DISSERTATIONES
MEDICINAE
UNIVERSITATIS
TARTUENSIS
382

TAAVI TORGA

Association of molecular markers CILP-2, DDR2 and C2C with the severity of tissue damage in knee osteoarthritis

DISSERTATIONES MEDICINAE UNIVERSITATIS TARTUENSIS
382

DISSERTATIONES MEDICINAE UNIVERSITATIS TARTUENSIS
382

TAAVI TORGA

Association of molecular markers CILP-2, DDR2 and C2C with the severity of tissue damage in knee osteoarthritis

UNIVERSITY OF TARTU
Press
1632

Department of Anatomy, University of Tartu, Estonia

Dissertation is accepted for the commencement of the degree of Doctor of Philosophy in Medicine on June 18th, 2025 by the Council of the Faculty of Medicine, University of Tartu, Estonia.

Supervisors: Andres Arend, MD, PhD, Professor of Histology and Embryology, Department of Anatomy, Institute of Biomedicine and Translational Medicine, University of Tartu, Estonia

Siim Suutre, PhD, Associate Professor of Anatomy and Histology, Department of Anatomy, Institute of Biomedicine and Translational Medicine, University of Tartu, Estonia

Kalle Kisand, MD, PhD, Professor in Laboratory Medicine, Department of Internal Medicine, Institute of Clinical Medicine, University of Tartu, Estonia

Marina Aunapuu, DVSc, Associate Professor of Histology, Department of Anatomy, Institute of Biomedicine and Translational Medicine, University of Tartu, Estonia

Reviewers: Marika Väli, MD, PhD, Professor of Forensic Medicine, Department of Pathological Anatomy and Forensic Medicine, Institute of Biomedicine and Translational Medicine, University of Tartu, Estonia

Priit Kaasik, PhD, Professor in Functional Morphology, Department of Exercise Biology, Institute of Sport Sciences and Physiotherapy, University of Tartu, Estonia

Opponent: Valērija Groma, PhD, Professor of Histology, Department of Morphology, Institute of Anatomy and Anthropology, Rīga Stradiņš University

Commencement: September 5th, 2025

The publication of this dissertation is granted by the University of Tartu.

ISSN 1024-395X (print)
ISBN 978-9916-27-954-0 (print)

ISSN 2806-240X (pdf)
ISBN 978-9916-27-955-7 (pdf)

University of Tartu Press
http://www.tyk.ee/

TABLE OF CONTENTS

LIST OF PUBLICATIONS

I. Torga, Taavi; Suutre, Siim; Kisand, Kalle; Aunapuu, Marina; Arend, Andres (2023). Expression of CILP-2 and DDR2 and ultrastructural changes in the articular cartilage of patients with knee osteoarthritis undergoing total knee arthroplasty: a pilot morphological study. Medical Molecular Morphology, 56 (1). DOI: 10.1007/s00795-022-00339-4.

II. Torga, Taavi; Suutre, Siim; Kisand, Kalle; Aunapuu, Marina; Arend, Andres (2024). Cartilage collagen neoepitope C2C expression in the articular cartilage and its relation to joint tissue damage in patients with knee osteoarthritis. Biomedicines, 12 (5), 1063. DOI: 10.3390/biomedicines12051063.

III. Torga, Taavi; Suutre, Siim; Kisand, Kalle; Aunapuu, Marina; Arend, Andres (2024). Comparison of Antigen Retrieval Methods for Immunohistochemical Analysis of Cartilage Matrix Glycoproteins Using Cartilage Intermediate Layer Protein 2 (CILP-2) as an Example. Methods and Protocols, 7 (5), 67. DOI: 10.3390/mps7050067.

Author's contribution:

I. TEM experiments, immunohistochemical experiments, writing the original draft and providing illustrative materials, such as photographs and tables.
II. Methodology, immunohistochemical experiments, histological analysis, writing the original draft and providing illustrative materials such as photographs and tables.
III. Methodology, different immunohistochemical antigen retrieval experiments, histological analysis, writing the original draft, and providing illustrative materials such as photographs and tables.

ABBREVIATIONS

AC	articular cartilage
ACL	anterior cruciate ligament
ADAMTS	a disintegrin and metalloproteinase with thrombospondin motifs
BMI	body mass index
BMP	bone morphogenetic protein
CI	confidence interval
CILP	cartilage intermediate layer protein
Col1	type I collagen
Col2	type II collagen
COMP	cartilage oligomeric matrix protein
COX2	cyclooxygenase-2
CTX-II	C-terminal telopeptide of type II collagen
C1,2C	collagen Type I and II cleavage product
C2C	collagen type-II C-terminal cleavage neoepitope
C2M	matrix metalloproteinase (MMP)-derived type II collagen neoepitope
DAB	3.3′ diaminobenzidine tetrahydrochloride
DDR2	discoidin domain receptor 2
DMOAD	disease-modifying OA drugs
ECM	extracellular matrix
EDTA	ethylene diamine tetraacetic acid
ELF3	E74-like factor 3
ER	endoplasmic reticulum
rER	rough endoplasmic reticulum
GBD	Global Burden of Disease Study
GDFs	growth/differentiation factors
HA	hyaluronic acid
HIER	heat-induced epitope retrieval
HIF-2α	hypoxia-inducible factor 2α
hsCRP	high-sensitivity C-reactive protein
IHC	immunohistochemistry
IL	interleukin
IPFP	Hoffa's infrapatellar fat pad
kOA	knee osteoarthritis
LFC	lateral femoral condyle
LTM	lateral tibial (meniscus-covered)
LTN	lateral tibia (no meniscus)
MFC	medial femoral condyle
MMP	matrix metalloproteinase
MSC	mesenchymal stem cell
MTM	medial tibia (meniscus-covered)
MTN	medial tibia (no meniscus)

NBF	neutral buffered formalin
NF-κB	nuclear factor-κB
NO	nitric oxide
NOS	nitric oxide synthase
NSAIDs	non-steroidal anti-inflammatory drugs
OA	osteoarthritis
OARSI	Osteoarthritis Research Society International
OR	odds ratio
PCM	pericellular matrix
PG	patellar groove
PGE2	prostaglandin E2
PIER	proteolytic-induced epitope retrieval
PTOA	post-traumatic osteoarthritis
RANKL	receptor activator of nuclear factor κB ligand
RUNX2	runt-related transcription factor 2
sCTX-II	C-terminal telopeptide of type II collagen in serum
STZ	superficial tangential zone
TEM	transmission electron microscopy
TGF-β	transforming growth factor-β
TC^{A}	type-II collagen α chain ¾ length fragment
TKA	total knee arthroplasty
TNF-α	tumor necrosis factor
uC2C	collagen type-II C-terminal cleavage neoepitope C2C in urine
uCTX-II	C-terminal telopeptide of type II collagen in urine

1. INTRODUCTION

Osteoarthritis (OA) is the most common form of arthritis and the most prevalent musculoskeletal disorder, causing pain. It primarily affects diarthrodial joints and is a progressive degenerative disease that mainly occurs in adults, leading to structural and functional deterioration of articular cartilage (AC) and periarticular tissues. The prevalence of OA increases significantly with age, with most cases occurring after the age of 40. The rising incidence of OA is largely attributed to the ageing population (Palazzo et al., 2016). OA is often associated with chronic pain and functional disability, both of which significantly impair the quality of life of patients. Despite being the most common progressive musculoskeletal condition, its pathophysiology remains not fully understood (O'Neill et al., 2018).

Traditionally, OA has been described as a 'wear and tear condition'. However, recent evidence highlights its complex and multifactorial nature. Although cartilage loss is the primary pathological feature, OA is now recognized as a disorder that affects the entire joint, causing inflammation and pathological changes in the bone and soft tissues, including the synovium, menisci, and ligaments (Wilder et al., 2002). OA is characterized by debilitating pain, joint stiffness, swelling, and loss of mobility. In addition to its physical impact, OA has been linked to an increased risk of mental health disorders and cardiovascular diseases. Several risk factors contribute to OA development, including ageing, traumatic knee injury, obesity, genetic predisposition, abnormal mechanical stress, and inflammation by infection or surgery (Niu et al., 2009; Palazzo et al., 2016; Long et al., 2022).

The knee is the most commonly affected joint, with an estimated global prevalence of 365 million, followed by OA of the hip and hand (Watt, 2021). Knee OA (kOA) is more prevalent in women than in men, with an estimated prevalence of 10% in men and 13% in women among individuals aged 60 years and older (O'Neill et al., 2018). Although the precise mechanisms underlying the initiation and progression of kOA are not fully elucidated, studies suggest a complex interplay of mechanical, biochemical, and genetic factors (Xin et al., 2021).

The diagnosis of OA is typically based on clinical symptoms and radiographic findings, which often reveal an advanced disease when surgical intervention is required (Felson & Hodgson, 2014). Molecular changes associated with OA occur much earlier than the noticeable clinical symptoms (Valdes, 2020). Consequently, there has been a growing interest in identifying biomarkers that can detect cartilage degradation, provide insights into disease-related biological activity, and predict disease progression (Mobasheri, 2012; Rodriguez-Merchan, 2023).

Despite extensive preclinical research, no definitive cure for OA currently exists. The limited understanding of the molecular mechanisms driving OA pathogenesis has hindered the development of effective therapeutic strategies. Various types of disease-modifying OA drugs (DMOAD) have been investigated, including anti-cytokine therapy (tanezumab, AMG 108, adalimumab, etanercept, anakinra), enzyme inhibitors (M6495, doxycycline, cindunistat, PG-116800),

growth factors (bone morphogenetic protein-7, sprifermin), gene therapy (micro ribonucleic acids, antisense oligonucleotides), peptide hormones (such as calcitonin) and others (SM04690, senolitic, transient receptor potential vanilloid 4, neural EGFL-like 1, TPCA-1, tofacitinib, lorecivivint and quercitrin), as well as stem cell therapies (e.g., spheroids of chondrocytes). However, these strategies have yet to demonstrate consistent efficacy and safety in OA treatment (Rodriguez-Merchan, 2023). Currently, total knee arthroplasty (TKA) remains the primary treatment for advanced kOA, a practice that has remained largely unchanged since its introduction in 1968. The continued reliance on surgical intervention highlights the urgent need for early-stage therapeutic strategies to halt OA progression before irreversible joint damage occurs (O'Neill et al., 2018). Furthermore, OA is increasingly recognized as a heterogeneous condition with distinct subtypes (endotypes), adding another layer of complexity to its pathogenesis and treatment. Surgery often does not prevent damage to the other knee, suggesting OA may be a more systemic disease than previously thought. Multiple joint involvement, such as both knees/hip combinations, is common (Metcalfe et al., 2012).

In this study, we aimed to investigate the expression of previously identified molecular markers associated with the pathogenesis of OA. Specifically, we examined cartilage intermediate layer protein 2 (CILP-2), discoidin domain receptor 2 (DDR2), and type II collagen (Col2) cleavage neoepitope (C2C) in OA cartilage. These biomarkers have the potential to improve our understanding of OA progression and aid in the development of targeted diagnostic and therapeutic strategies.

2. REVIEW OF LITERATURE

2.1. Prevalence of OA

Osteoarthritis (OA) is the most common form of arthritis and is considered the most frequent chronic joint disease affecting millions of people worldwide (Bijlsma et al., 2011; O'Neill et al., 2018; Pereira et al., 2015; Chen et al., 2024). However, the prevalence of OA varies depending on which joints are affected. The most commonly affected joint is the knee, followed by the hand and hip (Hunter & Bierma-Zeinstra, 2019).

Due to the ageing of the population, the incidence of OA is increasing significantly. OA represents a major public health concern as the progressive disease has a significant impact on mobility, strongly affecting the patients' quality of life. In 2020, approximately 595 million people were affected by OA, which is about 7.6% of the global population, and have been increased in total cases 132.2% since 1990 (GBD [Global Burden of Disease Study], 2023). From an economic perspective, arthritis-related hospitalizations ranked as the second most costly in the United States, amounting to approximately $20 billion in 2017 (Liang et al., 2020). While ageing is a key risk factor, the increasing prevalence of OA is also strongly associated with rising obesity rates (Badley et al., 2020). Different studies of prevalence have shown that OA generally develops after the age of 40 years, and most people over the age of 65 years experience this pathology (Buckwalter & Martin, 2006). The correlation between OA and age is well known, but the mechanisms leading to the development of OA are not clearly understood (Loeser, 2010). The notable trait is that OA occurs more in women than in men, with a global age-standardized prevalence in 2020 of 8,058.9 per 100,000 (95% uncertainty intervals: 7,251.9–8,867.9) for women and 5,780.1 per 100,000 (95% uncertainty intervals: 5,217.8–6,341.2) for men (Courties et al., 2024).

An analysis of skeletal remains from prehistoric times to the present revealed that kOA long existed at low frequency, but since the mid-20th century, the disease has doubled in prevalence (Wallace et al., 2017). This finding challenges the notion that the recent increase in kOA is solely attributable to longer life expectancy and higher obesity rates, suggesting that additional environmental and lifestyle factors unique to the postindustrial era contribute to disease pathogenesis.

Targeting the modern-day environmental factors in OA pathogenesis, it has been proposed that OA is an evolutionary mismatch disease, where humans are imperfectly adapted to modern environmental conditions. This hypothesis suggests that changes in physical activity patterns, prolonged sedentary behaviour, and the systemic and local low-grade inflammation associated with obesity may contribute to OA development (Berenbaum et al., 2018).

2.2. OA contributing risk factors

OA is a multifactorial disorder; the risk factors for OA can be categorized into person-level factors, such as age, gender, obesity, genetics, diet, and joint-level factors, including injury and abnormal loading of the joints (Johnson & Hunter, 2014). Understanding the interplay between these factors is essential for developing targeted prevention strategies and personalized treatment approaches to mitigate OA progression and improve patient outcomes.

Obesity, defined as a body mass index (BMI) greater than 30 kg/m^2, is strongly linked to kOA with 6.8 times more risk to the development of kOA than normal-weight controls (King et al., 2013). A meta-analysis of 22 studies found a pooled odds ratio (OR) of 2.66 (95% CI [confidence interval] 2.15–3.28) for this association. The authors reported a large amount of heterogeneity (I^2 statistic up to 98%) between the study findings, but all studies were generally consistent in reporting being obese as a risk factor for the onset of kOA. On the other hand, the relationship between being overweight (BMI > 25 kg/m2) and kOA is weaker but still significant, with a pooled OR of 1.98 (95% CI 1.57–2.20) (Silverwood et al., 2015). The ORs in Silverwood et al.'s meta-analysis for the association of each potential risk factor with knee pain were extracted (or calculated where information allowed) from each paper included in the final review and then combined into the pooled OR. The relationship between BMI and kOA is primarily linear, with the duration of increased joint loading or weight gain also being significant. 27% of hip arthroplasty cases and 69% of knee arthroplasty cases may be attributed to obesity (Grazio & Balen, 2009).

Ageing is one of the leading risk factors of OA (Felson et al., 2000). Ageing entails specific changes in joint morphology, biochemical composition, and cell signalling. Our joints experience repetitive loading throughout our lives, which can lead to tissue failure and progressive damage (O'Brien & McDougall, 2019). Due to ageing, various molecular pathways in the joint become deregulated, thereby affecting the homeostasis of the cartilage extracellular matrix (ECM). This leads to a disruption in its architecture and a progressive deterioration of its biomechanical properties. The exact mechanisms underlying joint damage in OA remain incompletely understood; however, they are believed to be multifactorial. Contributing factors may include oxidative stress, traumatic injuries that worsen with ageing, cartilage thinning, muscle weakness, and impaired proprioception, all of which play a role in joint dysfunction (Litwic et al., 2013).

As mentioned in the introduction, the prevalence of hip, knee, and hand OA is higher in women than in men, with the incidence increasing around menopause (Srikanth et al., 2005). It is suspected that hormonal factors play a role in the development of OA; nevertheless, the results in this field are conflicting, and a direct causal relationship with hormonal involvement has yet to be conclusively established (de Klerk et al., 2009). The difference between men and women might be attributed to other factors such as reduced cartilage volume, bone loss, or lack of muscle strength (Johnson & Hunter, 2014).

It is assumed that genetic factors account for approximately 40% of kOA development (Palazzo et al., 2016). Although OA is not a monogenic disease, multiple genes are believed to contribute to its onset and progression. Identifying these genetic factors could provide valuable insights into disease pathophysiology and serve as potential targets for future pharmacological interventions (e.g., genes encoding the vitamin D receptor, insulin-like growth factor 1, Col2, and growth differentiation factor 5, SMAD3, IL-6) (Palotie et al., 1989; Chapman et al., 2008; Evangelou et al., 2009; Valdes et al., 2010a; Valdes et al., 2010b).

Trauma is a significant contributing factor to the development of OA, with the knee being one of the most frequently injured joints. Knee injuries are well-established risk factors for OA. Major trauma, such as an anterior cruciate ligament (ACL) rupture, leads to post-traumatic osteoarthritis (PTOA) in 25–50% of patients (Evers et al., 2022). While primary OA typically affects older patients, it is crucial to note that the younger population faces a disproportionately high risk of knee injury and secondary PTOA. This heightened risk stems from the increased incidence of knee injuries among younger individuals (Le et al., 2021; McGuine et al., 2014; Lam & Markbreiter, 2019). Current knee injury treatment aims to relieve pain and symptoms with non-operative treatment or, in case of need, surgical repair. Although surgery can increase activity levels, it does not reduce the incidence of PTOA (Chalmers et al., 2014; Muthuri et al., 2011; Lohmander et al., 2007; Gelber et al., 2000; Lohmander et al., 2004).

2.3. Role of inflammation in OA

Inflammation plays an important role in the pathogenesis of OA, characterized by different interactions between tissues and molecular pathways (De Roover et al., 2023). Several molecules released by chondrocytes, synoviocytes, and infiltrating immune cells, including cytokines, chemokines, and matrix metalloproteinases (MMPs), participate in joint anabolism and catabolism regulation processes (Chow & Chin, 2020).

For long, OA has been characterized as a relatively simple ‘wear and tear’ disease leading to the breakdown of cartilage. The progress in molecular biology during the 1990s has significantly changed this paradigm. It was discovered that many soluble mediators, like cytokines or prostaglandins, can increase the production of MMPs by chondrocytes, and this understanding led to the first steps of an ‘inflammatory’ theory (Berenbaum, 2013). But it took a decade until synovitis was accepted as a critical feature of OA. Now, some studies are opening the possibility to view inflammation as the conditional driver of the OA process. Experimental data also suggests that subchondral bone may play a crucial role in the OA process. It acts as a mechanical damper and also serves as a source of inflammatory mediators involved in the OA pain process and the degradation of the deep layer of cartilage. The involvement of inflammatory processes in the

progression of OA and its associated pain is supported by the documented efficacy of glucocorticoids and non-steroidal anti-inflammatory drugs (NSAIDs) in providing symptomatic relief for OA patients.

Initially thought to be driven by cartilage, it has become clearer that OA is a much more complex disease involving the mediators released by cartilage, bone, and synovium (Kapoor et al., 2011; Loeser et al., 2012; Goldring & Otero, 2011).

2.4. Concept of OA as a whole joint disease

As emphasized above, OA has traditionally been considered a 'wear and tear' disease caused by injury or abnormal mechanical loading leading to an increased pressure of weight-bearing joints. Under normal conditions, articular chondrocytes exhibit low metabolic activity and a limited regenerative capacity, resulting in a very poor ability to repair the damage. In addition, unlike other tissue types, AC has avascular, aneural, and alymphatic matrix, further undermining regenerative capacities (Aigner & Fan, 2003).

It has now become clearer that not only cartilage but the entire joint is involved in the development of OA. A joint is a complex structure where different components, such as the AC, meniscus, subchondral bone, and synovial membrane, offer sufficient mechanical and functional support to maintain a healthy joint (Szponder et al., 2022). A detailed arrangement of a normal knee joint is illustrated in Figure 1.

The subchondral bone (consisting primarily of mineralized type I collagen [Col1]) helps the AC (consisting primarily of Col2 and proteoglycans) to provide a surface for joint movement (Giorgino et al., 2023). The menisci, composed of fibrocartilage, play an important role in the attenuation of mechanical forces due to their structural content of water, proteoglycans, and collagen. The synovial membrane produces synovial fluid (composed generally of hyaluronic acid [HA] and lubricin) to lubricate the articular space (Kuyinu et al., 2016).

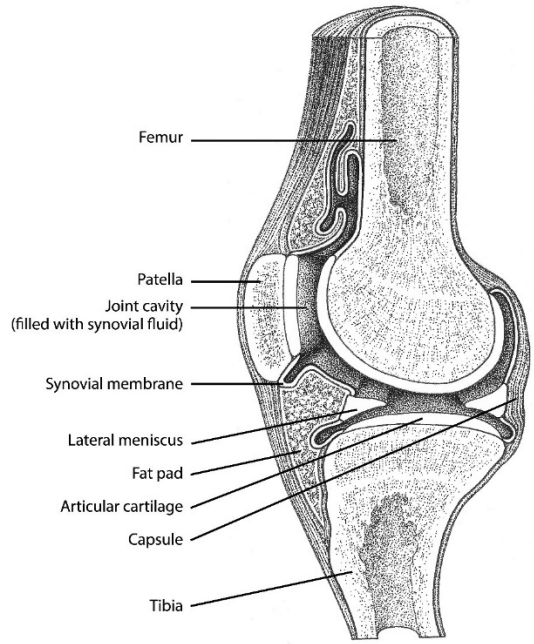

Figure 1. Healthy knee joint (*articulatio genus*) anatomy. The human body's largest and most complex joint is a synovial joint. This joint is articulated by three bones – the femur, tibia, and patella. The distal ends of the femur and tibia are significantly thickened and divided into lateral and medial condyles. The femur and tibia form the femorotibial joint (a weight-bearing component of the knee joint), and the femur and patella form the femoropatellar joint. The ends of the articulating bones are covered with smooth AC. Between the condyles of the femur and tibia are located semilunar-shaped fibrocartilaginous structures – the menisci. A dual-layer capsule surrounds the knee joint. The capsule consists of an external fibrous layer and an internal synovial membrane. The primary function of the synovial membrane is to create synovial fluid, which helps to reduce friction between the ACs of synovial joints during movement. Hoffa's infrapatellar fat pad (IPFP) is a structure located below the patella, between the joint capsule's external fibrous layer and internal synovial membrane (Drawing by R. T. Moss; Reproduced with permission).

2.4.1. Articular cartilage

AC is the highly specialized tissue covering synovial or diarthrodial joints at the end of the bones (Sophia Fox et al., 2009). The main function of AC is to provide a smooth, lubricated surface for low-friction articulation and to facilitate the transmission of loads with a low frictional coefficient (Blalock et al., 2015). AC comprises a sparse distribution of specialized cells known as chondrocytes and a dense ECM. The ECM primarily consists of tissue fluid, Col2, and proteoglycans (Xia et al., 2014). This tissue is known for its high water content – in normal AC tissue, fluid accounts for 65%–80% of total weight (Mow et al., 1992). Two major macromolecules of ECM are Col2 and the sulfated proteoglycan, aggrecan

(Vincent et al., 2022). Col2 together with proteoglycans contribute to retaining water in the ECM, which is essential for maintaining its unique mechanical properties (Sophia Fox et al., 2009). Col2 represents 15–22% and proteoglycans 4–7% of the wet weight of cartilage (Jackson & Gu, 2009). Other collagens including types V, VI, IX, X, XI, XII, and XIV (Eyre et al., 2002) and proteoglycans, such as decorin, biglycan, fibromodulin, lumican, epiphycan, and perlecan (Knudson & Knudson, 2001), constitute a small portion (less than 5%) of the normal cartilage composition. Cartilage-specific cells – chondrocytes, the only cell type present in this tissue, make up less than 5% of the total tissue volume (Yousefi et al., 2015). These cells are responsible for maintaining and generating ECM (Goldring & Marcu, 2009).

As AC is an avascular tissue, chondrocytes depend on the diffusion of nutrients and metabolites from the articular surface (Poole, 1997). The thickness of normal knee AC is from 1.79 ± 0.26 mm to 3.13 ± 0.54 mm (Guo et al., 2024).

2.4.1.1. The zones of articular cartilage

AC consists of fibres, composed primarily of Col2. The size and orientation of collagen fibres in AC depend on the tissue depth, which varies from the articular surface to the subchondral bone. This results in three distinct zones: the superficial zone, the middle zone, and the deep zone (Hamerman, 1989).

The superficial (tagential) zone (Figure 2) makes up about 10–20% of the cartilage thickness, and the main role of this layer is to protect the deeper layers from shear stresses (Chen et al., 2013). The collagen fibres of this zone (mainly Col2 and type IX collagen) are located parallel to the articular surface and packed tightly. This surface layer contains a relatively high number of flattened chondrocytes, and this layer's integrity is imperative in the protection and maintenance of deeper layers. The superficial zone is in contact with synovial fluid and is responsible for most tensile strength of cartilage, which allows it to resist the tensile, shear, and compressive forces imposed by articulation (Mansfield et al., 2015; Sophia Fox et al., 2009). The superficial layer of cartilage plays a critical role in regulating water content, thereby maintaining the tissue's structural integrity and biomechanical properties. Early damage to the superficial layer of the cartilage disrupts water homeostasis, leading to altered hydration levels and contributing to the formation of initial cartilage lesions. These changes subsequently compromise the load-bearing capacity of the cartilage, accelerating degenerative processes (Basso et al., 2020).

Next to the superficial zone is the middle or intermediate zone, which ensures a functional and anatomical bridge between the superficial and deep zones. The middle zone forms 40–60% of the total cartilage volume, and it contains thicker collagen fibrils and proteoglycans. The collagen fibres are organized obliquely in this layer, and the chondrocytes are at low density and spherical. The middle zone is functionally the first line of resistance to compressive forces (Ulrich-Vinter et al., 2003).

In the deep zone, collagen fibrils are arranged perpendicularly to the articular surface. This zone is responsible for providing the greatest resistance to compressive forces. The deep zone consists of the largest diameter collagen fibrils in a radial position, the lowest water content, and the highest proteoglycan content. The chondrocytes are squared in columnar orientation, perpendicular to the joint line and parallel to the collagen fibres. The deep zone forms approximately 30% of AC volume (Sophia Fox et al., 2009).

Additionally, there is a mineralized layer of calcified cartilage present at the interface between the cartilage matrix and subchondral bone – a tidemark (Arden & Nevitt, 2006). The tidemark separates the cartilage deep zone from the calcified cartilage. This zone is responsible for providing the greatest amount of resistance to compressive forces, containing high proteoglycan content. In this layer, the collagen fibrils are arranged perpendicular to the AC. The calcified layer has an essential role in securing cartilage to the bone by binding collagen fibrils of the deep zone to the subchondral bone. The cell population in this zone is scarce, and chondrocytes are hypertrophic (Sophia Fox et al., 2009).

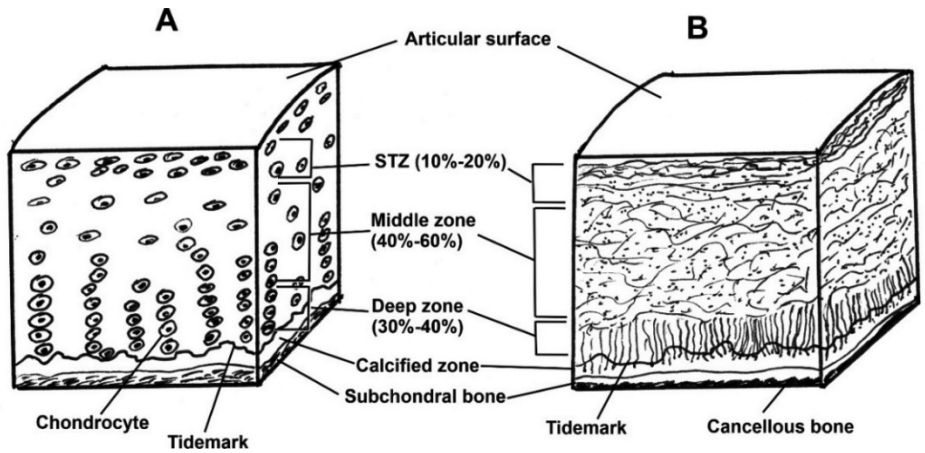

Figure 2. Structure of articular cartilage. A – Schematic diagram of chondrocyte organization in the three main zones of the uncalcified cartilage (STZ – superficial tangential zone), the tidemark, and the subchondral bone. B – Diagram of collagen fibre architecture and orientation (Drawing based on Buckwalter et al., 1994).

The unique arrangement of collagen fibres and the high osmotic pressure generated by proteoglycans provide the cartilage matrix with distinct biomechanical properties, allowing it to endure various mechanical stresses (Kafian-Attari et al., 2023).

2.4.1.2. Articular cartilage cells and extracellular matrix

As already mentioned, most of the cartilage tissue is composed of ECM, where the specific cells (chondrocytes) are enclosed (Karuppal, 2017). These cells proliferate and secrete ECM components to maintain and sustain the cartilage matrix (Akkiraju & Nohe, 2015). ECM separates chondrocytes from each other (Archer & Francis-West, 2003). Chondrocytes respond to external stimuli and tissue damage and play a responsible role in degenerative conditions like OA (Akkiraju & Nohe, 2015). The diameter of chondrocytes varies between 7 and 30 μm according to the anatomical layer (Şenol & Özer, 2020). Chondrocytes, derived from mesenchymal stem cells (MSCs), operate in an environment with low oxygen and low metabolic activity, containing in nature low mitochondrial numbers (Archer & Francis-West, 2003; Bhosale & Richardson, 2008). As all chondrocytes exhibit mechanosensitive properties, they ensure the mechanical and functional capacity to endure compressional, tensile, and shear forces across the diarthrodial joints (mainly through production of ECM) (Akkiraju & Nohe, 2015). In the superficial and middle zone, the main role of chondrocytes is to synthesize ECM consisting of Col2, type IX, and XI collagen, as well as proteoglycans. In the deep zone, the chondrocytes are irretrievably differentiated and actively synthesize type X collagen (Sophia Fox et al., 2009).

2.4.1.3. Chondrocytes and chondroptosis

Chondrocyte apoptosis, also referred to as chondroptosis, plays a significant role in the progression of AC degeneration in OA and is implicated in various cartilage disorders (Salucci et al., 2022). The survival of chondrocytes is crucial for maintaining the proper cartilage matrix. Compromising the function and survival of these cells would lead to AC failure (Hwang & Kim, 2015). Cartilage degeneration in ageing human AC is associated with a declining chondrocyte population, which impairs the tissue's ability to effectively regenerate and remodel (Adams & Horton, 1998). 'Chondroptosis' is the term that was coined to depict the type of cell death that is generally observed in chondrocytes, considering the fact that chondrocytes do not undergo apoptosis in a classical way (Aigner et al., 2004).

Chondroptosis has similar features to classical apoptosis, including the involvement of caspases (Matsuo et al., 2001), shrinkage of the cell, and condensed chromatin contained in the nucleus. However, condensed chromatin appearance in the nucleus is distinct. In chondroptosis, chromatin is scattered in patches throughout the nucleus, while in apoptosis, chromatin is aggregated in large masses and found at the margins of the nucleus membrane. Regarding cytoplasmic changes, drastic discrepancies distinguish chondroptosis from apoptosis. Different from apoptosis, where no increase of organelles is observed, chondroptosis cell death leads to the significant expansion of the rough endoplasmic reticulum (rER) and the Golgi apparatus (Charlier et al., 2016). In the chondroptotic process, cell elimination is phagocytosis-independent and involves self destruction mediated by three ultrastructural phenomena: (I) increased Golgi membrane and

ER create compartments where cytoplasmic components and organelles are digested (Pérez et al., 2005); (II) multiple autophagic vacuoles formation; and (III) cellular material extrusion into the lacunae space, leaving vesicular debris and remnants, which are distinct from the apoptotic bodies formed during apoptosis. In the final stage of chondroptosis, the lacunae are left empty (Roach et al., 2004).

2.4.1.4. Extracellular matrix and pericellular matrix

The ECM of the cartilage can be categorized into three distinct regions: pericellular, territorial, and interterritorial (Sophia Fox et al., 2009).

The pericellular matrix (PCM), clearly detectable by transmission electron microscopy (TEM), is a strict matrix region surrounding the chondrocytes that differs both biomechanically and biochemically from the other regions of cartilage ECM (Wilusz et al., 2014). The thickness of this specific region is approximately two to four μm (Guilak et al., 2018). Each chondrocyte, together with its surrounding PCM, is termed a chondron (Zhang, 2015). The term chondron is derived from chondrone, which was devised in the 19th century by Benninghoff, who observed altered matrix structure encircled around chondrocytes using for this purpose polarized light (Benninghoff, 1925). In later studies, it was found that chondrons contain a large volume of proteoglycans and Col2, type VI and IX collagens. Also, it was revealed that PCM is primarily defined by the presence of type VI collagen, fibronectin 1, and the proteoglycans – perlecan and biglycan (Poole et al., 1987; Poole et al., 1988; Kvist et al., 2008; Miosge et al., 1994). Since every chondrocyte is surrounded by PCM, it means that every chemical or physical signal that the chondrocyte receives is mediated by the PCM (Guilak et al., 2018). The significant role of the PCM has been emphasized in a review discussing its protective effects against the development of OA (Liu et al., 2018). It is assumed that the degradation of the PCM structure could be one of the first signs of the onset of OA. Increased interaction of Col2 fibrils with cell surface receptors, possibly via DDR2, has been suggested to influence chondrocyte metabolic activity and intracellular signalling pathways (Liu et al., 2018; Hu et al., 2006; Polur et al., 2010; Stevens et al., 2009). DDR2 binding to Col2 has been found to up-regulate matrix metalloproteinase 13 (MMP-13) production in chondrocytes (Xu et al., 2005). This process leads to the degradation of Col2 in the cartilage matrix (Holt et al., 2012). Although the osteoarthritic cartilage ECM has been the focus of extensive studies, increasing evidence indicates that numerous influences and characteristics of OA are demonstrated and potentially initiated in the PCM (Guilak et al., 2018).

The PCM is surrounded by the territorial matrix, which consists of a dense meshwork of thin collagen fibres that form a capsule-like structure around the cells, providing mechanical protection to the chondrocytes (Guilak & Mow, 2000). The territorial matrix is thicker than the PCM, and it is assumed that this matrix may protect cartilage cells from mechanical stresses. It may also contribute to the resiliency of the AC structure, enabling it to withstand substantial loads (Szirmai, 1969).

The interterritorial matrix is the largest of the three matrix regions and contributes the most to the biomechanical properties of AC (Mow & Guo, 2002). This region features differently oriented bundles of large collagen fibrils, arranged parallel to the surface in the superficial zone, obliquely in the middle zone, and perpendicular to the joint surface in the deep zone. Proteoglycans are abundant in this zone (Sophia Fox et al., 2009).

2.4.1.5. Collagens of articular cartilage

Collagens are the most abundant family of ECM proteins, which in healthy adult AC accounts for about two-thirds of the dry weight (Luo et al., 2017; Eyre, 2002). Mature AC principally consists of tissue fluid, Col2, and proteoglycans (Xia et al., 2014). Col2 and aggrecan are major ECM proteins in cartilage (Frazer et al., 1994). The Col2 molecule is the fundamental structural component of AC. It is composed of three identical α polypeptide chains of 1060 amino acid residues, arranged in a large triple helix and comparatively brief nonhelical telopeptides consisting of 19 amino acid residues in N-telopeptide and 27 amino acid residues in C-telopeptide (Dejica et al., 2012; Antipova & Orgel, 2010). In addition to Col2, type IX and XI collagens are considered the main collagens in AC of a healthy joint (Alcaide-Ruggiero et al., 2021). Other collagens, such as types I, III, IV, V, VI, X, XII, XIV, XVI, XXII, and XXVII, are minor collagens, represented in a small amount in the normal cartilage (Alcaide-Ruggiero et al., 2021). Currently, between 28 and 29 types of collagen have been identified (Sorushanova et al., 2019; Fidler et al., 2018; Regoli et al., 2020). In normal AC, Col2 constitutes 96% of the collagen content. Col1 constitutes only 0.2% of the healthy AC collagen content (Dickinson et al., 2005), but in cases of any damage or pathology of AC, an increase in Col1 can be found (Alcaide-Ruggiero et al., 2021). In mature AC, chondrocytes are responsible for synthesizing and maintaining cartilage matrix components, such as Col2 and proteoglycans, along with degrading enzymes, ensuring minimal turnover of cells and matrix (Xia et al., 2014). As explained above, collagen fibrils of AC mainly consist of Col2, accompanied with a lesser amount of minor collagens, which contribute to the physical properties of the matrix and help to provide cartilage with tensile strength (Luo et al., 2017; Heinegård & Saxne, 2011; Ichimura et al., 2000). Nevertheless, little is known about the processing of these minor collagens and how their turnover is induced by the progression of OA (Luo et al., 2017). It is detected that OA transforms Col2, the main collagen, to a mixture of types I, II, and III collagens. OA can prompt 100-fold upregulation in Col1 (Miosge et al., 2004), a 5-fold downregulation of Col2 (Lahm et al., 2010), and a 6-fold upregulation of type III collagen (Alcaide-Ruggiero et al., 2021; Bielajew et al., 2020). In addition, it has been noticed that a higher amount of type VI collagen is detected in osteoarthritic cartilage, which could be an outcome of a protective effect for chondrocytes (Alcaide-Ruggiero et al., 2021; Hosseininia et al., 2019).

2.4.1.5.1. Col2 neoepitopes (CTX-II, C1,2C, C2M and C2C)

As the Col2 macromolecule is the major structural component of AC (Martel-Pelletier et al., 2008), the breakdown of collagen is considered to be a critical and possibly irreversible point in the progression of OA (Eyre, 2002). Col2 undergoes breakdown assisted by proteolytic enzymes, including MMPs (Burrage et al., 2006). MMPs, like collagenases MMP-1, MMP-8, and MMP-13, possess the ability to cleave the natural triple-helix structure of collagen (Vincenti & Brinckerhoff, 2002). As a result of cleavage, two new peptides are formed, three-quarter-length and one-quarter-length of mature Col2, with specific neoepitopes at cleavage sites (Bay-Jensen et al., 2022; Bay-Jensen et al., 2008). Such neoepitopes include C-terminal telopeptide of Col2 (CTX-II) (Garnero et al., 2001), Col1 and Col2 cleavage products (COL2-3/4C Short; C1,2C) (Billinghurst et al., 1997), matrix metalloproteinase (MMP)-derived Col2 neoepitope (C2M) (Bay-Jensen et al., 2011), and Col2 C-terminal cleavage neoepitope (C2C) (Poole et al., 2004)

CTX-II presence has been detected in serum and urine. It is referred to as sCTX-II in serum and uCTX-II in urine (Oliviero & Ramonda, 2021; van Spil et al., 2010). Moreover, uCTX-II, shown to be associated with the progression of OA, is currently one of the most extensively studied OA biomarkers (Bay-Jensen et al., 2016). CTX-II has been found to be present not only in the damaged cartilage but also in the tidemark and calcified cartilage at the bone interface (Huebner et al., 2014). Furthermore, CTX-II is assumed to be a marker of subchondral bone degeneration (van Spil et al., 2013).

