unaffected and one affected section per right knee sample was picked, based on the previously applied cartilage erosion score of TB stained sections. Only tibial sides were used and the chondrocytes were analysed at 20x magnification (Figure 8). The relative numbers of chondrocytes in both cartilage layers displaying collagen type X were noted and statistically compared for the various experimental groups. Counting was conducted manually with the help of OsteoMeasure software.

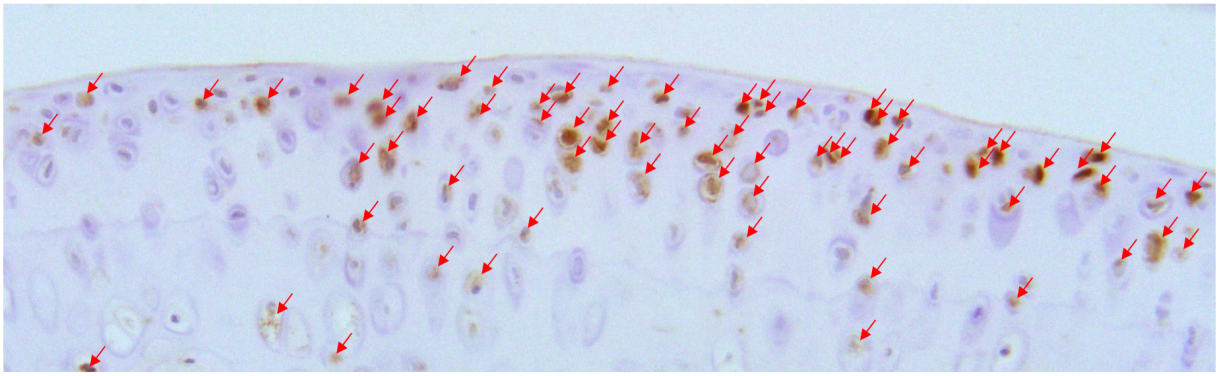


Figure 8: Collagen type X-positive chondrocytes (indicated by red arrows) were identified and counted manually. The ratio of positive chondrocytes to total number of chondrocytes was calculated. Harris hematoxylin counterstain. Magnification 20x.

3.6.4 Histomorphometric analysis of subchondral bone

Histomorphometric analysis of tibial subchondral bone was conducted for bone volume/tissue volume measurement. The nomenclature was based on the recommendations of the American Society of Bone and Mineral Research, where the ratio of cancellous bone volume (BV) and tissue volume (TV) is abbreviated as BV/TV (137).

Two consistent levels (distance from each other approximately 90 µm) from the centre of the medial joint half and one consistent level from the centre of the lateral joint half of each right knee joint were measured to acquire representative values of subchondral bone volume. The two values from the medial compartment were averaged. The region of interest (ROI) was defined as the subchondral bone covered by articular cartilage (or remnants) superiorly and limited by the growth plate inferiorly. Any peripheral osteophyte formation was not taken into account. Figure 9 demonstrates the way measurement was performed manually by means of several small rectangles to cover the entire ROI perfectly. Each rectangle was magnified separately, areas were circumscribed with a virtual pen and the summed up data was analysed afterwards.

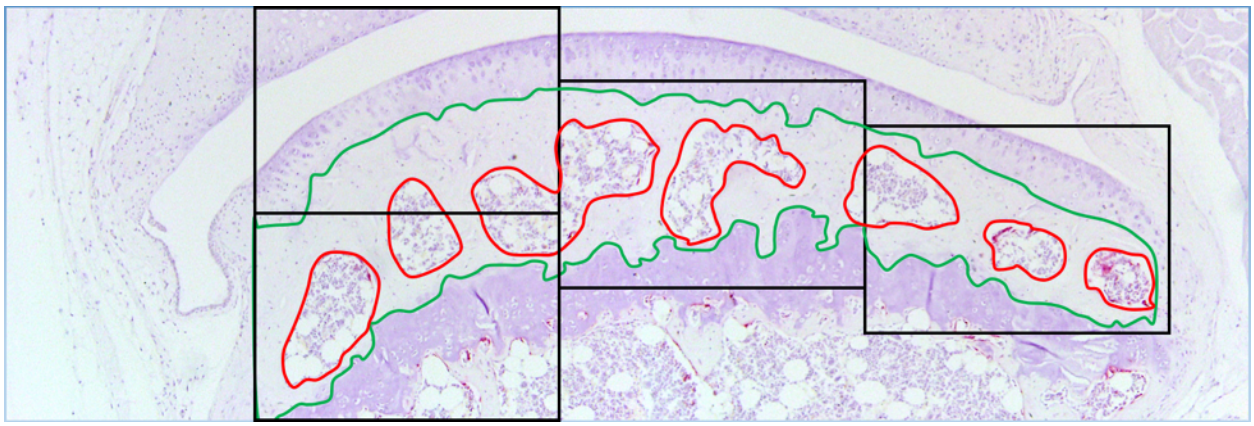


Figure 9: Diagram of histomorphometric analysis of subchondral bone. The black rectangles represent microscopic units of measurement to cover the entire region of interest between articular cartilage and the growth plate. The green perimeter encloses TV which is thus directly measured, while the red perimeter surrounds volume of bone marrow or cysts for the indirect calculation of BV (BV = TV - marrow or cyst volume). TRAP staining, composed image.

3.6.5 Histological analysis of osteophyte formation

Osteophyte formation was evaluated on toluidine blue stained sections, since this type of staining allows histological identification of osteophytes next to scoring of cartilage erosion. Accordingly, the slides which had already been used for erosion scoring were also scanned for osteophyte formation. Particular attention was payed to the joint margins where osteophytes are predisposed to occur. However, osteophytes were only found in one mouse. All other investigated samples displayed no osteophyte formation.

3.6.6 Histological analysis of synovitis

H&E stained sections were used for the evaluation of inflammatory signs, such as infiltration of inflammatory cells, soft tissue edema or synovial lining cell hyperplasia (85). Between 2 and 4 different joint levels of each mouse (always including slides adjacent to structurally affected and unaffected levels) were checked at 4 and 10 times magnification. There were no examined sections which revealed histological signs of inflammation.

3.7 Statistical analysis

Statistical analysis of entire data was conducted with GraphPad Prism, Version 5.02. Groups were compared using the Mann-Whitney U test for non-normally distributed values. Owing to the explorative character of this research project, significance level was defined as $p < 0.05$ and all p values confirm explorative data only. The significance level was not adjusted for multiple testing. In case of statistical significance, p values are expressed mathematically rounded to three decimal positions. All data in charts are displayed as box-and-whisker plots following Tukey's convention. Potential outliers were double-checked with regard to correctness of measurement and methodological implications. Unless stated otherwise, the group size (n) in each calculation refers to the experimental numbers declared in Table 3 in Chapter 3.3 (experimental design) on page 37.

4 RESULTS

4.1 Efficacy of glucocorticoid receptor knockout

To confirm that the glucocorticoid receptor is successfully and specifically deleted in chondrocytes (124), immunohistochemistry was performed on the knee joints of 1-year-old mice. Detection of GR was positive in WT mice samples. In young mice, approximately 90 % of articular chondrocytes expressed the GR protein (124). We observed reduced percentages (approximately 50 %) of GR positive chondrocytes in our 1-year-old WT mice (see Figure 10/GR WT). This can be attributed to the decline of GR levels in degenerating cartilage (138) and also in apoptotic chondrocytes that have to be expected in structurally unviolated regions of cartilage of 1-year-old mice (75).

Absence of GR and thus successful knockout was proved in KO mice. There were only isolated chondrocytes with GR positivity (compare Figure 10, GR KO), which is in line with previous studies and typical of this model (124).

