DISSERTATIONES MEDICINAE UNIVERSITATIS TARTUENSIS
200

DISSERTATIONES MEDICINAE UNIVERSITATIS TARTUENSIS
200

JAANIKA KUMM

Molecular markers of articular tissues in early knee osteoarthritis: a population-based longitudinal study in middle-aged subjects

UNIVERSITY OF TARTU
PRESS
1632

Department of Internal Medicine, University of Tartu, Estonia

This dissertation is accepted for the commencement of the degree of Doctor of Philosophy on 6th of July 2012 by the Council of the Faculty of Medicine, University of Tartu, Estonia.

Supervisors: Agu Tamm, MD, DMSci, Professor
Department of Internal Medicine, University of Tartu, Estonia

Kalervo Väänänen MD, PhD, Professor
Department of Cell Biology and Anatomy,
Institute of Biomedicine, University of Turku, Finland

Reviewers: Riina Kallikorm MD, PhD, Associate Professor
Department of Internal Medicine, University of Tartu, Estonia

Kai Kisand MD, PhD
Institute of General and Molecular Pathology,
University of Tartu, Estonia

Opponent: Ingemar Petersson MD, PhD, Professor
Departments of Orthopedics and Rheumatology,
Skåne University Hospital, Lund, Sweden

Commencement: 19th of October, 2012

This study was supported by the European Union through the European Social Fund.

ISSN 1024–395X
ISBN 978–9949–32–106–3 (print)
ISBN 978–9949–32–107–0 (pdf)

University of Tartu Press
www.tyk.ee
Order No. 416

TABLE OF CONTENTS

I. LIST OF PUBLICATIONS

I. Kumm J, Tamm A, Veske K, Lintrop M, Tamm A. Associations between cartilage oligomeric matrix protein and several articular tissues in early knee joint osteoarthritis. Rheumatology 2006; 45(10):1308–1309.

II. Kumm J, Ivaska KK, Rohtla K, Väänänen K, Tamm A. Urinary osteocalcin and other markers of bone metabolism: the effect of risedronate therapy. Scand J Clin Lab Invest 2008; 68(6):459–63

III. Kumm J, Tamm A, Lintrop M, Tamm A. The prevalence and progression of radiographic knee osteoarthritis over 6 years in a population-based cohort of middle-aged subjects. Rheumatol Int 2011, Nov 16; doi: 10.1007/s00296–011–2221–3

IV. Kumm J, Tamm A, Lintrop M, Tamm A. Association between ultrasonographic findings and bone/cartilage biomarkers in patients with early-stage knee osteoarthritis. Calcif Tissue Int 2009; 85: 514–522

V. Kumm J, Tamm A, Lintrop M, Tamm A. The value of cartilage biomarkers in progressive knee osteoarthritis: cross-sectional and 6-year follow-up study in middle-aged subjects. Rheumatol Int 2012, Jul 21; doi: 10.1007/s00296–012–463–8

VI. Kumm J, Tamm A, Lintrop M, Tamm A. Diagnostic and prognostic value of bone biomarkers in progressive knee osteoarthritis: a 6-year follow-up study in middle-aged subjects. Submitted.

Personal contribution

Jaanika Kumm was involved in the study planning, subject recruitment, obtainment of questionnaire data, collection of serum and urinary samples and radiographic data for all the papers. She performed laboratory measurements, statistical analysis and writing of all the papers.

2. ABBREVIATIONS

BMI	body mass index
JSN	joint space narrowing
JSW	joint space width
KL	Kellgren-Lawrence grading system
MMP	matrix metalloproteinase
MRI	magnetic resonance imaging
OA	osteoarthritis
Oph	osteophytes
PF	patellofemoral
ROI	region of interest
sCOMP	serum cartilage oligmeric matrix protein
sCTx-I	serum C-terminal cross-linked telopeptides of type I collagen
sOC	serum osteocalcin
sPINP	serum procollagen type I amino-terminal propeptide
sPIIANP	serum type II A procollagen amino-terminal propeptide
TF	tibiofemoral
uCTx-II	urinary C-telopeptide fragments of type II collagen
uMidOC	urinary midfragments of osteocalcin
US	ultrasonography

3. INTRODUCTION

Osteoarthritis (OA) is the most common form of arthritis and is still one of the few chronic diseases of the elderly for which there is little, if any, effective treatment [1]. It accounts for more mobility disability in the elderly than any other disease. The prevalence of symptomatic knee OA has ranged in different studies from 1.6–9.4% of adults and 10–15% of the elderly [2–3]. Since incidence and prevalence increase with age, longer life expectancy suggests even further increase in OA in the future [1].

OA has turned out to be a multifaceted and far more complex disease than initially believed. Nowadays, it is increasingly recognized that OA is a disease of the whole joint that affects all articular structures, including articular cartilage, subchondral bone, synovium, tendons, ligaments and menisci [4]. So far, the role of bone and articular soft tissues in the pathophysiology of OA has been widely overlooked. It is also not clear in whom the disease is likely to progress. It is assumed that the course of OA may not be constant but change over time. As the vast majority of studies on OA have been conducted on patients who have already reached end-stage disease, there is a significant lack of knowledge concerning the early phases of OA.

It is logical that subtle biochemical changes in articular cartilage and other joint tissues precede any macroscopic or radiographic evidence of joint destruction. Therefore, in recent years, an emphasis has been placed on developing serum and urinary molecular markers originating from different joint tissues, which would make it possible to evaluate the presence of OA metabolic changes during the earliest phases of the disease. These alterations are complex, as these involve not only joint tissue degradation but also the synthetic side of metabolism. In order to achieve a systematic overview of the pre-radiographic course of knee OA, it is necessary to assess biomarkers originating from different articular tissues, both cross-sectionally and longitudinally, in subjects much younger than those investigated previously, on a routine basis. This approach would make it possible to evaluate the diagnostic and potential predictive value of biomarkers for progressive OA.

The current gold standard for diagnosing OA and its progression is still plain radiography. Indeed, cartilage loss can be indirectly quantified from radiographic measurements of radiolucent joint space width (JSW). However, joint space narrowing (JSN) represents a late-stage indicator of already advanced cartilage destruction [5]. The importance of osteophytosis as an earlier radiographic sign of knee OA has been underestimated. Just recently, it was stated that as JSN and osteophytes represent different OA pathophysiology they should ideally be looked at individually [6]. Ultrasonography serves as an excellent method for the examination of articular soft tissues. However, the method has not yet been entirely standardized for its usage in OA.

Thus, the investigation of early-stage knee OA is not possible based on radiological methods alone, but requires a multifaceted diagnostic approach, including serum and urinary biomarkers originating from different joint tissues.

The globally increasing prevalence of OA calls for more detailed knowledge of the early phases of the disease. The investigation of OA pre-radiographic phases is utterly dependent on biomarkers as new diagnostic and/or prognostic tools for early OA management.

4. REVIEW OF THE LITERATURE

4.1. Osteoarthritis

4.1.1. Definition and the new concept of OA

OA was long considered a degenerative disease, the inevitable accompaniment of ageing, with "wear and tear" as the principle pathogenetic mechanism. However, in 1984, Kiss *et al.* declared that OA was neither a disease of ageing nor an inevitable consequence of the ageing of joints [7]. Indeed, according to the new concept, OA is now viewed as a metabolically active, dynamic process that may be triggered by a variety of biochemical and mechanical insults that destabilize the normal coupling of degradation and synthesis of all articular tissues, especially cartilage chondrocytes and the extracellular matrix [8]. Since 1994, OA has been defined as a group of overlapping distinct diseases, which may have different etiologies but similar biological, morphologic, and clinical outcomes. The disease process not only affects articular cartilage, but involves the entire joint, including the subchondral bone, ligaments, capsule, synovial membrane and periarticular muscles [8].

The OA disease process is now considered a continuum, beginning with an inciting event, such as genetic variation or injury, progressing through molecular, pre-radiographic and radiographic stages, and culminating in end-stage disease [9].

4.1.2. OA risk factors and pathogenesis

4.1.2.1. Risk factors for OA

Genetic predisposition

Twin studies have demonstrated that the hereditary component of knee OA may be on the order of 50% to 65% [10–11].

Gender

There is a marked increase in the prevalence of knee OA among women after the age of 50. The cause of this increase has been ascribed to an insufficiency of estrogen, which in normal conditions is related to the up-regulation of proteoglycan synthesis through its receptors on chondrocytes [12].

Age

Age-related morphologic changes in articular cartilage are due to a decrease in chondrocytes' ability to maintain and repair tissue as these cells undergo age-related decreases in mitotic and synthetic activity and exhibit decreased responsiveness to anabolic growth factors [13]. Age also appears to be an independent factor that predisposes chondrocytes to apoptosis, because the expression of proapoptotic genes is higher in aged cartilage [14–15].

Obesity

An increase in excessive load across knee joints is an important factor leading to cartilage breakdown. The majority of obese patients exhibit various deformities, which result in increased joint reactive forces in the medial compartment of the knee, thereby accelerating the degenerative process [16–17]. Emerging data show a crucial role for adipocytes in regulation of cells in bone, cartilage and soft tissues of the joint. It has been demonstrated that the adipocyte-derived factor interleukine-6 is procatabolic for chondrocytes [16].

It is clear that in any OA study the above-mentioned risk factors should be taken into consideration as important confounding factors.

4.1.2.2. OA pathogenesis – articular cartilage

Articular cartilage is comprised largely of an extracellular matrix synthesized by chondrocytes – the only cell type in cartilage [18]. Chondrocytes control the local environment of the cartilage extracellular matrix by performing both anabolic and catabolic activities [19]. The major part of the articular extracellular matrix is composed of collagen type II (60–70% of dry weight) and proteoglycans (10% of dry weight), of which aggrecan is the most abundant [20]. Other types of collagens are present in only minor amounts.

In OA, chondrocytes are activated by mechanical events (trauma) or genetic assault (mutation of a matrix molecule), to which they respond by increased metabolic activity, trying to repair the damaged matrix [21]. Although cartilage has a poor intrinsic reparative potential, there is an increase in aggrecan turnover, type II collagen synthesis and renewed chondrocyte proliferation [22]. This hyperanabolism has become a hallmark of OA cartilage – an attempt to repair the tissue that eventually fails [21]. These events finally end when the balance of catabolic events outstrips the anabolic events [21]. According to previous studies, temporally the first event in OA related cartilage degradation is the loss of aggrecan, which occurs prior to the breakdown of type II collagen [23–24]. The loss of cartilage matrix is mediated by proteolytic enzymes secreted by the chondrocytes and synoviocytes, including collagenases, the matrix metalloproteases (MMP`s) and the cysteine proteases [25–26]. Among them MMPs, especially MMP-13, play a central role [27–29]. There are, however, multiple pathways of chondrocyte triggering, which finally result in a common pathway of MMP-mediated collagen type II breakdown.

4.1.2.3. OA pathogenesis – osteophytes and changes in subchondral bone

Although it is now widely accepted that OA is a disease that can be initiated in any joint tissue, among them subchondral bone, research has long been focused predominantly on cartilage changes. However, there is evidence that remodelling of the bone-cartilage interface occurs early in the course of OA [30–32]. Moreover, even two decades ago it was suggested that changes in bone

might even precede changes in cartilage and might be responsible for the initial pathophysiological events in OA [30; 33].

Generally, in OA, the changes in periarticular bone are characterized by osteophyte formation and a sequence of subchondral changes finally leading to sclerosis [34].

Osteophytes are osteo-cartilaginous metaplastic tissues, protrusions of new cartilage which subsequently ossify, forming mostly at the margins of OA joints [35]. The cells that form osteophytes are considered to be mesenhymal stem cells residing in the periosteum [36]. Based on immunohistochemistry and in situ hybridization studies, the formation of osteophytes has been divided into five stages [35]:

Stage 0: Mesenchymal fibroblast-like cells in periosteal or synovial mesenhymal tissue produce a connective tissue consisting mainly of collagen types I and VI.

Stage I: Early chondrophyte, which is characterized by initial chondrometaplastic areas of deeper cell layers adjacent to the bone. Chondrogenic differentiation is detectable by the appearance of an extracellular matrix containing aggrecan and collagen type IIA.

Stage II: Fibrocartilage/chondrophyte, which is described as a structure with components of fibrous and cartilaginous tissue observed in parallel.