C1,2C levels have been found to be higher in OA cartilage compared to healthy samples. However, it has been noted that the accuracy of the C1,2C assay is compromised due to its tendency to cross-react with Col1, which is primarily indicative of bone turnover (Huebner et al., 2014).

Increased levels of uC2C (collagen type-II C-terminal cleavage neoepitope C2C in urine) have been correlated with knee pain and decreased lower limb functional abilities (Tamm et al., 2014). Several studies have demonstrated increased levels of uC2C in individuals with radiographically defined kOA (Poole et al., 2016; Cibere et al., 2009; Kuhi et al., 2020), and it has been discovered that uC2C is associated with the progression of kOA (Kraus et al., 2017; Kuhi et al., 2021). However, in a study conducted by Boeth et al. to trace OA progression, no significant difference between OA progression and urine content of C2C was detected (Boeth et al., 2019). Conrozier et al. compared serum concentrations of C2C in patients with multi-site OA to patients with only hip OA. They found that C2C levels were higher in patients suffering from solely hip OA (Conrozier et al., 2008).

2.4.1.6. Minor glycoproteins and cartilage intermediate layer protein

The cartilage matrix comprises up to 15% glycoproteins, and broad research has been done to elucidate their role in maintaining the functional structure of the AC and their involvement in various degenerative joint diseases (Sophia Fox et al., 2009). Changes in the content of glycoproteins in the cartilage matrix and different aspects of their biological functions have been associated with the decline of joint function and concomitant joint pain (Maldonado & Nam, 2013). The CILPs are one of several glycoproteins that have been in the focus of research on joint diseases, like OA (Lorenzo et al., 1998). CILPs are secreted by articular chondrocytes and deposited into the cartilage ECM. CILPs are large glycoproteins believed to play a role in cartilage scaffolding (Lorenzo et al., 1998). The main role of CILPs may be to modulate the cartilage matrix architecture (Tsuruha et al., 2001). CILPs expression has been demonstrated to change with ageing and in early stages of human OA (Lorenzo et al., 2004). Until now, two CILP isoforms, CILP-1 and CILP-2, have been described. These isoforms have been shown to be expressed in cartilage, but also in extra-skeletal tissues (Huang et al., 2020). These two CILP isoforms exhibit some structural similarity, with approximately 50% of their structure being homologous (Wu et al., 2019). However, these isoforms have been differently related to the progression of cartilage damage during OA, while the literature contains many contradictions. For example, in animal models of OA, CILP-1 and CILP-2 display different expression patterns, with CILP-1 being upregulated and CILP-2 downregulated (Bernardo et al., 2011). However, a proteomic analysis of cartilage samples from hip OA patients revealed elevated levels of CILP-2 (Hosseininia et al., 2019). As CILP-2 has been suggested to be more closely associated with the progression of OA compared to CILP-1, it has been proposed as a potential biomarker with diagnostic value in OA assessment (Bernardo et al., 2011; Lorenzo et al., 2014).

2.4.2. Other joint tissues (subchondral bone, synovium, infrapatellar fat pad, menisci, and ligaments)

OA is a chronic and progressive degenerative disease that affects not only the AC but also the entire joint, including subchondral bone, synovium, fat pad, menisci, and ligaments (Loeser et al., 2012; Martel-Pelletier et al., 2016; Swingler et al., 2019; Ilas et al., 2017). In the past, the most frequently discussed aspect of OA was the progressive damage to cartilage and its eventual loss. However, it is now widely acknowledged that changes in the subchondral bone and inflammation of the synovial membrane also have an impact on all other structures within the joint (Primorac et al., 2020). The structural alterations in these joint components represent key pathological characteristics of OA, further reinforcing the notion that OA affects the entire joint. Furthermore, comprehensive investigations suggest that interactions between cartilage and subchondral bone are essential for joint homeostasis, but also for OA progression (Xia et al., 2014; Goldring S. R. and

Goldring M. B., 2016). In the development of OA, the volume and thickness of the subchondral bone plate increase. These structural alterations are accompanied by distinct changes in the subchondral trabecular bone, characterized by bone deterioration in the early stages of OA and progressive sclerosis in the later stages of the disease (Burr & Gallant, 2012). Additionally, alterations in osteoblastic and osteoclastic activity lead to increased bone turnover, resulting in subchondral bone lesions, cysts, and osteophyte formation (Goldring, 2009).

The synovial membrane, which lines the inner surface of synovial joint capsules, plays a crucial role in maintaining metabolic homeostasis and the intra-articular environment, in conjunction with synovial fluid (Wenham and Conaghan, 2010). The synovial fluid plays a crucial role in providing nutrition to the cartilage (Primorac et al., 2020). The avascular cartilage relies on the synovial fluid both for essential nutrients and as a storage space for its degrading products (Schmidt et al., 2007). Synovitis is known to be the crucial factor in patients with OA and has been related both to symptoms and to structural progression. Besides, synovitis triggers the extensive production of proteolytic enzymes, leading to cartilage damage, while cartilage matrix catabolism produces molecules that promote synovial inflammation (Sellam & Berenbaum, 2010).

Hoffa's infrapatellar fat pad (IPFP) is the largest intra-articular adipose structure, placed in the anterior knee compartment, between the synovium and the joint capsule (Eymard & Chevalier, 2016; Paduszyński et al., 2018). IPFP is known to be a multifunctional tissue containing various types of cells: adipocytes, fibroblasts, leukocytes, macrophages, and other immune cells (Paduszyński et al., 2018). IPFP may excrete various cytokines and adipokines, contributing to local knee joint inflammation and the development of kOA (Zeng et al., 2020).

One of the most frequent risk factors of kOA development is meniscal lesions and injuries, because of decreased resistance to mechanical forces, like tension, compression, and shear stress (Englund et al., 2012). Loss of proper meniscus function contributes to OA by causing joint instability and abnormal mechanical loading (Hunter et al., 2006; Ding et al., 2007). In a normal healthy joint, the loaded collagen matrix of the meniscus can resist biomechanical stress, ensuring the stability of the knee during movements (Englund, 2010). Meniscus injury or damage often leads to damage to other joint structures, like cartilage loss, alterations in the subchondral bone, bone marrow lesions, and synovitis (Roemer et al., 2009).

Knee joint ligaments play a crucial role in stabilizing the joint by restricting excessive movement (Peters et al., 2022). Ligament injuries and tendon instabilities have been identified as significant risk factors for OA development. Studies show that after ligament injury, in around 50% of patients, OA develops in 10–20 years (Wu et al., 2020).

2.5. Changes in the OA-affected joint tissues

In the early stages of OA, the cartilage surface remains macroscopically intact, yet significant alterations occur at the molecular and structural levels within the ECM. These changes include disruptions in the collagen network, modifications in proteoglycans composition, and concurrent degenerative changes in the meniscus (Giorgino et al., 2023). Alterations in collagens and proteoglycans result in overcoming the compensatory mechanisms that limit the AC damage, eventually causing AC erosions and meniscal damage (Yunus et al., 2020). It should be noted that the proinflammatory role of cytokines affecting also the structural molecules in cartilage tissue has been confirmed as an important mechanism of joint damage in the early stages of OA (Chow & Chin, 2020). Responding to cartilage erosions, chondrocytes first undergo a phase of hypertrophic activity to increase the matrix synthesis, producing inflammatory mediators that promote cartilage breakdown (Figure 3). The superlative stage of cartilage destruction is chondrocyte apoptosis, which causes an imbalance of synthesis and catabolism of collagen and proteoglycans in favor of catabolism. Inflammatory mediators disperse to other joint structures, bringing along alterations in the synovial tissue and subchondral bone, causing bone sclerosis and increasing synovial membrane thickness and capsular structures (Aaron et al., 2017). As OA progresses, cartilage surface defects and voids develop, leading to the release of cartilage fragments into the joint space. These fragments contribute to synovial inflammation, which in turn disrupts the synthesis of key synovial molecules, such as lubricin and HA. Recent findings suggest that during chondrocyte growth, the expression of Col2 decreases; thus, mature chondrocytes are unable to produce Col2 *de novo* (Giorgino et al., 2023).

The onset of OA is a gradual process, which appears when the joint microenvironment is disturbed, leading to an imbalance between synthesis and destruction processes, pro-inflammatory and anti-inflammatory effects (Maldonado & Nam, 2013). AC, synovial membrane, and subchondral bone are the main components of the joint, which play crucial roles in the development of OA to varying degrees. This reflects various OA subgroups and phenotypes in patients (Berenbaum & Walker, 2020). Synovitis, in conjunction with mechanical factors, may serve as an initial trigger for immune system activation, playing a pivotal role in initiating and perpetuating the pathological cycle in the pathogenesis of OA (Zhu et al., 2020). The role of synovitis in the progression of OA, joint deterioration, and associated symptoms has been emphasized over the past decades (Hayashi et al., 2011; Atukorala et al., 2016). Changes have also been found to occur in the synovial fluid. Despite the scarcity of immune cells detected in joint tissues and synovial fluid in OA patients, the shifts in the biochemical profile of synovial fluid reflect alterations in cell metabolism in the joint (Zhu et al., 2020). Affected by joint diseases, many collagen turnover markers and cytokines accumulate in synovial fluid, including cartilage oligomeric matrix protein (COMP), MMPs, etc (Boffa et al., 2021).

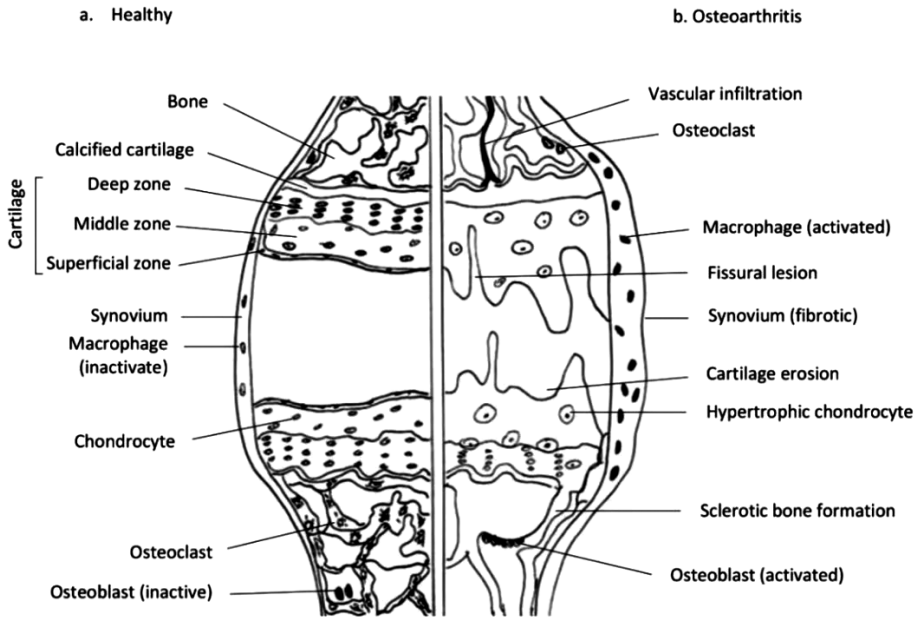

Figure 3. Comparative drawing of the differences between healthy (a. left side) and osteoarthritic (b. right side) knee joints. **a:** Healthy cartilage ECM is smooth, without fissures. Chondrocytes in the superficial zone are organized elongated, while in the deep zone, chondrocytes are larger, round in shape and oriented in columns. In the middle zone, chondrocytes are scattered. Superficial chondrocytes and cells in the synovial membrane produce substances such as lubricin and HA, which provide lubrication and reduce friction during joint movement. The anatomy of the subchondral bone in a healthy joint features a thin, dome-like subchondral plate supported by vertically oriented trabeculae. The healthy synovial membrane consists of a thin layer of synoviocytes and fibroblasts, which are inhabited by macrophages. Osteoblasts are inactivated, and osteoclasts are responsible for minimal subchondral bone turnover.
b: In osteoarthritic cartilage, chondrocytes become hypertrophic, and in cases of severe OA progression, they can become apoptotic. Hypertrophic chondrocytes are disoriented and produce increased levels of cytokines, such as interleukin (IL)-1, A Disintegrin and Metalloproteinase with Thrombospondin Motifs (ADAMTS)-4, ADAMTS-5, and Matrix Metallopeptidase (MMP)-1, MMP-13, which facilitate the remodeling of the cartilage ECM. The cartilage ECM is damaged – fissures, erosion, or even denudation are recognized. Osteoclasts in the subchondral bone become activated and begin to resorb bone, tunneling through the subchondral bone plate, which reduces its supportive function. Vascular ingrowth is supported, and osteoblasts begin to deposit significant amounts of bone, leading to the formation of a sclerotic bone. The synovial membrane is thickened, and activated synovial macrophages produce large quantities of cytokines that promote osteophyte formation, synovial fibrosis, and enhance cartilage degradation (Drawing based on Glyn-Jones et al., 2015; Siebelt, 2015).

The changes in AC altered by OA involve changes in cartilage ECM and its cellular components. The disruption of the matrix cellular organization entails tissue fibrillation, vertical fissures, cartilage degradation, and endochondral ossification (Boffa et al., 2021). Decreased quantity and quality of Col2, the loss of aggrecan, Col1 accumulation, and disorders of chondrocytes are observed at the molecular level (Hunter & Bierma-Zeinstra, 2019). The alterations of the ECM affect the composition of PCM (Guilak et al., 2018). The loss of microfibrillar type VI collagen, the primary component of the PCM, along with type IV collagen, perlecan, fibronectin 1, nidogens, and laminins, are related to disturbed integrity of PCM (Wilusz et al., 2014). The loss of PCM components in OA contributes to the increased deposition of Col1 and promotes cartilage endochondral ossification (Schminke et al., 2016).

One of the pathological hallmarks of kOA is the presence of osteophytes. Osteophytes are the bony outgrowths at the joint margin, which can contribute to reduced joint range of motion and pain (Hsia et al., 2018). Osteophytes start as cartilaginous growths, which afterward undergo intramembranous and endochondral ossification. Mature osteophytes are mineralized, fully integrated with native bone, and covered by AC that extends from the articular surface (van der Kraan & van den Berg, 2007; Zoricic et al., 2003). While the exact mechanisms of osteophyte formation and growth remain unknown, it is considered a remodeling and reparative feature of OA. Some authors suggest that in experimental joint damage, the synthesis of chondrocytes and the formation of osteophytes are influenced by growth factors (van Osch et al., 1996). Periosteal and synovial MSCs are believed to be the cellular source of osteophyte precursors, with developing osteophytes consisting of fibroblasts, mesenchymal prechondrocytes, maturing chondrocytes, hypertrophic chondrocytes, and osteoblasts (van der Kraan & van den Berg, 2007). Transforming growth factor-β (TGF-β) seems to be the most potent factor in initiating chondrogenesis in osteophytes (Uchino et al., 2000), while Bone morphogenetic protein 2 (BMP 2) plays an important role in the terminal differentiation of chondrocytes and endochondral ossification of the osteophytes (van Beuningen et al., 1998). While osteophyte formation is believed to be a repair mechanism that helps stabilize joints, osteophytes also bring along negative effects such as pain and reduced mobility (Blom et al., 2004).

Taken together, the pathologic changes seen in osteoarthritic joints have common features that affect the entire joint structure, resulting in loss of function, deformity, and pain (Loeser et al. 2012). Though activity-related knee pain is one of the most frequent symptoms of kOA, eventually enforcing patients to undertake TKA, it has to be stressed that the discordance between radiographic severity and pain in kOA is widely debated in the literature (Bedson & Croft, 2008; Finan et al., 2013).

2.6. Pathways of OA pathogenesis

Recent strategies for OA treatment focus on early prevention and delaying its progression before significant damage occurs. Considerable efforts have been made to understand the signalling pathways and molecules crucial in the onset and development of OA (Yao et al., 2023). Evidence suggests that cartilage degradation caused by abnormal loading stimulates the production of MMPs by chondrocytes. More exactly, abnormal loads are thought to trigger mechanoreceptors on the chondrocyte surface (Millward-Sadler & Salter, 2004), followed by activation of intracellular signalling pathways, including nuclear factor-kB (NF-κB) and TGF-β superfamily.

NF-κB is a group of transcription factors whose pathways can be activated by various pro-inflammatory cytokines (Jimi & Ghosh, 2005). The NF-κB signalling pathway plays a significant role in the pathology of OA through a variety of patterns (Yao et al., 2023). NF-κB signalling promotes the secretion of degrading enzymes, including MMPs such as MMP1, MMP2, MMP3, MMP7, MMP8, MMP9, and MMP13, as well as ADAMTS4 and ADAMTS5, resulting in the degradation of AC (Ulivi et al., 2008). Numerous cytokines and chemokines mediated by NF-κB, including receptor activators of TNF-α (tumor necrosis factor), IL-1β, IL-6, and NF-κB ligands (RANKL), are expressed in osteoarthritic articular chondrocytes. These factors can upregulate the production of MMPs, decrease the cellular synthesis of collagen and proteoglycan, and create a positive feedback loop that further enhances NF-κB signalling (Kapoor et al., 2011). NF-κB can intensify joint injury by inducing PGE2 (prostaglandin E2), NOS (nitric oxide synthase), NO (nitric oxide), and COX2 (cyclooxygenase-2), which promotes tissue inflammation, the synthesis of catabolic factors, and apoptosis of articular chondrocytes (Ulivi et al., 2008). NF-κB enhances the activation of other transcription factors, including HIF-2α (hypoxia-inducible factor 2α), ELF3 (E74-like factor 3), and RUNX2 (runt-related transcription factor 2) (Goldring et al., 2011; Li et al., 2022; Zheng et al., 2022; Lai et al., 2022). These transcription factors stimulate the expression of ADAMTS5 and MMP13 proteases, facilitating the differentiation of pre-hypertrophic articular chondrocytes into terminally differentiated hypertrophic chondrocytes (Goldring & Marcu, 2009).

TGF-β-group growth factors are essential for cartilage development, maintenance, and repair (Blaney Davidson et al., 2007; Song et al., 2009; van der Kraan et al., 2009). These growth factors play a crucial role in regulating cell proliferation, differentiation, apoptosis, and migration, while also managing the synthesis and degradation of the ECM. Deficiency of TGF-β or abnormal TGF-β signalling leads to a cartilage phenotype resembling the pathology seen in OA (Blaney Davidson et al., 2007). The TGF-β superfamily includes BMPs, TGF-βs, growth/differentiation factors (GDFs), and other related factors, which play a role in the development and differentiation of musculoskeletal structures (Suutre et al., 2009; Salazar et al., 2016; Zhang & Que, 2020). TGF-β and BMP signalling play a protective role in AC by regulating cartilage homeostasis. However, their

dysregulation is implicated in chondrocyte hypertrophy and ECM degradation (Zhong et al., 2015).

DDR2 is a cell membrane tyrosine kinase receptor located on the surface of chondrocytes. It plays a crucial role in cell-ECM interaction during the ongoing degeneration of AC in OA (Kumar et al., 2019). The activation of DDR2 triggers a cascade of cellular processes that eventually result in the release and activation of matrix-degrading proteinases, especially MMP13, leading to the development of OA (Xu et al., 2005). Activation of DDR2 through its interaction with native Col2 leads to upregulated receptor expression in chondrocytes and induces the expression of MMP13 (Xu et al., 2011). It is suggested that DDR2 could be a good therapeutic target for the development of DMOADs (Xiao et al., 2021). Studies have proposed that mice with a heterozygous knockdown of the DDR2 gene exhibit partial protection against experimental OA (Xu et al., 2010). Moreover, small molecule antagonist for DDR2 receptor in chondrocytes blocks DDR2 activation through TGF-β and Col2. This leads to downregulation of MT1-MMP13 via IL-1β and TNF-α, thereby ensuring repairment of damaged cartilage (Kumar et al., 2019). The IHC study between DDR2 expression and AC degeneration in OA showed the expression of DDR2 in human AC to increase with advancing degree of tissue injury. Likewise, AC degradation with elevated DDR expression resulted in a more significant release of MMP13 and Col2 breakdown products (Sunk et al., 2007). This renders DDR2 a compelling target for investigation in the context of OA pathogenesis.

2.7. Antigen retrieval methods in OA articular cartilage studies

Evaluating protein expression in tissue sections is essential for biomedical research. Nevertheless, this is often difficult to achieve with skeletal tissues, such as cartilage, that require prolonged decalcification and tend to have poor adhesion to slides (Dou et al., 2021). Also, the specificity of cartilage lies in an extensive dense ECM, which inhibits antibody penetration. In IHC investigations, various critical points can affect the results of the trials. Attention must be given to each step, from tissue fixation, decalcification, to antigen retrieval (Bord, 2003). Cartilage fixation takes longer than in the case of soft tissues. The standard fixative used for paraffin embedding is 10% neutral buffered formalin (NBF). After fixation, the following crucial step is decalcification. The purpose of decalcification is to soften the tissue by lowering the calcium content (Idleburg et al., 2021). Ethylene diamine tetraacetic acid (EDTA) is the most common solution used for decalcifying in IHC.

Like most fixatives, NBF solutions preserve tissue by cross-linking proteins. As a result, tissues typically require antigen retrieval before incubation with a primary antibody. The retrieval process is often necessary because epitopes can be masked during formalin fixation, caused by the cross-linking of amino groups on adjacent molecules (Fox et al., 1985). One crucial point in IHC is selecting the

appropriate antigen retrieval method, as this significantly impacts the most critical step of the procedure – antibody binding to its epitope (Kim et al., 2016). The selection of a retrieval method will depend on the specific antigens and antibodies being used. There are commonly two types of antigen retrieval methods: heat-induced epitope retrieval (HIER) and proteolytic-induced epitope retrieval (PIER). The latter is the most common method presented in the mid-1970s, with trypsin, proteinase K, pronase, ficin, and pepsin as the most frequently used enzymes in PIER. The main objective of PIER is to degrade protein crosslinks by protein digestion, where the effectiveness depends on different factors: the concentration and type of the enzyme, incubation parameters (time, temperature, and pH), and the length of fixation (Ramos-Vara & Miller, 2014). Later, in the 1990s, the second method, HIER, was also introduced, which is now a regularly used pre-treatment procedure in IHC to modify the molecular conformation of 'target' proteins of slide-mounted specimens by heated buffer solution. The common problem of HIER is the potential destruction of the antigenicity of the epitopes (Kim et al., 2016). The heat resistance of proteins has been suggested to be related to their solubility, and as most proteins are heat-labile, heat treatment may cause proteins to be irreversibly denatured and precipitate (Kim et al., 2000). Heat resistance can also depend on the glycosylation of the protein (Sola & Griebenow, 2009); therefore, caution is particularly advised to optimize a suitable IHC protocol for the evaluation of cartilage proteins.

Thus, every retrieval method has its own challenges and requires optimization depending on specimen types. In PIER, enzyme digestion requires precise control over pH and treatment duration, as different tissues digest at varying rates. The main challenge in HIER is to ensure that the tissue is treated long enough to facilitate antigen retrieval, without causing it to lift off the slide – a common issue when working with cartilage and bone (Idleburg et al., 2021).

In this study, antigen retrieval optimization was necessary for effective detection of CILP-2 using the HPA041847 polyclonal antibody (Atlas Antibodies), but not for monoclonal DDR2 (Abcam ab63337) or C2C (IBEX Pharmaceutical Inc.) antibodies. The need for retrieval was guided primarily by the manufacturers' instructions: antigen retrieval was explicitly recommended for the CILP-2 antibody, whereas no such step was required for DDR2 and C2C, both of which performed effectively without it.

The likely reason for antigen retrieval in the case of CILP-2 is conformational epitope masking due to formalin-induced cross-linking, particularly within the dense ECM of cartilage. In contrast, the DDR2 antibody targets a membrane protein with a linear epitope that remains accessible following fixation, and the C2C antibody detects a Col2 degradation neoepitope, which is also expected to stay exposed post-fixation. Notably, both DDR2 and C2C antibodies produced distinct and reproducible staining patterns, supporting their reactivity and specificity under the applied conditions.

2.8. Summary of the literature review

There is a great need for molecular markers reflecting alterations during the pathogenesis of kOA. The discovery and characterization of reliable biomarkers could facilitate the recommendation of individualized lifestyle modifications, assist in clinical decision-making in patient management, and support the development of targeted rehabilitation strategies following joint replacement surgery. Furthermore, the identification of specific molecules or molecular pathways involved in the pathogenesis of kOA may contribute to the development of disease-modifying OA drugs.

As can be concluded from the literature discussed above, extensive research has been done in the field of OA biomarkers, but still very few candidate molecules have been proposed. Although the markers investigated in our study were discovered years ago, studies in the field of histology can give inconsistent results, and the role of these markers in the pathogenesis of kOA remains controversial. Therefore, further investigation of these markers is still necessary. Numerous studies have been conducted to determine markers in urine and serum, but there are few studies on the local determination of such markers that can be determined using IHC, or, moreover, the determination of different markers in one sample. Besides, when evaluating molecular markers using IHC, it is very important to focus on the quality of antibodies and optimization of antigen retrieval methods. This study aims to employ validated or extensively characterized antibodies to compare immunohistochemical staining patterns with the degree of tissue damage, as assessed by a well-established histopathological grading system. The inclusion of relatively younger patients (under 69 years of age) provides a rationale for assuming a more active involvement of molecular mechanisms in the pathogenesis of osteoarthritis in this cohort, thereby making them particularly suitable candidates for research focused on the identification and evaluation of molecular markers.

3. AIMS OF THE STUDY

General Aim:
The general aim of this study was to clarify whether the molecular markers detected in osteoarthritic cartilage correlate with the morphological changes observed in tissue samples. This will help to determine their potential role as biomarkers for osteoarthritis (OA) pathogenesis.

Hypotheses:

I. The expression of Cartilage Intermediate Layer Protein -2 (CILP-2), discoidin domain receptor 2 (DDR-2) and type II collagen cleavage neoepitope (C2C) in osteoarthritic knee cartilage correlate with the degree of cartilage damage, as assessed by OARSI (Osteoarthritis Research Society International) histopathology grades and macroscopic joint surface damage scores. These markers could serve as reliable indicators of OA-related tissue damage.

II. Detailed structural description of CILP-2, DDR-2, and C2C staining patterns, along with the comparative histological tissue damage description, will provide additional insights into the pathogenesis of cartilage degradation.

Specific Aims:

I. **Optimize Antigen Retrieval Protocols**: To evaluate and compare three different antigen retrieval protocols for CILP-2 immunohistochemistry (IHC) in OA AC samples. The goal is to optimize the protocol for skeletal tissues, given the significant variability in CILP-2 staining dependent on the antigen retrieval method employed.

II. **Evaluate CILP-2 Expression**: To assess the expression of CILP-2 in knee AC of patients with OA and determine its correlation with the degree of cartilage damage, as assessed by OARSI histopathology grades and macroscopic joint surface damage scores. This will help evaluate its potential as a biomarker for OA-related tissue damage.

III. **Assess C2C Expression**: To assess the expression of C2C across different regions of osteoarthritic cartilage. This will help determine its potential as a biomarker for OA by evaluating its correlation with OARSI indicators of cartilage and joint damage.

IV. **Investigate DDR2 Expression**: To investigate the expression pattern of DDR2 in osteoarthritic cartilage and its relationship with CILP-2. This includes comparing their respective correlations with OA severity to determine the more reliable candidate for disease research.

V. **Analyse Ultrastructural Changes**: To analyse ultrastructural changes in chondrocytes and the pericellular matrix (PCM) of osteoarthritic cartilage using transmission electron microscopy (TEM). This will focus on chondroptosis and potential PCM involvement in OA pathogenesis in samples from the most affected superficial layer.

4. MATERIALS AND METHODS

4.1. Ethical statement

All the methods used in this study were in accordance with the Declaration of Helsinki. The research project was approved by the Ethics Review Committee on Human Research of the University of Tartu (Protocol no. 265T-22, 19 December 2016). Written informed consent was obtained from all subjects who were involved in this study.

4.2. Patient selection

In this study, we used for analysing the Total Knee Arthroplasty (TKA) Cohort. We obtained cartilage tissue samples from 43 patients undergoing primary unilateral TKA due to end-stage kOA at the Traumatology and Orthopedics Clinic of the University of Tartu between 2017 and 2018 (Table 1).

Table 1. A distribution of the subjects.

	Subjects involved
Paper I	20
Paper II	27
Paper III	4

The overall number of involved patients is 43 as five patients were enrolled both in Papers I and II, and three patients of Paper III were also enrolled in Papers I and II.

The inclusion criteria of the subjects were radiologically diagnosed end-stage kOA (Kellgren/Lawrence grade 3–4) before the age of 70 years. Kellgren/Lawrence grading system categorize kOA into five grades (0–4) (Table 2).

Table 2. Kellgren/Lawrence grading system (Pai et al., accessed on 11 Apr 2025).

Kellgren/Lawrence grading system	
Grade 0	Normal
Grade 1	Doubtful joint space narrowing and possible osteophytic lipping
Grade 2	Definite osteophytes and possible joint space narrowing
Grade 3	Moderate multiple osteophytes, definite narrowing of joint space, some sclerosis and possible deformity of bone ends
Grade 4	Large osteophytes, marked narrowing of joint space, severe sclerosis and definite deformity of bone ends

Patients with the signs of acute infection in the past three months were excluded. Other exclusion criteria were secondary OA caused by trauma, gout, infections, or congenital and developmental problems affecting the knee joints, as well as rheumatoid arthritis.

Paper I. This study involved 20 subjects. Eight men and 12 women aged 46 to 68 years were enrolled. Tissue punches from the two different load-bearing sites of the tibia plateau were analysed in each subject. Analysed punches were collected from meniscus-covered medial (MTM) and meniscus-covered lateral tibial plateau (LTM). A subset of patients formed a group for transmission electron microscopy studies. Samples for ultrastructural studies of the superficial layer of AC from ten randomly selected patients were taken from the tibial plateau (MTM or LTM; for details see 4.6 Transmission Electron Microscopy).

Paper II. This study involved 27 subjects. 10 men and 17 women aged 55 to 66 years were enrolled. Tissue punches from the two different load-bearing sites of the tibia plateau (MTM and LTM) were analysed in each subject.

Paper III. This study involved four subjects. Two men and two women aged 58 to 65 years were enrolled. Tissue punches from load-bearing sites of the tibia plateau were analysed in three patients. In one case, to ensure a sufficient amount of material for testing and to check the site specificity of different staining protocols, three samples from different locations were analysed in one patient.
It should be pointed out that only five patients were enrolled in both Paper I and Paper II. Two patients were enrolled in all three papers. One patient was enrolled in both Paper II and Paper III.

4.3. Sampling

Tissue punches, which included all cartilage layers and the subchondral bone, were obtained from the load-bearing sites of the tibial plateau and femur in each subject (Figure 4). Cylindrical osteochondral explants of seven mm diameter were drilled perpendicular to the articular surface immediately after TKA.

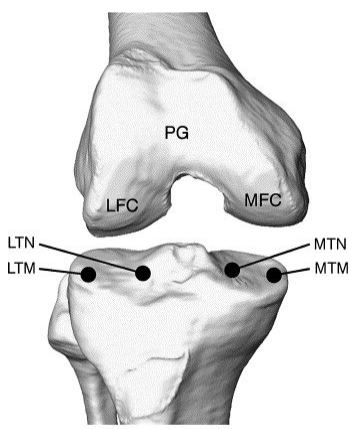

Figure 4. An anatomical drawing of the human knee joint (distal femur and tibial plateau) with indications of seven different anatomical locations. In each subject, tissue punches were collected from meniscus-covered medial (MTM) and meniscus-covered lateral tibial plateau (LTM) (Quinn T, et al., 2005; Reproduced with permission).

4.4. H&E and Safranin O staining

For histopathological assessment, samples were fixed in 10% buffered formalin solution for at least 48 hours. Decalcified with Decalcifier Solution (Sakura Finetek Europe, Alphen aan den Rijn, The Netherlands) in Sakura TDE™ 30 Decalcifier System (Sakura Finetek Europe, Alphen aan den Rijn, The Netherlands) for 24 hours. Thereafter, the samples were embedded in paraffin with a vacuum infiltration processor Sakura Tissue-Tek® VIP 5 Jr (Sakura Finetek Japan, Tokyo, Japan). Specimens were cut to a thickness of four to seven µm using the microtome Microm Ergostar HM200 (Microm GmbH, Walldorf, Germany), mounted on microscope slides, and dried overnight on a slide dryer heating plate at 37 °C. Subsequently, the sections were deparaffinized with xylene, rehydrated in a series of graded ethanol solutions, and stained using H&E and the Safranin O (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) staining methods (Kiernan, 2001). Finally, the sections were dehydrated in graded ethanol solutions, in xylene, and mounted with Eukitt® medium (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) before applying the coverslips.

4.5. Histological analysis

The OARSI OA Cartilage Histopathology Assessment System was used for evaluation of the level of local cartilage pathology, ranging from grade 0 (normal cartilage architecture) to grade 6 (severe damage) (Table 3).

Table 3. OARSI OA Cartilage Histopathology Assessment System (Pritzker et al., 2006).

OARSI OA Cartilage Histopathology Assessment System	
Grade 0	Normal matrix architecture and smooth surface; cells are intact and in appropriate orientation
Grade 1	Only the superficial zone is affected. The surface of the cartilage is generally intact but may show superficial fibrillation. The middle and deep zones are not affected.
Grade 2	Discontinuity of the cartilage superficial zone by focal fibrillation. Small portions of cartilage from the superficial zone are detached due to abrasion from shear forces.
Grade 3	Vertical fissures extending from the superficial zone into the middle zone or even into the deep zone.
Grade 4	The key feature is erosion. If, in the earliest stage, cartilage matrix loss leads to delamination of the superficial zone, then more extensive erosion causes excavation and loss of matrix in fissured domains.
Grade 5	The key feature is denudation. Unmineralized cartilage is completely eroded to the level of mineralized cartilage or bone.
Grade 6	Recognized by deformation. The contour of the AC is changed by processes of microfracture, repair and bone remodeling.

An advanced grade within the primary grade is indicated by adding 0.5 to the primary grade as specified in the OARSI grading system (Pritzker et al., 2006). The macroscopic stage of OA was assessed by visually evaluating the tibial plateau removed during the operation. To evaluate the extent of articular surface involved with OA, the staging method ranging from stage 1 (50% of involvement) to stage 4 (>50% of involvement) was used. For OA scoring, a simple formula was used: OA score = OA grade × OA stage (Pritzker et al., 2006). Two independent assessors evaluated the AC damage. In the case of the few evaluation disagreements, the specimens were revised to formulate the final assessment.

4.6. Transmission Electron Microscopy (TEM)

The specimens for TEM investigations were taken from the superficial layer of AC of the MTM or LTM, as indicated with an asterisk in Table 4. The samples were fixed in 2.5% glutaraldehyde solution (Sigma-Aldrich Chemie GmbH, Germany) buffered with sodium cacodylate buffer (Sigma-Aldrich Chemie GmbH, Germany) at pH 7.4 for two hours at 4°C and postfixed for one hour in 1% osmium tetroxide solution (Agar Scientific, England). Thereafter, the samples were dehydrated in ethanol (50°→70°→90°→96°→ absolute ethanol) and embedded in Epon-812 (Fluka, Sigma-Aldrich Chemie GmbH, Switzerland) according to the standard methods. Semi-thin sections were cut at two µm and stained with methylene blue-azure II. The slides were observed and photographed using a Zeiss Axiophot-2 microscope (Carl Zeiss AG, Jena, Germany). The ultrathin sections were cut at 70 nm on the Reichert Om U3 ultratome (Reichert, Germany) with a diamond knife (Diatome Ltd, Biel/Benne, Switzerland). After that, the sections were mounted on copper grids of mesh size 200 (Sigma-Aldrich Chemine GmbH, Germany), stained with uranyl acetate (Agar Scientific, England) and lead citrate (Agar Scientific, England) according to standard methods. Viewing and photographing were done using a Transmission Electron Microscope Philips Tecnai-10 (Philips, Netherlands) with Veleta camera (Olympus Soft Imaging Solutions GmbH, Germany).

4.7. Immunohistochemistry (IHC)

4.7.1. CILP-2 and DDR2 (Paper I)

For IHC analysis, paraffin-embedded specimens were cut at four µm thickness using the microtome Microm Ergostar HM200 (Microm GmbH, Walldorf, Germany), mounted on Menzel-Gläser Superfrost® Plus slides (Gerhard Menzel GmbH, Braunschweig, Germany), and dried overnight on a slide dryer heating plate at a temperature of 37 °C. After that, the sections were treated with 30 µg/ml Proteinase K (Invitrogen, USA) in 50 mM Tris, pH 6.0, 5 mM $CaCl_2$ at 37°C for 90 minutes and with 0.4% bovine hyaluronidase in HEPES-buffered medium (Fertipro, Belgium) for three hours to ensure antibody penetration (see Paper III).

To block peroxidase activity, the sections were treated with 0.6% H_2O_2 (Fluka, France) in distilled water for 15 minutes. Thereafter, the sections were washed in 1× Gibco™ PBS (Thermo Fisher Scientific, Landsmeer, The Netherlands) solution for 3 × 5 minutes. After washing, the sections were incubated in Dako Antibody Diluent S2022 (Dako Denmark A/S, Denmark) to block nonspecific antibody binding. Then, the sections were incubated overnight at 4 °C with the primary CILP-2 antibody (Atlas Antibody, HPA041847, Sweden) in dilution of 1:100 or with the primary DDR2 antibody (Abcam, [3B11E4] ab63337, UK) in dilution of 1:200; both antibodies were diluted in Dako Antibody Diluent (Dako Denmark A/S, Denmark). The treatment with Proteinase K and hyaluronidase was not performed in the case of DDR2 immunostaining, as there did not appear to be any need for it. The omission of antigen retrieval for DDR2 was justified, as tissue fixation appears to have a limited effect on epitope accessibility in this case. On the next day, the sections were incubated with biotinylated secondary antibody (REAL™ EnVision™ Detection System, Dako Denmark A/S, Denmark) for one hour at room temperature. Then, the sections were incubated with 3.3′ diaminobenzidine tetrahydrochloride (DAB) solution (REAL™ EnVision™ Detection System, Dako Denmark A/S, Denmark) for 10 minutes at room temperature in the dark and counterstained with toluidine blue. Washing 3 × 5 minutes between each step after incubation with the primary antibody was performed in 1× Gibco™ PBS (pH 7.4) solution (Thermo Fisher Scientific, Landsmeer, The Netherlands) containing 0.07% Tween 20 detergent (BioTop, Naxo, Tartu, Estonia). IHC negative controls were performed by omitting the primary antibody. The extent of staining was evaluated by tissue staining incidence ranging from 1 to 5 (‘1’: no staining, ‘2’: 1–25% stained, ‘3’: 26–50% stained, ‘4’: 51–75% stained, ‘5’: 76–100% stained) (Meyerholz & Beck, 2018).