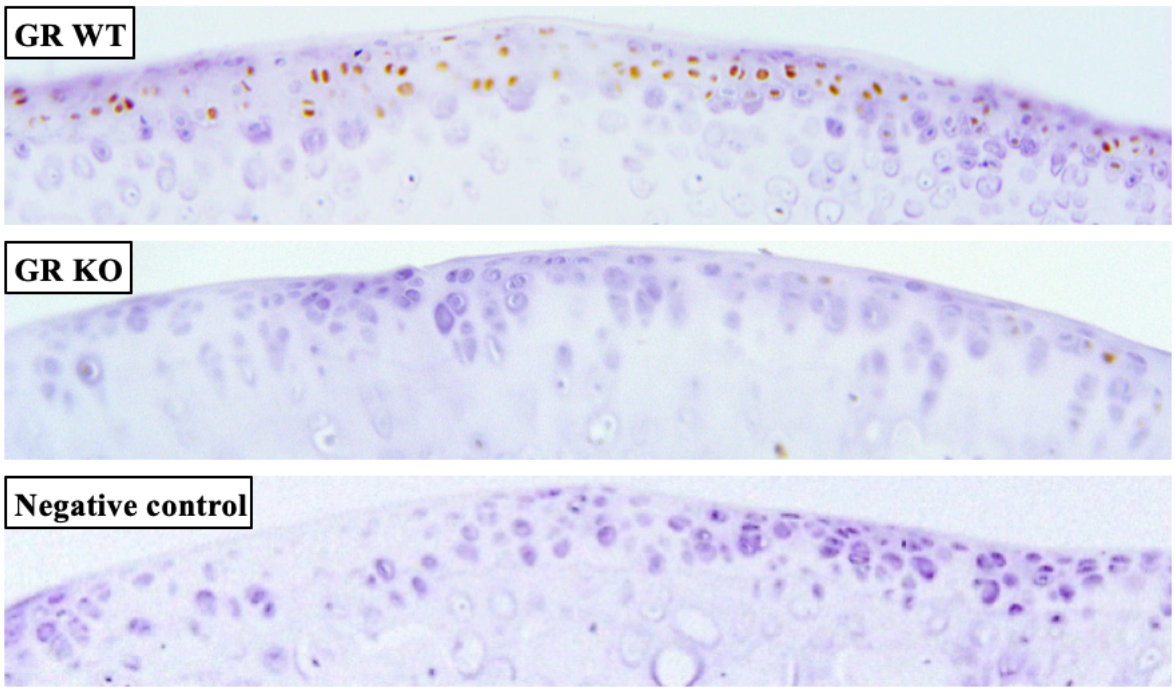


Figure 10: Detection of GR in chondrocytes of articular cartilage in 1-year-old GR WT vs. GR KO mice via IHC. Negative control sections showed no false-positive staining. Samples were counterstained with Harris hematoxylin. Magnification 20x.

4.2 Body weights of the experimental mice

In order to consider body weight as a potential confounder for the development of OA, mice were weighed on the day of harvesting. Unfortunately, the body weight data of 7 male mice (2 WT, 5 KO), with 27 male mice harvested in total, was not recorded prior to my analysis. Thus, the numbers of male WT and male KO for body weight analysis shown here were reduced to 11 and 9, respectively. Figure 11 shows that there was no statistically significant difference between WT and KO mice of the same sex ($p = 0.224$ for males, $p = 0.190$ for females). However, male WT mice were significantly heavier than their female counterparts ($p = 0.0003$).

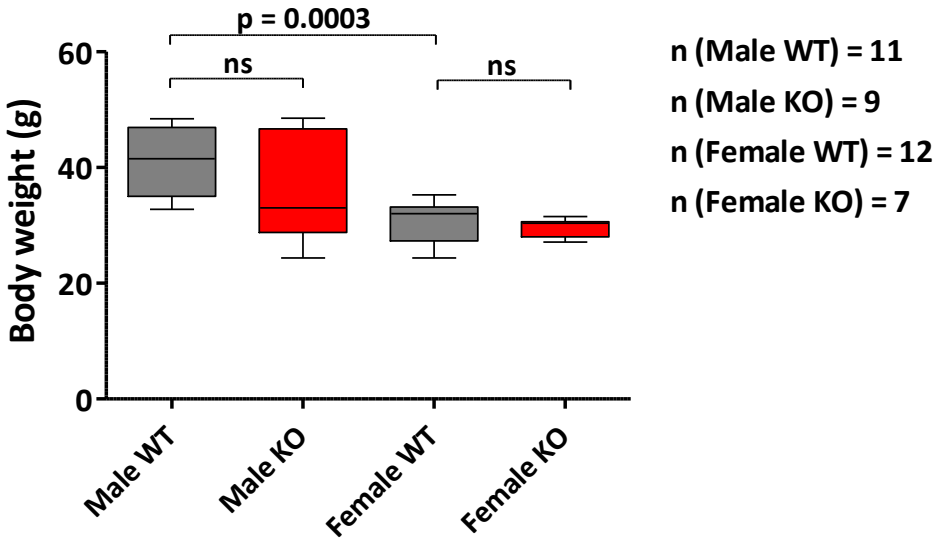


Figure 11: Body weight (g) on day of harvesting in the various experimental groups. Ns = not significant ($p \geq 0.05$). Data shown here are incomplete, which accounts for altered group sizes.

4.3 Cartilage erosion

Articular cartilage erosion was scored to look for signs of pathological changes which may be attributable to OA. The different experimental groups displayed a high prevalence of beginning structural cartilage erosion, ranging from 79 % to 100 %. A mouse was counted positive if at least one joint level from this mouse (from on average 8 assessed joint levels per mouse) exhibited a degree of cartilage erosion > 0. The exact number of mice with at least one positively graded joint level per group can be viewed in Table 5. The highest prevalence was found in male GR WT and female GR KO mice (all mice from these groups had developed some kind of cartilage erosion), while the lowest prevalence was found in male GR KO mice (11 of 14 male GR KO mice had developed cartilage erosion in at least one joint level).

Genotype \ Sex	♂	♀
GR WT	13/13 (100.0 %)	11/12 (91.7 %)
GR KO	11/14 (78.6 %)	7/7 (100.0 %)

Table 5: Ratio of mice with at least one positively scored joint level from on average 8 assessed joint levels per mouse, separated for the experimental groups (positively scored mice/total number of mice in the group). Calculated prevalence (in %) is provided in brackets.

To convey an impression of the investigated pictures and the mild degree of damage that had occurred despite the high group prevalence, Figure 12 provides representative images. In the following, scoring results are presented as maximum score (Figure 13A) and average score (Figure 13B), which were described previously in the methods section (Chapter 3.6.1).

Looking at the maximum score (Figure 13A), significantly more severe erosion was observed in male WT in comparison to male KO mice ($p = 0.027$). There was one extreme outlier mouse among the male WT group which exhibited complete erosion down to subchondral bone, being graded with a maximum score of "4". If this outlier mouse was excluded from statistical analysis, the p value between WT and KO mice would still have been 0.049. Additionally, male WT mice showed more pronounced damage than their female counterparts ($p = 0.043$). On the other hand, a significant difference between the two genotypes of female mice could not be found ($p = 0.114$), although there was a trend towards the opposite direction with a higher maximum score in female KO mice.

The observation of more intense cartilage erosion in male WT vs. male KO mice was also confirmed by the average score (Figure 13B). The P-value was 0.031 when the outlier mouse was included, but 0.054 without it. No statistically significant difference was found between the male WT and female WT group when the average score was applied ($p = 0.068$). Consistent with the maximum score, there was no genotype difference within the female groups ($p = 0.099$). Apart from enabling comparison between different groups, the average score allowed for a general overview of the mean grade of cartilage erosion. The median of all experimental groups was below 0.5 and the arithmetic mean smaller than 0.6. Consequently, almost all mice had only developed rather mild cartilage erosion.

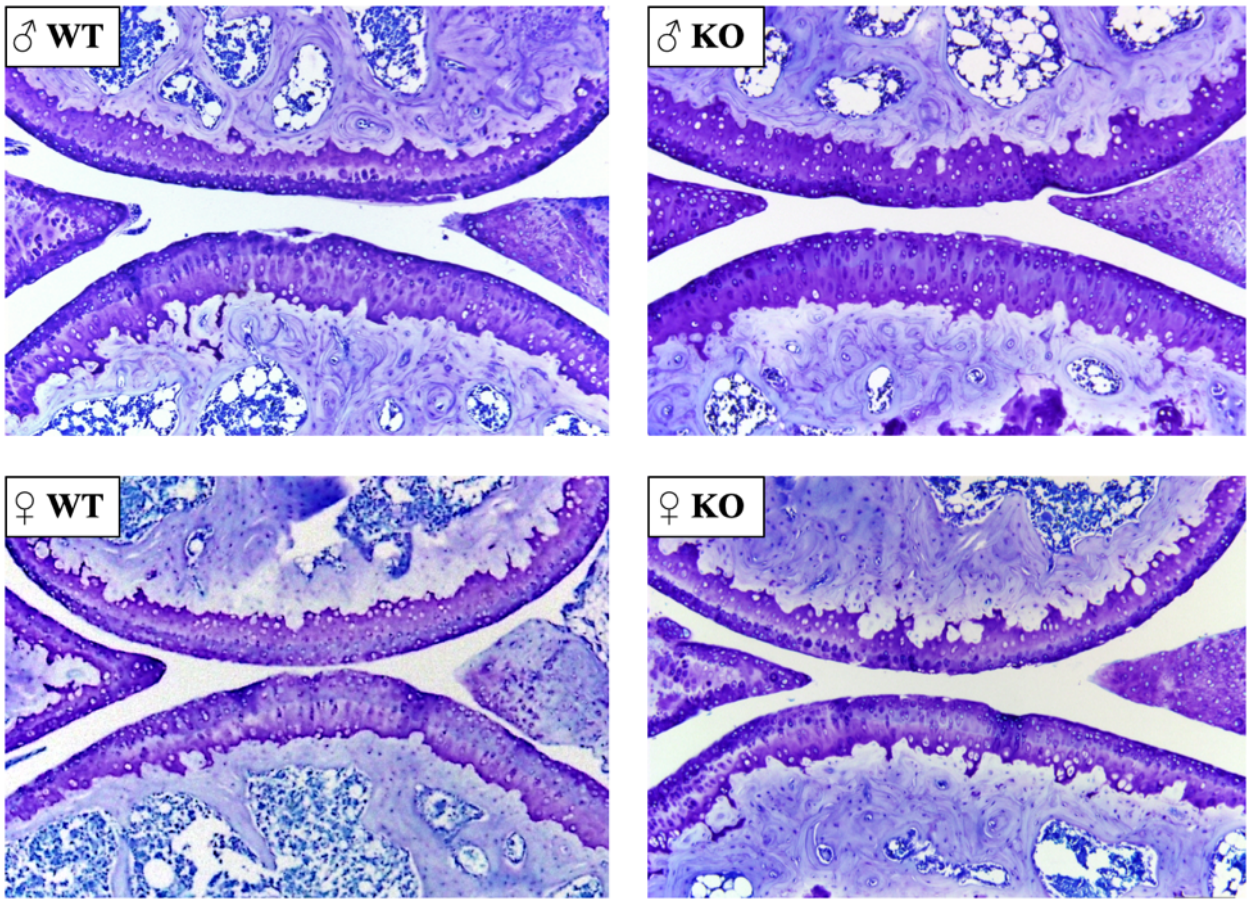


Figure 12: Representative images of cartilage erosion in correlation to mean scores of the respective experimental mouse group (approximatively). TB staining. Magnification 10x.