Stage III: Early osteophyte, consisting of aggrecan, collagen type II and chondrocytes. In this phase, active endochondral bone formation is evident with osteoblastic synthesis of collagen type I.

Stage IV: Mature osteophyte with extended ossification in the central core, although the predominant tissue is still hyaline cartilage.

The actual role of osteophytes during the process of OA is still unknown. They seem to be a way of compensating for instability but, apparently, they can be a side effect of elevated levels of growth factors [36]. This hypothesis is supported by the fact that osteophytes are usually found in non-weight bearing areas; therefore, their mechanical stability and biological benefit are questionable [35]. So far, in OA studies, the role of osteophytes has been widely overlooked. However, there is some evidence that during the spatial course of knee OA, osteophyte formation may even precede JSN [7], indicating their importance especially in the process of early knee OA.

By scintigraphic studies of elderly subjects with advanced knee OA, it was shown that subchondral bone turnover – both formation and degradation – is increased [38–39] and accompanied by decreased mineralization [40]. At the same time, there is still a remarkable knowledge gap concerning the metabolic shifts in subchondral bone in early-stage OA. It is well known that in established OA, the thickening of the subchondral plate and an increase in trabecular volume results in subchondral sclerosis [34]. On the other hand, micro-CT examinations have recently revealed that, in contrast to changes found in end-stage OA, at an early stage the subchondral plate thins [34;41–42]. These findings suggest that bone remodelling in OA is a biphasic phenomenon:

an early decrease in subchondral plate thickness is followed by a phase in which the subchondral bone becomes denser and stiffens.

The above-mentioned understanding of OA pathology indicates that there is inadequate data about the metabolic processes taking place in subchondral bone during early-stage knee OA. However, there is some radiologic evidence that morphological alterations in bone may not develop in a constant manner, as perceived before, but seem to have a biphasic course. This calls for further evaluation on the biochemical level and on much younger patients with early-stage OA.

4.1.2.4. The role of inflammation in OA

Articular cartilage, being avascular, aneural and alymphatic, presents no classic features of inflammation. However, at the molecular level, activated chondrocytes perpetuate disease progression by the production of a cascade of inflammatory mediators [43]. These inflammatory mediators drive catabolic pathways, inhibit matrix synthesis, and promote cellular apoptosis [16].

It is generally accepted that activated synovial tissue contributes to OA cartilage pathology [44]. In the osteoarthritic knee, the synovium often develops lining cell hyperplasia and hypertrophy and, in some cases, becomes infiltrated with subsynovial inflammatory cells [16; 45]. Activated synovial cells secrete excess synovial fluid, proteases and cytokines, which accelerate OA progression [46]. It is suggested that cartilage breakdown products can also provoke the release of collagenases from synovial cells and macrophages and result in mononuclear cell infiltration, as well as vascular hyperplasia in the synovial membrane.

This knowledge indicates that to achieve a systematic overview of OA processes inflammatory changes in knee joint soft tissues should also be addressed, in addition to the alterations in articular cartilage and subchondral bone. Nevertheless, so far, the role of knee joint soft tissue changes has been widely overlooked.

4.1.3. Clinical criteria of knee OA

In 1986, the Subcommittee on Osteoarthritis of the American College of Rheumatology Diagnostic and Therapeutic Criteria Committee published classification criteria for knee OA [47]. These criteria sets were modified into algorithms by Altman, facilitating their use in clinical research and population-based studies [48]. These criteria were based on the presence and duration of knee pain, age, morning stiffness, crepitus in active joint motion, radiographically detected osteophytes and laboratory findings of synovial fluid changes. Because the major parameter of these criteria is joint pain, these criteria help to identify patients with clinically important OA and are therefore useful for differentiating patients with OA from those with inflammatory joint diseases [49]. The sensitivity of these criteria is rather limited in discriminating

patients with early OA from healthy controls [49], because at the individual level there is poor correlation between the severity of radiographic changes and clinical symptomatology [50]. Thus, in epidemiological studies, radiographic criteria remain the basis for classifying subjects as having OA [4; 51].

4.1.4. Imaging methods for diagnosing knee OA

Up to now, conventional radiography, as an inexpensive and readily available imaging modality, has remained the method of choice in assessing the structural changes of OA and monitoring disease progression [52–53]. The most commonly used OA radiographic grading system was developed by Kellgren and Lawrence (KL) in 1957 [54]. This system is based on a global assessment combining several features of OA, such as JSN, osteophytes, subchondral sclerosis and subchondral cysts, and continues to be widely used even today. This system divides OA into five grades (0–4), in which a score ≥2 has traditionally been considered to be a definitive radiographic diagnosis of OA [49]. However, evidence suggests that KL grade 1 is *bona fide* OA and distinct from KL grade 0, based on the subsequent risk of progression [49]. There has been a great deal of criticism of the KL system for its relative insensitivity to dynamic changes, poor reproducibility [55] and its global assessment of OA, which has advantages only for more severe disease [56–57]. Nowadays, it has become clear that increased detail is much more appropriate in the interpretation of joint radiographs. In a recent review, it was stated that OA features – JSN and osteophytes – involve different pathways and pathophysiologies and are, therefore, inappropriately conflated by KL grades. Therefore, ideally these two aspects of OA should be looked at individually [6]. It has even been suggested that osteophytes alone may be a more reliable indicator of early disease than a grade 1 JSN [55; 58]. At the same time, there has been some doubt as to whether to treat small osteophytes as a grade 1 OA. In a 10-year follow-up study, Hart and Spector demonstrated that 62% of women having small tibiofemoral (TF) osteophytes at baseline went on to develop true osteophytic knee OA, compared with only 22% of controls with no sign of disease [59]. Therefore, small osteophytes cannot be ignored and should be treated as a subgroup of early disease.

In recent decades, there has been an effort to develop radiographic atlases that can be used as guides in the evaluation of individual features of OA [57]. In 2000, a line drawing atlas was developed by Nagaosa et al that allowed for the grading of JSN and osteophyte in TF, as well as in the patellofemoral (PF) compartment, and it had several advantages over previous scoring systems [56]. This atlas is based on the mathematical calculation of grades from normal JSW and the maximum size of osteophytes, giving excellent face validity, separate illustrations for grading of JSN for men and women, good reproducibility compared with previous atlases, and ease of use.

Nevertheless, in the majority of knee OA studies conducted so far, the presence and progression of the disease have still been assessed radiographically

on the basis of global KL grade or solely on JSN [38; 60–61], while only a few studies have assessed the development of osteophytes as an initial feature of OA or its progression [5; 62–63].

The natural course of knee OA progression is assumed to be continuous. However, a single report by Sharif *et al.* has suggested that this might not be true, as in their study knee OA progression in patients with a mean age of 64 years turned out to be non-linear over a period of five years [64].

Although ultrasonography (US) is a less expensive, easily available, well-established and sensitive diagnostic tool for soft tissue examinations, its role in knee OA is widely underestimated, except in patients with acute arthritis. US clearly has limitations in terms of assessing articular cartilage pathology; nevertheless, it is a reliable method for the demonstration of synovial pathology, synovial fluid and bony cortex abnormalities [65].

There have been only a few systematic investigations dedicated to US findings in the case of advanced knee OA [66–68]. DeMiguel Mendieta *et al.* investigated US findings in patients with painful knee OA and found that the most frequent US finding was suprapatellar effusion, whereas in patients without pain the most common finding was meniscal lesions [66]. In another knee OA study, knee pain was associated with ultrasonographically detected effusion, protrusion of the medial meniscus, and displacement of the medial collateral ligament [67]. Baker's cysts have been frequent US findings in individuals with painful knee OA [66–68].

There is currently no data available on the role and benefits afforded by the use of this assessment modality in patients with early knee OA.

Among the new imaging techniques, magnetic resonance imaging (MRI) is the most promising and sensitive imaging modality for use in the immediate future. MRI is superior in assessing the structure of articular cartilage, subchondral bone and soft tissues, and changes in the disease over time. However, its use is limited due to a current lack of accepted and validated scoring systems, long examination and interpretation times, and its high cost [5;69–71].

So far, ultrasound and MRI have not yet been included in any set of diagnostic criteria for OA.

Taking the above facts into consideration, it seems to be necessary to evaluate early-stage OA radiographically separately by osteophytes and JSN, and to focus on the presence of osteophytes as potentially earlier radiographic signs of OA. There is still no data available on the natural progressive course (continuous or phasic) of early-stage knee OA in middle-aged subjects as well as on the benefits of US in assessing soft tissue changes.

4.1.5. The prevalence and progression of radiographic knee OA in middle-aged subjects

The vast majority of studies of OA have been conducted on elderly subjects with already advanced or even end-stage disease. There is limited data about the prevalence of radio-graphic knee OA in subjects younger than 50 years.

In a Dutch study, radiographic TFOA was found in 7.7%–14.3% of people aged 45–49 [72]. Whereas, in a Swedish study, Petersson *et al.* detected radiographic TFOA (KL grade ≥ 2) in 1.5% of individuals aged 35–54 [73]. The same cohort of middle-aged subjects, followed by Thorstensson *et al.* over 12 years, revealed that 86% of middle-aged subjects with chronic knee pain developed TFOA (KL>or 1) over 12 years and 31% developed incident PFOA over five years [74].

In the Estonian cohort, the prevalence rate of an advanced (grade ≥2) radiographic knee OA, including TFOA and/or PFOA, among 35–55-year-old subjects was 3.7% [3]. Among them, the prevalence of advanced TFOA was 1.7% and of advanced PFOA slightly higher – 2.9% [3]. Schouten *et al.* followed 142 subjects aged 46–68 with radiographic knee OA (KL grade >2) over 12 years and observed JSN in 34% of cases [75].

Thus, in previous studies, the prevalence and progression of radiographic knee OA has been assessed in different ways: based on either TF JSN or KL grade level, it is difficult to compare the results of different study groups. There is also no conclusive documentation of inter-individual variations in the speed and course of knee OA progression. There is a considerable lack of knowledge concerning the prevalence and progression of early-stage radiographic knee OA.

4.2. Molecular markers of articular tissues in OA

To date, the diagnosis of any disease has usually required the presence of clinical symptoms [49]. However, there is significant evidence that there are often early, pre-symptomatic biomarkers of disease, which, if detected, may allow for earlier diagnosis and treatment. Therein lies the power and importance of applying biomarkers to OA, a disease often characterized by a prolonged asymptomatic molecular phase, a pre-radiographic phase, and a later radiographic phase, with visible structural joint changes (**Figure 1**) [49]. Indeed, studies with animal models have shown that subtle biochemical changes in joint tissues precede any clinical and radiographic evidence of joint destruction [76–77].

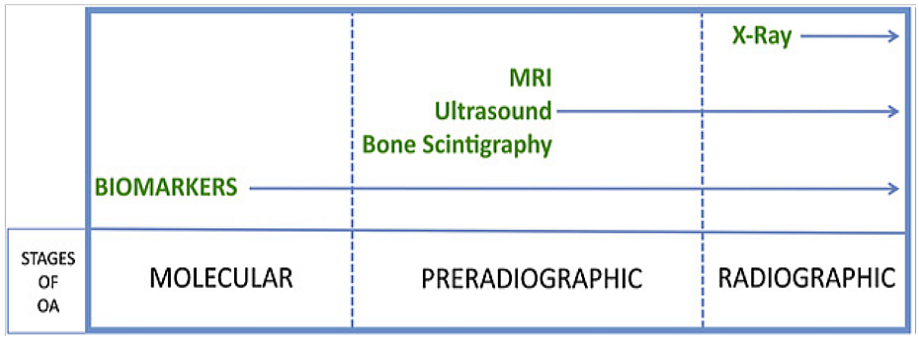

Figure 1. Different stages in the course of OA
Figure 1 presents different stages of OA and possible diagnostic means for each stage. It is evident that early molecular phase of OA can be assessed only by biomarkers.
By Virginia Kraus *et al*.; from Osteoarthritis Cartilage 2011;19:515-42.