4.7.2. Cartilage Collagen Neoepitope C2C (Paper II)

Paraffin-embedded specimens were cut at a four µm thickness using the microtome Microm Ergostar HM200 (Microm GmbH, Walldorf, Germany), mounted on AutoFrost® Microscope Slides (Cancer Diagnostics, Inc., Durham, NC, USA), and dried overnight on a slide dryer heating plate at a temperature of 37 °C. Thereafter, the sections were deparaffinized in xylene and rehydrated in a series of graded ethanol solutions. Endogenous peroxidase activity was inactivated by incubating the sections for 15 minutes with freshly prepared 0.6% H_2O_2 (Honeywell Fluka™, Seelze, Germany) dissolved in distilled water. Thereafter, the sections were washed for 3 × 5 minutes in 1× Gibco™ Phosphate Buffered Saline (PBS, pH 7.4) solution (Thermo Fisher Scientific, Landsmeer, The Netherlands). To block nonspecific antibody binding, the sections were incubated with 100 µL Dako Antibody Diluent (cat no S2022) (Dako Denmark A/S, Glostrup, Denmark) for 30 minutes. After that, the sections were incubated with 100 µL C2C monoclonal antibody (product no 50-1018; Ab 5109; produced by IBEX Pharmaceutical Inc., Mount Royal, QC, Canada) in a dilution of 1:10,000 for one hour at room temperature. To visualize the primary antibody, the sections were

incubated with the commercial kit Dako Real™ Envision™ Detection System, Peroxidase/ DAB+, Rabbit/Mouse (cat no K5007) (Dako Denmark A/S, Glostrup, Denmark) – the sections were incubated with secondary antibody for one hour and after that with DAB for 10 minutes. Washing 3 × 5 minutes between each step after incubation with the primary antibody was performed in 1× Gibco™ Phosphate Buffered Saline (PBS, pH 7.4) solution (Thermo Fisher Scientific, Landsmeer, The Netherlands) containing 0.07% Tween 20 detergent (BioTop, Naxo, Tartu, Estonia). To counterstain the cartilage proteoglycans, the sections were stained for one minute with Toluidine blue. Negative controls were performed by omitting the primary antibody. Eventually, the sections were dehydrated in a series of graded ethanol solutions, in xylene, and mounted with Eukitt® medium (Sigma-Aldrich GmbH, Taufkirchen, Germany) before applying the coverslips. For analysis, the tissue slides were fully scanned using a Leica SCN400 (Leica Microsystems, Wetzlar, Germany) with a magnification of 20×. The expression levels of the analysed proteins were quantified as the proportion of stained tissue within the sample. This assessment was performed using scanned histological images and analysed semi-quantitatively with ImageJ 2.14.0 software, utilizing the color threshold function in accordance with the established protein expression determination protocol (Crowe & Yue, 2019).

4.7.3. Comparison of Antigen Retrieval Methods for Immunohistochemical Analysis of Cartilage Matrix Glycoproteins Using Cartilage Intermediate Layer Protein 2 (CILP-2) as an Example (Paper III)

Paraffin-embedded specimens were cut at a four μm thickness using the microtome Microm Ergostar HM200 (Microm GmbH, Walldorf, Germany), mounted on TOMO® Adhesion Microscope Slides (Matsunami Glass, Kishivada, Japan), and dried overnight on a slide dryer heating plate at a temperature of 37 °C. Four different protocols were compared from the aspect of antigen retrieval: (1) heat-induced epitope retrieval (HIER) without proteolytic-induced epitope retrieval (HIER group), (2) proteolytic-induced epitope retrieval (PIER) without heat-induced epitope retrieval (PIER group), (3) HIER combined with PIER (samples treated with this antigen retrieval method formed the group designated as HIER/ PIER group), and (4) no retrieval at all (control group). Heat retrieval was performed at 95 °C for 10 minutes using a specific heat retrieval (decloaking) solution (Reveal Decloaker, Biocare Medical, Pacheco, CA, USA). Proteolytic retrieval was performed with 30 μg/mL Proteinase K solution (Merck KGaA, Darmstadt, Germany) in 50 mM Tris/HCl, 5 mM $CaCl_2$ solution (pH 6.0) for 90 minutes at 37 °C. Thereafter, the same sections were treated with 0.4% bovine hyaluronidase in HEPES-buffered medium (Fertipro NV, Beernem, Belgium) for three hours at 37 °C. The sections were deparaffinized in xylene and rehydrated in a series of graded ethanol solutions. Sections in groups PIER, HIER, and HIER/PIER received antigen retrieval protocols as described above. At the same time, the control group sections were kept in distilled water. Subsequently, the

sections of all groups were treated similarly. Endogenous peroxidase activity was inactivated by incubating the sections for 15 minutes with freshly prepared 0.6% H_2O_2 (Honeywell Fluka™, Seelze, Germany) dissolved in distilled water. Thereafter, the sections were washed for 3 × 5 minutes in 1 × Gibco™ Phosphate-Buffered Saline (PBS, pH 7.4) solution (Thermo Fisher Scientific, Landsmeer, The Netherlands) and with Dako REAL Antibody Diluent (S2022, Dako Denmark A/S, Glostrup, Denmark) for 30 minutes, to block non-specific binding. Then, the sections were incubated overnight at 4 °C with rabbit polyclonal antibody to CILP-2 (Atlas Antibody, Sweden, anti-CILP-2 cat no HPA041847, dilution 1:100 in Dako REAL Antibody Diluent). To visualize the primary antibody, the sections were incubated with the commercial kit Dako Real™ Envision™ Detection System, Peroxidase/DAB+, Rabbit/Mouse (cat no K5007) (Dako Denmark A/S, Glostrup, Denmark) – the sections were incubated with secondary antibody for one h and after that with DAB for 10 min. Washing 3 × 5 minutes between each step after incubation with the primary antibody was performed in 1× Gibco™ Phosphate Buffered Saline (PBS, pH 7.4) solution (Thermo Fisher Scientific, Landsmeer, The Netherlands) containing 0.07% Tween 20 detergent (BioTop, Naxo, Tartu, Estonia). Negative controls were performed by omitting the primary antibody. To counterstain the cartilage proteoglycans, the sections were stained for one minute with Toluidine blue solution. Finally, the sections were dehydrated with ethanol and xylene and mounted with Eukitt® medium (Sigma-Aldrich GmbH, Steinheim, Germany) before applying the coverslips. For scoring the results, semi-quantitative staining assessment was used, based on the CILP-2 staining extent. The staining scores ranged from 1 to 5 (‘1’: no staining; ‘2’: 1–25% of the slide stained; ‘3’: 26–50% stained; ‘4’: 51–75% stained; ‘5’: 76–100% stained) (Meyerholz & Beck, 2018). Evaluation of the staining scores was performed under a microscope at 400× magnification by two independent observers in a blinded fashion; in the case of the six discrepancies, the specimens were reviewed together to formulate the final assessment.

4.8. Statistical Analysis

Statistical analysis was performed using the GraphPad InStat software version 3.10 or GraphPad Prism version 10.2.3 (GraphPad Software, San Diego, CA, USA). The level of significance was set at $p < 0.05$. In Paper I, Spearman´s rank correlation coefficient was applied to analyse the correlations between the extension of expression of CILP-2 and DDR2 and the degree of cartilage damage (both OARSI histopathology grade and OARSI score). Similarly, in Paper II, Spearman’s rank correlation analysis was used to analyse the correlations between the expression of C2C in the AC and the degree of cartilage damage. In addition, in Paper I, we compared the CILP-2 and DDR2 expression between LTM and MTM in each patient by Wilcoxon’s matched-pairs test, where lower OARSI grade specimens were compared with higher grade counterparts from the same knee.

Mann–Whitney U test was performed to analyse the difference between OA patients' samples with either dominant medial or lateral side damage. In Paper II, we compared the OARSI histological grade, stage, and C2C staining of LTM and MTM by the Wilcoxon signed-rank test. In Paper III, evaluations of CILP-2 staining scores were analysed by applying the Mann–Whitney U-test.

5. RESULTS

5.1. Histopathologic results (Paper I)

20 patients (mean age 61.25±5.32 years) were involved in the first set of the study. The clinical characteristics and histopathology assessment results have been pointed out in Table 4. Advanced OARSI histopathology grades (grades 3 and above) were detected in the samples of eleven patients, while the grades below three were detected in the samples of nine patients. We compared the two sides of the knee joint – the medial and lateral tibial plateaus of each individual. The OARSI histopathology grades were higher in the medial tibial plateau of 12 patients, while in the lateral tibial plateau, higher grades were seen in seven patients. In one instance (patient 20), the histopathology grades were the same for both medial and lateral sites (Table 4). To analyse the differences between samples from OA patients with either dominant medial or lateral side damage, two groups were established: medial tibia (MT) group with higher OARSI grade of the medial tibial plateau (12 first patients in Table 4) and lateral tibia (LT) group with higher OARSI grade of the lateral tibial plateau (patients No 13–19 in Table 4). A tendency to the greater difference between the medial and lateral side of OARSI grades was observed in the MT group compared to the LT group (median difference 1.75 (IQR 0.5–3) and 0.5 (IQR 0.5–1), respectively; Mann–Whitney U test $p = 0.061$).

Table 4. Patients' characteristics and results of histopathology assessments and CILP-2 and DDR2 stainings

No of patient	Gen-der	Age (years)	Macro-scopic damage (stage)	Tibial plateau site	OARSI histo-pathol grade	OARSI score (grade × stage)	Assesment of CILP-2 staining				Assesment of DDR2 staining			
							Super-ficial zone	Middle zone	Deep zone	Average of all zones	Super-ficial zone	Middle zone	Deep zone	Average of all zones
1	F	62	1	LTM	1	1	3	2	2	2.3	2	1	2	1.7
				MTM*	3	3	4	1	1	2	4	2	2	2.7
2	F	57	2	LTM*	2.5	5	3	2	2	2.3	4	3	2	3
				MTM	3	6	5	3	2	3.3	5	4	2	3.7
3	F	65	2	LTM*	1.5	3	2	2	2	2	4	2	2	2.7
				MTM	2	4	5	4	4	4.3	3	2	2	2.3
4	F	63	1	LTM*	2	2	3	2	2	2.3	3	2	1	2
				MTM	2.5	2.5	4	3	1	2.7	5	4	3	4
5	F	66	1	LTM	0.5	0.5	3	1	1	1.7	2	1	1	1.3
				MTM*	2.5	2.5	4	3	2	3	2	1	1	1.3
6	F	65	2	LTM	2.5	5	5	4	3	4	3	2	2	2.3
				MTM	4	8	5	4	3	4	5	2	2	3
7	M	60	2	LTM	2	4	4	2	1	2.3	3	2	2	2.3
				MTM	5	10	-	-	5	5	-	-	3	3
8	F	59	1	LTM	1.5	1.5	5	3	3	3.7	5	4	3	4
				MTM	2.5	2.5	4	2	1	2.3	3	1	1	1.7
9	F	63	2	LTM	1.5	3	3	2	1	2	4	4	3	3.7
				MTM	3	6	3	1	1	1.7	3	1	1	1.7
10	M	65	4	LTM	2.5	10	2	2	1	1.7	3	2	2	2.3
				MTM	5	20	-	5	5	5	-	5	5	5
11	F	64	3	LTM	1.5	4.5	4	3	2	3	4	3	2	3
				MTM	4.5	13.5	4	4	2	3.3	3	2	1	2
12	M	58	4	LTM	2	8	4	3	2	3	3	2	2	2.3
				MTM	5.5	22	-	-	5	5	-	-	5	5

No of patient	Gen-der	Age (years)	Macro-scopic damage (stage)	Tibial plateau site	OARSI histo-pathol grade	OARSI score (grade × stage)	Assesment of CILP-2 staining				Assesment of DDR2 staining			
							Super-ficial zone	Middle zone	Deep zone	Average of all zones	Super-ficial zone	Middle zone	Deep zone	Average of all zones
13	M	46	1	LTM*	2.5	2.5	4	3	2	3	3	2	1	2
				MTM	2	2	4	3	2	3	4	3	2	3
14	M	58	1	LTM*	2.5	2.5	4	2	2	2.7	4	2	2	2.7
				MTM	2	2	4	3	2	3	5	3	1	3
15	M	53	2	LTM*	4	8	4	2	1	2.3	2	2	1	1.7
				MTM	3	6	5	3	1	3	5	4	2	3.7
16	F	64	3	LTM*	4	12	4	3	4	3.7	4	3	2	3
				MTM	1.5	4.5	2	1	1	1.3	3	2	2	2.3
17	M	68	4	LTM	2.5	10	4	3	2	3	4	3	2	3
				MTM*	2	8	3	1	1	1.7	3	2	2	2.3
18	F	58	2	LTM	3	6	5	3	2	3.3	4	2	2	2.7
				MTM	2.5	5	5	2	2	3	3	1	1	1.7
19	M	68	1	LTM	1.5	1.5	5	3	2	3.3	4	2	1	2.3
				MTM	1	1	4	2	2	2.7	3	1	1	1.7
20	F	63	2	LTM	2	4	2	4	3	3	2	2	2	2
				MTM	2	4	2	4	2	2.7	3	1	1	1.7

F – female patients; M – male patients; LTM – lateral tibial plateau, meniscus covered; MTM – medial tibial plateau, meniscus covered; * Site of samples taken for transmission electron microscopy studies

5.1.1. CILP-2 and DDR2 expression in OA cartilage

CILP-2 and DDR2 staining was observed in all the studied samples; the staining appeared predominantly localized in the superficial and intermediate zones (Figures 5, 6). In the deep zone, minimal staining was observed in most instances (Table 4). Nevertheless, in samples with OARSI grade 5 and above (samples 7, 10, and 12; Table 4), which are characterized by the loss of the superficial zone, very extensive CILP-2 and DDR2 staining was noted in the remaining deeper zones of the cartilage. In the deep zone, CILP-2 staining was primarily noted to be localized in PCM (Figure 6).

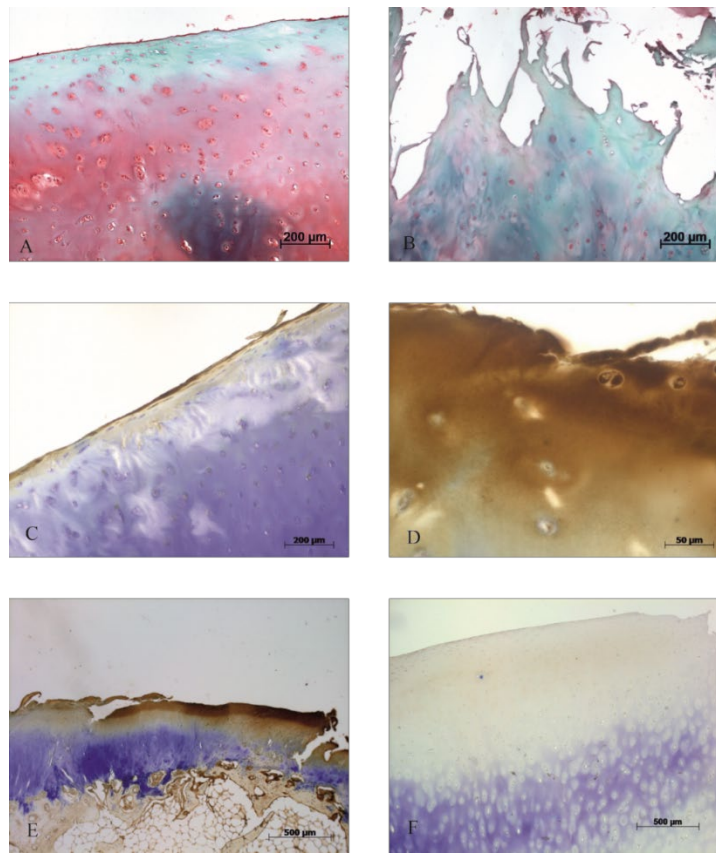

Figure 5. A and B: OA cartilage pathology evaluated according to the OARSI grading system (Safranin O staining). A – Grade 1.5, the AC demonstrates superficial fibrillation. B – Grade 3, vertical fissures extend into the middle zone. C to E: OA AC immunohistochemistry for discoidin domain receptor 2 (DDR-2), counterstained with Toluidine blue. C – Moderate immunostaining in the superficial zone (OARSI grade 2). D – Abundant immunostaining in the superficial and the middle zone (OARSI grade 2.5). E – Abundant immunostaining in the superficial and the upper part of the middle zone (OARSI grade 4). F – No staining in negative control (during the staining antibody to DDR2 was omitted).

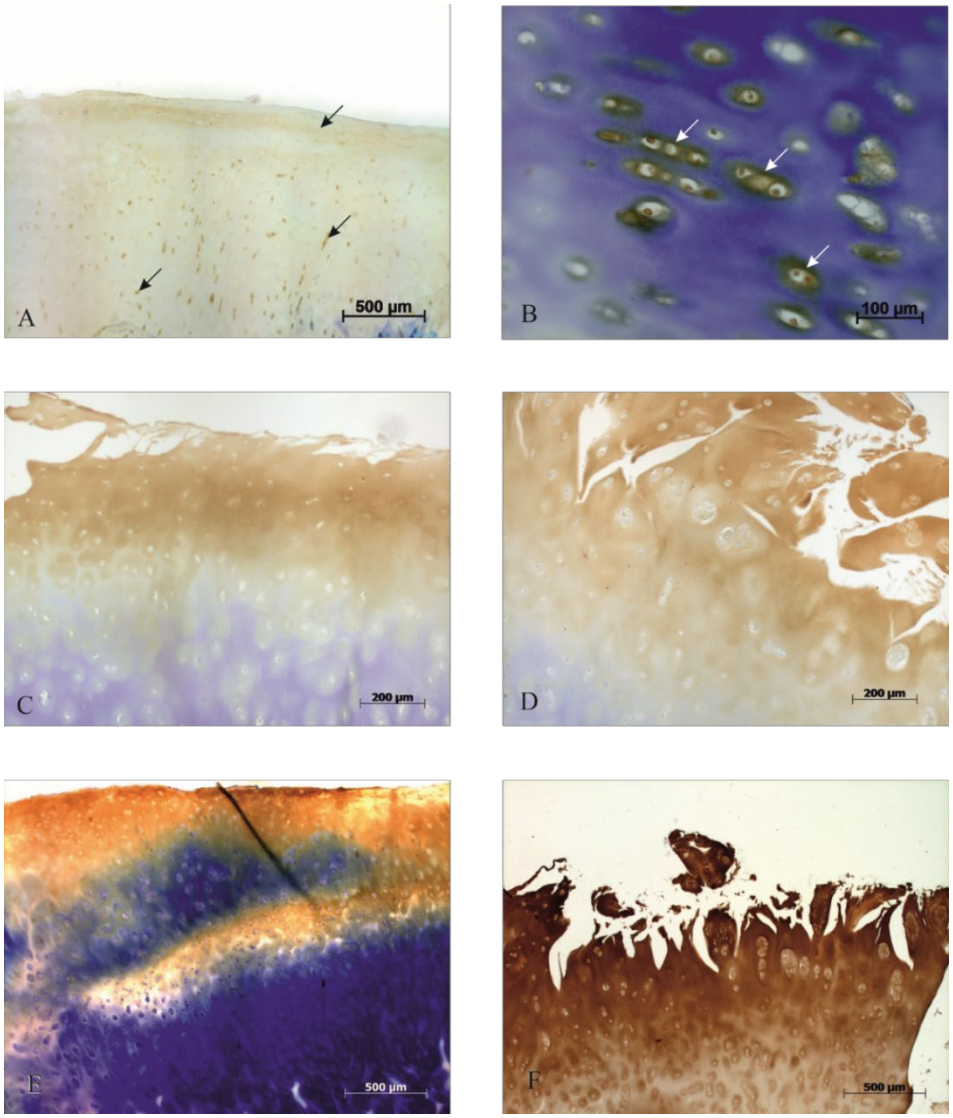

Figure 6. CILP-2 immunohistochemical staining in the OA AC, counterstained with Toluidine blue. A – Arrows indicate moderate staining in the middle and the deep zone (OARSI grade 2). B – Abundant pericellular staining (arrows) in the deep zone (OARSI grade 2). C – Abundant staining in the superficial, the middle, and the upper part of the deep zone (OARSI grade 3). D – Abundant staining in the superficial, the middle, and the upper part of the deep zone (OARSI grade 4). E – Moderate staining in the superficial and middle zone (OARSI grade 0.5). F – Abundant staining in the superficial and middle zone (OARSI grade 3.0).

5.1.1.1. Comparison of CILP-2 and DDR2 staining with the degree of cartilage damage

We first evaluated the correlation between CILP-2 and DDR2 expression (average of IHC staining estimates of all zones) with the corresponding OARSI histopathology grades and OARSI scores (which combine the histopathology grade and the stage of macroscopic tissue damage) in all 40 samples. We indicated a statistically significant medium-strength positive correlation between the extent of CILP-2 staining and OARSI grades $p = 0.005$; Spearman rho = 0.436). Besides, OARSI scores correlated positively but more modestly with CILP-2 staining ($p = 0.044$; Spearman rho = 0.320). No statistically significant correlation was found between DDR2 staining assessments and OARSI grades of 40 samples ($p = 0.079$; Spearman rho = 0.281). However, OARSI scores of joint damage that also includes the macroscopic evaluation of the extent of the joint involvement demonstrated a tendency to positive correlation with the extent of DDR2 staining ($p = 0.0543$; Spearman rho = 0.307).

Unfortunately, we could not obtain knee AC samples without pathological changes to form a comparative 'control' group. Therefore, we divided the specimen pairs, medial and lateral probes of the same knee, into two groups based on OARSI histopathology grades. The hypothesis was that the expression of DDR2 and CILP-2 differs in samples with high OARSI grade compared to the counter side low-grade samples, i.e. DDR2 and CILP-2 expression pattern reflects the severity of cartilage damage. Thus, two groups were formed, which consisted of medial and lateral probes of the same knee. The specimen with a lower OARSI histopathology grade of the pairs was included in the low OARSI grade group, and the specimen with a higher OARSI grade was assigned to the high OARSI grade group. For instance, the LTM sample of patient no. 1 with grade 1 was included in the low OARSI grade group, while the MTM sample of the same patient with grade 3 was included in the high OARSI grade group. The high grade group with median grade 3 (grades remained between 2.5–4) and the low grade group with median grade 2 (grades remained between 1.5–2.5) differ significantly as the Wilcoxon matched-pairs signed-ranks test p value is < 0.0001.

The groups differed by median OARSI grades significantly (Wilcoxon matched-pairs signed-rank test p value <0.0001). Patient 20, who had equal OARSI histopathology grades on both the medial and lateral sides, was not included in the estimation. In samples from three patients (No 7, 10, and 12), the superficial zone and samples from two patients (No 7 and 12), the middle zone was not possible to assess. We observed a more abundant CILP-2 staining in the samples with a high OARSI grade group in the superficial and middle zones, as well as in all layers in sum compared to the low OARSI grade group (Figure 7). No statistically significant difference in DDR2 expression between the low and high OARSI grade groups was found.

Besides, we analysed the correlation between CILP-2 and DDR2 expression (based on the average IHC staining estimates across all zones) in the samples of all 40 patients. Our findings revealed a statistically significant but medium-

strength positive correlation (p = 0.006; Spearman rho = 0.422). This demonstrates the concurrent upregulation of both markers in advanced stages of kOA.

5.1.1.2. CILP-2 and DDR2 expression in MT and LT groups

We analysed CILP-2 and DDR2 expression comparatively in groups of predominant medial (MT group; n = 12) or lateral side damage (LT group; n = 7). In the groups, both medial and lateral tibial plateau sites were analysed. Therefore, 24 samples were evaluated in the MT group, and 14 samples were assessed in the LT group. In MT group, the extent of CILP-2 staining had a positive correlation with OARSI grades (p = 0.018; Spearman rho = 0.480) and stages (p = 0.023; Spearman rho = 0.463), while no correlations with OARSI grades or stages were found with DDR2 staining (p = 0.147; Spearman rho = 0.305 and p = 0.070; Spearman rho = 0.377, respectively). In the LT group, no correlation of OARSI grades and OARSI stages with CILP-2 (p = 0.244; Spearman rho = 0.335 and p = 0.857; Spearman rho = 0.053, respectively) or DDR2 staining (p = 0.417; Spearman rho = 0.236 and p = 0.438; Spearman rho = 0.225, respectively) were found.

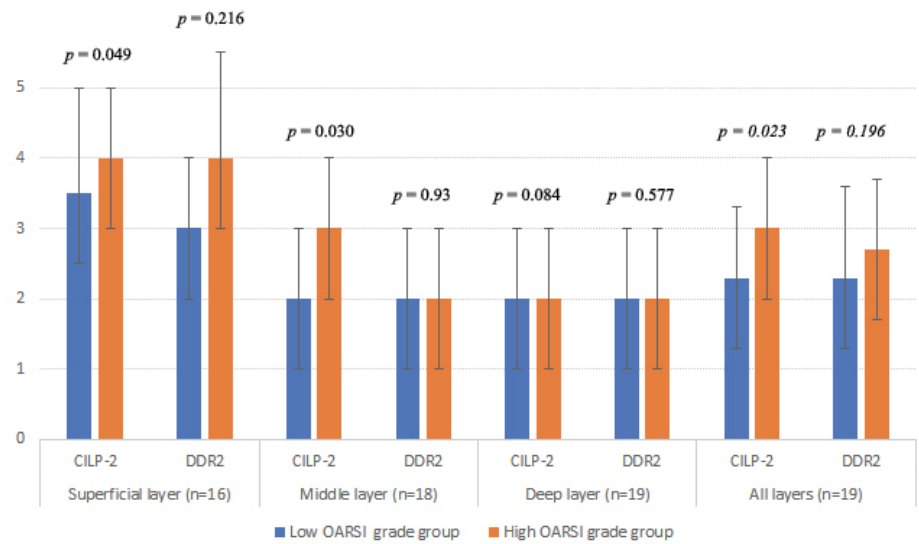

Figure 7. Comparisons of assessments of CILP-2 and DDR2 immunohistochemical (IHC) staining in groups with low (median grade 2, interquartile range 1.5–2.5) and high (median grade 3, interquartile range 2.5–4) OARSI histopathology grades. To compare the lower OARSI grade specimens with higher grade counterparts of the same knee, the specimens of the medial and lateral sides were divided into low and high OA grade groups. The X-axis presents the medians of IHC (error bars indicate 25% and 75% interquartile range). *P*-value of Wilcoxon's signed rank test is shown for each comparison.

5.1.2. Transmission electron microscopy of OA cartilage (Paper I)

10 patients' samples from 20 patients were randomly selected for TEM investigations (sampling site is indicated by an asterisk in Table 4). Specimens were obtained from the superficial zone of the AC. TEM investigations indicated that most cells had a rounded or elongated shape, while spindle-shaped cells were rarely observed (Figures 8, 9). The cells were often found as single units, with only a few isogenic groups observed (Figure 8). The cell membrane of chondrocytes was generally smooth, although some cells exhibited short cytoplasmic processes (Figure 8B). The nuclei of chondrocytes were either spherical or ovoid, with visible nuclear indentations in some cells. Disrupted location of chromatin or chromatin-poor nuclei were often seen (Figures 8, 9). Autophagic vacuoles and lipid droplets were noticed in many cells (Figures 8, 9). Mitochondria appeared swollen and rounded, exhibiting damaged cristae, the rER was observed in varying amounts, regularly arranged stacks of cisterns of rER were observed in some cells, and in some cells, cisterns were irregularly enlarged (Figures 8, 9). Shifts in the cytoskeleton were observed in several cells; in remarkable cases, the cytoplasm adjacent to the nucleus contained large aggregates of cytoskeletal filaments occupying significant areas of the cell (Figure 8). The features described above can be considered characteristic of chondroptosis. It was noteworthy that degenerative, highly vacuolated cells with a round or ovoid shape were frequently observed. In specimens with advanced OARSI grades (Grade 4), completely disintegrated cells were often observed (Figure 8). In the ECM, numerous short collagen fibres were oriented in various directions. However, the fibres observed to run parallel often alternated with fibres encountered in the section plane transversely. The collagen fibres in the PCM were oriented both in parallel and in an irregular pattern, creating a densely intertwined network (Figure 2). Moreover, in various samples, enlargement of the PCM was seen, which in some cases resulted in highly variable expansions. For example, in the AC sample with QARSI grade 4, heavily vacuolated cells were seen to be surrounded by very irregular PCM (Figure 9). Thus, the rearrangement of PCM seems to be a prominent feature of OA cartilage.

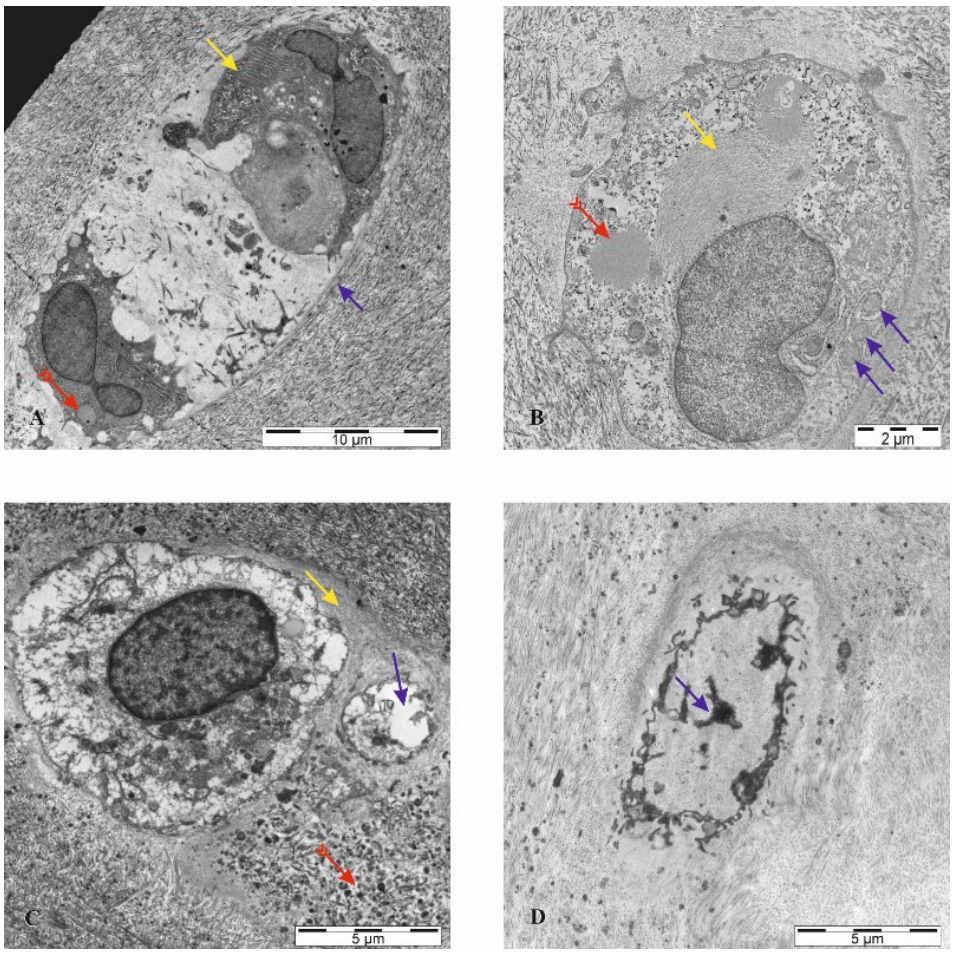

Figure 8. Transmission electron micrographs of OA chondrocytes. A – Chondrocyte isogenic group. Collagen fibres around an isogenic group of cartilage cells (blue arrow), endoplasmic reticulum (ER) (yellow arrow), autophagic vacuoles (two-tailed red arrow) (OARSI grade 2.5). B – Autophagic vacuoles in chondrocyte (blue arrow), well-developed cytoskeleton (yellow arrow), and lipid droplets (two-tailed red arrow) (OARSI grade 2.5). C – Remnant of a chondrocyte (blue arrow), collagen fibres around an isogenic group of cartilage cells (yellow arrow), two-tailed red arrow indicates chondrocyte debris within lacunae (OARSI grade 2.5). D – Completely disintegrated chondrocyte (blue arrow) (OARSI grade 4).

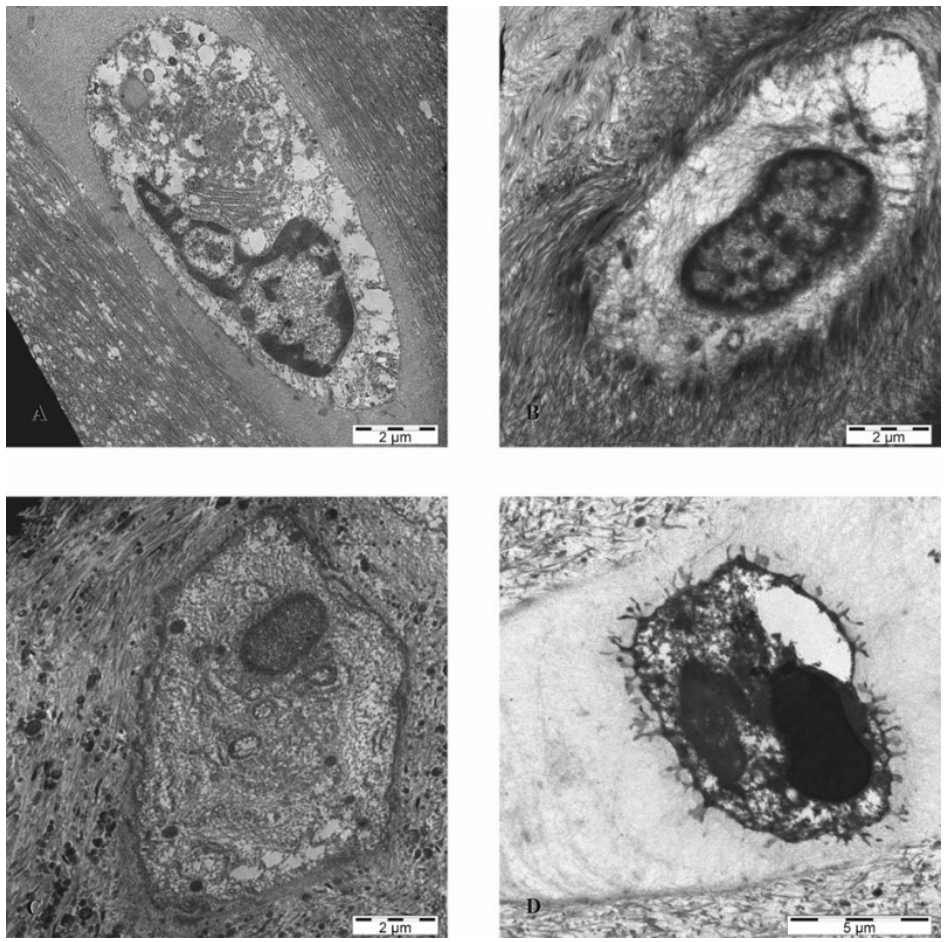

Figure 9. Transmission electron micrographs of OA chondrocytes. A – Highly vacuolated cytoplasm, reduced organelles, pericellular matrix (PCM) expansion (OARSI grade 4). B – Complete vacuolization of the cytoplasm with vanished organelles, fibrillar rearrangement of the pericellular matrix (OARSI grade 4). C – Vacuolated cytoplasm, reduced organelles (OARSI grade 2). D – Enlargement of the PCM (OARSI grade 4).

5.2. Results of histopathology assessments and C2C staining (Paper II)

In this study, we correlated the degree of AC damage in OA patients with C2C expression in histological samples of tissues removed during TKA. The key findings of this study include the histopathology grade of the tissue damage, the stage of macroscopic damage of the tibial plateau, the kOA score, and the proportion of the tissue sample stained positively for C2C depending on the tissue damage detected (summarized in Table 5).

The histopathology grades of kOA, based on OARSI indicators, ranged from 0 to 6.5. When comparing the LTM and MTM samples of the same patient, the OARSI histopathology grades differed in most cases (24 from 27; 89%); equal grades were observed in only three patients. Higher histopathology grades were mostly observed for the medial side of the joint – in 17 cases, MTM samples showed higher grades. In only six cases, LTM samples had higher grades as compared to the opposite side. The OARSI histopathology grades and scores of MTM side samples were significantly higher than on the LTM side ($p = 0.021$ and $p = 0.010$, respectively, Wilcoxon signed-rank test). Moreover, the percentage of C2C staining on the MTM side was larger than that on the LTM side ($p = 0.022$, Wilcoxon signed-rank test) (Table 5).

Representative micrographs are presented in Figure 10 to constitute histopathology grades 0, 1.5, 3.5, and 6. Additionally, a positive weak correlation between the percentage of C2C immunohistochemical expression and the OARSI histopathology grade (Spearman rho = 0.289 (corrected for ties); $p = 0.0356$; Figure 10, Table 5) was discovered. Nevertheless, this correlation did not remain significant when calculated only for females (n = 17) (Spearman rho = 0.189 (corrected for ties); $p = 0.285$) or for males (n = 10) (Spearman rho = 0.369 (corrected for ties); $p = 0.121$). Moreover, a significant positive correlation was found between the OA score (combining the histological grade of damage with the OA macroscopic stage) and the percentage of C2C staining in the samples (Spearman rho = 0.388 (corrected for ties); $p = 0.0041$). This correlation remained positive among the female participants (Spearman rho = 0.349 (corrected for ties); $p = 0.043$), but not in the male subgroup (Spearman rho = 0.382 (corrected for ties); $p = 0.107$). Our findings indicate indeed that the amount of tissue stained for C2C may be associated with cartilage damage (see Figure 10). Besides, it is worth noting that the OARSI histopathology grade and OARSI score correlated with patients' ages (Spearman rho = 0.293 (corrected for ties); $p = 0.033$ and Spearman rho = 0.358 (corrected for ties); $p = 0.0085$, respectively), however the percentage of C2C staining in the samples did not (Spearman rho = 0.169 (corrected for ties); $p = 0.227$).

No correlation was found between the proportions of C2C tissue staining of LTM and MTM samples (Spearman rho = 0.039 (corrected for ties); $p = 0.84$). It could be because more profound damage and, therefore, more intense staining are usually on one (lateral or medial) side of the tibial plateau.

There was no correlation between the extent of C2C staining and the Kellgren/Lawrence score.

Table 5. Results of histopathology assessments and C2C staining of samples from 27 patients (10 male and 17 female patients; in each subject, two load-bearing sites of the tibial plateau were analysed).