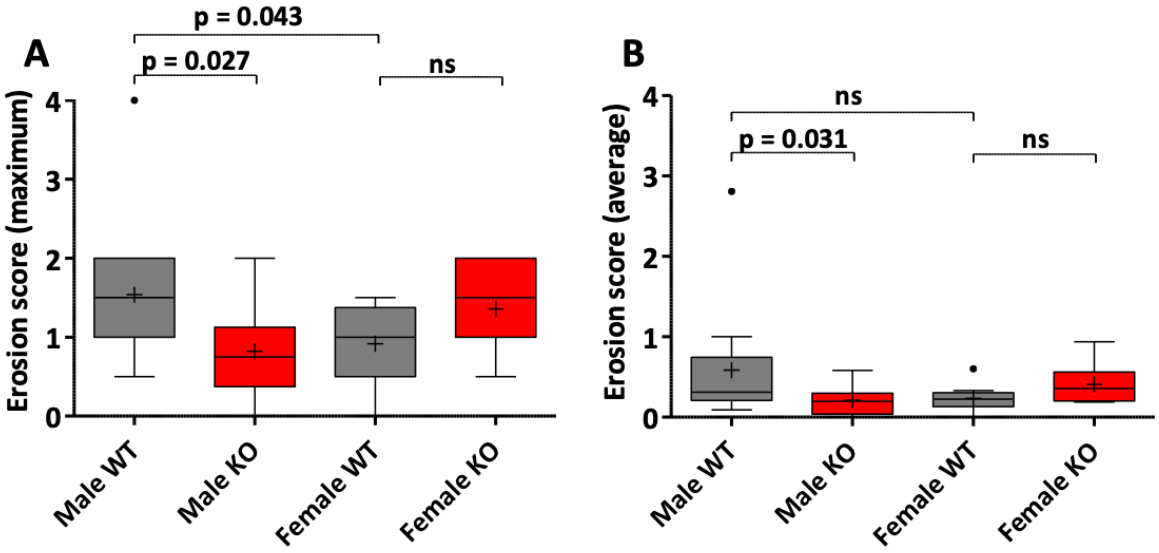


Figure 13: Cartilage erosion score results based on maximum (A) or average (B) approach. Arithmetic means are shown as "+" symbols. Group sizes as described (male WT 13, male KO 14, female WT 12, female KO 7).

4.4 Histomorphometric analysis of cartilage layers

On basis of the significant difference in cartilage erosion between male WT and male KO mice, the ratio of hyaline to calcified cartilage area (HC/CC ratio) in these groups was measured using histomorphometric analysis in the medial compartment of the right knee joints. This was done to identify pathological calcification processes within cartilage layers.

The HC/CC ratio can decline for two reasons: either on account of aging (age-associated calcification) or on account of degenerative processes (OA-associated calcification) (47). In order to investigate if age-associated calcification had taken place in our old experimental mice, younger 12-week-old male WT mice from a previous project (124) were included and compared to the old male mice with regard to HC/CC ratio. Statistical analysis revealed no significant difference between young male WT and old male WT mice ($p = 0.442$), nor between young male WT and old male KO mice ($p = 0.396$). Hence, there was no significant age-associated calcification in the 1-year-old mice compared to 12-week-old mice.

Furthermore, old male WT mice were compared to old male KO mice to see if the higher scores of cartilage erosion in old male WT mice were accompanied by pathological, degeneration-associated calcification. Since the previously mentioned “outlier mouse” in the male WT group had no cartilage tissue left in the medial compartment that could possibly be measured, it had to be excluded from analysis. Therefore, the size of the male WT group is reduced to $n = 12$ in the present statistics. Direct comparison between male WT and male KO mice showed no significant difference in their distribution ($p = 0.939$, Figure 15). Consequently, old male WT mice revealed no increased percentage of cartilage calcification in comparison to their KO littermates. Commensurate with absent calcification, we also did not note any histologically visible tidemark duplication.

The corresponding images and results of both the age-related and genotype-related comparisons are provided in Figures 14 and 15 on the next page.

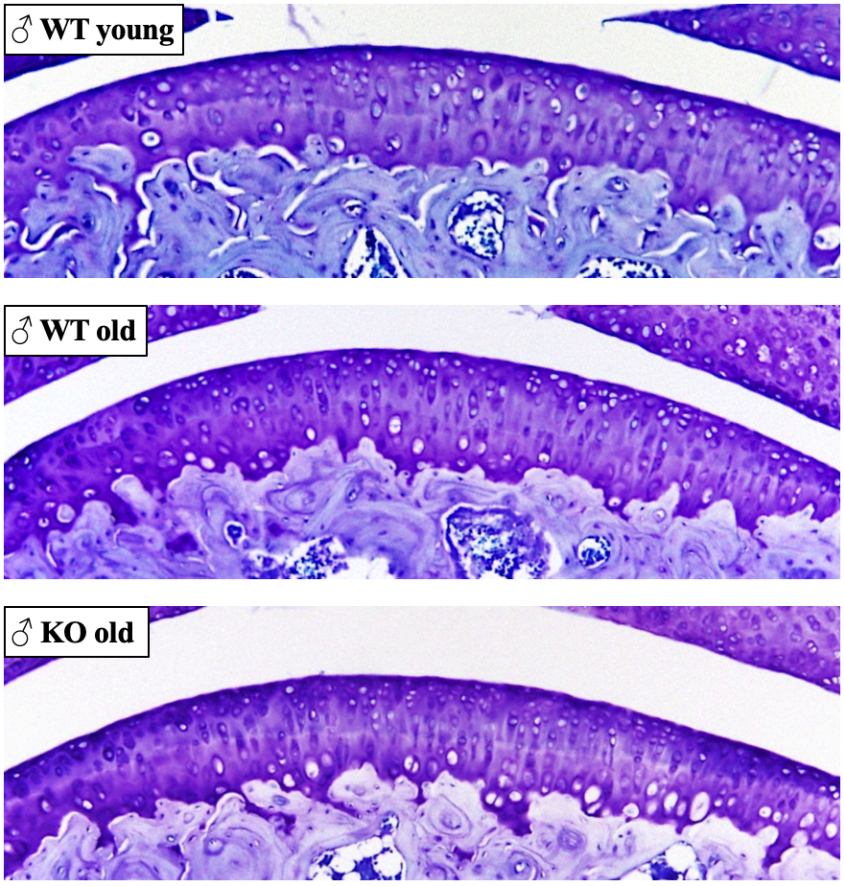


Figure 14: Representative images of cartilage layers used for HC/CC measurement, commensurate with mean scores of the respective experimental group (young male WT, old male WT and KO). HC and CC layers are clearly distinguishable. TB staining. Magnification 10x.

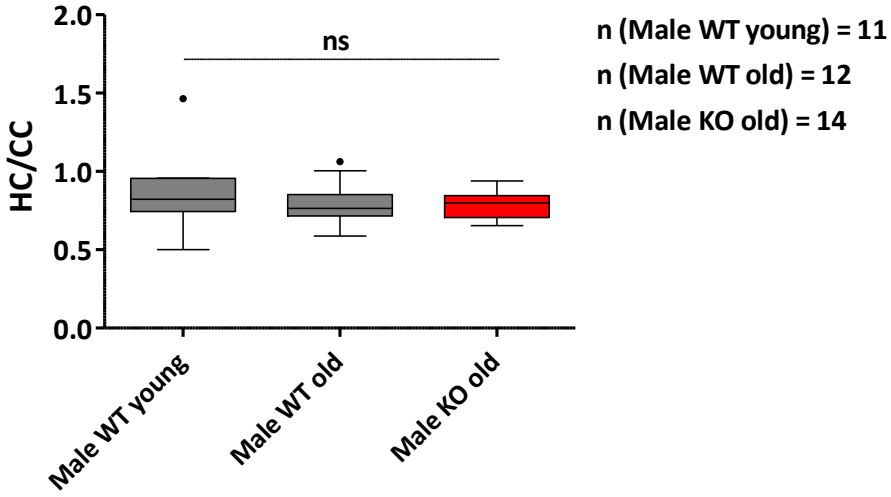


Figure 15: Ratio of hyaline cartilage layer to calcified cartilage layer (derived from area measurement). Young male WT (12 weeks), old male WT and old male KO (1 year) were compared. The statistics revealed no significant difference between groups with regard to HC/CC ratio.