Biochemical markers of articular tissue metabolism are molecules derived from cartilage, bone or soft tissues that are released into synovial fluid, serum and/or urine during tissue turnover, at which point they can be assayed by laboratory means [78–79]. Biomarkers have the potential to provide an early warning of the initiation of articular matrix breakdown, which in future could lead to earlier treatment to prevent joint destruction that leads to disability [49]. Thus, molecular markers of joint tissue metabolism have opened novel possibilities for earlier diagnosis and monitoring of OA, and are expected to offer means of finding subjects with a higher risk for OA progression [49;80–81]. Nevertheless, identification of OA before it becomes evident on radiographs remains a challenge. Whether biochemical markers are useful in this process is still unclear. So far, the majority of studies on biomarkers have been conducted on patients with already advanced radiographically evident structural changes in OA. There is a crucial need to evaluate biomarkers much earlier – in the molecular stage of OA, when biochemical changes may be most strikingly evident. There is currently a remarkable knowledge gap concerning the benefits afforded by the use of biomarkers in early stage OA.

Metabolic changes in OA involve the interaction of several tissues and different pathophysiology pathways and are therefore not adequately represented by the measurement of a single biomarker. Every single biomarker represents a distinct side of metabolism, i.e. the synthesis or break-down of bone, or the synthesis or degradation of cartilage. In addition, there are biomarkers available that reflect the metabolism of articular tissues in general. The measurement of a single biomarker is therefore insufficiently sensitive to be useful for the diagnosis or monitoring of OA. Nevertheless, in previous studies only single biomarkers originating from cartilage or bone tissue have been used, which does not offer a full overview of the complex metabolic changes in OA. Therefore, the investigation of OA calls for a simultaneous usage of a whole set of biomarkers originating from different joint tissues.

In recent years, several biomarkers have been studied for their potential use in advanced knee OA. Some of the most promising biomarkers, as well as their tissue origin, are presented in **Table 1**. Our study is the first to simultaneously evaluate the role of these biomarkers presented in Table 1 in early-stage knee OA.

Table 1. Molecular markers of articular tissues

Tissue origin	Biomarker	Marker characteristics	BIPED category
Cartilage	PIIANP	Synthesis of type II collagen	BPD
Cartilage	CTx-II	Degradation of type II collagen	BPED
Cartilage, tendon, ligament, synovium, bone	COMP	Non-collagenous marker. Mainly cartilage degradation	BPD
Bone, tendon	PINP	Synthesis of type I collagen	Not categorized
Bone, tendon	CTx-I	Degradation of type I collagen	B
Bone	OC	Non-collagenous marker. Osteoblast activity, bone mineralization	BPED
Bone	MidOC	Non-collagenous marker. Degradation of bone matrix	Not categorized

Molecular markers are here presented according to their articular tissue origin and characteristic metabolic processes reflected by them.
Abbreviations: PIIANP – type II A procollagen amino terminal propeptide; CTx-II – C-telopeptide fragments of type II collagen; COMP – cartilage oligmeric matrix protein; PINP – procollagen type I amino-terminal propeptide; CTx-I – C-terminal cross-linked telopeptides of type I collagen; OC – osteocalcin; MidOC – midfragments of osteocalcin.
BIPED – a classification system developed to categorize biomarkers into five categories corresponding to their utility as follows: **B**urden of Disease, **I**nvestigational, **P**rognostic, **E**fficacy of Intervention, and **D**iagnostic, for detailes see Text.

BIPEDS classification of biomarkers

Van Spil *et al.* developed a classification system called BIPED, which categorizes biomarkers into five categories corresponding to their utility [82]. Recently, the Osteoarthritis Research Society International Federal Drug Administration (OARSI FDA) Biomarkers Working Group added a Safety category to the BIPED system and, as a result, the acronym for this classification was updated to BIPEDS [49].

The categories of the BIPEDS system are as follows:

(i) **B**urden of disease biomarkers indicate the extent or severity of disease and can be considered tools for the staging of the disease.

(ii) **I**nvestigative biomarkers are those that show sufficient promise to be incorporated into research to determine their utility for subsequent use.

(iii) **P**rognostic biomarker indicates whether a patient's disease is likely to progress and may also indicate how quickly the progression will occur.
(iv) Biomarkers of the **e**fficacy of intervention are expected to demonstrate that a drug or intervention has the desired biochemical effect.
(v) A **d**iagnostic biomarker indicates whether an individual has the disease or a specific subtype of the disease, but may not reflect disease severity.
(vi) **S**afety biomarkers can be used to detect pathological changes and cytotoxicity. There are currently no studies that have specifically explored this aspect of joint tissue related biomarkers.

4.2.1. Molecular markers of cartilage turnover

Molecular markers reflecting collagen type II synthesis
Type II collagen is a triple helix composed of three identical alpha-chains. The synthesis of type II collagen involves several unique post-translational modifications. A large precursor molecule, termed procollagen, is processed soon after its release from chondrocyte. Type II procollagen is synthesized in two splice forms, type IIA and type IIB. Type IIA contains an additional 69-amino-acid cysteine-rich domain of the N-propeptide; it is expressed mainly by fetal tissues but can be re-expressed by adult chondrocytes of human OA cartilage, suggesting the potential reversion of the cells to a chondroprogenitor cellular phenotype in OA [83–84]. Type IIB is expressed at high levels in well-differentiated chondrocytes forming the framework of normal adult cartilage.

Before the incorporation of type II collagen molecules into fibrils, its non-helical extensions, termed the amino-terminal type II and carboxy-terminal type II procollagen propetides (PIIANP or PIIBNP and PIICP), are cleaved by specific N- and C-propeptidases. N- and C-terminal propeptides diffuse out of the cartilage matrix into the synovial fluid and are cleared into the blood, where their concentration can be assayed. The serum levels of these propeptides are thus believed to represent the rate of type II collagen synthesis in cartilage [81;83].

Molecular markers reflecting collagen type II degradation
Type II collagen is degraded by proteolytic enzymes, including the MMPs, the cysteine proteases and collagenases [25–26]. MMPs, such as MMP-9 and MMP-13, and a series of cystine dependent cathepsins, such as cathepsin K, B, L and S, attack collagen at the level of its propeptides [19]. MMP-13 is responsible for the generation of the fragment carboxy-terminal telopeptide of type II collagen (CTx-II), which is released to joint fluid and subsequently into serum and urine, where it can be assayed as a measure of articular cartilage breakdown [29]. Immunochemistry studies have indicated that CTx-II is abundantly present at the cartilage surface and at the bone-to-cartilage interface of the calcified region [19]. Therefore, it has been suggested that the excretion of CTx-II may be greater among patients with more advanced knee OA, which includes cartilage defects that have full penetration to the bone [85].

Collagenases are able to cleave the native triple helix of collagen, which results in the generation of specific neoepitopes at cleavage sites [19]. Among them, the neoepitopes C2C and C1,2C have been found to be elevated in OA cartilage and body fluids, serving as an another possible laboratory measure of cartilage breakdown [86–87].

Molecular markers of noncollagenous cartilage matrix turnover

Noncollagenous extracellular matrix proteins in cartilage have roles in modulating the assembly of structural proteins and cross-linking formed networks, as well as providing feedback to the chondrocytes on tissue structure and function [88]. Among these, the cartilage oligomeric matrix protein (COMP) is a biomarker that has been most thoroughly investigated for its potential role for OA [83]. COMP is a 524 kDa homopentameric glycoprotein consisting of five identical units of 755 amino acid, which belongs to the thrombospondin family. [89–90]. Originally considered to be entirely cartilage-specific, COMP has now been identified in all articular structures, including ligaments, menisci, tendons, synovium, osteoblasts and even vascular smooth muscle cells [91–92]. However, the concentration of COMP in synovial tissue, ligaments and tendons has been shown to be low, even up to 100-fold less than in cartilage and menisci [91;93]. Therefore, the major part of circulating COMP most probably originates from cartilage and has been demonstrated to be representative of cartilage catabolism [94]. There is, however, no data available on the exact contribution of soft tissue changes to the systemic values of COMP in early stage knee OA.

4.2.2. Molecular markers of bone turnover

Bone turnover is characterized by two opposite activities: the formation of new bone by osteoblasts and the resorption of old bone by osteoclasts [83]. Most of the collagen in the organic matrix of bone is type I collagen, which is synthesized by osteoblasts and provides a well-organized scaffold for the deposition of minerals [95]. Although most of the type I collagen is located in the skeleton, this protein is also the most abundant collagen in soft tissues.

Molecular markers reflecting bone synthesis

The type I collagen molecule is a heterotrimer of two alpha-1 chains and one alpha-2 chain, which are wrapped around each other in a triple helix. The two additional bulky domains at both ends of the molecule are called the amino-terminal (PINP) and the carboxy-terminal (PICP) propeptides of type I procollagen: specific by-products that are cleaved from procollagen by two specific endoproteinases once the molecule has reached the extracellular space [95]. These procollagen propetides are released into the systemic circulation and provide an opportunity to assess quantitatively the rate of newly synthesized type I collagen [95–96]. In practice, PINP has been shown to have greater diagnostic value than PICP as a specific biomarker for bone formation [97–98].

Molecular markers of noncollagenous bone matrix turnover

Serum osteocalcin (OC), also called bone Gla protein, is a vitamin K dependent hydroxyapatite-binding bone specific noncollagenous protein consisting of 49 amino acids [99–100]. OC is exclusively synthesized by osteoblasts, odontoblasts and hypertrophic chondrocytes during the process of bone matrix formation and accounts for approximately 15% of the total noncollagenous protein in bone. Although most of the newly synthesized OC is captured by bone matrix, a small fraction is released into the blood, where it can be detected by immunoassays [83]. In serum, the intact OC molecule is further metabolized by the proteases, among them cathepsins, except for the mid-portion of the molecule, which is shown to be resistant to degradation *in vitro* [101–103]. Circulating OC is therefore constituted of different immunoreactive forms, including the intact molecule and various fragments [104–105]. In addition to the newly synthesized OC derived from osteoblasts, the circulating OC pool also includes fragments derived from the resorption process when OC embedded in the bone matrix is released [102; 106–107]. Importantly, OC is known to be involved in the inhibition of bone matrix mineralization [108]. Hence, in recent years, OC, initially considered to be a marker of bone synthesis, is now believed to be a noncollagenous marker of bone turnover and mineralization.

Molecular markers reflecting bone resorption

The majority of bone resorption markers are degradation products of collagen type I, except for tartrate resistant acid phosphatase (TRACP) isoenzyme 5b, which mainly reflects the number of osteoclasts, some specific fragments of osteocalcin (e.g. the mid-fragment) and bone sialoprotein (BSP) [83].

During bone resorption, osteoclasts secrete different factors, such as acid, matrix MMPs and cathepsin K. These enzymes degrade type I collagen into several products, including the hydroxypyridinium cross-links of collagen, pyridinoline (PYD) and deoxypyridinoline (DPD), the MMP product carboxy-terminal telopeptide of type I collagen (ICTP) and the combined MMP and cathepsin K products type I cross-linked N-and C-telopeptides (NTx-I and CTx-I) [83]. These cross-linked telopeptides are cleaved specifically from type I collagen during bone resorption and are shown to be specific markers of bone resorption [83].

There is emerging evidence that OC detected from serum or urine gives qualitatively different information concerning bone turnover [107]. Urinary OC represents a heterogeneous pool of different OC fragments that consist mainly of the middle portion of the molecule; according to different studies, the predominant fragment consists of the sequence Asp^{14}-Asp^{28} [106] and of the residues Gly^{7}-Glu^{31}[109]. There is evidence that urinary OC fragments serve as an index of bone resorption [107]. It has been shown that the mid-molecule fragments of OC were suppressed in post-menopausal women with osteoporosis who received bisphosphonate alendronate therapy [106; 110]. It has even been suggested that urinary OC fragments may be more specific for bone resorption than type I collagen-related markers [83]. In 2005, Kaisa Ivaska *et al.* (Univer-

sity of Turku, Finland) developed a novel immunoassay for the detection of mid-fragments of urinary OC (MidOC) [107]. There is currently no data available on the potential value of urinary OC mid-fragments in subjects with knee OA.

4.3. Molecular markers of joint tissue metabolism in incident and/or progressive knee OA

Several biomarkers have been investigated in association with incident radiographic knee OA and disease progression, often with conflicting results. The study designs of previous investigations have been variable, with differences in the number of investigated subjects, gender profile, age, follow-up time and especially the radiographic criteria to define OA and its progression. This may explain the discordant results observed with biomarkers across the different OA studies [83]. The vast majority of OA studies have been focused only on elderly patients (with a mean age $\geq$ 65 years) with already advanced knee OA (KL grade $\geq$ 2). Therefore, only limited data is available on the early stages of OA and the potential value of biochemical markers in early disease.