Patients' Age (in Years)	Tibial Plateau Site	OARSI Histo-Grade	OARSI Score (Grade × Stage)	Assessment of C2C Staining, %
59 (58–64) Min–max = 55–66	LTM + MTM (n = 53) *	3.0 (2.5–4.3) [a] Min–max = 0–6.5	8.0 (5.0–10.5) [b] Min–max = 0–26	73.2 (53.2–89.5) [a,b] Min–max = 16.2–100
	LTM (n = 27)	2.5 (2.0–3.5) [c] Min–max = 0–6	6.85 (4.6–10.4) [d] Min–max = 0–13.5	60.4 (49.0–84.6) [e] Min–max = 16.2–96.1
	MTM (n = 26) *	3.7 (2.6–4.5) [c] Min–max = 1.5–6.5	8.0 (5.3–13.5) [d] Min–max = 2–26	79.6 (68.1–94.2) [e] Min–max = 42.3–100

Data are presented as median with interquartile range in brackets; min–max—minimal and maximal values. LTM—lateral tibial plateau, meniscus-covered area biopsy; MTM—medial tibial plateau, meniscus-covered area biopsy. [a] Positive correlation of the percentage of C2C immunohistochemical expression with the OARSI histopathology grade (Spearman rho = 0.289, corrected for ties, $p = 0.0356$) and [b] with the OARSI score (Spearman rho = 0.388, corrected for ties, $p = 0.0041$). [c] Significant differences of OARSI histo-grades between LTM and MTM (the two-tailed $p = 0.021$; Wilcoxon signed-rank test), [d] significant differences of OARSI score between LTM and MTM (the two-tailed $p = 0.010$; Wilcoxon signed-rank test) and [e] significant difference of C2C staining between LTM and MTM (the two-tailed $p = 0.022$; Wilcoxon signed-rank test). * Due to technical problems, no MTM sample was obtained from one patient.

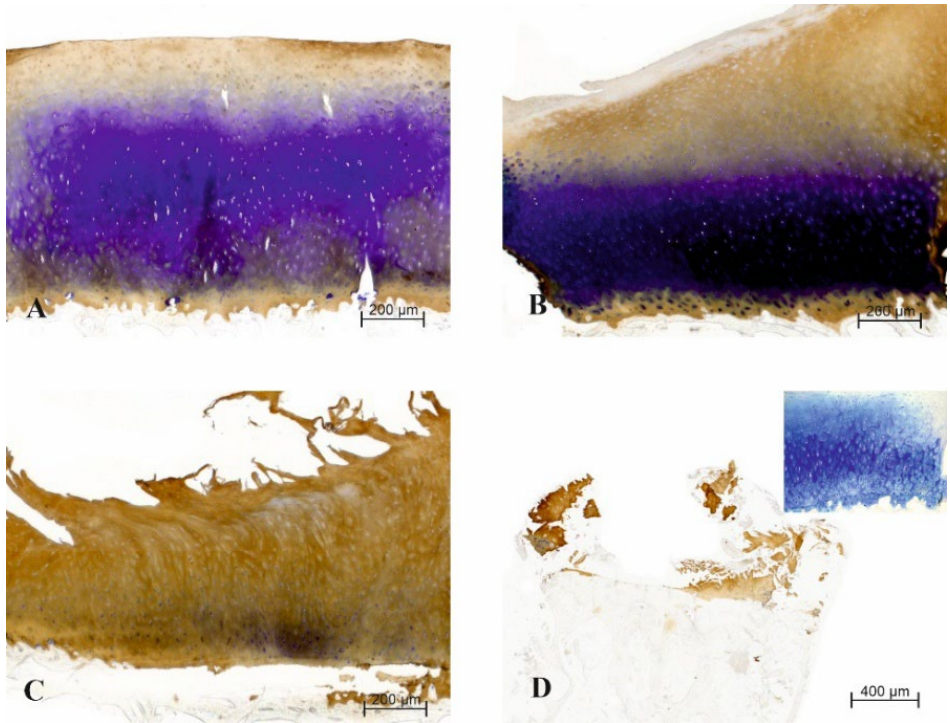

Figure 10. A – Micrograph of a sample with histopathology grade 0: weak C2C immunostaining (brown) in the superficial and deep zones (counterstaining of proteoglycans with Toluidine blue). The cartilage content of proteoglycans, determined by the amount of Toluidine blue staining, was decreased in the tissue samples with more advanced OARSI tissue damage grade. B – Micrograph of a sample with histopathology grade 1.5: moderate C2C immunostaining (brown) in the superficial and upper parts of the middle zone (counterstaining of proteoglycans with Toluidine blue). C – Micrograph of a sample with histopathology grade 3.5: overall abundant C2C immunostaining (brown), weak counterstaining in the deep zone (counterstaining of proteoglycans with Toluidine blue). D – Micrograph of a sample with histopathology grade 6: abundant C2C immunostaining (brown) in the remaining cartilage; subchondral bone, which occupies most of the specimen, is virtually not stained (counterstaining of proteoglycans with Toluidine blue). A sample picture of negative control, where the primary antibody was omitted, is presented as an inset in the upper-right corner of micrograph D. All micrographs were taken using a 5× objective.

5.3. Comparison of Antigen Retrieval Methods for Immunohistochemical Analysis of Cartilage Matrix Glycoproteins Using Cartilage Intermediate Layer Protein 2 (CILP-2) as an Example (Paper III)

In this study, we compared different antigen retrieval methods to improve the outcome of IHC performed on OA cartilage obtained from TKA. We tested four different antigen retrieval protocols. The study showed clearly that CILP-2 IHC scores differ significantly depending on various combinations of antigen retrieval methods (Figure 11).

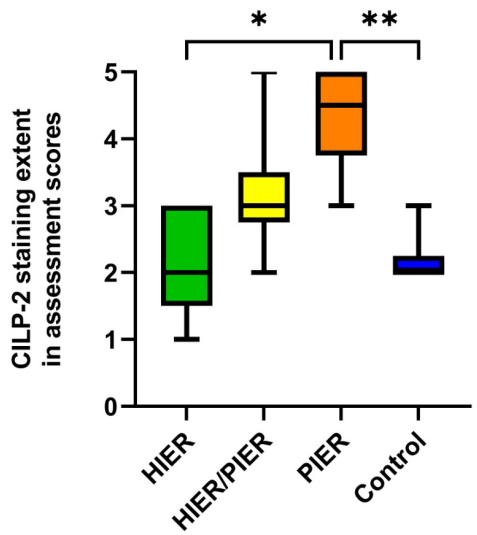

Figure 11. Comparative assessment of CILP-2 IHC staining in four study groups. The groups were formed as follows: HIER group (heat-induced epitope retrieval without proteolytic-induced epitope retrieval), HIER/PIER group (proteolytic-induced epitope retrieval with heat-induced epitope retrieval), PIER group (proteolytic-induced epitope retrieval without heat-induced epitope retrieval), and control group (no antigen retrieval). Grades represent CILP-2 staining extent in assessment scores. Box–whiskers plot with 5th–95th percentiles. Significant differences by the Mann–Whitney U-test: * PIER group vs. HIER group ($p = 0.012$), and ** PIER group vs. control group ($p = 0.004$).

The PIER group exhibited the highest staining scores, with extensive IHC staining of CILP-2 observed throughout the slide (Figure 12C). Regarding CILP-2 staining grades, the PIER group exhibited the highest levels, followed by the HIER/PIER group, which showed moderate CILP-2 staining (Figure 11 and Figure 12A, C, respectively). In the residual two groups, HIER and control, CILP-2 staining grades were similarly low (Figure 11). Immunostaining was visible only in the HIER group's superficial layer and in the control group's superficial and middle layers (Figure 12 B, D).

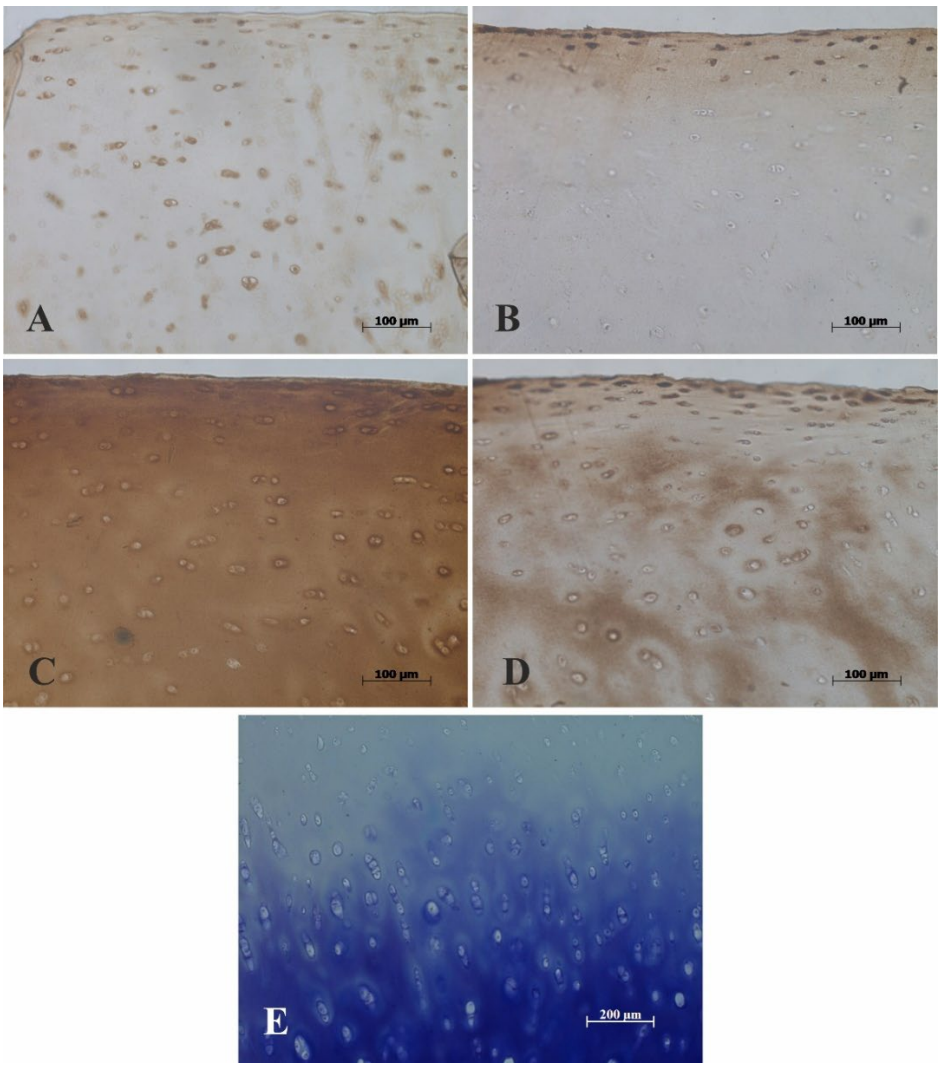

Figure 12. Examples of the effect of different antigen retrieval methods on CILP-2 immunohistochemical staining. A – HIER/PIER group, some immunostaining in chondrocytes, imperceptible immunostaining in ECM. B – HIER group, moderate immunostaining in the superficial zone. C – PIER group, abundant immunostaining. D – Control group (no retrieval at all)—moderate immunostaining in the superficial zone and the middle zone. Toluidine blue was used for counterstaining. E – Image of negative control where the primary antibody was omitted.

The CILP-2 staining grades for the two groups that did not undergo proteinase treatment during epitope retrieval were significantly lower compared to the PIER group ($p = 0.004$ vs. control group and $p = 0.012$ vs. HIER group; Mann–Whitney U-test, Figure 11). No statistical differences were observed between groups HIER/PIER and HIER ($p = 0.069$) and groups HIER/PIER and control ($p = 0.084$). Therefore, in the settings of this study, heat induction did not improve antigen availability, as the results of CILP-2 immunostaining scores were similar in the control and HIER groups. Besides, it is important to note that heat retrieval with the application of high temperature caused the frequent loosening of sections, which did not occur with proteinase retrieval.

One complementary morphological finding was the distinctive appearance of CILP-2 staining observed in the territorial matrix around the isogenous groups of chondrocytes in the PIER and HIER/PIER groups (see Figure 12 A, C, and Figure 13). Such staining was not present in only the HIER-treated and control sections.

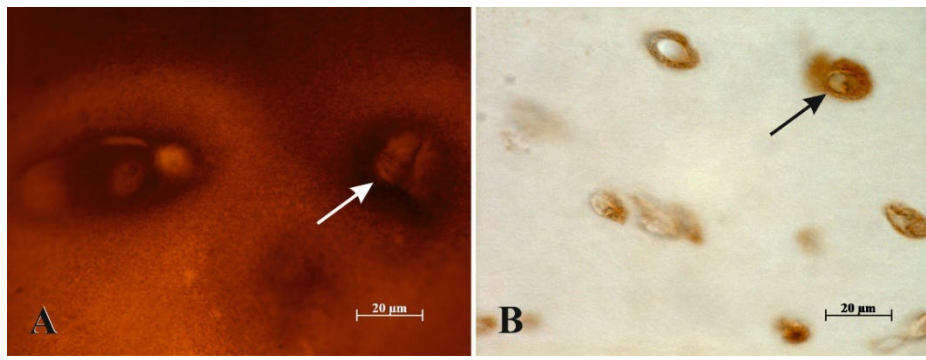

Figure 13. CILP-2 immunohistochemical staining – brown indicates positive staining. A – PIER antigen retrieval. B – HIER/PIER antigen retrieval. CILP-2 staining is noted in the territorial matrix around the isogenous groups of chondrocytes in images A and B. The examples are indicated by arrows. The staining of the rest of the tissue matrix is lost in the group that also received heat treatment (image B), but intensive staining around the isogenous groups remains evident in both images.

No specific staining was observed in the negative controls where the primary antibody was omitted.

6. DISCUSSION

The present study was undertaken to correlate potential OA markers in the AC with the degree of cartilage damage in kOA, applying both OARSI grading and staging. In recent years, several cartilage matrix breakdown products as OA markers have been evaluated in readily accessible biological samples, such as urine and blood serum. However, there is a clear need to detect putative biomarkers first in the AC to ensure that the tissue-level changes correspond to the actual tissue damage and the grade of OA. It is important to acknowledge that some ECM degradation products are not exclusively specific to cartilage. Although these molecules are found in AC, they may also be present in various other tissues, and consequently, the excreted biomarkers may not originate solely from cartilage.

It has to be emphasised that very few histopathological studies specifically address the tissue-level expression of putative markers in the knee AC. Although biopsy-level assessment of cartilage damage for OA evaluation is not feasible in clinical practice, we propose that research on potential OA biomarkers should prioritise investigations at the tissue level. This approach would provide a more accurate representation of the biomarkers' involvement and role in the pathogenesis of cartilage degradation. Therefore, in the present study, we evaluated selected OA markers such as CILP-2, DDR2 (Paper I), and COL2-C2C (Paper II) in the AC. We compared the proportion of tissue staining of these biomarkers with OARSI histopathology results, which reflected morphological changes in AC. Besides, in Paper I, we compared OARSI histopathology grades obtained from the medial and lateral tibial plateaus of each individual and observed ultrastructural changes in the AC using TEM. In Paper III, we compared different antigen retrieval methods to improve the outcome of IHC performed on AC, using CILP-2 as an example.

6.1 OARSI histopathology grades and predominant side of tibial plateau damage of OA patients undergoing total knee arthroplasty

We analysed samples from twenty patients undergoing TKA to characterise OA-induced changes in the AC. Advanced OARSI histopathology grades (grades 3 and above) were determined in most of the patients' samples. However, in nine patients' samples, OARSI grades did not exceed grade 3, demonstrating that not all patients undergoing TKA have far-advanced cartilage damage. Persistent pain, forcing patients to seek TKA, is not linearly dependent on the extent of cartilage destruction, as OA pain is known to be initiated from free axonal endings located in the synovium, periosteum, subchondral bone, and tendons, but not in the cartilage (Perrot, 2015).

We obtained two punches from each subject from the load-bearing sites of the medial and lateral tibial plateau, allowing us to conduct side comparisons. The analysis of the OARSI histopathology grades of the two sides demonstrated higher grades more frequently in the medial tibial plateau than in the lateral tibial plateau (12 vs seven cases; in one case, histopathology grades were equal for both sides). Predominant damage of the medial tibial plateau of OA patients is commonly reported (Raju et al., 2012; Stoddart et al., 2021; Piccolo et al., 2023), whereas Barrett et al in a radiographic study of kOA reported that in 63% of cases, degenerative changes were found in the medial compartment (Barrett et al., 1990). Furthermore, our findings indicated a trend toward an increasing disparity in OARSI histopathology grades between the medial and lateral compartments in patients exhibiting predominant medial compartment degeneration. Conversely, in patients with predominant lateral compartment degeneration, the disparity between the two sides was less pronounced. Therefore, the predominant lateral side damage group with a small difference between the sides may represent the 'non-classical pattern' of kOA. The differential contribution of the medial and lateral sides of the joint to symptom manifestation in kOA has also been shown in a study, which found that lateral compartment kOA was associated with a higher risk of pain and increased inflammation assessed by high-sensitivity C-reactive protein (hsCRP) (Nigoro et al., 2021). Moreover, in two large prospective cohort studies of kOA, risk factor profiles for medial and lateral compartment radiographic kOA were found to be different – obesity and varus malalignment were found to be more strongly associated with medial compartment kOA, whereas older age, female sex, and valgus knee malalignment were more strongly associated with lateral compartment kOA (Wei et al., 2019).

6.2. CILP-2 as a potential marker of OA progression

In our study, we correlated CILP-2 and DDR2 expression in the AC with the degree of OA-induced histopathological damage. If DDR2 is already known to correlate with the degree of human knee cartilage destruction (Sunk et al., 2007; Suutre et al., 2015), then CILP-2 is considered a promising novel biomarker shown in human kOA to relate to radiographically detected cartilage thickness loss (Boeth et al., 2019). CILP-2 is a novel ECM protein which is expressed by articular chondrocytes (Bernardo et al., 2011). Bernardo et al found that CILP-2 appeared to be more localized in the deeper intermediate zone of the AC ECM at maturity (Bernardo et al., 2011). In our study, we found CILP-2 expressed in the superficial zone, in the middle zone, and in the deep zone, predominantly in the PCM. Therefore, CILP-2 staining in our samples of advanced OA was not restricted to the intermediate layer as described in the original studies that gave the specific name to the protein. Nevertheless, we found more abundant CILP-2 staining in the middle zone in samples with high OARSI grades, i.e. equal or exceeding grade 3. More importantly, we found extensive CILP-2 staining in samples with advanced AC damage and detected a positive correlation of

CILP-2 expression with both OARSI histopathology grades and stages. Furthermore, differences in CILP-2 staining were found when patients were divided into low and high OARSI grade groups: a significantly increased CILP-2 staining was noted in the superficial and middle zones but not in the deep zone in the group of high OARSI grade. Also, differences in CILP-2 staining proportions were observed when patients were categorized based on the predominant site of tibial plateau damage. In the group with predominant medial compartment damage (MT group), CILP-2 staining exhibited a positive correlation with both OARSI grades and macroscopic joint surface damage scores. However, no such correlation was seen in the group of predominant lateral side damage (LT group). Thus, it can be further speculated that CILP-2 expression differences in these groups may support the notion of a 'classical pattern' of the kOA associated with medial tibial plateau involvement, whereas lateral tibial plateau involvement may represent a 'non-classical pattern'.

The already acknowledged OA marker DDR2 is considered a cell surface signalling molecule, accelerating the process of AC damage by upregulating the expression of MMPs (Sunk et al., 2007; Xiao et al., 2021). We found DDR2 staining predominantly in the superficial and middle layers of cartilage of the TKA patient. However, in samples of advanced OARSI grades, extensive DDR2 staining was seen in the deep layer, similar to the staining pattern of CILP-2. The tendency of increased proportion of cartilage staining for DDR2 was seen in the case of higher cartilage damage; however, the difference between the low and high OARSI grade groups was not significant. Also, no correlations were found between DDR2 staining and OARSI grades ($p = 0.079$) or scores ($p = 0.054$). Furthermore, no differences in DDR2 expression were recorded when patients were divided into the MT and LT groups with predominant medial or lateral side damage, respectively. Still, the positive correlation found between DDR2 and CILP-2 expression supports the similar potential of these two OA markers.

Based on these findings, CILP-2 may be considered a better potential marker of OA-related tissue damage than DDR2. This is supported by other researchers suggesting that CILP-2 may be associated with the progression of OA (Bernardo et al., 2011; Boeth et al., 2019). However, contradictory results have been reported. Bernardo et al found that CILP-2 is reduced during cartilage degeneration in a mouse OA model (Bernardo et al., 2011). Vice versa, the proteomics studies revealed an increased amount of CILP-2 in OA hip and knee AC samples obtained during joint replacement operations (Hosseininia et al., 2019; Hsueh et al., 2016). Boeth et al proposed CILP-2 to be a collagen degeneration serum biomarker indicating early development of kOA, as serum CILP-2 levels were found to be related to MRI-detected cartilage thickness loss in individuals with a 30-year history of high-impact training in weight-bearing sports (Boeth et al., 2019). On the other hand, Lorenzo et al (Lorenzo et al., 2004) demonstrated an upregulation of CILP, likely CILP-1, in both early and late stages of human kOA. Hence, more studies are needed to understand the functional role of CILPs in OA AC. As CILP-1 and CILP-2 are approximately 50% homologous in their structure, it is important to note that early reports regarding CILP expression in tissues can

sometimes complicate the differentiation between the two CILP isoforms. CILP protein was first described in 1998 (Lorenzo et al., 1998), and only in 2003 existence of two CILP isoforms, CILP-1 and CILP-2 was reported (Johnson et al., 2003). Thus, it may not always be clear which CILP isoform was analysed in tissues in studies published before 2003. This is particularly relevant in the context of immunological methods, as the two isoforms are similar and may share the same epitope for the antibody (Bernardo et al., 2011; Johnson et al., 2003). The information in these publications about specific epitopes used for producing the antibodies is limited.

6.4. Possible role of pericellular matrix in the initiation of OA and characteristic ultrastructural changes in the osteoarthritic articular cartilage

It is assumed that the pathogenetic processes of OA cartilage start in the ECM, and it has been proposed that the onset of OA may be initiated in the PCM, a thin layer surrounding chondrocytes, which mediates biochemical and biomechanical signals (Guilak et al., 2018). The molecular cascade of events leading to degradation of the PCM is believed to involve upregulation of different molecules, such as TGF-β1, DDR2, and MMP13, an ECM degrading enzyme (Xu & Li, 2020). In our TEM investigations, we observed characteristic dilatations or irregular arrangement of the PCM in samples with higher OARSI grades. Similarly, Lee and co-authors, who utilized enzymatically isolated chondrons form normal and OA human cartilage, reported the higher incidence of enlarged PCM in OA cartilage (Lee et al., 2000). Regarding CILP-2 IHC staining, we detected it in the ECM but specially also in the PCM. These findings align with the ultrastructural analysis conducted by Bernardo et al., who proposed that CILP-2 may be associated with collagen VI microfibrils in the PCM, as CILP-2 was shown to co-localized with collagen VI suprastructures, allowing to hypothesize that CILP-2 may mediate interactions between matrix components in the territorial and inter-territorial AC matrix (Bernardo et al., 2011).

Our TEM studies of the superficial zone of the AC demonstrated strongly damaged chondrocytes being more obvious in specimens with higher OARSI grades. Many cells were highly vacuolated, lipid droplets were frequently seen, and the organelles were often diminished or focally aggregated, e.g. large areas with enlarged cisterns of rER and expanded areas of aggregated cytoskeletal filaments were seen. It has been proposed that alterations in the cytoskeleton may be related to the modification of the cell's synthetic activity, which in turn leads to a change in the cell's shape. The transformation of the spindle-shaped chondrocytes in the superficial layer of the cells to a rounded or elongated shape is thought to represent the acquisition of the phenotype characteristic of the chondrocytes in the reparative or degradative phase of OA progression (Kouri & Lavalle, 2006). This hypothesis is supported by the findings of our study where most of the

chondrocytes from the superficial zone of the cartilage had a rounded or elongated shape, and as stated above, the cells had several characteristic features of degenerative cells. This may also be associated with the loss of the superficial layer, as described by Basso et al. (Basso et al., 2020). Reorganization of the cytoskeleton (Kouri & Lavalle, 2006), as well as dilatation of cisterns of rER referred to as ER stress (Rellmann et al., 2021), are thought to activate chondroptosis, a variant of classical apoptosis which leads to chondrocytes' death (Roach et al., 2004). In our samples, many cells were seen with enlarged areas of rER and cytoskeletal filaments; the nuclei of these cells were with patchy chromatin condensation, features considered characteristic of chondroptosis. Furthermore, we saw cell remnants and vesicular debris in the cell lacunae, another feature typical of the final stages of chondroptosis (Roach et al., 2004).

6.5. Cartilage collagen neoepitope C2C as a molecular marker of the severity of knee osteoarthritis

The breakdown of collagen in the AC is considered a critical and possibly irreversible point in the progression of OA (Eyre, 2002). MMPs can cleave the natural triple-helix structure of collagen (Vincenti & Brinckerhoff, 2002), producing two new peptides, three-quarter-length and one-quarter-length of mature Col2, with specific neoepitopes at cleavage sites (Bay-Jensen et al., 2022; Bay-Jensen et al., 2008). Such neoepitopes include the C-terminal telopeptide of type II collagen (CTX-II), COL2-3/4C Short (C1,2C), matrix metalloproteinase (MMP)-derived Col2 neoepitope (C2M), and Col2 C-terminal cleavage neoepitope (C2C). The latter neoepitope was of interest to us as increased urine levels of C2C were correlated with radiographic grade of kOA in several studies (Tamm et al., 2014; Poole et al., 2016; Cibere et al., 2009; Kraus et al., 2017), suggesting that C2C may serve as a potential diagnostic marker for OA development. However, it is important to examine the expression and localization of the C2C neoepitope directly in damaged cartilage, considering that other tissues in the body may contribute to its excretion. Such a type of investigation is of significance not only for C2C but also for other biomarkers to establish their pathogenetic relevance in the context of the disease (Poole et al., 2016; Torga et al., 2024).

When assessing C2C as a potential marker, it is crucial to demonstrate the protein in histological samples, which offers the strongest evidence for establishing a correlation between the actual tissue damage and the levels of the biomarker in the cartilage. It should be pointed out that it was the first time this C2C antibody was used in IHC-P experiments. Thanks to collaboration with IBEX, we managed to get the antibody employed in the IB-C2C-Human Urine Sandwich Assay (IB-C2C-HUSA™) for our immunohistochemical experiments. As this antibody has not been commercially available for IHC studies, we had a unique situation to demonstrate C2C expression in tissues. Several studies have effectively utilised IB-C2C-HUSA™ to trace C2C in the urine of kOA patients, revealing a positive association with the degree of cartilage degradation,

reporting also increased levels to positively associate with the risk of emergent kOA, its onset and progression, particularly in females (Poole et al., 2016; Kuhi et al., 2020; Kuhi et al., 2021). The antibody specific to this type of peptide has been validated through Western blot analysis (Poole et al., 2004). The appropriate staining pattern of the cartilage tissue matrix, observed with the antibody, along with the finding that the proportion of tissue stained for C2C correlated with the tissue damage, ensured evidence that in IHC experiments, the proper epitope was detected with the antibody. C2C expression was detected in all the regions of AC, including the superficial zone, the mid zone, the deep zone, the tidemark area, and the zone of calcified cartilage. The subchondral bone was generally not stained.

It is worth emphasizing that, even though detected and measured in body fluids, the investigation and quantification of neoepitopes in AC have been the subject of limited attention within the existing literature, resulting in a restricted pool of studies available for consideration. A correlation between another neo-epitope, CTX-II, staining and tissue damage has been described in patients with femoral head necrosis (He et al., 2019). Neoepitope COL2-3/4C Short (C1,2C) has been detected in the AC of mice, while neoepitope C2M has been identified in the AC of humans. However, there is limited evidence regarding alterations in their expression and their relationship to tissue damage (He et al., 2019; Thomas et al., 2015). Poole et al. introduced an antibody designed to target neoepitope C2C, but their study did not undertake an exploration of potential immunohisto-chemical applications, as focused on the quantification of the C2C in patients' serum and urine samples using the ELISA test (Poole et al., 2004). In a paper by Dejica et al., C2C and C1,2C were detected and described in AC, but the results of immunohistochemistry were not quantified (Dejica et al., 2012). An additional neoepitope of the C-terminus of the Col2 TC^A fragment (type-II collagen α chain ¾ length fragment) was assessed in equine AC, but the staining patterns were characterized solely by their intensity, lacking direct quantification (Billinghurst et al., 2001). Thus, there has been limited investigation into quantifying the neo-epitopes at the tissue structural level, and, therefore, it is important to describe the local immunohistochemical expression pattern of C2C in AC.

In our study, we show that it is possible to detect C2C in articular osteoarthritic cartilage, and we demonstrated a correlation between C2C expression and both the grade of cartilage damage and the overall knee joint damage score as evaluated by OARSI indicators. This implies that C2C may possess indicative properties for the development of kOA on the local level. It is important to note that an ideal biomarker should demonstrate a strong correlation with the advance-ment of tissue damage, both at the tissue level and in more readily accessible biomaterials like plasma or urine. Subsequent investigations should ascertain whether local tissue alterations of C2C content are reflected in the C2C levels found in urine samples – an aspect explored to some extent by other researchers (Kuhi et al., 2021).

6.6. Antigen retrieval methods to enhance results of CILP-2 immunohistochemical staining

In Paper III, we also studied the expression of CILP-2 in OA cartilage, but at the same time, specifically addressed methodological challenges in unmasking epitopes for ICH staining. As epitope retrieval methods affect the most crucial step – antibody binding to its epitope, we focused on the testing of different antigen retrieval methods.

If a tissue sample is fixed in formalin, as is the case with most of the routinely collected pathoanatomical specimens, the cross-linking of protein amino acid residues occurs, involving formaldehyde-derived methylene bridges, which may block the antibody binding and therefore mask the antigen availability for IHC, necessitating the implementation of effective epitope retrieval techniques (Ezaki, 1996; McNicol & Richmond, 1998). There are essentially two methods to retrieve the epitope in the tissue so that it can bind to the antibody. One possibility is to break the methylene bridges by heating, applying a method designated as heat-induced epitope retrieval (HIER), and the other option is the digestion of structures surrounding the epitope with the help of enzymes – a method designated as proteolytic-induced epitope retrieval (PIER) (Dou et al., 2021). It is usually expected that HIER would give better results, as there is a risk that PIER may fail to unmask the epitope or destroy the tissue morphology and the antigen of interest, but it has also been suggested that a combination of HIER and PIER would give the best results (Shi et al., 2001; Ezaki, 2000). An added effect of HIER combined with PIER was also our working hypothesis when we started tests of antigen retrieval for the IHC in the AC. However, as we soon discovered, the optimal results were achieved with the enzymatic treatment. We found no beneficial retrieval effect of heating compared to non-treated AC specimens, and furthermore, when heating was added to enzymatic retrieval, the final staining was significantly reduced compared to the use of only proteinase K/hyaluronidase treatment. Moreover, heating resulted in the frequent detachment of sections from the slides. We also found a distinctive appearance of CILP-2 staining in the territorial matrix around the isogenous groups of chondrocytes in groups that received the proteolytic treatment. This was especially distinctive in the HIER/PIER group, as the rest of the cartilage matrix seemed to lose the staining, while it was still preserved around the isogenous groups. This was noted in superficial, intermediate, and deep layers of AC, and it could be hypothesized that CILP-2 molecules in closer proximity of the chondrocytes were synthesized relatively recently compared to the rest of the tissue matrix and were therefore perhaps more resistant to the heat treatment.

Interpreting these results is challenging due to the unique characteristics of cartilage. This tissue is distinguished by a dense and highly organized ECM, which can impede antibody penetration and affect immunohistochemical analyses. This makes it important to choose an appropriate retrieval method for immunohistochemical research of the cartilage. Compared to other tissues, where the application of HIER or a combination of HIER and PIER is optimal, in

cartilage, the use of proteinases for antigen retrieval may be more relevant. This is supported by studies on skeletal tissues where PIER yielded better results than HIER (Dou et al., 2021; Amitham et al., 2020). Furthermore, PIER seems particularly useful as it does not cause as extensive detachment of cartilage sections from glass slides as HIER. The poor adhesion of cartilage sections to microscope slides has been a challenge with HIER, as reported by others working on skeletal tissues (Idleburg et al., 2021; Dou et al., 2021). It should also be noted that osteochondral tissues usually require proper decalcification. To protect the antigens of interest throughout the extended decalcification step, the skeletal tissues need to be thoroughly fixed (usually no less than 24 to 48 hours), which again emphasizes the necessity of selecting effective antigen retrieval methods for skeletal tissues once the IHC has to be performed. Yet another factor in determining the optimal antigen retrieval method and, therefore, the successful binding of the antibody to its antigen is the nature of the antigen molecule itself. The temperatures involved in the HIER protocols can be rather extreme, and the factors determining the thermostability of a protein (such as glycosylation) could have a significant effect on the outcome of IHC. To address the limitations of our study, we have to acknowledge that we cannot rule out the possibility that OA-induced changes in the AC may impact the availability of epitopes as well as the outcome of the antigen retrieval. Since it has been shown that glycosylation increases the stability of a protein (Solá & Griebenow, 2009), the structure of the glycoproteins in OA-affected cartilage does not need to be the same as in metabolically and functionally normal tissue. To reduce the effect of the advancement of the OA on our results, sections from all the samples were treated with all three retrieval protocols and a control protocol (no retrieval) to have a comparison within the sample. A dedicated chemical characterization of the heat sensitivity of the molecules in pathological tissues should be performed to further prove our hypothesis. Also, while this study focuses on OA cartilage, the findings may not apply to other types of tissues or conditions. However, it is important to acknowledge that immunohistochemical analysis of cartilage exhibits unique characteristics that differentiate it from other tissues, often necessitating the use of cartilage-specific IHC protocols.

6.7. Strengths and limitations of the study

As a strength of the study, it should be emphasized that the enrolled patients formed a cohort of OA patients with the age below 70 years (age from 46 to 68 years – Paper I; 55–66 – Paper II; 55–65 – Paper III), i.e. a middle-age population cohort in terms of OA investigations, where the factor of ageing is not pronounced, representing patients with rapid OA progression.

Another strength of the study is that the evaluations of immunohistochemical staining were performed with computer assistance, reducing the subjectivity of the estimations.

Among the limitations of the study, it should be drawn out that the study groups were relatively small, not allowing to prove all possible statistical associations. Secondly, obtaining an appropriate comparison group of healthy cartilage specimens from normal knee joints was not feasible due to ethical and logistical constraints. Thirdly, not the same patients were enrolled in all studies; as only five patients were enrolled in both Paper I and Paper II, it was not possible to correlate the expression of CILP-2 and C2C.

7. CONCLUSIONS

The study demonstrated that CILP-2 and C2C are promising biomarkers for kOA, showing a correlation with the degree of local tissue damage as assessed by OARSI grades and joint surface damage in samples from patients undergoing total knee arthroplasty (TKA).

I. The comparative evaluation of three antigen retrieval protocols for CILP-2 immunohistochemistry (IHC) revealed that the optimal results were achieved using proteolytic-induced epitope retrieval (PIER), which combined proteinase K with hyaluronidase.
II. Assessing the expression of CILP-2 in the knee AC of OA patients showed a positive correlation between CILP-2 levels and the degree of cartilage damage, as determined by OARSI histopathology grades and macroscopic joint surface damage scores.
III. C2C expression was found to correspond with the severity of OA, showing positive correlations between the proportion of cartilage stained for C2C and both the grades of cartilage damage and the overall knee joint damage scores, as evaluated by OARSI indicators. Thus, C2C holds promise as a biomarker for monitoring OA-induced histopathological changes of AC.
IV. The expression pattern of DDR2 in the AC was similar to that of CILP-2, showing a positive correlation between DDR2 and CILP-2 expression. However, unlike CILP-2, DDR2 did not exhibit statistically significant correlation with OARSI histopathology grades and scores, making CILP-2 a more reliable marker of OA-related cartilage damage than DDR2.
V. TEM investigations of the superficial zone of the AC revealed severely damaged chondrocytes with several features of degenerative cells and chondroptosis, especially in specimens with higher OARSI grades. Irregular arrangements of the pericellular matrix (PCM) were observed, and since CILP-2 IHC staining was predominantly localized in the PCM, this supports the hypothesis of the importance of PCM in OA.

8. REFERENCES

Aaron, R. K., Racine, J., & Dyke, J. P. (2017). Contribution of Circulatory Disturbances in Subchondral Bone to the Pathophysiology of Osteoarthritis. *Current rheumatology reports*, *19*(8), 49. https://doi.org/10.1007/s11926-017-0660-x

Adams, C. S., & Horton, W. E., Jr (1998). Chondrocyte apoptosis increases with age in the articular cartilage of adult animals. *The Anatomical record*, *250*(4), 418–425. https://doi.org/10.1002/(SICI)1097-0185(199804)250:4<418::AID-AR4>3.0.CO;2-T

Aigner, T., & Fan, Z. (2003). Anatomy and Biochemistry of Articular Cartilage. In: Hendrich, C., Nöth, U., Eulert, J. (eds) Cartilage Surgery and Future Perspectives. Springer, Berlin, Heidelberg. https://doi.org/10.1007/978-3-642-19008-7_1

Aigner, T., Kim, H. A., & Roach, H. I. (2004). Apoptosis in osteoarthritis. *Rheumatic diseases clinics of North America*, *30*(3), 639–xi. https://doi.org/10.1016/j.rdc.2004.04.002

Akkiraju, H., & Nohe, A. (2015). Role of Chondrocytes in Cartilage Formation, Progression of Osteoarthritis and Cartilage Regeneration. *Journal of developmental biology*, *3*(4), 177–192. https://doi.org/10.3390/jdb3040177

Alcaide-Ruggiero, L., Molina-Hernández, V., Granados, M. M., & Domínguez, J. M. (2021). Main and Minor Types of Collagens in the Articular Cartilage: The Role of Collagens in Repair Tissue Evaluation in Chondral Defects. *International journal of molecular sciences*, *22*(24), 13329. https://doi.org/10.3390/ijms222413329

Amirtham, S. M., Ozbey, O., Kachroo, U., Ramasamy, B., & Vinod, E. (2020). Optimization of immunohistochemical detection of collagen type II in osteochondral sections by comparing decalcification and antigen retrieval agent combinations. *Clinical anatomy (New York, N.Y.)*, *33*(3), 343–349. https://doi.org/10.1002/ca.23441

Antipova, O., & Orgel, J. P. (2010). In situ D-periodic molecular structure of type II collagen. *The Journal of biological chemistry*, *285*(10), 7087–7096. https://doi.org/10.1074/jbc.M109.060400

Archer, C. W., & Francis-West, P. (2003). The chondrocyte. *The international journal of biochemistry & cell biology*, *35*(4), 401–404. https://doi.org/10.1016/s1357-2725(02)00301-1

Arden, N., & Nevitt, M. C. (2006). Osteoarthritis: epidemiology. *Best practice & research. Clinical rheumatology*, *20*(1), 3–25. https://doi.org/10.1016/j.berh.2005.09.007

Atukorala, I., Kwoh, C. K., Guermazi, A., Roemer, F. W., Boudreau, R. M., Hannon, M. J., & Hunter, D. J. (2016). Synovitis in knee osteoarthritis: a precursor of disease?. *Annals of the rheumatic diseases*, *75*(2), 390–395. https://doi.org/10.1136/annrheumdis-2014-205894

Badley, E. M., Wilfong, J. M., Yip, C., Millstone, D. B., & Perruccio, A. V. (2020). The contribution of age and obesity to the number of painful joint sites in individuals reporting osteoarthritis: a population-based study. *Rheumatology (Oxford, England)*, *59*(11), 3350–3357. https://doi.org/10.1093/rheumatology/keaa138

Barrett, J. P., Jr, Rashkoff, E., Sirna, E. C., & Wilson, A. (1990). Correlation of roentgenographic patterns and clinical manifestations of symptomatic idiopathic osteoarthritis of the knee. *Clinical orthopaedics and related research*, (253), 179–183.