4.5 Chondrocyte hypertrophy

In addition to cartilage erosion scores and histomorphometric analysis of the HC/CC ratio, chondrocyte hypertrophy was measured to identify a different sign of cartilage degeneration. Expression of collagen type X, detected through IHC, served as a marker of chondrocyte hypertrophy. As reasoned in Chapter 4.4, only male WT and KO mice were included. Since three male KO mice had shown no signs of cartilage erosion at all, i.e. all sections from these mice were unaffected, they were excluded from collagen type X analysis, as a comparison of structurally affected and unaffected sections from the same mice was intended. Therefore, the group size of KO mice in chondrocyte hypertrophy analysis was reduced to n = 11.

Representative images of collagen type X in chondrocytes are presented in Figure 16 on the next page. The obtained results can be studied in Figure 17. Collagen type X was mainly detected in and around chondrocytes from the hyaline, but also from the calcified cartilage layer, which is consistent with previous observations (131). In addition, a fine brownish line at the articular surface was noted, especially in the proximity of superficial fibrillations, which is in line with other researchers (139). Chondrocyte hypertrophy was found both in microscopically unaffected and affected sections, with an expectedly higher amount of positive cells in sections with features of cartilage erosion, but no significant p-value comparing unaffected and affected sections of the same genotype ($p = 0.505$ considering unaffected and affected male WT mice, $p = 0.057$ comparing unaffected and affected male KO mice). Although the average percentage of collagen type X positive cells was slightly higher in WT than in KO mice (24.2 % in unaffected WT vs. 17.2 % in unaffected KO sections, 31.4 % in affected WT vs. 26.5 % in affected KO sections), no statistically significant difference was observed for the respective subgroups ($p = 0.271$ comparing unaffected WT and unaffected KO sections, $p = 0.685$ comparing affected WT and affected KO sections). In general, the percentage of chondrocyte hypertrophy seemed to be variable within the experimental groups and did not clearly correlate with cartilage erosion status. The negative control for IHC showed no false-positive staining.

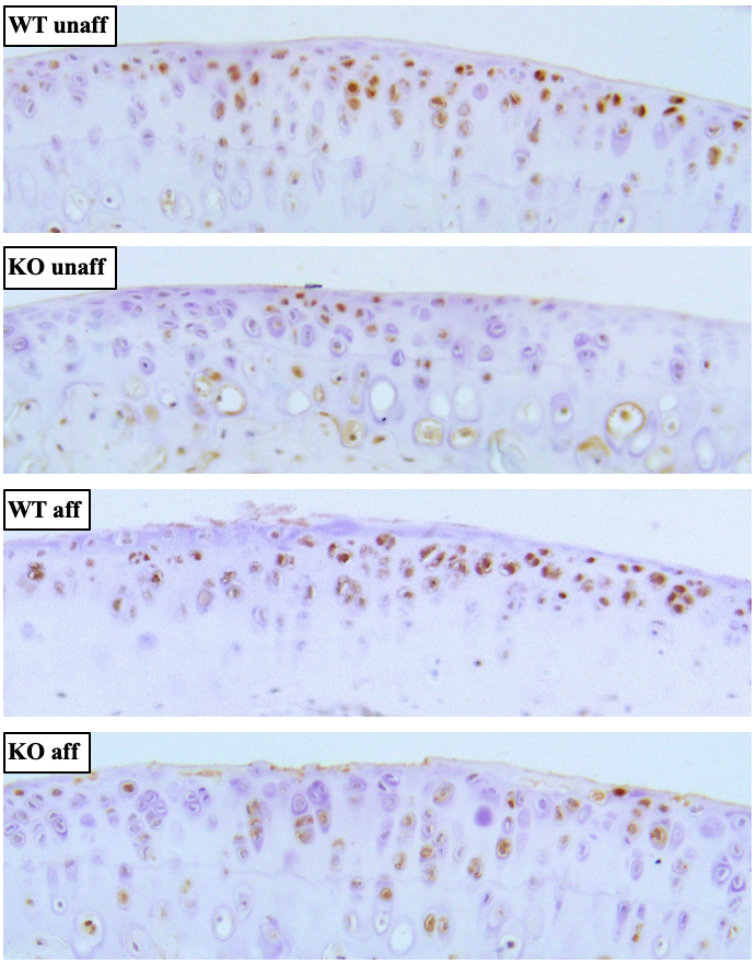


Figure 16: Detection of collagen type X in chondrocytes via IHC in different experimental groups. The affected sections show signs of superficial cartilage erosion. Abbreviations: unaff = unaffected, aff = affected. Harris hematoxylin counterstain. Magnification 20x.

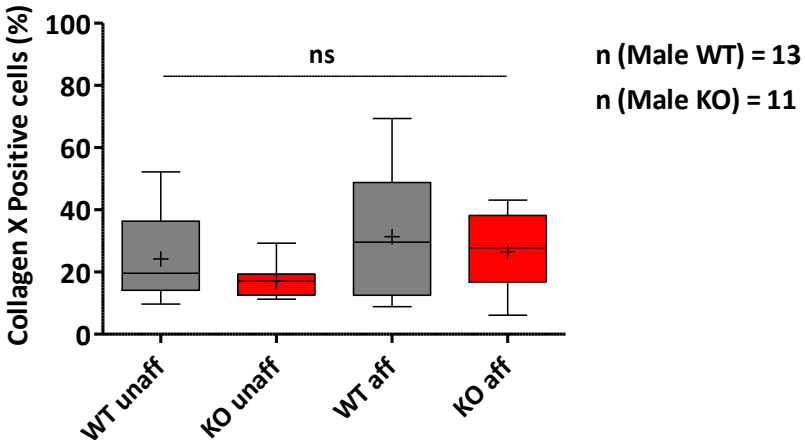


Figure 17: Percentage of chondrocytes in articular cartilage expressing collagen type X in unaffected and affected sections (as defined by cartilage erosion) of male WT and male KO mice. Arithmetic means shown as "+" symbols. Abbreviations: unaff = unaffected, aff = affected.

4.6 Histomorphometric analysis of subchondral bone

Apart from cartilage degradation, subchondral bone sclerosis or cysts belong to the main features of OA (140). Hence, histomorphometric analysis of subchondral bone was undertaken. There is debate about the natural time course of occurring OA features (1). Since the sequence of pathological mechanisms is not clearly defined and early changes can take place both in cartilage and subchondral bone (1), male and female mice were included in the following measurement.

The central medial and central lateral joint compartment were evaluated separately for bone volume measurement, as shown in Figure 18. Statistical analysis of the medial half (Figure 18A) did not reveal any significant difference between male WT and KO mice ($p = 0.544$), or between female WT and KO mice ($p = 0.057$). Female mice exhibited higher BV/TV in comparison to their male counterparts ($p = 0.002$ between male WT and female WT mice). The same constellation of results was confirmed in the lateral compartment (Figure 18B), where BV/TV was generally lower than in the medial part. No statistically significant difference was found with regard to genotypes of the same sex ($p = 0.865$ in male mice, $p = 0.767$ in female mice), but female mice showed higher BV/TV values than male mice ($p = 0.021$ between male WT and female WT mice).

Due to the absence of visible sclerosis or significant discrepancies in BV/TV between genotypes, analysis of bone formation (osteoblasts) and bone resorption (osteoclasts) was not conducted. Furthermore, the measured BV/TV ratio did not correlate with the observed cartilage erosion score. The "outlier mouse" with complete cartilage erosion down to the level of subchondral bone was the only mouse with obvious sclerosis; an image of this extraordinary finding is provided in Figure 19 in the following chapter (4.7).

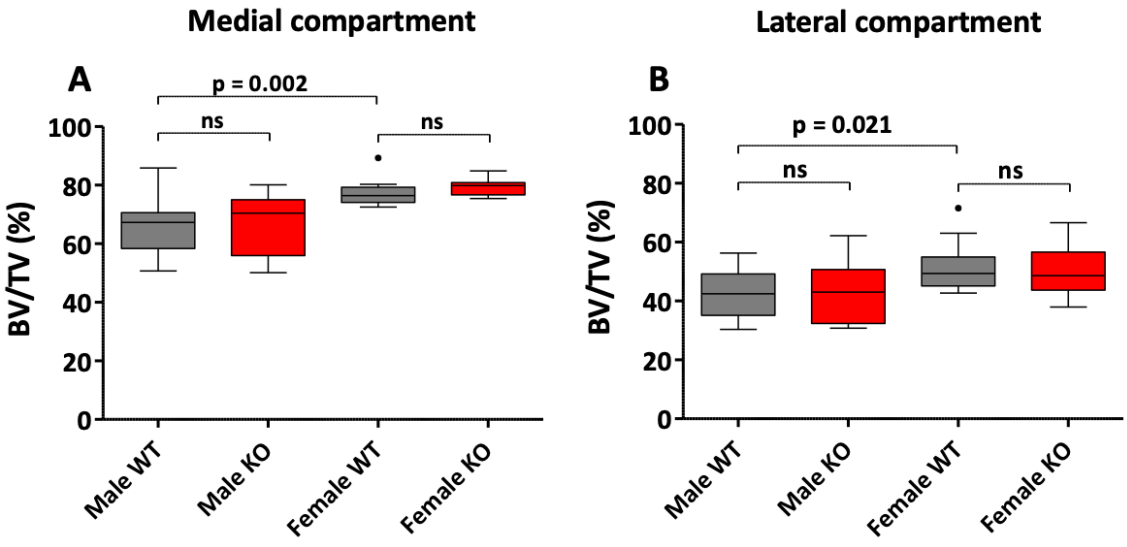


Figure 18: Ratio of bone volume to tissue volume (in %) in the medial (A) and lateral (B) tibial subchondral bone of all experimental groups.