4.3.1. Cartilage markers in incident and/or progressive knee OA

Through cross-sectional studies, several associations have been demonstrated between cartilage turnover markers and radiographic knee OA. Garnero *et al.* have shown that the serum values of COMP, as well as urinary output of CTx-II, were increased in elderly subjects with advanced TFOA (based on JSN) compared to age- and sex-matched controls [79]. The same conclusion was reached by Jordan *et al.* and Dam *et al.* for uCTx-II [111–112]. They demonstrated that uCTx-II values were associated with the presence and severity of knee OA as measured by osteophyte formation, JSN and overall KL grade in TF and PF joints [111], or by MRI-detected loss in cartilage volume [112]. Serum values of COMP have been shown to be significantly higher in the case of TFOA compared to PFOA [113], and associated with the presence of clinically diagnosed synovitis [114]. Conflicting data have been published by Cibere *et al.,* who found that the risk of radiographic knee OA was higher with increasing urinary levels of CTx-II, C2C and C1,C2, but no correlation with levels of sCOMP was observed [115]. Also, the serum values of PIIANP, a marker for the synthesis of type II collagen, have been shown to be decreased in patients with knee OA compared to controls [116].

In several longitudinal studies using radiographic KL and/or semi-quantitative MRI scores, a predictive value of sCOMP and/or uCTx-II for subsequent knee OA progression has been demonstrated [5; 112;114;117–119]. Garnero *et al*. have shown that patients with low levels of cartilage synthesis marker sPIIANP and high levels of the cartilage degradation marker uCTx-II had an

eight-fold increased risk of OA progression over 12 months, and that the ratio of the values of given markers could make the difference between OA progressors and non-progressors [81]. In contrast, in a study by Bruyere *et al.,* no correlation was found between the baseline values of sCOMP and subsequent one-year MRI-detected change in cartilage volume and thickness in subjects with advanced knee OA [120]. On the other hand, a longitudinal study by Hunter *et al.* revealed a prognostic value of serum COMP for subsequent cartilage loss on MRI over 2.5 years, but no association with the urinary output of CTx-II, C2C or C1,2C was found [121].

The limited number of previous studies conducted on middle-aged subjects has indicated that cartilage markers seem to have both diagnostic [122] and predictive [123–124] roles in radiographic knee OA progression at an early stage. This finding, however, relies on only three biomarkers: insulin-like growth factor-1 (IGF-1) [123], COMP [122] and PIICP [124]. In these studies, the follow-up period varied from three to 12 years and assessment of OA progression was based mainly on TF or PF JSN, except in the study by Schouten *et al*. [123], who also examined the growth of osteophytes and overall OA grade progression.

4.3.2. Bone markers in incident and/or progressive knee OA

Much less data is available for the value of bone markers in the case of knee OA. In 1995, Sharif *et al.* demonstrated a correlation between abnormal bone scintigraphic scans and synovial fluid levels of bone turnover marker OC in patients with knee OA [125]. Nevertheless, there is still limited knowledge about the value of bone markers in knee OA and its progression. It is even unclear whether OA is characterized by increased or decreased bone turnover. So far, the studies available have yielded conflicting data [79]. Except for the work by Petersson *et al.,* studies on bone turnover markers have been focused on elderly subjects with already advanced-stage knee OA [122]. In the study by Bettica *et al.,* it was shown that the urinary levels of bone resorption markers CTx-I and NTx-I were higher in patients with progressive knee OA when compared to controls [62], indicating the prognostic value of these markers. The predictive value of bone markers was also demonstrated by the recent study by Berry *et al.,* who found that higher baseline values of the bone formation marker PINP and resorption markers CTx-I and NTx-I, as well as the marker of bone turnover and mineralization OC, were associated with two-year MRI-detected reduction in cartilage loss [126]. Moreover, they observed that in the subgroup of patients with high sPINP, there was a significant link between increased values of CTx-I and NTx-I and a reduced rate of cartilage loss. At the same time, Bruyere *et al.* found no predictive association between the baseline values of OC or CTx-I and a 12-month loss in cartilage thickness and volume using MRI [120]. Interestingly, cross-sectional studies on advanced knee OA have even indicated a decrease in the values of sCTx-I, uCTx-I and OC [79], or no significant change at all [111;115].

In the only bone marker study available for middle-aged subjects, with a mean age of 47 years, increased serum levels of BSP were found in individuals who developed radiographic TF and PF knee OA over the following three years [122].

Except for the study by Berry *et al*. [126], previous investigations of bone markers have focused mainly on the resorption of bone. The other part of bone turnover – the synthesis of type I collagen (reflected in the levels of PINP) – has been overlooked. Instead of PINP, serum levels of a noncollagenous marker OC were assayed as a measure of bone formation [79;111;120]. However, OC as a general marker of bone turnover reflects the formation and resorption of bone matrix in combination [107], and might be more useful as a marker reflecting the inhibition of bone matrix mineralization [108].

There is still a significant lack of knowledge regarding the diagnostic and prognostic utility of bone markers in early OA, and no data are available on the possible value of the specific bone formation marker PINP in patients with early knee OA.

Even less data is available on potential associations between biomarkers and ultrasonographically detectable changes in knee joint soft tissues during OA [127]. A single study by Jung *et al.* established that the serum levels of hylauronic acid (HA) and COMP increased in elderly patients with longer medial osteophytes and capsular distension of the knee joint and, in the case of HA, also with effusion and/or synovial proliferation.

There are, however, no data available on the possible associations between biomarkers and US-detected soft tissue changes of the knee joints in middle-aged subjects with early-stage OA.

The conflicting data from the above studies indicate that the complexity of the OA process calls for more extensive studies and a more systematic approach. Therefore, a panel of biomarkers reflecting the formation and degradation of both collagenous and noncollagenous parts of both cartilage and bone would allow for a more systematic look at complicated metabolic processes. There are still minimal data available on articular turnover markers in middle-aged subjects with early-stage knee OA – a stage when biochemical alterations of joint tissues are now assumed to be most active.

5. AIMS OF THE STUDY

To enhance the knowledge of the long-term radiographic and biochemical behaviour of knee OA in middle-aged subjects at an early stage of the disease.

Specific aims:

- To specify what kind of lesions or processes in the OA-affected knee joint are reflected by the values of serum COMP and the novel bone marker urinary MidOC.
- To determine the prevalence and progression of radiographic features of OA in middle-aged subjects with chronic knee joint complaints.
- To clarify the potential relationships between molecular markers of cartilage and bone metabolism and ultrasonographically detected changes in knee joint soft tissues in subjects with early-stage knee OA.
- To examine the potential diagnostic and prognostic value of cartilage biomarkers in progressive cases of knee OA.
- To examine the potential diagnostic and prognostic value of bone biomarkers in progressive cases of knee OA.

6. MATERIALS AND METHODS

6.1. Study subjects

Subjects with persistent (> three-month duration) knee complaints were identified using a questionnaire sent to a random sample of individuals aged 35–55 from the register of a general practitioner in the southern Estonian town of Elva [3]. An initial invitation was sent to 559 randomly selected subjects to participate in the study. Of those invited, 348 (62%) responded. Out of the 348 responders, 220 admitted either knee pain (60%) or other knee symptoms (40%), such as crepitation and stiffness. Out of 220 subjects, 161 (73% of those with knee complaints; 101 women and 60 men) agreed to participate in the longitudinal study and were examined at three different time points, at baseline (in 2002), after three years (in 2005) and after six years (in 2008). Over the six years, 33 subjects out of the 161 (20%) were lost to follow-up. The reasons for participants being not eligible or available for follow-up included refusal (n=21), leaving the study area (n=9), death (n=1), and inability to contact (n=2).

Knee joint radiographs were available for at least two time points, the baseline and at the end of the study, for 128 subjects out of the 161, and these subjects were included in the present study. The patient characteristics are presented in **Table 2**. The distribution of age, gender and BMI of these 128 subjects did not differ significantly from the initial group of 220 individuals.

Table 2. Characteristics of the study participants

Characteristics	**Study group at baseline (n=161)**		
	Returned (n=128)	**Lost to follow-up (n=33)**	**p** *
Baseline age, mean +/- SD years	45.0 +/- 6.2	46.9 +/- 5.4	0.116
Baseline BMI, mean +/- SD kg/m^3	27.6 +/- 5.1	27.7 +/- 5.4	0.928
Women	85/128 (66%)	16/33 (48%)	0.417
Baseline radiographic knee OA †	73/128 (57%)	21/33 (64%)	0.752
Baseline radiographic knee OA grade 2-3 ‡	7/128 (5.5%)	1/33 (3%)	0.100

Except where indicated otherwise values denote the number/total number (%) of patients.

* By the chi-square test for categorical variables and by the Mann-Whitney U-test for continuous variables

BMI – body mass index

† Knee OA grades 1, 2 and 3 according to the grading system of Nagaosa.

‡ Knee OA grades 2 and 3 according to the grading system of Nagaosa.

Out of the 348 responders, 108 reported no knee joint complaints, and among them 73 agreed to participate in the longitudinal study. Out of the 73 subjects,

40 (15 males and 25 females) had no radiographic signs of knee OA and served as controls.

There were no statistically significant differences in mean age or BMI between the female and the male subjects. The proportion of post-menopausal women was 35% at baseline and 45% at the end of the study. Subjects with radiographic evidence of rheumatoid arthritis or other inflammatory arthropathies in the knees were not included in the study.

To investigate the potential value of a novel bone biomarker, MidOC, 19 post-menopausal women with osteoporosis, aged 49–66 (mean 60), receiving bisphosphonate treatment with risedronate 35 mg weekly for 12 months were followed. Osteoporosis was diagnosed if the T score of lumbar spine bone mineral density (BMD) (L2-L4) was less than –2.5 by dual-energy X-ray absorptiometry, DXA (DPX-IQ, Lunar Corp). These patients were examined for BMD at baseline and after 12 months. Women were considered responders to the therapy if the 12-month change in BMD exceeded +3% from the baseline value. The levels of bone markers, among them MidOC, were investigated in relation to the changes in BMD at five different time points (0, 1, 3, 6 and 12 months) during therapy.

Written informed consent for participation was obtained from each subject according to the Declaration of Helsinki. The study protocol was approved by the Ethics Committee for Human Research of the University of Tartu.

The study subject allocation, according to different papers, is presented in **Figure 2**.

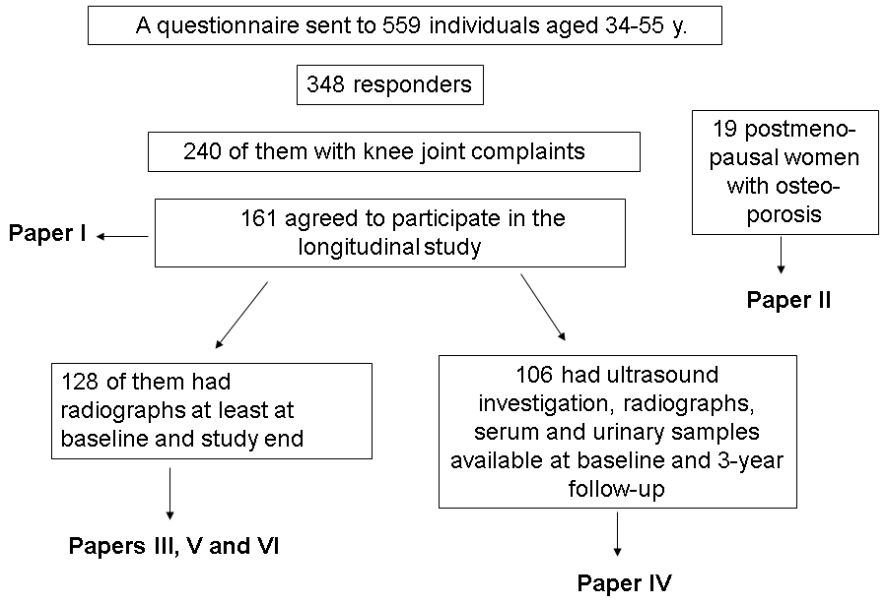

Figure 2. Study subjects
Study subjects allocation according to different papers.