Basso, P. R., Carava', E., Protasoni, M., Reguzzoni, M., & Raspanti, M. (2020). The synovial surface of the articular cartilage. *European journal of histochemistry : EJH*, *64*(3), 3146. https://doi.org/10.4081/ejh.2020.3146

Bay-Jensen, A. C., Andersen, T. L., Charni-Ben Tabassi, N., Kristensen, P. W., Kjaersgaard-Andersen, P., Sandell, L., Garnero, P., & Delaissé, J. M. (2008). Biochemical markers of type II collagen breakdown and synthesis are positioned at

specific sites in human osteoarthritic knee cartilage. *Osteoarthritis and cartilage*, *16*(5), 615–623. https://doi.org/10.1016/j.joca.2007.09.006

Bay-Jensen, A. C., Liu, Q., Byrjalsen, I., Li, Y., Wang, J., Pedersen, C., Leeming, D. J., Dam, E. B., Zheng, Q., Qvist, P., & Karsdal, M. A. (2011). Enzyme-linked immunosorbent assay (ELISAs) for metalloproteinase derived type II collagen neoepitope, CIIM – increased serum CIIM in subjects with severe radiographic osteoarthritis. *Clinical biochemistry*, *44*(5-6), 423–429. https://doi.org/10.1016/j.clinbiochem.2011.01.001

Bay-Jensen, A. C., Reker, D., Kjelgaard-Petersen, C. F., Mobasheri, A., Karsdal, M. A., Ladel, C., Henrotin, Y., & Thudium, C. S. (2016). Osteoarthritis year in review 2015: soluble biomarkers and the BIPED criteria. *Osteoarthritis and cartilage*, *24*(1), 9–20. https://doi.org/10.1016/j.joca.2015.10.014

Bay-Jensen, A. C., Mobasheri, A., Thudium, C. S., Kraus, V. B., & Karsdal, M. A. (2022). Blood and urine biomarkers in osteoarthritis – an update on cartilage associated type II collagen and aggrecan markers. *Current opinion in rheumatology*, *34*(1), 54–60. https://doi.org/10.1097/BOR.0000000000000845

Bedson, J., & Croft, P. R. (2008). The discordance between clinical and radiographic knee osteoarthritis: a systematic search and summary of the literature. *BMC musculoskeletal disorders*, *9*, 116. https://doi.org/10.1186/1471-2474-9-116

Benninghoff, A. (1925). Form und Bau der Gelenkknorpel in ihren Beziehungen zur Funktion. *Zeitschrift für Zellforschung und mikroskopische Anatomie*, *2*(5), 783-862.

Berenbaum F. (2013). Osteoarthritis as an inflammatory disease (osteoarthritis is not osteoarthrosis!). *Osteoarthritis and cartilage*, *21*(1), 16–21. https://doi.org/10.1016/j.joca.2012.11.012

Berenbaum, F., Wallace, I. J., Lieberman, D. E., & Felson, D. T. (2018). Modern-day environmental factors in the pathogenesis of osteoarthritis. *Nature reviews. Rheumatology*, *14*(11), 674–681. https://doi.org/10.1038/s41584-018-0073-x

Berenbaum, F., & Walker, C. (2020). Osteoarthritis and inflammation: a serious disease with overlapping phenotypic patterns. *Postgraduate medicine*, *132*(4), 377–384. https://doi.org/10.1080/00325481.2020.1730669

Bernardo, B. C., Belluoccio, D., Rowley, L., Little, C. B., Hansen, U., & Bateman, J. F. (2011). Cartilage intermediate layer protein 2 (CILP-2) is expressed in articular and meniscal cartilage and down-regulated in experimental osteoarthritis. *The Journal of biological chemistry*, *286*(43), 37758–37767. https://doi.org/10.1074/jbc.M111.248039

Bhosale, A. M., & Richardson, J. B. (2008). Articular cartilage: structure, injuries and review of management. *British medical bulletin*, *87*, 77–95. https://doi.org/10.1093/bmb/ldn025

Bielajew, B. J., Hu, J. C., & Athanasiou, K. A. (2020). Collagen: quantification, biomechanics, and role of minor subtypes in cartilage. *Nature reviews. Materials*, *5*(10), 730–747. https://doi.org/10.1038/s41578-020-0213-1

Bijlsma, J. W., Berenbaum, F., & Lafeber F. P. (2011). Osteoarthritis: an update with relevance for clinical practice. *Lancet (London, England)*, *377*(9783), 2115–2126. https://doi.org/10.1016/S0140-6736(11)60243-2

Billinghurst, R. C., Dahlberg, L., Ionescu, M., Reiner, A., Bourne, R., Rorabeck, C., Mitchell, P., Hambor, J., Diekmann, O., Tschesche, H., Chen, J., Van Wart, H., & Poole, A. R. (1997). Enhanced cleavage of type II collagen by collagenases in osteoarthritic articular cartilage. *The Journal of clinical investigation*, *99*(7), 1534–1545. https://doi.org/10.1172/JCI119316

Billinghurst, R. C., Buxton, E. M., Edwards, M. G., McGraw, M. S., & McIlwraith, C. W. (2001). Use of an antineoepitope antibody for identification of type-II collagen

degradation in equine articular cartilage. *American journal of veterinary research*, *62*(7), 1031–1039. https://doi.org/10.2460/ajvr.2001.62.1031

Blalock, D., Miller, A., Tilley, M., & Wang, J. (2015). Joint instability and osteoarthritis. *Clinical medicine insights. Arthritis and musculoskeletal disorders*, *8*, 15–23. https://doi.org/10.4137/CMAMD.S22147

Blaney Davidson, E. N., van der Kraan, P. M., & van den Berg, W. B. (2007). TGF-beta and osteoarthritis. *Osteoarthritis and cartilage*, *15*(6), 597–604. https://doi.org/10.1016/j.joca.2007.02.005

Blom, A. B., van Lent, P. L., Holthuysen, A. E., van der Kraan, P. M., Roth, J., van Rooijen, N., & van den Berg, W. B. (2004). Synovial lining macrophages mediate osteophyte formation during experimental osteoarthritis. *Osteoarthritis and cartilage*, *12*(8), 627–635. https://doi.org/10.1016/j.joca.2004.03.003

Boeth, H., Raffalt, P. C., MacMahon, A., Poole, A. R., Eckstein, F., Wirth, W., Buttgereit, F., Önnerfjord, P., Lorenzo, P., Klint, C., Pramhed, A., & Duda, G. N. (2019). Association between changes in molecular biomarkers of cartilage matrix turnover and changes in knee articular cartilage: a longitudinal pilot study. *Journal of experimental orthopaedics*, *6*(1), 19. https://doi.org/10.1186/s40634-019-0179-3

Boffa, A., Merli, G., Andriolo, L., Lattermann, C., Salzmann, G. M., & Filardo, G. (2021). Synovial Fluid Biomarkers in Knee Osteoarthritis: A Systematic Review and Quantitative Evaluation Using BIPEDs Criteria. *Cartilage*, *13*(1_suppl), 82S–103S. https://doi.org/10.1177/1947603520942941

Bord, S. (2003). Protein localization in wax-embedded and frozen sections of bone using immunohistochemistry. *Methods in molecular medicine*, *80*, 237–247. https://doi.org/10.1385/1-59259-366-6:237

Buckwalter, J. A., Mow, V. C., & Ratcliffe, A. (1994). Restoration of Injured or Degenerated Articular Cartilage. *The Journal of the American Academy of Orthopaedic Surgeons*, *2*(4), 192–201. https://doi.org/10.5435/00124635-199407000-00002

Buckwalter, J. A., & Martin, J. A. (2006). Osteoarthritis. *Advanced drug delivery reviews*, *58*(2), 150–167. https://doi.org/10.1016/j.addr.2006.01.006

Burr, D. B., & Gallant, M. A. (2012). Bone remodelling in osteoarthritis. *Nature reviews. Rheumatology*, *8*(11), 665–673. https://doi.org/10.1038/nrrheum.2012.130

Burrage, P. S., Mix, K. S., & Brinckerhoff, C. E. (2006). Matrix metalloproteinases: role in arthritis. *Frontiers in bioscience: a journal and virtual library*, *11*, 529–543. https://doi.org/10.2741/1817

Chalmers, P. N., Mall, N. A., Moric, M., Sherman, S. L., Paletta, G. P., Cole, B. J., & Bach, B. R., Jr (2014). Does ACL reconstruction alter natural history?: A systematic literature review of long-term outcomes. *The Journal of bone and joint surgery. American volume*, *96*(4), 292–300. https://doi.org/10.2106/JBJS.L.01713

Chapman, K., Takahashi, A., Meulenbelt, I., Watson, C., Rodriguez-Lopez, J., Egli, R., Tsezou, A., Malizos, K. N., Kloppenburg, M., Shi, D., Southam, L., van der Breggen, R., Donn, R., Qin, J., Doherty, M., Slagboom, P. E., Wallis, G., Kamatani, N., Jiang, Q., Gonzalez, A., … Ikegawa, S. (2008). A meta-analysis of European and Asian cohorts reveals a global role of a functional SNP in the 5' UTR of GDF5 with osteoarthritis susceptibility. *Human molecular genetics*, *17*(10), 1497–1504. https://doi.org/10.1093/hmg/ddn038

Charlier, E., Relic, B., Deroyer, C., Malaise, O., Neuville, S., Collée, J., Malaise, M. G., & De Seny, D. (2016). Insights on Molecular Mechanisms of Chondrocytes Death in Osteoarthritis. *International journal of molecular sciences*, *17*(12), 2146. https://doi.org/10.3390/ijms17122146

Chen, T., Hilton, M. J., Brown, E. B., Zuscik, M. J., & Awad, H. A. (2013). Engineering superficial zone features in tissue engineered cartilage. *Biotechnology and bioengineering*, *110*(5), 1476–1486. https://doi.org/10.1002/bit.24799

Chen, C., Yang, F., Chen, R., Yang, C., Xiao, H., Geng, B., & Xia, Y. (2024). TRPV Channels in Osteoarthritis: A Comprehensive Review. *Biomolecules*, *14*(3), 292. https://doi.org/10.3390/biom14030292

Chow, Y. Y., & Chin, K. Y. (2020). The Role of Inflammation in the Pathogenesis of Osteoarthritis. *Mediators of inflammation*, *2020*, 8293921. https://doi.org/10.1155/2020/8293921

Cibere, J., Zhang, H., Garnero, P., Poole, A. R., Lobanok, T., Saxne, T., Kraus, V. B., Way, A., Thorne, A., Wong, H., Singer, J., Kopec, J., Guermazi, A., Peterfy, C., Nicolaou, S., Munk, P. L., & Esdaile, J. M. (2009). Association of biomarkers with pre-radiographically defined and radiographically defined knee osteoarthritis in a population-based study. *Arthritis and rheumatism*, *60*(5), 1372–1380. https://doi.org/10.1002/art.24473

Conrozier, T., Poole, A. R., Ferrand, F., Mathieu, P., Vincent, F., Piperno, M., Verret, C., Ionescu, M., & Vignon, E. (2008). Serum concentrations of type II collagen biomarkers (C2C, C1, 2C and CPII) suggest different pathophysiologies in patients with hip osteoarthritis. *Clinical and experimental rheumatology*, *26*(3), 430–435.

Courties, A., Kouki, I., Soliman, N., Mathieu, S., & Sellam, J. (2024). Osteoarthritis year in review 2024: Epidemiology and therapy. *Osteoarthritis and cartilage*, *32*(11), 1397–1404. https://doi.org/10.1016/j.joca.2024.07.014

Crowe, A. R., & Yue, W. (2019). Semi-quantitative Determination of Protein Expression using Immunohistochemistry Staining and Analysis: An Integrated Protocol. *Bio-protocol*, *9*(24), e3465. https://doi.org/10.21769/BioProtoc.3465

Dejica, V. M., Mort, J. S., Laverty, S., Antoniou, J., Zukor, D. J., Tanzer, M., & Poole, A. R. (2012). Increased type II collagen cleavage by cathepsin K and collagenase activities with aging and osteoarthritis in human articular cartilage. *Arthritis research & therapy*, *14*(3), R113. https://doi.org/10.1186/ar3839

Dickinson, S. C., Sims, T. J., Pittarello, L., Soranzo, C., Pavesio, A., & Hollander, A. P. (2005). Quantitative outcome measures of cartilage repair in patients treated by tissue engineering. *Tissue engineering*, *11*(1–2), 277–287. https://doi.org/10.1089/ten.2005.11.277

Ding, C., Martel-Pelletier, J., Pelletier, J. P., Abram, F., Raynauld, J. P., Cicuttini, F., & Jones, G. (2007). Meniscal tear as an osteoarthritis risk factor in a largely non-osteoarthritic cohort: a cross-sectional study. *The Journal of rheumatology*, *34*(4), 776–784.

de Klerk, B. M., Schiphof, D., Groeneveld, F. P., Koes, B. W., van Osch, G. J., van Meurs, J. B., & Bierma-Zeinstra, S. M. (2009). No clear association between female hormonal aspects and osteoarthritis of the hand, hip and knee: a systematic review. *Rheumatology (Oxford, England)*, *48*(9), 1160–1165. https://doi.org/10.1093/rheumatology/kep194

De Roover, A., Escribano-Núñez, A., Monteagudo, S., & Lories, R. (2023). Fundamentals of osteoarthritis: Inflammatory mediators in osteoarthritis. *Osteoarthritis and cartilage*, *31*(10), 1303–1311. https://doi.org/10.1016/j.joca.2023.06.005

Dou, Z., Chau, M., Muder, D., Vedung, T., & Nilsson, O. (2021). Optimized protocols for in situ hybridization, immunohistochemistry, and immunofluorescence on skeletal tissue. *Acta histochemica*, *123*(5), 151747. https://doi.org/10.1016/j.acthis.2021.151747

Englund M. (2010). The role of biomechanics in the initiation and progression of OA of the knee. *Best practice & research. Clinical rheumatology*, *24*(1), 39–46. https://doi.org/10.1016/j.berh.2009.08.008

Englund, M., Roemer, F. W., Hayashi, D., Crema, M. D., & Guermazi, A. (2012). Meniscus pathology, osteoarthritis and the treatment controversy. *Nature reviews. Rheumatology*, *8*(7), 412–419. https://doi.org/10.1038/nrrheum.2012.69

Ezaki T. (1996). *Kaibogaku zasshi. Journal of anatomy*, *71*(6), 615–628.

Ezaki T. (2000). Antigen retrieval on formaldehyde-fixed paraffin sections: its potential drawbacks and optimization for double immunostaining. *Micron (Oxford, England: 1993)*, *31*(6), 639–649. https://doi.org/10.1016/s0968-4328(99)00064-5

Evangelou, E., Chapman, K., Meulenbelt, I., Karassa, F. B., Loughlin, J., Carr, A., Doherty, M., Doherty, S., Gómez-Reino, J. J., Gonzalez, A., Halldorsson, B. V., Hauksson, V. B., Hofman, A., Hart, D. J., Ikegawa, S., Ingvarsson, T., Jiang, Q., Jonsdottir, I., Jonsson, H., Kerkhof, H. J., … Ioannidis, J. P. (2009). Large-scale analysis of association between GDF5 and FRZB variants and osteoarthritis of the hip, knee, and hand. *Arthritis and rheumatism*, *60*(6), 1710–1721. https://doi.org/10.1002/art.24524

Evers, B. J., Van Den Bosch, M. H. J., Blom, A. B., van der Kraan, P. M., Koëter, S., & Thurlings, R. M. (2022). Post-traumatic knee osteoarthritis; the role of inflammation and hemarthrosis on disease progression. *Frontiers in medicine*, *9*, 973870. https://doi.org/10.3389/fmed.2022.973870

Eyre D. (2002). Collagen of articular cartilage. *Arthritis research*, *4*(1), 30–35. https://doi.org/10.1186/ar380

Eyre, D. R., Wu, J. J., Fernandes, R. J., Pietka, T. A., & Weis, M. A. (2002). Recent developments in cartilage research: matrix biology of the collagen II/IX/XI heterofibril network. *Biochemical Society transactions*, *30*(Pt 6), 893–899. https://doi.org/10.1042/bst0300893

Eymard, F., & Chevalier, X. (2016). Inflammation of the infrapatellar fat pad. *Joint bone spine*, *83*(4), 389–393. https://doi.org/10.1016/j.jbspin.2016.02.016

Felson, D. T., Lawrence, R. C., Dieppe, P. A., Hirsch, R., Helmick, C. G., Jordan, J. M., Kington, R. S., Lane, N. E., Nevitt, M. C., Zhang, Y., Sowers, M., McAlindon, T., Spector, T. D., Poole, A. R., Yanovski, S. Z., Ateshian, G., Sharma, L., Buckwalter, J. A., Brandt, K. D., & Fries, J. F. (2000). Osteoarthritis: new insights. Part 1: the disease and its risk factors. *Annals of internal medicine*, *133*(8), 635–646. https://doi.org/10.7326/0003-4819-133-8-200010170-00016

Felson, D. T., & Hodgson, R. (2014). Identifying and treating preclinical and early osteoarthritis. *Rheumatic diseases clinics of North America*, *40*(4), 699–710. https://doi.org/10.1016/j.rdc.2014.07.012

Fidler, A. L., Boudko, S. P., Rokas, A., & Hudson, B. G. (2018). The triple helix of collagens – an ancient protein structure that enabled animal multicellularity and tissue evolution. *Journal of cell science*, *131*(7), jcs203950. https://doi.org/10.1242/jcs.203950

Finan, P. H., Buenaver, L. F., Bounds, S. C., Hussain, S., Park, R. J., Haque, U. J., Campbell, C. M., Haythornthwaite, J. A., Edwards, R. R., & Smith, M. T. (2013). Discordance between pain and radiographic severity in knee osteoarthritis: findings from quantitative sensory testing of central sensitization. *Arthritis and rheumatism*, *65*(2), 363–372. https://doi.org/10.1002/art.34646

Fox, C. H., Johnson, F. B., Whiting, J., & Roller, P. P. (1985). Formaldehyde fixation. The journal of histochemistry and cytochemistry: official journal of the Histochemistry Society, 33(8), 845–853. https://doi.org/10.1177/33.8.3894502

Frazer, A., Bunning, R. A., Thavarajah, M., Seid, J. M., & Russell, R. G. (1994). Studies on type II collagen and aggrecan production in human articular chondrocytes in vitro and effects of transforming growth factor-beta and interleukin-1beta. *Osteoarthritis and cartilage*, *2*(4), 235–245. https://doi.org/10.1016/s1063-4584(05)80075-5

Garnero, P., Piperno, M., Gineyts, E., Christgau, S., Delmas, P. D., & Vignon, E. (2001). Cross sectional evaluation of biochemical markers of bone, cartilage, and synovial tissue metabolism in patients with knee osteoarthritis: relations with disease activity and joint damage. *Annals of the rheumatic diseases*, *60*(6), 619–626. https://doi.org/10.1136/ard.60.6.619

GBD 2019 Diseases and Injuries Collaborators (2020). Global burden of 369 diseases and injuries in 204 countries and territories, 1990–2019: a systematic analysis for the Global Burden of Disease Study 2019. *Lancet (London, England)*, *396*(10258), 1204–1222. https://doi.org/10.1016/S0140-6736(20)30925-9

GBD 2021 Osteoarthritis Collaborators (2023). Global, regional, and national burden of osteoarthritis, 1990-2020 and projections to 2050: a systematic analysis for the Global Burden of Disease Study 2021. *The Lancet. Rheumatology*, *5*(9), e508–e522. https://doi.org/10.1016/S2665-9913(23)00163-7

Gelber, A. C., Hochberg, M. C., Mead, L. A., Wang, N. Y., Wigley, F. M., & Klag, M. J. (2000). Joint injury in young adults and risk for subsequent knee and hip osteoarthritis. *Annals of internal medicine*, *133*(5), 321–328. https://doi.org/10.7326/0003-4819-133-5-200009050-00007

Giorgino, R., Albano, D., Fusco, S., Peretti, G. M., Mangiavini, L., & Messina, C. (2023). Knee Osteoarthritis: Epidemiology, Pathogenesis, and Mesenchymal Stem Cells: What Else Is New? An Update. *International journal of molecular sciences*, *24*(7), 6405. https://doi.org/10.3390/ijms24076405

Glyn-Jones, S., Palmer, A. J., Agricola, R., Price, A. J., Vincent, T. L., Weinans, H., & Carr, A. J. (2015). Osteoarthritis. *Lancet (London, England)*, *386*(9991), 376–387. https://doi.org/10.1016/S0140-6736(14)60802-3

Goldring, M. B., & Marcu, K. B. (2009). Cartilage homeostasis in health and rheumatic diseases. *Arthritis research & therapy*, *11*(3), 224. https://doi.org/10.1186/ar2592

Goldring, M. B., & Otero, M. (2011). Inflammation in osteoarthritis. *Current opinion in rheumatology*, *23*(5), 471–478. https://doi.org/10.1097/BOR.0b013e328349c2b1

Goldring, M. B., Otero, M., Plumb, D. A., Dragomir, C., Favero, M., El Hachem, K., Hashimoto, K., Roach, H. I., Olivotto, E., Borzì, R. M., & Marcu, K. B. (2011). Roles of inflammatory and anabolic cytokines in cartilage metabolism: signals and multiple effectors converge upon MMP-13 regulation in osteoarthritis. *European cells & materials*, *21*, 202–220. https://doi.org/10.22203/ecm.v021a16

Goldring S. R. (2009). Role of bone in osteoarthritis pathogenesis. *The Medical clinics of North America*, *93*(1), 25–xv. https://doi.org/10.1016/j.mcna.2008.09.006

Goldring, S. R., & Goldring, M. B. (2016). Changes in the osteochondral unit during osteoarthritis: structure, function and cartilage-bone crosstalk. *Nature reviews. Rheumatology*, *12*(11), 632–644. https://doi.org/10.1038/nrrheum.2016.148

Grazio, S., & Balen, D. (2009). Debljina: cimbenik rizika i prediktor razvoja osteoartritisa [Obesity: risk factor and predictor of osteoarthritis]. *Lijecnicki vjesnik*, *131*(1–2), 22–26.

Guilak, F., & Mow, V. C. (2000). The mechanical environment of the chondrocyte: a biphasic finite element model of cell-matrix interactions in articular cartilage. *Journal of biomechanics*, *33*(12), 1663–1673.

Guilak, F., Nims, R. J., Dicks, A., Wu, C. L., & Meulenbelt, I. (2018). Osteoarthritis as a disease of the cartilage pericellular matrix. *Matrix biology: journal of the International Society for Matrix Biology*, *71–72*, 40–50. https://doi.org/10.1016/j.matbio.2018.05.008

Guo, J., Yan, P., Qin, Y., Liu, M., Ma, Y., Li, J., Wang, R., Luo, H., & Lv, S. (2024). Automated measurement and grading of knee cartilage thickness: a deep learning-based approach. *Frontiers in medicine*, *11*, 1337993. https://doi.org/10.3389/fmed.2024.1337993

Hamerman D. (1989). The biology of osteoarthritis. *The New England journal of medicine*, *320*(20), 1322–1330. https://doi.org/10.1056/NEJM198905183202006

Hayashi, D., Roemer, F. W., Katur, A., Felson, D. T., Yang, S. O., Alomran, F., & Guermazi, A. (2011). Imaging of synovitis in osteoarthritis: current status and outlook. *Seminars in arthritis and rheumatism*, *41*(2), 116–130. https://doi.org/10.1016/j.semarthrit.2010.12.003

He, M., Gong, S. D., Chen, X. J., Yang, F., Pang, F. X., Chen, Z. Q., Huang, J. Y., Zhou, Y. C., Qin, Y. X., He, W., & Wei, Q. S. (2019). Plasma C-terminal cross-linking telopeptide of type II collagen as a biomarker in advanced stages of femoral head osteonecrosis. *Biomedicine & pharmacotherapy = Biomedecine & pharmacotherapie*, *111*, 1213–1220. https://doi.org/10.1016/j.biopha.2019.01.013

Heinegård, D., & Saxne, T. (2011). The role of the cartilage matrix in osteoarthritis. *Nature reviews. Rheumatology*, *7*(1), 50–56. https://doi.org/10.1038/nrrheum.2010.198

Holt, D. W., Henderson, M. L., Stockdale, C. E., Farrell, J. T., Kooyman, D. L., Bridgewater, L. C., & Seegmiller, R. E. (2012). Osteoarthritis-like changes in the heterozygous sedc mouse associated with the HtrA1-Ddr2-Mmp-13 degradative pathway: a new model of osteoarthritis. *Osteoarthritis and cartilage*, *20*(5), 430–439. https://doi.org/10.1016/j.joca.2011.11.008

Hosseininia, S., Önnerfjord, P., & Dahlberg, L. E. (2019). Targeted proteomics of hip articular cartilage in OA and fracture patients. *Journal of orthopaedic research: official publication of the Orthopaedic Research Society*, *37*(1), 131–135. https://doi.org/10.1002/jor.24158

Hsia, A. W., Emami, A. J., Tarke, F. D., Cunningham, H. C., Tjandra, P. M., Wong, A., Christiansen, B. A., & Collette, N. M. (2018). Osteophytes and fracture calluses share developmental milestones and are diminished by unloading. *Journal of orthopaedic research: official publication of the Orthopaedic Research Society*, *36*(2), 699–710. https://doi.org/10.1002/jor.23779

Hsueh, M. F., Khabut, A., Kjellström, S., Önnerfjord, P., & Kraus, V. B. (2016). Elucidating the Molecular Composition of Cartilage by Proteomics. *Journal of proteome research*, *15*(2), 374–388. https://doi.org/10.1021/acs.jproteome.5b00946

Hu, K., Xu, L., Cao, L., Flahiff, C. M., Brussiau, J., Ho, K., Setton, L. A., Youn, I., Guilak, F., Olsen, B. R., & Li, Y. (2006). Pathogenesis of osteoarthritis-like changes in the joints of mice deficient in type IX collagen. *Arthritis and rheumatism*, *54*(9), 2891–2900. https://doi.org/10.1002/art.22040

Huang, F., Peng, Y., Ye, Q., Chen, J., Li, Y., Liu, S., Xu, Y., & Huang, L. (2020). CILP2 overexpression correlates with tumor progression and poor prognosis in patients with colorectal cancer in The Cancer Genome Atlas (TCGA) study. *World journal of surgical oncology*, *18*(1), 274. https://doi.org/10.1186/s12957-020-02049-6

Huebner, J. L., Bay-Jensen, A. C., Huffman, K. M., He, Y., Leeming, D. J., McDaniel, G. E., Karsdal, M. A., & Kraus, V. B. (2014). Alpha C-telopeptide of type I collagen is associated with subchondral bone turnover and predicts progression of joint space

narrowing and osteophytes in osteoarthritis. *Arthritis & rheumatology (Hoboken, N.J.)*, *66*(9), 2440–2449. https://doi.org/10.1002/art.38739

Hunter, D. J., Zhang, Y. Q., Niu, J. B., Tu, X., Amin, S., Clancy, M., Guermazi, A., Grigorian, M., Gale, D., & Felson, D. T. (2006). The association of meniscal pathologic changes with cartilage loss in symptomatic knee osteoarthritis. *Arthritis and rheumatism*, *54*(3), 795–801. https://doi.org/10.1002/art.21724

Hunter, D. J., & Bierma-Zeinstra, S. (2019). Osteoarthritis. *Lancet (London, England)*, *393*(10182), 1745–1759. https://doi.org/10.1016/S0140-6736(19)30417-9

Hwang, H. S., & Kim, H. A. (2015). Chondrocyte Apoptosis in the Pathogenesis of Osteoarthritis. *International journal of molecular sciences*, *16*(11), 26035–26054. https://doi.org/10.3390/ijms161125943

Ichimura, S., Wu, J. J., & Eyre, D. R. (2000). Two-dimensional peptide mapping of cross-linked type IX collagen in human cartilage. *Archives of biochemistry and biophysics*, *378*(1), 33–39. https://doi.org/10.1006/abbi.2000.1805

Idleburg, C., Lorenz, M. R., DeLassus, E. N., Scheller, E. L., & Veis, D. J. (2021). Immunostaining of Skeletal Tissues. *Methods in molecular biology (Clifton, N.J.)*, *2221*, 261–273. https://doi.org/10.1007/978-1-0716-0989-7_15

Ilas, D. C., Churchman, S. M., McGonagle, D., & Jones, E. (2017). Targeting subchondral bone mesenchymal stem cell activities for intrinsic joint repair in osteoarthritis. *Future science OA*, *3*(4), FSO228. https://doi.org/10.4155/fsoa-2017-0055

Jackson, A., & Gu, W. (2009). TRANSPORT PROPERTIES OF CARTILAGINOUS TISSUES. *Current rheumatology reviews*, *5*(1), 40. https://doi.org/10.2174/157339709787315320

Jimi, E., & Ghosh, S. (2005). Role of nuclear factor-kappaB in the immune system and bone. *Immunological reviews*, *208*, 80–87. https://doi.org/10.1111/j.0105-2896.2005.00329.x

Johnson, K., Farley, D., Hu, S. I., & Terkeltaub, R. (2003). One of two chondrocyte-expressed isoforms of cartilage intermediate-layer protein functions as an insulin-like growth factor 1 antagonist. *Arthritis and rheumatism*, *48*(5), 1302–1314. https://doi.org/10.1002/art.10927

Johnson, V. L., & Hunter, D. J. (2014). The epidemiology of osteoarthritis. *Best practice & research. Clinical rheumatology*, *28*(1), 5–15. https://doi.org/10.1016/j.berh.2014.01.004

Kafian-Attari, I., Nippolainen, E., Bergmann, F., George, A., Paakkari, P., Mirhashemi, A., Foschum, F., Kienle, A., Töyräs, J., & Afara, I. O. (2023). Broadband scattering properties of articular cartilage zones and their relationship with the heterogenous structure of articular cartilage extracellular matrix. *Journal of biomedical optics*, *28*(12), 125003. https://doi.org/10.1117/1.JBO.28.12.125003

Kapoor, M., Martel-Pelletier, J., Lajeunesse, D., Pelletier, J. P., & Fahmi, H. (2011). Role of proinflammatory cytokines in the pathophysiology of osteoarthritis. *Nature reviews. Rheumatology*, *7*(1), 33–42. https://doi.org/10.1038/nrrheum.2010.196

Karuppal R. (2017). Current concepts in the articular cartilage repair and regeneration. *Journal of orthopaedics*, *14*(2), A1–A3. https://doi.org/10.1016/j.jor.2017.05.001

Kiernan J. (2001). Histological and histochemical methods: Theory and practice. 12.

Kim, T. D., Ryu, H. J., Cho, H. I., Yang, C. H., & Kim, J. (2000). Thermal behavior of proteins: heat-resistant proteins and their heat-induced secondary structural changes. *Biochemistry*, *39*(48), 14839–14846. https://doi.org/10.1021/bi001441y

Kim, S. W., Roh, J., & Park, C. S. (2016). Immunohistochemistry for Pathologists: Protocols, Pitfalls, and Tips. *Journal of pathology and translational medicine*, *50*(6), 411–418. https://doi.org/10.4132/jptm.2016.08.08

King, L. K., March, L., & Anandacoomarasamy, A. (2013). Obesity & osteoarthritis. *The Indian journal of medical research*, *138*(2), 185–193.

Knudson, C. B., & Knudson, W. (2001). Cartilage proteoglycans. *Seminars in cell & developmental biology*, *12*(2), 69–78. https://doi.org/10.1006/scdb.2000.0243

Kolasinski, S. L., Neogi, T., Hochberg, M. C., Oatis, C., Guyatt, G., Block, J., Callahan, L., Copenhaver, C., Dodge, C., Felson, D., Gellar, K., Harvey, W. F., Hawker, G., Herzig, E., Kwoh, C. K., Nelson, A. E., Samuels, J., Scanzello, C., White, D., Wise, B., … Reston, J. (2020). 2019 American College of Rheumatology/Arthritis Foundation Guideline for the Management of Osteoarthritis of the Hand, Hip, and Knee. *Arthritis care & research*, *72*(2), 149–162. https://doi.org/10.1002/acr.24131

Kouri, J. B., & Lavalle, C. (2006). Do chondrocytes undergo "activation" and "transdifferentiation" during the pathogenesis of osteoarthritis? A review of the ultrastructural and immunohistochemical evidence. *Histology and histopathology*, *21*(7), 793–802. https://doi.org/10.14670/HH-21.793

Kraus, V. B., Collins, J. E., Hargrove, D., Losina, E., Nevitt, M., Katz, J. N., Wang, S. X., Sandell, L. J., Hoffmann, S. C., Hunter, D. J., & OA Biomarkers Consortium (2017). Predictive validity of biochemical biomarkers in knee osteoarthritis: data from the FNIH OA Biomarkers Consortium. *Annals of the rheumatic diseases*, *76*(1), 186–195. https://doi.org/10.1136/annrheumdis-2016-209252

Kuhi, L., Tamm, A. E., Tamm, A. O., & Kisand, K. (2020). Cartilage collagen neoepitope C2C in urine as an integrative diagnostic marker for early knee osteoarthritis. *Osteoarthritis and cartilage open*, *2*(4), 100096. https://doi.org/10.1016/j.ocarto.2020.100096

Kuhi, L., Tamm, A. E., Tamm, A. O., & Kisand, K. (2021). Risk Assessment of the Progression of Early Knee Osteoarthritis by Collagen Neoepitope C2C: A Longitudinal Study of an Estonian Middle-Aged Cohort. *Diagnostics (Basel, Switzerland)*, *11*(7), 1236. https://doi.org/10.3390/diagnostics11071236

Kumar, A., Dutta Choudhury, M., Ghosh, P., & Palit, P. (2019). Discoidin domain receptor 2: An emerging pharmacological drug target for prospective therapy against osteoarthritis. *Pharmacological reports: PR*, *71*(3), 399–408. https://doi.org/10.1016/j.pharep.2019.01.007

Kuyinu, E. L., Narayanan, G., Nair, L. S., & Laurencin, C. T. (2016). Animal models of osteoarthritis: classification, update, and measurement of outcomes. *Journal of orthopaedic surgery and research*, *11*, 19. https://doi.org/10.1186/s13018-016-0346-5

Kvist, A. J., Nyström, A., Hultenby, K., Sasaki, T., Talts, J. F., & Aspberg, A. (2008). The major basement membrane components localize to the chondrocyte pericellular matrix--a cartilage basement membrane equivalent? *Matrix biology: journal of the International Society for Matrix Biology*, *27*(1), 22–33. https://doi.org/10.1016/j.matbio.2007.07.007

Lahm, A., Mrosek, E., Spank, H., Erggelet, C., Kasch, R., Esser, J., & Merk, H. (2010). Changes in content and synthesis of collagen types and proteoglycans in osteoarthritis of the knee joint and comparison of quantitative analysis with Photoshop-based image analysis. *Archives of orthopaedic and trauma surgery*, *130*(4), 557–564. https://doi.org/10.1007/s00402-009-0981-y

Lai, Y., Zheng, W., Qu, M., Xiao, C. C., Chen, S., Yao, Q., Gong, W., Tao, C., Yan, Q., Zhang, P., Wu, X., & Xiao, G. (2022). Kindlin-2 loss in condylar chondrocytes causes

spontaneous osteoarthritic lesions in the temporomandibular joint in mice. *International journal of oral science*, *14*(1), 33. https://doi.org/10.1038/s41368-022-00185-1

Lam, K. C., & Markbreiter, J. G. (2019). The Impact of Knee Injury History on Health-Related Quality of Life in Adolescent Athletes. *Journal of sport rehabilitation*, *28*(2), 115–119. https://doi.org/10.1123/jsr.2017-0226

Le, C. Y., Toomey, C. M., Emery, C. A., & Whittaker, J. L. (2021). What Does the Future Hold? Health-Related Quality of Life 3-12 Years Following a Youth Sport-Related Knee Injury. *International journal of environmental research and public health*, *18*(13), 6877. https://doi.org/10.3390/ijerph18136877

Lee, G. M., Paul, T. A., Slabaugh, M., & Kelley, S. S. (2000). The incidence of enlarged chondrons in normal and osteoarthritic human cartilage and their relative matrix density. *Osteoarthritis and cartilage*, *8*(1), 44–52. https://doi.org/10.1053/joca.1999.0269

Leifer, V. P., Katz, J. N., & Losina, E. (2022). The burden of OA-health services and economics. *Osteoarthritis and cartilage*, *30*(1), 10–16. https://doi.org/10.1016/j.joca.2021.05.007

Li, W., Wu, N., Wang, J., Wang, Y., Wu, M., & Wang, H. (2022). Role of HIF-2α/NF-κB pathway in mechanical stress-induced temporomandibular joint osteoarthritis. *Oral diseases*, *28*(8), 2239–2247. https://doi.org/10.1111/odi.13986

Liang, L., Moore, B., & Soni, A. (2020). National Inpatient Hospital Costs: The Most Expensive Conditions by Payer, 2017. In *Healthcare Cost and Utilization Project (HCUP) Statistical Briefs*. Agency for Healthcare Research and Quality (US).

Litwic, A., Edwards, M. H., Dennison, E. M., & Cooper, C. (2013). Epidemiology and burden of osteoarthritis. *British medical bulletin*, *105*, 185–199. https://doi.org/10.1093/bmb/lds038

Liu, C., Wang, B., Xiao, L., Li, Y., Xu, L., Zhao, Z., & Zhang, L. (2018). Protective effects of the pericellular matrix of chondrocyte on articular cartilage against the development of osteoarthritis. *Histology and histopathology*, *33*(8), 757–764. https://doi.org/10.14670/HH-11-967

Loeser R. F. (2010). Age-related changes in the musculoskeletal system and the development of osteoarthritis. *Clinics in geriatric medicine*, *26*(3), 371–386. https://doi.org/10.1016/j.cger.2010.03.002

Loeser, R. F., Goldring, S. R., Scanzello, C. R., & Goldring, M. B. (2012). Osteoarthritis: a disease of the joint as an organ. *Arthritis and rheumatism*, *64*(6), 1697–1707. https://doi.org/10.1002/art.34453

Lohmander, L. S., Ostenberg, A., Englund, M., & Roos, H. (2004). High prevalence of knee osteoarthritis, pain, and functional limitations in female soccer players twelve years after anterior cruciate ligament injury. *Arthritis and rheumatism*, *50*(10), 3145–3152. https://doi.org/10.1002/art.20589

Lohmander, L. S., Englund, P. M., Dahl, L. L., & Roos, E. M. (2007). The long-term consequence of anterior cruciate ligament and meniscus injuries: osteoarthritis. *The American journal of sports medicine*, *35*(10), 1756–1769. https://doi.org/10.1177/0363546507307396

Long, H., Liu, Q., Yin, H., Wang, K., Diao, N., Zhang, Y., Lin, J., & Guo, A. (2022). Prevalence Trends of Site-Specific Osteoarthritis From 1990 to 2019: Findings From the Global Burden of Disease Study 2019. *Arthritis & rheumatology (Hoboken, N.J.)*, *74*(7), 1172–1183. https://doi.org/10.1002/art.42089

Lorenzo, P., Bayliss, M. T., & Heinegård, D. (1998). A novel cartilage protein (CILP) present in the mid-zone of human articular cartilage increases with age. *The Journal of biological chemistry*, *273*(36), 23463–23468. https://doi.org/10.1074/jbc.273.36.23463

Lorenzo, P., Bayliss, M. T., & Heinegård, D. (2004). Altered patterns and synthesis of extracellular matrix macromolecules in early osteoarthritis. *Matrix biology: journal of the International Society for Matrix Biology*, *23*(6), 381–391. https://doi.org/10.1016/j.matbio.2004.07.007

Lorenzo, P., Saxne, T., & Heinegård, D. Cartilage Intermediate Layer Protein 2 C1 and Its Use to Differentiate Osteoarthritis from Rheumatoid Arthritis and Non-Disease Conditions. U.S. Patent US 2011 0076708A1, 4 March 2014. Available online: https://patents.google.com/patent/US20110076708A1/en (accessed on 18 June 2024).