4.7 Osteophyte formation

Formation of osteophytes was absent in all investigated sections and mice, apart from the single male WT mouse exhibiting severe signs of cartilage erosion, which has previously been mentioned several times. A picture of the outlier mouse and its osteophyte can be found below this paragraph (Figure 19). Not only osteophyte formation, but also severe cartilage erosion, subchondral bone sclerosis and fibrillated or ruptured menisci are present in this knee joint. Consequently, it was the only mouse characterised by all classical histological features of OA. To investigate whether this pathology was just a unilateral phenomenon, the left leg was cut in addition, displaying a very similar degree of damage. The structure of the osteophyte, which started to develop at the posterior part of the medial tibial plateau, is marked with a yellow broken line. Its maturity can probably be classified as medium, since the tissue is composed of both cartilaginous and bony elements as a result of pathological endochondral ossification.

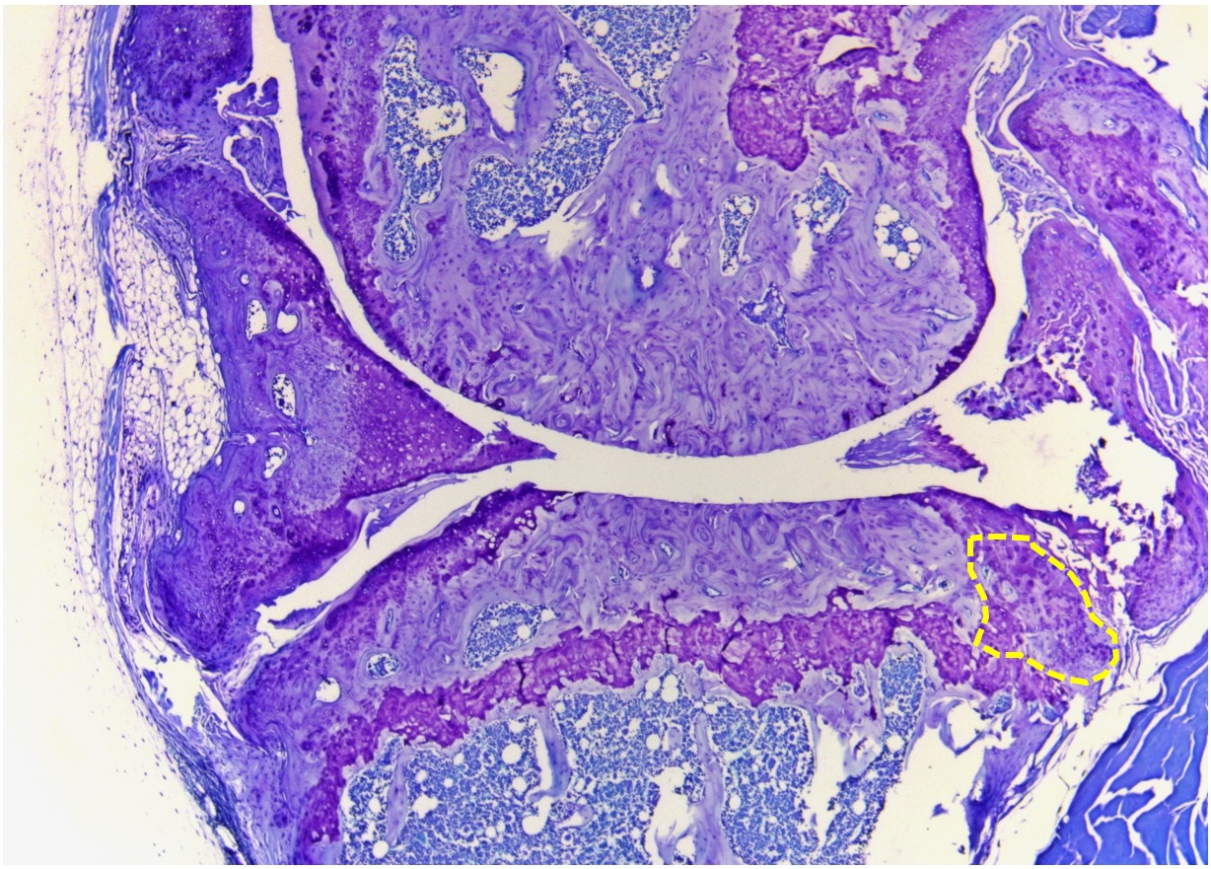


Figure 19: Osteophyte formation on the edge of the tibial plateau (yellow broken line). Apart from osteophyte formation, the knee joint also exhibits other classical signs of advanced OA: high-grade cartilage erosion, subchondral bone sclerosis and damaged menisci. TB stain. Magnification 4x.

4.8 Inflammation

Hardly any microscopic signs of synovitis were found in all experimental groups. Not even the above-mentioned mouse with severe joint degeneration displayed inflammation. Consequently, processes which could be assigned to activated OA were ruled out at the time of observation. Proof of evidence for the absence of inflammation in the OA-affected knee joint is provided in Figure 20.

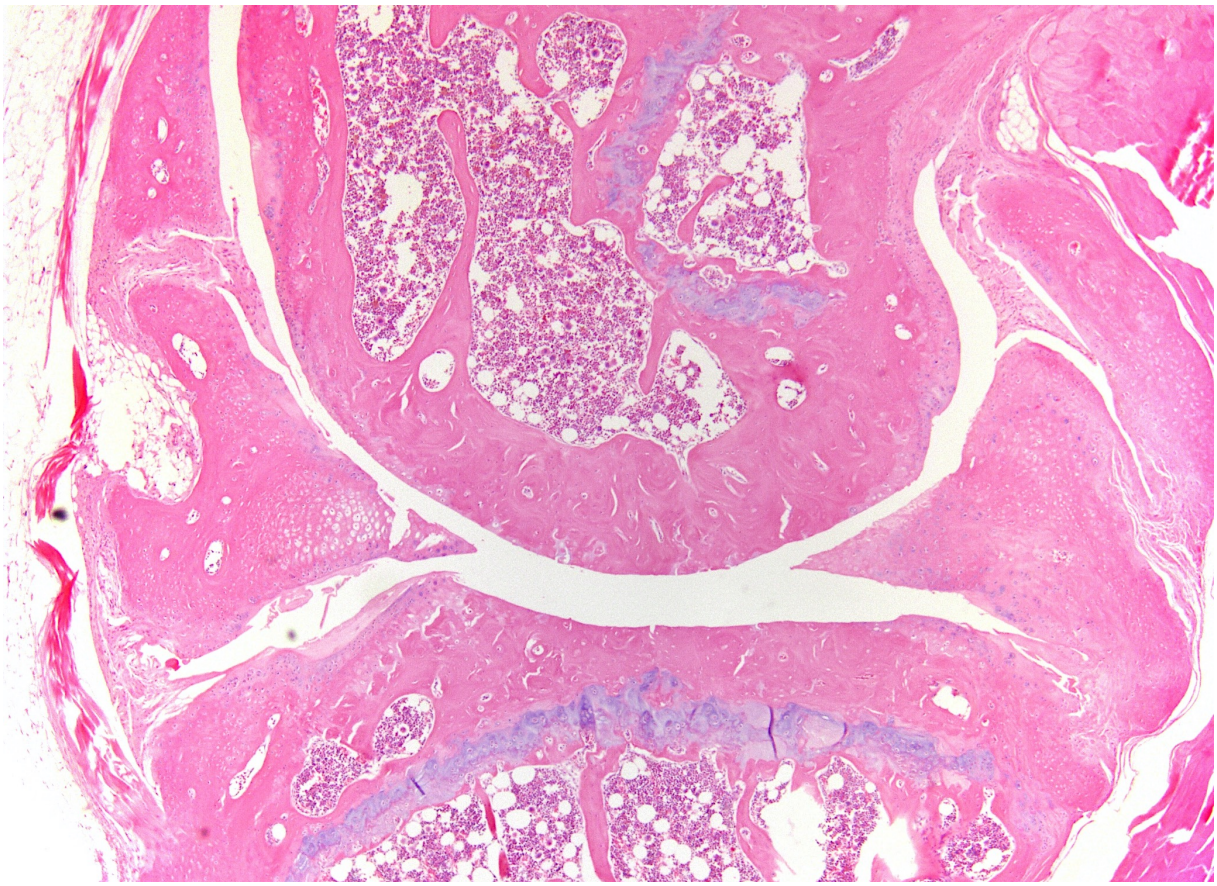


Figure 20: Absence of microscopic indicators of inflammation in the mouse with pronounced features of OA. H&E stain. Magnification 4x.