6.2. Standardized radiographic investigation

The TF and PF joints of both knees were radiographed separately. Standardized radiographs of the TF joints were taken with the subject in a standing frontal antero-posterior position, with the knee joints in full extension and with equal weight on both legs. Axial views of the PF joints were taken with the subject in a standing position, with knee joints at 60° of flexion according to the technique described by Boegard *et al.* [128]. JSN and osteophyte (Oph) size were classified on four-point scales (grades 0–3) according to the system of Nagaosa *et al.* [56]. JSW were measured separately for the medial and lateral compartments. Osteophyte size was determined in eight distinct regions of interest (ROIs) in each knee joint: medial and lateral femoral condyle, medial and lateral tibial plateau, medial and lateral patella, and medial and lateral trochlea. The presence of subchondral sclerosis was not considered for OA grading. TFOA and PFOA grades were defined as the highest grade documented in any ROI. The global grade of knee OA for each subject was based on the status of TF and PF joints for both knees. The radiographic OA grade 1 was diagnosed if at least a single osteophyte (grade 1) and/or JSN (grade 1) was assessed in any ROI. OA grade 2 was diagnosed if an osteophyte with grade 2 and/or JSN (grade 2) was assessed in any ROI. OA grade 3 was considered to be present if an osteophyte with grade 3 and/or JSN (grade 3) was assessed in any ROI.

The criteria for radiographic OA grade progression were defined as:

(i) the presence of osteophytes and/or JSN in subjects with no previous radiographic evidence of OA or

(ii) an increase in the grade and/or number of already existing osteophytes and/or JSN grade over three and/or six follow-up years.

In addition, a subset of patients was identified whose OA grade did not change over the follow-up period, but who developed JSN or osteophytes in new locations: “minimal progressors”.

All radiographs were interpreted by an experienced radiologist who was blind to clinical details. To assess radiographic progression, radiographs from different time points were examined in pairs to ensure consistent evaluation over time.

6.3. Ultrasonographic examination

US of both knees of all subjects was performed by a single experienced musculoskeletal radiologist blind to the results of clinical, radiographic and laboratory examinations, using a multi-frequency linear 7.5 MHz probe. The presence of osteophytes, thickness of tendons, cartilaginous structure, meniscal changes, synovial hyperperfusion, effusion, and the presence of Baker’s cysts was assessed according to EULAR guidelines [129] and graded on a 0/1 scale. In addition, calcification in the quadriceps and patellar tendons and defects in the subchondral bone contour were documented.

US data were available for 106 individuals (33 men and 73 women), who constituted a subset of an initial population-based cohort of 240 subjects with persistent (> three months) pain in one or both knees. The mean subject age was 49 years (range, 36–55 years).

6.4. Laboratory investigation

Serum samples were obtained at all three time-points, at baseline, after three and six years, and second-morning urinary void samples were collected at the three- and six-year follow-ups. All samples were stored frozen at –70°C until measured.

Specific synthesis and degradation markers were implemented separately for bone and cartilage. In addition, two markers – OC and COMP were used to reflect the metabolism of bone and articular tissue in general.

Cartilage formation was estimated by measuring serum concentrations of type II A procollagen amino-terminal propeptide (sPIIANP). Serum PIIANP was measured by enzyme-linked immunosorbent assay (ELISA), using a polyclonal antibody raised against recombinant GST-human type II procollagen exon 2 fusion protein (Synarc, Lyon, France). The assay was based on the competition between circulating PIIANP and recombinant fusion protein GST-exon 2 for the binding to the polyclonal antibody immobilized on a micro-titreplate. Standards and serum samples were incubated for 4 h with shaking at room temperature with 100 µl/well of IIA antiserum. After washing, the peroxidase conugated anti-rabbit (diluted: 1/8000, Sigma, St Louis, MO) was added (100 µl/well) and incubated at room temperature for 1 h with shaking. After washing, 100 µl H_2O_2/Tetramethylbenzidine substrate-indicator solution (Sigma, St Louis, MO) was added. After incubation at room temperature for 30 min with shaking, the colour reaction was stopped by the addition of 100 µl 2M H_2SO_4/well and the optical density was read at 450 nm in a Dynatech MR 7000. Each sample was run in duplicate.

Cartilage degradation was estimated by assaying the urinary concentrations of the C-telopeptide fragments of type II collagen (uCTx-II). The concentrations of CTx-II were determined in a competitive ELISA, using a monoclonal antibody mAbF46 raised against a linear six-amino-acid epitope of the type II collagen C telopeptide (Urine CartiLaps®, Immunodiagnostic Systems, Herlev, Denmark). A biotinylated CTX-II C-telopeptide derived peptide (EKGPDP) was coated on a streptavidine microtitre plate, and sample and the primary antibody (mAbF46) were added. After overnight incubation, the amount of bound antibody was quantified using a peroxidase-labelled secondary antibody and a chromogenic peroxidase substrate. The concentration of the CartiLaps ELISA (ng/l) was normalized against urinary creatinine concentrations (mmol/l), which were measured by Jaffe's kinetic method (as ng/mmolCrea). For this, the following formula was used: corrected CTX-II value (ng/mmol) = 1000 x urine CartiLaps (µg/L) / creatinine (mmol/L).

A serum concentration of the general joint tissue marker sCOMP was assayed by a solid-phase, two-site enzyme immunoassay based on the direct sandwich technique, in which two monoclonal antibodies are directed against separate antigenic determinants on the COMP molecule [COMP® ELISA, AnaMar Medical, Göteborg, Sweden]. During incubation, COMP in the samples reacted with peroxidase-conjugated anti-COMP antibodies and anti-COMP antibodies bound to the microtitration well. Using a single washing step, unbound enzyme-labeled antibody was removed. The bound conjugate was then detected by a reaction with 3,3`5,5`-tetramethylbenzidine (TMB). This reaction was stopped by adding acid to give a colorimetric endpoint that was read spectophotometrically.

Bone formation was assessed by serum concentration of procollagen type I amino-terminal propeptide (sPINP). Bone resorption was estimated by the serum level of the C-terminal cross-linked telopeptides of type I collagen (sCTx-I) and by the urinary level of MidOC. The serum concentrations of PINP and CTx-I, and of the general marker of bone turnover and its mineralization – serum OC – were assayed by an automated electrochemiluminescence immunoassay (ECLIA, Elecsys), according to the directions for use by Roche.

Urinary MidOC were assayed by ELISA implementing two monoclonal antibodies: Mab6F9, which binds to the residues Gly^{7}-Arg^{19}, and Mab3H8, which recognizes the fragment Arg^{20}-Arg^{43}. The values of uMidOC were normalized for urinary creatinine, which was measured by Jaffe`s kinetic method.

The intra-assay variations for PINP, CTx-I, OC, MidOC, COMP, CTx-II and PIIANP were 3%, <4%, <4%, <3%, <10%, 4% and <9%, respectively, and the inter-assay variations were 3%, 12%, 5%, 8%, 13%, 13% and 14%, respectively.

6.5. Statistical analysis

Descriptive statistics were calculated for sex, age and presence of radiographic knee OA grades and compared between subjects who had returned and those who were lost to follow-up by a chi-square test for categorical variables and by the Mann-Whitney U-test for continuous variables.

As biomarkers did not follow normal distribution, non-parametric methods were used for statistical evaluation.

The associations between the biomarker values and radiographic progression of the knee OA features (osteophytes and JSN) were assessed by Spearman's rank correlations.

The diagnostic and prognostic value of each biomarker for radiographic knee OA progression was assessed by the Mann-Whitney U-test, comparing the values of the biomarkers for the OA progressors and the non-progressors.

The risk of radiographic knee OA progression with increasing bone marker values was calculated by logistic regression analysis (odds ratios), with adjustment for age, gender and BMI.

Multiple linear regression analysis was employed to assess the ability of US parameters to predict the variability of biomarker values. Several models of US parameters were tested to find the best set of US parameters for each given marker.

A p value <0.05 was considered statistically significant. For statistical computations, we used the software STATISTICA 9.1.

7. RESULTS

7.1. Serum COMP and urinary MidOC – specifications for their usage as molecular markers of joint tissue metabolism (Papers I–II)

7.1.1. Serum COMP – a marker reflecting metabolic changes in several articular tissues

Serum COMP was initially considered an entirely cartilage-specific macromolecule reflecting cartilage degradation. Later evidence was gathered that in OA patients serum COMP might also be released from such joint soft tissues as tendons, ligaments, menisci and synovium.

Using cross-sectional analysis, we investigated what kind of knee joint structures might be associated with increased levels of sCOMP in subjects with early knee OA. Our findings, based on the correlations between serum COMP values and radiographic and ultrasonographic changes, revealed that in early-stage knee OA there is an important contribution of TF osteophytosis, as well as soft tissue changes in the values of COMP (**Table 3**). Among the latter, meniscal changes made the largest contribution to the systemic level of COMP. Based on the above findings, we could confirm the view that COMP is a general marker of joint tissues. Compared to the entirely cartilage-specific biomarkers, COMP might reflect even better the changes in the OA joint, as it consists of the contributions of different articular tissues (**Paper I**).

Table 3. Comparison of serum levels of COMP between the groups with and without radiographic and ultrasonographic findings

Variable	The median values of S-COMP					
	Females			Males		
	With lesions	Without lesions	p value	With lesions	With-out lesions	p value
TF osteophytes (by X-ray) n= 26/12*	10.7	9.5	**0.029**	12.4	12.0	0.904
PF osteophytes (by X-ray) n= 16/28*	9.8	9.8	0.343	11.9	12.2	0.443
Tibial osteophytes (by US) n=5/4*	12.8	9.7	**0.005**	13.0	11.7	0.268
Femoral osteophytes (by US) n=6/3*	12.4	9.7	**0.019**	12.3	11.7	0.564
Meniscal changes (left, by US) n= 5/3*	12.8	9.7	**0.043**	14.6	11.6	**0.022**
Meniscal changes (right, by US) n= 8/6*	10.1	9.8	0.446	11.2	11.8	0.369

Differences between groups by Mann-Whitney U-test.
US – ultrasonography
* – the number of females/males with lesions

7.1.2. Urinary MidOC – a novel marker of bone resorption

Serum OC is derived from osteoblasts and is therefore widely used as an index of bone formation. The intact OC molecule accounts for approximately one-third of total OC, while the rest is composed of fragments. The presence of multiple immunoreactive OC fragments was demonstrated in the urine as early as 1990 [102]. There was evidence that some urinary fragments of OC might reflect bone matrix resorption, not formation. So far, urinary OC mid-fragments have not been tested in OA patients.

To evaluate the possible resorptive origin of urinary OC mid-fragments, we assayed these fragments along with other markers of bone resorption (sCTx-I), synthesis (sPINP) and mineralization (sOC) in a 12-month follow-up study on 19 post-menopausal women receiving bisphosphonate – risedronate treatment (**Paper II**).

A parallel significant decrease was observed in the levels of uMidOC and the well-characterized bone resorption marker sCTx-I after the first month of risedronate treatment, while the levels of sOC and sPINP did not differ from the pre-treatment values at this time point (**Figure 3**). In the course of the first six months of therapy, the correlation between uMidOC and sCTx-I ($r(s)=0.511$, $p=0.0003$, $n=45$) was more pronounced than the correlation between uMidOC and sPINP ($r(s)=0.312$, $p=0.039$, $n=44$). These results provide further evidence that urinary OC mid-fragments and serum total OC reflect different aspects of bone turnover. The quick response of uMidOC to antiresorptive therapy indicates its mainly resorptive origin (**Paper II**). Based on these follow-up data, we succeeded in validating uMidOC as a new marker of bone matrix resorption, with the aim of its further evaluation in knee OA patients.