Luo, Y., Sinkeviciute, D., He, Y., Karsdal, M., Henrotin, Y., Mobasheri, A., Önnerfjord, P., & Bay-Jensen, A. (2017). The minor collagens in articular cartilage. *Protein & cell*, *8*(8), 560–572. https://doi.org/10.1007/s13238-017-0377-7

Maldonado, M., & Nam, J. (2013). The role of changes in extracellular matrix of cartilage in the presence of inflammation on the pathology of osteoarthritis. *BioMed research international*, *2013*, 284873. https://doi.org/10.1155/2013/284873

Mansfield, J. C., Bell, J. S., & Winlove, C. P. (2015). The micromechanics of the superficial zone of articular cartilage. *Osteoarthritis and cartilage*, *23*(10), 1806–1816. https://doi.org/10.1016/j.joca.2015.05.030

Martel-Pelletier, J., Boileau, C., Pelletier, J. P., & Roughley, P. J. (2008). Cartilage in normal and osteoarthritis conditions. *Best practice & research. Clinical rheumatology*, *22*(2), 351–384. https://doi.org/10.1016/j.berh.2008.02.001

Martel-Pelletier, J., Barr, A. J., Cicuttini, F. M., Conaghan, P. G., Cooper, C., Goldring, M. B., Goldring, S. R., Jones, G., Teichtahl, A. J., & Pelletier, J. P. (2016). Osteoarthritis. *Nature reviews. Disease primers*, *2*, 16072. https://doi.org/10.1038/nrdp.2016.72

Matsuo, M., Nishida, K., Yoshida, A., Murakami, T., & Inoue, H. (2001). Expression of caspase-3 and -9 relevant to cartilage destruction and chondrocyte apoptosis in human osteoarthritic cartilage. *Acta medica Okayama*, *55*(6), 333–340. https://doi.org/10.18926/AMO/32000

McGuine, T. A., Winterstein, A. P., Carr, K., & Hetzel, S. (2014). Changes in Health-Related Quality of Life and Knee Function After Knee Injury in Young Female Athletes. *Orthopaedic journal of sports medicine*, *2*(4), 2325967114530988. https://doi.org/10.1177/2325967114530988

McNicol, A. M., & Richmond, J. A. (1998). Optimizing immunohistochemistry: antigen retrieval and signal amplification. *Histopathology*, *32*(2), 97–103. https://doi.org/10.1046/j.1365-2559.1998.00342.x

Metcalfe, A. J., Andersson, M. L., Goodfellow, R., & Thorstensson, C. A. (2012). Is knee osteoarthritis a symmetrical disease? Analysis of a 12 year prospective cohort study. *BMC musculoskeletal disorders*, *13*, 153. https://doi.org/10.1186/1471-2474-13-153

Meyerholz, D. K., & Beck, A. P. (2018). Principles and approaches for reproducible scoring of tissue stains in research. *Laboratory investigation; a journal of technical methods and pathology*, *98*(7), 844–855. https://doi.org/10.1038/s41374-018-0057-0

Millward-Sadler, S. J., & Salter, D. M. (2004). Integrin-dependent signal cascades in chondrocyte mechanotransduction. *Annals of biomedical engineering*, *32*(3), 435–446. https://doi.org/10.1023/b:abme.0000017538.72511.48

Miosge, N., Flachsbart, K., Goetz, W., Schultz, W., Kresse, H., & Herken, R. (1994). Light and electron microscopical immunohistochemical localization of the small proteoglycan core proteins decorin and biglycan in human knee joint cartilage. *The Histochemical journal*, *26*(12), 939–945.

Miosge, N., Hartmann, M., Maelicke, C., & Herken, R. (2004). Expression of collagen type I and type II in consecutive stages of human osteoarthritis. *Histochemistry and cell biology*, *122*(3), 229–236. https://doi.org/10.1007/s00418-004-0697-6

Mobasheri A. (2012). Osteoarthritis year 2012 in review: biomarkers. *Osteoarthritis and cartilage*, *20*(12), 1451–1464. https://doi.org/10.1016/j.joca.2012.07.009

Mori, M., Nakajima, M., Mikami, Y., Seki, S., Takigawa, M., Kubo, T., & Ikegawa, S. (2006). Transcriptional regulation of the cartilage intermediate layer protein (CILP) gene. *Biochemical and biophysical research communications*, *341*(1), 121–127. https://doi.org/10.1016/j.bbrc.2005.12.159

Mow, V. C., Ratcliffe, A., & Poole, A. R. (1992). Cartilage and diarthrodial joints as paradigms for hierarchical materials and structures. *Biomaterials*, *13*(2), 67–97. https://doi.org/10.1016/0142-9612(92)90001-5

Mow, V. C., & Guo, X. E. (2002). Mechano-electrochemical properties of articular cartilage: their inhomogeneities and anisotropies. *Annual review of biomedical engineering*, *4*, 175–209. https://doi.org/10.1146/annurev.bioeng.4.110701.120309

Muthuri, S. G., McWilliams, D. F., Doherty, M., & Zhang, W. (2011). History of knee injuries and knee osteoarthritis: a meta-analysis of observational studies. *Osteoarthritis and cartilage*, *19*(11), 1286–1293. https://doi.org/10.1016/j.joca.2011.07.015

Nigoro, K., Ito, H., Kawata, T., Ishie, S., Morita, Y., Nishitani, K., Aoyama, T., Tabara, Y., Matsuda, F., Narumiya, S., & Matsuda, S. (2021). Differential Contribution of the Medial and the Lateral Side of the Joint to Symptoms in Knee Osteoarthritis: A Radiographic and Laboratory Analysis in the Nagahama Study. *Cartilage*, *13*(1_suppl), 1648S–1657S. https://doi.org/10.1177/19476035211025817

Niu, J., Zhang, Y. Q., Torner, J., Nevitt, M., Lewis, C. E., Aliabadi, P., Sack, B., Clancy, M., Sharma, L., & Felson, D. T. (2009). Is obesity a risk factor for progressive radiographic knee osteoarthritis? *Arthritis and rheumatism*, *61*(3), 329–335. https://doi.org/10.1002/art.24337

O'Brien, M. S., & McDougall, J. J. (2019). Age and frailty as risk factors for the development of osteoarthritis. *Mechanisms of ageing and development*, *180*, 21–28. https://doi.org/10.1016/j.mad.2019.03.003

Oliviero, F., & Ramonda, R. (2021). Cartilage-derived biomarkers in osteoarthritis. *The Indian journal of medical research*, *153*(4), 413–415. https://doi.org/10.4103/ijmr.IJMR_436_20

O'Neill, T. W., & Felson, D. T. (2018) Mechanisms of Osteoarthritis (OA) Pain. *Current osteoporosis reports*, *16*(5), 611–616. https://doi.org/10.1007/s11914-018-0477-1

O'Neill, T. W., McCabe, P. S., & McBeth, J. (2018). Update on the epidemiology, risk factors and disease outcomes of osteoarthritis. *Best practice & research. Clinical rheumatology*, *32*(2), 312–326. https://doi.org/10.1016/j.berh.2018.10.007

Paduszyński, W., Jeśkiewicz, M., Uchański, P., Gackowski, S., Radkowski, M., & Demkow, U. (2018). Hoffa's Fat Pad Abnormality in the Development of Knee Osteoarthritis. *Advances in experimental medicine and biology*, *1039*, 95–102. https://doi.org/10.1007/5584_2017_77

Pai V, Knipe H, Rock P, et al. Kellgren and Lawrence system for classification of osteoarthritis. Reference article, Radiopaedia.org (Accessed on 11 Apr 2025) https://doi.org/10.53347/rID-27111

Palazzo, C., Nguyen, C., Lefevre-Colau, M. M., Rannou, F., & Poiraudeau, S. (2016). Risk factors and burden of osteoarthritis. *Annals of physical and rehabilitation medicine*, *59*(3), 134–138. https://doi.org/10.1016/j.rehab.2016.01.006

Palotie, A., Väisänen, P., Ott, J., Ryhänen, L., Elima, K., Vikkula, M., Cheah, K., Vuorio, E., & Peltonen, L. (1989). Predisposition to familial osteoarthrosis linked to type II collagen gene. *Lancet (London, England)*, *1*(8644), 924–927. https://doi.org/10.1016/s0140-6736(89)92507-5

Pereira, D., Ramos, E., & Branco, J. (2015). Osteoarthritis. *Acta medica portuguesa*, *28*(1), 99–106. https://doi.org/10.20344/amp.5477

Perrot S. (2015). Osteoarthritis pain. *Best practice & research. Clinical rheumatology*, *29*(1), 90–97. https://doi.org/10.1016/j.berh.2015.04.017

Peters, A. E., Geraghty, B., Bates, K. T., Akhtar, R., Readioff, R., & Comerford, E. (2022). Ligament mechanics of ageing and osteoarthritic human knees. *Frontiers in bioengineering and biotechnology*, *10*, 954837. https://doi.org/10.3389/fbioe.2022.954837

Pérez H, E., Luna M, J., Rojas M, L., & Kouri, J. B. (2005). Chondroptosis: an immunohistochemical study of apoptosis and Golgi complex in chondrocytes from human osteoarthritic cartilage. *Apoptosis: an international journal on programmed cell death*, *10*(5), 1105–1110. https://doi.org/10.1007/s10495-005-0649-1

Piccolo, C. L., Mallio, C. A., Vaccarino, F., Grasso, R. F., & Zobel, B. B. (2023). Imaging of knee osteoarthritis: a review of multimodal diagnostic approach. *Quantitative imaging in medicine and surgery*, *13*(11), 7582–7595. https://doi.org/10.21037/qims-22-1392

Polur, I., Lee, P. L., Servais, J. M., Xu, L., & Li, Y. (2010). Role of HTRA1, a serine protease, in the progression of articular cartilage degeneration. *Histology and histopathology*, *25*(5), 599–608. https://doi.org/10.14670/HH-25.599

Poole, C. A., Flint, M. H., & Beaumont, B. W. (1987). Chondrons in cartilage: ultrastructural analysis of the pericellular microenvironment in adult human articular cartilages. *Journal of orthopaedic research: official publication of the Orthopaedic Research Society*, *5*(4), 509–522. https://doi.org/10.1002/jor.1100050406

Poole, C. A., Ayad, S., & Schofield, J. R. (1988). Chondrons from articular cartilage: I. Immunolocalization of type VI collagen in the pericellular capsule of isolated canine tibial chondrons. *Journal of cell science*, *90 (Pt 4)*, 635–643. https://doi.org/10.1242/jcs.90.4.635

Poole C. A. (1997). Articular cartilage chondrons: form, function and failure. *Journal of anatomy*, *191 (Pt 1)* (Pt 1), 1–13. https://doi.org/10.1046/j.1469-7580.1997.19110001.x

Poole, A. R., Ionescu, M., Fitzcharles, M. A., & Billinghurst, R. C. (2004). The assessment of cartilage degradation in vivo: development of an immunoassay for the measurement in body fluids of type II collagen cleaved by collagenases. *Journal of immunological methods*, *294*(1–2), 145–153. https://doi.org/10.1016/j.jim.2004.09.005

Poole A. R. (2012). Osteoarthritis as a whole joint disease. *HSS journal: the musculoskeletal journal of Hospital for Special Surgery*, *8*(1), 4–6. https://doi.org/10.1007/s11420-011-9248-6

Poole, A. R., Ha, N., Bourdon, S., Sayre, E. C., Guermazi, A., & Cibere, J. (2016). Ability of a Urine Assay of Type II Collagen Cleavage by Collagenases to Detect Early Onset and Progression of Articular Cartilage Degeneration: Results from a Population-based Cohort Study. *The Journal of rheumatology*, *43*(10), 1864–1870. https://doi.org/10.3899/jrheum.150917

Primorac, D., Molnar, V., Rod, E., Jeleč, Ž., Čukelj, F., Matišić, V., Vrdoljak, T., Hudetz, D., Hajsok, H., & Borić, I. (2020). Knee Osteoarthritis: A Review of Pathogenesis and State-Of-The-Art Non-Operative Therapeutic Considerations. *Genes*, *11*(8), 854. https://doi.org/10.3390/genes11080854

Pritzker, K. P., Gay, S., Jimenez, S. A., Ostergaard, K., Pelletier, J. P., Revell, P. A., Salter, D., & van den Berg, W. B. (2006). Osteoarthritis cartilage histopathology: grading and staging. *Osteoarthritis and cartilage*, *14*(1), 13–29. https://doi.org/10.1016/j.joca.2005.07.014

Quinn, T. M., Hunziker, E. B., & Häuselmann, H. J. (2005). Variation of cell and matrix morphologies in articular cartilage among locations in the adult human knee. *Osteoarthritis and cartilage*, *13*(8), 672–678. https://doi.org/10.1016/j.joca.2005.04.011

Raju, P. K., Kini, S. G., & Verma, A. (2012). Wear patterns of tibiofemoral articulation in osteoarthritic knees: analysis and review of literature. *Archives of orthopaedic and trauma surgery*, *132*(9), 1267–1271. https://doi.org/10.1007/s00402-012-1547-y

Ramos-Vara, J. A., & Miller, M. A. (2014). When tissue antigens and antibodies get along: revisiting the technical aspects of immunohistochemistry – the red, brown, and blue technique. *Veterinary pathology*, *51*(1), 42–87. https://doi.org/10.1177/0300985813505879

Regoli, M., Tosi, G. M., Neri, G., Altera, A., Orazioli, D., & Bertelli, E. (2020). The Peculiar Pattern of Type IV Collagen Deposition in Epiretinal Membranes. *The journal of histochemistry and cytochemistry: official journal of the Histochemistry Society*, *68*(2), 149–162. https://doi.org/10.1369/0022155419897258

Rellmann, Y., Eidhof, E., & Dreier, R. (2021). Review: ER stress-induced cell death in osteoarthritic cartilage. *Cellular signalling*, *78*, 109880. https://doi.org/10.1016/j.cellsig.2020.109880

Roach, H. I., Aigner, T., & Kouri, J. B. (2004). Chondroptosis: a variant of apoptotic cell death in chondrocytes? *Apoptosis: an international journal on programmed cell death*, *9*(3), 265–277. https://doi.org/10.1023/b:appt.0000025803.17498.26

Rodriguez-Merchan E. C. (2023). The Current Role of Disease-modifying Osteoarthritis Drugs. *The archives of bone and joint surgery*, *11*(1), 11–22. https://doi.org/10.22038/ABJS.2021.56530.2807

Roemer, F. W., Guermazi, A., Hunter, D. J., Niu, J., Zhang, Y., Englund, M., Javaid, M. K., Lynch, J. A., Mohr, A., Torner, J., Lewis, C. E., Nevitt, M. C., & Felson, D. T. (2009). The association of meniscal damage with joint effusion in persons without radiographic osteoarthritis: the Framingham and MOST osteoarthritis studies. *Osteoarthritis and cartilage*, *17*(6), 748–753. https://doi.org/10.1016/j.joca.2008.09.013

Salazar, V. S., Gamer, L. W., & Rosen, V. (2016). BMP signalling in skeletal development, disease and repair. *Nature reviews. Endocrinology*, *12*(4), 203–221. https://doi.org/10.1038/nrendo.2016.12

Salucci, S., Falcieri, E., & Battistelli, M. (2022). Chondrocyte death involvement in osteoarthritis. *Cell and tissue research*, *389*(2), 159–170. https://doi.org/10.1007/s00441-022-03639-4

Schipplein, O. D., & Andriacchi, T. P. (1991). Interaction between active and passive knee stabilizers during level walking. *Journal of orthopaedic research: official publication of the Orthopaedic Research Society*, *9*(1), 113–119. https://doi.org/10.1002/jor.1100090114

Schmidt, T. A., Gastelum, N. S., Nguyen, Q. T., Schumacher, B. L., & Sah, R. L. (2007). Boundary lubrication of articular cartilage: role of synovial fluid constituents. *Arthritis and rheumatism*, *56*(3), 882–891. https://doi.org/10.1002/art.22446

Schminke, B., Frese, J., Bode, C., Goldring, M. B., & Miosge, N. (2016). Laminins and Nidogens in the Pericellular Matrix of Chondrocytes: Their Role in Osteoarthritis and Chondrogenic Differentiation. *The American journal of pathology*, *186*(2), 410–418. https://doi.org/10.1016/j.ajpath.2015.10.014

Sellam, J., & Berenbaum, F. (2010). The role of synovitis in pathophysiology and clinical symptoms of osteoarthritis. *Nature reviews. Rheumatology*, *6*(11), 625–635. https://doi.org/10.1038/nrrheum.2010.159

Semenistaja, S., Skuja, S., Kadisa, A., & Groma, V. (2023). Healthy and Osteoarthritis-Affected Joints Facing the Cellular Crosstalk. *International journal of molecular sciences*, *24*(4), 4120. https://doi.org/10.3390/ijms24044120

Sharma, L., Kapoor, D., & Issa, S. (2006). Epidemiology of osteoarthritis: an update. *Current opinion in rheumatology*, 18(2), 147–156. https://doi.org/10.1097/01.bor.0000209426.84775.f8

Shi, S. R., Cote, R. J., & Taylor, C. R. (2001). Antigen retrieval techniques: current perspectives. *The journal of histochemistry and cytochemistry: official journal of the Histochemistry Society*, *49*(8), 931–937. https://doi.org/10.1177/002215540104900801

Siebelt, Michiel. (2015). Osteoarthritis: pathogenesis and therapeutic interventions for a whole joint disease. 10.13140/RG.2.1.3549.9683.

Silverwood, V., Blagojevic-Bucknall, M., Jinks, C., Jordan, J. L., Protheroe, J., & Jordan, K. P. (2015). Current evidence on risk factors for knee osteoarthritis in older adults: a systematic review and meta-analysis. *Osteoarthritis and cartilage*, *23*(4), 507–515. https://doi.org/10.1016/j.joca.2014.11.019

Solá, R. J., & Griebenow, K. (2009). Effects of glycosylation on the stability of protein pharmaceuticals. *Journal of pharmaceutical sciences*, *98*(4), 1223–1245. https://doi.org/10.1002/jps.21504

Song, B., Estrada, K. D., & Lyons, K. M. (2009). Smad signaling in skeletal development and regeneration. *Cytokine & growth factor reviews*, *20*(5-6), 379–388. https://doi.org/10.1016/j.cytogfr.2009.10.010

Sophia Fox, A. J., Bedi, A., & Rodeo, S. A. (2009). The basic science of articular cartilage: structure, composition, and function. *Sports health*, *1*(6), 461–468. https://doi.org/10.1177/1941738109350438

Sorushanova, A., Delgado, L. M., Wu, Z., Shologu, N., Kshirsagar, A., Raghunath, R., Mullen, A. M., Bayon, Y., Pandit, A., Raghunath, M., & Zeugolis, D. I. (2019). The Collagen Suprafamily: From Biosynthesis to Advanced Biomaterial Development. *Advanced materials (Deerfield Beach, Fla.)*, *31*(1), e1801651. https://doi.org/10.1002/adma.201801651

Srikanth, V. K., Fryer, J. L., Zhai, G., Winzenberg, T. M., Hosmer, D., & Jones, G. (2005). A meta-analysis of sex differences prevalence, incidence and severity of osteoarthritis. *Osteoarthritis and cartilage*, *13*(9), 769–781. https://doi.org/10.1016/j.joca.2005.04.014

Szirmai, J. A. (1969). Aging of Connective and Skeletal Tissue. *Structure of cartilage*, 163–200

Szponder, T., Latalski, M., Danielewicz, A., Krać, K., Kozera, A., Drzewiecka, B., Nguyen Ngoc, D., Dobko, D., & Wessely-Szponder, J. (2022). Osteoarthritis: Pathogenesis, Animal Models, and New Regenerative Therapies. *Journal of clinical medicine*, *12*(1), 5. https://doi.org/10.3390/jcm12010005

Stevens, A. L., Wishnok, J. S., White, F. M., Grodzinsky, A. J., & Tannenbaum, S. R. (2009). Mechanical injury and cytokines cause loss of cartilage integrity and upregulate proteins associated with catabolism, immunity, inflammation, and repair. *Molecular & cellular proteomics: MCP*, *8*(7), 1475–1489. https://doi.org/10.1074/mcp.M800181-MCP200

Stoddart, J. C., Dandridge, O., Garner, A., Cobb, J., & van Arkel, R. J. (2021). The compartmental distribution of knee osteoarthritis – a systematic review and meta-analysis. *Osteoarthritis and cartilage*, *29*(4), 445–455. https://doi.org/10.1016/j.joca.2020.10.011

Sunk, I. G., Bobacz, K., Hofstaetter, J. G., Amoyo, L., Soleiman, A., Smolen, J., Xu, L., & Li, Y. (2007). Increased expression of discoidin domain receptor 2 is linked to the degree of cartilage damage in human knee joints: a potential role in osteoarthritis pathogenesis. *Arthritis and rheumatism*, *56*(11), 3685–3692. https://doi.org/10.1002/art.22970

Suutre, S., Toom, A., Arend, A., & Selstam, G. (2009). Bone tissue content of TGF-beta2 changes with time in human heterotopic ossification after total hip arthroplasty. *Growth factors (Chur, Switzerland)*, *27*(2), 114–120. https://doi.org/10.1080/08977190802703976

Suutre, S., Kerna, I., Lintrop, M., Tamm, H., Aunapuu, M., Arend, A., & Tamm, A. (2015). Evaluation of correlation of articular cartilage staining for DDR2 and proteoglycans with histological tissue damage and the results of radiographic assessment in patients with early stages of knee osteoarthritis. *International journal of clinical and experimental pathology*, *8*(5), 5658–5665.

Swingler, T. E., Niu, L., Smith, P., Paddy, P., Le, L., Barter, M. J., Young, D. A., & Clark, I. M. (2019). The function of microRNAs in cartilage and osteoarthritis. *Clinical and experimental rheumatology*, *37 Suppl 120*(5), 40–47.

Şenol, M. S., Özer, H. (2020). Chapter 7 – Architecture of cartilage tissue and its adaptation to pathological conditions. Comparative Kinesiology of the Human Body *Academic Press*, 91–100. https://doi.org/10.1016/B978-0-12-812162-7.00007-2.

Zeng, N., Yan, Z. P., Chen, X. Y., & Ni, G. X. (2020). Infrapatellar Fat Pad and Knee Osteoarthritis. *Aging and disease*, *11*(5), 1317–1328. https://doi.org/10.14336/AD.2019.1116

Zhang, Z. (2015). Chondrons and the pericellular matrix of chondrocytes. *Tissue engineering. Part B, Reviews*, *21*(3), 267–277. https://doi.org/10.1089/ten.teb.2014.0286

Zhang, Y., & Que, J. (2020). BMP Signaling in Development, Stem Cells, and Diseases of the Gastrointestinal Tract. *Annual review of physiology*, *82*, 251–273. https://doi.org/10.1146/annurev-physiol-021119-034500

Zheng, T., Li, Y., Zhang, X., Xu, J., & Luo, M. (2022). Exosomes Derived From miR-212-5p Overexpressed Human Synovial Mesenchymal Stem Cells Suppress Chondrocyte Degeneration and Inflammation by Targeting ELF3. *Frontiers in bioengineering and biotechnology*, *10*, 816209. https://doi.org/10.3389/fbioe.2022.816209

Zhong, L., Huang, X., Karperien, M., & Post, J. N. (2015). The Regulatory Role of Signaling Crosstalk in Hypertrophy of MSCs and Human Articular Chondrocytes. *International journal of molecular sciences*, *16*(8), 19225–19247. https://doi.org/10.3390/ijms160819225

Zhu, W., Zhang, X., Jiang, Y., Liu, X., Huang, L., Wei, Q., Huang, Y., Wu, W., & Gu, J. (2020). Alterations in peripheral T cell and B cell subsets in patients with osteoarthritis. *Clinical rheumatology*, *39*(2), 523–532. https://doi.org/10.1007/s10067-019-04768-y

Zoricic, S., Maric, I., Bobinac, D., & Vukicevic, S. (2003). Expression of bone morphogenetic proteins and cartilage-derived morphogenetic proteins during osteophyte formation in humans. *Journal of anatomy*, *202*(Pt 3), 269–277. https://doi.org/10.1046/j.1469-7580.2003.00158.x

Tamm, A. O., Kumm, J., Tamm, A., Lintrop, M., Kukner, A., Saluse, T., Rips, L., Tein, T., Pintsaar, A., & Kukner, A. (2014) Cartilage collagen neoepitope c2c and clinical parameters in middle-aged patients with knee problems. Correlations of urinary output of C2C with cartilage lesions, koos values and functional abilities of lower limb. *Osteoarthritis and cartilage*, 22:S70–S71. doi: 10.1016/j.joca.2014.02.144.

Thomas, N. P., Li, P., Fleming, B. C., Chen, Q., Wei, X., Xiao-Hua, P., Li, G., & Wei, L. (2015). Attenuation of cartilage pathogenesis in post-traumatic osteoarthritis (PTOA) in mice by blocking the stromal derived factor 1 receptor (CXCR4) with the specific inhibitor, AMD3100. *Journal of orthopaedic research: official publication of the Orthopaedic Research Society*, *33*(7), 1071–1078. https://doi.org/10.1002/jor.22862

Torga, T., Suutre, S., Kisand, K., Aunapuu, M., & Arend, A. (2024). Cartilage Collagen Neoepitope C2C Expression in the Articular Cartilage and Its Relation to Joint Tissue Damage in Patients with Knee Osteoarthritis. *Biomedicines*, *12*(5), 1063. https://doi.org/10.3390/biomedicines12051063

Tsuruha, J., Masuko-Hongo, K., Kato, T., Sakata, M., Nakamura, H., & Nishioka, K. (2001). Implication of cartilage intermediate layer protein in cartilage destruction in subsets of patients with osteoarthritis and rheumatoid arthritis. *Arthritis and rheumatism*, *44*(4), 838–845. https://doi.org/10.1002/1529-0131(200104)44:4<838::AID-ANR140>3.0.CO;2-C

Uchino, M., Izumi, T., Tominaga, T., Wakita, R., Minehara, H., Sekiguchi, M., & Itoman, M. (2000). Growth factor expression in the osteophytes of the human femoral head in osteoarthritis. *Clinical orthopaedics and related research*, (377), 119–125. https://doi.org/10.1097/00003086-200008000-00017

Ulivi, V., Giannoni, P., Gentili, C., Cancedda, R., & Descalzi, F. (2008). p38/NF-kB-dependent expression of COX-2 during differentiation and inflammatory response of chondrocytes. *Journal of cellular biochemistry*, *104*(4), 1393–1406. https://doi.org/10.1002/jcb.21717

Ulrich-Vinther, M., Maloney, M. D., Schwarz, E. M., Rosier, R., & O'Keefe, R. J. (2003). Articular cartilage biology. *The Journal of the American Academy of Orthopaedic Surgeons*, *11*(6), 421–430. https://doi.org/10.5435/00124635-200311000-00006

Valdes, A. M., Arden, N. K., Tamm, A., Kisand, K., Doherty, S., Pola, E., Cooper, C., Tamm, A., Muir, K. R., Kerna, I., Hart, D., O'Neil, F., Zhang, W., Spector, T. D., Maciewicz, R. A., & Doherty, M. (2010). A meta-analysis of interleukin-6 promoter polymorphisms on risk of hip and knee osteoarthritis. *Osteoarthritis and cartilage*, *18*(5), 699–704. https://doi.org/10.1016/j.joca.2009.12.012

Valdes, A. M., Spector, T. D., Tamm, A., Kisand, K., Doherty, S. A., Dennison, E. M., Mangino, M., Tamm, A., Kerna, I., Hart, D. J., Wheeler, M., Cooper, C., Lories, R. J., Arden, N. K., & Doherty, M. (2010). Genetic variation in the SMAD3 gene is associated with hip and knee osteoarthritis. *Arthritis and rheumatism*, *62*(8), 2347–2352. https://doi.org/10.1002/art.27530

Valdes A. M. (2020). Metabolic syndrome and osteoarthritis pain: common molecular mechanisms and potential therapeutic implications. *Osteoarthritis and cartilage*, *28*(1), 7–9. https://doi.org/10.1016/j.joca.2019.06.015

van Beuningen, H. M., Glansbeek, H. L., van der Kraan, P. M., & van den Berg, W. B. (1998). Differential effects of local application of BMP-2 or TGF-beta 1 on both articular cartilage composition and osteophyte formation. *Osteoarthritis and cartilage*, *6*(5), 306–317. https://doi.org/10.1053/joca.1998.0129

van Spil, W. E., DeGroot, J., Lems, W. F., Oostveen, J. C., & Lafeber, F. P. (2010). Serum and urinary biochemical markers for knee and hip-osteoarthritis: a systematic review applying the consensus BIPED criteria. *Osteoarthritis and cartilage*, *18*(5), 605–612. https://doi.org/10.1016/j.joca.2010.01.012

van Spil, W. E., Drossaers-Bakker, K. W., & Lafeber, F. P. (2013). Associations of CTX-II with biochemical markers of bone turnover raise questions on its tissue origin: data

from CHECK, a cohort study of early osteoarthritis. *Annals of the rheumatic diseases*, *72*(1), 29–36. https://doi.org/10.1136/annrheumdis-2011-201177

van der Kraan, P. M., & van den Berg, W. B. (2007). Osteophytes: relevance and biology. *Osteoarthritis and cartilage*, *15*(3), 237–244. https://doi.org/10.1016/j.joca.2006.11.006

van der Kraan, P. M., Blaney Davidson, E. N., Blom, A., & van den Berg, W. B. (2009). TGF-beta signaling in chondrocyte terminal differentiation and osteoarthritis: modulation and integration of signaling pathways through receptor-Smads. *Osteoarthritis and cartilage*, *17*(12), 1539–1545. https://doi.org/10.1016/j.joca.2009.06.008

van Osch, G. J., van der Kraan, P. M., van Valburg, A. A., & van den Berg, W. B. (1996). The relation between cartilage damage and osteophyte size in a murine model for osteoarthritis in the knee. *Rheumatology international*, *16*(3), 115–119. https://doi.org/10.1007/BF01409983

Vincent, T. L., McClurg, O., & Troeberg, L. (2022). The Extracellular Matrix of Articular Cartilage Controls the Bioavailability of Pericellular Matrix-Bound Growth Factors to Drive Tissue Homeostasis and Repair. *International journal of molecular sciences*, *23*(11), 6003. https://doi.org/10.3390/ijms23116003

Vincenti, M. P., & Brinckerhoff, C. E. (2002). Transcriptional regulation of collagenase (MMP-1, MMP-13) genes in arthritis: integration of complex signaling pathways for the recruitment of gene-specific transcription factors. *Arthritis research*, *4*(3), 157–164. https://doi.org/10.1186/ar401

Wallace, I. J., Worthington, S., Felson, D. T., Jurmain, R. D., Wren, K. T., Maijanen, H., Woods, R. J., & Lieberman, D. E. (2017). Knee osteoarthritis has doubled in prevalence since the mid-20th century. *Proceedings of the National Academy of Sciences of the United States of America*, *114*(35), 9332–9336. https://doi.org/10.1073/pnas.1703856114

Watt F. E. (2021). Posttraumatic osteoarthritis: what have we learned to advance osteoarthritis? *Current opinion in rheumatology*, *33*(1), 74–83. https://doi.org/10.1097/BOR.0000000000000760

Wei, J., Gross, D., Lane, N. E., Lu, N., Wang, M., Zeng, C., Yang, T., Lei, G., Choi, H. K., & Zhang, Y. (2019). Risk factor heterogeneity for medial and lateral compartment knee osteoarthritis: analysis of two prospective cohorts. *Osteoarthritis and cartilage*, *27*(4), 603–610. https://doi.org/10.1016/j.joca.2018.12.013

Wenham, C. Y., & Conaghan, P. G. (2010). The role of synovitis in osteoarthritis. *Therapeutic advances in musculoskeletal disease*, *2*(6), 349–359. https://doi.org/10.1177/1759720X10378373

Wilusz, R. E., Sanchez-Adams, J., & Guilak, F. (2014). The structure and function of the pericellular matrix of articular cartilage. *Matrix biology: journal of the International Society for Matrix Biology*, *39*, 25–32. https://doi.org/10.1016/j.matbio.2014.08.009

Wu, T., Zhang, Q., Wu, S., Hu, W., Zhou, T., Li, K., Liu, D., Gu, H. F., Zheng, H., Zhu, Z., Li, L., & Yang, G. (2019). CILP-2 is a novel secreted protein and associated with insulin resistance. *Journal of molecular cell biology*, *11*(12), 1083–1094. https://doi.org/10.1093/jmcb/mjz016

Wu, C. L., Harasymowicz, N. S., Klimak, M. A., Collins, K. H., & Guilak, F. (2020). The role of macrophages in osteoarthritis and cartilage repair. *Osteoarthritis and cartilage*, *28*(5), 544–554. https://doi.org/10.1016/j.joca.2019.12.007

Yao, Q., Wu, X., Tao, C., Gong, W., Chen, M., Qu, M., Zhong, Y., He, T., Chen, S., & Xiao, G. (2023). Osteoarthritis: pathogenic signaling pathways and therapeutic targets. *Signal transduction and targeted therapy*, *8*(1), 56.

https://doi.org/10.1038/s41392-023-01330-w
Yoon, D. S., Lee, K. M., Choi, Y., Ko, E. A., Lee, N. H., Cho, S., Park, K. H., Lee, J. H., Kim, H. W., & Lee, J. W. (2022). TLR4 downregulation by the RNA-binding protein PUM1 alleviates cellular aging and osteoarthritis. *Cell death and differentiation*, *29*(7), 1364–1378. https://doi.org/10.1038/s41418-021-00925-6
Yousefi, A. M., Hoque, M. E., Prasad, R. G., & Uth, N. (2015). Current strategies in multiphasic scaffold design for osteochondral tissue engineering: A review. *Journal of biomedical materials research. Part A*, *103*(7), 2460–2481. https://doi.org/10.1002/jbm.a.35356
Yunus, M. H. M., Nordin, A., & Kamal, H. (2020). Pathophysiological Perspective of Osteoarthritis. *Medicina (Kaunas, Lithuania)*, *56*(11), 614. https://doi.org/10.3390/medicina56110614
Xia, B., Di Chen, Zhang, J., Hu, S., Jin, H., & Tong, P. (2014). Osteoarthritis pathogenesis: a review of molecular mechanisms. *Calcified tissue international*, *95*(6), 495–505. https://doi.org/10.1007/s00223-014-9917-9
Xiao, L., Liu, C., Wang, B., Fei, W., Mu, Y., Xu, L., & Li, Y. (2021). Targeting Discoidin Domain Receptor 2 for the Development of Disease-Modifying Osteoarthritis Drugs. *Cartilage*, *13*(2_suppl), 1285S–1291S. https://doi.org/10.1177/1947603519852401
Xin, X., Tan, Q., Li, F., Chen, Z., Zhang, K., Li, F., Yang, B., Xing, Z., Zhou, F., Tian, Y., Lv, Y., & Zhu, T. (2021). Potential Value of Matrix Metalloproteinase-13 as a Biomarker for Osteoarthritis. *Frontiers in surgery*, *8*, 750047. https://doi.org/10.3389/fsurg.2021.750047
Xu, L., Peng, H., Wu, D., Hu, K., Goldring, M. B., Olsen, B. R., & Li, Y. (2005). Activation of the discoidin domain receptor 2 induces expression of matrix metalloproteinase 13 associated with osteoarthritis in mice. *The Journal of biological chemistry*, *280*(1), 548–555. https://doi.org/10.1074/jbc.M411036200
Xu, L., Servais, J., Polur, I., Kim, D., Lee, P. L., Chung, K., & Li, Y. (2010). Attenuation of osteoarthritis progression by reduction of discoidin domain receptor 2 in mice. *Arthritis and rheumatism*, *62*(9), 2736–2744. https://doi.org/10.1002/art.27582
Xu, L., Polur, I., Servais, J. M., Hsieh, S., Lee, P. L., Goldring, M. B., & Li, Y. (2011). Intact pericellular matrix of articular cartilage is required for unactivated discoidin domain receptor 2 in the mouse model. *The American journal of pathology*, *179*(3), 1338–1346. https://doi.org/10.1016/j.ajpath.2011.05.023
Xu, L., & Li, Y. (2020). A Molecular Cascade Underlying Articular Cartilage Degeneration. *Current drug targets*, *21*(9), 838–848. https://doi.org/10.2174/1389450121666200214121323

9. SUMMARY IN ESTONIAN

Põlve osteoartroosi molekulaarsete markerite CILP-2, DDR2 ja C2C seos koekahjustuse raskusastmega

Sissejuhatus

Osteoartroos (OA) on kõige levinum liigesekahjustuse vorm ja kõige sagedamini esinev luu- ja lihaskonna valu põhjustav haigus. See haigus mõjutab peamiselt sünoviaalliigeseid. See on progresseeruv degeneratiivne haigus, mis esineb peamiselt täiskasvanuil ja hõlmab endas liigeskõhre ja periartikulaarsete kudede ehitust ja funktsiooni mõjutavaid patoloogilisi muutusi. OA levimus suureneb märkimisväärselt vanusega – valdav enamus juhtudest esineb pärast 40. eluaastat. OA esinemissageduse suurenemine on suuresti tingitud vananevast elanikkonnast (Palazzo et al., 2016). See on haigus, mis on tihti seotud valuga, ning valu kui ka liigese funktsionaalsuse vähenemine halvendavad oluliselt patsientide elukvaliteeti. Kuigi OA on kõige levinum luu- ja lihaskonna haigus, ei ole selle patofüsioloogia veel täpselt teada (O'Neill et al., 2018).

Pikka aega on OA-d kirjeldatud kui "kulumishaigust". Uuemad OA-d puudutavad uuringud aga viitavad sellele, et tegemist on keerulise ja mitmetest teguritest sõltuva seisundiga. Kuigi kõhrkoe kulumine ja kadu on OA puhul peamine patoloogiline tunnus, on nüüdseks leitud, et OA on haigus, mis mõjutab kogu liigest, põhjustades põletikku ja patoloogilisi muutusi subkondraalses luus ja pehmetes kudedes, nagu sünooviumis, meniskides ja sidemetes (Wilder et al., 2002). OA peamised tunnused on kurnav valu, liigesejäikus, turse ja liikuvuse piiratus. Lisaks füüsilisele mõjule on OA-d seostatud vaimse tervise häirete ja südame-veresoonkonna haiguste suurenenud riskiga. OA arengut soodustavad mitmed riskifaktorid nagu vananemine, traumaatiline põlvevigastus, rasvumine, geneetiline eelsoodumus, ebanormaalne mehaaniline stress ja muu infektsioonist või operatsioonist põhjustatud põletik (Niu et al., 2009; Palazzo et al., 2016; Long et al., 2022).

Põlv on OA poolt kõige sagedamini mõjutatud liiges hinnangulise üleilmse levimusega 365 miljonit haigusjuhtu, millele järgnevad puusa- ja käeliigese OA (Watt, 2021). Põlveliigese OA (pOA) esineb rohkem naistel kui meestel – hinnanguline levimus on 60-aastaste ja vanemate inimeste seas 10% meestest ja 13% naistest (O'Neill et al., 2018). Kuigi pOA tekke ja progresseerumise täpsed mehhanismid ei ole täielikult välja selgitatud, viitavad uuringud mehaaniliste, biokeemiliste ja geneetiliste tegurite koosmõjule (Xin et al., 2021).

OA diagnoosimine põhineb tavaliselt kliinilistel sümptomitel ja radioloogilistel leidudel, mis sageli kinnitavad kaugelearenunud OA-d, kui on vaja kirurgilist sekkumist (Felson & Hodgson, 2014). On leitud, et OA-ga seotud molekulaarsed muutused tekivad palju varem kui märgatavad kliinilised sümptomid (Valdes, 2020). Seetõttu on kasvanud huvi biomarkerite vastu, mis suudavad

tuvastada kõhre lagunemist, peegeldada haigusprotsessi aktiivsust ja ennustada haiguse progresseerumist (Mobasheri, 2012; Rodriguez-Merchan, 2023).

Vaatamata ulatuslikele prekliinilistele uuringutele ei ole OA-le täna põhjuslikku ravi olemas. OA patogeneesi mõjutavate molekulaarsete mehhanismide piiratud mõistmine on takistanud tõhusate ravistrateegiate väljatöötamist. Praegu on põlve endoproteesimine endiselt peamine ravi kaugelearenenud pOA korral, mis on suures osas muutumatuna püsinud alates selle kasutusele võtmisest 1968. aastal. Seetõttu on pakiline vajadus OA varajase staadiumi ravistrateegiate järele, et peatada haiguse progresseerumine enne pöördumatute liigesekahjustuste tekkimist (O'Neill et al., 2018). Lisaks tuntakse OA-d üha enam heterogeense seisundina, millel on erinevad alatüübid (endotüübid), mis lisab selle patogeneesile ja ravile täiendavat keerukust. Lisaks on oluline mainida, et operatsioon ei pruugi teise põlve kahjustusi ära hoida – OA võib olla süsteemsem haigus, kui arvatakse. Sageli esineb OA puhul mitme liigese kombinatsioon, mõlemad põlved, põlv/puus (Metcalfe et al., 2012).

Uuringu eesmärgid

Selles töös uuriti OA patogeneesiga seotud molekulaarsete markerite: kõhre vahekihi valk 2 (inglisk – *cartilage intermediate layer protein* (CILP-2)), diskoidiini domeeni retseptor 2 (inglisk – *discoidin domain receptor 2* (DDR2)) ja II tüüpi kollageeni (Col2) lõhustumise neoepitoop (inglisk – *collagen type-II C-terminal cleavage neoepitope* (C2C)) ekspressiooni OA kõhres. Uuringu eesmärk oli analüüsida ja hinnata, kas põlveliigese osteoartroosiga (pOA) patsientide kõhrkoes tuvastatud molekulaarsete markerite immunohistokeemilise värvingu ulatus ja paigutus korreleeruvad morfoloogiliste muutustega, näidates seeläbi nende markerite potentsiaalset rolli OA patogeneesis.

Töö hüpoteesid olid järgmised:

1. Biomarkerite CILP-2, DDR2 ja C2C avaldumine pOA kõhrkoes korreleerub lokaalse OARSI (Rahvusvahelise Osteoartriidi Uurimise Ühingu) hindamissüsteemi järgi määratud kõhrekahjustuse astme ja makroskoopilise liigespinna kahjustuse skooriga. Need markerid võivad olla usaldusväärseteks indikaatoriteks OA-ga seotud kõhrekahjustuste korral.
2. CILP-2, DDR2 ja C2C värvumismustrite detailne strukturaalne kirjeldus ja võrdlus histoloogiliselt iseloomustatud koekahjustusega võib anda täiendavaid teadmisi kõhre lagunemise patogeneesist.

Uuringu konkreetsed eesmärgid olid järgmised

Antigeeni esile toomise protokollide optimeerimine: hinnata ja võrrelda kolme erinevat CILP-2 antigeeni esile toomise protokolli OA liigeskõhre proovides eesmärgiga optimeerida skeletikudedele mõeldud protokolli, kuna CILP-2 puhul kasutatud antigeeni esile toomise meetodid näitavad olulist varieeruvust immunohistokeemilisel värvimisel.

CILP-2 ekspressiooni hindamine: hinnata CILP-2 ekspressiooni pOA patsientide kõhrkoes ja määrata selle korrelatsioon kõhrekahjustuse astmega, mida hinnati OARSI histopatoloogiliste astmete ja makroskoopiliste liigespinna kahjustuste skooridega, ning seeläbi analüüsida ja hinnata CILP-2 potentsiaali OA-ga seotud koekahjustuse biomarkerina.

C2C ekspressiooni hindamine: hinnata C2C ekspressiooni pOA kõhrkoe erinevates piirkondades ja määrata selle potentsiaal OA progresseerumise biomarkerina, hinnates selle korrelatsiooni OARSI kõhrekahjustuse astmete ja skooridega.

DDR2 ekspressiooni uurimine: Uurida DDR2 ekspressioonimustrit pOA kõhrkoes ja võrrelda seda CILP-2 ekspressiooniga, hõlmates nende vastavaid korrelatsioone OA raskusastmega, et määrata usaldusväärsem kandidaat haiguse ning selle võimalike progresseerumise markerite uurimiseks.

Ultrastruktuursete muutuste analüüs: Analüüsida ultrastruktuurseid muutusi pOA pindmise kihi kõhrkoe kondrotsüütides ja peritsellulaarses maatriksis, kasutades transmissioonelektronmikroskoopiat (TEM), keskendudes eelkõige kondroptoosile ja võimalikule peritsellulaarse maatriksi osalusele OA patogeneesis.

Uuritavad ja meetodid

Uuringusse kaasati kuni 70 aastat vanad patsiendid Tartu Ülikooli Kliinikumi traumatoloogia ja ortopeedia kliinikust, kellel oli radioloogiliselt diagnoositud lõppstaadiumis põlveliigese osteoartroos (Kellgren/Lawrence radioloogine skoor 3-4) ja kellele teostati esmane ühepoolne täielik põlveliigese asendusoperatsioon. Välja jäeti patsiendid, kellel olid viimase kolme kuu jooksul esinenud ägeda infektsiooni tunnuseid. Samuti välistati patsiendid, kellel oli diagnoositud reumatoidartriit, sekundaarne osteoartriit, mis oli põhjustatud traumast, podagrast, infektsioonidest, või põlveliigeseid mõjutavatest kaasasündinud ja arengulistest probleemidest. Ajavahemikul 2017–2018 patsientidelt kogutud materjalist analüüsiti 43 patsiendi proove. Patsientidest 26 olid naised ja 17 mehed vanuses 46-68 eluaastat.

Biomarkerite CILP-2 ja DDR2 uuringusse kaasati 20 patsienti, kellest 8 olid mehed ja 12 naised vanusevahemikus 46-68 eluaastat. Võrreldi sääreluu mediaalselt ja lateraalselt platoolt kogutud proove. Biomarkerite määramiseks kõhrkoes kasutasime immunohistokeemilist uuringut. 10 patsiendi puhul teostati ka TEM uuringud ultrastruktuursete muutuste uurimiseks. OARSI kahjustusastmete hindamiseks valmistati histoloogilised preparaadid ja värviti safraniin O värvinguga. Makroskoopiline kahjustusaste määrati visuaalselt hinnates patoloogiliste muutuste ulatust sääreluu platool vastavalt OARSI parameetritele.

Biomarker C2C uuringusse kaasati 27 patsienti, kellest 10 olid mehed ja 17 naised vanusevahemikus 55-66 eluaastat. Võrreldi sääreluu mediaalset ja

lateraalset platoolt kogutud proove. Biomarkerite määramiseks kõhrkoes kasutasime immunohistokeemilist uuringut. OARSI kahjustusastmete hindamiseks valmistati histoloogilised preparaadid ja värviti safraniin O värvinguga. Makroskoopiline kahjustusaste määrati visuaalselt, hinnates patoloogiliste muutuste ulatust sääreluu platool vastavalt OARSI parameetritele.

Antigeeni esile toomise meetodite optimeerimise uuringusse kaasati 4 patsienti, kellest 2 olid mehed ja 2 naised vanusevahemikus 58-65 eluaastat. Kolmelt patsiendilt võeti kõhrebiopsiad sääreluu platoo koormust kandvatest kohtadest. Ühelt patsiendilt võeti kolm proovi erinevatest kohtadest, et tagada testimiseks piisav materjali hulk ja kontrollida erinevate värvimisprotokollide kohaspetsiifilisust. Katsetati kolme erinevat antigeeni esile toomise protokolli.

Statistiliseks andmetöötluseks kasutati mitteparameetrilisi meetodeid (Spearmani'i astakkorrelatsiooni, Wilcoxon'i sobitatud paaride testi, Mann-Whitney U-testi).

Uuringute läbiviimiseks oli saadud luba Tartu Ülikooli inimuuringute eetikakomiteelt.

Tulemustest lähtuvad järeldused

Uuring näitas, et CILP-2 ja C2C on paljulubavad pOA biomarkerid. Mõlemate biomarkerite värvingu ulatus korreleerus OA lokaalse koekahjustuse raskusega, nii kõhrekahjustuse sügavust näitava OARSI hindamissüsteemi astmetega kui ka OARSI skooridega, mis hinnangus arvestavad täiendavalt kogu kõhrepinna makroskoopilise kahjustuse ulatust.

1. Kolme CILP-2 immunohistokeemilisele värvimisele eelnenud antigeeni esile toomise protokolli võrdlev hindamine näitas, et optimaalsed tulemused saavutati PIER-meetodil ehk proteolüütiliselt indutseeritud epitoobi esile toomisega, mis hõlmas proteinaas K kombineerimist hüaluronidaasiga.

   Liigeskõhrede CILP-2 immunohistokeemilistel uuringutel ilmnes vajadus antigeeni esiletoomise järele. Kolme antigeeni esile toomise meetodite võrdlusest ilmnes, et immunohistokeemiliste uuringute tulemus varieerub nimetatud markeri puhul sõltuvalt antigeeni esiletoomise meetodist äärmiselt suurel määral ning liigeskõhre koeproovides andis parima tulemuse proteolüütilisi ensüüme rakendav PIER-meetod. Sageli antigeenide esile toomiseks rakendatav koelõikude kuumtöötlus (HIER-meetod; kuumutamisega indutseeritud antigeeni esile toomine) andis selgelt nõrgema CILP-2 värvingu ja põhjustas sagedast koelõikude lahtitulekut alusklaasilt. Ka kahe meetodi kombineerimine (PIER + HIER) ei andnud paremaid tulemusi. Võrdluskatsed näitasid selget antigeeni esile toomise protokollide adapteerimise vajadust liigeskõhrede biopsiate immunohistokeemilistel uuringutel.

2. CILP-2 ekspressiooni hindamine OA-patsientide põlveliigese kõhres näitas, et CILP-2 korreleerub positiivselt kõhrekahjustuse astmega, hinnatuna OARSI histopatoloogiliste astmete ja skooride abil.

   Kuivõrd CILP-2 korreleerus positiivselt nii OARSI kõhrekahjustuse hindamissüsteemi astmete kui ka skooridega, siis võib CILP-2 pidada arvestatavaks OA-st põhjustatud liigeskõhre lagunemise markeriks. Seda kinnitavad ka tulemused, mis saadi patsientide jagamisel kõrgema ja madalama OARSI raskusastmetega gruppidesse (OARSI astmed vastavalt üle või alla 3), kus kõrgema raskusastmega gupis ilmnes CILP-2 oluliselt ulatuslikum värvumus. CILP-2 ekspressioonis tuli erinevus välja ka patsientide jagamisel sõltuvalt sellest kas domineeris sääreluu platoo mediaalse või lateraalse poole kahjustus. Positiivne korrelatsioon ilmnes CILP-2 värvumise ja liigeskõhre kahjustusastmete vahel domineerivalt mediaalse poole kahjustusega patsientidel. Sääreluu platoo mediaalse poole domineeriv kahjustus on pOA patsientide seas kõige sagedasemaks esinemisvormiks.

3. C2C ekspressioon vastas OA raskusastmele, mida tõendavad C2C-ga värvunud kõhre osakaalu positiivsed korrelatsioonid nii kõhrekahjustuse astme kui ka põlveliigese kahjustuse üldise skoori vahel. Seega on C2C paljulubav biomarker OA-st põhjustatud liigeskõhre patoloogiliste muutuste jälgimiseks.

   Uuringus kasutati kommertsiaalselt mitte kättesaadavat C2C antikeha, mida seni on edukalt kasutatud *sandwich*-tüüpi ELISA testides (IB-C2C-HUSA™, IBEX Pharmaceuticals). Tänu IBEX-i vastutulelikkusele õnnestus eksklusiivselt hangitud C2C antikehaga teostada immunohistokeemilised uuringud pOA liigeskõhredel, kus ilmnes positiivne korrelatsioon C2C värvingu ulatuse ning OARSI histopatoloogiliste astmete ja skooride vahel. Samuti leiti erinev C2C värvumus sääreluu platoo mediaalse ja lateraalse poole vahel – enam kahjustunud mediaalsel poolel oli C2C ekspressioon kõrgem. Seega võib C2C pidada heaks OA-ga seotud koekahjustuse markeriks ja käesolev töö annab lootust, et uriinis IB-C2C-HUSA™ abil määratud C2C tase võiks peegeldada koetasandil toimuvaid muutusi.

4. DDR2 ekspressioonimuster liigeskõhredes oli sarnane CILP-2 omaga, mida tõendab DDR2 ja CILP-2 ekspressiooni vaheline positiivne korrelatsioon. Erinevalt CILP-2-st näitas DDR2 ekspressioon aga vaid nõrka korrelatsioonitendentsi. Sellest võib järeldada, et CILP-2 on parem kõhrekahjustuse marker kui DDR2.

   DDR2 on juba tunnustatud OA markeriks, mistõttu sai võrdlevalt uuritud DDR2 ja CILP-2 ekspressiooni. Uuritud patsientidel näitas DDR2 ainult nõrka korrelatsioonitendentsi OARSI histopatoloogiliste raskusastmete ja skooridega. Kuigi DDR2 ekspressioon suurenes kõr-

gema raskusastmega patsientide rühmas, siis see muutus ei olnud statistiliselt tõepärane. Erinevalt CILP-2 ei ilmnenud DDR2 värvumises vahet kui patsiendid jagati sääreluu platoo mediaalse või lateraalse poole kahjustusega rühmadeks. Samas DDR2 ja CILP-2 vahel ilmnenud positiivne korrelatsioon viitab mõlema OA marker sarnasele potentsiaalile. Seega antud uuringu valguses võib CILP-2 pidada DDR2-ga võrreldes paremaks OA markeriks.

5. Liigeskõhre pindmise tsooni TEM-uuringutel täheldati tugevalt kahjustatud kondrotsüüte, millel esinesid mitmed degeneratiivsete rakkude ja kondroptoosi tunnused. Need tunnused esinesid enim kõrgema OARSI astmega proovides. Lisaks täheldati peritsellulaarse maatriksi tavatut paigutust, ning kuna leiti, et CILP-2 immunohistokeemiline värvimine lokaliseerus peamiselt peritsellulaarses maatriksis, siis toetab see hüpoteesi peritsellulaarse maatriksi olulisusest OA korral.

   TEM uuringutel leiti pindmise kihi kondrotsüütides tugevaid muutusi, organellid olid kas tugevalt redutseerunud või koldeliselt koondunud, näiteks esines tugevalt liitunud tsütoskeleti filamentidega alasid. Tsütoskeleti muutused võivad viia ka rakukuju muutusele, mis võib põhjendada miks enamik pindmise kihi käävjaid rakke olid omandanud ovaalse või ümara kuju. Paljudel rakkudel oli kõhrerakkude apoptoosi ehk kondroptoosi tunnuseid, näiteks suured alad laienenud endoplasmavõrgustiku tsisternidega, rakutuumad ebaühtlaselt kondenseerunud kromatiiniga ja vesikulaarset lagumaterjali sisaldavad rakulakuunid. Iseloomulik leid oli kondrotsüüte vahetult ümbritseva maatriksi (peritsellulaarse maatriksi) kohatine laienemine või ebakorrapärane ümberehitumine. Kuivõrd CILP-2 immunohistokeemilistel uuringutel ilmnes tugev peritsellulaarne värvumumine, siis need leiud toetavad hüpoteesi peritsellulaarse maatriksi olulisust OA korral.

10. ACKNOWLEDGEMENTS

This study was carried out in the Department of Anatomy, University of Tartu. This work was supported by University of Tartu's core funding of research.

- My deepest gratitude goes to my supervisors, Professor Andres Arend, Professor Kalle Kisand, associate professor Siim Suutre and associate professor Marina Aunapuu for guidance, support and motivation.
- I would like to express my sincere gratitude to Professor Emeritus Agu Tamm for initiating research on kOA biomarkers in Estonia and for his invaluable contribution to the initial planning and organization of these TKA studies.
- Special thanks to Prof Aare Märtson, Head of Traumatology and Orthopaedics Clinic, for conducting the collection of biopsies, and orthopaedic surgeons Eiki Strauss, Anna Helena Kase, Jaan Laos, Mart Parv, Sigrid Paul, Egon Puuorg, Alo Rull, and Viktor Šapovalov for collection of biopsies during the TKA.
- I am very grateful to all the patients who participated in the study.
- I want to thank Niina Puusaag for the teaching in the field of histology.
- I am very thankful to Anu Kõiveer for making and teaching TEM samples preparation and Rein Laiverik for his assistance with the electron microscope.
- I want to thank the Department of Anatomy for assistance in my research.
- I am very grateful to my family and friends.

PUBLICATIONS

CURRICULUM VITAE

Name: Taavi Torga
Date of Birth: June 26th 1989, Tartu, Estonia
Citizenship: Estonian
Address: Chair of Histology and Embryology, Department of Anatomy, Institute of Biomedicine and Translational Medicine, University of Tartu, Ravila 19, 50411, Tartu, Estonia
Phone: +372 7374255
E-mail: taavi.torga@ut.ee

Education

2018–	University of Tartu, Faculty of Medicines, PhD studies
2012–2015	Estonian University of Life Sciences, The Institute of Veterinary Medicine and Animal Sciences, meat technology, masters
2009–2012	Estonian University of Life Sciences, The Institute of Veterinary Medicine and Animal Sciences, meat and milk technology, bachelor
1996–2008	Tartu Tamme Gymnasium

Professional employment

01.09.2023–	University of Tartu, Faculty of Medicine, Institute of Biomedicine and Translational Medicine, specialist (0.5)
15.09.2023–31.08.2025	University of Tartu, Faculty of Medicine, Institute of Biomedicine and Translational Medicine, junior research fellow in histology (0.5)
01.09.2022–31.08.2023	University of Tartu, Faculty of Medicine, Institute of Biomedicine and Translational Medicine, specialist (1.0)
01.02.2020–31.08.2022	University of Tartu, Faculty of Medicine, Institute of Biomedicine and Translational Medicine, specialist (0.5)

My field of research: Study of bone and cartilage diseases with a focus on changes in the structure of the knee articular cartilage.

ELULOOKIRJELDUS

Nimi: Taavi Torga
Sünniaeg: 26. juuni 1989, Tartu, Eesti
Kodakonsus: Eesti
Aadress: Histoloogia ja embrüoloogia õppetool, Anatoomia osakond, Bio- ja siirdemeditsiini instituut, Tartu Ülikool, Ravila 19, 50411
Telefon: +372 7374255
E-post: taavi.torga@ut.ee

Haridus

2018–	Tartu Ülikool, meditsiiniteaduste valdkond, doktorantuur
2012–2015	Eesti Maaülikool, Veterinaarmeditsiini ja loomakasvatuse instituut, lihatehnoloogia, magistriõpe
2009–2012	Eesti Maaülikool, Veterinaarmeditsiini ja loomakasvatuse instituut, liha- ja piimatehnoloogia, bakalaureuseõpe
1996–2008	Tartu Tamme Gümnaasium

Erialane teenistuskäik

01.09.2023–	Tartu Ülikool, Meditsiiniteaduste valdkond, bio- ja siirdemeditsiini instituut, spetsialist (0.5)
15.09.2023–31.08.2025	Tartu Ülikool, Meditsiiniteaduste valdkond, bio- ja siirdemeditsiini instituut, histoloogia nooremteadur (0.5)
01.09.2022–31.08.2023	Tartu Ülikool, Meditsiiniteaduste valdkond, bio- ja siirdemeditsiini instituut, spetsialist (1.0)
01.02.2020–31.08.2022	Tartu Ülikool, Meditsiiniteaduste valdkond, bio- ja siirdemeditsiini instituut, spetsialist (0.5)

Peamised uurimisvaldkonnad:
Luu- ja kõhrehaigustega seotud uuringud, kus põhifookus on suunatud põlveliigese osteoartroosiga patsientide liigeskõhre struktuurimuutustele.

DISSERTATIONES MEDICINAE UNIVERSITATIS TARTUENSIS

1. **Heidi-Ingrid Maaroos**. The natural course of gastric ulcer in connection with chronic gastritis and *Helicobacter pylori*. Tartu, 1991.
2. **Mihkel Zilmer**. Na-pump in normal and tumorous brain tissues: Structural, functional and tumorigenesis aspects. Tartu, 1991.
3. **Eero Vasar**. Role of cholecystokinin receptors in the regulation of behaviour and in the action of haloperidol and diazepam. Tartu, 1992.
4. **Tiina Talvik**. Hypoxic-ischaemic brain damage in neonates (clinical, biochemical and brain computed tomographical investigation). Tartu, 1992.
5. **Ants Peetsalu**. Vagotomy in duodenal ulcer disease: A study of gastric acidity, serum pepsinogen I, gastric mucosal histology and *Helicobacter pylori*. Tartu, 1992.
6. **Marika Mikelsaar**. Evaluation of the gastrointestinal microbial ecosystem in health and disease. Tartu, 1992.
7. **Hele Everaus**. Immuno-hormonal interactions in chronic lymphocytic leukaemia and multiple myeloma. Tartu, 1993.
8. **Ruth Mikelsaar**. Etiological factors of diseases in genetically consulted children and newborn screening: dissertation for the commencement of the degree of doctor of medical sciences. Tartu, 1993.
9. **Agu Tamm**. On metabolic action of intestinal microflora: clinical aspects. Tartu, 1993.
10. **Katrin Gross**. Multiple sclerosis in South-Estonia (epidemiological and computed tomographical investigations). Tartu, 1993.
11. **Oivi Uibo**. Childhood coeliac disease in Estonia: occurrence, screening, diagnosis and clinical characterization. Tartu, 1994.
12. **Viiu Tuulik**. The functional disorders of central nervous system of chemistry workers. Tartu, 1994.
13. **Margus Viigimaa**. Primary haemostasis, antiaggregative and anticoagulant treatment of acute myocardial infarction. Tartu, 1994.
14. **Rein Kolk**. Atrial versus ventricular pacing in patients with sick sinus syndrome. Tartu, 1994.
15. **Toomas Podar**. Incidence of childhood onset type 1 diabetes mellitus in Estonia. Tartu, 1994.
16. **Kiira Subi**. The laboratory surveillance of the acute respiratory viral infections in Estonia. Tartu, 1995.
17. **Irja Lutsar**. Infections of the central nervous system in children (epidemiologic, diagnostic and therapeutic aspects, long term outcome). Tartu, 1995.
18. **Aavo Lang**. The role of dopamine, 5-hydroxytryptamine, sigma and NMDA receptors in the action of antipsychotic drugs. Tartu, 1995.
19. **Andrus Arak**. Factors influencing the survival of patients after radical surgery for gastric cancer. Tartu, 1996.

20. **Tõnis Karki**. Quantitative composition of the human lactoflora and method for its examination. Tartu, 1996.
21. **Reet Mändar**. Vaginal microflora during pregnancy and its transmission to newborn. Tartu, 1996.
22. **Triin Remmel**. Primary biliary cirrhosis in Estonia: epidemiology, clinical characterization and prognostication of the course of the disease. Tartu, 1996.
23. **Toomas Kivastik**. Mechanisms of drug addiction: focus on positive reinforcing properties of morphine. Tartu, 1996.
24. **Paavo Pokk**. Stress due to sleep deprivation: focus on $GABA_A$ receptor-chloride ionophore complex. Tartu, 1996.
25. **Kristina Allikmets**. Renin system activity in essential hypertension. Associations with atherothrombogenic cardiovascular risk factors and with the efficacy of calcium antagonist treatment. Tartu, 1996.
26. **Triin Parik**. Oxidative stress in essential hypertension: Associations with metabolic disturbances and the effects of calcium antagonist treatment. Tartu, 1996.
27. **Svetlana Päi**. Factors promoting heterogeneity of the course of rheumatoid arthritis. Tartu, 1997.
28. **Maarike Sallo**. Studies on habitual physical activity and aerobic fitness in 4 to 10 years old children. Tartu, 1997.
29. **Paul Naaber**. *Clostridium difficile* infection and intestinal microbial ecology. Tartu, 1997.
30. **Rein Pähkla**. Studies in pinoline pharmacology. Tartu, 1997.
31. **Andrus Juhan Voitk**. Outpatient laparoscopic cholecystectomy. Tartu, 1997.
32. **Joel Starkopf**. Oxidative stress and ischaemia-reperfusion of the heart. Tartu, 1997.
33. **Janika Kõrv**. Incidence, case-fatality and outcome of stroke. Tartu, 1998.
34. **Ülla Linnamägi**. Changes in local cerebral blood flow and lipid peroxidation following lead exposure in experiment. Tartu, 1998.
35. **Ave Minajeva**. Sarcoplasmic reticulum function: comparison of atrial and ventricular myocardium. Tartu, 1998.
36. **Oleg Milenin**. Reconstruction of cervical part of esophagus by revascularised ileal autografts in dogs. A new complex multistage method. Tartu, 1998.
37. **Sergei Pakriev**. Prevalence of depression, harmful use of alcohol and alcohol dependence among rural population in Udmurtia. Tartu, 1998.
38. **Allen Kaasik**. Thyroid hormone control over β-adrenergic signalling system in rat atria. Tartu, 1998.
39. **Vallo Matto**. Pharmacological studies on anxiogenic and antiaggressive properties of antidepressants. Tartu, 1998.
40. **Maire Vasar**. Allergic diseases and bronchial hyperreactivity in Estonian children in relation to environmental influences. Tartu, 1998.
41. **Kaja Julge**. Humoral immune responses to allergens in early childhood. Tartu, 1998.

42. **Heli Grünberg**. The cardiovascular risk of Estonian schoolchildren. A cross-sectional study of 9-, 12- and 15-year-old children. Tartu, 1998.
43. **Epp Sepp**. Formation of intestinal microbial ecosystem in children. Tartu, 1998.
44. **Mai Ots**. Characteristics of the progression of human and experimental glomerulopathies. Tartu, 1998.
45. **Tiina Ristimäe**. Heart rate variability in patients with coronary artery disease. Tartu, 1998.
46. **Leho Kõiv**. Reaction of the sympatho-adrenal and hypothalamo-pituitary-adrenocortical system in the acute stage of head injury. Tartu, 1998.
47. **Bela Adojaan**. Immune and genetic factors of childhood onset IDDM in Estonia. An epidemiological study. Tartu, 1999.
48. **Jakov Shlik**. Psychophysiological effects of cholecystokinin in humans. Tartu, 1999.
49. **Kai Kisand**. Autoantibodies against dehydrogenases of α-ketoacids. Tartu, 1999.
50. **Toomas Marandi**. Drug treatment of depression in Estonia. Tartu, 1999.
51. **Ants Kask**. Behavioural studies on neuropeptide Y. Tartu, 1999.
52. **Ello-Rahel Karelson**. Modulation of adenylate cyclase activity in the rat hippocampus by neuropeptide galanin and its chimeric analogs. Tartu, 1999.
53. **Tanel Laisaar**. Treatment of pleural empyema — special reference to intrapleural therapy with streptokinase and surgical treatment modalities. Tartu, 1999.
54. **Eve Pihl**. Cardiovascular risk factors in middle-aged former athletes. Tartu, 1999.
55. **Katrin Õunap**. Phenylketonuria in Estonia: incidence, newborn screening, diagnosis, clinical characterization and genotype/phenotype correlation. Tartu, 1999.
56. **Siiri Kõljalg**. *Acinetobacter* – an important nosocomial pathogen. Tartu, 1999.
57. **Helle Karro**. Reproductive health and pregnancy outcome in Estonia: association with different factors. Tartu, 1999.
58. **Heili Varendi**. Behavioral effects observed in human newborns during exposure to naturally occurring odors. Tartu, 1999.
59. **Anneli Beilmann**. Epidemiology of epilepsy in children and adolescents in Estonia. Prevalence, incidence, and clinical characteristics. Tartu, 1999.
60. **Vallo Volke**. Pharmacological and biochemical studies on nitric oxide in the regulation of behaviour. Tartu, 1999.
61. **Pilvi Ilves**. Hypoxic-ischaemic encephalopathy in asphyxiated term infants. A prospective clinical, biochemical, ultrasonographical study. Tartu, 1999.
62. **Anti Kalda**. Oxygen-glucose deprivation-induced neuronal death and its pharmacological prevention in cerebellar granule cells. Tartu, 1999.
63. **Eve-Irene Lepist**. Oral peptide prodrugs – studies on stability and absorption. Tartu, 2000.

64. **Jana Kivastik**. Lung function in Estonian schoolchildren: relationship with anthropometric indices and respiratory symptomas, reference values for dynamic spirometry. Tartu, 2000.
65. **Karin Kull**. Inflammatory bowel disease: an immunogenetic study. Tartu, 2000.
66. **Kaire Innos**. Epidemiological resources in Estonia: data sources, their quality and feasibility of cohort studies. Tartu, 2000.
67. **Tamara Vorobjova**. Immune response to *Helicobacter pylori* and its association with dynamics of chronic gastritis and epithelial cell turnover in antrum and corpus. Tartu, 2001.
68. **Ruth Kalda**. Structure and outcome of family practice quality in the changing health care system of Estonia. Tartu, 2001.
69. **Annika Krüüner**. *Mycobacterium tuberculosis* – spread and drug resistance in Estonia. Tartu, 2001.
70. **Marlit Veldi**. Obstructive Sleep Apnoea: Computerized Endopharyngeal Myotonometry of the Soft Palate and Lingual Musculature. Tartu, 2001.
71. **Anneli Uusküla**. Epidemiology of sexually transmitted diseases in Estonia in 1990–2000. Tartu, 2001.
72. **Ade Kallas**. Characterization of antibodies to coagulation factor VIII. Tartu, 2002.
73. **Heidi Annuk**. Selection of medicinal plants and intestinal lactobacilli as antimicrobil components for functional foods. Tartu, 2002.
74. **Aet Lukmann**. Early rehabilitation of patients with ischaemic heart disease after surgical revascularization of the myocardium: assessment of health-related quality of life, cardiopulmonary reserve and oxidative stress. A clinical study. Tartu, 2002.
75. **Maigi Eisen**. Pathogenesis of Contact Dermatitis: participation of Oxidative Stress. A clinical – biochemical study. Tartu, 2002.
76. **Piret Hussar**. Histology of the post-traumatic bone repair in rats. Elaboration and use of a new standardized experimental model – bicortical perforation of tibia compared to internal fracture and resection osteotomy. Tartu, 2002.
77. **Tõnu Rätsep**. Aneurysmal subarachnoid haemorrhage: Noninvasive monitoring of cerebral haemodynamics. Tartu, 2002.
78. **Marju Herodes**. Quality of life of people with epilepsy in Estonia. Tartu, 2003.
79. **Katre Maasalu**. Changes in bone quality due to age and genetic disorders and their clinical expressions in Estonia. Tartu, 2003.
80. **Toomas Sillakivi**. Perforated peptic ulcer in Estonia: epidemiology, risk factors and relations with *Helicobacter pylori*. Tartu, 2003.
81. **Leena Puksa**. Late responses in motor nerve conduction studies. F and A waves in normal subjects and patients with neuropathies. Tartu, 2003.
82. **Krista Lõivukene**. *Helicobacter pylori* in gastric microbial ecology and its antimicrobial susceptibility pattern. Tartu, 2003.

83. **Helgi Kolk**. Dyspepsia and *Helicobacter pylori* infection: the diagnostic value of symptoms, treatment and follow-up of patients referred for upper gastrointestinal endoscopy by family physicians. Tartu, 2003.
84. **Helena Soomer**. Validation of identification and age estimation methods in forensic odontology. Tartu, 2003.
85. **Kersti Oselin**. Studies on the human MDR1, MRP1, and MRP2 ABC transporters: functional relevance of the genetic polymorphisms in the *MDR1* and *MRP1* gene. Tartu, 2003.
86. **Jaan Soplepmann**. Peptic ulcer haemorrhage in Estonia: epidemiology, prognostic factors, treatment and outcome. Tartu, 2003.
87. **Margot Peetsalu**. Long-term follow-up after vagotomy in duodenal ulcer disease: recurrent ulcer, changes in the function, morphology and *Helicobacter pylori* colonisation of the gastric mucosa. Tartu, 2003.
88. **Kersti Klaamas**. Humoral immune response to *Helicobacter pylori* a study of host-dependent and microbial factors. Tartu, 2003.
89. **Pille Taba**. Epidemiology of Parkinson's disease in Tartu, Estonia. Prevalence, incidence, clinical characteristics, and pharmacoepidemiology. Tartu, 2003.
90. **Alar Veraksitš**. Characterization of behavioural and biochemical phenotype of cholecystokinin-2 receptor deficient mice: changes in the function of the dopamine and endopioidergic system. Tartu, 2003.
91. **Ingrid Kalev**. CC-chemokine receptor 5 (CCR5) gene polymorphism in Estonians and in patients with Type I and Type II diabetes mellitus. Tartu, 2003.
92. **Lumme Kadaja**. Molecular approach to the regulation of mitochondrial function in oxidative muscle cells. Tartu, 2003.
93. **Aive Liigant**. Epidemiology of primary central nervous system tumours in Estonia from 1986 to 1996. Clinical characteristics, incidence, survival and prognostic factors. Tartu, 2004.
94. **Andres, Kulla**. Molecular characteristics of mesenchymal stroma in human astrocytic gliomas. Tartu, 2004.
95. **Mari Järvelaid**. Health damaging risk behaviours in adolescence. Tartu, 2004.
96. **Ülle Pechter**. Progression prevention strategies in chronic renal failure and hypertension. An experimental and clinical study. Tartu, 2004.
97. **Gunnar Tasa**. Polymorphic glutathione S-transferases – biology and role in modifying genetic susceptibility to senile cataract and primary open angle glaucoma. Tartu, 2004.
98. **Tuuli Käämbre**. Intracellular energetic unit: structural and functional aspects. Tartu, 2004.
99. **Vitali Vassiljev**. Influence of nitric oxide syntase inhibitors on the effects of ethanol after acute and chronic ethanol administration and withdrawal. Tartu, 2004.

100. **Aune Rehema**. Assessment of nonhaem ferrous iron and glutathione redox ratio as markers of pathogeneticity of oxidative stress in different clinical groups. Tartu, 2004.
101. **Evelin Seppet**. Interaction of mitochondria and ATPases in oxidative muscle cells in normal and pathological conditions. Tartu, 2004.
102. **Eduard Maron**. Serotonin function in panic disorder: from clinical experiments to brain imaging and genetics. Tartu, 2004.
103. **Marje Oona**. *Helicobacter pylori* infection in children: epidemiological and therapeutic aspects. Tartu, 2004.
104. **Kersti Kokk**. Regulation of active and passive molecular transport in the testis. Tartu, 2005.
105. **Vladimir Järv**. Cross-sectional imaging for pretreatment evaluation and follow-up of pelvic malignant tumours. Tartu, 2005.
106. **Andre Õun**. Epidemiology of adult epilepsy in Tartu, Estonia. Incidence, prevalence and medical treatment. Tartu, 2005.
107. **Piibe Muda**. Homocysteine and hypertension: associations between homocysteine and essential hypertension in treated and untreated hypertensive patients with and without coronary artery disease. Tartu, 2005.
108. **Külli Kingo**. The interleukin-10 family cytokines gene polymorphisms in plaque psoriasis. Tartu, 2005.
109. **Mati Merila**. Anatomy and clinical relevance of the glenohumeral joint capsule and ligaments. Tartu, 2005.
110. **Epp Songisepp**. Evaluation of technological and functional properties of the new probiotic *Lactobacillus fermentum* ME-3. Tartu, 2005.
111. **Tiia Ainla**. Acute myocardial infarction in Estonia: clinical characteristics, management and outcome. Tartu, 2005.
112. **Andres Sell**. Determining the minimum local anaesthetic requirements for hip replacement surgery under spinal anaesthesia – a study employing a spinal catheter. Tartu, 2005.
113. **Tiia Tamme**. Epidemiology of odontogenic tumours in Estonia. Pathogenesis and clinical behaviour of ameloblastoma. Tartu, 2005.
114. **Triine Annus**. Allergy in Estonian schoolchildren: time trends and characteristics. Tartu, 2005.
115. **Tiia Voor**. Microorganisms in infancy and development of allergy: comparison of Estonian and Swedish children. Tartu, 2005.
116. **Priit Kasenõmm**. Indicators for tonsillectomy in adults with recurrent tonsillitis – clinical, microbiological and pathomorphological investigations. Tartu, 2005.
117. **Eva Zusinaite**. Hepatitis C virus: genotype identification and interactions between viral proteases. Tartu, 2005.
118. **Piret Kõll**. Oral lactoflora in chronic periodontitis and periodontal health. Tartu, 2006.
119. **Tiina Stelmach**. Epidemiology of cerebral palsy and unfavourable neurodevelopmental outcome in child population of Tartu city and county, Estonia Prevalence, clinical features and risk factors. Tartu, 2006.