5 DISCUSSION

5.1 Discussion of experimental design

The idea of the present thesis was to study the impact of chondrocyte-specific endogenous GC signalling on articular cartilage degeneration in mice of advanced age, which were thereby prone to development of primary (idiopathic) OA. To our knowledge, the direct effects of endogenous GCs on cartilaginous degeneration have not been studied before. For this reason, we designed an OA experiment with aging as the driving disease force and investigated the different phenotypes of mice with intact vs. disrupted chondrocytic GC signalling (GR WT vs. GR KO mice).

5.1.1 Aetiological factors of osteoarthritis apart from aging

Since OA has a multifactorial disease aetiology (1), and because we intentionally wanted to focus on spontaneously developed joint degeneration, we tried to minimize the influence of other aetiological aspects of OA by ensuring homogenous conditions in all mouse subgroups. The aetiological factors which were taken into consideration prior to experimental setup and analysis consisted of 1) body weight/nutrition, 2) physical activity, 3) sex-related dimorphism and 4) genetical predisposition to OA.

First of all, the *ad libitum* food access of our experimental mice was not associated with significant body weight differences in GR WT vs. GR KO mice of the same sex (see Chapter 4.2). Accordingly, we were able to exclude body weight as a potential between-genotypes confounder of OA promotion (considering available body weight data only). Excessive body mass index is associated with increased odds of knee OA in humans (141). Similarly, studies conducted by Silberberg et al. about the role of obesity and food intake in murine OA came to conclusion that heavier mice are more susceptible to disease incidence compared to lighter animals receiving the identical diet and, more importantly, that the nutritional composition (namely the ratio of saturated fats) influences the risk of knee OA onset (142, 143). Since all of our mice received the same standard diet, no between-group differences on account of nutrition were to be expected.

Secondly, all experimental animals were kept in normal-sized cages and free movement was not restricted or supported by any means. This is important as encouraged running can promote the development of OA in mice (144). The cage size in our experiment was standardised for all mice. Each mouse was granted between 85 and 170 cm^2 of the base area. However, the natural activity level was not monitored, so we cannot totally exclude any inter-animal variation in spontaneous movement that might have led to individual or group-specific differences of cartilage damage.

Thirdly, we intentionally included animals of both sexes and clearly distinguished their outcomes. Male mice have been known for a long time to be more predisposed to spontaneous OA than their female counterparts (145, 146). Ma et al. investigated the impact of sexual hormones in a DMM model of OA and observed the same constellation, noticing the protective effects of ovarian hormones and detrimental actions of male dihydrotestosterone (147). Furthermore, as mentioned earlier, sexual hormones are suspected to interfere with GR expression or GR signalling, for instance through sex-dependent co-regulators (148). This made it desirable to examine the role of endogenous GCs separately for males and females. With a maximum age of 13.8 months, female mice in our experiment were still in their pre-menopausal life phase, as the murine menopause sets in between 17 and 18 months of age (149). Hence, inconsistencies owing to differences in the female sexual hormone status were not to be anticipated.

Last but not least, any experimental constellation involving mice requires thoughtful choice of the appropriate mouse strain, since not all strains develop OA spontaneously and some are genetically more prone to joint degeneration than others (150). C57BL/6 mice, the mice we utilized, are among the strains which develop knee OA unforcedly with age (146). More importantly, we relied on this particular mouse strain as the chondrocyte-specific GR KO modification has so far only been established in C57BL/6 mice. Post-natal GR knockout induction in this strain does not influence the subsequent growth phase and results in identical chondroskeletal phenotypes of young GR WT and GR KO mice (124). Hence, any inborn mechanoskeletal predispositions to joint degeneration, such as malalignment or malformation, are unlikely to bias primary OA studies in these mice.

5.1.2 Age-related mouse models for the study of osteoarthritis

Conducting research on age-related OA by means of C57BL/6 mice offers numerous advantages, but also some disadvantages. Age-related “induction” of joint degeneration is presumably the closest imitation of primary (idiopathic) OA in human beings due to its gradual progression, polygenic origin and independence of (macro-)injury (140, 151). This is the major advantage of naturally occurring OA models in comparison to artificial OA induction techniques, namely surgical, chemical, mechanical or genetical methods (including combinations of these methods) (140). Many invasive induction techniques, for instance the surgical DMM model or chemical collagenase-induced OA (CiOA) model, mimic post-traumatic disease aetiologies which develop within a shorter period of time and follow a different pathophysiological pattern. Instable mechanics lead to highly accelerated joint destruction, therefore these models are more suitable for studying advanced disease stages (151). The slow progress of spontaneously developed OA, in contrast, allowed us to investigate the impact of endogenous GCs in earlier disease stages. Since

disruption of endogenous GC signalling in our experiment was targeted at chondrocytes, between-groups differences were primarily expected to manifest themselves in cartilage. Earlier disease stages (instead of end-stage OA with total or subtotal cartilage erosion) allowed us to compare cartilage layers morphologically and to measure additional cartilaginous parameters. However, predicting the age-dependent OA disease course in mice can be difficult. Inconsistency and reproducibility issues are an acknowledged disadvantage of spontaneous OA models (1, 140, 151). Not only is there remarkable interstrain dependency (152, 153), but also variability of disease onset in each particular mouse strain itself. After reviewing the literature with regard to spontaneous age-related OA in C57BL/6 mice, the discrepancy of OA prevalences becomes obvious. Wilhelmi et al. reported knee OA prevalences of 39 to 61 % in 17-month-old mice, whereas prevalence in animals at 15.5 months of age was only 19 %. In line with the observations in my thesis, female mice in Wilhelmi's study were characterised by lower OA prevalences than their male counterparts (146). Stoop et al. worked with 24-month-old mice and found distinct cartilage erosion in (only) 50 % of the samples (152), and other labs confirmed mild to moderate degrees of OA in 21-month-old mice (154). In view of these research results, the absence of OA-defining changes in our one-year-old mice appears less surprising. By contrast, other scientists reported manifest OA at the age of one year. A study by O'Conor et al., using a similar experimental design as our project, euthanised C57BL/6 mice at 12 months of age and observed advanced cartilage degeneration, synovitis and osteophyte formation (155) – signs which were mainly missing in our animals. In support of this earlier harvesting time point, a general recommendation by Poole et al. proposed to evaluate spontaneous murine OA at the age of 9 to 12 months, although this was not specified for the various mouse strains (156). As we ourselves had also noticed OA-defining alterations in a different batch of one-year-old C57BL/6 mice, the decision to euthanise mice after approximately one year in the present project was based on reasonable and valid arguments, being aware of the potential intra-strain variability at the same time.

Although age-related mouse OA models share many features with human age-related OA, it is clear that biomechanical conditions in mice are different from humans, not least on account of the quadrupedal posture and gait (151). Also, whereas the architecture of human cartilage is more complex, murine cartilage only displays two clearly distinguishable layers, of which the calcified proportion is higher than in other species (135). Nevertheless, mice are among the most commonly used and accepted animal species for *in vivo* research of OA (151).

5.2 Chondrocyte-specific glucocorticoid receptor knockout

Background Cre activity in absence of externally administered tamoxifen is a point for consideration when utilizing the Cre/LoxP system, since spontaneous Cre activity in Cre-ER^{T2} mice without tamoxifen injections has been reported (157). That is why tissue-targeted and tamoxifen-dependent expression of Cre recombinase was proven with the help of Col2a1-CreER^{T2};Rosa26R reporter mice and subsequent X-Gal staining during the establishing phase of our chondrocyte-specific glucocorticoid receptor knockout mouse line. Successful deletion of exon 3 of the GR gene in Col2a1-Cre$^{+/-}$ER^{T2};GR$^{flox/flox}$ mice after intraperitoneal administration of tamoxifen, detected by PCR, was shown to occur only in cartilaginous samples from spine (intervertebral discs), xiphoid and knee joints, but not in other organs or tissues (124).

The expected GR KO efficiency (ratio of GR-negative chondrocytes in GR KO mice) was confirmed to be roughly 90 % via IHC in my experiment, which reflects the corresponding Cre-recombination efficiency. Characteristic of this model, not all tamoxifen-treated chondrocytes express active Cre, presumably owing to subtotal penetration of tamoxifen especially into deeper cartilage layers (124, 158), hence the GR KO efficiency is usually below 100 % (124). GR WT samples, on the other hand, showed numerous GR-positive chondrocytes, but overall GR positivity was markedly lower in comparison to young GR WT cartilage samples which usually show more than 90 % GR positivity (124). The phenomenon of declined GR levels in degenerating chondrocytes was first described by DiBattista and colleagues in 1993. They assumed that impaired cellular gene expression at sites of lesions accounts for reduced GR synthesis (138). This assumption was more recently reinforced by Collins-Racie et al. in a wide-ranging analysis of the nuclear receptor superfamily expression in OA vs. healthy cartilage. Collins-Racie et al. discovered dysregulated gene transcription of multiple nuclear receptor proteins in OA cartilage, including GR, MR and ER. There was a significant decline in the expression of the above-mentioned (and many other) nuclear receptors (159). Even though cartilage samples from my project presented rather early signs of cartilaginous OA, the degenerative processes were apparently already influencing GR protein levels, as detected via IHC. Furthermore, apoptotic chondrocytes are to be expected in mice of approximately one year of age (75), which interferes with their normal protein expression profile (72). According to McNulty et al., apoptotic chondrocytes also occur in structurally unviolated regions of aged cartilage (75), which could explain why cartilage samples without clear erosion were characterised by diminished GR levels in my project.

Disruption of endogenous GC signalling aims at GR deletion and thereby genomic GC actions in the first place. After reconsidering alternative routes of GC-mediated effects, namely the non-

genomic GC actions, they were considered unlikely to play a role in our experiment. Following the proposed three-part classification system by Stahn et Buttgereit for non-genomic mechanisms (102), non-genomic actions of the cytosolic GR and of membrane-bound GRs, which are presumably splicing variants of the GR, are directly stopped by the created knockout at a translational level. Immediate effects on biological membranes are also very unlikely since they are only known to occur at high GC concentrations, classically after therapeutical interventions (102).

5.3 Discussion of methods and results

Histological assessment of the knee joints revealed beginning structural cartilage degeneration in 42 out of 46 one-year-old mice. The degree of erosion was relatively mild and rarely exceeded the non-calcified cartilage layer, and average erosion scores underlined the limited extent of damage. No bony changes or inflammatory infiltrates were observed in mice with this superficial dimension of structural cartilage lesions. Reconsidering the four major OA features (cartilage degeneration, subchondral bone sclerosis, osteophyte formation and synovitis (1)), we therefore preferred to speak of age-related cartilage degeneration instead of manifest OA.

The applied semi-quantitative cartilage erosion scoring scheme by Kamekura et al. (131) appeared to be best suitable for lesion assessment in our study, taking into account the mild degree of damage. It covered all degrees of existing erosion in our samples and consists of five qualitative categories, which is optimal for ordinally scaled data in terms of reproducibility and sensitivity (160). Unlike the popular and widely-used OARSI scoring system by Glasson et al. (135), Kamekura's concept does not rely on (potentially imprecise) percentage estimation of the affected articular surface. Furthermore, the Glasson system especially differentiates erosion degrees at the level of calcified cartilage, which would not have been useful for our samples since vertical clefts down to this layer were rarely seen. Another common alternative scoring system to Kamekura was introduced by Mankin et al., which is known as the Histological-Histochemical Grading System (161). Similar to Glasson's grading scheme, the Mankin system is especially weak when it comes to differentiating mild and moderate lesions, causing overestimation and underestimation of the pathological severity, respectively (162). On a further note, Mankin's system was originally invented to evaluate human OA samples and transferred to rodents later, but the architecture of murine cartilage differs from the layers found in humans. Therefore, it is problematic to apply the Mankin system in mice (135). Since scoring grades according to Kamekura are clearly related to the histological layers of *murine* cartilage, this approach seemed superior.

Nevertheless, what all semi-quantitative scoring systems, including the system by Kamekura et al. (131), have in common is to simplify (that is, due to categorizing) reality and to be susceptible to inter- and intra-observer variability. I would strongly support the idea of software-aided quantification of lesion depth and width in order to acquire objective and more reliable data, but the status quo of histopathological research is built on semi-quantitative tools (163). Moreover, I would also like to emphasize that the debate about ordinal data (acquired from semi-quantitative grading schemes) which are treated like interval data (by summing and averaging) was taken into consideration. In order to not insinuate anything, all data were therefore intentionally analysed by the non-parametric Mann-Whitney U test and shown as box-whisker plots with medians and interquartile ranges instead of arithmetic means with standard error of means (164).

The finding of promoted cartilage degeneration in male GR WT mice, which indicates a negative impact of endogenous GCs, is commensurate with related published OA experiments by my research group (128). Both the maximum and average score revealed significantly more severe structural cartilage damage in male WT compared to male KO mice ($p = 0.027$ and $p = 0.031$, respectively). To check whether the "male WT outlier mouse" was what had tipped the scales, the Mann-Whitney test was repeated without it and confirmed significance for the maximum ($p = 0.049$), but not for the average score ($p = 0.054$). In this respect, the observation of promoted cartilage degeneration in male WT mice seems somewhat robust for the former, though not for the latter score. To our knowledge, nothing pointed towards any suspect features predisposing the male WT "outlier mouse" to OA, but it is interesting that the left leg of this mouse, which had additionally been evaluated, exhibited a similar degree of damage. Therefore, the mouse had developed bilateral knee OA, which makes a specific local reason less likely.

In line with other studies (146, 147), female GR WT mice in our experiment had developed less severe cartilage erosion than male GR WT mice (when comparing maximum scores ($p = 0.043$), no average scores – despite a clear trend ($p = 0.068$)). Interestingly, we did not observe promoted cartilage erosion in female GR WT compared to female GR KO mice. On the contrary, the trend suggested a higher degree of damage in the female KO mice, but this finding was not significant ($p = 0.114$ for maximum score, $p = 0.099$ for average score). What biological explanations are conceivable? Theoretically, sex-specific alterations of GC signalling might be suitable to account for our observation, for instance through interactions of estrogen receptor (ER) signalling and the downstream pathways of GR signalling, as they both belong to the nuclear receptor superfamily (106). Functionally intact ERs were shown to be present in articular cartilage and to influence ECM synthesis (165). It would be interesting to know if female GR WT mice are, by certain interactions of estrogen and glucocorticoid signalling in chondrocytes, naturally less responsive to

endogenous GCs and thereby do not exhibit a significantly different phenotype in comparison to GR KO mice. The idea of less effective GC signalling in females was expressed by Duma et al. (166) and by Quinn et al. (148). One possible clue for variable downstream actions of GC signalling in females compared to males is the glucocorticoid-induced leucine zipper (GILZ), which was shown to be suppressed by estrogens in uterine cells (108, 148). Malaise et al. have shown that GILZ facilitates GC-induced leptin secretion in OA synovial fibroblasts (167). Hypothetically, if activated ERs were able to interfere with GC-dependent pathways like GILZ in *chondrocytes* and counteract the respective signalling transduction, this could make females less sensitive to endogenous and exogenous GCs during the pathogenesis of OA. Estrogens were not only reported to interfere with the leucine zipper gene, but also to modulate GR activity via post-translational modification, in particular to antagonise GR function as a result of dephosphorylation (166, 168), so further arguments might exist to support impaired GC actions in female cartilage. One has to admit, however, that these are purely hypothetical assumptions, since the role of GILZ in chondrocytes and interactions between GC signalling and sexual hormones in cartilage have to date not been described.

While cartilage erosion was the only parameter which suggested significantly pronounced joint degeneration in male GR WT compared to male GR KO mice, all other cartilaginous, bony and synovial parameters were non-significant.

Firstly, no measurable signs of cartilage calcification as a result of age-related or OA-related processes were detected. Initially, the missing difference between one-year-old and 12-week-old mice seemed unexpected, as it is contradictory to observations in other mouse strains (69). On the other hand, this is most likely explainable by the inter- and intra-strain variability of age-related processes (140, 151), indicating that age-related calcification had simply not yet occurred in our batch of C57/BL6 mice. Closely associated with the missing cartilage calcification, the chondrocyte hypertrophy showed neither a genotype- nor erosion-corresponding increase. Although IHC demonstrated the distinct presence of collagen type X in the various subgroups, and although there was a trend of more hypertrophic chondrocytes in structurally damaged compared to structurally undamaged regions, with slightly higher average percentages in WT vs. KO mice, all statistics were non-significant. Notably, van der Kraan et al., who investigated the occurrence of collagen type X in C57/BL6 mice of different ages and in the context of cartilage degeneration, also did not observe age- or damage-associated increase of collagen type X expression in tibiofemoral joint parts. Therefore, they questioned whether expression of collagen type X indicates imminent or actual cartilage erosion (139). Explanatory support of this observation is

provided by additional researchers, who showed that presence of collagen type X and cartilage erosion (including MMP-13 upregulation) are not necessarily linked with each other (48, 169). Commensurate with these results, the higher degree of cartilage erosion in male GR WT in our experiment was obviously not translatable to an equivalent increase of collagen type X. An explicit impact of endogenous GC signalling on chondrocyte hypertrophy was not found.