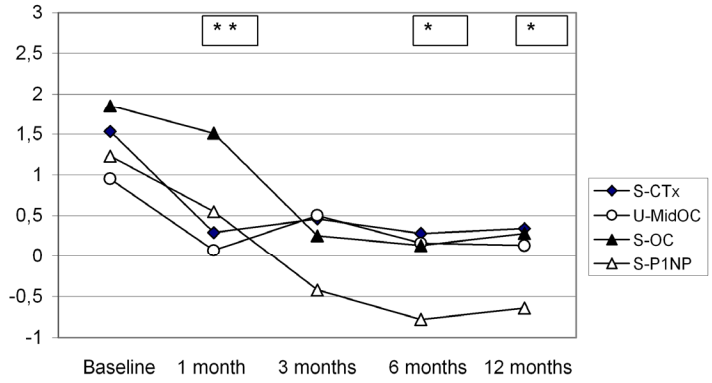

Figure 3. Longitudinal changes in bone turnover markers in response to 12 months risedronate therapy.
The results are presented as T-scores for each marker compared to the control group. T-scores were calculated as: (observed result – mean of controls) / SD of premenopausal controls. Changes greater than one T-score (1 SD) were considered as response to therapy. The response of uMidOC to risedronate compared to the pre-treatment T-score value is marked by: * $p< 0.05$, ** $p<0.002$.

7.2. Results of radiographic investigations

To determine the prevalence and progression of early-stage radiographic knee OA, we prospectively evaluated a population-based cohort of middle-aged subjects over six consecutive years.

7.2.1. The prevalence of radiographic knee OA (Paper III)

At baseline, less than half of the studied subjects (44%) had no radiographic sign of OA (grade 0), and the rest had mild OA in different combinations. The details are presented in **Table 4**. Only seven subjects out of 128 (5%) presented with OA grade 2 or 3. It is important to note that as many as 20% of the subjects with OA grade 1 had isolated PF involvement.

Table 4. The prevalence of radiographic knee osteoarthritis at baseline and 6- year follow-up

OA grade	At baseline	At 6-yr follow-up
OA grade 0	56 (44%)	23 (18%)
OA grade 1	65 (51%)	86 (67%)
TFOA=1	20	24
PFOA=1	25	16
TFOA + PFOA=1	**20**	**46**
OA grade 2	6 (4%)	16 (13%)
TFOA=2	3	7
PFOA=2	1	6
TFOA + PFOA=2	2	3
OA grade 3	1 (<1%)	3 (2%)
TOTAL	**128**	**128**

Here are presented the numbers and percentages for different radiographic knee OA grades.

When the features of OA were analysed separately, we found that in 57% of the subjects with OA the diagnosis was based on osteophytes alone, in 13% on isolated JSN, and in 30% on both.

After six years, we observed a remarkable decrease in the number of subjects with no radio-graphic knee OA (grade 0). Of the subjects with OA grade 1, approximately 60% had both TFOA and PFOA in combination, twice the percentage as at baseline (**Figure 4**). Relative to baseline, 12 new subjects were diagnosed with OA grade 2–3. The distribution of knee OA grade did not differ significantly between males and females.

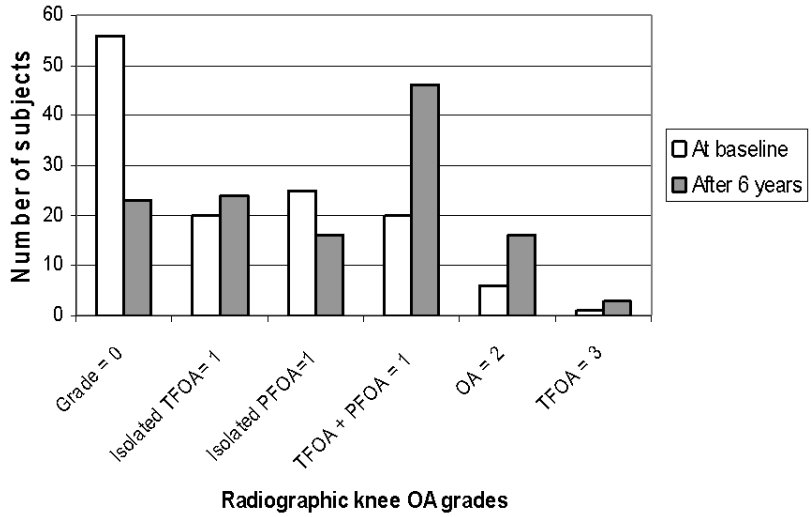

Figure 4. Distribution of radiographic knee OA grades at baseline and after 6 years. Here are presented the radiographic data of 128 subjects at baseline and after 6 years. At baseline, the majority of subjects with radiographic knee OA had only grade 1 (65 out of 72). Note that 25 of them had isolated PFOA grade 1. In 56 subjects no radiographic OA was present at baseline. At 6-yrs follow-up we observed 86 subjects with OA grade 1 and 19 with grade 2–3. Relative to baseline, 12 additional subjects were diagnosed with OA grade 2–3.

7.2.2. Radiographic knee OA progression over six years (Paper III)

Over six years, we observed radiographic OA grade progression in 71 subjects out of 128 (56%); of these, 35 had progression only in TFOA, 22 only in PFOA and 14 in both (**Figure 4**). Thus, we observed radiographic OA progression in more than half of the studied middle-aged subjects. However, in the majority of progressive cases it was an early stage OA: progression from OA grade 0 to grade 1. Moreover, the progression was largely based on the development of osteophytes alone (31/71), or osteophytes in combination with JSN (26/71). At the same time, progression based on JSN alone accounted for only 20% of the cases (14/71). Again, the pattern of progression did not differ between men and women.

7.2.2.1. Radiographic knee OA progression in two consecutive three-year follow-up periods

During the first three years of follow-up, 49 of 119 subjects (41%) showed OA grade progression, including 30 with progression only in TFOA, 15 only in PFOA and four in both.

During the second three-year period, only 28 of 119 subjects (24%) showed radiographic OA progression, including 10 with TFOA progression, 15 with PFOA progression, and three with both.

The number of TFOA progressors was significantly higher over the first three years of follow-up when compared to the last three years (Chi square 12.0, p=0.0005), whereas the number of PFOA progressors was the same over both periods. In the majority of cases, TFOA progression did not proceed during the second period of observation. Thus, the radiographic course of OA over six years followed a non-continuous pattern with periods of progression and stabilization.

7.2.2.2. Individual pattern and types of knee OA progression over six years

A more detailed analysis, based on the individual course of OA progression over six years, revealed five distinct groups of OA progression (**Table 5**). Of the total of 120 subjects, 40% showed no knee OA progression (subdivided as "stable OA" and "no OA" groups). Most of the remainder (41/120) were classified as "first phase progressors" – progression only in the first three years of follow-up; 21 as "second phase progressors" – progression only in the last three years of follow-up; and seven as "continuous progressors" – progression during both periods.

Table 5. Patterns of radiographic OA progression over 6 years

Radiographic progression group	n	First 3 years of follow-up	Last 3 years of follow-up
First phase progression *	41	**progression**	**no progression**
Second phase progression †	13	**no progression**	**progression**
Continuous progression ‡	7	**progression**	**progression**
Stable OA §	28	no progression	no progression
No OA	20	no OA	no OA

***** Including 26 subjects with TFOA progression, 11 with PFOA progression and 4 with both.

† Including 4 subjects with TFOA progression, 7 with PFOA progression and 2 with both. Of the initial group of 21 subjects with late progression, 8 were excluded because they were of significantly younger age and did not have OA at baseline.

‡ Including 4 subjects with TFOA progression and 3 with PFOA progression over the first 3 years of follow-up, and 3 with TFOA progression and 4 with PFOA progression from the last 3 years of follow-up.

§ Including 8 subjects with TFOA grade 1, 5 with PFOA grade 1, 13 with TFOA+PFOA grade 1, 1 with TFOA grade 1 + PFOA grade 2 and 1 with TFOA grade 2 + PFOA grade 1.

The results of this detailed analysis revealed again that the overall OA progression is non-continuous and heterogeneous, as in half of the cases there were periods of progression and stabilization. In three-fourths of the cases, the OA process either did not progress or progressed with intermittent stops. Therefore, the overall OA progression proved to be slow. However, at the same time, there were a few cases with continuous progression of OA.

7.3. Effect of age, gender, BMI and menopausal status on serum and urinary values of biomarkers

In every studied subject, specific synthesis and degradation markers of bone (sPINP, sCTx-I and uMidOC) and cartilage (sPIIANP and uCTx-II) were assayed. In addition, sOC was used to assess changes in bone mineralization and sCOMP – to reflect the metabolism of articular tissues, among them soft tissues in general.

We did not find any statistically significant differences in the median values of biomarkers between male and female control subjects. However, in subjects with chronic knee complaints, we observed significantly higher median values of four biomarkers (COMP, PIIANP, CTx-I, and MidOC) in males when compared to females. The median values for males and females are presented in **Table 6**.

Table 6. Median concentrations of biomarkers in subjects with chronic knee complaints

Biomarker	Males	Females	Unit	p value
	Median +/- SD	Median +/- SD		
sCOMP	11.8 (3.2)	9.7 (2.6)	U/L	0.001
uCTx-II	121.2 (121.3)	172.4 (128.3)	ng/mmol Crea	ns.
sPIIANP	766.2 (370.1)	670.2 (212.0)	ng/mL	0.016
sPINP	43.1 (14.3)	41.4 (18.1)	µg/L	ns.
sCTx-I	0.43 (0.2)	0.38 (0.2)	µg/L	0.039
sOC	22.7 (7.1)	22.2 (10.1)	µg/L	ns.
uMidOC	1.45 (0.7)	1.01 (0.9)	µg/mmol Crea	0.025

By Mann-Whitney U-test.
SD – standard deviation.

Only in the case of PIIANP were the median values significantly higher in subjects with chronic knee pain when compared with controls. The respective median values for males and male controls were 766.2 and 437.2 ng/ml and for females and female controls 670.2 and 357.6 ng/ml ($p<0.0001$ for both males and females).

As expected the levels of biomarkers were significantly dependent on the ages, BMI and menopausal status of the subjects.

In female patients, COMP, CTx-II, PINP and OC values were closely associated with age, CTx-II and PIIANP were significantly associated with BMI and all studied biomarkers except PIIANP were affected by menopausal status. In males the input of BMI seemed to be the most important confounder for the systemic values of biomarkers – all studied bone markers (PINP, CTx-I, OC, MidOC) were closely associated with BMI.

Therefore, all the following results presented in the present study are adjusted for gender, age, menopausal status and BMI.

7.4. Associations between joint tissue biomarkers and radiographic knee OA at three different time-points (Paper V)

In our study, OA was radiographically assessed in two different ways: by global OA grades and separately by OA features: osteophytes and JSN. There was no significant correlation between the values of bone markers and global knee OA grades (TFOA and PFOA) at any studied time point. However, several statistically significant associations were observed between biomarkers and JSN and/or osteophytes if they were analyzed separately (**Tables 7** and **8** for cartilage and bone markers, respectively).

Table 7. Correlations between cartilage markers and radiographic features of OA at three different time points (baseline, 3- and 6-year follow-up)

Cartilage marker	Baseline		3-year follow-up						6-year follow-up
	TFOA	TF oph	TFOA	PFOA	Medial TF JSN	Medial PF JSN	TF oph	PF oph	PF oph
COMP	F *	F *		M *			M * F ***	M ** F *	NA
CTx-II	NA	NA	F ***				F ***		M *
PIIANP	NA	NA			M *INV	M *INV		F *	NA

Cross-sectional associations between the values of cartilage markers and features of knee OA examined at three different time points.
By Spearman's rank correlations.
Presented data are adjusted for age, BMI and menopausal status.
Abbreviations:
M – males, F – females, INV – inverse correlation, NA – biomarker not assayed at given time point, Oph – osteophytes, JSN – joint space narrowing
* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Table 8. Correlations between bone markers and radiographic features of OA at three different time points (baseline, 3- and 6-year follow-up)

Bone marker	Baseline	3-year follow-up	6-year follow-up			
	Medial TF JSN	PF JSN	Medial TF JSN	Lateral PF JSN	TF oph	PF oph
PINP			M * inv			F *
CTx-I	F *	F *				
OC				F * *	M * inv	
MidOC		F * *				

Cross-sectional associations between the values of bone markers and features of knee OA examined at three different time points.
By Spearman`s rank correlations.
Presented data are adjusted for age, body mass index, and menopausal status.
Abbreviations: M – males, F– females, INV – inverse correlation, Oph – osteophytes, JSN – joint space narrowing,
* $p < 0.05$, ** $p < 0.01$

It is remarkable that the observed associations between biomarkers and radiographic OA features were different over the course of six years. For example, in females, at baseline and at the three-year follow-up, bone resorption (by CTx-I) was associated with medial TF JSN, whereas at the six-year follow-up the correlation turned out to be non-significant. At the six-year follow-up, a significant association (not observed before) was evident between OC and PF JSN. At the same time, at the three-year follow-up, cartilage degradation (by CTx-II) was strongly associated with TF osteophytes, a correlation that was not seen later at the six-year follow-up, whereas at the three-year follow-up cartilage synthesis (by PIIANP) was inversely associated with TF and PF JSN only in males.