120. **Katrin Pudersell**. Tropane alkaloid production and riboflavine excretion in the field and tissue cultures of henbane (*Hyoscyamus niger* L.). Tartu, 2006.
121. **Külli Jaako**. Studies on the role of neurogenesis in brain plasticity. Tartu, 2006.
122. **Aare Märtson**. Lower limb lengthening: experimental studies of bone regeneration and long-term clinical results. Tartu, 2006.
123. **Heli Tähepõld**. Patient consultation in family medicine. Tartu, 2006.
124. **Stanislav Liskmann**. Peri-implant disease: pathogenesis, diagnosis and treatment in view of both inflammation and oxidative stress profiling. Tartu, 2006.
125. **Ruth Rudissaar**. Neuropharmacology of atypical antipsychotics and an animal model of psychosis. Tartu, 2006.
126. **Helena Andreson**. Diversity of *Helicobacter pylori* genotypes in Estonian patients with chronic inflammatory gastric diseases. Tartu, 2006.
127. **Katrin Pruus**. Mechanism of action of antidepressants: aspects of serotoninergic system and its interaction with glutamate. Tartu, 2006.
128. **Priit Põder**. Clinical and experimental investigation: relationship of ischaemia/reperfusion injury with oxidative stress in abdominal aortic aneurysm repair and in extracranial brain artery endarterectomy and possibilities of protection against ischaemia using a glutathione analogue in a rat model of global brain ischaemia. Tartu, 2006.
129. **Marika Tammaru**. Patient-reported outcome measurement in rheumatoid arthritis. Tartu, 2006.
130. **Tiia Reimand**. Down syndrome in Estonia. Tartu, 2006.
131. **Diva Eensoo**. Risk-taking in traffic and Markers of Risk-Taking Behaviour in Schoolchildren and Car Drivers. Tartu, 2007.
132. **Riina Vibo**. The third stroke registry in Tartu, Estonia from 2001 to 2003: incidence, case-fatality, risk factors and long-term outcome. Tartu, 2007.
133. **Chris Pruunsild**. Juvenile idiopathic arthritis in children in Estonia. Tartu, 2007.
134. **Eve Õiglane-Šlik**. Angelman and Prader-Willi syndromes in Estonia. Tartu, 2007.
135. **Kadri Haller**. Antibodies to follicle stimulating hormone. Significance in female infertility. Tartu, 2007.
136. **Pille Ööpik**. Management of depression in family medicine. Tartu, 2007.
137. **Jaak Kals**. Endothelial function and arterial stiffness in patients with atherosclerosis and in healthy subjects. Tartu, 2007.
138. **Priit Kampus**. Impact of inflammation, oxidative stress and age on arterial stiffness and carotid artery intima-media thickness. Tartu, 2007.
139. **Margus Punab**. Male fertility and its risk factors in Estonia. Tartu, 2007.
140. **Alar Toom**. Heterotopic ossification after total hip arthroplasty: clinical and pathogenetic investigation. Tartu, 2007.

141. **Lea Pehme**. Epidemiology of tuberculosis in Estonia 1991–2003 with special regard to extrapulmonary tuberculosis and delay in diagnosis of pulmonary tuberculosis. Tartu, 2007.
142. **Juri Karjagin**. The pharmacokinetics of metronidazole and meropenem in septic shock. Tartu, 2007.
143. **Inga Talvik**. Inflicted traumatic brain injury shaken baby syndrome in Estonia – epidemiology and outcome. Tartu, 2007.
144. **Tarvo Rajasalu**. Autoimmune diabetes: an immunological study of type 1 diabetes in humans and in a model of experimental diabetes (in RIP-B7.1 mice). Tartu, 2007.
145. **Inga Karu**. Ischaemia-reperfusion injury of the heart during coronary surgery: a clinical study investigating the effect of hyperoxia. Tartu, 2007.
146. **Peeter Padrik**. Renal cell carcinoma: Changes in natural history and treatment of metastatic disease. Tartu, 2007.
147. **Neve Vendt**. Iron deficiency and iron deficiency anaemia in infants aged 9 to 12 months in Estonia. Tartu, 2008.
148. **Lenne-Triin Heidmets**. The effects of neurotoxins on brain plasticity: focus on neural Cell Adhesion Molecule. Tartu, 2008.
149. **Paul Korrovits**. Asymptomatic inflammatory prostatitis: prevalence, etiological factors, diagnostic tools. Tartu, 2008.
150. **Annika Reintam**. Gastrointestinal failure in intensive care patients. Tartu, 2008.
151. **Kristiina Roots**. Cationic regulation of Na-pump in the normal, Alzheimer's and CCK_2 receptor-deficient brain. Tartu, 2008.
152. **Helen Puusepp**. The genetic causes of mental retardation in Estonia: fragile X syndrome and creatine transporter defect. Tartu, 2009.
153. **Kristiina Rull**. Human chorionic gonadotropin beta genes and recurrent miscarriage: expression and variation study. Tartu, 2009.
154. **Margus Eimre**. Organization of energy transfer and feedback regulation in oxidative muscle cells. Tartu, 2009.
155. **Maire Link**. Transcription factors FoxP3 and AIRE: autoantibody associations. Tartu, 2009.
156. **Kai Haldre**. Sexual health and behaviour of young women in Estonia. Tartu, 2009.
157. **Kaur Liivak**. Classical form of congenital adrenal hyperplasia due to 21-hydroxylase deficiency in Estonia: incidence, genotype and phenotype with special attention to short-term growth and 24-hour blood pressure. Tartu, 2009.
158. **Kersti Ehrlich**. Antioxidative glutathione analogues (UPF peptides) – molecular design, structure-activity relationships and testing the protective properties. Tartu, 2009.
159. **Anneli Rätsep**. Type 2 diabetes care in family medicine. Tartu, 2009.
160. **Silver Türk**. Etiopathogenetic aspects of chronic prostatitis: role of mycoplasmas, coryneform bacteria and oxidative stress. Tartu, 2009.

161. **Kaire Heilman**. Risk markers for cardiovascular disease and low bone mineral density in children with type 1 diabetes. Tartu, 2009.
162. **Kristi Rüütel**. HIV-epidemic in Estonia: injecting drug use and quality of life of people living with HIV. Tartu, 2009.
163. **Triin Eller**. Immune markers in major depression and in antidepressive treatment. Tartu, 2009.
164. **Siim Suutre**. The role of TGF-β isoforms and osteoprogenitor cells in the pathogenesis of heterotopic ossification. An experimental and clinical study of hip arthroplasty. Tartu, 2010.
165. **Kai Kliiman**. Highly drug-resistant tuberculosis in Estonia: Risk factors and predictors of poor treatment outcome. Tartu, 2010.
166. **Inga Villa**. Cardiovascular health-related nutrition, physical activity and fitness in Estonia. Tartu, 2010.
167. **Tõnis Org**. Molecular function of the first PHD finger domain of Autoimmune Regulator protein. Tartu, 2010.
168. **Tuuli Metsvaht**. Optimal antibacterial therapy of neonates at risk of early onset sepsis. Tartu, 2010.
169. **Jaanus Kahu**. Kidney transplantation: Studies on donor risk factors and mycophenolate mofetil. Tartu, 2010.
170. **Koit Reimand**. Autoimmunity in reproductive failure: A study on associated autoantibodies and autoantigens. Tartu, 2010.
171. **Mart Kull**. Impact of vitamin D and hypolactasia on bone mineral density: a population based study in Estonia. Tartu, 2010.
172. **Rael Laugesaar**. Stroke in children – epidemiology and risk factors. Tartu, 2010.
173. **Mark Braschinsky**. Epidemiology and quality of life issues of hereditary spastic paraplegia in Estonia and implemention of genetic analysis in everyday neurologic practice. Tartu, 2010.
174. **Kadri Suija**. Major depression in family medicine: associated factors, recurrence and possible intervention. Tartu, 2010.
175. **Jarno Habicht**. Health care utilisation in Estonia: socioeconomic determinants and financial burden of out-of-pocket payments. Tartu, 2010.
176. **Kristi Abram**. The prevalence and risk factors of rosacea. Subjective disease perception of rosacea patients. Tartu, 2010.
177. **Malle Kuum**. Mitochondrial and endoplasmic reticulum cation fluxes: Novel roles in cellular physiology. Tartu, 2010.
178. **Rita Teek**. The genetic causes of early onset hearing loss in Estonian children. Tartu, 2010.
179. **Daisy Volmer**. The development of community pharmacy services in Estonia – public and professional perceptions 1993–2006. Tartu, 2010.
180. **Jelena Lissitsina**. Cytogenetic causes in male infertility. Tartu, 2011.
181. **Delia Lepik**. Comparison of gunshot injuries caused from Tokarev, Makarov and Glock 19 pistols at different firing distances. Tartu, 2011.
182. **Ene-Renate Pähkla**. Factors related to the efficiency of treatment of advanced periodontitis. Tartu, 2011.

183. **Maarja Krass**. L-Arginine pathways and antidepressant action. Tartu, 2011.
184. **Taavi Lai**. Population health measures to support evidence-based health policy in Estonia. Tartu, 2011.
185. **Tiit Salum**. Similarity and difference of temperature-dependence of the brain sodium pump in normal, different neuropathological, and aberrant conditions and its possible reasons. Tartu, 2011.
186. **Tõnu Vooder**. Molecular differences and similarities between histological subtypes of non-small cell lung cancer. Tartu, 2011.
187. **Jelena Štšepetova**. The characterisation of intestinal lactic acid bacteria using bacteriological, biochemical and molecular approaches. Tartu, 2011.
188. **Radko Avi**. Natural polymorphisms and transmitted drug resistance in Estonian HIV-1 CRF06_cpx and its recombinant viruses. Tartu, 2011, 116 p.
189. **Edward Laane**. Multiparameter flow cytometry in haematological malignancies. Tartu, 2011, 152 p.
190. **Triin Jagomägi**. A study of the genetic etiology of nonsyndromic cleft lip and palate. Tartu, 2011, 158 p.
191. **Ivo Laidmäe**. Fibrin glue of fish (*Salmo salar*) origin: immunological study and development of new pharmaceutical preparation. Tartu, 2012, 150 p.
192. **Ülle Parm**. Early mucosal colonisation and its role in prediction of invasive infection in neonates at risk of early onset sepsis. Tartu, 2012, 168 p.
193. **Kaupo Teesalu**. Autoantibodies against desmin and transglutaminase 2 in celiac disease: diagnostic and functional significance. Tartu, 2012, 142 p.
194. **Maksim Zagura**. Biochemical, functional and structural profiling of arterial damage in atherosclerosis. Tartu, 2012, 162 p.
195. **Vivian Kont**. Autoimmune regulator: characterization of thymic gene regulation and promoter methylation. Tartu, 2012, 134 p.
196. **Pirje Hütt**. Functional properties, persistence, safety and efficacy of potential probiotic lactobacilli. Tartu, 2012, 246 p.
197. **Innar Tõru**. Serotonergic modulation of CCK-4- induced panic. Tartu, 2012, 132 p.
198. **Sigrid Vorobjov**. Drug use, related risk behaviour and harm reduction interventions utilization among injecting drug users in Estonia: implications for drug policy. Tartu, 2012, 120 p.
199. **Martin Serg**. Therapeutic aspects of central haemodynamics, arterial stiffness and oxidative stress in hypertension. Tartu, 2012, 156 p.
200. **Jaanika Kumm**. Molecular markers of articular tissues in early knee osteoarthritis: a population-based longitudinal study in middle-aged subjects. Tartu, 2012, 159 p.
201. **Kertu Rünkorg**. Functional changes of dopamine, endopioid and endocannabinoid systems in CCK2 receptor deficient mice. Tartu, 2012, 125 p.
202. **Mai Blöndal**. Changes in the baseline characteristics, management and outcomes of acute myocardial infarction in Estonia. Tartu, 2012, 127 p.

203. **Jana Lass**. Epidemiological and clinical aspects of medicines use in children in Estonia. Tartu, 2012, 170 p.
204. **Kai Truusalu**. Probiotic lactobacilli in experimental persistent *Salmonella* infection. Tartu, 2013, 139 p.
205. **Oksana Jagur**. Temporomandibular joint diagnostic imaging in relation to pain and bone characteristics. Long-term results of arthroscopic treatment. Tartu, 2013, 126 p.
206. **Katrin Sikk**. Manganese-ephedrone intoxication – pathogenesis of neurological damage and clinical symptomatology. Tartu, 2013, 125 p.
207. **Kai Blöndal**. Tuberculosis in Estonia with special emphasis on drug-resistant tuberculosis: Notification rate, disease recurrence and mortality. Tartu, 2013, 151 p.
208. **Marju Puurand**. Oxidative phosphorylation in different diseases of gastric mucosa. Tartu, 2013, 123 p.
209. **Aili Tagoma**. Immune activation in female infertility: Significance of autoantibodies and inflammatory mediators. Tartu, 2013, 135 p.
210. **Liis Sabre**. Epidemiology of traumatic spinal cord injury in Estonia. Brain activation in the acute phase of traumatic spinal cord injury. Tartu, 2013, 135 p.
211. **Merit Lamp**. Genetic susceptibility factors in endometriosis. Tartu, 2013, 125 p.
212. **Erik Salum**. Beneficial effects of vitamin D and angiotensin II receptor blocker on arterial damage. Tartu, 2013, 167 p.
213. **Maire Karelson**. Vitiligo: clinical aspects, quality of life and the role of melanocortin system in pathogenesis. Tartu, 2013, 153 p.
214. **Kuldar Kaljurand**. Prevalence of exfoliation syndrome in Estonia and its clinical significance. Tartu, 2013, 113 p.
215. **Raido Paasma**. Clinical study of methanol poisoning: handling large outbreaks, treatment with antidotes, and long-term outcomes. Tartu, 2013, 96 p.
216. **Anne Kleinberg**. Major depression in Estonia: prevalence, associated factors, and use of health services. Tartu, 2013, 129 p.
217. **Triin Eglit**. Obesity, impaired glucose regulation, metabolic syndrome and their associations with high-molecular-weight adiponectin levels. Tartu, 2014, 115 p.
218. **Kristo Ausmees**. Reproductive function in middle-aged males: Associations with prostate, lifestyle and couple infertility status. Tartu, 2014, 125 p.
219. **Kristi Huik**. The influence of host genetic factors on the susceptibility to HIV and HCV infections among intravenous drug users. Tartu, 2014, 144 p.
220. **Liina Tserel**. Epigenetic profiles of monocytes, monocyte-derived macrophages and dendritic cells. Tartu, 2014, 143 p.
221. **Irina Kerna**. The contribution of *ADAM12* and *CILP* genes to the development of knee osteoarthritis. Tartu, 2014, 152 p.

222. **Ingrid Liiv**. Autoimmune regulator protein interaction with DNA-dependent protein kinase and its role in apoptosis. Tartu, 2014, 143 p.
223. **Liivi Maddison**. Tissue perfusion and metabolism during intra-abdominal hypertension. Tartu, 2014, 103 p.
224. **Krista Ress**. Childhood coeliac disease in Estonia, prevalence in atopic dermatitis and immunological characterisation of coexistence. Tartu, 2014, 124 p.
225. **Kai Muru**. Prenatal screening strategies, long-term outcome of children with marked changes in maternal screening tests and the most common syndromic heart anomalies in Estonia. Tartu, 2014, 189 p.
226. **Kaja Rahu**. Morbidity and mortality among Baltic Chernobyl cleanup workers: a register-based cohort study. Tartu, 2014, 155 p.
227. **Klari Noormets**. The development of diabetes mellitus, fertility and energy metabolism disturbances in a Wfs1-deficient mouse model of Wolfram syndrome. Tartu, 2014, 132 p.
228. **Liis Toome**. Very low gestational age infants in Estonia. Tartu, 2014, 183 p.
229. **Ceith Nikkolo**. Impact of different mesh parameters on chronic pain and foreign body feeling after open inguinal hernia repair. Tartu, 2014, 132 p.
230. **Vadim Brjalin**. Chronic hepatitis C: predictors of treatment response in Estonian patients. Tartu, 2014, 122 p.
231. **Vahur Metsna**. Anterior knee pain in patients following total knee arthroplasty: the prevalence, correlation with patellar cartilage impairment and aspects of patellofemoral congruence. Tartu, 2014, 130 p.
232. **Marju Kase**. Glioblastoma multiforme: possibilities to improve treatment efficacy. Tartu, 2015, 137 p.
233. **Riina Runnel**. Oral health among elementary school children and the effects of polyol candies on the prevention of dental caries. Tartu, 2015, 112 p.
234. **Made Laanpere**. Factors influencing women's sexual health and reproductive choices in Estonia. Tartu, 2015, 176 p.
235. **Andres Lust**. Water mediated solid state transformations of a polymorphic drug – effect on pharmaceutical product performance. Tartu, 2015, 134 p.
236. **Anna Klugman**. Functionality related characterization of pretreated wood lignin, cellulose and polyvinylpyrrolidone for pharmaceutical applications. Tartu, 2015, 156 p.
237. **Triin Laisk-Podar**. Genetic variation as a modulator of susceptibility to female infertility and a source for potential biomarkers. Tartu, 2015, 155 p.
238. **Mailis Tõnisson**. Clinical picture and biochemical changes in blood in children with acute alcohol intoxication. Tartu, 2015, 100 p.
239. **Kadri Tamme**. High volume haemodiafiltration in treatment of severe sepsis – impact on pharmacokinetics of antibiotics and inflammatory response. Tartu, 2015, 133 p.

240. **Kai Part**. Sexual health of young people in Estonia in a social context: the role of school-based sexuality education and youth-friendly counseling services. Tartu, 2015, 203 p.
241. **Urve Paaver**. New perspectives for the amorphization and physical stabilization of poorly water-soluble drugs and understanding their dissolution behavior. Tartu, 2015, 139 p.
242. **Aleksandr Peet**. Intrauterine and postnatal growth in children with HLA-conferred susceptibility to type 1 diabetes. Tartu. 2015, 146 p.
243. **Piret Mitt**. Healthcare-associated infections in Estonia – epidemiology and surveillance of bloodstream and surgical site infections. Tartu, 2015, 145 p.
244. **Merli Saare**. Molecular Profiling of Endometriotic Lesions and Endometria of Endometriosis Patients. Tartu, 2016, 129 p.
245. **Kaja-Triin Laisaar**. People living with HIV in Estonia: Engagement in medical care and methods of increasing adherence to antiretroviral therapy and safe sexual behavior. Tartu, 2016, 132 p.
246. **Eero Merilind**. Primary health care performance: impact of payment and practice-based characteristics. Tartu, 2016, 120 p.
247. **Jaanika Kärner**. Cytokine-specific autoantibodies in AIRE deficiency. Tartu, 2016, 182 p.
248. **Kaido Paapstel**. Metabolomic profile of arterial stiffness and early biomarkers of renal damage in atherosclerosis. Tartu, 2016, 173 p.
249. **Liidia Kiisk**. Long-term nutritional study: anthropometrical and clinico-laboratory assessments in renal replacement therapy patients after intensive nutritional counselling. Tartu, 2016, 207 p.
250. **Georgi Nellis**. The use of excipients in medicines administered to neonates in Europe. Tartu, 2017, 159 p.
251. **Aleksei Rakitin**. Metabolic effects of acute and chronic treatment with valproic acid in people with epilepsy. Tartu, 2017, 125 p.
252. **Eveli Kallas**. The influence of immunological markers to susceptibility to HIV, HBV, and HCV infections among persons who inject drugs. Tartu, 2017, 138 p.
253. **Tiina Freimann**. Musculoskeletal pain among nurses: prevalence, risk factors, and intervention. Tartu, 2017, 125 p.
254. **Evelyn Aaviksoo**. Sickness absence in Estonia: determinants and influence of the sick-pay cut reform. Tartu, 2017, 121 p.
255. **Kalev Nõupuu**. Autosomal-recessive Stargardt disease: phenotypic heterogeneity and genotype-phenotype associations. Tartu, 2017, 131 p.
256. **Ho Duy Binh**. Osteogenesis imperfecta in Vietnam. Tartu, 2017, 125 p.
257. **Uku Haljasorg**. Transcriptional mechanisms in thymic central tolerance. Tartu, 2017, 147 p.
258. **Živile Riispere**. IgA Nephropathy study according to the Oxford Classification: IgA Nephropathy clinical-morphological correlations, disease progression and the effect of renoprotective therapy. Tartu, 2017, 129 p.

259. **Hiie Soeorg**. Coagulase-negative staphylococci in gut of preterm neonates and in breast milk of their mothers. Tartu, 2017, 216 p.
260. **Anne-Mari Anton Willmore**. Silver nanoparticles for cancer research. Tartu, 2017, 132 p.
261. **Ott Laius**. Utilization of osteoporosis medicines, medication adherence and the trend in osteoporosis related hip fractures in Estonia. Tartu, 2017, 134 p.
262. **Alar Aab**. Insights into molecular mechanisms of asthma and atopic dermatitis. Tartu, 2017, 164 p.
263. **Sander Pajusalu**. Genome-wide diagnostics of Mendelian disorders: from chromosomal microarrays to next-generation sequencing. Tartu, 2017, 146 p.
264. **Mikk Jürisson**. Health and economic impact of hip fracture in Estonia. Tartu, 2017, 164 p.
265. **Kaspar Tootsi**. Cardiovascular and metabolomic profiling of osteoarthritis. Tartu, 2017, 150 p.
266. **Mario Saare**. The influence of AIRE on gene expression – studies of transcriptional regulatory mechanisms in cell culture systems. Tartu, 2017, 172 p.
267. **Piia Jõgi**. Epidemiological and clinical characteristics of pertussis in Estonia. Tartu, 2018, 168 p.
268. **Elle Põldoja**. Structure and blood supply of the superior part of the shoulder joint capsule. Tartu, 2018, 116 p.
269. **Minh Son Nguyen**. Oral health status and prevalence of temporomandibular disorders in 65–74-year-olds in Vietnam. Tartu, 2018, 182 p.
270. **Kristian Semjonov**. Development of pharmaceutical quench-cooled molten and melt-electrospun solid dispersions for poorly water-soluble indomethacin. Tartu, 2018, 125 p.
271. **Janne Tiigimäe-Saar**. Botulinum neurotoxin type A treatment for sialorrhea in central nervous system diseases. Tartu, 2018, 109 p.
272. **Veiko Vengerfeldt**. Apical periodontitis: prevalence and etiopathogenetic aspects. Tartu, 2018, 150 p.
273. **Rudolf Bichele**. TNF superfamily and AIRE at the crossroads of thymic differentiation and host protection against *Candida albicans* infection. Tartu, 2018, 153 p.
274. **Olga Tšuiko**. Unravelling Chromosomal Instability in Mammalian Preimplantation Embryos Using Single-Cell Genomics. Tartu, 2018, 169 p.
275. **Kärt Kriisa**. Profile of acylcarnitines, inflammation and oxidative stress in first-episode psychosis before and after antipsychotic treatment. Tartu, 2018, 145 p.
276. **Xuan Dung Ho**. Characterization of the genomic profile of osteosarcoma. Tartu, 2018, 144 p.
277. **Karit Reinson**. New Diagnostic Methods for Early Detection of Inborn Errors of Metabolism in Estonia. Tartu, 2018, 201 p.

278. **Mari-Anne Vals**. Congenital N-glycosylation Disorders in Estonia. Tartu, 2019, 148 p.
279. **Liis Kadastik-Eerme**. Parkinson's disease in Estonia: epidemiology, quality of life, clinical characteristics and pharmacotherapy. Tartu, 2019, 202 p.
280. **Hedi Hunt**. Precision targeting of intraperitoneal tumors with peptide-guided nanocarriers. Tartu, 2019, 179 p.
281. **Rando Porosk**. The role of oxidative stress in Wolfram syndrome 1 and hypothermia. Tartu, 2019, 123 p.
282. **Ene-Ly Jõgeda**. The influence of coinfections and host genetic factor on the susceptibility to HIV infection among people who inject drugs. Tartu, 2019, 126 p.
283. **Kristel Ehala-Aleksejev**. The associations between body composition, obesity and obesity-related health and lifestyle conditions with male reproductive function. Tartu, 2019, 138 p.
284. **Aigar Ottas**. The metabolomic profiling of psoriasis, atopic dermatitis and atherosclerosis. Tartu, 2019, 136 p.
285. **Elmira Gurbanova**. Specific characteristics of tuberculosis in low default, but high multidrug–resistance prison setting. Tartu, 2019, 129 p.
286. **Van Thai Nguyeni**. The first study of the treatment outcomes of patients with cleft lip and palate in Central Vietnam. Tartu, 2019, 144 p.
287. **Maria Yakoreva**. Imprinting Disorders in Estonia. Tartu, 2019, 187 p.
288. **Kadri Rekker**. The putative role of microRNAs in endometriosis pathogenesis and potential in diagnostics. Tartu, 2019, 140 p.
289. **Ülle Võhma**. Association between personality traits, clinical characteristics and pharmacological treatment response in panic disorder. Tartu, 2019, 121 p.
290. **Aet Saar**. Acute myocardial infarction in Estonia 2001–2014: towards risk-based prevention and management. Tartu, 2019, 124 p.
291. **Toomas Toomsoo**. Transcranial brain sonography in the Estonian cohort of Parkinson's disease. Tartu, 2019, 114 p.
292. **Lidiia Zhytnik**. Inter- and intrafamilial diversity based on genotype and phenotype correlations of Osteogenesis Imperfecta. Tartu, 2019, 224 p.
293. **Pilleriin Soodla**. Newly HIV-infected people in Estonia: estimation of incidence and transmitted drug resistance. Tartu, 2019, 194 p.
294. **Kristiina Ojamaa**. Epidemiology of gynecological cancer in Estonia. Tartu, 2020, 133 p.
295. **Marianne Saard**. Modern Cognitive and Social Intervention Techniques in Paediatric Neurorehabilitation for Children with Acquired Brain Injury. Tartu, 2020, 168 p.
296. **Julia Maslovskaja**. The importance of DNA binding and DNA breaks for AIRE-mediated transcriptional activation. Tartu, 2020, 162 p.
297. **Natalia Lobanovskaya**. The role of PSA-NCAM in the survival of retinal ganglion cells. Tartu, 2020, 105 p.

298. **Madis Rahu**. Structure and blood supply of the postero-superior part of the shoulder joint capsule with implementation of surgical treatment after anterior traumatic dislocation. Tartu, 2020, 104 p.
299. **Helen Zirnask**. Luteinizing hormone (LH) receptor expression in the penis and its possible role in pathogenesis of erectile disturbances. Tartu, 2020, 87 p.
300. **Kadri Toome**. Homing peptides for targeting of brain diseases. Tartu, 2020, 152 p.
301. **Maarja Hallik**. Pharmacokinetics and pharmacodynamics of inotropic drugs in neonates. Tartu, 2020, 172 p.
302. **Raili Müller**. Cardiometabolic risk profile and body composition in early rheumatoid arthritis. Tartu, 2020, 133 p.
303. **Sergo Kasvandik**. The role of proteomic changes in endometrial cells – from the perspective of fertility and endometriosis. Tartu, 2020, 191 p.
304. **Epp Kaleviste**. Genetic variants revealing the role of STAT1/STAT3 signaling cytokines in immune protection and pathology. Tartu, 2020, 189 p.
305. **Sten Saar**. Epidemiology of severe injuries in Estonia. Tartu, 2020, 104 p.
306. **Kati Braschinsky**. Epidemiology of primary headaches in Estonia and applicability of web-based solutions in headache epidemiology research. Tartu, 2020, 129 p.
307. **Helen Vaher**. MicroRNAs in the regulation of keratinocyte responses in *psoriasis vulgaris* and atopic dermatitis. Tartu, 2020, 242 p.
308. **Liisi Raam**. Molecular Alterations in the Pathogenesis of Two Chronic Dermatoses – Vitiligo and Psoriasis. Tartu, 2020, 164 p.
309. **Artur Vetkas**. Long-term quality of life, emotional health, and associated factors in patients after aneurysmal subarachnoid haemorrhage. Tartu, 2020, 127 p.
310. **Teele Kasepalu**. Effects of remote ischaemic preconditioning on organ damage and acylcarnitines' metabolism in vascular surgery. Tartu, 2020, 130 p.
311. **Prakash Lingasamy**. Development of multitargeted tumor penetrating peptides. Tartu, 2020, 246 p.
312. **Lille Kurvits.** Parkinson's disease as a multisystem disorder: whole transcriptome study in Parkinson's disease patients' skin and blood. Tartu, 2021, 142 p.
313. **Mariliis Põld.** Smoking, attitudes towards smoking behaviour, and nicotine dependence among physicians in Estonia: cross-sectional surveys 1982–2014. Tartu, 2021, 172 p.
314. **Triin Kikas**. Single nucleotide variants affecting placental gene expression and pregnancy outcome. Tartu, 2021, 160 p.
315. **Hedda Lippus-Metsaots**. Interpersonal violence in Estonia: prevalence, impact on health and health behaviour. Tartu, 2021, 172 p.

316. **Georgi Dzaparidze.** Quantification and evaluation of the diagnostic significance of adenocarcinoma-associated microenvironmental changes in the prostate using modern digital pathology solutions. Tartu, 2021, 132 p.
317. **Tuuli Sedman.** New avenues for GLP1 receptor agonists in the treatment of diabetes. Tartu, 2021, 118 p.
318. **Martin Padar.** Enteral nutrition, gastrointestinal dysfunction and intestinal biomarkers in critically ill patients. Tartu, 2021, 189 p.
319. **Siim Schneider.** Risk factors, etiology and long-term outcome in young ischemic stroke patients in Estonia. Tartu, 2021, 131 p.
320. **Konstantin Ridnõi.** Implementation and effectiveness of new prenatal diagnostic strategies in Estonia. Tartu, 2021, 191 p.
321. **Risto Vaikjärv.** Etiopathogenetic and clinical aspects of peritonsillar abscess. Tartu, 2021, 115 p.
322. **Liis Preem.** Design and characterization of antibacterial electrospun drug delivery systems for wound infections. Tartu, 2022, 220 p.
323. **Keerthie Dissanayake.** Preimplantation embryo-derived extracellular vesicles: potential as an embryo quality marker and their role during the embryo-maternal communication. Tartu, 2022, 203 p.
324. **Laura Viidik.** 3D printing in pharmaceutics: a new avenue for fabricating therapeutic drug delivery systems. Tartu, 2022, 139 p.
325. **Kasun Godakumara.** Extracellular vesicle mediated embryo-maternal communication – A tool for evaluating functional competency of preimplantation embryos. Tartu, 2022, 176 p.
326. **Hindrek Teder.** Developing computational methods and workflows for targeted and whole-genome sequencing based non-invasive prenatal testing. Tartu, 2022, 138 p.
327. **Jana Tuusov.** Deaths caused by alcohol, psychotropic and other substances in Estonia: evidence based on forensic autopsies. Tartu, 2022, 157 p.
328. **Heigo Reima.** Colorectal cancer care and outcomes – evaluation and possibilities for improvement in Estonia. Tartu, 2022, 146 p.
329. **Liisa Kuhi.** A contribution of biomarker collagen type II neoepitope C2C in urine to the diagnosis and prognosis of knee osteoarthritis. Tartu, 2022, 157 p.
330. **Reeli Tamme.** Associations between pubertal hormones and physical activity levels, and subsequent bone mineral characteristics: a longitudinal study of boys aged 12–18. Tartu, 2022, 118 p.
331. **Deniss Sõritsa.** The impact of endometriosis and physical activity on female reproduction. Tartu, 2022, 152 p.
332. **Mohammad Mehedi Hasan.** Characterization of follicular fluid-derived extracellular vesicles and their contribution to periconception environment. Tartu, 2022, 194 p.
333. **Priya Kulkarni.** Osteoarthritis pathogenesis: an immunological passage through synovium-synovial fluid axis. Tartu, 2022, 268 p.

334. **Nigul Ilves.** Brain plasticity and network reorganization in children with perinatal stroke: a functional magnetic resonance imaging study. Tartu, 2022, 169 p.
335. **Marko Murruste.** Short- and long-term outcomes of surgical management of chronic pancreatitis. Tartu, 2022, 180 p.
336. **Marilin Ivask.** Transcriptomic and metabolic changes in the WFS1-deficient mouse model. Tartu, 2022, 158 p.
337. **Jüri Lieberg.** Results of surgical treatment and role of biomarkers in pathogenesis and risk prediction in patients with abdominal aortic aneurysm and peripheral artery disease. Tartu, 2022, 160 p.
338. **Sanna Puusepp.** Comparison of molecular genetics and morphological findings of childhood-onset neuromuscular disorders. Tartu, 2022, 216 p.
339. **Khan Nguyen Viet.** Chemical composition and bioactivity of extracts and constituents isolated from the medicinal plants in Vietnam and their nanotechnology-based delivery systems. Tartu, 2023, 172 p.
340. **Getnet Balcha Midekessa.** Towards understanding the colloidal stability and detection of Extracellular Vesicles. Tartu, 2023, 172 p.
341. **Kristiina Sepp.** Competency-based and person-centred community pharmacy practice – development and implementation in Estonia. Tartu, 2023, 242 p.
342. **Linda Sõber.** Impact of thyroid disease and surgery on patient's quality of voice and swallowing. Tartu, 2023, 114 p.
343. **Anni Lepland.** Precision targeting of tumour-associated macrophages in triple negative breast cancer. Tartu, 2023, 160 p.
344. **Sirje Sammul.** Prevalence and risk factors of arterial hypertension and cardiovascular mortality: 13-year longitudinal study among 35- and 55-year-old adults in Estonia and Sweden. Tartu, 2023, 158 p.
345. **Maarjaliis Paavo**. Short-Wavelength and Near-Infrared Autofluorescence Imaging in Recessive Stargardt Disease, Choroideremia, *PROM1*-Macular Dystrophy and Ocular Albinism. Tartu, 2023, 202 p.
346. **Kaspar Ratnik.** Development of predictive multimarker test for preeclampsia in early and late pregnancy. Tartu, 2023, 134 p.
347. **Kärt Simre.** Development of coeliac disease in two populations with different environmental backgrounds. Tartu, 2023, 161 p.
348. **Qurat Ul Ain Reshi.** Characterization of the maternal reproductive tract and spermatozoa communication during periconception period via extracellular vesicles. Tartu, 2023, 182 p.
349. **Stanislav Tjagur.** *Mycoplasma genitalium* and other sexually transmitted infections causing urethritis – their prevalence, impact on male fertility parameters and prostate health. Tartu, 2023, 225 p.
350. **Lagle Lehes.** The first study of voice and resonance related treatment outcomes of Estonian cleft palate children. Tartu, 2023, 126 p.
351. **Liis Ilves.** Metabolomic profiling of chronic inflammatory skin diseases. Tartu, 2023, 146 p.

352. **Marina Šunina**. Flow cytometric analysis of T and B cell properties in healthy donors and subjects with vitiligo. Tartu, 2023, 164 p.
353. **Jaanus Suumann.** Gastric biomarkers and their dynamics as a less invasive method to evaluate stomach health in bariatric surgery patients. Tartu, 2023, 122 p.
354. **Ele Hanson.** Clinical and biochemical markers for the prediction and early diagnosis of pregnancy related complications. Tartu, 2023, 145 p.
355. **Priit Pauklin.** Hemodynamic and biochemical characteristics of patients with atrial fibrillation and anticoagulation of ≥65-year-old patients with atrial fibrillation in Estonia. Tartu, 2023, 144 p.
356. **Triinu Keskpaik.** Quality Indicators and Non-Ischemic Myocardial Injury in Emergency Medicine. Tartu, 2023, 121 p.
357. **Laura Roht.** Hereditary colorectal cancer syndromes in Estonia. Tartu, 2023, 178 p.
358. **Norman Ilves.** Risk factors and onset time of periventricular hemorrhagic infarction in preterm born children and periventricular venous infarction in term born children. Tartu, 2024, 177 p.
359. **Edgar Lipping.** Postoperative antibacterial therapy in complicated appendicitis and appendectomy in pregnancy. Tartu, 2024, 121 p.
360. **Celia Teresa Pozo Ramos.** Preparation and assessment of antimicrobial electrospun matrices for prospective applications in wound healing. Tartu, 2024, 203 p.
361. **Karl Kuusik.** Effects of remote ischaemic preconditioning on arterial stiffness, organ damage and metabolomic profile in patients with lower extremity artery disease. Tartu, 2024, 173 p.
362. **Kelli Somelar-Duracz.** The molecular and cellular mechanisms of brain plasticity impairing factors. Tartu, 2024, 245 p.
363. **Aleksei Baburin.** Breast cancer incidence, mortality and survival in Estonia in the context of health care system changes and screening. Tartu, 2024, 130 p.
364. **Marina Loid.** Molecular and cellular determinants of healthy receptive and aged endometrium. Tartu, 2024, 159 p.
365. **Ulvi Vaher.** Epilepsy after ischemic perinatal stroke in term born children: neuroimaging predictors, clinical course and cognitive outcome. Tartu, 2024, 160 p.
366. **Allan Tobi.** Development of Smart Nanoparticles for Experimental Treatment of Cancer. Tartu, 2024, 160 p.
367. **Leho Rips.** The influence of vitamin D on the physical performance of conscripts in the Estonian Defence Forces. Tartu, 2024, 147 p.
368. **Kati Kärberg.** Factors and markers predicting subclinical atherosclerosis in type 2 diabetes. Tartu, 2024, 161 p.
369. **Valeria Sidorenko.** Novel anthracycline-loaded nanoparticles for precision cancer therapy. Tartu, 2024, 183 p.

370. **Kadri Kõivumägi.** Acute gastroenteritis hospitalizations in Estonia after implementation of universal mass vaccination against rotavirus. Tartu, 2024, 150 p.
371. **Ingrid Oit-Wiscombe.** Genetic markers of enzymatics in the pathogenesis of chronic obstructive pulmonary disease as a systemic disease and the effects of antioxidant peptides. Tartu, 2025, 170 p.
372. **Gerli Mõts.** Ethical issues in nursing before and during the COVID-19 pandemic: a multi-method study. Tartu, 2025, 150 p.
373. **Annika Valner.** Changes in structure and function of extremities in early rheumatoid arthritis. Tartu, 2025, 129 p.
374. **Meruert Sarsenova.** Molecular and cellular landscape of endometriosis. Tartu, 2025, 157 p.
375. **Mailis Liiv.** Role of mitochondrial dynamics in Wolfram syndrome. Tartu, 2025, 180 p.
376. **Marta Velgan.** Addressing the family physician shortage: Career and migration intentions in Estonia and Europe. Tartu, 2025, 161 p.
377. **Ihor Filippov.** Single-cell data analysis in immunology: from technology to applications. Tartu, 2025, 204 p.
378. **Anna Tisler.** HPV-related cancers among people living with HIV and transition towards risk-based prevention. Tartu, 2025, 122 p.
379. **Priit Paluoja.** Computational methods for NIPT-based aneuploidy and microdeletion screening. Tartu, 2025, 154 p.
380. **Ere Uibu.** Utilisation and outcomes of patient safety incident reporting and learning in hospitals from a nursing perspective: a multi-method study. Tartu, 2025, 142 p.
381. **Jane Idavainu.** Health effects of environmental contamination in the oil shale industry region of Estonia. Tartu, 2025, 164 p.