Secondly, pathological processes within the bony compartment, although one of the characteristic features of joint degeneration (1), were not discovered, except in the single male WT mouse with total cartilage erosion. Cartilage erosion scores and the BV/TV ratio were not positively correlated in our experiment. Male WT mice had a similar BV/TV ratio in comparison to male KO mice, and the same was found between female WT and KO mice. The only significant result was a sex-dependent difference, with relatively higher BV/TV values in female mice both in the medial and lateral subchondral bone of tibia ($p = 0.002$ and $p = 0.021$, respectively). Reconsidering the mechanistic theory of Radin et al., who thought of subchondral bone as a shock absorber (80), it might seem counter-intuitive that female WT mice display higher BV/TV ratios, but lower cartilage erosion scores than their male counterparts. This indicates a complex relationship between sex hormones, bone structure and cartilage. As previously stated, epidemiological studies suggest a link between increased bone density and the risk for OA development in humans (81). Yamada et al. described higher tibial subchondral bone density in women compared to men, but did not observe a positive correlation between subchondral bone density and cartilage degradation (170). Experimental data regarding sex-specific subchondral bone density in C57BL/6 mice and their relation to cartilage degeneration are scarce. What has been reported, both for humans (171) and mice (133), is that the medial tibial compartment is characterised by higher BV/TV (or bone mineral density) values than the lateral side, as was also found in our study. Looking at the values from our research project, one can conclude that promoted cartilage degeneration in male WT mice was not accompanied by increased subchondral bone density. Furthermore, our results suggest the onset of structural cartilage erosion prior to bony changes. Subchondral bone sclerosis might actually be a late phenomenon in the pathology of OA, developing only once cartilage is extensively eroded (170). Our own results support this viewpoint. However, the ongoing debate about the sequence of OA-related alterations certainly depends on the sensitivity and validity of methods used for investigating cartilaginous breakdown or bony remodelling (3).

Thirdly, the observed absence of inflammation in our samples is commensurate with the mild degree of cartilage erosion and OA-related changes. Although inflammation is not considered to be the main disease driver in OA (1), it is recommended to scan mice from OA experiments for potential cellular, vascular or biochemical inflammatory signs (156). Investigations with regard to

the coincidence of histological inflammation and overall OA disease stage have been limited, however, and do not express a clear correlation (172). There is some unofficial agreement that histopathological scoring for synovitis in OA usually reveals only mild to moderate levels of inflammation (85, 172) and that these signs are more likely detectable in advanced murine OA (135, 173). It is therefore not really unexpected to find no inflammatory infiltrates or synovial hyperplasia in our mice. One needs to bear in mind that our samples only represent the histological situation at the time of harvesting. Previous intermittent stages of joint inflammation, which may have contributed to the development of cartilage breakdown, cannot be excluded.

5.4 Limitations

Although this research project consisted of several methods focusing on OA-related changes and had a high number of evaluated sections, most parameters of joint degeneration in our experiment either revealed non-significant group differences or were actually absent. The initial presumption that endogenous GCs promote development of OA was successfully verified in terms of cartilage erosion scoring in male mice. Admittedly, it was the only finding supportive of our hypothesis. Other cartilaginous, or bone- or soft tissue-related outcomes did not point towards reinforced OA severity in GR WT mice. In fact, we have to concede that almost no mouse developed manifest OA. Although it might be tempting to conclude causality between intact endogenous GC signalling and enhanced structural cartilage damage, this interpretation requires particular caution. Semi-quantitative scoring systems ultimately rely on subjective assessment by humans, and thus implicate a relevant source of error. Despite close inter-observer reproducibility, overrating – independent of the experimental group – is a potential issue, since we were obviously looking, and subconsciously wishing, for more pronounced degenerative changes (observer-expectancy bias). The absence of other OA-related changes limits the power of our experiment with regard to hypothesis testing. Since our significance level and p-value was not adjusted for multiple testing, the single values below 0.05 have to be viewed critically (multiple comparisons problem).

In retrospect, the question emerged if it would have been better to let all mice grow older in order to ensure a phenotype more closely resembling OA, which could have potentially supported the finding of additional between-genotype differences. This conjecture becomes especially meaningful when taking into account the hypothesized degeneration-*attenuating* effect of GR knockout. Accordingly, we relied on a distinct disease phenotype in wildtype mice for optimal comparison. Speaking of advanced age as the "inductor" of OA, it should be noted that one-year-old mice should not be regarded as murine "Methuselah" in view of their mean lifespan of 2 years

and recorded maximum ages of 3 to 4 years (149). A later harvesting time point in spontaneous age-related OA models seems therefore legitimate.

Last but not least, the methodological repertoire of this project was broad, but not entirely exhaustive. Both mice legs had been decalcified, which is why micro-CT analysis of subchondral bone was not part of our experiment. Furthermore, no other biological material, for instance synovial fluid, serum or urine, was saved to analyse OA biomarkers like the C-terminal telopeptide of collagen type II (CTX-II), which might be a promising research field for the prediction of OA in mice (174). Finally, the whole range of histological techniques was not exploited (for instance TUNEL assays for the detection of chondrocytic apoptosis as an early OA feature (75)), though it is debatable whether additional parameters would have been useful in testing our hypothesis considering the pre-osteoarthritic phenotype of our one-year-old mice.

5.5 Prospect

This explorative research project was the first one to investigate the role of chondrocyte-specific endogenous GC signalling in a murine model of spontaneously developed, age-related degeneration of the knee joint. We were able to show that endogenous GCs influence the integrity of articular cartilage and promote structural breakdown at an early disease stage, and that they do this in a sex-specific manner, apparently. Subsequent experiments are necessary to evaluate the impact of chondrocyte-specific endogenous GC signalling in manifest OA. If age-related models are applied, awareness should be especially raised of the inter- and intra-strain variability of disease onset and progression. A later rather than earlier harvesting time-point should preferably be chosen, since the chondrocyte-specific GR knockout is presumably a disease-*preventing* genetic modification. Refinement of the experimental design, for instance via implementation of a controlled exercise regimen plus restriction of spontaneous movement in confined cages, could improve inter-animal reproducibility of OA severity (175). Timeline concepts (with several harvesting time points) could increase understanding of the sequence of OA-related pathologies and be beneficial for anticipating adequate stages of disease severity. Indeed, it would also be favourable to utilize OA detection methods prior to euthanisation, such as biomarkers (174), *in vivo* fluorescence-based imaging (176) or high-resolution MRI (177), but naturally these amendments would be very costly or are still under development. Alternatively, artificial mechanisms of OA induction could be applied in order to ensure a more consistent and predictable disease phenotype. This has already been done fur the surgical DMM model (128), but not for chemically-induced techniques like collagenase-induced OA. From my experience, spontaneous

models take too long and imply the risk of pre-osteoarthritic phenotypes, which suggests that artificially induced mouse models might be more practical and preferable. Experiments with manifest OA would provide reasons for further investigation into the underlying signalling pathways (e.g. Wnt, TGF-β, FOXO transcription factors), helping to understand the link between GC signalling and observed phenotypical outcomes. Also, in any future project, both male and female mice should be part of the experimental design to study the sexually dimorphic response to GC-mediated signalling.

The current state of knowledge points towards a negative impact of both exogenous (121) and endogenous GCs (128) on OA pathogenesis. In the long run, transition from observational to explanatory research could help to identify the precise biomolecular mechanisms through which endogenous GC signalling affects cartilage integrity. If the degeneration-promoting effects of endogenous GCs are confirmed, antagonisation of endogenous GC signalling (e.g. by local administration of the GR antagonist mifepristone) could potentially delay disease progression. This could be a step forward in the development of DMOADs. For the time being, sharpened awareness of the Janus-headed properties of commonly administered GC hormones in OA patients should be the rationale.

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7 APPENDIX

7.1 Statutory Declaration

I, Sebastian Mohr, by personally signing this document in lieu of an oath, hereby affirm that I prepared the submitted dissertation on the topic "**The Role of Chondrocyte-specific Endogenous Glucocorticoid Signalling in Age-Related Cartilage Degeneration**" ("Die Bedeutung chondrozyten-spezifischer endogener Glucocorticoid-vermittelter Signaltransduktion für die altersbedingte Knorpeldegeneration") , independently and without the support of third parties, and that I used no other sources and aids than those stated.
All parts which are based on the publications or presentations of other authors, either in letter or in spirit, are specified as such in accordance with the citing guidelines. The sections on methodology (in particular regarding practical work, laboratory regulations, statistical processing) and results (in particular regarding figures, charts and tables) are exclusively my responsibility.

Furthermore, I declare that I have correctly marked all of the data, the analyses, and the conclusions generated from data obtained in collaboration with other persons, and that I have correctly marked my own contribution and the contributions of other persons (cf. declaration of contribution). I have correctly marked all texts or parts of texts that were generated in collaboration with other persons.

My contributions to any publications to this dissertation correspond to those stated in the below joint declaration made together with the supervisor. All publications created within the scope of the dissertation comply with the guidelines of the ICMJE (International Committee of Medical Journal Editors; www.icmje.org) on authorship. In addition, I declare that I shall comply with the regulations of Charité – Universitätsmedizin Berlin on ensuring good scientific practice.

I declare that I have not yet submitted this dissertation in identical or similar form to another Faculty.

The significance of this statutory declaration and the consequences of a false statutory declaration under criminal law (Sections 156, 161 of the German Criminal Code) are known to me.

Berlin, 08th of April 2020

Date Signature

7.2 Curriculum vitae

For data protection reasons my curriculum vitae is not published in the electronical version of my thesis.

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