The above results indicated that over the course of six years the associations between biomarkers and radiographic knee OA were largely dependent on (i) gender, (ii) time point and (iii) OA feature.

7.5. Associations between biomarkers and ultrasonographic features in OA (Paper IV)

In middle-aged subjects with knee complaints, nine different parameters were ultrasonographically differentiated (**Table 9**). Among them, the most prevalent findings were calcification in the patellar and quadriceps tendons, synovial thickening, suprapatellar effusion, Baker's cysts, changes in the structure of the femoral cartilage, and meniscal changes. Several US parameters were influenced by age and BMI (**Table 9**). The associations between US findings and

BMI were found to be gender-specific, with most applying only to women. Such US features as tendon calcification, meniscal changes and femoral osteophytes were associated with age.

Table 9. Ultrasonographic findings in early stage knee OA and associations between US features, age, and BMI

Ultrasonographic features	**n**	**Age**	**BMI**
Calcification in tendons #	65	0.42 ***	0.21 *
Thickening of synovia	33	– 0.05	0.21 *
Suprapatellar effusion	27	0.10	– 0.24 *
Baker's cysts	24	0.12	0.07
Changes in cartilage structure	20	0.11	0.08
Meniscal changes	14	0.21 *	0.23 *
Defects in subchondral bone	12	0.21 *	0.20 *
Femoral osteophytes	10	0.26 **	0.23 *
Synovial hyperperfusion	6	– 0.06	– 0.23 *

* $p<0.05$, ** $p<0.01$, *** $p<0.001$ by Spearman's rank correlation.
# – here are presented the summary cases of tendon calcifications in all locations. Calcifications in quadriceps tendons and patellar tendons were documented separately in 50 and 40 cases, respectively.

Surprisingly, all of the studied bone markers were associated with at least some US findings. In pre-menopausal women, thickness of the synovium and the presence of Baker's cysts predicted up to 20% of the overall variability of bone collagen synthesis (by PINP; **Table 10**). The serum values of OC were substantially influenced by the presence of suprapatellar effusion. In men, a strong association was observed between tendinal calcifications and bone collagen synthesis (by PINP; **Table 11**).

The presence of tendinal calcifications was accompanied by the degradation of cartilage collagen (by CTx-II). In addition to routinely ultrasonographically followed suprapatellar effusion, meniscal changes and the presence of Baker`s cysts turned out to play substantial roles in the prediction of cartilage marker values.

The associations between soft tissue changes and cartilage markers were most strongly expressed in post-menopausal women (description of COMP up to 29 %, and CTx-II up to 35%, **Table 10**). In males, both sides of cartilage metabolism (synthesis and resorption) were enhanced in the presence of soft tissue changes (prediction of CTx-II up to 25 %, and PIIANP up to 38%, **Table 11**).

Table 10. Correlations between ultrasonographic findings and biomarkers of bone and cartilage in women.

Marker	Subjects	Calcif. pat. Tend.	Calcif. quad. Tend.	Thick. cart.	Oph	Sub. bone	Eff.	TC	BC	MC	Contribut. to variability (%) #
PINP	F		**	* inv.							13.0
	Prem.							***	*		20.3
CTx-I	F		*								7.7
OC	F		*								7.1
	Postm.						*				11.6
COMP	F			* inv.		***					16.0
	Postm.					*	*			*	29.4
U-CTx-II	F	***			*				**		26.6
	Prem.		*								15.9
	Postm.	**							**		35.5
PIIANP	Prem.						* inv.		* inv.		7.7
	Postm.									*	11.7

* $p<0.05$, ** $p<0.01$, *** p level <0.001 by Spearman's rank correlation.
US parameters that showed significant correlation with the levels of a given marker were included in the regression model.
Contribution of these US findings to the total variability of the marker as a dependent parameter in multiple linear regression analysis.
Abbreviations: BC – Baker's cysts, Calcif. pat. tend. – calcification in the patellar tendon, Calcif. quad. tend. – calcification in the quadriceps tendon, Eff. – suprapatellar effusion, F – female, MC – meniscal changes, Oph – femoral osteophytes, Postm. – postmenopausal women, Prem. – premenopausal women, Sub. bone – subchondral bone defects, TC – thickening of synovia, Thick.cart. – thickness of the femoral cartilage.

Table 11. Correlations between ultrasonographic findings and biomarkers of bone and cartilage in men

Marker	Calcif. pat. Tend.	Calcif. quad. Tend.	Thick. cart.	Oph	Cart. struct. def.	TC	Contribut. to variability (%)
PINP	*						13.7
U-CTx-II			***		*	*	24.5
PIIANP	*	**		*			38.1

* p<0.05, ** p<0.01, *** p<0.001 by Spearman's rank correlation.
US parameters that showed significant correlation with the levels of a given marker were included in the regression model to describe the contribution of such findings to the total variability of the marker, using multiple linear regression analysis.
Abbreviations: Calcif. pat. tend – calcification in the patellar tendon, Calcif. quad. tend – calcification in the quadriceps tendon, Cart. struct.def. – defect in cartilaginous structure, Oph – femoral osteophytes, TC – thickening of synovia, Thick. cart. – thickness of the femoral cartilage.

US-detected soft tissue changes were common in early-stage knee OA. The variability of bone and cartilage biomarkers was substantially dependent on the accompanying status of soft tissues. The above associations were substantially influenced by the patient's gender.

7.6. Associations between cartilage and bone biomarkers and radiographic knee OA progression (Papers V–VI)

7.6.1. The diagnostic and prognostic value of cartilage markers in radiographic knee OA progression (Paper V)

7.6.1.1. Cartilage markers over the six-year follow-up if knee OA progression was expressed by global OA grades

In general, there was no statistically significant difference in the values of the cartilage markers (sCOMP, PIIANP and uCTx-II) between the progressors and non-progressors when we focused only on the changes in global OA grades (TFOA, PFOA). However, an exception was noted for uCTx-II, whose three-year follow-up concentrations (in 2005) were significantly higher in males who developed PFOA progression over the first three years of follow-up (2002–2005) when compared to subjects without radiographic knee OA; the median concentrations for the progressors and "no OA group" were 342 and 220 ng/mmolCrea, respectively, p=0.007. The three-year follow-up concentrations of uCTx-II (in 2005) were also higher in subjects who developed a combined progression in TFOA+PFOA over six years when compared to the individuals without radiographic evidence of knee OA; the medians for progressors and "no OA group" were 247.6 and 188.1 ng/mmolCrea, respectively, p=0.045.

Several associations were found when the radiographic progression of OA was assessed separately for JSN and osteophytes.

7.6.1.2. The diagnostic and prognostic values of the cartilage markers if radiographic OA progression was expressed separately by osteophytes and JSN over two three-year periods

As the radiographic course of knee OA over six years was non-continuous (see the results of the radiographic investigation), the values of the biomarkers were assessed separately for the two three-year periods (2002 to 2005 and 2005 to 2008).

The values of COMP (assayed at baseline and at the three-year follow-up) were associated with progressive osteophytosis over the first three years of follow-up (2002–2005), especially in female patients (**Table 12**). At the same time, no association was observed between cartilage markers and JSN in any location.

Quite the opposite was found three years later (2005–2008), when the values of another cartilage degradation marker – uCTx-II (assayed at the three- and six-year follow-ups) – were associated with progressive JSN in TF, as well as the PF compartment (details in **Table 12**). It seems that the two cartilage degradation markers (COMP and CTx-II) reflect different aspects of the early process of OA. COMP, reflecting a non-collagenous part of cartilage degradation, seems to respond to the disease earlier in the OA phase, which is predominantly expressed by osteophytosis. In summary, both of these markers had some diagnostic, as well as prognostic, value for radiographic progression of knee OA.

Table 12. Associations between cartilage markers and radiographic progression of OA features – osteophytes and JSN over 6-year follow-up

Cartilage biomarkers	**Progression of radiographic features First 3 years of follow-up**		**Progression of radiographic features Last 3 years of follow-up**	
	TF Oph	**PF Oph**	**TF JSN**	**PFJSN**
COMP at baseline	F *	n.s.	n.s.	n.s.
COMP at 3-yr follow-up	M+F ***	F *	n.s.	n.s.
CTx-II at 3-yr follow-up	n.s.	n.s.	F *	M+F **
CTx-II at 6-yr follow-up	M+F *	n.s.	M+F *	M+F **

Associations between cartilage markers and radiographic progression of OA features were analyzed separately for the two consecutive 3-year periods.
* $p<0.05$, ** $p<0.01$, *** $p<0.001$ by Spearman's rank correlation.
Presented data are adjusted for age, BMI and menopausal status
Abbreviations:
M – males, F – females
M+F – males and females together, Oph – osteophytes, JSN – joint space narrowing, n.s. – statistically not significant

7.6.1.3. The diagnostic and prognostic value of the cartilage markers if distinct forms of progressive OA were differentiated

In the following more detailed analysis, we differentiated between specific radiographic subgroups of progressive OA with an *isolated* progression of only JSN (without progressive osteophytosis), of only osteophytes (without progressive JSN), with progression in only the TF compartment, or with simultaneous progression of osteophytes and JSN in several knee joint compartments ("extensive progression"). This approach revealed more *diagnostic* as well as *prognostic* associations between cartilage markers and progressive knee OA.

First three years of follow-up (2002 to 2005):
The values of all studied cartilage markers – COMP, CTx-II and PIIANP (assayed at the three-year follow-up) – were higher in subjects with progressive osteophytosis when compared to subjects without OA (**Table 13**). Serum COMP values had a significant positive correlation with isolated TF osteophyte progression (cases without JSN; r(s)=0.449, p=0.003; medians in **Table 13**). The uCTx-II values correlated with isolated progression in the TF compartment (TF osteophytes in combination with TF JSN; no PF involvement; r(s)=0.411, p=0.022; medians in **Table 13**). As expected, uCTx-II output was higher in patients with an extensive progression of OA (i.e. progressive osteophytosis and JSN simultaneously in several knee compartments; r(s)=0.415, p=0.004; medians in **Table 13**). The same finding was observed also for sPIIANP (r(s)=0.291, p=0.05). Several other associations between the values of sPIIANP and radiographic knee OA progression did not remain significant after adjustment for age, gender and BMI (data not shown), as sPIIANP had a strong correlation with BMI, especially in females (r(s)=0.309, p=0.005).

Table 13. Median concentrations of cartilage markers in specific subgroups of radiographic OA progression over 3-year follow-up

Marker *	Subgroup of OA progression	Progression	No OA	p value
sCOMP	**Isolated TF osteophytes**	**13.8 (n=16)**	**11.9 (n=25)**	**0.003**
uCTx-II	**Extensive progression** †	**229 (n=22)**	**150 (n=25)**	**0.004**
uCTx-II	**Isolated TF osteophytes + TF JSN** ‡	**228 (n=6)**	**150 (n=25)**	**0.023**
sPIIANP	**Extensive progression** †	**758.5 (n=21)**	**602.7 (n=25)**	**0.050**

The differences in medians between the progressive OA and no OA groups were calculated by Mann-Whitney U-test.
***** Serum and urinary markers assayed at 3-year follow-up.
† Simultaneous progressive osteophytosis with or without progressive JSN in multiple knee joint compartments.
‡ OA progression only in the TF compartment (TF osteophytes and/or TF JSN), without simultaneous PF involvement.
The numbers in the parentheses represent the number of patients with a distinct type of radiographic progression and the number of subjects without radiographic knee OA.

According to the above results, all three cartilage markers had some diagnostic value for early-stage radiographic knee OA progression. The latter analysis offered more precise evidence that progressive osteophytosis is accompanied by an enhanced metabolism of articular cartilage.

Subsequent three years of follow-up (2005 to 2008):
The values of serum COMP and urinary CTx-II, assayed at the three-year follow-up (in 2005), had some predictive value for OA progression during the subsequent three years (2005–2008). However, the association was limited only to the subset of patients with extensive progression of OA (i.e. progressive osteophytosis and JSN simultaneously in several knee compartments; for COMP r(s)=0.444, p=0.009; n=34; and for CTx-II r(s)=0.408, p=0.016, n=34). The median sCOMP values for progressors and non-progressors were 11.9 and 13.7 U/L, respectively (p=0.016); and for uCTx-II 243 and 172 ng/mmolCrea, respectively (p=0.013).

Significantly increased values of sCOMP and uCTx-II were found in subjects who subsequently developed more severely expressed radiographic OA, indicating some predictive value of these biomarkers. At the same time, no predictive value for subsequent radiographic knee OA progression was observed for the serum levels of PIIANP.

7.6.2. The diagnostic and prognostic value of bone markers in radiographic knee OA progression (Paper VI)

7.6.2.1. Bone markers over a six-year follow-up if knee OA progression was expressed by global OA grades

In general, there was no statistically significant difference in the values of studied bone markers (sPINP, sCTx-I, sOC and uMidOC) between the progressors and non-progressors when we focused only on changes in global OA grades (TFOA, PFOA). However, a single exception was noted for the levels of sPINP, whose baseline concentrations were significantly higher in subjects who developed subsequent simultaneous progression in TFOA and PFOA grades over six years; the median concentrations for the progressors and non-progressors were 59.3 (n=9) and 44.6 (n=21) ng/mL, respectively; p=0.009. A significant positive correlation between TFOA+PFOA grade progression and baseline values of PINP was also observed (r(s) =0.481, n=30, p=0.008).

Several associations were found when the radiographic progression of OA was assessed separately for JSN and osteophytes.

7.6.2.2. The diagnostic and prognostic values of the bone markers if radiographic OA progression was expressed separately by osteophytes and JSN

No statistically significant association was observed between values of bone markers and progressive JSN for any knee joint location. However, a significant positive correlation was observed between baseline values of sPINP and subsequent progressive TF and PF osteophytosis over the three-year follow-up (2002–2005) (r(s)=0.33, n=44, p=0.029). The medians of PINP for the progressors and non-progressors were 45.2 (n=29) and 39.4 (n=15) ng/mL, respectively; p=0.030. This correlation indicates the prognostic value of the bone marker PINP for progressive osteophytosis.

At the same time, no diagnostic association was observed between values of PINP and progressive osteophytosis. In addition, no statistically significant diagnostic or prognostic association between values of the other studied bone markers, CTx-I, OC and MidOC, and progressive osteophytosis was found.

7.6.2.3. The diagnostic and prognostic value of the bone markers if distinct forms of progressive OA were differentiated

In the following analysis, we differentiated between specific radiographic subgroups of progressive OA with *isolated* progression of only JSN (without progressive osteophytosis), of only osteophytes (without progressive JSN), with progression in only the TF compartment, or with simultaneous progression of osteophytes and JSN in several knee joint compartments (so called "extensive progression"). As already seen for cartilage markers, this approach also revealed several *diagnostic* and *prognostic* associations between bone markers and progressive knee OA.

First three years of follow-up (2002 to 2005):
Prognostic value: The baseline serum values of PINP were significantly higher in the subgroup of patients with more extensive progression of OA compared to the "no OA group" (r(s)=0.460, n=35, p=0.005; **Table 14**).

Diagnostic value: The serum values of PINP and OC (assayed at the three-year follow-up, in 2005) had a significant positive correlation with isolated progressive TF osteophytosis during the first three years of follow-up (r(s)= 0.350, n=33, p=0.046 and r(s)=0.458, n=33, p=0.007, respectively). The median concentrations of PINP and OC for the osteophyte progressors versus "no OA group" are presented in **Table 14**. A similar association was observed for the values of OC (assayed at the three-year follow-up) in subjects in whom disease progression was only confined to the TF compartment (TF osteophytes and/or TF JSN; without PF involvement; r(s)=0.355, n=42, p=0.007; **Table 14**).

At the same time, no statistically significant correlation was observed between the values of sCTx-I or uMidOC and the above-described distinct forms of progressive knee OA.

Table 14. Median concentrations of the bone markers for specific subgroups of radiographic OA progression over the first 3 years of follow-up

Bone marker	Type of OA progression	Gender	Progression	No OA	p value	Observed role
PINP assayed at baseline	**Extensive progression ***	**M + F**	58.2 (n=11)	42.3 (n=24)	**0.006**	Predictive
		F	64.0 (n=11)	38.1 (n=12)	**0.0002**	
PINP assayed at 3-yr follow-up	**Isolated TF osteophytes †**	**M + F**	50.2 (n=16)	36.4 (n=18)	**0.036**	Diagnostic
OC assayed at 3-yr follow-up	**Isolated TF osteophytes †**	**M + F**	26.1 (n=16)	18.6 (n=18)	**0.005**	Diagnostic
		F	34.2 (n=11)	18.6 (n=12)	**0.013**	
OC assayed at 3-yr follow-up	**Isolated TF involvement ‡**	**M + F**	24.5 (n=38)	18.6 (n=13)	**0.008**	Diagnostic
		F	24.3 (n=29)	18.1 (n=13)	**0.012**	

Differences in medians between the progressive OA and no OA groups were calculated by Mann-Whitney U-test.

* Simultaneous progressive osteophytosis with or without progressive JSN in multiple knee joint compartments.

† OA progression expressed only by tibiofemoral osteophytes (without simultaneous progressive TF JSN or PF involvement)

‡ OA progression only in the TF compartment (TF osteophytes and/or TF JSN), without simultaneous PF involvement.

The numbers in the parentheses represent the number of patients with a distinct type of radiographic progression and the number of subjects without radiographic knee OA.

Subsequent three years of follow-up (2005 to 2008):

Over the following three years, only a single association was found between bone markers and progressive knee OA: baseline values of sPINP were positively correlated with subsequent extensive progression of knee OA (r(s)=0.457, n=25, p=0.022). The median values of sPINP for the progressors compared to the non-progressors were 58.2 (n=7) and 39.8 (n=18) ng/mL, respectively; p=0.013. The latter association again indicates the prognostic value of the bone marker PINP for radiographic knee OA progression when the subgrouping of the patients was applied. At the same time, no diagnostic value of PINP for knee OA progression was observed over the last three years of the follow-up. Moreover, no diagnostic or prognostic value of the other bone markers, CTx-I, OC and MidOC, was found.

7.7. Simultaneous assessment of the associations between cartilage and bone biomarkers and knee OA progression

In the present study, an additional statistical computation – logistic regression analysis – was performed to quantitatively assess (by odd ratios) the potential risk associations between knee OA progression and increasing levels of cartilage and bone biomarkers.

We observed that the risk of isolated TF osteophyte progression was significantly increased with the increasing levels of sCOMP, sOC and uMidOC, in comparison with the non-progressors (OR=1.41, 95% CI 1.056–1.870, p=0.020 for COMP; 1.34, 95% CI 1.05–1.72, p=0.021 for OC and 5.32, 95% CI 1.41–20.06, p=0.014 for MidOC; **Figure 5**). These findings demonstrate that, among all studied biomarkers, the risk for progressive osteophytosis was highest in the case of increased levels of the bone resorption marker uMidOC. Increased risk became evident only if a radiographic sub-grouping of patients was applied.

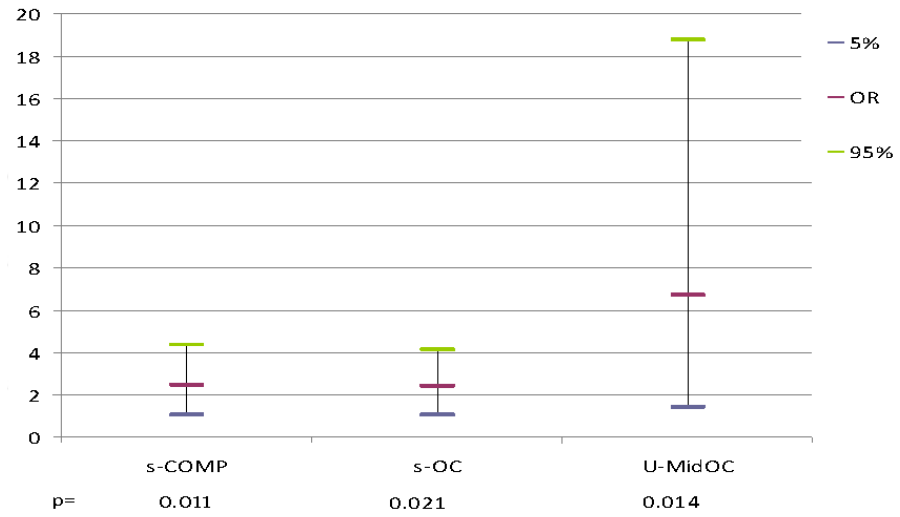

Figure 5. Associations between biomarker values and progressive tibiofemoral osteophytosis.
By logistic regression analysis.
OR – odds ratios; 95 % CI – 95% confidence interval.
Cartilage and bone markers assayed at 3-year follow-up.
Progressive tibiofemoral osteophytosis (without simultaneous progressive TF JSN or PF involvement) over first three years of follow-up.
Note that the risk of TF osteophyte progression was significantly increased with the increasing levels of sCOMP, sOC and uMidOC, in comparison with the non-progressors. However, among the studied biomarkers, the risk for progressive osteophytosis was highest in case of the increasing levels of bone resorption marker – uMidOC.
All analyses were adjusted for age, gender, and BMI.

The above results are summarized in **Table 15** to demonstrate the diagnostic and prognostic value of all studied cartilage and bone markers for progressive knee OA. The behaviour of biomarkers was clearly different over the six follow-up years.

Table 15. The diagnostic and prognostic value of cartilage and bone biomarkers for radiographic knee OA progression over two 3-year follow-up periods

Biomarker	First 3 years of follow-up (2002–2005)		Last 3 years of follow-up (2005–2008)	
	Diagnostic value	Prognostic value	Diagnostic value	Prognostic value
sCOMP	**Yes** †	**Yes** *	NA	**Yes** †
UCTx-II	**Yes** †	NA	**Yes** ‡	**Yes** †
sPIIANP	**Yes** †	NA	NA	NA
sPINP	**Yes** †	**Yes** *	No	**Yes** *
sOC	**Yes** †	No	No	No
sCTx-I	No	No	No	No
uMidOC	**Yes** †	No	No	No

* Biomarker assayed at baseline (2002)
† Biomarker assayed at 3-year follow-up (2005)
‡ Biomarker assayed at 6-year follow-up (2008)
NA – the biomarker was not assayed at given time point to evaluate its predictive or diagnostic value.

All three studied cartilage markers – COMP, CTx-II and PIIANP – expressed the diagnostic value for radiographic knee OA progression. Among them, CTx-II had diagnostic value for progressive OA over both studied time periods (the first and last three years of follow-up). Two cartilage markers out of three – COMP and CTx-II – had a prognostic value for radiographic knee OA progression and, of the two, COMP had prognostic value during both studied time periods.

Three bone markers out of four – PINP, OC and MidOC – expressed the diagnostic value for progressive knee OA over the first three years of follow-up. Interestingly, an association between bone resorption and progressive knee OA was observed for the non-collagenous bone resorption marker MidOC, but not for the bone collagen (type I) degradation marker CTx-I. Over the last three years of follow-up, however, no diagnostic association was observed between the studied bone markers and progressive knee OA, which indicates a metabolically non-continuous course of knee OA. This is in accordance with the data on the radiographic course of knee OA over six years, which revealed a non-continuous pattern with periods of progression and intermittent stops. Only one

of the studied bone markers, the bone formation marker PINP, had a prognostic value for OA progression over both assessed time periods.

There was, however, no prognostic or diagnostic association between the collagen type I degradation marker CTx-I and progressive knee OA at any time point.

8. DISCUSSION

During the past two decades, there have been significant developments in the scientific understanding of OA. It is now widely accepted that the OA process may arise from any kind of articular tissue: cartilage, subchondral bone, synovial tissue, menisci, tendons, ligaments or even periarticular muscles or nerves. Moreover, OA is now characterized as a disease with a prolonged asymptomatic molecular phase, a pre-radiographic phase and later a radiographic stage, with evident structural joint changes, pain and loss of function [49]. