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ABBREVIATIONS

ACR	the American College of Rheumatology
AKOA	accelerated knee osteoarthritis
AUC	area under the curve
BL	baseline
BMI	body mass index
CI	confidence interval
Col2	type II collagen
COMP	cartilage oligomeric matrix protein
Crea	creatinine in urine
DMOAD	disease modifying OA drug
EKOA	early knee osteoarthritis
EULAR	European League against Rheumatism
GAG	glycosaminoglycan
GLM	generalized linear model
GP	general practitioners
gOA	global grade of radiographic kOA by Nottingham system for classification
IL	interleukin
JSN	joint space narrowing
JSW	joint space width
KL	radiographic knee osteoarthritis by Kellgren and Lawrence scoring system for classification
kOA	knee osteoarthritis
KOOS	The Knee injury and Osteoarthritis Outcome Score
KOOSsympt	KOOS subscale of knee symptoms
KOOSpain	KOOS subscale of knee pain
KOOSadl	KOOS subscale of activities of daily living
KOOSsp/recr	KOOS subscale of sport and recreation
KOOSqol	KOOS subscale of knee-related quality of life
LM	linear regression model
MJOA	multiple joint osteoarthritides
MMP	matrix metalloproteinase
MRI	magnet resonance imagine
n	numbers
NSy	radiographic knee osteoarthritis by Nottingham scoring system for classification
OA	osteoarthritis
OARSI	Osteoarthritis Research Society International
Oph	osteophyte
OR	odds ratio
PF	patellofemoral
PROM	patient-reported outcome measure

rkOA	radiographic knee osteoarthritis
SCB	substantial clinical benefit
SD	standard deviation
SF	synovial fluid
SFA	(medial) *Société Francaise d'Arthroscopie* score
sHA	serum hyaluronic acid
sumVAS	summary VAS pain of different joints
TF	tibiofemoral
TKR	total knee replacement
TNF-α	tumor necrosis factor alpha
uC2C	collagen type II C-terminal cleavage neoepitope C2C in urine
uCTX-II	C-terminal telopeptide of type II collagen in urine
VAS pain	Visual Analog Scale for Pain
WOMAC	Western Ontario and McMaster Universities osteoarthritis index
X-ray	radiographic examinatiom
Yrs	years

1. INTRODUCTION

Osteoarthritis (OA) is the most frequent musculoskeletal disorder and the single most common cause of disability in elderly people; therefore, the global impact of this chronic disease is a major worldwide challenge for healthcare systems in the 21st century (Allen *et al.* 2021). In 2019, OA affected population was ~500 million people worldwide, which was about 7% of the global population. (Vos *et al.* 2020), and the incidence of the disease is increasing annually by approximately 0.3% (Jin *et al.* 2020). OA amounts a tremendous burden globally, especially in aging and increasingly obese populations (Hunter *et al.* 2020). In 2015, the overall cost of OA represents 1.1% of the United States´ gross domestic product (GDP), making it the second most expensive disease (Zhao *et al.* 2019). It should be underlined that OA affects not only pain and physical function but also has many other outcomes such as a decline in mental health, quality of life, and even mortality from cardiovascular disease (CVD) (Veronese *et al.* 2016).

Although OA was first discovered among Palaeolithic hunter-gatherers, an approximate doubling of knee OA (kOA) prevalence has occurred since the mid-20th century (Berenbaum *et al.* 2018; Wallace *et al.* 2017). This tremendous rise in the prevalence of OA can be attributed at least in part to the increase in known OA risk factors, including longevity, obesity, physical inactivity, and joint injury. From 2000 to 2013, global all-age obesity increased by 26%, and the proportion of adults with low physical activity grew by 20%. However, these factors are insufficient to explain the increase in OA prevalence indicating that the additional, yet unexplained, risk factors are contributing (Hawker, 2019; Berenbaum *et al.* 2018). The analysis has shown that by 2032, more than 26,000 new OA consultations per 1,000,000 people aged ≥45 will take place mainly in the primary care system, therefore a potential "tsunami" in OA cases is expected (Turkiewicz *et al.* 2014; Mobasheri *et al.* 2015).

OA may affect any synovial joint; however, knees, hips, hands (carpometacarpal joints, distal or proximal interphalangeal joints), and spine are most commonly involved. A prevalence of radiographic OA in the knee joint ranges from 15 to 76% depending on investigated population (D. T. Felson *et al.* 1987; Dillon *et al.* 2006; Jordan *et al.* 2007; Turkiewicz *et al.* 2014; Blanco *et al.* 2021). Worldwide, a pooled global prevalence of kOA is 22.9% in people aged 40 years and over, while a pooled global incidence was 203 per 10000 person-years in a recent review (Cui *et al.* 2020). In Estonia, the prevalence of kOA was 6.6% in 2019 (Vos *et al.* 2020). However, the prevalence of kOA is most likely underestimated worldwide, including in Estonia, as the disease in the patellofemoral (PF) joint is usually overlooked. Moreover, there is an evidence that knee pain and severity of radiographic OA features do not correlate (Bedson & Croft, 2008). Therefore, the assessment of OA prevalence depends significantly on the used diagnostic criteria. All these facts reveal that OA is a comprehensive and expensive disorder that puts stress on the health and social

care systems across the globe. Despite its considerable social-economic toll, OA was generally neglected until the year 2000 (Veronese *et al.* 2016). There is an enormous unmet need to avoid this disability. The Bone and Joint Decade 2000–2010, a global campaign, was arranged to achieve a greater awareness of the growing burden of musculoskeletal disorders, including OA, supported by the United Nations and WHO (Woolf, 2000). In 2016, the Osteoarthritis Research Society International (OARSI), the leading global organization in OA research, submitted a White Paper, Osteoarthritis: A serious disease (https://oarsi.org/education/oarsi-resources/oarsi-white-paper-oa-serious-disease), to raise an understanding of the importance of OA.

Since the year 2000, there has been an extensive research in the field of knee OA in Estonia. Notably, the Estonian middle-age population kOA cohort was created. These subjects were investigated longitudinally using novel methods of radiographic evaluation of kOA in different joint compartments by Nothingam system (Nagaosa *et al.* 2000), for the duration of three-years, and follow-up of a relevant proportion of subjects lasted as long as 12 years. This large kOA repository has provided an opportunity for extensive research and involvement in several international consortia (e.g. TREAT-OA, NanoDiaRA) to discover the causes and solutions to the complexity of this serious disease (Kerkhof *et al.* 2011). Several papers have been published based on the data of Estonian cohorts, including the studies on the prevalence and progression of radiographic kOA (rkOA) (Tamm *et al.* 2008b; Kumm *et al.* 2012; Kumm *et al.* 2013a), the studies on rkOA in association with cartilage (Kumm *et al.* 2006; Kerna *et al.* 2012; Kumm *et al.* 2013b), bone (Kumm *et al.* 2008; Kumm *et al.* 2013c), genetic markers (Kerna *et al.* 2009; Valdes *et al.* 2010a; Valdes *et al.* 2010b; Kerna *et al.* 2010; Valdes *et al.* 2011; Kerna *et al.* 2013), and cytokines (Kisand *et al.* 2018), patient-reported assessment and performance-based knee function test (Tamm *et al.* 2008a; Tamm *et al.* 2011; Tamm *et al.* 2012; A.E. Tamm *et al.* 2014), and the studies on knee ultrasound (Kumm *et al.* 2009; Kumm *et al.* 2010).

Years ago, OA was characterized as a degenerative joint disease, or hypertrophic arthritis affecting an irrelevant proportion of the population (Katz *et al.* 2021; Mobasheri *et al.* 2021). However, the approach to OA as a single entity of cartilage damage changed greatly in recent decades. In 1986, the American of College Rheumatology (ACR), suggested the definition of OA as, ‘a heterogeneous group of conditions that lead to the joint symptoms and signs which are associated with the defective integrity of articular cartilage, in addition to related changes in the underlying bone at the joint margins’ (Hutton, 1987).

Nowadays, OA is considered to have a complex pathophysiology affecting multiple joint tissues from molecular to structural level (Katz *et al.* 2021; Lv & Shi, 2021). In 2015, OARSI proposed a new definition of OA as, ‘the disease manifests first as a molecular derangement (abnormal joint tissue metabolism) followed by anatomic, and/or physiologic derangements (characterized by cartilage degradation, bone remodelling, osteophyte formation, joint inflammation

and loss of normal joint function), that can culminate in illness.' (Kraus *et al.* 2015).

Currently, there is no cure for OA; this means, we do not know how to prevent the disease or to reverse its pathogenic processes (Hunter & Bierma-Zeinstra, 2019). However, some progress in the development of disease-modifying OA drugs (DMOADs) can be noticed, for example, the preliminary evidence of substantial structure-protective action of fibroblast growth factor 18 has been established (FGF-18, named Sprifermin) (Oo *et al.* 2021). OA management is targeted to relieve the symptoms, primarily pain. Total knee replacement (TKR) at the terminal-stage of the disease is expensive and does not always give the expected results. Several important challenges such as a limited understanding of the OA joint biology, etc. have slowed the development of OA treatment, and as a result, it is considerably lagging as compared to the advances in other rheumatic diseases (Mobasheri *et al.* 2021). Moreover, recently it is recognized that OA can have several endotypes (subtypes) with certain differences in the pathogenesis, risk factors (including genetics), and the disease trajectory (Mobasheri *et al.* 2019). The three main molecular or mechanistic OA endotypes are supposed to be cartilage-driven, synovitis-driven, and bone-driven endotypes (Oo *et al.* 2021). It has been proposed that the best "window of opportunity" exists in the early phase of the disease before a serious remodelling of the joint tissues (Mahmoudian *et al.* 2021). However, reliable biomarkers for the early diagnosis of OA patients are needed for appropriate surrogate endpoints in clinical trials.

The new definition of OA highlights a molecular derangement as a primary disorder in the pathogenesis of this disease; therefore, the detection of early biomarkers is paramount in searching for more sensitive diagnostic possibilities in kOA. In the current study, we investigated a urinary biomarker for OA, type II collagen (Col2) cleavage neoepitope (uC2C) as a potential biomarker of kOA for diagnosis, prognosis, and outcome throughout the disease progression.

2. REVIEW OF THE LITERATURE

2.1. Epidemiology of OA

OA is one of the most frequent chronic progressive disorders in the world that causes a notable disease burden in middle-aged and elderly population (Turkiewicz *et al.* 2014; Jin *et al.* 2020; Allen *et al.* 2021). The incidence of OA rises precipitously with age and tends to be higher in females, especially after the age of 50 years (Cui *et al.* 2020; Prieto-Alhambra *et al.* 2014). However, the estimated OA prevalence varies depending on the definition (radiographic, symptomatic, etc.) and severity used to categorize the disease (Bedson & Croft, 2008), as well as the characteristics of study population taken into consideration (age, sex, ethnicity, genetics, diet, overweight/obesity, smoking, physical activity, and joint injuries) (Hunter & Bierma-Zeinstra, 2019), for example, in the Framingham Osteoarthritis Study, the prevalence of radiographic OA found elevated from 33% among the subjects aged 60–70 years to 44% among those aged more than 80 years (Felson *et al.* 1987). The prevalence of OA in Estonia as estimated in the year 2019 is 150 800 cases (12% of all causes), including the prevalence of kOA (88100 cases, 7% of all causes) (Vos *et al.* 2020).

There is limited data on the prevalence of kOA in a population younger than 50 years. In a Dutch study, rkOA (grade 2 or higher) was found in 8% of men and 12.5% of females aged 45–49 years (van Saase *et al.* 1989). Similarly, chronic knee pain (> 3 months) was reported by 15% of people aged 35–54 years in Sweden, however, rkOA was diagnosed in only about 10% of them (1.5% prevalence in the age group) (Petersson *et al.* 1997). A small population study in South Estonia demonstrated that knee problems are common among the Estonian middle-aged population (34–55 years of age). 60% of the participants reported knee pain and 40% other knee symptoms. However, early rkOA (grade 1 according to the Nottingham system (NSy) (Nagaosa *et al.* 2000)) was found in 55.6% and grade 2 or 3 in 8.1% of the subjects, resulting in the prevalence of 46% and 3.7%, respectively (Tamm *et al.* 2008b).

Functionally, the knee includes 2 articulations – tibiofemoral (TF) and patellofemoral (PF) joints which share the common capsule, making the knee the largest synovial joint (Brandt *et al.* 2003; Flandry & Hommel, 2011). Although the medial TF compartment is most frequently disturbed, OA is more common in PF joint and occurs even in the absence of the disease in TF joint (McAlindon *et al.* 1992). The meta-analysis of PF OA revealed that overall crude prevalence of OA in PF joint was 25% in the population-based cohorts (aged ≥20 years) and 39% in the symptom-based cohorts (aged ≥30 years) (Kobayashi *et al.* 2016). Unlike to the population-based cohorts, women appeared to have a higher prevalence of PF OA (41%) than men (23%) in the symptom-based cohorts. The prevalence of PF OA classified by osteophytes was 48%. A study on the Estonian middle-aged people population-based cohort (438 participants: aged 35–57 years) (Kerna *et al.* 2013) was comprised in this meta-analysis and reported the lowest prevalence estimate (8%) (Kobayashi *et*

al. 2016). Younger cohort could explain the lower prevalence of kOA in the Northern European studies.

The kOA could be unilateral or bilateral, with or without multi-joint involvement (Sharma, 2021). Established and symptomatic kOA is often radiographically bilateral (Ledingham *et al.* 1993; Bihlet *et al.* 2019). The analysis of a 12-year prospective cohort study revealed that 26% of patients had bilateral disease at baseline, whereas this percentage was found increased to 52% at the 5^{th} year and 70% at the 12^{th} year follow-up (Metcalfe *et al.* 2012). The most common pattern was an involvement of medial compartment in both knees (Metcalfe *et al.* 2012). Unilateral and isolated medial TF kOA was more common in men (Ledingham *et al.* 1993). The examination of both knees (and not restricted to only the symptomatic knee) is always indicated, and the different conditions of knees could influence the results of biomarkers.

Involvement of multiple joints in OA is frequent (Nelson *et al.* 2014; Kraus *et al.* 2007). Generalized or multiple joint OA (MJOA), in particular MJOA of hand joints, is more prevalent in women than men (Nelson *et al.* 2014). However, men as compared to women are more likely to have lumbosacral spine OA as reported (Nelson *et al.* 2013). A higher frequency of MJOA or elevated risk of MJOA progression is associated with age (Nelson *et al.* 2014).

Therefore, the analyses of kOA biomarkers should take into account the different knee compartments and possible MJOA.

2.2. Pathogenesis of OA

OA is a heterogeneous disease that results from a combination of mechanical, inflammatory, genetic, and metabolic factors (Johnson & Hunter, 2014; Hunter & Bierma-Zeinstra, 2019). This multifactorial pathogenesis involves a wide range of underlying pathways leading to a common final pathway of joint destruction (Martel-Pelletier *et al.* 2016; Deveza & Loeser, 2018). OA is previously referred to as passive degenerative cartilage-limited disease or so-called 'wear-and-tear' disease (Loeser *et al.* 2012). However, the modern concept describes OA as an active dynamic alteration arising from an imbalance between the repair and destruction of joint tissues (Hunter & Bierma-Zeinstra, 2019). Initially considered cartilage-driven, OA is a much more complex disease with different inflammatory and tissue mediators released by the joint tissues (cartilage, bone, synovium, etc.; Fig. 1) (Berenbaum *et al.* 2017).

There is now a wealth of evidence that inflammation plays an important role in OA pathogenesis (Scanzello, 2017) and the involvement of all branches of immune system is demonstrated (Haseeb & Haqqi, 2013). Inflammation can be triggered within the joint because of tissue damage and stress responses, and obesity-related systemic inflammation might enhance these local responses. Generated low-grade inflammation disturbs tightly regulated anabolic and catabolic processes responsible for the maintenance of cartilage homeostasis (Wojdasiewicz *et al.* 2014).

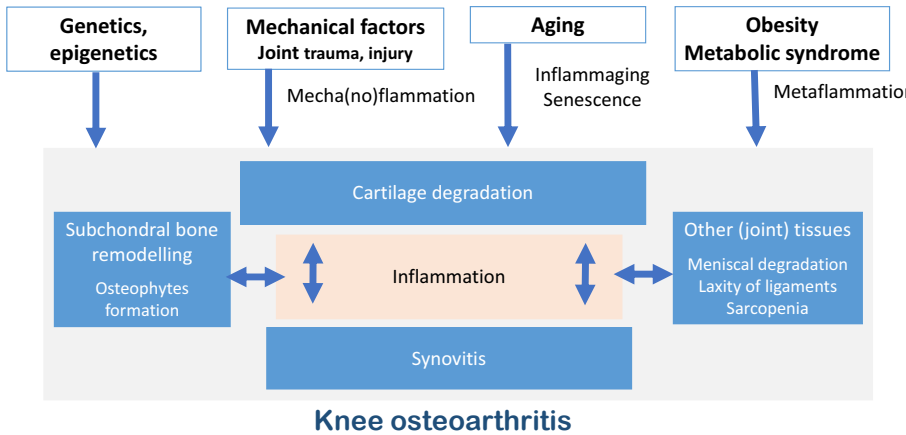


Figure 1. Pathogenic interactions involving different joint tissues in knee osteoarthritis.

Dysregulation of several cytokines and chemokines has been described in the blood and joint tissues of OA patients (Haseeb & Haqqi, 2013; Primorac *et al.* 2020; Endres *et al.* 2020). Interleukin-1 beta (IL-1β) and tumor necrosis factor alpha (TNF-α) are the two major synergistically acting pro-inflammatory cytokines in OA causing degradation of the cartilage (Haseeb & Haqqi, 2013). They are also involved in pain generation. IL-1 and TNF-α upregulate the expression of inducible nitric oxide synthase (iNOS), soluble phospholipase A2, cyclooxygenase 2 (COX-2), and microsomal prostaglandin E synthase 1, and also stimulate the release of nitric oxide (NO) and prostaglandin E2 (PGE2) (Kapoor *et al.* 2011). Moreover, these cytokines increase the expression of metalloproteases (collagenases and aggrecanases) in chondrocytes and synovial fibroblasts and inhibit the synthesis of proteoglycan and Col2 in chondrocytes (Saklatvala, 1986; Goldring *et al.* 1988; Kobayashi *et al.* 2005; Fan *et al.* 2005). In response to IL-1β and TNF-α, the joint cells release IL-6, which leads to amplifying the catabolic effect (Goldring & Goldring, 2007; Mabey & Honsawek, 2015). In OA, also the other pro-inflammatory cytokines, including IL-15, IL-17, IL-18, IL-21, and leukaemia inhibitory factor (LIF) has been implicated (Kapoor *et al.* 2011).

Several chemokines are also involved in the OA pathology and were reported to be expressed in synovium or chondrocytes including IL-8/ chemokine (C-X-C motif) ligand 8 (CXCL-8), growth-regulated oncogene α (GROα)/ CXCL-1, monocyte chemoattractant protein-1 (MCP-1)/ chemokine (CC-motif) ligand 2 (CCL-2), regulated upon activation, normal T cell expressed and presumably secreted (RANTES)/CCL-5, macrophage inflammatory protein-1α (MIP-1α)/CCL-3, and MIP-1β/CCL-4 (Borzi et al. 1999). Chondrocytes also express chemokine receptors including C-X-C chemokine receptor 3 (CXCR3), CXCR4, CXCR5, CC chemokine receptor 1 (CCR1), CCR3, CCR5, and CCR6

(Houard et al. 2013). Pro-inflammatory cytokine IL-1β was reported to stimulate the expression of the chemokines in OA chondrocytes (Akhtar & Haqqi, 2011). In addition, several pro-inflammatory cytokines and adipokines (like leptin and adiponectin) are produced by adipocytes and tissue-infiltrating macrophages in overweight people (Lago *et al.* 2007). Recently, it was shown in the Estonian cohort that an association between angiogenic cytokines and kOA severity exists since a very early phase of the disease. Interestingly, this important pathogenic aspect was predominantly expressed in females (Kisand *et al.* 2018).

2.2.1. Role of obesity and meta-inflammation

Obesity is one of the most clinically significant and modifiable risk factors for OA (Felson *et al.* 1997; Muthuri *et al.* 2011). The mechanisms by which obesity leads to the onset and progression of OA are debatable due to the complex interactions among metabolic, biomechanical, and inflammatory factors that accompany overweight and adiposity (Collins *et al.* 2021).

Traditionally, it has been suggested that weight gain predisposes people to OA simply because of mechanical loading. However, obesity-associated metabolic factors have been found to be associated with a higher incidence of OA in non-weight bearing joints as well (Yusuf *et al.* 2010; Gandhi *et al.* 2012; Visser *et al.* 2014). Therefore, obesity and overweight are now regarded as low-grade systemic inflammatory states, named as 'metaflammation' with elevated inflammatory markers (Lago *et al.* 2007; Choi *et al.* 2013).

During obesity, tissue macrophages adopt a metabolically activated phenotype in response to the altered metabolic environment of white adipose tissue (WAT) in this setting, particularly to an increased concentration of free fatty acids, and high insulin and glucose concentration during an emerging state of insulin resistance. WAT is considered as an endocrine organ secreting a large variety of peptides named as 'adipokines', including leptin, visfatin, adiponectin, resistin, and others (Wang *et al.* 2015). Adipokines can also be produced by the joint cells including chondrocytes when induced by inflammatory stimuli (Conde *et al.* 2011). Leptin seems to be a possible link between obesity and OA; high leptin levels are found in the synovial fluid of obese patients. Also, leptin receptor is expressed in cartilage and its sensitivity was found enhanced. Leptin induces the production of matrix metalloproteinases (MMPs), pro-inflammatory mediators, and nitric oxide (NO) in chondrocytes (Vuolteenaho *et al.* 2014). A recent study on lipodystrophic mice demonstrated a direct relationship between adipose tissue and cartilage damage, independent of the mechanical aspect of joint loading – implantation of fat tissue to fat-free animals restores the susceptibility to OA (Collins *et al.* 2021).

In addition to WAT, an infrapatellar (Hoffa) fat pad (IPFP) may act as a modulator in OA (X. Wang *et al.* 2015). IPFP is a very sensitive tissue composed of adipocytes, immune cells (primarily macrophages and lymphocytes), fibroblasts, blood vessels, and collagen matrix (Tu *et al.* 2019). Thus, IPFP

serves both as a local source of adipokine and a local modulator of inflammatory responses contributing to initiation and progression of knee OA (Belluzzi *et al.* 2017). IPFP-derived adipokines exhibit unique patterns of secretion and distribution, imparting a direct impact on articular cartilage degeneration (Richter *et al.* 2015). For instance, except leptin and resistin, the other adipokines are more actively secreted by IPFP than by subcutaneous WAT in the same OA individual (Ioan-Facsinay & Kloppenburg, 2013).

Obesity also has an effect on synovium and it causes synovial adipocyte hypertrophy, macrophage accumulation, fibrosis, and increased expression of TNF-α and toll-like receptor 4 (TLR4) (Hamada *et al.* 2016; Harasymowicz *et al.* 2017; Eymard *et al.* 2017) supporting innate immune signalling via TLR4 in obesity-induced OA (Kalaitzoglou *et al.* 2019). Some levels of lipopolysaccharides (LPS) are routinely detectable even in the absence of infection, presumably due to disturbances in the gut microbiome of obese patients. However, whether the relationship between LPS and OA severity is directly mediated by TLR4 interaction is unclear (Scanzello, 2017). A number of questions remain about which factors are associated with obesity and directly modulate synovial inflammation. Studies indicate that multiple factors could be involved, including synovial insulin resistance (Hamada *et al.* 2016), dietary fatty acid composition (Sekar *et al.* 2017), and gut microbiome composition, which is related to circulating LPS levels, body fat percentage, fat cell apoptosis, and OA manifestations (Collins *et al.* 2015). To sum up the aspects of fatty tissue, obesity is an important confounding factor in OA biomarker research and should be taken into account in the analysis of clinical groups.

2.2.2. Role of biomechanics

High risk of OA after knee injury demonstrates a crucial role of biomechanical factors in the initiation of the disease in susceptible individuals (Englund, 2010). Although all joint tissues are affected to some degree as a result of joint trauma, injury of articular cartilage appears substantial, as it is largely irreversible and may initiate a subsequent development of OA. The injury drives active mechanosensitive intracellular signalling which modulates the biochemical activity of chondrocytes by the process called ‘mechanotransduction’ (Primorac *et al.* 2020). Two principal pathways are proposed – the one that can lead to the release of growth factors from the matrix and stimulate repair; the another can trigger an inflammatory response to mechanical injury, referred also as ‘mechanoflammation’ (Vincent, 2019). The upstream activator of mechanoflammation remains unknown, but it involves the activation of several transcription factors controlling the expression of target genes such as *MMP13*, *NOS2*, *COX2*, *ADAMTS*, and *IL1B* (Houard *et al.* 2013). Repair-promoting pathways appear to be largely driven by the release of growth factors such as transforming growth factor β (TGFβ) and fibroblast growth factor 2. Both the factors are sequestered in the pericellular matrix of cartilage and released immediately in response to injury (Vincent, 2013).

A precise relationship between mechanoflammation and cartilage repair is currently unclear but it is likely that chronic mechanoflammation contributes to the disease by suppressing intrinsic tissue repair (Vincent, 2019) and changing a balance between pro-degenerative and pro-repair pathways.

2.2.3. Role of aging

Aging of the joint tissues increases the incidence of OA substantially but aging and OA are two independent processes (Loeser *et al.* 2016). While OA is not an inevitable consequence of aging, aging-related changes in the joint tissues contribute to OA development along with some other risk factors (Anderson & Loeser, 2010). Aging is characterized by changes in metabolic and mitotic activity and decreased sensitivity of chondrocytes to growth factors such as TGF-β signalling (van der Kraan & van den Berg, 2008). Common biological changes seen in senescence are genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, deregulated nutrient-sensing, mitochondrial dysfunction, cellular senescence associated with cell cycle arrest, and unresponsiveness to mitogenic stimuli, stem cell exhaustion, and altered intercellular communication (López-Otín *et al.* 2013). 'Chondrosenescence' is defined as an age-dependent deterioration of chondrocyte function (Mobasheri *et al.* 2015). Aging-related changes in the cartilage matrix, including formation of advanced glycation end-products (AGEs) and development of the senescence-associated secretory phenotype (SAPS) of chondrocytes with the production of plethora of soluble signalling factors (inflammatory cytokines and chemokines, growth and angiogenic factors, proteases, bioactive lipids, matrix metalloproteinases) are considered to be involved in OA pathogenesis (Anderson & Loeser, 2010). Aging and inflammation are major contributing factors to the development and progression of arthritic diseases (Mobasheri *et al.* 2015). 'Inflammaging' is a term for describing low-grade chronic inflammation that occurs during physiological aging as a consequence of the adaption and counteraction to the different stressors, i.e, lifelong antigenic burden (Franceschi & Bonafè, 2003). An increase in fat mass, aging-related metabolic changes, and epigenetic regulation of age-dependent gene expression can also result in inflammaging (Loeser *et al.* 2016; M. Zhang *et al.* 2019). This condition is suggested to be a result of an imbalance between inflammatory and anti-inflammatory networks (Franceschi *et al.* 2007). In chondrosenescence a proliferative and synthetic capacity of cells decreases, while a production of pro-inflammatory mediators and matrix-degrading enzymes is maintained (Loeser, 2009). Inflammaging and chondrosenescence are intimately linked with each other in the osteoarthritic joint contributing to a decrease in the efficacy of articular cartilage repair (Mobasheri *et al.* 2015). Non-enzymatic crosslinking of collagen by AGEs that occurs with aging alters mechanical properties of cartilage, and the resulting changes to mechanotransduction pathways reduce extracellular matrix synthesis by chondrocytes (Loeser *et al.* 2016). With normal joint aging, articular cartilage remains intact but loses its

thickness and glycosaminoglycan (GAG) content, however in OA, fibrillation of the cartilage surface occurs in focal areas and can be associated with a complete loss of GAG (Lotz & Loeser, 2012).

In conclusion, several hallmarks of senescence are associated with OA. However, it remains uncertain that which factors and mechanisms contribute to the distinct OA phenotypes and in particular to the disease progression (Coryell *et al.* 2021).

2.3. Pathological changes in the joint tissues

OA is a whole joint disease, involving structural alterations in the hyaline articular cartilage, subchondral bone, ligaments, capsule, synovium, and periarticular muscles (Hunter & Bierma-Zeinstra, 2019; Martel-Pelletier *et al.* 2016). Despite the multifactorial nature of OA, the pathologic changes seen in osteoarthritic joints have common features that affect the entire joint structure, resulting in pain, deformity, and loss of function (Loeser *et al.* 2012).

2.3.1. Articular cartilage

Normal articular cartilage is avascular, alymphatic, and aneural connective tissue which is composed of chondrocytes and extracellular matrix (ECM) (Martel-Pelletier *et al.* 2008). ECM consists of water (65–80 % of the wet mass), inorganic salts, and organic components in which collagens (15–22% of the cartilage wet weight) and proteoglycans (aggrecans) are the main macromolecules (Martel-Pelletier *et al.* 2008; Xia *et al.* 2014). All cartilage components and proteolytic enzymes are synthesized by chondrocytes, providing a minimal and balanced cartilage turnover between anabolic and catabolic processes during the life (Goldring & Marcu, 2009). In recent years, it has been reported that mature articular cartilage contains a small population of mesenchymal stem cells (MSC)-like progenitors that are capable of differentiating into mature chondrocytes. Furthermore, these cells exist in greater numbers in OA cartilage than in normal cartilage (Jayasuriya *et al.* 2018).

The unique properties of cartilage are related to the composition and structure of its components, which are highly ordered from the cartilage surface to its deepest layers. Cartilage consists of four zones with different architecture and functions – superficial, middle, deep, and calcified cartilage zones (Martel-Pelletier *et al.* 2008). The calcified zone is separated from the unmineralized upper cartilage layers by a histologically defined zone called ‘tidemark’, which separates the cartilage from the underlying subchondral bone (Martel-Pelletier *et al.* 2016; Goldring & Goldring, 2016). The layers are characterized by chondrocyte shape and positioning, as well as collagen fibrils’ orientation (Korhonen *et al.* 2008).

Collagen fibrils compose a network providing a shape and form that provides tensile stiffness and strength to the cartilage. Fibril diameters vary from

20 nm in the superficial zone to 70–120 nm in the deep zone. Col2(90–98% of the total tissue collagen) is specific to cartilage (Martel-Pelletier *et al.* 2008). Majority of the cartilage proteoglycans are in complex supramolecular aggregates (aggregans), which consist of a centrally placed/ satiated hyaluronic acid (HA) filament and multiple monomers are attached to it. The monomers are made up of negatively charged GAG molecules (polymeric saccharides chondroitin-4-sulfate, chondroitin-6-sulfate, and keratan sulfate) bound to the aggrecan core protein (Goldring & Goldring, 2016). Aggregans, together with other matrix components, are entrapped in a collage network. GAG molecules are responsible for osmotic properties of the cartilage matrix.

In OA, the earliest changes in the cartilage appear at the joint surface areas, where mechanical forces, in particular shear stress, are greatest (Andriacchi *et al.* 2004). One of the initial changes is an increase in water content in the superficial zone of articular cartilage, with aggrecan degradation, presenting as a superficial cartilage fibrillation (Pratta *et al.* 2006). As OA advances, a process known as 'matrix swelling' expands to the deep zone (Goldring & Goldring, 2010). Also, chondrocytes become 'activated', characterized by cell proliferation, cluster formation, and increased production of both matrix proteins and matrix-degrading enzymes (Goldring & Marcu, 2009; Houard *et al.* 2013). The matrix-degrading enzymes found in OA joints include members of ADAMTSs (a disintegrin and metalloproteinase with thrombospondin motifs) and MMP families (Loeser, 2006; Troeberg & Nagase, 2012). Matrix degradation in early OA may be due to ADAMTS-5 (aggrecanase) which degrades aggrecan (Stanton *et al.* 2005), followed by increased activity of collagenases, MMP-1, MMP-8, MMP-13 (Loeser *et al.* 2012). MMP-13 (collagenase 3) is highly efficient in the cleavage of Col2 participating in cartilage degradation (Mitchell *et al.* 1996; Houard *et al.* 2013). The collagen fragments (gelatins) can be denatured by further cleavage by gelatinases (MMP-2 and MMP-9) (Martel-Pelletier *et al.* 2008).

Once the collagen network is degraded, it reaches the irreversible state (Loeser *et al.* 2012). Degenerative changes lead to a diminished cartilage thickness and fissuring, which could be observed by arthroscopy. On the other side, transformation in the cellular composition along with the cartilage calcification takes place. This process involves osteochondral angiogenesis, the penetration of calcified cartilage by vascular elements and sensory nerve fibres that extend from the subchondral bone (Walsh *et al.* 2007). These processes could be associated with the development of chronic pain. All these tissue transformations result in duplication of the tidemark and advancement of the calcified cartilage into the deep zone of articular cartilage, leading to local cartilage thinning (Loeser *et al.* 2012). These changes become more pronounced with time, when articular cartilage reaches total destruction, eventually leaving the underlying subchondral bone plate completely exposed (Goldring & Goldring, 2007). The advanced signs of cartilage damage are seen by imaging and arthroscopic methods but identification of early signs and sensitive monitoring of

dynamics can be provided by soluble biomarkers (see section 2.7. OA biomarkers).

2.3.2. Subchondral bone

Subchondral bone is divided into two layers: a plate-like layer of cortical bone beneath the calcified cartilage, also known as a 'subchondral bone plate', and a deeper layer of subchondral trabecular or cancellous bone with bone marrow space (Li *et al.* 2013; Funck-Brentano & Cohen-Solal, 2015). The structure of subchondral bone is mostly dependent on two types of cells —osteoblasts and osteoclasts. Osteoblasts synthesize new bone, while osteoclasts resorb the old (Funck-Brentano & Cohen-Solal, 2015). The structural and functional properties of subchondral bone are defined by a composition of an organic bone matrix and mineral content that represent a dynamic adaptation to biomechanical factors as well as the effects of biochemical factors. A state of bone mineralization is highly dependent on the rate of bone remodeling (Day *et al.* 2001; Donnelly *et al.* 2010). Higher rate of bone remodelling is associated with a state of relative hypomineralization. Subchondral bone adapts its architecture and structure more rapidly than the cartilage in order to respond to the changes in the mechanical environment via cell-mediated processes of modelling (Goldring & Goldring, 2007).

The OA related changes in the volume and density of subchondral bone are caused by previous trauma or excessive load (Frost, 2003). Bone remodelling may be initiated at the sites of local bone damage resulting from excessive repetitive loading. In early OA, alterations of bone turnover initiate a deterioration of subchondral trabecular bone such as bone marrow lesions (BML), cysts, and osteophyte (Oph) formation (Goldring, 2008; Burr & Gallant, 2012). BML represents a micro-damage to subchondral bone and is characterized by marrow fibrosis at various stages of healing, fat necrosis, and a local increase in bone remodelling that results in microfractures of the trabecular bone (Taljanovic *et al.* 2008; Driban *et al.* 2012). As a putative additional skeletal adaptive mechanism, Ophs are formed at the joint margins via endochondral ossification (Loeser *et al.* 2012). Oph formation is also indicated as a result of abnormal healing response of subchondral trabeculae, or blood vessels and nerve fibres' penetration into the degrading cartilage (Gilbertson, 1975). A growth factor TGF-β is involved in the Oph formation (Uchino *et al.* 2000). Although Oph remains controversial regarding their functional role; they may serve to stabilize the joint rather than to contribute to OA progression (van der Kraan & van den Berg, 2007). Anyway, Ophs are simply recognizable features by imaging methods that can help to identify OA progression in the studies of biomarkers´ classification.

Subchondral bone cysts development, a hallmark of the advanced OA, depends on osteoclast-mediated bone resorption, a process initiated by bone damage and necrosis at sites of former BMLs (Crema *et al.* 2010). In established OA, the subchondral bone plate increases in volume and thickness. The

sclerosis of subchondral trabecular bone is a characteristic of terminal OA (Burr & Gallant, 2012).

2.3.3. Synovium

Synovium is a specialized connective tissue that lines diarthrodial joints, surrounds tendons, and forms a lining of bursae and fat pads (Mathiessen & Conaghan, 2017). It is responsible for the maintenance of synovial fluid (SF), which is a source of nutrients for cartilage. SF is also a reservoir for cartilage degrading products and is a valuable material for OA studies (Sellam & Berenbaum, 2010).

Synovial inflammatory infiltrates are found in many OA patients in ultrasound or magnet resonance imagine (MRI) investigations (Loeser *et al.* 2012). Macrophages and T-cells are the most predominant immune cells in OA synovium, whereas mast cells, B cells, and plasma cells are also noticed (Haseeb & Haqqi, 2013). Histological pattern of the synovium in OA patients is characterized by hyperplasia, sub-lining fibrosis, and stromal vascularization (Scanzello & Goldring, 2012). Angiogenesis in the synovium is closely associated with chronic synovitis and is observed at all stages of OA. Synovitis can occur even in early OA. Specific aspects of inflammation, such as a higher number of infiltrating macrophages (Benito *et al.* 2005) and co-location of inflamed synovium and cartilage degradation areas, were also noticed (Ayral *et al.* 2005). However, the prevalence of synovitis increases with the disease progression (Scanzello *et al.* 2011; Krasnokutsky *et al.* 2011), and synovial inflammation is diffuse in the late stage (Ene *et al.* 2015).

2.3.4. Menisci and ligaments

Pathologic changes in the menisci include matrix disruption, fibrillation, cell clusters, calcification, and cell death (Katsuragawa *et al.* 2010; Pauli *et al.* 2011). Degeneration of menisci is initiated within the tissue substance rather than the surface. Tissue fibrillation and disruption are first seen at the inner rim, which spreads into articular surfaces of the meniscus over time and progresses to a total disruption or loss of meniscus tissue mainly in the avascular zone (Pauli *et al.* 2011). Col1 content decreases gradually from the surface to the middle and the deep zone of osteoarthritic meniscus (Sun *et al.* 2012). Unlike Col1, a decrease in Col2 content is severe in the surface zone, and also prominent in the middle and deep zones of osteoarthritic meniscus (Sun *et al.* 2012). In turn, proteoglycan content increase in osteoarthritic menisci, when compared to normal menisci (Sun *et al.* 2012). All these intrameniscal changes correlated with perimeniscal synovitis contributing to the degeneration and reduction in the tensile strength of the meniscus (Grainger *et al.* 2007). The meniscus is rarely able to withstand loading and force transmission during normal movements of the joint, further leading to degenerative tear in it (Bhattacharyya *et al.* 2003). Moreover, a meniscal tear can be considered as the

first feature of emerging OA (Englund *et al.* 2012). An increased vascular penetration accompanied by increased sensory nerve densities has been noted in OA menisci and could be associated with pain (Ashraf *et al.* 2011).

Degenerative changes are commonly seen in the ligaments in the late stage of OA. Similar to the meniscus, histologic changes include matrix disruption, collagen fibres disorganization, and mucoid degeneration (Hasegawa *et al.* 2012).

2.3.5. Cross-talk between the joint tissues in OA

Synovial joint acts as a functional unit, where all parts support each other (Goldring & Goldring, 2016). The synovium and subchondral bone help chondrocytes with nutrition, as articular cartilage has no direct supply (Scanzello & Goldring, 2012). Because of relatively slower turnover rate (as compared to articular cartilage), subchondral bone undergoes more rapid modelling and remodelling to respond to the changes in the mechanical environment (Goldring, 2012). Furthermore, in the knee joint, meniscal lesions and injuries decrease resistance to mechanical forces, often leading to the structural progression of the disease (Englund *et al.* 2012). A molecular crosstalk between cartilage and bone increases with OA progression and newly formed vessels together with nerves infiltrate the subchondral bone and invade the overlying cartilage tissue, creating a communication channel for an exchange of biologic factors such as cytokines (Funck-Brentano & Cohen-Solal, 2015).

A fact remains controversial if initial structural and composition alterations related to OA, first take place in the bone or articular cartilage (Goldring, 2012). The study by Yang *et al.* involving a rat model found that the changes in the subchondral bone precedes cartilage degeneration, while cartilage changes were noted before the subchondral bone changes in the collagenases induced model (Yang *et al.* 2020). It is also unclear whether the morphological changes that occur in osteoarthritic synovial membrane are primary or secondary (Sutton *et al.* 2009). Synovitis is believed to be induced at first by the ECM degradation products and soluble cartilage-specific neo-antigens, as well as other factors including microcrystals and abnormal mechanical stress (Sellam & Berenbaum, 2010). These components are released into synovial fluid and phagocyted by synovial lining macrophages, triggering an immune response with the synthesis of mediators. The whole process creates a vicious circle, with increased cartilage degradation, subsequently producing more inflammation and pain (Berenbaum, 2013). Although inflammation may not be the initiator of disease, at some point it becomes a driver of disease progression.

2.4. Phenotypes of OA

OA is well accepted as a multifaceted disease and of heterogeneous nature; therefore, it can be considered a complex syndrome rather than a single disease (Deveza & Loeser, 2018; van Spil *et al.* 2020). However, there are many

discrepancies about OA phenotypes as they could be created for different purposes, such as treatment choice or prognosis prediction (Bierma-Zeinstra & van Middelkoop, 2017). Another proposal is to differentiate clinical phenotypes and molecular/mechanistic endotypes (Mobasheri *et al.* 2019). However, the task is rather complicated as the same patient may have overlapping phenotypes and endotypes of OA (Mobasheri *et al.* 2019a; Mobasheri *et al.* 2019b). Clinical phenotype could be defined as a subgroup of patients with similar clinical characteristics (Mobasheri *et al.* 2019a; Dell'Isola *et al.* 2016). The systematic review by Dell'Isola *et al.* (2016) proposed six clinical phenotypes of OA as – inflammatory, metabolic syndrome, bone and cartilage metabolism, malaligned biomechanical, minimal joint disease, and chronic pain (Dell'Isola *et al.* 2016). Molecular endotypes congregate cases with distinct pathophysiological mechanisms and/or molecular signalling pathways (Mobasheri *et al.* 2019a). Also, different mechanistic osteoarthritis endotypes have been described, for example, inflammatory (local and systemic), metabolic syndrome driven, aging driven, mechanical injury driven, endocrine (oestrogen deficiency), etc. (Mobasheri *et al.* 2019b; Henrotin, 2021). Recently, in an international project the provision of consensus-based definitions and recommendations was proposed (van Spil *et al.* 2020). In this project, OA phenotypes were defined as subtypes of OA that share distinct underlying pathobiological, pain mechanisms, and their structural and functional consequences. OA phenotypes are a very promising but challenging area of research that should determine the underlying mechanisms and discover a combination of sensitive biomarkers for differentiation of distinct OA phenotypes already in the molecular stage of disease (Mobasheri *et al.* 2019a; Van Spil *et al.* 2019).

2.5. Classification criteria of kOA

The most often used criteria for established kOA are the American College of Rheumatology (ACR) criteria for the classification of OA (Altman *et al.* 1986) and the European League against Rheumatism (EULAR) recommendations for diagnosis (W. Zhang *et al.* 2010). Both the criteria define kOA by a combination of clinical symptoms and radiographic findings. It is important because of the discordance between symptoms and radiography, although these discrepancies decrease with more severe radiographic disease (Duncan *et al.* 2007). Classification criteria aim for differentiating kOA patients from those with other arthritic diseases to achieve a homogeneous kOA patient group for research to assess the effect of novel treatment or appropriateness of biomarkers (Aggarwal *et al.* 2015).

Due to the frequent discrepancies between symptoms and radiographic findings in kOA, a combined use of both, imaging and clinical findings in the diagnosis of kOA is justified to achieve higher specificity for kOA and to get uniform kOA severity groups (Anderson & Loeser, 2010; Kraus *et al.* 2011). Moreover, imaging features and patient-reported outcome measures (PROMs)

are proposed as reliable outcome measures for OA patients, wherein: PROMs are used for the assessment of knee complaints and physical function, while imaging can be used for the detection of the structural progression of kOA (Emery *et al.* 2019).

2.5.1. Imaging in the diagnosis of kOA

Radiography (X-ray) is a gold standard for the morphological assessment of OA. Major OA-associated radiographic hallmarks include a progressive loss of articular cartilage and bony features, such as Oph-s, subchondral sclerosis, and subchondral cysts (Altman & Gold, 2007). Joint space narrowing (JSN) is still the only structural end point currently approved by the U.S. Food and Drug Administration (FDA) to demonstrate an efficacy of disease-modifying OA drugs (DMOAD) in phase-III clinical trials (Roemer *et al.* 2014). However, a precise determination of cartilage is not possible by radiography; joint space width (JSW) is an indirect measure, which depends on cartilage thickness and meniscal integrity, extrusion, or subluxation (Gale *et al.* 1999; Hunter *et al.* 2006). Therefore, Oph on knee radiographs are both sensitive (91%) and fairly specific (83%) (Altman *et al.* 1986; Katz *et al.* 2021).

While the knee joint is a multicompartmental, a weight-bearing posteroanterior view to visualize the TF joint and skyline view for axial projection or lateral view for the PF joint is used to confirm all the relevant OA-related radiographic changes which may not be parallel in each compartment (Fig. 2) (Sharma, 2021). However, the skyline view has a better reproducibly assessing PF compartment than the lateral view (Jones *et al.* 1993).

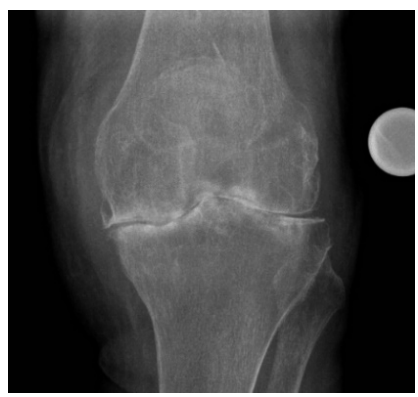
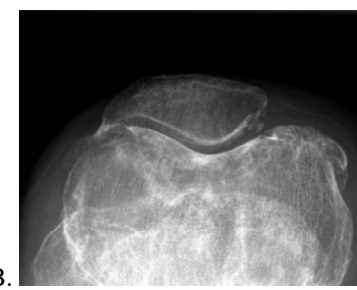
A.  B. 

Figure 2. Knee radiographs for examination of osteoarthritis-related changes (from an unpublished personal archive of professor Aare Märtson). A. Weight-bearing posteroanterior view to visualize the tibiofemoral (TF) joint; B. Skyline view of axial projection of the patellofemoral (PF) joint. Radiographs are from a female (aged 82 years) with radiographic OA grade 4 according to Kellgren and Lawrence (KL) grading system.

Low sensitivity and specificity for the detection of OA-associated cartilage damage, a poor correlation between radiographic findings and symptoms of the disease (e.g. pain), lack of ability to detect synovitis and bone marrow lesions, variations in semi-flexed knee positioning are major limitations of radiography (Bedson & Croft, 2008; Schiphof *et al.* 2008; Roemer *et al.* 2014; Mobasheri *et al.* 2019b). Despite these limitations, radiography remains the most widely used, well accessible, and affordable imaging modality.

To standardize the evaluation of kOA severity, several semi-quantitative imaging-based scoring systems have been worked out (Roemer *et al.* 2014). The most widely used radiographic scoring system to define classification criteria for clinical trials is Kellgren and Lawrence (KL) grading system (Roemer *et al.* 2014; Hayashi *et al.* 2018). This system categorize kOA into five grades (0–4) (Grade 0 = normal; Grade 1 = presence of equivocal osteophyte; Grade 2 = presence of definite osteophyte without joint space narrowing (JSN), Grade 3 = presence of JSN; Grade 4 = complete loss of joint space). KL score 2 or more is traditionally accepted as a definitive criterion for kOA and the inclusion criterion for OA research (Kraus *et al.* 2011).

However, the KL grading has some limitations – the definition of OA is based on the TF joint, and does not estimate the status of the PF joint. Several knees with KL Grade 0 revealed OA features detectable by MRI (Guermazi *et al.* 2012). Although KL grade 1 is not commonly used in the definitive diagnosis of kOA, its inclusion in epidemiologic studies may be useful; this is because, KL 1 had a higher subsequent risk of OA progression than KL grade 0 knees (Hart & Spector, 2003). KL 3 expresses a large scale of JSN severity and is insensitive to longitudinal changes (Felson *et al.* 2011). KL Grade 4 is considered as the end-stage of kOA, but MRI can detect further progression in non-bony structures (Guermazi *et al.* 2015). Because the KL scoring system is based predominantly on the presence of Oph, an atrophic form of OA with JSN may remain underestimated (Kraus *et al.* 2011). Finally, the results of OA assessment of radiographic severity by the KL grading system are relatively variable among observers (Spector *et al.* 1993).

Therefore, complementary radiographic scoring systems for OA were developed. One of the most frequently used is, the OA Research Society International system, published as an OARSI photographic atlas of radiographs (Altman & Gold, 2007). In this scoring system, kOA can be assessed separately on JSN (graded as 0 (none), 1 (mild), 2 (moderate), and 3 (severe), and Oph-s (divided similarly into grades 0–3) both in TF and PF joint. As the severity of osteophytosis and JSN is not proportional (Jones *et al.* 2004), separate grading of the two illustrates the severity and progression of OA in a better way. Intra- and interobserver reproducibility are suitable for clinical practices (Lanyon *et al.* 1998).

However, in radiographs, the grades for JSN and Oph do not strictly increase geometrically, and unusual shapes are found. To overcome these disadvantages, a user-friendly atlas of line drawings for grading principal features of OA has been produced by Nottingham University scientists (Nagaosa *et al.* 2000). Dra-

wings were presented separately for JSN and Oph in the TF and PF compartments and separately for men and women (in the case of JSN). Grade 0 means normal joint widths, grade 1 and 2 were calculated as two-third and one-third of the widths of grade 0, respectively. Grade 3 being bone on bone and accords to the maximum sized osteophyte; grades 1 and 2 are two-thirds and one-third decrease of grade 3 Ophs. Nottingham atlas sustained the reproducibility with OARSI atlas but has more advantages in practical use.

Magnetic resonance imaging (**MRI**) is indicated only in special cases (e.g., in doubt of displaced meniscal tear, tumour) in routine diagnostics of kOA (Katz *et al.* 2021; Sharma, 2021). It is useful for research studies due to its ability to evaluate the knee as a whole organ, while multiple tissues can be visualized simultaneously over several time-points (Roemer *et al.* 2014). MRI can detect changes in other knee tissues such as cartilage, menisci, bone, synovium, capsular structures, and ligaments. Of note, sometimes MRI images may be affected by artifacts that mimic pathological findings (Roemer *et al.* 2014). The technique has its semiquantitative scoring systems, e.g. MRI OA Knee Score (MOAKS) (Hayashi *et al.* 2018). At present, MRI is not recommended as an aid to identify early kOA (EKOA) in routine clinical practice due to the absence of validated consensus criteria and the high frequency of MRI-detected structural joint changes in the population (Luyten *et al.* 2018).

Ultrasound imaging enables the detection of synovial pathology, which is a major advantage over radiography; it can visualize joint effusion, synovitis, Oph, cortical erosive changes, and other features (Katz *et al.* 2021; Roemer *et al.* 2014). It is less expensive and more portable than MRI but not as accurate as MRI in the assessment of JSN (Podlipská *et al.* 2016). Ultrasound is frequently used in imaging of hands in OA research (Hayashi *et al.* 2018), however, our research group has demonstrated valuable additional findings of this imaging technology in EKOA patients (Kumm *et al.* 2009).

Arthroscopy provides a direct visualization and palpation of intra-articular soft tissues (Chu *et al.* 2012). It enables to study radiographically invisible pathologies such as meniscal tears, articular cartilage lesions, and cruciate ligament tears. Moreover, articular cartilage softening, called also chondromalacia, detected by a surgeon through subjective palpation is the earliest detectable clinical sign of pre-OA (Outerbridge, 1961). A modified Outerbridge system for arthroscopy evaluates cartilage damage in five grades (0–4); Grade 0: smooth, firm articular cartilage, Grade 1: articular cartilage is surface-intact, but softened, Grade 2: articular cartilage with a damaged surface <50% of tissue depth, Grade 3: articular cartilage with a damaged surface >50% of tissue depth Grade 4: articular cartilage with full-thickness tissue disruption extending to the subchondral bone.

2.5.2. Patient-reported outcome measures in kOA

Patient-reported outcome measures (PROMs) are preferable methods for a regular assessment of health status and outcomes in OA to follow up the disease dynamics and even the response to treatment (O'Neill *et al.* 2018). A standard set of outcome measures includes joint pain, function, and quality of life scales (Rolfson *et al.* 2016). The most widely used disease-specific questionnaires in kOA are The Western Ontario and McMaster Universities osteoarthritis index (WOMAC) and Knee injury and Osteoarthritis Outcome Score (KOOS) (Bellamy *et al.* 1988; Roos *et al.* 1998). While WOMAC was developed focusing on the elderly people to assess OA status, KOOS was made as an extent of WOMAC for younger and more active patients with knee injuries or kOA (O'Neill *et al.* 2018). WOMAC consists of three domains – pain, stiffness, and physical function (activities of daily living [ADLs]) including 24 items with a recall period previous 48 hours (McAlindon *et al.* 2015). The scoring range in individual domains varies as -pain from 0 to 20, stiffness from 0 to 8, and physical function from 0 to 68. A total score is obtained by the summation of all the three individual scores. A higher score shows worse disease status.

On the other hand, KOOS involves five domains – pain, symptoms, ALD function, Sport and Recreation Function, and quality of life, including 42 items. The self-evaluation covers the past week. In contrast to WOMAC, the total KOOS score should not be calculated. 5-point Likert scale scores are transformed to a 0–100 scale for each domain, with 0 meaning extreme knee problems and 100 representing no knee problems. Similar to WOMAC scores, KOOS scores are influenced significantly by age, sex, and BMI in the general population (Marot *et al.* 2019).

2.6. Course of kOA

Process of OA development can be viewed as a multi-stage continuum from health to joint failure (Kraus *et al.* 2011). Staging of kOA development starts with pre-kOA, when the first molecular biomarkers appear in the absence of symptoms and signs, followed by symptomatic early-stage kOA, established kOA, and finally end-stage kOA (Mahmoudian *et al.* 2021) (Fig. 3). Spectrum of joint tissue damage levels can be divided into molecular, pre-radiographic, and radiographic stages, defined by the techniques' capabilities in distinguishing damaged from healthy tissue (Migliore & Massafra, 2014).

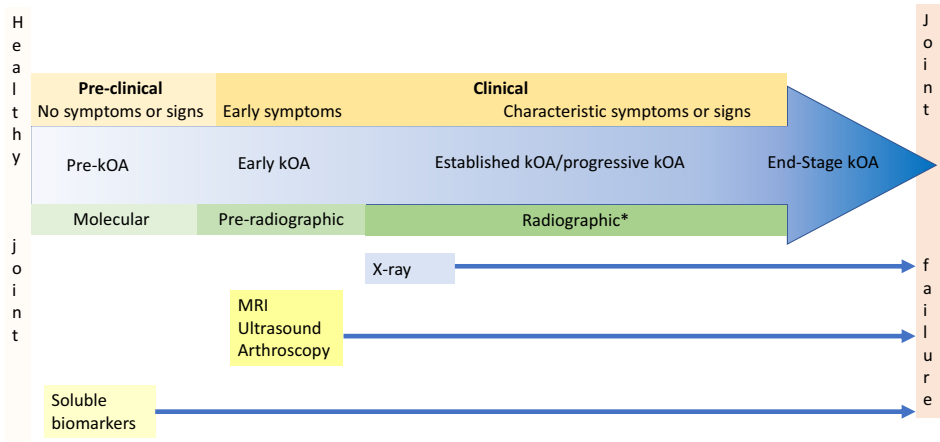


Figure 3. A course of knee osteoarthritis: staging and diagnostic capabilities according to the stage. *Radiographic kOA stage is defined as KL grade ≥2. The figure is inspired by the works of V. Kraus *et al*. (2011), A. Mahmoudian *et al.* (2021), and Z. Lv *et al.* (2021).

2.6.1. Pre-kOA

The novel, pre-kOA stage, was proposed for a characterization of the disease stage where cellular processes have been triggered but no structural changes can be detected by standard MRI, arthroscopy, or X-ray (Ryd *et al.* 2015). The risk factors-related molecules that contribute to a long transition from healthy to early symptomatic kOA, can act as biomarkers for the disease prediction and evaluation (Lv *et al.* 2021).

2.6.2. Early-stage kOA

Although a common understanding about EKOA involves the stage where the first relevant OA findings only emerge, the definite, harmonized definition is not established, and a validated diagnostic criteria are not available for EKOA at present (Mahmoudian *et al.* 2021). The study population in distinct EKOA groups includes either symptoms such as pain and stiffness (the CHECK study), or the presence of risk factors such as a previous knee surgery or injury and overweight (the OAI study) (Wesseling *et al.* 2009). The middle-aged individuals of the Estonian kOA cohort were recruited from the general list of family doctors. The identification of EKOA is thought to be problematic, because the characteristic clinical signs and symptoms of kOA may still be limited and occur sporadic (Luyten *et al.* 2012). The first symptoms and structural changes on imaging can appear often discordant (Roemer *et al.* 2015). The first symptoms could be vague knee pain on bending activities such as using stairs, twisting and pivoting, swelling of the knee, or difficulty to the knee in taking off socks/stockings (Hensor *et al.* 2015). Thus, early symptomatic kOA can be detected with reliable PROMs like KOOS. These prodromal symptoms can last for 2–3 years before the onset of radiographic kOA (Case *et al.*

2015). Patients could also experience stiffness after a period of inactivity (e.g. after awakening), which normally disappears with an exercise for a few minutes, known as 'gelling phenomenon' (Madry *et al.* 2016).

Clinical examination mostly reveals almost normal range of mobilization, joint-line tenderness, crepitus, or mild joint effusion (Mahmoudian *et al.* 2021). Radiographic evaluation is of limited value in EKOA and by the time the first definite Ophs as per the KL classification are visible by radiography, the loss of cartilage reaches more than 10% (Jones *et al.* 2004).

For clinical research studies, the reliable discrimination of the patients with symptomatic EKOA from the patients with knee symptoms due to other pathologies a set of classification criteria is proposed. These consensus-based criteria consist of three classes: (1) pain, symptoms/signs, self-reported function, and quality of life using PROMs (KOOS) (scoring ≤85% in at least 2 out of these 4 categories); (2) clinical examination – at least 1 criterion should be present out of joint line tenderness or crepitus; (3) knee radiographs – Kellgren & Lawrence (KL) grade 0 or 1 (Luyten *et al.* 2018). Biomarkers may have future utility in EKOA classification, but no individual or set of biomarkers is yet robust enough (Luyten *et al.* 2018).

2.6.3. Established kOA and progression of the disease

The next stage, established kOA, is characterized by typical kOA symptoms and/or signs, although a severity of the symptoms and structural damages as assessed on imaging are often discordant (Sharma, 2021). A majority (90%) of the subjects with chronic knee pain but without radiographic changes developed knee OA over a period of 12 years (Thorstensson *et al.* 2009).

Clinical progression was defined as an increase in pain (using scales such as WOMAC or KOOS), worsening in physical function by lower limb performance test results, or knee joint surgery as an outcome (Bastick *et al.* 2015a). Although large variation in definitions to characterize structural worsening of kOA is existing, the studies with structural progression have commonly used two criteria: decrease in joint space width (JSW) or increase in radiographic grade (generally KL grade) (Bastick *et al.* 2015b). However, the definitions using both Oph and JSN provide the most precise estimation of an association between the most known OA risk factors and kOA progression (LaValley *et al.* 2001).

Systematic reviews of kOA prognostic factors found that age, BMI, ethnicity, co-morbidity count, MRI-detected infrapatellar synovitis, joint effusion, and baseline OA severity (both radiographic and clinical) are associated with clinical kOA progression (Bastick, Runhaar, *et al.* 2015); whereas baseline knee pain, presence of Heberden nodes, varus alignment, and high levels of serum hyaluronic acid (sHA) and TNF-α were able to predict radiographic kOA progression (Bastick *et al.* 2015b).

Although OA is generally a slowly progressive disease, there are variabilities in trajectories of symptoms and structural changes, and patients with kOA (Katz *et al.* 2021). While some individuals have a trajectory of slowly worsening

symptoms and experience an intermittent pain over many years, whereas some undergo a rapid disease worsening (Katz *et al.* 2021; Mahmoudian *et al.* 2021). An overview of different trajectories of structural progression is rather complicated because there is no universal definition of kOA progression and drawing the trajectory is somewhat subjective (Collins *et al.* 2021). Felson *at al.* (2013) has proposed a concept that progression of kOA follows a state of inertia – the stable knees tend to remain stable, whereas knees with recent worsening would be expected to continue (Felson *et al.* 2013). However, 8-year longitudinal data in a patient with kOA structural changes, indicated that a stable trajectory is prevalent (over 85%) (Collins *et al.* 2021).

Moreover, at the stage of translation from EKOA to established kOA, a separate assessment of TF and PF joints is recommended. The result of our Estonian population-based cohort of middle-aged subjects presented that the radiographic course of kOA was non-consistent with intermittent periods of progression and stabilization. The follow-up study over 6 years showed that 40% of subjects had no kOA progression but 6% of subjects showed a continuous radiographic progression (Kumm *et al.* 2012). It turned out that the progression rate of radiographic kOA over 9 years was 69%, of which approximately one-third of progression was only in PF joint (Kumm *et al.* 2013a).

Accelerated kOA (AKOA) is defined as a process characterized by a transition between no radiographic kOA to advanced-stage kOA in less than 4 years (Driban *et al.* 2014). Approximately 3.4% of adults and at least 1 in 7 cases of emerging kOA were found to develop AKOA (Driban *et al.* 2014). Two out of three adults that develop AKOA will experience a sudden onset and progression within 12 months. To identify the patients who follow an accelerated track is a high priority in OA research because these patients are expected to experience the best effect of appropriate DMOAD therapy (Collins *et al.* 2021). Importantly, identifying risk factors/prognostic biomarkers for rapid progression could uncover the targets for preventive management (Conaghan *et al.* 2014).

2.6.4. End-stage kOA

End-stage kOA is an advanced stage of the disease with persistent severe pain and functional limitations, accompanied by complications, such as flexion contractures and joint laxity, restricting normal joint function (Driban *et al.* 2016). Radiographically, end-stage kOA generally corresponds to KL grade 4. Currently, as the licensed DMOADs are missing, the OA therapy focuses on pain management and improving disability and quality of life with non-pharmacologic methods such as physical activity/exercise and weight management coupled with self-management strategies (Hawker, 2019). At this stage, TKR surgery is the treatment option if non-surgical management fails to provide any relief (Hunter & Bierma-Zeinstra, 2019). However, TKR is an expensive management. The study by Losina *et al.* (2015) showed that the costs of primary TKR accounted for ~60% of OA-related total direct medical costs (Losina et al. 2015). If current eligibility criteria for TKR will continue, over

50% of symptomatic kOA patients in the US are expected to receive TKR in their lifetimes (Losina *et al.* 2015). Whereas a minor percentage of the typical kOA patient (0.3%) undergo TKR over 8 years, 1 in 7 AKOA cases (14%) are performed TKR during a span of 9-years. More seriously, 7% of AKOA patients may undergo TKR even within 2.3 years after the onset of radiographic progression (Davis *et al.* 2018). However, the expected outcome of TKR is not always achieved and 20–30% of TKR recipients have reported no benefit from TKR one year after surgery (Hawker, 2019). Therefore, a discovery of predictive biomarkers as a part of TKR appropriateness tool to identify the individuals with the best improvement after TKR is urgently needed.

Taking together, this kind of staging of OA would enable a discovery of the most appropriate biomarkers for each stage, and eventually an identification of diagnostic and management algorithms that could fit the right patient in the corresponding stage.

2.7. OA biomarkers: renewed approach

A biomarker is defined by the National Institutes of Health (NIH) Biomarkers Definitions Working Group as "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention" (Biomarkers Definitions Working Group, 2001). There is a clear need for biomarkers that could select a patient for personalized and eventually a safer treatment (Karsdal *et al.* 2014; Bay-Jensen *et al.* 2016). However, it is important to understand that one biomarker may not fit for all purposes and one has to find the appropriate area of use for each biomarker (Kraus & Karsdal, 2021).

Biomarkers can be divided into two major groups- the non-soluble or 'dry' or 'in vivo' biomarkers (patient-reported outcome measures (PROMs), performed tasks, or imaging) and the soluble or 'wet' or 'in vitro' biomarkers, which are usually endogenous molecules measured in a selected body fluid such as blood (serum, plasma), urine, or synovial fluid (SF) (Kraus *et al.* 2011). Biomarker measurement in SF is a more direct study of joint tissue metabolism and structural changes than in serum or urine (Rousseau *et al.* 2021; Mobasheri *et al.* 2017; Elsaid & Chichester, 2006). However, it is not feasible in all patients because sampling involves an invasive procedure, and standardizing protocols for analysis are needed (Rousseau *et al.* 2021). On this background, urine values showed a better correlation with structural changes when compared to serum values (Elsaid & Chichester, 2006).

While OA may be long-time asymptomatic in its early stages, biomarkers that reflect a tissue turnover and preclinical disease activity provide an early warning of the onset of tissue damage, enabling earlier diagnosis of OA (Kraus *et al.* 2011; Kraus *et al.* 2015; Kraus & Karsdal, 2021). These biomarkers may allow earlier management to prevent cartilage and bone destruction that leads to disability (Kraus *et al.* 2011). Instead of commonly used PROMs, the develop-

ment of disease-modifying OA drugs (DMOADs) requires objective endpoints such as measures of joint structure (imaging biomarkers), joint tissue homeostasis (molecular biomarkers), and/or joint survival (joint replacement frequency) (Kraus & Karsdal, 2021).

Imaging biomarkers such as radiographs or MRI may provide a sensitive measure of knee joint status, whereas molecular biomarkers are often produced during the pathophysiological process (Kraus & Karsdal, 2021). Thus, molecular biomarkers, especially neo-epitopes, are sensitive and dynamic markers for joint tissues' turnover, reflecting more disease activity and thereby rate of disease progression, than the current status (Karsdal *et al.* 2010; Siebuhr *et al.* 2014a). They are potentially more useful treatment efficacy markers as compared to conventional imaging methods because of their fast reaction to the pathological changes (Karsdal *et al.* 2019). They have a unique property to detect changes within a few weeks (Rousseau *et al.* 2021). However, the early and end-stage disease may be associated with different activity periods, therefore imaging may not correlate to molecular biomarkers (Kraus & Karsdal, 2021). OA is a slowly progressive disease for which molecular biomarkers could provide a more rapid indication of an active period of the disease and therapeutic response than other biomarkers (Kraus *et al.* 2011). Identification of prognostic markers that predict rapid progression, is necessary for patient stratification, while the preventive management and early interventional treatment are justified to target a high-risk group (Favero *et al.* 2015). In summary, molecular and imaging markers complement each other and their combined utility may be necessary (Dam *et al.* 2009; Kraus & Karsdal, 2021).

Biomarkers require a validation as well as a qualification for their use as a surrogate endpoint (Wagner, 2002). The NIH-funded OA Biomarkers Network in 2006 proposed a classification of molecular biomarkers, named by the acronym BIPED (Bauer *et al.* 2006). This classification scheme includes five categories based on the key parameters of utility – Burden of Disease (B), Investigative (I), Prognostic (P), Efficacy of intervention (E), and Diagnostic (D). In 2011, the OARSI FDA Osteoarthritis Biomarkers Working Group added "S" into the categories, which stands for safety (BIPEDS) (Kraus *et al.* 2011). In 2016, the FDA-NIH Biomarkers Working Group published the BEST (Biomarkers, EndpointS, and other Tools) glossary which includes more detailed descriptions of biomarker functions and surrogate endpoints (FDA-NIH Biomarker Working Group, 2016). While BIPED criteria are more suitable for early biomarker development, the BEST criteria may be valuable at the phase of approval, qualification, or labelling of that biomarker for clinical usage (Mobasheri *et al.* 2017). An overview of both criteria is presented in Table 1.

Many of the existing OA-related biomarkers are associated with radiographic severity, progression, and pharmacodynamics (Bay-Jensen *et al.* 2022). However, biomarkers at the level of *in vitro* diagnostics (IVD) in all categories are lacking (Bay-Jensen *et al.* 2022). A potential use of biomarkers in personalized medicine and their role in monitoring drug efficacy are most promising (Mobasheri *et al.* 2017; Rousseau *et al.* 2021). A very few bio-

markers have been tested to identify and define specific endotypes of OA and should be a focus of future research efforts (Mobasheri *et al.* 2017; Bay-Jensen *et al.* 2022).

Table 1. BIPEDS and BEST biomarker classification criteria (Mobasheri *et al*, 2017).

BIPEDS		BEST	
Diagnostic Biomarker	classifies subjects as – diseased or non-diseased.	**Diagnostic Biomarker**	detects or confirms the presence of a disease or condition or identifies subjects with a subtype of the disease.
Prognostic Biomarker	predicts a future onset of OA among those without OA at baseline or OA progression among those with the existing disease.	**Susceptibility/ Risk Biomarker**	indicates a potential of developing a disease or condition in a subject, who does not currently have a clinically apparent disease or condition.
		Prognostic Biomarker	identifies a likelihood of a clinical event, disease recurrence, or progression in patients who have the disease or condition.
Burden of Disease Biomarker	assesses the severity or extent of disease, typically at a single point in time, among OA subjects.	**Monitoring Biomarker**	is measured serially for assessing the status of a disease or condition or as evidence of exposure to (or effect of) a medical product or an environmental agent.
Efficacy of Intervention Biomarker	provides information about the efficacy of treatment among OA patients or those at high risk of developing OA.	**Pharmaco-dynamic/ Response Biomarker**	shows that a biological response occurred in a subject who has been exposed to a medical product or an environmental agent.
		Predictive Biomarker	identifies subjects who are more likely to experience a favourable effect from exposure to a medical product or an environmental agent than similar subjects without the biomarker.
Safety Biomarker	monitors the health status of the joint tissue or general cytotoxic status in response to treatment.	**Safety Biomarker**	measured before or after exposure to a medical product or an environmental agent, indicates the likelihood, presence, or extent of toxicity as an adverse effect.
Investigative Biomarker	insufficient information to allow inclusion into one of the existing categories.		

2.7.1. Inflammatory markers

Although synovitis may not initiate OA, it may become the driver of the disease at some later phase (Karsdal *et al.* 2014; Kraus *et al.* 2015). Majority of subjects with radiographic kOA have inflammation, including effusion in 70–81% of patients and synovial thickening in 34–50% (Tarhan & Unlu, 2003). The cytokines levels in SF, such as IL-1α, IL-18, and TNF-α, were associated with OA severity grades (Daghestani & Kraus, 2015). Moreover, a baseline level of IL-18 can predict OA progression. Recently, the association of synovial MMP-3, soluble vascular cell adhesion molecule-1 (sVCAM-1), soluble intercellular adhesion molecule-1 (sICAM-1), vascular endothelial growth factor (VEGF), tissue inhibitor of metalloproteinase-1 (TIMP-1), and monocyte chemoattractant protein-1 (MCP-1) with synovial inflammation, clinical symptoms, and radiographic severity of kOA has been shown (Haraden *et al.* 2019).

Identifying systemic biomarkers that can reflect localized joint inflammation in OA is a rather complicated task (Kraus & Karsdal, 2021). C-reactive protein (CRP) is a commonly used inflammatory marker, but it does not specify a location of the tissue (Skjøt-Arkil *et al.* 2012). Erythrocyte sedimentation rate, IL-1β, IL-6, TNF-α, and fibrinogen have the same disadvantages in addition to high variation (Siebuhr *et al.* 2016). Moreover, a meta-analysis of 32 studies on CRP revealed its significant association with OA pain and decreased physical function, but not radiographic OA (X. Jin *et al.* 2015). Interestingly, the higher CRP was associated with greater kOA pain in women, but not in men (Perruccio *et al.* 2017).

Another opportunity to identify a status of joint inflammation is to study the tissue turnover biomarkers, which are influenced by inflammation. MMP-mediated type I and III collagen degradation markers C1M and C3M, the markers of connective tissue destruction, are highly correlated with CRP (Siebuhr *et al.* 2013; Siebuhr *et al.* 2016). CRPM is a metabolite of CRP that is generated in joint tissues by MMP (Siebuhr *et al.* 2014) and serve as a promising marker of multi-joint inflammation (Alexander *et al.* 2021). High level of C1M is positively associated with KL score, CRP, and CRPM, (Siebuhr *et al.* 2014; Alexander *et al.* 2021).

A growing body of evidence indicates that a subset of OA patients with inflammation has the highest risk of progression and symptoms (Daghestani & Kraus, 2015). Although the role of inflammation in OA pathogenesis is strongly recognized, clinical trials with several anti-inflammatory agents have failed (McAlindon *et al.* 2017; Deyle *et al.* 2020). One of the promising ways could be an identification and classification of molecular inflammatory markers for better subdivision of heterogeneous groups of patients into the homogeneous subset of inflammatory phenotype for effective clinical trials testing of anti-inflammatory therapeutics (Lv *et al.* 2021).

2.7.2. Bone remodelling markers

Although alterations in subchondral bone are not evident in all patients with kOA, they can be the earliest pathological changes in a fraction of patients (Hu *et al.* 2021). OA changes in bone occur more rapidly and therefore are recognizable at initial stage as compared to cartilage abnormalities (Goldring & Goldring, 2010). So, early-stage kOA displays a typical activation of bone resorption, since an increased formation and decreased mineralization of subchondral bone occurs in late-stage kOA (Funck-Brentano & Cohen-Solal, 2011).

Type I collagen (Col1) is the major protein in bone and its fragments can be used for the characterization of bone turnover (Siebuhr *et al.* 2014b; Karsdal *et al.* 2019). N-terminal propeptide of type I procollagen (P1NP), the product of posttranslational cleavage of Col1, is a marker of bone formation (Eastell & Szulc, 2017). In the Estonian middle-aged cohort study, the authors reported that higher baseline values of serum P1NP are associated with kOA progression, especially the progression of osteophytosis (Kumm *et al.* 2013c).

C-telopeptide of Col1 (CTX-I) and N-telopeptide of Col1 (NTX-I) are degradation products of Col1 and indicate osteoclast activity (Siebuhr *et al.* 2014b). They can be assessed as markers of bone resorption in serum (s) and urine (u). uNTX-I and uCTX- are significantly increased in the patients with progressive kOA as compared to controls (Bettica *et al.* 2002). CTX-I epitope exists in two forms – non-isomerized form of CTX-Iα is an indicator of newly formed bone, while isomerized CTX-Iβ is a consequence of aging (Huebner *et al.* 2014). Data from the FNIH OA Biomarkers Consortium study showed that time-integrated concentrations over 24 months of sCTX-I, sNTX-I, uNTX-I, uCTX-Iα, and uCTX-Iβ can predict clinically relevant kOA progression (Kraus *et al.* 2017). In summary, bone remodelling markers can reflect high bone turnover suggesting the kOA progression.

2.7.3. Cartilage markers

While Col2 is the main structural component of cartilage, numerous tests of several distinct degradation fragments of Col2 have been developed for non-invasive and objective assessment of OA. In addition to aggrecan which is another main component (next to Col2), cartilage consists of minor collagens and non-collagenous proteins such as cartilage oligomeric matrix protein (COMP), fibulin, cartilage intermediate layer protein (CILP), follistatin-like protein 1 (FSTIL-1), etc. (Karsdal *et al.* 2019; Kumavat *et al.* 2021). These proteins are targeted as OA potential biomarkers in a range of studies.

2.7.3.1. Aggrecans

The structure of aggrecan is extremely complex and multiple cleavage sites create neoepitopes, for example, peptides with amino acid sequences of FFGV (AGNx-2), NITEGE (AGNx-1), and ARGS (Bay-Jensen *et al.* 2022). ARGS is the most robust in this group of biomarkers and commonly considered as an

indicator of cartilage degradation. However, it also reveals cartilage remodelling (Bay-Jensen *et al.* 2022)., ARGS levels in SF are reported to be associated with WOMAC stiffness scores in end-stage OA as well as improved KOOS pain and symptoms scores in knee trauma (Struglics *et al.* 2015; Wasilko *et al.* 2016). Moreover, low serum levels of ARGS were reported to predict fast radiographic kOA progression over 2 years (He *et al.* 2021). Chondroitin sulfate 846 (CS846), the fragment of aggrecan, is considered to be a marker of cartilage turnover, although proposed to use as a marker of aggrecan formation (Mazzuca *et al.* 2006). The potential of CS846 as an independent diagnostic marker of radiographic OA was shown in the OAI-FNIH cohort study (Liem *et al.* 2020a).

2.7.3.2. Cartilage oligomeric protein (COMP)

Meta-analyses confirmed that elevated serum COMP was indicative of kOA severity and predicted the kOA progression (Hoch *et al.* 2011; Hao *et al.* 2019). A high level of serum COMP was detected after traumatic injury (Bjerre-Bastos *et al.* 2021; Bay-Jensen *et al.* 2022). In an Estonian middle-aged cohort study, serum COMP was associated with meniscal changes and progressive osteophytosis in early-stage kOA (Kumm *et al.* 2013b). However, this level was rather associated with clinical parameters of kOA than the radiographic severity in late-stage kOA (Riegger *et al.* 2020). Another study also supported the finding that the COMP level was increased in the early stages but declined in later stages of kOA. The same study demonstrated a positive correlation of COMP with pain and no correlation with radiographic grading (Verma & Dalal, 2013).

2.7.3.3. Type II collagen (Col2)

Col2, a major structural and characteristic protein of articular cartilage is composed of three identical alpha 1 chains, arranged in a triple helix (Poole *et al.* 2003). It creates a meshwork that receives stabilization from other proteins, providing cartilage with a tensile strength (Gly-Jones *et al.* 2015). In normal cartilage, there is a strict regulation of matrix turnover; this means, there is a delicate balance between synthesis and degradation of matrix proteins. In OA, this balance is disturbed; both degradation and synthesis of Col2 are usually increased (Sandell & Aigner, 2001), followed by a loss of articular cartilage matrix. The breakdown of the Col2 meshwork is considered a crucial point in the pathogenesis of OA (Henrotin *et al.* 2007). Proteolytic enzymes, including collagenases and gelatinases, participate in this breakdown process. MMP-13 may be a key enzyme in OA (Abramson & Attur, 2009). A cleavage of the triple helix produces two fragments that are 3/4 and 1/4 the length of the mature Col2 (Aurich *et al.* 2017).

Col2 is a substantial source of epitopes (Henrotin *et al.* 2007). Several potential biomarkers of Col2 fragments that reflect a cartilage turnover are developed and intensively studied (Bay-Jensen et al. 2022). Col2 biomarkers may be divided into four main groups, according to the localization and expres-

sion of epitopes (Henrotin *et al.* 2007; Fig. 4). The first group of epitopes of Col2 propeptide protein fragments that are released during collagen synthesis represents cartilage formation markers (e.g., PIIANP, PRO-C2). The other three groups are markers of cartilage degradation. The second group includes epitopes localized at the telopeptides of Col2 (like uCTX-II). The third group consists of the denaturation epitopes, which are localized in the N-terminal triple-helical region (for example, Coll2-1, Coll2-1-NO2). The final group includes the markers formed by processes of protease cleavage, denoted as cleavage neoepitopes, and localized at the cleavage site (for example, uC2C, C1,2C, TIINE) (Henrotin *et al.* 2007; Karsdal *et al.* 2010).

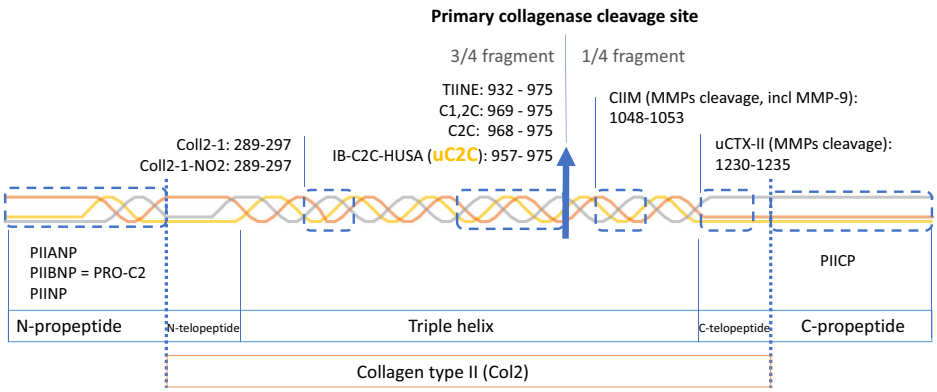


Figure 4. A schematic localization of epitopes of human type II procollagen (UniProtKB n^{o} P02458). The arrow indicates a primary site of cleavage leading to the neoepitopes (The design is based on the work by Rousseau *et al*. 2021).

Biomarkers of type II collagen formation

Serum levels of propeptides at the C- and N-termini (PIICP and PIINP, respectively, Fig. 4) were used for the evaluation of Col2 formation (Rousseau *et al.* 2021). These propeptides are cleaved by specific proteases during a maturation of Col2. Two main splice variants, N-propeptide of collagen IIA (PIIANP), and N-propeptide of collagen IIB (PIIBNP or termed also PRO-C2) are generated and their measurement in serum was developed (Luo et al. 2018), (Bay-Jensen *et al.* 2022). Both PIIANP and PRO-C2 levels were lower in the subjects with established kOA than in the controls (Luo *et al.* 2018). Considering a fact that high serum PIIANP predicts decreased odds of clinically relevant kOA progression PIIANP may own some prognostic value for kOA, (Kraus, Collins, *et al.* 2017). Very recently, a study revealed that low levels of baseline serum PRO-C2 were associated with a higher (3.4-fold) possibility of rkOA progression as compared to high PRO-C2 (Luo *et al.* 2021). Thus, PRO-C2 seems to be an indicator of low cartilage repair endotype in OA (Bay-Jensen *et al.* 2022).

Biomarkers of type II collagen degradation

Urinary C-terminal cross-linked telopeptide of type II collagen (uCTX-II)

uCTX-II, currently one of the most evaluated OA biomarkers, is a neoepitope generated by MMP-9 and MMP-13 (Bay-Jensen *et al.* 2016). Besides the damaged articular cartilage, CTX-II is also present in the tidemark and calcified cartilage at the bone interface (Huebner *et al.* 2014; Bay-Jensen *et al.* 2016). In addition to being a marker of cartilage degradation, uCTX-II is supposed to be a marker of subchondral bone degradation (Spil *et al.* 2013). It is a proven promising diagnostic and prognostic biomarker for OA in multiple clinical studies as summarized in the meta-analyses (Valdes *et al.* 2014; Huang *et al.* 2017). Elevated baseline uCTX-II predicts an increased risk of both symptomatic and radiographic kOA progression over 4 years, as well as a higher risk of undergoing a total knee replacement (TKR) in several OA studies (Kraus, Collins, *et al.* 2017; Garnero *et al.* 2020; Bihlet *et al.* 2020). Levels of CTX-II were reported to be associated with radiographic severity of OA and the number of affected skeletal sites (knees, hip, spine, and hands), and to be able to differentiate between slow and rapid OA progressors (Reijman *et al.* 2004; Meulenbelt *et al.* 2006; Dam *et al.* 2009). A recent meta-analysis demonstrated that uCTX-II levels were higher in the patients with severe kOA than in moderate kOA (Cheng *et al.* 2020). Moreover, this study revealed that uCTX-II had a better diagnostic performance in females than in males. In addition, uCTX-II has been applied in clinical trials for testing the efficacy of DMOADs such as risedronate, oral salmon calcitonin (sCT), and strontium ranelate (Rousseau *et al.* 2021). Despite a lack of visible treatment effect on X-ray progression, an early decline in the levels of uCTX-II was reported along with a significant response to the treatment (Bingham III *et al.* 2006; Karsdal *et al.* 2015; Alexandersen *et al.* 2011). However, prospective studies could show a true role of uCTX-II in evaluating the treatment effect (Rousseau *et al.* 2021).

Coll2-1 and Coll2-1-NO2

The assays for measuring Coll2-1 and its nitrated form, Coll2-1-NO2, were developed as another version of markers of cartilage degradation (Deberg *et al.* 2005). Coll2-1 is susceptible to nitration resulting in the production of Coll2-1-NO2, thereby reflecting an oxidative-related Col2 degradation due to local inflammation (Bay-Jensen *et al.* 2022), (Mobasheri *et al.* 2019). Serum levels of both Coll2-1 and Coll2-1-NO2 were found significantly elevated in OA patients (Deberg *et al.* 2005). Moreover, baseline levels of both markers in urine were associated with the clinical activity of kOA. Furthermore, the one-year increase in uColl2-1 levels was predictive of kOA radiological progression (Deberg *et al.* 2005). A study with the FNIH cohort reported that Coll2-1-NO2 was independently associated with radiographic OA severity (Liem *et al.* 2020b).

Cleavage neoepitopes

A cleavage at the primary cleavage site in Col2 creates a variety of neoepitope that is located on the carboxy (C)-terminus of the ¾ fragment and several assays

were developed to measure these neoeptiopes (Billinghurst *et al.* 1997) (Fig. 4). A 45-mer peptide fragment of Col2 is the most abundant neoepitope peptide in urine (Nemirovskiy *et al.*, 2007). The level of C1,2C, the utmost neoepitope in the C-terminus of the 3/4 fragment, was higher in OA cartilage than in the healthy samples; however, the C1,2C assay was relatively unspecific due to cross-reactivity of Col1 fragments (Billinghurst *et al.* 1997). The type II collagen neoepitope (TIINE) assay targets a Col2 neoepitope upstream of the 3/4 cleavage sites, but monoclonal 9A4 antibodies in TIINE assay can recognize both type I and II C-terminal cleavage neoepitope–containing 45-mer collagen fragments. Nevertheless, uTIINE assay targets a Col2 epitope upstream of the 3/4 cleavage sites and was shown to distinguish between OA patients and healthy subjects, as well as between symptomatic and asymptomatic patients with radiographic OA (Nemirovskiy *et al.* 2007). C2C assay measures an elongated version of C1,2C in serum and urine, using specific monoclonal C2C antibodies. However, the old C2C assay can detect smaller fragments than the dominant 45-mer fragment and has a limited capability in detecting an initiation and progression of OA (Poole *et al.* 2004; Cahue *et al.* 2007; Cibere *et al.* 2009). Therefore, a new C2C assay, denoted also as C2C-HUSA, was designed to detect urinary C-terminal 45-mer fragment and any larger fragments of Col2 that contain both neoepitope C2C and intrachain epitope (Poole *et al.* 2016). Thus, the new uC2C assay is more specific and can identify a subpopulation of fragments that are associated with cartilage degradation. Previously our study group has shown that an increased output of uC2C correlates with knee pain, a decline in the functional abilities of a lower limb, and results of lower limb performance tests (A.O. Tamm *et al.* 2014). A recent study the patients with knee pain showed that uC2C levels were increased in subjects with KL grade 2, a definitive for the radiographic diagnosis of OA. A baseline urinary uC2C level was significantly elevated in kOA progressors in comparison with nonprogressors and was associated with an increased risk of progression of knee cartilage degradation during the next 3 years (Poole *et al.* 2016). The OAI-FNIH study endorsed that uC2C was associated with kOA progression (Kraus *et al.* 2017). However, a role of uC2C as an OA biomarker still remains unclear because of very scarce clinical data. Quite a small number of studies have used a new, more specific uC2C method, IB-C2C-HUSA, and a complete potential of this biomarker as an indicator of distinct aspects of kOA is undiscovered and unconfirmed yet.

2.8. Summary of the literature review

There is a need to a great extent for biomarkers that could support a personalized management in kOA patients to achieve the best outcome at each stage of kOA. Currently, the development of efficient and safe OA treatment faces several important challenges such as a limited understanding of the OA joint biology, a limited biomarker toolbox for characterization of molecular proces-

ses in different joint tissues, and a lack of tools for the faster prediction of disease progression in high-risk individuals, gender differences, etc. Despite the above-described intensive research in the field of OA biomarkers, there are a few very promising candidates and none of the best candidate biomarkers has entered into clinical use as *in vitro* diagnostic tests (IVD). Published reviews underlined a typical shortage – a lack of consistent evidence due to differences in sample collection, implemented diagnostic criteria, investigated populations, and the used methods. Thus, there remains a clear need for more research in the field in terms of validation of existing markers and identifying new candidates applying the BIPED and BEST classifications. Moreover, such research should involve further exploration of the underlying mechanisms of OA. Since a breakdown of Col2, the major structural cartilage protein, is considered a crucial point in the pathogenesis of OA and a substantial source of epitopes, several biomarkers of Col2 fragments have been developed. The assay of uC2C detects the most abundant Col2 cleavage fragments in urine. Thus, uC2C is a potential, yet incompletely studied biomarker of kOA.

3. AIM AND HYPOTHESES

Overall aim:
To evaluate collagen type II C-terminal cleavage neoepitope C2C in urine (uC2C) as a potential biomarker for diagnosis, prognosis, and outcome of knee osteoarthritis (kOA) through its course.

Hypotheses:

I. uC2C reflects an involvement of osteophytes (Ophs) and joint space narrowing (JSN) in the different compartments of the knee joint.
II. uC2C is an early-stage biomarker of radiographic kOA.
III. uC2C is higher in progressors than in non-progressors in the same stage of radiographic kOA.
IV. Total knee replacement (TKR) reduces the level of uC2C in the 12th month after the surgery. The change in uC2C level and the Knee injury and Osteoarthritis Outcome Score (KOOS) scores after the surgery depends on its preoperative level.
V. In presence of kOA, uC2C level can behave differently in men and women.

Specific tasks:

I. To measure uC2C levels in healthy subjects (Paper I), early-grade (pre-radiographic) kOA (Paper I), advanced kOA (Paper I, III), and to specify associations of uC2C with Ophs and JSN in the tibiofemoral (TF) and the patellofemoral (PF) joint (Paper I).
II. To compare uC2C baseline values in different groups – subjects with emerging of kOA and without emerging of kOA in 3 years and controls for a prediction of onset of kOA (Paper II).
III. To compare uC2C baseline values at distinct kOA stages in progressors and non-progressors of the disease (Paper II).
IV. To assess preoperative uC2C and dynamics of uC2C levels after TKR during 12 months period (Paper III).
V. To compare uC2C baseline values in the subjects with and without postoperative improvement in KOOS scores (in12 months) for a prediction of a subjective outcome of TKR (Paper III).
VI. To compare the results of the above-mentioned tasks in different genders (Paper I–III).

4. MATERIALS AND METHODS

4.1. Study subjects

We used the subsets from three different study cohorts: Estonian Early Knee OA Study, The Arthroscopy, and Total Knee Replacement Cohort. First two cohorts consisted of a middle-aged population; this age-group could presumably be in an early stage of kOA and can provide an opportunity for a better description of soluble biomarkers to find high-risk persons for kOA and to ensure the expected DMOAD treatment outcome for the future.

A. Estonian Early Knee OA Study Cohort. A population-based cohort was recruited by three general practitioners (GP) in southern Estonia (Kumm 2012, Kerna 2013). The relevant questionnaire was sent to randomly selected 1793 subjects and the response was received from 964 of them (54%). Out of 964 responders, 506 reported kOA complaints (knee pain in 65%, stiffness, and crepitus in 35%), and the remaining 458 had no kOA problems. Out of the 964 responders, 475 (67% females) agreed to in-depth investigations. Among them, 308 study patients indicated some subjective knee complaints, while the remaining 167 were without any complaints. Two subgroups were formed as follows:
    a. *Longitudinal group* (n=388; 69% females). In addition to the subjects with knee complaints, this group consisted of knee complaints-free subjects with radiographic kOA signs or KOOS scores <85%. They were examined at least two different time points, at baseline and three years after. In 62 subjects, some data were missing and were excluded from futher analysis.
    b. *Long-term control group* (n=25; 44% females). Among the study subjects without knee complaints, 35 individuals (57% females) had KOOS ≥85% and no radiographic signs of kOA at baseline. After 12 years of follow-up of these persons, 25 subjects did not develop radiographic signs of kOA and they formed a long-term control group.
B. The Arthroscopy Cohort consisted of 109 patients (50% females), who were examined and treated during this visit at the Department of Traumatology and Orthopedics, Tartu University Hospital (Estonia) in 2007−2010. This cohort consistently recruited all subjects aged <65 years, who were indicated for arthroscopic surgery due to chronic knee complaints (duration – several months to years). Men and women were recruited separately; therefore, the gender groups were similar in size. The types of knee impairments were different in the arthroscopy cohort but the clinical data were available only for 71 cases. Twenty-six subjects from 71 cases (36%) in the arthroscopy cohort had isolated degeneration of the meniscus, 23% had isolated knee trauma and 41% had a combination of degeneration and knee trauma. 84 subjects agreed

to attend the longitudinal study and were examined at the second time-point three years later.

In both cohorts, demographic, clinical data (including KOOS questionnaire and a visual analog scale for pain (VAS Pain)), and radiographs of both knees were obtained for each subject at every time point. The subjects with radiographic evidence of rheumatoid arthritis or other inflammatory arthropathies in the knees, history of knee arthroplasty, or technically unsuitable radiographs were excluded. The knee injury was not an exclusion criterion.

C. Total Knee Replacement (TKR) Cohort. A prospective study cohort consisted of 105 patients with end-stage kOA (KL 3–4) and undergoing primary unilateral TKR between January 2017 and October 2019 at the Department of Orthopedics, Tartu University Hospital (Estonia). All the subjects under 70 years of age (mostly <65 years) targeted for knee arthroplasty were sequentially enrolled in the TKR cohort, as the progression of kOA was expected to be particularly rapid in this age group. The records of men and women were kept separately so that the groups of men and women were equal.

Paper I. A cross-sectional study involved 302 subjects from the Estonian Early Knee OA Study Cohort and The Arthroscopy Cohort, from whom the urine sample was collected and were available for assessment (Fig. 5). In a small number of subjects from the arthroscopy cohort [n = 14 (5 males and 9 females)], orthopedic surgeons performed a direct visual assessment of knee articular cartilage according to the evaluation system of *Société Francaise d'Arthroscopie* (SFA score).

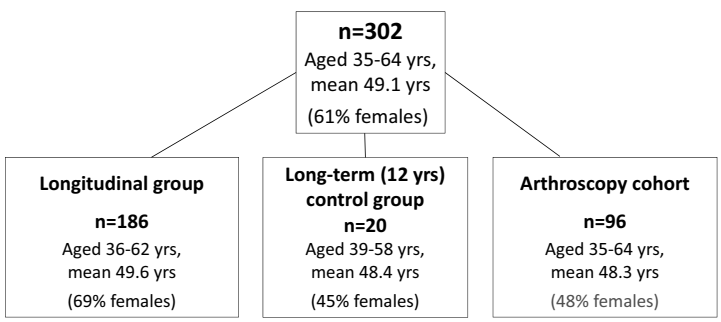


Figure 5. A distribution and characteristics of the subjects from different cohorts in Paper I. Abbreviation: yrs – years.

Paper II. In this longitudinal study, a total of 330 subjects from the Estonian Early Knee OA Study Cohort and The Arthroscopy Cohort (Fig. 6) were investigated at two time points – at baseline (T0) and a follow-up visit three years later (T3) (mean follow-up period 38 ± 5 months). Urine samples were collected and assessed at T0.

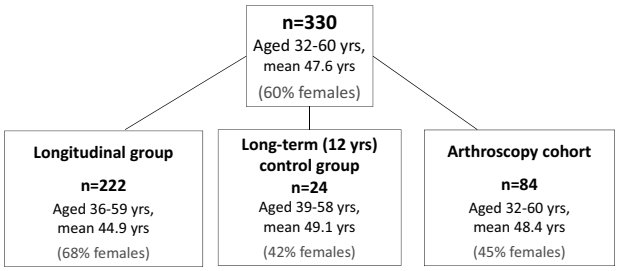


Figure 6. A distribution and characteristics of the subjects from different cohorts in Paper II. Abbreviation: yrs – years.

Paper III. A subset of the Total Knee Replacement (TKR) Cohort consisted of 86 patients. The exclusion criteria were rheumatoid arthritis or other inflammatory arthropathies, history of knee arthroplasty, or technically unsuitable radiographs. Also the patients who had signs of acute infections in the previous three months were excluded. Other exclusion criteria were an evidence of secondary OA, such as trauma, gout, infection, or congenital and developmental disorders affecting the knee joints. Relevant clinical data, including urine samples, were collected at three time points – 1–2 days before TKR and 3 months, and 12 months after TKR (Fig. 7). A clinical status of the subjects was established by PROMs such as KOOS and SF-36 questionnaires and a visual analog scale for pain (VAS Pain), along with performance tests and knee radiographs.

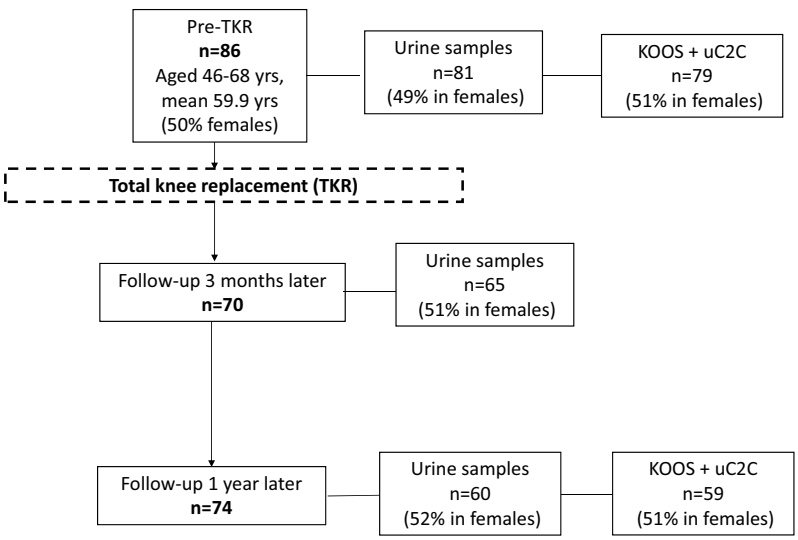


Figure 7. A flowchart that describes the total knee replacement (TKR) study groups at different time points in Paper III. Out of 86 patients at baseline, clinical and radiographic data of 70 patients was obtained 3 months after TKR, while the same data of 74 patients was collected after one year after TKR. Abbreviations: Pre-TKR – 1–2 days before total knee replacement; KOOS – Knee Injury and Osteoarthritis Outcome Score; n – numbers.

4.2. Urinary collagen type-II C-terminal cleavage neoepitope (uC2C) measurement

The second-morning void urine was collected. The collected urine samples were aliquoted and stored at −80 °C on the same day.

The level of uC2C was determined using the IBEX C2C human urine sandwich assay (IB-C2C-HUSA™) by IBEX Pharmaceuticals (IBEX Pharmaceuticals Inc., Montreal, Quebec, Canada) according to the manufacturer's instructions in duplicates (Poole et al. [2016] and https://www.ibex.ca/product-catalog/, accessed on 1 July 2020). In IB-C2C-HUSA™, two different antibodies are used to increase a specificity of the detection of Col2 fragments; capture antibodies are specific for the intrachain epitope with sequence GEPGDDGPS, and HRP-labeled tracer antibodies recognize neoepitope EGPPGPOG on Col2 fragments (Figure 8). The assay detects fragments longer than 20 amino acids including the most abundant 45-mer peptide containing the C2C neoepitope. Intraassay and interassay variations of the method were ≤ 4.8% and ≤ 6.7%, respectively. We corrected C2C concentrations with the concentration of creatinine in the same urine sample for better consideration of a urine dilution factor (A. O. Tamm *et al.* 2014) and the results were expressed in units such as ng/mmol. The creatinine was measured by QuantiChrom™ Creatinine Assay kit (DICT-500; BioAssay Systems, Hayward, USA) in Paper I and II, and by Cobas® Creatinine plus ver.2 (CREP2) kits (08057524190; Roche Diagnostics, Indianapolis, IN, USA) using a Roche Cobas c501 Analyzer in Paper III.

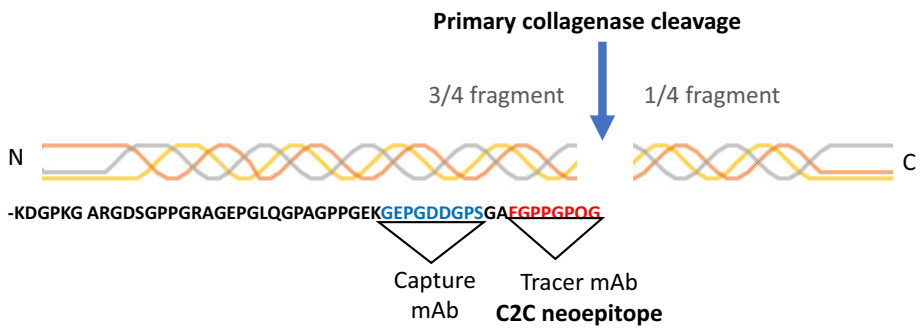


Figure 8. The epitopes of the Col2 fragments are detectable by antibodies in the IB-C2C-HUSA assay™. Abbreviation: mAb – monoclonal antibodies.

4.3. Standardized radiographic investigation

Two grading systems — the Nottingham system (NSy) (Paper I-III), and the classic KL system (Paper III) were used for the assessment of kOA (Nagaosa *et. al* 2000; Kellgren & Lawrence, 1957). Two radiologists independently graded kOA severity by both systems. A final decision was made by a consensus between the two radiologists.

For NSy, a standardized anteroposterior radiographs of TF compartments and radiographs of PF compartments with the knees, flexed at 60° were used (Nagaosa *et. al* 2000; Kumm et. al 2012). The OA changes such as JSN and Oph in the TF and PF joints were graded on a four-point scale (grades 0–3). For bilateral cases, the knee with more severe OA served as the study knee. The highest grade of JSN or Oph was regarded as the grade of OA in the corresponding joint (TFOA or PFOA). The highest grade of OA in both compartments was defined as a radiographic global grade of the disease (gOA). A distribution of the subjects in the cross-sectional study was based on the radiographic evaluation as presented in Figure 9.

For the KL system, a five-grade classification of kOA severity (grades 0–4) was done using the same anteroposterior X-rays in Paper III. A severity of the formation of Oph, JSN, pseudocystic areas with sclerotic walls in the sub-chondral bone, and altered shape of bone ends was evaluated.

In Paper III, KL and NSy were used parallelly. A correspondence of a radiographic KL grade to NSy grade for the TKR cohort is presented in Table 2.

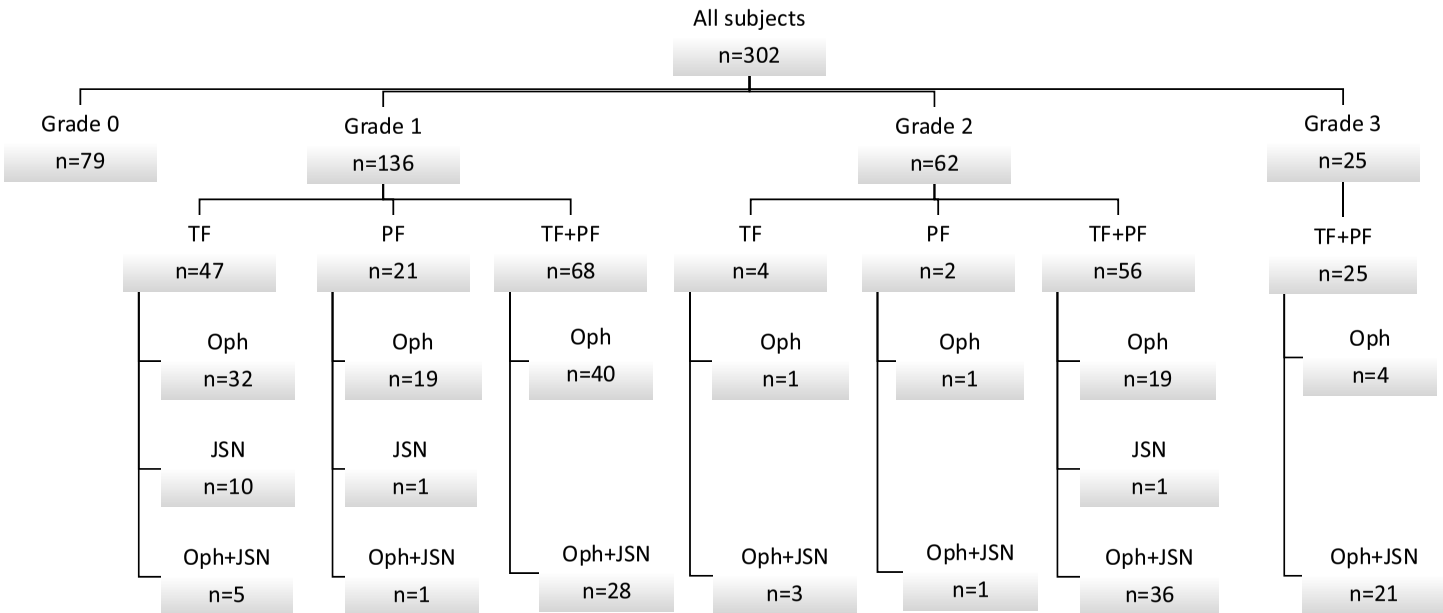


Figure 9. A distribution of the subjects of the cross-sectional study based on the grade, location, and type of radiographic changes. (Paper I). Abbreviations: Grade – radiographic global knee osteoarthritis (gOA) grade; TF – OA in a tibiofemoral joint; PF – OA in a patellofemoral joint; TF+PF – OA concurrently in TF and PF joints; JSN – joint space narrowing; Oph – osteophytes; Oph+JSN – osteophytes and joint space narrowing at the same joint; *n* – numbers.

Table 2. A distribution of the study cohort of Paper III by radiographic grades of kOA evaluated in 86 cases by two different systems: the Nottingham (NSy) system and the KL system.

Radiographic kOA grades/number of cases	gOA by NSy	
KL	Grade 2	Grade 3
Grade 2	3	1
Grade 3	11	28
Grade 4	2	41

Abbreviations: KL – A severity grade of knee OA assessed by Kellgren–Lawrence system; gOA – the highest grade of OA changes in the tibiofemoral and/or patello-femoral joints assessed by the Nottingham system (NSy).

4.3.1. Definitions of radiographic progression and distribution of the progression groups (Paper II)

kOA progression was evaluated by comparing radiographic changes in the subjects of the longitudinal study at time points – T0 and T3. Two main outcome groups were defined (Fig. 6): (1) the progressors' group, which was formed by the subjects with signs of radiographic kOA progression within 3 years; and (2) the non-progressors' group, which consisted of the subjects lacking the radiographic progression of kOA. The *progressors' group* was further divided into two subgroups according to the extent of the changes during 3 years: (a) gr(ade)-progressors, which included the subjects with radiographic worsening by at least one grade of gOA (≥1 grade); (b) the min(imal)-progressors, which contained the subjects with radiographic worsening (addition or increasing in the grade of Oph or JSN) within the same gOA grade. The subjects in gOA grade 0 at baseline but developing kOA grade 1 or more during the 3 years, were named the emerging kOA group. The subjects without radiographic findings of kOA during the whole study period (grade 0 of gOA) were defined as the without-kOA group. A long-term control group with a twelve-year follow-up period without the development of kOA formed a distinct group. A distribution of the subjects of the longitudinal study cohort (Paper II) by the progression status is demonstrated in Figure 10, and in addition, a correspondence to gOA grades in both sexes is presented in Table 3.

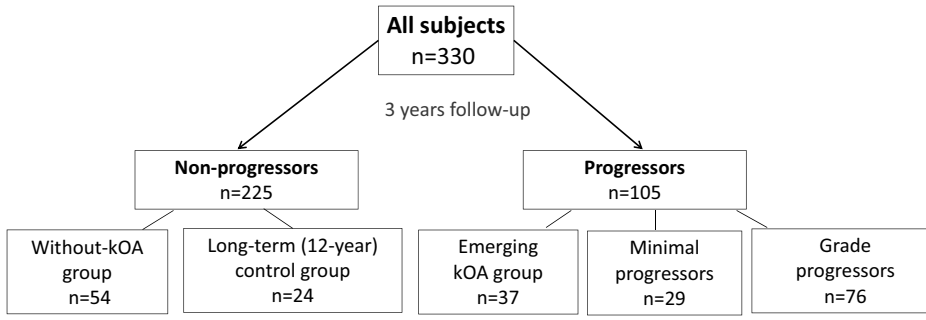


Figure 10. A distribution of the radiographic groups in Paper II. Abbreviation: kOA – knee osteoarthritis.

Table 3. A distribution of the study cohort by the progression status, gOA grades, and sex (Paper II) at T0.

	All grades	**Grade 0**	**Grade 1**	**Grade 2**	**Grade 3**
Subjects, *n*, (% all subjects)	330	91 (27.6)	169 (51.2)	55 (16.7)	15 (4.5)
Progressors, *n*, (%)	105	37 (41)	40 (24)	23 (42)	5 (33)
gr-progressors, *n*, (%)	76	37 (41)	24 (14)	15 (27)	-
min-progressors, *n*, (%)	29	-	16 (10)	8 (15)	5 (33)
Non-progressors, *n,* (%)	225	54 (59)	129 (76)	32 (58)	10 (67)
Males, *n*, (%)	131	41 (45)	61 (36)	21 (38)	8 (53)
Male progressors, *n*	37	15	12	7	3
Male gr-progressors, *n*	26	15	7	4	-
Male min-progressors, *n*	11	-	5	3	3
Male non-progressors, *n*	94	26	49	14	5
Females, *n*, (%)	199	50 (55)	108 (64)	34 (62)	7 (47)
Female progressors, *n*	68	22	28	16	2
Female gr-progressors, *n*	50	22	17	11	-
Female min-progressors, *n*	18	-	11	5	2
Female non-progressors, *n*	131	28	80	18	5

Abbreviations: gOA grade – global grade of knee osteoarthritis (kOA); Progressors – the subjects with signs of radiographic kOA progression during 3 years; Non-progressors – subjects without radiographical changes of kOA during 3 years; gr-progressors – grade-progressors, subjects with kOA progression of gOA ≥1 grade; min-progressors – minimal progressors, subjects with radiographic worsening of kOA within the same gOA grade; *n* – numbers.

4.4. Evaluation of articular cartilage lesions (Paper I)

Orthopedic surgeons performed a direct visual assessment and reported a status of articular cartilage of the knee (lesions' location, depth, and extent) by using a modified Outerbridge system (Ayral, 2005). The chondral area of interest was graded 0–IV (0=normal). The extent of the lesion (%) of the respective grades was evaluated on the two articular surfaces of the medial TF compartment, medial femoral condyle, and tibial plateau. The surgeons' findings (drawings on paper) were used to calculate *Société Francaise d'Arthroscopie* (*SFA*) scores for the medial TF compartments (Dougados *et al.* 1994; Ayral *et al.* 2005). The SFA score (continuous variable, scale 0–100) was obtained as follows: SFA score = extent (%) of grade I lesions x 0.14 + extent (%) of grade II lesions x 0.34 + extent (%) of grade III lesions x 0.65 + extent (%) of grade IV lesions x 1.00. A distribution of the subjects of the arthroscopic group with SFA score data by radiographic signs is shown in Table 4.

Table 4. The distribution of the subjects of the arthroscopic group with medial SFA score data (n=14) according to the presence of JSN and Oph.

Radiographic signs and grades by NSy	Presence of medial SFA score (*n*=14)	Values of medial SFA score
JSN grade 0 and Oph grade 0	4	0.1, 1.2, 1.4, 14
JSN grade 0 and Oph grade ≥2	3	9, 22, 44
JSN grade ≥2 and Oph grade ≥2	7	13, 15, 33, 38, 42, 46, 69

Abbreviations: JSN – joint space narrowing; Oph – osteophyte; NSy – radiographic grades by Nottingham system; SFA score – *Société Francaise d'Arthroscopie* score for cartilage visual evaluation.

4.5. Patient-relevant outcome questionnaires

4.5.1. Knee Injury and Osteoarthritis Outcome Score (KOOS)

The knee joint complaints of the recruited subjects' were evaluated using the Estonian version of the KOOS questionnaire (www.koos.nu). The five patient-relevant subscales were evaluated separately – symptoms (7 items); pain (9 items); ADL function (17 items); sport and recreation function (5 items), and quality of life (4 items). The scores express the health status of knees on 0–100% scale, wherein 0 represents extreme knee problems and 100 represents no knee problems. KOOS scores ≥85% were considered as 'healthy knees' (Roos *et al.* 1998).

The change in KOOS after one year of TKR was calculated by subtracting the preoperative score value from the postoperative one. To evaluate substantial clinical benefit (SCB) of KOOS, defined as a change by more than ±20 units, we divided patients into SCB subgroups – worsening, no change, and improvement after TKR (Glassman *et al.* 2008; Ogura *et al.* 2020).

4.5.2. Visual analog scale for pain (VASpain) in joints of different skeletal areas

VASpain was self-completed by the study subjects. Fourteen joints (skeletal areas) were evaluated: right and left hip, right and left knee, right and left ankle, right and left shoulder, right and left elbow, right and left hand (wrist, metacarpal, and finger joints included), and upper (head, neck, and thorax) and lower (lumbar spine) back. The responders were asked to place a perpendicular line at a 100 mm scale on paper. The drawings were measured and transposed to a 0–10 pain score (0 = no pain; 10 = worst imaginable pain). Separate VAS scores were calculated for knee joints (mean knee VAS score) and for other joints (median summary VAS score; sumVAS). The number of noticeably affected joints was presented as sumJoint (VASpain > 5 was evaluated as noticeable involvement of a joint).

4.6. Statistical analysis

The sample size for power 80% was calculated using a sample size calculator (https://clincalc.com/stats/samplesize.aspx) for Mann–Whitney Test U (Wilcoxon Rank Sum Test) with a confidence interval set at 95%.

The other data were analyzed using R (Free Software Foundation, Boston, MA, USA; http://www.r-project.org) and GraphPad Prism 8 (GraphPad Software, San Diego, CA, USA). Descriptive variables (age, BMI, etc.) are presented as a number or mean with standard deviation (SD) and analyzed using parametric tests (e.g., chi-squared test, t-test, ANOVA). As the uC2C concentrations and KOOS subscale values were not distributed normally (checked by Shapiro–Wilk test), nonparametric tests (e.g., Kruskal–Wallis test, Mann–Whitney U test) were used for their evaluation. P-values < 0.05 were considered significant. Bonferroni correction was used for multiple comparisons.

In addition, the linear regression models (LM) were created to adjust the analysis of uC2C concentration for the confounding factors (age, sex, BMI) that can influence the associations. To determine the ability of OA features alone and in combinations to predict uC2C concentration. R-squared (R^2) as a goodness-of-fit measure for linear regression models was used/applied (Paper I).

A forward method for multiple logistic regression (GLM) with the calculation of odds ratios (ORs) and 95% confidence intervals was used to assess the association between uC2C concentrations and gOA grades or OA progression or clinical improvement in kOA after TKR. GLM models were adjusted for age, sex, and BMI (Paper I-III). For the prediction of different gOA grades in Paper I, calculated OR reflected an increase in the odds of more severe gOA grade in case of 2-fold rise of uC2C. For prediction of kOA progression (Paper II), the models containing the subjects of all grades were adjusted additionally for gOA. In Paper II, the OR showed an increase in the odds of kOA progression in case of 2-fold rise of uC2C. The analysis was performed separately for the whole

group and the sexes. A discriminative ability of GLM models was assessed using the c statistic (area under the curve, AUC).

4.7. Ethics

Research Ethics Committee of the University of Tartu approved all the studies (protocol code 140/41, 22.08.2005; 156/8, 22.01.2007; 166/M-21 26.10.2009; 219/M-10 22.10.2012; and 265/T–22, 19.12.2016), which were conducted according to the precepts of the Declaration of Helsinki. All the subjects provided a written informed consent for the study participation.

5. RESULTS

5.1. Association of uC2C and kOA radiographic features (Paper I and III)

5.1.1. Prevalence of radiographic features according to clinical study groups

Two different groups were used in this set of the study: a subset from the Estonian Early Knee OA Study Cohort and The Arthroscopy Cohort (Paper I, *n*=302, Table 5) and TKR patients (Paper III, *n*=86, Table 6).

Table 5. Description of the study cohort of Paper I divided by radiographic grades of gOA.

	All grades	**Grade 0**	**Grade 1**	**Grade 2**	**Grade 3**	P-value
Subjects, *n*	302	79	136	62	25	<0.0001*
Mean Age, years ±SD	49.1 ±6.5	46.6 ±6.1	48.8 ±6.1	52.0 ±6.1	51.5 ±7.0	<0.0001*
BMI, kg/m^2 ±SD	28.4 ± 5.2	26.6 ±4.9	27.8 ±4.5	31.9 ±5.3	29.1 ±5.6	<0.0001*
Unilateral/bilateral gOA cases, *n*	51/172	-	48/88	3/59	-/25	
Females, %	61	58	60	66	60	0.8**

Abbreviations: gOA – global grade of kOA; *n* – numbers; SD – standard deviation; BMI – body mass index; * difference between grades by ANOVA; ** difference between grades by Chi-squared test.

Neither mean age (48.4 ± 5.8 years) nor mean BMI (25.9 ± 4.6 kg/m^2) of the subjects in the long-term control group showed any significant difference from the subjects with gOA grade 0. Age and BMI were similar in females and males in the same gOA groups.

Isolated Oph was the most common radiographic finding (*n*=116) in the whole study group (Fig. 7). Among the cases with Ophs, JSN was more frequently seen in females than in males (52% vs. 34%; chi-squared test, $p = 0.01$). Mean age and BMI did not differ significantly between the sexes, although BMI was slightly higher in females (mean BMI: 33.1 vs. 31.5 kg/m2 for males; $p = 0.07$, t-test).

Table 6. Clinical characteristics of the total knee replacement (TKR) cohort by timepoints (Paper III).

Clinical Characteristics/ Timepoints	Pre-TKR	3 Months Post-TKR	12 Months Post-TKR
Subjects, *n* (%)	86 (100)	70 (81)	74 (86)
Mean age, years ± SD	59.9 ± 4.7	60.2 ± 4.7	60.9 ± 4.7
Mean BMI, kg/m^2 ± SD	32.3 ± 4.2	32.3 ± 4.2	32.4 ± 4.2
Previous TKR of opposite knee, *n* (%)	20 (23)	16 (23)	15 (20)
Females, %	50	53	49
Obesity (BMI ≥ 30), *n* (%)	59 (69)	49 (70)	50 (68)

Abbreviations: *n* – numbers; SD – standard deviation; BMI – body mass index; TKR – total knee replacement.

5.1.2. Association of uC2C level with gOA

Median uC2C level was the lowest in gOA grade 0 group and gradually elevated until grade 3 as assessed by NSy (Fig. 11A, Paper I). Similarly, a gradual increase in uC2C level was associated with the radiographic severity of kOA, when assessed by the KL system (Fig. 11B, Paper III).

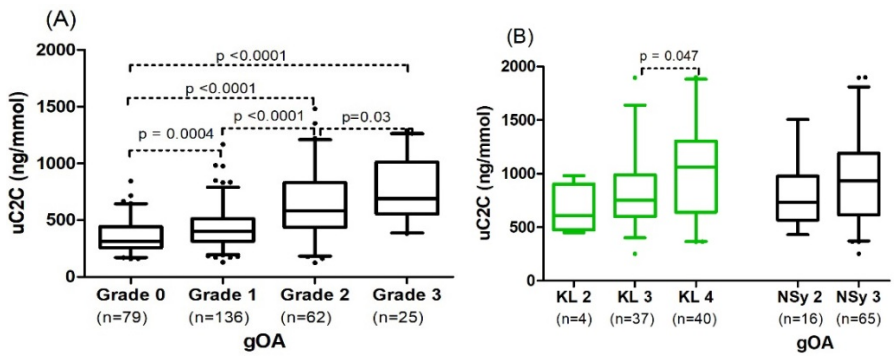


Figure 11. An association of uC2C with radiological grades of knee osteoarthritis (kOA): (A) Paper I. uC2C and gOA (*n*=302), (B) Paper III. uC2C and gOA by NSy or severity by KL system in the preoperative period (*n*=86). Box-whiskers plot with 5th-95th percentiles, the *p*–value determined by Mann–Whitney U-test. Abbreviations: KL – grades of radiographic knee osteoarthritis by the Kellgren–Lawrence scoring system; gOA – the highest grade of knee OA changes in two knee joint compartments (tibiofemoral and/or patellofemoral joints) by the Nottingham system (NSy), *n* – numbers.

As age, BMI and sex are confounders for uC2C analysis, we used GLM for further analysis. In GLM models adjusted for age, sex and BMI, the elevation of uC2C level was associated with a gradual increase in the risk of a more severe grade of kOA (adjusted OR per a grade change = 2.14–3.7; Fig. 12A, Paper I).

Similar to the unadjusted data, a significant increase in uC2C level was predicted gOA grade 1 (grade 1 vs. 0: adjusted OR = 2.14, AUC = 0.66, p = 0.01). However, the increase in uC2C level was better predicted the next gOA levels (grade 2 vs. 1: adjusted OR = 2.84, AUC = 0.79, p = 0.0007; grade 3 vs. 2: adjusted OR = 3.7, AUC = 0.74, p = 0.008). BMI had a significant prediction value only for grade 2 vs. 1 and grade 3 vs. 2, but not for grade 1 vs. 0. Overall, uC2C levels excellently predicted a presence of radiographic gOA (grade 3 vs. 0, AUC = 0.934; grade 2 vs. 0, AUC = 0.861, Fig. 12B).

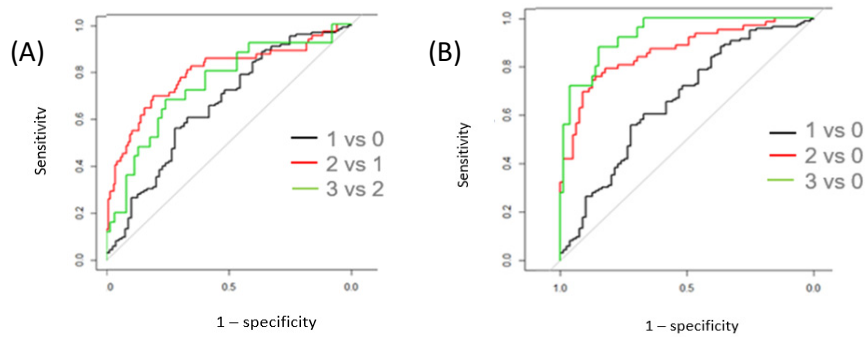


Figure 12. Receiver operating characteristic (ROC) curves of uC2C for predicting a presence of more severe gOA grade (Paper I): (A) Comparisons between consecutive gOA grades (0–3); (B) Comparisons between higher (1–3) grades of gOA and gOA grade 0. Abbreviations: gOA – the highest grade of knee OA changes in two knee joint compartments by the Nottingham system (NSy); AUC – area under the curve.

The uC2C level did not differ between the long-term (12 y) control group (gOA grade 0, without symptoms) and the subjects with knee symptoms but with radiographically normal knees (gOA grade 0, Paper I).

We detected no difference in uC2C level between unilateral and bilateral kOA cases (Paper I). There was no association between uC2C and the average pain scores of other joints (sumVAS pain) as well as with the status of previous TKR of the opposite knee (Paper III). Nevertheless, a tendency of higher uC2C levels in bilateral kOA as compared to unilateral cases could be demonstrated in females (Fig. 13, Paper III).

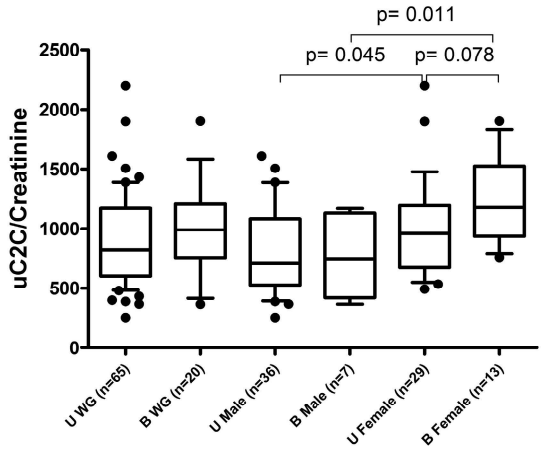


Figure 11. An association of preoperative uC2C levels with unilateral or bilateral kOA status. Boxplot with 10th–90th percentiles, Wilcoxon test for multiple comparisons was used to determine *p*–values. Abbreviations: U WG – unilateral kOA without previous TKR in the whole group; B WG – bilateral kOA with previous TKR in the whole group); U Male – unilateral kOA without previous TKR in males; B Male – bilateral kOA with previous TKR in males; U Female – unilateral kOA without previous TKR in females; B Female – bilateral kOA with previous TKR in females.

5.1.3. Association of uC2C level with TFOA and PFOA

The uC2C level was significantly higher in the combined TFOA and PFOA cases and isolated TFOA cases as compared to the cases without radiographic change (Fig. 14, Paper I). Moreover, we demonstrated a significantly higher uC2C in combined TFOA+PFOA cases as compared to isolated TFOA or PFOA.

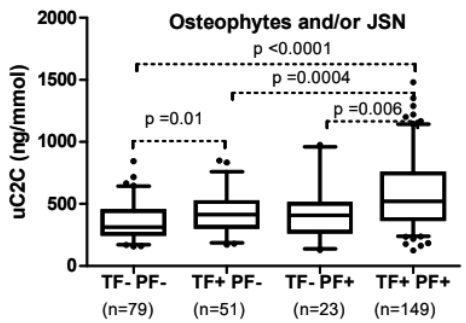


Figure 14. An association of uC2C with radiographic features of OA (osteophytes and/or JSN) in TF and/or PF joints. Box-whiskers plot with 5^{th}-95^{th} percentiles, the *p*-value was determined by Mann–Whitney U test. Abbreviations: TF+ – OA in a tibiofemoral joint; PF+ – OA in a patellofemoral joint; TF- no OA in a tibiofemoral joint; PF- – no OA concurrently in tibiofemoral and patellofemoral joints; JSN – joint space narrowing; n – numbers.

In linear regression analysis (Paper I), we demonstrated that gOA, TFOA, and PFOA equally predicted uC2C level (adjusted R^2 = 0.33–0.36). Also, a combined use of TFOA and PFOA in the same model did not increase the predictive power. Higher age and BMI, and male gender predicted higher uC2C levels.

5.1.4. Association of uC2C level with Ophs, JSN, and the SFA score

A presence of Oph in the TF joint or both, TF and PF joints, was associated with an increase in the uC2C level as compared to the cases without radiographic features of kOA (Fig. 15A, Paper I). The level of uC2C was higher in the patients with osteophytosis in both joint compartments. However, a presence of JSN did not increase the uC2C level within the same Oph grade (Fig. 15B, Paper I).

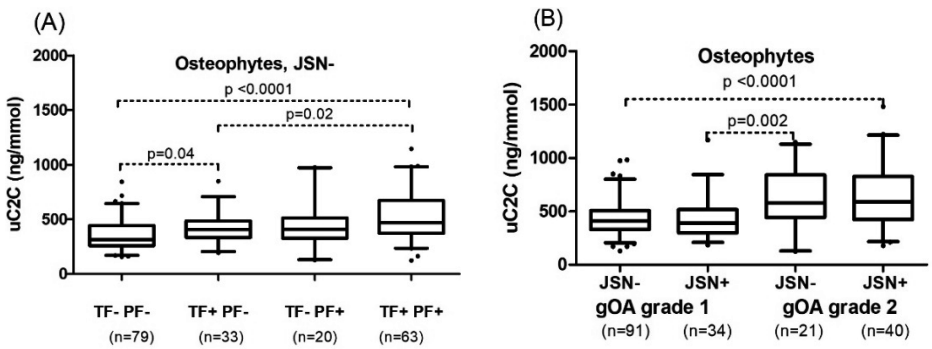


Figure 15. An association of uC2C with different combinations of radiographic features of knee OA: (A) osteophytes in TF and/or PF joints among subjects without joint space narrowing (JSN-); (B) osteophytes in gOA grade 1 and 2 in the cases with (JSN+) and without (JSN-) joint space narrowing.Box-whiskers plot with 5th-95th percentiles, the *p*–value was determined by Mann–Whitney U-test. Abbreviations: TF+ – OA in a tibiofemoral joint; PF+ – OA in a patellofemoral joint, TF- no OA in a tibiofemoral joint; PF- – no OA in patellofemoral joints; gOA – the highest grade of knee OA changes in two knee joint compartments by the Nottingham system; n – numbers.

Using a linear regression analysis, we demonstrated that Oph predict the uC2C concentrations better than JSN (Table 7); Oph was the most important predictor for uC2C in TF joint.

Table 7. Lm-models adjusted for age, sex, and body mass index, predicting an increase of uC2C by radiographic features and their combinations (Paper I).

Model (main parameter)	Adjusted R^2
TFOph	0.35
PFOph	0.33
TFOph+PFOph	0.35
TFJSN	0.27
PFJSN	0.27
TFJSN+PFJSN	0.29
TFOph+TFJSN+PFOph+PFJSN	0.36

Abbreviations: TF+ – OA in a tibiofemoral join; TFJSN, PFJSN – joint space narrowing (JSN) score in tibiofemoral (TF) or patellofemoral (PF) joint; TFOph, PFOph – osteophytes score in TF or PF joint.

We demonstrated that medial SFA scores ranged from 0.1 – 69 (median value of 18.6) in the study group (Paper I). Also, we found that macroscopic cartilage lesions were presented in four cases without radiographic change (Fig. 16). Generally, higher SFA scores are associated with the severity of gOA grade (p = 0.03, Kruskal-Wallis test). We demonstrated that the SFA score well predicted the uC2C level in regression models (adjusted R^2 = 0.59). The best prediction models for uC2C level (adjusted R^2 = 0.75) formed by the addition values of different OA signs: macroscopic cartilage lesion expressed by medial SFA, Ophs in the TF joint, and JSN in the PF joint. Both, the presence of Ophs and cartilage damage in TF were significantly associated with each other on statistical scale ($p < 0.05$) and independently with the uC2C level in the model.

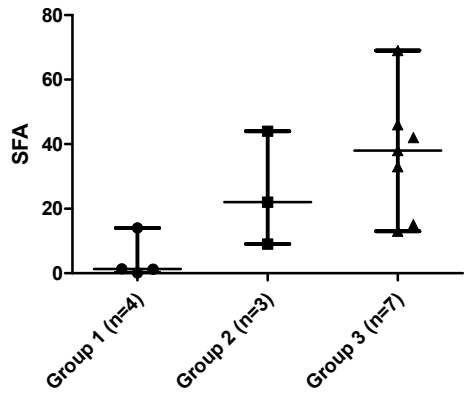


Figure 16. An association of *Société Francaise d'Arthroscopie* (SFA) score and radiographic features of knee osteoarthritis; Group 1: joint space narrowing (JSN) grade 0 and osteophytes (Oph) grade 0; Group 2: JSN grade 0 and Oph grade ≥2; Group 3: JSN grade ≥2 and Oph grade ≥2. Median and range are shown.

5.1.5. Sex-related differences in associations with uC2C and kOA features

The biomarker level did not differ between males and females in the same gOA grades 0–2 (Paper I). However, the uC2C level was significantly higher in females than in males during the preoperative period of advanced kOA (grade 3; p = 0.0039, Mann–Whitney U-test, Paper III). Moreover, a gradual increase in uC2C level was associated with the radiological grades in females (Fig. 17, Paper III; Fig. 20A, Paper II). In males, the uC2C level association was found only with the progressors of more severe radiological grades (Fig. 17, Paper III; Fig. 20B, Paper II).

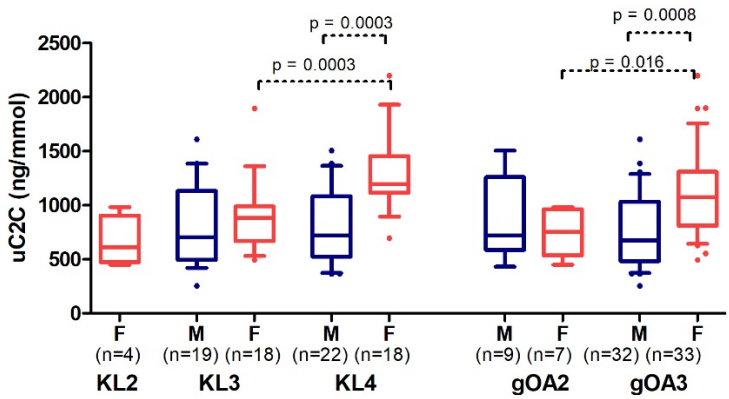


Figure 17. An association of uC2C levels with the radiological severity of knee osteoarthritis in the preoperative period in males (M) and females (F) (boxplot with 10th–90th percentiles, Mann–Whitney U-test). Abbreviations: KL – grades of radiographic knee osteoarthritis by the Kellgren–Lawrence scoring system; gOA – the highest grade of knee OA changes in two knee joint compartments (tibiofemoral and/or patello-femoral joints) by the Nottingham system (NSy); *n* – numbers.

Prominent sex-related differences were also demonstrated in the results of linear regression analysis combining different radiographic and clinical features. The prediction of uC2C levels by radiographic features, particularly OA features in TF joint, was better in females than in males (adjusted R^2 = 0.35–0.42 vs. 0.09–0.27, Paper I). Moreover, the best uC2C prediction model worked better in females as compared to males. In females, it was comprised of a combination of Oph in the TF joint and JSN in both joint compartments (adjusted R^2 = 0.43). On the other hand, the prediction model in males was the combination of JSN in the TF joint and Ophs in both compartments (adjusted R^2 = 0.28).

5.2. uC2C level as a marker of progression of kOA (Paper II)

5.2.1. Clinical characteristics of the longitudinal study group at baseline

The subjects of the longitudinal study group were middle-aged and overweight (Table 8).

Table 8. Clinical characteristics of the subjects of the longitudinal study group at baseline in Paper II.

Groups	All gOA grades	gOA grade 0	Long-term control group
Subjects, *n*	330	91	24
Females, *n* (%)	199 (60)	50 (55)	10 (42)
	Mean age, years ± SD		
Subjects	47.6 ±6.5	46.1±6.5	49.1 ± 5.7
Males	46.6±6.9	46.3±6.4	48.9 ± 6.3
Females	48.2±6.1	45.9±6.6	49.4 ± 5.1
p-value*	0.04	0.8	0.8
	Body mass index (BMI), kg/m^2 ± SD		
Subjects	28.0 ± 5.3	26.2±5.1	26.0 ± 4.6
Males	28.1 ± 4.7	27.5±5.6	27.7 ± 5.0
Females	27.9 ± 5.6	25.2±4.4	23.6 ± 2.4
p-value*	0.7	0.03	0.01

Abbreviations: gOA – global grade of knee osteoarthritis; *n* – numbers; SD – standard deviation; BMI – body mass index; *difference between sexes by t-test.

The BMI of investigated subgroups (progressors/non-progressors) did not differ in males. In contrast, females presented higher BMI in progressors compared with non-progressors (29.5 ± 6.1 kg/m^2 vs. 27.1 ± 5.2kg/m^2; $p = 0.008$, t-test). The characteristics of the control group and gOA grade 0 had no significant difference. The VAS pain score of other joints (knees excluded) did not differ between progressors and non-progressors ($p = 0.3$, t-test).

5.2.2. Association of uC2C at baseline level with the three-year follow-up of kOA progression

The level of uC2C was significantly higher in the progressors as compared to the non-progressors at baseline (T0) (16% difference in the median; $p = 0.0008$; Mann–Whitney U-test, Fig. 18). This association was found in both progression subgroups: gr-progressors (19% higher as compared to non-progressors; $p = 0.003$) and min-progressors (12% higher as compared to non-progressors;

p = 0.04). However, no significant difference in the uC2C levels was detected between the min-progressors and the gr-progressors.

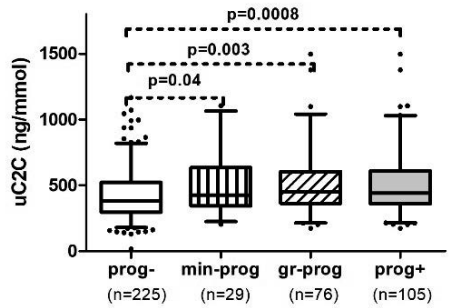


Figure 18. An association of uC2C with radiographic progression of knee osteoarthritis (kOA) in the whole study group. Box-whiskers plot with 5th–95th percentiles, the *p*-value determined by Mann–Whitney U test. Abbreviations: (prog-) – non-progressors, subjects without kOA radiographical changes during 3 years; (prog+) – progressors, subjects with the progression of kOA; (min-prog) – minimal progressors, subjects with radiographic worsening of kOA within the same gOA grade; (gr-prog) – grade progressors, the subjects with the progression of gOA ≥1 grade; n – numbers.

Using GLM models, we demonstrated that uC2C level successfully predicted kOA progression within three years in all the progressors (adjusted OR = 2.34 (1.48–3.68) for uC2C, p = 0.0003, AUC = 0.67), and even better in gr-progressors subgroup (adjusted OR = 2.8 (1.66–4.72) for uC2C, p = 0.0001, AUC = 0.73). Two confounding factors in the model contributed to the kOA progression risk – higher BMI slightly magnified a risk of kOA progression (adjusted OR = 1.07 (1.02–1.12); p = 0.006), but higher gOA grade showed a mild protective effect (adjusted OR = 0.67 (0.47–0.96); p = 0.03).

5.2.3. A prognostic value of uC2C for kOA progressors in distinct gOA

We presented that the level of uC2C of the progressors at gOA ≥1 was higher than the levels of the non-progressors at T0 (Fig. 19). We also found that the gr-progressors had a 25–33% higher median uC2C level as compared to the non-progressors (33% difference in grade 1; p = 0.001 and 25% difference in grade 2/3, p = 0.03; Mann–Whitney U test). However, no difference in the uC2C levels was found between the emerging kOA group and the non-progressors of kOA in grade 0. Interestingly, the uC2C levels of the emerging kOA subjects were rather similar to the non-progressors in gOA grade 1. Also, there was no significant difference between the uC2C values of the progressors in grade 1 and the non-progressors in grade 2/3 (p = 0.72, Mann–Whitney U test).

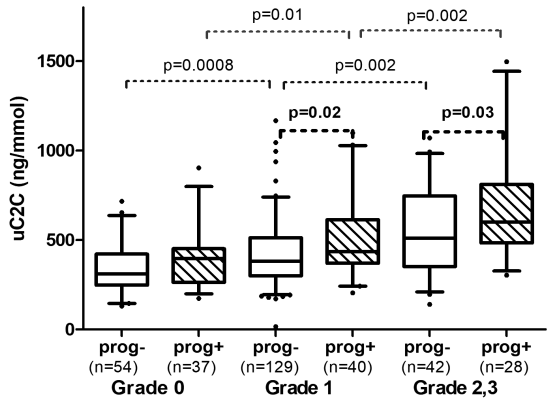


Figure 19. An association of uC2C with radiographic progression of knee osteoarthritis (kOA in all the subjects by global grade of kOA (gOA). Box-whiskers plot with 5th–95th percentiles, the *p*–value determined by Mann–Whitney U test. Abbreviations: (prog-) – non-progressors, the subjects without radiographical changes of kOA during 3 years; (prog+) – progressors, the subjects with kOA progression; *n* – numbers.

However, we demonstrated that the uC2C levels quite efficiently predicted the onset of kOA in GLM models given that the emerging kOA group was compared to the without-kOA-group, and the model was adjusted for age, gender, and BMI (adjusted OR = 2.58 (1.08–6.16) for uC2C, $p = 0.03$; AUC = 0.64). The best kOA prediction model was achieved when the emerging kOA was compared with the long-term control group (adjusted OR = 5.87 (1.71–20.22) for uC2C; $p = 0.005$; AUC = 0.79). In the same model, females demonstrated a significant risk for the developing of kOA (adjusted OR = 4.01 (1.12–14.39) for uC2C; $p = 0.03$), while older age revealed some protective effect against emerging of kOA (adjusted OR = 0.86 (0.77–0.96) per year of age; $p = 0.009$). Although a significant prediction power of the uC2C for the progression of the disease was demonstrated at baseline grade 1 (adjusted OR = 2.36 (1.19–4.67); $p = 0.01$; AUC = 0.67), we could not prove the same in the case of established kOA (gOA grade 2/3).

5.2.4. Sex-related differences between associations of uC2C and kOA progression

Albeit the uC2C level predicted the kOA progression in both sexes, several differences were observed.

In females, the levels of uC2C were 24–30% higher in the progressors as compared to the non-progressors ($p = 0.0009$ for the progressors and $p = 0.001$ for the gr-progressors, Mann–Whitney U test). Moreover, the uC2C was approximately 72% higher in the females with emerging kOA as compared to the long-term control group ($p = 0.009$, Mann–Whitney U test). The level of uC2C demonstrated a significantly higher efficacy to predict emerging kOA in the

GLM model as compared to the long-term control group in females when adjusted the confounders like age and BMI (OR = 23.0 (2.2–245) for uC2C; AUC = 0.91). In the advanced grades of the disease, a difference in the uC2C level between the progressors and the non-progressors was found decreased; this means, it was significant in gOA grade 1 and was found disappeared in gOA grade 2/3 (Fig.20A). This finding was also supported by GLM model analysis – the uC2C level had a significant prediction power of detecting the kOA progression in gOA grade 1 (OR = 2.67 (1.18–6.04) of uC2C; AUC = 0.68), but this ability was lost in gOA grade 2/3.

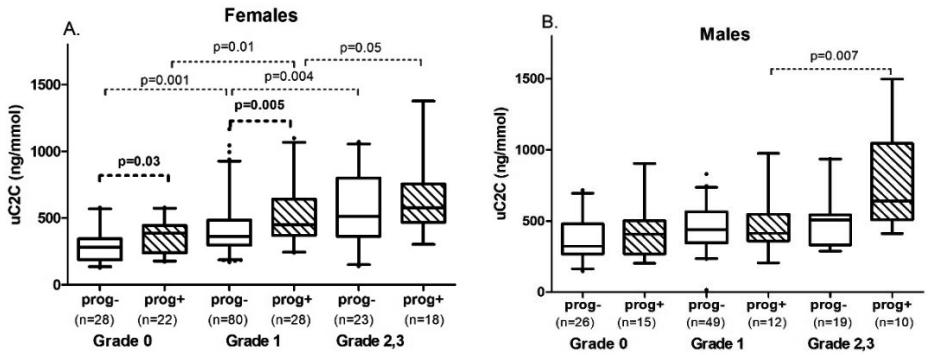


Figure 20. An association of uC2C with radiographic progression of knee osteoarthritis (kOA) in females (A) and males **(**B). Box-whiskers plot with 5th–95th percentiles, the *p*-value determined by Mann–Whitney U test. Abbreviations: (prog-) – non-progressors, the subjects without radiographical changes of kOA during 3 years; (prog+) – progressors, the subjects with the progression of kOA.

In contrast, uC2C levels in progressor and non-progressor males were not significantly different (Fig. 18B). Exception was the min-progressors, a sub-group of males with gOA grade 2/3, which demonstrated a significantly higher uC2C in comparison with the non-progressors of the same baseline gOA (833.0 (606.0–1019.5) vs. 507.0 (345.0–541.5) ng/mmol, p = 0.02, Mann–Whitney U-test). We did not find any significant predictive value of uC2C in males by GLM analysis. Interestingly, mean values of uC2C in the non-progressor males were significantly higher in comparison with the values of non-progressor females (p=0.03, Mann–Whitney U-test); however, the uC2C levels did not differ in the progressors of both sexes.

5.3. uC2C and total knee replacement (Paper III)

5.3.1. The dynamics of uC2C levels after TKR

We demonstrated that although median level of uC2C was significantly elevated after three months of the TKR as compared to the pre-TKR level (p = 0.012, Mann–Whitney U test), it was found declined to the preoperative level after 12 months (Fig. 21).

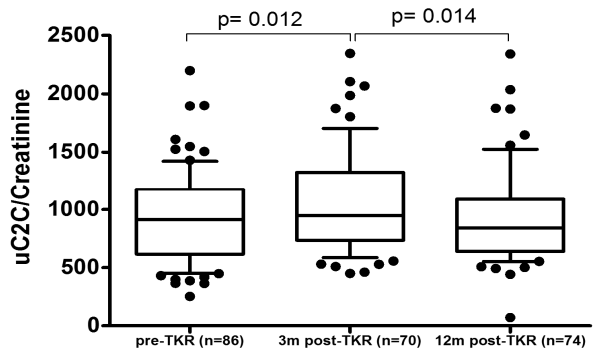


Figure 21. Dynamics of uC2C during the study period. Boxplot with 10th–90th percentiles, Mann–Whitney U test. Abbreviations: TKR – total knee replacement; pre-TKR – 1–2 days before TKR; 3m post-TKR – 3 months after TKR; 12m post-TKR – 12 months after TKR; *n* – numbers.

At the same time, a variety of individual changes existed – an excretion of uC2C could decrease, increase, or remain constant. The individual dynamics of uC2C over 12 months ranged from a decline of 87% to a rise of 120% in the uC2C levels (Fig. 22). Furthermore, a remarkable (~20%) increase in the postoperative uC2C levels was obvious in the cases with lower preoperative uC2C levels (16 out of the first 30 ranked cases). Conversely, several cases (12 out of the last 29 ranked cases) with a significant decrease in the post-TKR period were among those with higher preoperative uC2C levels.

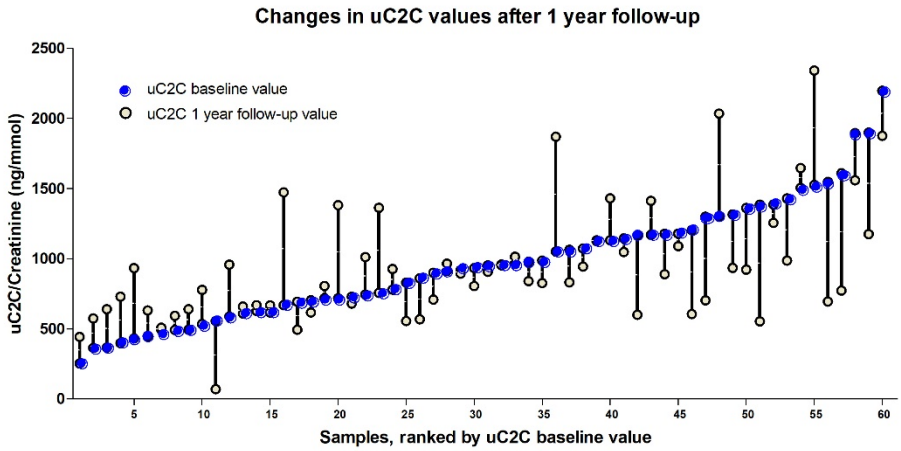


Figure 22. Individual dynamics of uC2C values (pre-TKR and 12 months after TKR) are ranked by preoperative (baseline) uC2C level. Abbreviation: TKR – total knee replacement.

Accounting a 20% change in the biomarker as a basis of grouping, we demonstrated that 28% of patients (17 out of 60) were "descenders", 32% were "ascenders" (19 out of 60), and the remaining 40% belonged to a "stable" group. A statistically significant difference between the uC2C values of "ascenders" and "descenders" was demonstrated in both pre-TKR and 12 months after TKR ($p = 0.00022$ and $p = 0.021$, respectively, Mann–Whitney U test; Fig. 21). However, the subgroups did not differ in the uC2C levels at the three-month postoperative timepoint.

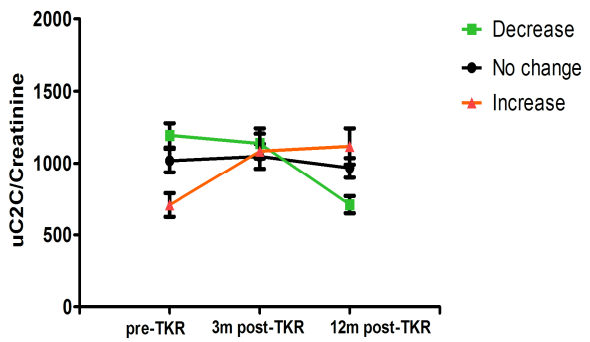


Figure 23. A line plot of uC2C dynamics in the whole group based on postoperative uC2C changes (decrease or increase means, > 20% decrease (green) or increase (red) in the 12-month post-TKR uC2C value as compared to the preoperative one; mean and standard error of the mean (SEM) shown). Abbreviations: pre-TKR – before total knee replacement; 3m post-TKR – 3 months after TKR; 12m post –TKR 12 months after TKR.

5.3.2. Sex-related differences in the dynamics of uC2C levels after TKR

In males, median uC2C was significantly increased after three months of TKR as compared to the preoperative value, and a decline, comparable to the pre-operative level, was observed after 12 months, resembling the dynamic of the whole group. On the contrary, in females, the uC2C was lower for a year after surgery as compared to preoperative levels (Fig. 24).

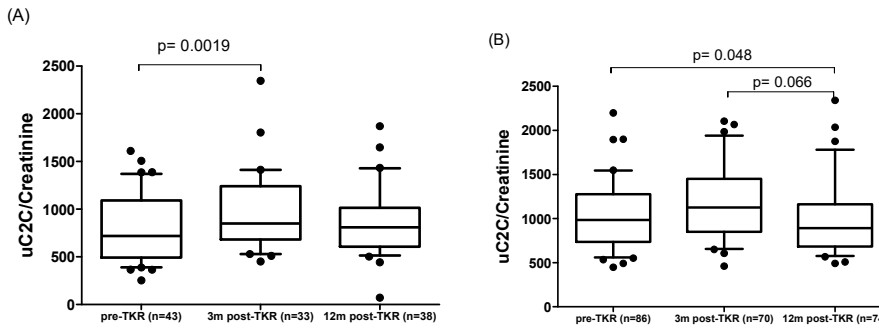


Figure 24. Dynamics of uC2C during the study period in (A) males, and (B) females (boxplot with 10th–90th percentiles, Mann–Whitney U-test). Abbreviations: pre-TKR – 1–2 days before total knee replacement; 3m post-TKR – 3 months after TKR; 12m post-TKR 12 months after TKR.

We found a statistically significant difference between "ascenders" and "descenders" males (p = 0.019 for pre-TKR and p = 0.035 for one-year post-TKR, Mann–Whitney U test; Fig. 25A), but not in females (p = 0.10 for pre-TKR and p = 0.053 for one-year post-TKR, Mann–Whitney U test; Fig. 25B).

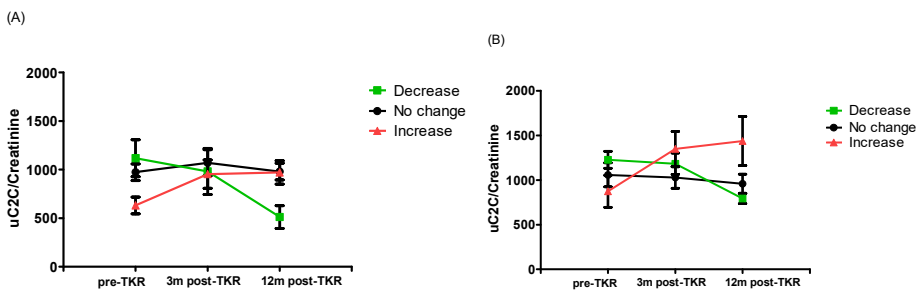


Figure 25. A line plot of uC2C dynamics in (A) males, and (B) females, grouped on the basis of postoperative uC2C changes (decrease or increase means, > 20% decrease (green) or increase (red) in the 12-month post-TKR uC2C/Crea value as compared to its preoperative value; mean and standard error of the mean (SEM) shown). Abbreviations: pre-TKR – before total knee replacement; 3m post-TKR – 3 months after TKR; 12m post-TKR 12 months after TKR.

5.4. uC2C level and patient's self-assessment outcomes (Paper III)

5.4.1. Associations between uC2C and pain

uC2C levels were weakly correlated with VAS pain of the knee joint in the whole group (Spearman's rho = 0.22, $p = 0.045$); however, there was not any significant association of uC2C with the sexes separately. A weak correlation between uC2C levels and KOOS pain was also observed in the whole group (Spearman's rho = –0.31, $p = 0.006$, for KOOS pain) and in the males (Spearman's rho = –0.33, $p = 0.04$), but it was missing in the females (Spearman's rho = –0.20, $p = 0.21$).

5.4.2. An association of uC2C levels with the improvement of KOOS subscales in the post-TKR

Significant improvements in all KOOS subscales were observed after one year of TKR in comparison with their pre-TKR scores ($p < 0.0001$, Mann–Whitney U test; Fig. 26). However, none of the scores of any subscale rose to the score of 85 (=healthy).

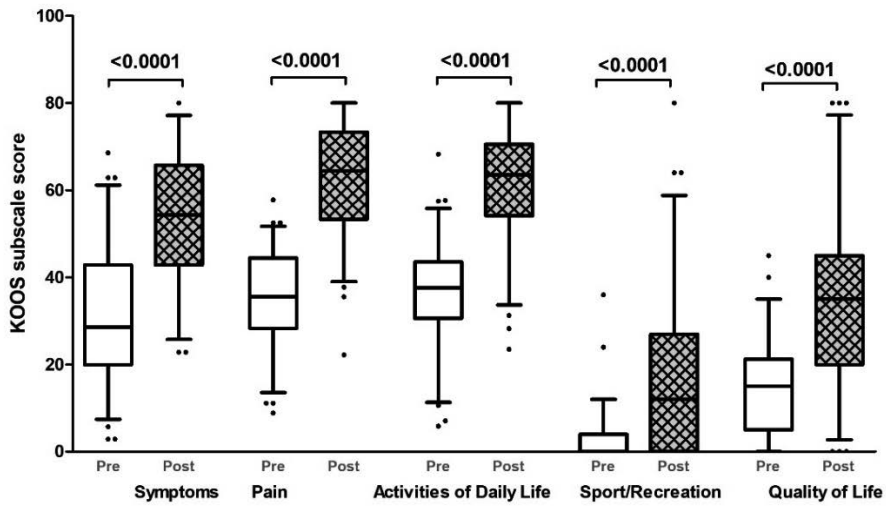


Figure 26. KOOS profiles before and after 12 months of TKR. Boxplot with 10th–90th percentiles. KOOS subscales: pain; symptoms; activities of daily living; sport and recreation; quality of life (Mann–Whitney U test *p*-values). Abbreviations: TKR – total knee replacement; KOOS – Knee Injury and Osteoarthritis Outcome Score; Pre – preoperative status; Post – postoperative (1 year after TKR) status.

Difficulties were more prominent in the activities such as squatting, kneeling, running, jumping, and twisting, as expressed by the function in the sport and recreation subscale.

A significant clinical worsening of KOOS was very rare (only one patient in this TKR study group). Approximately two-thirds of the patients reported significant clinical improvements in the pain and the function in daily living subscales (70% vs. 30% and 65% vs. 35%, respectively; Table 9). However, improvement in the KOOSsp/recr subscale was minor as compared to the no-change subgroup (36% vs. 64%).

Table 9. Substantial clinical benefit (SCB) in Knee Injury and Osteoarthritis Outcome Score (KOOS) subscales at 12-month follow-up after total knee replacement.

Clinically Important KOOS Change *	Substantial Worsening, *n* (%)	No Change, *n* (%)	Substantial Improvement, *n* (%)	Total **
KOOSsymp	1 (1%)	30 (42%)	40 (56%)	71
KOOSpain	0 (0%)	21 (30%)	49 (70%)	70
KOOSadl	0 (0%)	25 (35%)	46 (65%)	71
KOOSsp/recr	0 (0%)	46 (65%)	25 (35%)	71
KOOSqol	1 (1%)	32 (46%)	37 (56%)	70

Abbreviation: *n* – numbers; * Changes of more than ±20 units were counted as SCB; **: number change due to missing data. Knee Injury and Osteoarthritis Outcome Score (KOOS) subscales: symptoms (KOOSsymp), pain (KOOSpain), activities of daily living (KOOSadl), sport and recreation (KOOSsp/recr), and knee-related quality of life (KOOSqol).

The preoperative uC2C level was significantly higher in the improvement subgroup of KOOSsymp as compared to the no-change subgroup of the same subscale (Table 9; $p = 0.01$, Mann–Whitney U test). There were no significant differences in uC2C concentrations in the other KOOS subscales.

Table 10. A comparison of preoperative uC2C between SCB subgroups of KOOS subscales.

KOOS Subscale	Substantial Improvement Group *		No Change Group		*p*-value (between Groups) **
	n	uC2C ***, ng/mmol	*n*	uC2C ***, ng/mmol	
KOOSsymp	38	1016.9(782.8–1204.9)	29	718.8(570.5–899.7)	**0.01**
KOOSpain	46	935.8(637.7–1178.9)	21	752.7(703.1–1315.5)	0.94
KOOSadl	44	974.0(626.3–1216.2)	24	731.9(668.7–978.0)	0.16
KOOSsp/recr	25	917.3(608.8–1203.5)	44	923.6(687.9–1178.7)	0.70
KOOSqol	36	956.3(626.3–1216.2)	30	839.6(685.4–1132.0)	0.55

Abbreviation: *n* – numbers; * Knee Injury and Osteoarthritis Outcome Score (KOOS) subscales changes of more than 20 units between the 12-month postoperative and preoperative periods were counted as SCB (substantial clinical benefit); ** Mann–Whitney U test; *** Median value (1st-3rd quantiles) of uC2C. KOOS subscales: symptoms (KOOSsymp), pain (KOOSpain), activities of daily living (KOOSadl), sport and recreation (KOOSsp/recr), and knee-related quality of life (KOOSqol).

In GLM models, the preoperative uC2C levels predicted a significant clinical improvement in KOOSsymp in the whole group (OR = 2.79; CI 95% 1.19–6.53, $p = 0.02$), albeit an adjustment of the confounders (age, BMI, and sex) removed the statistical significance of the model. However, in females, the power of uC2C to predict the improvement of KOOSsympt remained significant even after adjustment of cofounders (adjusted OR = 9.43; CI 95% 1.19–74.73, $p = 0.03$). The prediction value of uC2C was missing in males. Moreover, no associations were found between uC2C and other KOOS subscales in the whole group or separately in both sexes.

6. DISCUSSION

Despite the recent advantages in the field of OA research and joint biology, still many challenges persist in OA-related studies. This has slowed the development of OA treatment, and as a result, it has remained considerably behind the advances in other rheumatic diseases. Several aspects can be underlined in addressing the challenges – a limited biomarker toolbox and lack of appropriate surrogate endpoints in OA clinical trials, a lack of tools to identify a faster OA progression, and a poor correlation between structural progression and pain progression. New biomarkers may help to solve several among the listed problems and many aspects related to it are needed to be considered, including a proposed clinical value of new tests. Therefore, BIPEDS classification (Burden of disease, Investigational, Prognostic, Efficacy of intervention, Diagnostics, and Safety) of biomarkers (Bauer *et al.* 2006) or the BEST criteria (Diagnostic, Susceptibility/Risk, Prognostic, Monitoring, Pharmacodynamic/Response, Predictive and Safety Biomarkers) (FDA-NIH Biomarker Working Group, 2016) is worse to follow. There are a number of promising candidates, such as COMP, aggregan, and uCTX-II, although none is sufficiently discriminatory to distinguish the patients with different stages of OA (including emerging), to predict the individuals at a high risk of progression of the disease or to operate consistently so that it could act as a surrogate in clinical trials.

In the current thesis, we focused on exploring a potential role of uC2C, a new Col2 degradation biomarker.

6.1. uC2C: a risk and an early diagnostic biomarker of radiographic kOA

Early radiographic diagnosis of knee OA continues to be a challenge. Due to several diagnostic methods and approaches, it is quite difficult to compare different studies. To our knowledge, our studies were the first to show that increased uC2C values can predict the early development (emergence) of knee OA. Therefore, we believe that uC2C has a potential to be a good marker for a diagnosis of a minimal radiographic kOA (gOA grade 1 vs. 0, Paper I). Previous work in this aspect was lacking, partly due to a fact that the presence of JSN and Oph are often merged as a combined scale for OA severity in many studies. Moreover, KL grade 2 is more traditionally accepted as a definitive criterion for kOA, while OA grade 1 (early) is generally considered to indicate a ‘doubtful’ presence of Ophs underestimating its significance as reported in many publications and clinical practice (Hart & Spector, 2003). Recently, researchers specified that MRI-detected Ophs were associated with OA changes over time (Zhu *et al.* 2017; Katsuragi *et al.* 2015). Despite advances in MRI techniques in recent decades, radiography still remains as a primary imaging modality for the definition of inclusion and exclusion criteria for OA-related clinical trials

(Hayashi *et al.* 2018). Our data demonstrate that a use of the NSy system (Nagaosa *et al.* 2000) helps to overcome the limitations of the KL system in the evaluation of early stages of the disease. Therefore, our study underlines a need of careful evaluation of early stages of radiographic OA for biomarker studies.

Moreover, our study clearly showed that an appropriate well-defined control group is essential for the correct evaluation of OA biomarkers. This is particularly important because the incidence of the disease is high all over the world, and its early stages are often underdiagnosed. The question 'who is healthy' is still essential to correctly validate the results of OA studies. We demonstrated that the ability of uC2C to predict an onset of the disease was particularly prominent, when the long-term control group (twelve years without any sign of kOA) was taken into consideration.

It is important to note that because of recent methodological development, a use of two different well-characterized C2C-specific antibodies allowed to increase a specificity of IB-C2C-HUSA™ for the measurement of C2C neoepitope fragments in urine. The first antibody effectively captures C2C fragments and the second, tracer antibody, provides a higher specificity by binding to the C2C neoepitope. There are several other *in vitro* ELISA methods available in the market for uC2C estimation that does not provide any information on the utilized antibodies, therefore, a comparison of uC2C levels in different publications is still quite tricky and the methods of estimation need to be compared. Moreover, standardization of the C2C concentrations with the concentration of creatinine in the same urine sample is important for a better consideration of urine dilution factor (A. O. Tamm *et al.* 2014).

The most investigated Col2 degradation marker, uCTX-II, was also observed as a biomarker of EKOA, however, the evidence in this regard is still inconclusive (Cibere *et al.* 2009; Saberi Hosnijeh *et al.* 2015; van Spil *et al.* 2015; P. Wang *et al.* 2019; Liu *et al.* 2020). Possible reasons for this could be, the use of KL classification (insensitive for early rkOA), control group selection criteria, or modest study groups (as discussed above). Moreover, comparative observations revealed that uC2C and uCTX-II assays have significant differences and the levels of uC2C correlated weakly with uCTX-II (Cibere *et al.* 2009; Kraus *et al.* 2017). Several other markers (serum COMP, sHA, and NTX-I) have been investigated in early-stage kOA, however, no definite conclusion was reached; furthermore, many of these studies have not been reproduced in other populations (Ren & Krawetz, 2018; Zhang, 2018). In addition, a combined usage of Coll2-1NO2, CS846, COMP, and urinary CTX-II provided an additional prediction power for early rkOA over the common demographic predictors such as age, BMI, and sex in the study using the Osteoarthritis Initiative (OAI) database (Liem *et al.* 2020a). The new approaches to study molecular alterations in EKOA, especially at the pre-kOA stage, are using epigenomics, transcriptomics, proteomics, and metabolomic and lipidomic platforms, a machine learning method to construct predictive models, and monitoring changes in the chondrocyte secretome (Steinberg *et al.* 2017; Lazzarini *et al.* 2017; Sanchez *et al.* 2017). Recently, a specific panel of serum

autoantibodies was detected at baseline in the subjects developing an emerging rkOA (Camacho-Encina *et al.* 2019).

In conclusion, our results demonstrated that uC2C could be a potential biomarker for early-stage kOA studies. Of course, further clinical validation of uC2C is needed. Availability of well-characterized biomarkers on the 'stage of molecular events' certainly opens up an opportunity for the development of new DMOAD and treating a disease in early stages, where metabolic perturbations are frequently considered as reversible (Chu *et al.* 2012).

6.2. uC2C: an early integrative biomarker of different kOA features in distinct joint compartments

As direct examination of cartilage is not suitable at early-stage, and a performance of multiple radiological or MRI examinations of the knee joint is time-consuming and/or expensive, we here present the data that support a use of uC2C as an early dynamic marker for the evaluation of cartilage status and associated processes in the context of kOA. Investigation of several radiological features together in a model clearly demonstrated that the mechanisms underlying uC2C excretion are complex and are associated with the status of Oph and JSN in both knee joint compartments, TF and PF.

Similar to the present study, the previous publications (Wancket *et al.* 2005; van der Kraan & van den Berg, 2007) showed that Oph formation is an important feature of early OA. Moreover, we found that the uC2C level was associated significantly not only with JSN but also with the status of Oph. Irrespective of the presence of JSN, the radiographic presence of marginal Ophs has a high sensitivity, specificity, and positive predictive value for the presence of MRI-detected cartilage defects in the TF joint and meniscal abnormalities (Boegard *et al.* 1998). Histomorphologically, Ophs appear as fibrocartilage with an admixture of cartilaginous and fibrous matrix components, such as Col2 and aggrecan on the one hand and Col1 on the other hand (Gelse *et al.* 2003). At the same time, osteophytic chondrocytes demonstrate an increased expression of tissue-remodelling enzymes (MMP-9, MMP-13, and hyaluronan synthase 1) (Gelse *et al.* 2012), possibly causing a generation of C2C during endochondral ossification in Oph.

It is well-known that radiographic JSN is an insensitive marker in the assessment of early-stage OA (Favero *et al.* 2015; Gly-Jones *et al.* 2015), and a direct examination of the cartilage status may eliminate a risk of underdiagnosis of early cartilage damage. Arthroscopic investigation of articular cartilage have shown that cartilage damage develops years before radiographic JSN (Kijowski *et al.* 2006). We demonstrated in our similarly aged cohort that the presence of cartilage lesions, indicated by the SFA score, might precede radiographic JSN. Furthermore, we also demonstrated that uC2C excretion is substantially associated with the presence of knee cartilage lesions, as determined by macroscopic examination. This finding is consistent with immunohistochemical

results indicating that areas of cartilage damage significantly increased levels of C2C (Wancket *et al.* 2005). The low values of medial SFA scores were characteristic of the subjects with early stages of kOA in our study.

In addition to cartilage thickness on radiographs, a position and degeneration of the meniscus account substantially for the explained variance in JSN (Hunter *et al.* 2006). Convincing evidence indicates that degenerative meniscal lesion is often suggestive of early-stage kOA (Englund *et al.* 2009). The menisci are structurally analogous to the surfaces of articular cartilage (Andrews *et al.* 2017) and are composed of Col1 as well as Col2 (Kambic & McDevitt, 2005). Therefore, we suggest that in addition to cartilage damage, meniscal degeneration is one of the sources of uC2C during OA development.

In recent years, kOA has been conceptualized as a multicompartmental disease (van der Esch *et al.* 2014). Although PFOA may occur in the absence of TFOA (McAlindon *et al.* 1992), a few studies focused only on the PF compartment. The Nottingham scoring system (Nagaosa *et al.* 2000) has a clear advantage here, as it enables a detailed and standardized radiographic evaluation of both knee compartments.

In our study, the presence of OA in an isolated compartment (TF or PF) was a characteristic of early stages of the disease. In advanced cases, a parallel involvement of both compartments was demonstrated. This aspect complicated the analysis of the association between uC2C and each joint compartment. However, we demonstrated a significant association between uC2C level and TFOA. On the other hand, we just found a tendency toward the association with PFOA, probably due to a small number of isolated PFOA cases. Another possible reason could be the distinct biochemical properties (water and proteoglycan content) of patellar cartilage as compared to the tibial and femoral cartilage (Hinman & Crossley, 2007). Nevertheless, the inclusion of the status of both compartments in the models increased the power of prediction of the uC2C level. Thus, while interpreting the uC2C results, especially in early-stage kOA and when the isolated presence is more common, an influence of the OA processes occurring in both compartments (as sources of uC2C) cannot be ignored. Moreover, not only Oph formation but minor cartilage lesions in different knee compartments may occur in EKOA. A combined use of different kOA features formed the basis of our best prediction model. Prediction of uC2C concentration was the best with the SFA score in combination with Ophs in the TF joint and JSN in PF joint.

Taken together, uC2C could serve as a parameter that integrates several important simultaneously on-going features of EKOA and may reflect an early disturbance of Col2 in any knee joint relevant structure.

6.3. uC2C: a biomarker to monitor a severity of disease

To the best of our knowledge, this study was also the first to show that uC2C production continuously increases with the aggravation of structural changes in kOA. We found that the uC2C level could be used to discriminate between the subjects with early and advanced gOA. Moreover, uC2C levels were higher in rapidly progressive cases of the TKR study cohort (Paper III) as compared to the levels of the same disease severity group of our cross-sectional study subset (Paper I). Thus, high levels of uC2C could help us to assess the Col2 degradation preoperatively as well. This phenomenon clearly contradicts to the old understanding that OA is the disease of 'wear and tear'.

In normal cartilage, matrix turnover is strictly regulated by a delicate balance between synthesis and degradation. In OA, this balance is disturbed, usually with enhanced Col2 degradation and synthesis (Sandell & Aigner, 2001). OA is characterized by an excessive damage of collagen fibrillar network, which appears to be primarily mediated by collagenases, especially, MMP-1 and MMP-13 (Poole *et al.* 2003). Thus, the uC2C level reflects the severity of OA changes and indirectly can reflect an activity of these MMPs (Li *et al.* 2007). In the meta-analysis it was revealed that the uCTX-II levels are positively correlated with the OA severity (Huang *et al.* 2017), supporting the finding of the same alteration in Col2 metabolism.

Considering the results of our studies, we can state that, uC2C is a marker of disease severity and we suggest that it could be useful for monitoring of disease activity. Hopefully, it will be reflected in some possible DMOAD treatment effects in the future. However, the fact needed to be evaluated in clinical studies that a decline in CTX-II did not associate with a slowing of radiographic progression in treatment studies (e.g. biphosphonate) as reported in the previous studies (Bingham III *et al.* 2006). This also underlines a need to expand our understanding about the molecular subtypes of the disease.

6.4. uC2C: a prognostic biomarker of kOA radiographic progression

Our observations revealed that the uC2C levels were positively associated with kOA radiographic progression assessed by the NSy (Nagaosa *et al.* 2000). This result is in line with the previous investigations on other populations, where the same uC2C detection method was used (A. Tamm *et al.* 2013; Poole *et al.* 2016; Kraus *et al.* 2017). Unmatching many previously published studies, the majority of the subjects from our study population were middle-aged and had mild kOA radiographic findings. Therefore, osteophytosis was the main radiographic feature at early grades as well as the main additional finding among the kOA progressors. The NSy allowed a better evaluation of osteophytes and was a clear advantage of our studies. One previous study revealed that the patients having KL grade I on radiographs are at a higher risk of structural progression than the

patients with KL grade 0 (Hart & Spector, 2003). Due to the heterogeneity of both OA-phenotypes and evaluated radiographic signs among the publications, a meta-analysis of the studied biochemical markers was not possible, as clearly mentioned in the systematic review (Hosnijeh *et al.* 2015). A lack of clear consensus on a definition of radiographic progression of OA or clinical endpoint creates a huge challenge for defining and validating the biomarkers.

In contrast with previous studies that focused on the radiographic changes only in the TF compartment, we also assessed the progression in the PF compartment. Therefore, we could evaluate the two different levels of radiographic changes – within-the-same grade and intergrade. We observed that higher uC2C values were associated with minimal radiographic progression of kOA within the same gOA grade. When the process expanded into the other joint compartments of the knee, as expected, uC2C predicted a better over-the-grade progression.

These results are more remarkable for the three-year follow-up period, as it is relatively shorter for evaluating ongoing kOA processes radiographically and the progression of kOA may be nonlinear with intermittent periods of stabilization (Kumm *et al.* 2012). Moreover, we revealed that the predictive value of uC2C was less pronounced in more advanced kOA (grades 2 and 3). Although this result may be explained because of a smaller size of the advanced kOA group, it may be a characteristic feature for uC2C, as a biomarker.

Several other biomarkers were associated with the progression of OA and are mentioned in the literature. Out of these the most promising were, cartilage-derived Col2 markers and aggregan markers (Bay-Jensen *et al.* 2022). Recently, a predictive validity of 18 biomarkers was investigated in a large USA biomarker project (FNIH study) and several Col2 synthesis (e.g. PIIANP) or degradation markers (e.g. CTXII) were found associated with two-year radiographic progression of OA (Kraus *et al.* 2017). The PIIANP assay targets type IIA form (embryonic) of the splice variant of N-terminal propeptide of Col2 and characterizes the Col2 syntesis (Rousseau *et al.* 2004). The low level of Col2 synthesis predicted the progression of JSN or a combination of pain and JSN progression (Kraus *et al.* 2017). In addition, OA progression is associated with elevated Col2 degradation markers, mainly CTXII. Eight catabolic biomarkers including uC2C, were shown as the best predictors of pain and radiographic progression over two years. The combination of the eight best biomarkers resulted in the best model for progression including uCTXII, sHA, and serum NTXI. However, the prediction power of the best model was relatively low (AUC up to 0.668), possibly due to the heterogeneity of the kOA study group.

In addition, OA-associated cofounders must be taken into account – an increase in BMI slightly magnified the risk of kOA progression. This finding seems to be consistent with other research studies, which reported that the individuals with higher BMIs had a higher rate of osteophyte progression (Zhu *et al.* 2018).

In summary, the uC2C values appear to be dependent on at least two factors – severity of gOA grade and the existence of radiographic kOA

progression. It is therefore likely that for estimating a risk of kOA progression, the baseline uC2C level in gOA grade must be considered; in the same stage of radiographic kOA, uC2C was found higher in progressors than in non-progressors.

However, further studies are warranted to clarify clinical implications of uC2C.

Genetic markers of OA have not been addressed in this work because they form a completely separate and very large topic, which is not associated directly with the thesis. However, an interesting topic in OA research is opened with small RNA molecules. MicroRNAs, small and stable noncoding RNA molecules, have emerged as powerful candidates for biomarkers in musculoskeletal disorders because of their impact on the expression of tissue proteins. (Ali *et al.* 2022). MicroRNA profiling, also called as 'liquid biopsies' identified that miR-320 family members are associated with fast-progressing radiographic kOA in the Osteoarthritis Initiative cohort.

6.5. uC2C: a biomarker to monitor pre-TKR and post-TKR response

To date, only a limited number of studies have investigated the behaviour of biomarkers after TKR – probably due to the understanding that the operation is the 'end-stage' of OA. Our study with the TKR cohort is the first to present the dynamics of uC2C levels in the subjects in pre- and post-TKR periods. Surprisingly, the uC2C level of the whole group was still high (not decreased) after three months of TKR but reverted to the baseline level after one year of the operation. Thus, the postoperative uC2C levels remained relatively high and did not drop to the level of the subjects without kOA or with EKOA. However, we found that the individual dynamics of uC2C levels were rather heterogeneous, this means, some patients had an increase, while others had a decrease.

To our knowledge, four similar studies evaluating the dynamics of cartilage biomarkers in serum (keratan sulfate, COMP, and Col2-derived fragments Coll2–1 and Coll2–1NO2) were performed in TKR patients (Sweet *et al.* 1988; Sharif *et al.* 2004; Deberg *et al.* 2008; Endres *et al.* 2020). A quite old study by Sweet *et al.* reported a short preliminary decrease in keratan sulphate levels one week after TKR, which subsequently increased up to preoperative levels after six months (Sweet *et al.* 1988). Sharif *et al.* demonstrated a substantial rise in serum COMP levels after TKR in all patients, persisting up to 12 months following the surgery (Sharif *et al.* 2004). Recently, Endres *et al.* showed a short reduction in COMP levels after a week of total hip replacement; later on, these levels returned to the preoperative baseline (Endres *et al.* 2020). These results were unexpected, as a decrease in COMP levels was expected, and the authors were forced to consider a possibility that COMP could also originate from the sources other than the operated joint alone. It was assumed that there might be an increased production or degradation of COMP in the contralateral

knee, or an increased release of COMP from other tissues – such as tendons, ligaments, and capsules – of the TKR joint. Endres *et al*. attributed the results to changes in physical activity – patients with recovery were much more mobile than pre-TKR. Similarly, a study of Coll2–1 and Coll2–1NO2 showed that the pathogenic metabolism of Col2 persisted after TKR (Deberg *et al.* 2008).

Summarizing the conclusions from these few studies on variations in biomarkers after total joint replacement, the level of several degradation biomarkers (e.g., uC2C, Coll2–1NO2, COMP) do not decrease after joint replacement. Thus, in general, there is scope for interpretation that TKR does not stop the breakdown of Col2 and possible synovitis (as reflected by COMP levels). It has been suggested that a sustained level of Col2 degradation markers after TKR can be attributed to a persistent Col2 degradation in different structures of a synovial joint, although a presence of OA lesions in other joints cannot be ruled out and could be considered as a secondary source. We presented a quite common involvement of both knees (in a quarter of cases); however, we found no associations between uC2C levels and previous history of TKR.

Nevertheless, OA has been proposed as a systemic disease (Visser *et al.* 2015), and its burden can be thought of in terms of severity and the number of joints involved (Henrotin *et al.* 2007). However, in a cross-sectional study (Paper I) we found that the uC2C level did not differ between unilateral and bilateral OA cases. Moreover, we did not prove that the uC2C level is significantly influenced by the involvement of other joints assessed by pain. Of course, a more detailed investigation – not just of pain – is needed to measure the total burden of OA (Addison *et al.* 2009). In addition, several authors have demonstrated that rather a large proportion of kOA patients may have a generalized joint disease (Stürmer *et al.* 1998; Kraus *et al.* 2010).

In conclusion, though we did not find a significant reduction in the uC2C level after 12 months of TKR, our study can be considered as the first attempt to investigate the behaviour of uC2C in monitoring the treatment effect of kOA.

6.6. uC2C: a predictive biomarker for clinical outcome of TKR

An improvement in quality of life and knee function is expected after TKR, however, a great heterogeneity is seen. Here, we demonstrated only a partial improvement in KOOS subscales after 1 year of TKR. This was particularly apparent in terms of restrictions in the use of legs for more demanding functions (expressed by KOOSsp/recr). Thus, a substantial proportion of these patients require post-surgery rehabilitation – especially exercise therapy – also years after TKR.

We demonstrated that higher preoperative uC2C level could predict the improvement in the KOOSsymp scale after surgery in women (OR > 9). There is no good explanation for why the reduction of pain did not associate with a drop in uC2C, but the pain and other symptoms of kOA probably present different

aspects of the disease. A possible explanation is that pain in OA could be multifactorial (Neogi, 2013), and may arise from multiple structural changes in the knee joint (Yusuf *et al.* 2011).

Interestingly, we presented that the preoperative uC2C values could predict its postoperative status; this means, if the preoperative value was low, Coll2 degradation had become more intense, and vice versa was true for high preoperative values. Likewise, in the patients with preoperative Coll2–1NO2 (a biomarker of oxidative damage) levels above the median showed a significant and progressive decrease postoperatively but, they tended to increase in the patients with preoperative Coll2–1NO2 values below the median (Deberg *et al.* 2008). If this phenomenon is confirmed by other studies, there may be an opportunity for an additional grouping of the patients undergoing TKR surgery. The possibility of a more precise categorization of patients became apparent here.

In total, this observation may support the hypothesis that the postoperative change in uC2C levels depends on its preoperative level.

6.7. Sex-related differences of uC2C level in the course of kOA

Although there was no general difference found in the uC2C excretion between the sexes in the cross-sectional study, several special sex-based features of uC2C in the incidence and course of kOA were noted.

At first, the mean value of uC2C in the male non-progressors was significantly higher than the female non-progressors, including the results in the long-term control group. This finding contrasts with a large FNIH/OARSI biomarker consortium study suggesting no effect of sex on reference levels of uC2C (Kraus *et al.* 2017). The reason for this contradiction is unclear, but it might be related to different subject characteristics, especially age, which was significantly lower in our study. Moreover, higher BMI in the progressors as compared to the non-progressors females was the characteristic of our longitudinal study.

Secondly, the best model for uC2C prediction emerged by a combination of Ophs and JSN, and especially in TFOA; it was almost 2-fold better in females than in males. Moreover, a combination of the radiographic signs like, Ophs and JSN were more common in females. In addition, we demonstrated that uC2C is a promising prognostic marker for emerging kOA in females but not in males; a gradual increase of uC2C level was associated with the radiological grades only in females, except the male progressors with more severe kOA grades.

Thirdly, we observed that the preoperative uC2C values could predict the postoperative trends more prominently in females than males. We found that low preoperative value was probably related to intense Col2 degradation in postoperative period, and vice versa was true for high preoperative values. Unlike in males, there was a significant decrease in the uC2C levels in females after one year of TKR.

Finally, the preoperative uC2C levels could predict an improvement in the KOOS symptoms score only in females. At the same time, the KOOS pain score was correlated with the uC2C value in men during the preoperative period.

The sex-related differences in the course of OA and probably in its pathogenesis, should be highlighted. Albeit sex-based differences in OA have been reported, they might be often overlooked by researchers (Boyan *et al.* 2013; Bihlet *et al.* 2019). Women are associated with a higher prevalence and severity of OA as compared to men, particularly following menopause (Srikanth *et al.* 2005). Gender discrepancies may be caused due to differences in hormones, bone strength, alignment, ligament laxity, and neuromuscular strength (Johnson & Hunter, 2014). A systematic review of risk factors for the onset of knee pain showed that one of the strongest factors associated with knee pain was female sex (Silverwood *et al.* 2015). A recent study in the Estonian Early Knee OA Study Cohort demonstrated a presence of significant sex-dependent differences in cytokine production, with predominant angiogenesis in females with grade 1 kOA, whereas activation of tissue remodelling was predominated in males (Kisand *et al.* 2018), indicating sex-related differences in the pathways of kOA pathogenesis. Recognition of the sex-based differences related to uC2C may aid the assessment of the efficacy of novel therapeutic agents that directly suppress MMP gene expression (Malemud, 2019) or angiogenesis (Hamilton *et al.* 2016). Nevertheless, further studies with larger sample sizes are needed to clarify the role of sex-related differences in uC2C throughout the course of OA.

According to these data, we can infer that the uC2C level behaves differently in males and females in kOA and could be a suitable kOA marker for females exclusively.

6.8. Limitations of the study

The study had several limitations. Main limitation was a small number of subjects in some sub-groups, which decreased a statistical power of inter-group comparison and models and could have reduced the chance of detecting a true effect of the results. It prevented drawing definitive conclusions about the utility of uC2C in some special aspects of kOA. Firstly, SFA scores and uC2C concentrations were determined simultaneously in a small number of cross-sectional study subjects, therefore a statistical power of the models including SFA determination was limited (Paper I). Secondly, a prevalence of the cross-sectional study subjects with isolated OA changes, especially JSN, was low (Paper I). Moreover, we had a limited number of cases with exclusive JSN progression; thus, we could indirectly interpret that the increased level of uC2C reflected the risk of subsequent cartilage damage (Paper II). Despite this, the association of a high level of uC2C with growing Oph is considered a prognostic of the progression of kOA. Thirdly, a low prevalence of the individuals with advanced kOA cases (gOA grade 2–3), especially in separate gender groups, complicated the conclusions at this stage of the disease. (Paper II, III). In order to confirm the results, future work should evaluate uC2C in

several directions such as, using a larger population cohort with a higher prevalence of advanced kOA cases and recording the observations at intermediate timepoints with different imaging modalities. Additionally, an observation period more than three years would be required. Moreover, although the TKR study group was sufficient in number of subjects to allow a general assessment of uC2C behaviour in surgical patients, a larger cohort is needed for the evaluation of sex-related differences and additional relationships in the pathogenesis of kOA (Paper III). Also, one year may be too short period to assess far-reaching changes in OA biomarkers after TKR.

Next, we exclusively investigated uC2C as a single biomarker for an integration of several possible signs of kOA (Paper I-III) and did not measure the Col2 synthesis at the same time; therefore, we could not evaluate a balance between the synthesis and degradation of Col2. A combination of various biomarkers with uC2C would probably improve a prediction of the disease progression (Kraus *et al.* 2017). Investigation of a different set of biomarkers would help to explore certain phenotypes of OA (Mobasheri *et al.* 2019a; van Spil *et al.* 2019).

The following limitation should be considered regarding the adjustments. We adjusted the LM and GLM models for age, sex, and BMI as generally accepted OA risk factors, but not for the other possible confounders, e.g. menopausal status (Paper I-III). As uC2C was also observed in deep calcified cartilage (Wancket *et al.* 2005), it may be influenced by menopause like the markers of bone turnover.

Finally, although we used sumVAS for the assessment of pain in other joints (excluding the knee joint) (Paper II, III), the presence and progression of osteoarthritis at other sites were not excluded because the radiographs of other joints were not available (Paper I-III). It could cause confounding effects with regard to uC2C levels.

In summary, although the findings of our study demonstrate the potential roles of uC2C as a risk, diagnostic and prognostic marker, further validation and qualification are needed for its clinical use (*in vivo* diagnostics). In future studies, sex-specific differences in the pathogenesis of kOA should also be addressed.

7. CONCLUSIONS

- uC2C is an integrative marker for kOA; it is simultaneously associated with the main pathological processes of OA like, cartilage degradation and osteophytes (Ophs) formation development in the PF and TF compartments.

  By regression analysis, we demonstrated that Oph describe uC2C level better than joint space narrowing (JSN). However, the best prediction was achieved by a combination of both radiographic features assessed separately in TF and PF joints, in the model. Replacement of radiographic TF JSN with macroscopically assessed cartilage lesions of the arthroscopy (SFA score) further improved the prediction power of the model for the uC2C level.

- uC2C is a good candidate for the development of an early diagnostic test for kOA.

  We have shown that an increase in uC2C concentration already exists in the early stages of the disease (radiographic grade 1 by NSy), which is generally considered as a pre-radiological stage according to the KL evaluation system.

- uC2C is a potential kOA risk prediction marker in females.

  Generalized linear models (GLM) analysis showed that a higher baseline value of uC2C is an excellent predictor of kOA initiation in women (gOA 0 becomes gOA 1) over the follow-up of 3 years. The best prediction value for the model (> 90%) was obtained by comparing the emerging kOA group with the long-term (12 years) control group.

- uC2C is higher in progressors than in non-progressors in the same radiographic severity stage kOA. For a proper clinical evaluation of uC2C, two aspects are important: 1) uC2C levels are positively associated with severity of kOA (uC2C is higher in higher radiographic grades); and 2) higher uC2C is associated with ongoing disease progression, particularly in females. The values of uC2C are highest in rapid progressors like pre-operative TKR cases.

  We found that median uC2C values gradually elevate with the gOA grade, indicating a moderate diagnostic performance at each grade as compared to the previous grade. At each severity stage, a higher value of uC2C predicts the progression of kOA over the next 3 years. We found that uC2C is a sensitive marker of progression; this means, it predicts minimal radiographic changes within the same gOA grade like the addition of Oph or the worsening of JSN in any compartment of the knee joint.

- Following total knee replacement (TKR), the dynamics of uC2C are quite heterogeneous; excretion of uC2C may decrease, increase, or can remain unchanged.

  We found that relatively higher baseline uC2C values were associated with a declining trend after TKR, and in contrast, the degradation of Col2 was accelerated after surgery in the subjects with low preoperative uC2C values. Thus, TKR could not stop or reduce the degradation of Col2 in the majority of cases. Baseline uC2C was also shown to predict postoperative improvement in KOOS symptom scores in females. We found no association between uC2C and pain score.
- uC2C appears as a better diagnostic and prognostic biomarker in females than in males.

  We found that uC2C predicts the progression of kOA more accurately in females than in males; female uC2C values showed a positive correlation with radiographic severity grade. In males, this association was weak and less significant on the statistical scale. However, it should be noted, that the sample size of the male group was relatively smaller and might be a reason for low statistical power. Therefore, these results need to be confirmed on a larger sample size and different populations.

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9. SUMMARY IN ESTONIAN

II tüüpi kollageeni neoepitoop C2C uriinis kui põlve osteoartriidi diagnoosimise ja kulu prognoosimise biomarker

Osteoartriit (OA) on tõsine haigus – kõige sagedasem lihasskeleti häire ja olulisim puude põhjustaja eakatel (Allen *et al.* 2021). OA haarab ligi 500 miljonit inimest maailmas (Vos *et al.* 2020), haigestumus suureneb umbes 0,3% aastas (Z. Jin *et al.* 2020). Põlveliiges on üks peamisi OA-st tabatud liigestest. Haigus võib kahjustada ühte või mõlemat põlve, kuid on kirjeldatud ka hulgiliigeste OA-d (Sharma, 2021). Põlve OA (pOA) võib haarata eraldi või mõlemat liigese funktsionaalset osa: tibiofemoraal- (TF) ja patellofemoraalliigest (PF) (Brandt *et al.* 2003).

Kuigi OA-le viitavad tunnused on avastatud juba küttide-korilaste skelettidel, siis haiguse levimuse kiire, kuni kahekordne kasv on toimunud alates 20. sajandi keskpaigast (Wallace *et al.* 2017; Berenbaum *et al.* 2018). OA tohutu sagenemise põhjuseks peetakse selliste teadaolevate riskifaktorite osakaalu tõusu nagu kõrge iga (rahvastiku vananemine), ülekaal ja rasvumine, vähene liikumine ning liigesevigastused.

Varasemalt on OA-d peetud liigese degeneratiivseks haiguseks, kõhre „kulumiseks“ või hüpertroofiliseks artriidiks (Mobasheri *et al.* 2021). Kaasaja käsitluse alusel on OA patofüsioloogia kompleksne, haarates mitmeid liigesekudesid (kõhr, luu, sünoovium, menisk, ligament) molekulaarsetest muutustest kuni kudede struktuuri muutusteni (Lv & Shi, 2021). OA on etioloogiliselt heterogeenne hulgiteguriline haigus, mis kujuneb geneetiliste, mehaaniliste, metaboolsete ja põletikuliste faktorite kombineerumisel (Johnson & Hunter, 2014). Arvatakse, et OA tuleneb liigesekudede lammutus- ja paranemisprotsesside tasakaalustamatusest, mis viib liigese hävimiseni (Deveza & Loeser, 2018; Hunter & Bierma-Zeinstra, 2019).

2015. a esitas rahvusvaheline OA uurimise ühing OARSI OA definitsiooni, mille kohaselt „haigus avaldub algselt molekulaarse ümberkorraldusega (liigesekudede ainevahetuse häirumine), millele järgnevad anatoomilised ja/või füsioloogilised ümberkorraldused (kõhre lammutamine, luu remodelleerumine, osteofüütide moodustumine, liigese põletik ja liigesefunktsiooni häirumine), mis võib kulmineeruda haigustunnustena“ (Kraus *et al.* 2015). See definitsioon juhib tähelepanu vajadusele diagnoosida OA-d juba presümptomaatilises, nn molekulaarses staadiumis. Arvatakse, et haigust modifitseerivate ravimite toime on kõige efektiivsem just haiguse varajastes staadiumites enne tõsiste struktuursete muutuste teket.

Peamised pOA-ga seotud radiograafilised tunnused on liigesepilu kitsenemine (LPK) ja luulised muutused, sh osteofüütide (OF) teke, kuid need väljenduvad alles haiguse hilisemas faasis ja on aeglase dünaamikaga (Altman & Gold, 2007). Molekulaarsed markerid võiksid olla tundlikumad ja dünaamilisemad kudede ainevahetuse markerid, peegeldades rohkem haiguse aktiivsust ja seeläbi ka haiguse progressiooni kiirust (Karsdal *et al.* 2010; Siebuhr *et al.*

2014a; Kraus & Karsdal, 2021). Kuna OA võib kulgeda varajases järgus kaua asümptomaatilisena, hoiatavad kudede ainevahetuse aktiivsust peegeldavad molekulaarsed markerid varakult koekahjustuse tekke eest ning seega võimaldavad OA varajasemat kliinilist diagnoosi (Kraus *et al.* 2011; Kraus & Karsdal, 2021). Samas on OA ebaühtlaselt progresseeruv haigus, mille puhul stabiilsemad perioodid vahelduvad kiiremate muutustega (Kumm *et al.* 2012; Kumm *et al.* 2013a). Seetõttu on molekulaarsed biomarkerid olulised ka haiguse kulu ja ravivastuse hindamisel (Kraus *et al.* 2011).

Kuna II tüüpi kollageen (Col2) on kõhre peamine struktuurne komponent, on OA mitteinvasiivseks ja objektiivseks hindamiseks välja töötatud üsna palju analüüse mitme erineva Col2 fragmendi kohta (uCTX-II, Coll2, Coll2-1, C1,2C jne) (Bay-Jensen *et al.* 2022). Ükski pOA molekulaarne marker ei ole veel piisavalt valideeritud kliiniliseks kasutamiseks (st ei ole saanud *in vitro* diagnostika märgist). Käesolevas uurimuses hindasime hiljuti tähelepanu pälvinud OA biomarkeri – II tüüpi kollageeni (Col2) lõhustumise neoepitoobi uriinis (uC2C) (Poole *et al.* 2016; Kraus *et al.* 2017) – kasutusvõimalusi pOA korral.

Uurimuse eesmärk ja hüpoteesid

Üldine eesmärk oli hinnata uC2C-d ja selgitada selle molekulaarse biomarkeri potentsiaali pOA diagnoosimisel ja kulu prognoosimisel haiguse erinevates staadiumites.

Töö hüpoteesid olid:

1. uC2C on radiograafilise pOA varajase staadiumi biomarker.
2. uC2C peegeldab samaaegselt OF-de ja LPK esinemist põlveliigese eri osade, TF-liigese ja PF-liigese haaratuse korral.
3. Sama radiograafilise pOA staadiumi korral on uC2C väärtus kõrgem isikutel, kellel haigus progresseerub.
4. Põlve asendusoperatsioon vähendab uC2C taset 12 kuud pärast operatsiooni. uC2C ja põlvespetsiifilise küsimustiku KOOS skooride muutus pärast operatsiooni sõltub nende operatsioonieelsest tasemest.
5. pOA ajal tekkivad uC2C muutused (biomarkeri dünaamika) on meestel ja naistel erinevad.

Töö spetsiifilised ülesanded:

1. Määrata uC2C tase tervetel isikutel ja haigetel varajases (preradiograafilises) ja väljakujunenud pOA staadiumis ning analüüsida uC2C seoseid haiguse erinevate radiograafiliste tunnustega (OF-d ja LPK) TF- ja PF-liigestes.
2. Hindamaks biomarkeri võimet ennustada pOA teket, võrrelda uC2C algtasemeid kolmel uuritaval rühmal: 1) kontrollrühma isikud, kellel ei tekkinud 12 a jooksul pOA-d; 2) isikud, kellel ei teki 3 a jooksul pOA-d; 3) isikud, kellel tekib 3 a jooksul pOA.
3. Võrrelda uC2C baastaset pOA eri staadiumides olevatel progresseeruva haigusega ja mitteprogresseeruva haigusega isikutel.

4. Määrata operatsioonieelne uC2C väärtus ja biomarkeri dünaamika 12 kuu jooksul pärast põlve asendusoperatsiooni.
5. Võrrelda uC2C algväärtusi operatsioonijärgse KOOS-skoori muutusega, et hinnata biomarkeri võimet ennustada operatsiooni subjektiivset tulemit.
6. Võrrelda eelnevate ülesannete tulemusi meestel ja naistel võimalike sooliste erisuste tuvastamiseks.

Uuritavad isikud ja meetodid

Uurimuses kasutasime valimeid kolmest erinevast kohordist: Eesti varajase põlveliigese OA uuringu, artroskoopia ja põlveliigese täieliku asendamise kohordist. Kahe esimese kohordi uuritavad kuulusid nii läbilõikelise kui ka longitudinaalse uuringu valimitesse. Mõlema kohordi korral koguti igas uuringupunktis demograafilised ja kliinilised andmed, sh patsiendipõhised hinnangud: põlvespetsiifiline küsimustik KOOS (0–100% skaalal) ja valu subjektiivne hinnang (visuaalse analoogskaala (VAS) järgi, valuskoor 0–10). Lisaks põlvedele hinnati ka teisi liigeseid. Uuritavatele tehti mõlema põlve röntgenuuring, millel hinnati eraldi tibiofemoraalse (TF) ja patellofemoraalse (PF) osa OA muutusi (liigesvahemiku kitsenemist (LPK) ja osteofüüte (OF)) neljaastmelisel skaalal (astmed 0–3) Nottinghami süsteemi järgi (Nagaosa *et. al,* 2000). Mõlema põlve haaratuse korral võeti uuritavaks suurema OA raskusastmega põlv.

Põlve asendusoperatsiooni kohordi moodustasid pOA lõppstaadiumis olevad patsiendid (vanus <70 aastat), kellele tehti TÜ kliinikumi ortopeedia osakonnas esmane ühepoolne täielik põlveliigese asendusoperatsioon. Uuritavatelt koguti demograafilised ja kliinilised andmed ning uriiniproovid kolmes ajapunktis: 1–2 päeva enne operatsiooni, 3 kuud ja 12 kuud pärast operatsiooni. Kliinilise seisundi hindamiseks kasutati KOOS ja SF-36 küsimustikke ning valu visuaalset analoogskaalat, funktsionaalseid sooritusteste ja põlvede röntgenuuringuid. pOA preoperatiivseks radiograafiliseks hindamiseks kasutati kahte hindamissüsteemi: Nottinghami süsteemi ja klassikalist Kellgren-Lawrence'i (KL) süsteemi. KL-i korral tehti pOA raskusastme viieastmeline klassifikatsioon (astmed 0–4). Hinnati OF, LPK-d, subkondraalse luu sklerootiliste seintega pseudotsüstiliste piirkondade ja luuotste muutunud kuju raskusastmeid. KOOS-i muutusena määratleti muutumine rohkem kui ±20 ühiku võrra ühe aasta jooksul pärast põlveoperatsiooni.

uC2C määrasime 3 valimis järgmiselt:

1. Läbilõikeuuringusse kaasati 302 uuritavat, kellelt oli kogutud uriiniproov. Neist 14 uuritaval (5 mehel ja 9 naisel) hindasid ortopeedid artroskoopia ajal põlveliigese kõhre seisundit visuaalselt Outerbridge'i (modifitseeritud) süsteemi järgi. Kahjustuse pindala ja sügavuse põhjal arvutati Société Francophone d'Arthroscopie (SFA) skoor (skaala 0–100).
2. Longitudinaaluuringu valimisse kuulus 330 isikut, keda uuriti kahes ajapunktis: algpunktis ja järelkontrollis kolm aastat hiljem (keskmine

jälgimisperiood 38±5 kuud). uC2C määrati algpunktis kogutud uriinis. pOA progresseerumist hinnati algus- ja jälgimispunkti radiograafiliste leidude võrdlemise alusel. Progresseerujate rühma moodustasid 105 uuritavat, kellel esinesid põlveliigese radiograafilise OA progresseerumise tunnused (OF-d ja/või LPK) 3 a jooksul. See rühm jagunes omakorda kolmeks alarühmaks: (a) tekkiva pOA rühm, kuhu kuuluvatel isikutel algtasemel pOA radiograafilised tunnused puudusid, kuid need tekkisid 3 a jooksul; (b) minimaalsed progresseerujad (n=29), kellel radiograafilised tunnused süvenesid sama radiograafilise astme piires (OF-de või LPK lisandumine või suurenemine teises liigeseosas); (c) astme võrra progresseerujad (n=76), kellel radiograafiline aste süvenes vähemalt ühe astme võrra.

3. Operatsiooni prospektiivse uuringu valimisse kuulusid 86 uuritavat põlveliigese asendusoperatsiooni kohordist (n=105).

uC2C kontsentratsioon määrati hommikuses teises uriiniproovis *sandwich*-tüüpi ELISA-ga (IB-C2C-HUSA™, IBEX Pharmaceuticals Inc., Montreal, Quebec, Kanada). Uriini lahjendusteguri arvessevõtmiseks väljendasime tulemused C2C kontsentratsiooni ja kreatiniini suhtena (mõõdetud samast uriiniproovist).

Kliiniliste andmete (vanus, sugu, KMI jt) statistilisel töötlemisel kasutasime hii-ruudu testi, t-testi ja ANOVA meetodit. uC2C kontsentratsioonide seoseid analüüsisime mitteparameetriliste testidega (Kruskal-Wallise test, Mann-Whitney U-test). Segavate faktorite (vanus, sugu, KMI) arvesse võtmiseks kasutasime regressioonanalüüsi.

Uuring oli kooskõlastatud Tartu Ülikooli inimuuringute eetikakomiteega.

Tulemused ja järeldused

- uC2C on integreeriv pOA marker ja on seotud haiguse mitme põhiprotsessiga: kõhre lammutamise ja osteofüütide moodustumisega emmaskummas põlveliigese osas – PF- ja TF-liigestes.

  Demonstreerisime regressioonanalüüsiga, et OF-id kirjeldavad uC2C väärtust paremini kui LPK, kuid uC2C tase on kõige paremini prognoositud, kui mudelisse lisada mõlemad OA radiograafilised tunnused, hinnatuna põlveliigese mõlemas osas eraldi (TF- ja PF-liiges). Mudel ennustab uC2C taset veelgi paremini, kui selles asendada radiograafiline tunnus (TF-liigese LPK) artroskoopias hinnatava liigeskõhre makroskoopilise kahjustuse näitajaga (SFA skoor). Arvame, et mudelit võiks veelgi parandada meniski leid, kuid selle tõestuseks on vaja täiendavaid uuringuid.
- uC2C on hea kandidaat pOA varajase diagnostilise testi väljaarendamiseks.

Näitasime, et uC2C kontsentratsiooni tõus uriinis esineb juba haiguse varajases staadiumis (radiograafiline aste 1), mida paljud peavad haiguse preradioloogiliseks staadiumiks.

- uC2C on naistel võimalik pOA riskimarker.

  Analüüs üldistatud lineaarsete mudelitega (GLM) näitas, et C2C kõrgem algväärtus ennustab naistel väga hästi pOA teket (aste 0 muutub astmeks 1) järgneva 3 a jooksul. Parim mudeli ennustusväärtus (>90%) ilmnes tekkiva pOA grupi võrdlemisel pikaajalise (12 aastat pOA muutusteta) kontrollrühmaga.

- uC2C hindamisel on olulised kaks aspekti: 1) uC2C tase on seotud pOA raskusastmega (uC2C on kõrgem suuremates astmetes); 2) kõrgem uC2C on seotud haiguse käimasoleva progressiooniga (eeskätt kehtib see naiste kohta). uC2C väärtused on kõige kõrgemad kiiresti progresseeruva haigusega isikutel enne TKR kirurgiat.

  Leidsime, et uC2C mediaanväärtused kasvavad järk-järgult koos gOA astmega, näidates igas astmes mõõdukat diagnostilist võimekust eelmise astme suhtes. Iga astme väärtuste hulgas ennustab uC2C kõrgem väärtus pOA progressiooni järgneva 3 a jooksul. Lisaks tuvastasime, et uC2C on tundlik progressioonimarker: see ennustab minimaalseid radiograafilisi muutusi sama summaarse radiograafilise astme sees, milleks võib olla OF-de lisandumine või LPK süvenemine põlveliigese mistahes osas ilma astme muutuseta.

- Pärast põlveliigese asendamist on uC2C dünaamika üsna heterogeenne: uC2C eritumine uriiniga võib väheneda, suureneda või jääda muutumatuks.

  Leidsime, et suhteliselt kõrgemad uC2C algväärtused seostuvad operatsioonijärgse langustrendiga ja vastupidi: Col2 lagunemine kiirenes peale operatsiooni madalate operatsioonieelsete uC2C väärtustega isikutel. Seega ei peata liigeseasendus paljudel juhtudel Col2 lagundamist organismis. Samuti selgus, et uC2C algväärtus ennustab operatsioonijärgset KOOS sümptomite skoori paranemist naistel, kuid me ei leidnud seost uC2C ja valu skoori vahel.

- uC2C näib olevat naistel võrreldes meestega parem diagnostiline ja prognostiline biomarker.

  Me leidsime, et uC2C ennustab pOA progressiooni teket naistel täpsemini kui meestel ning naiste uC2C väärtused suurenevad koos haiguse raskusastme süvenemisega. Meestel olid nimetatud seosed biomarkeriga nõrgemad ja statistiliselt vähem olulised. Peab siiski mainima, et uuritud meesterühmade suurus oli suhteliselt väike, mistõttu tegemist võib olla statistilise analüüsi väikese võimsusega ja need tulemused tuleb üle kontrollida suuremal valimil.

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PUBLICATIONS

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Publications

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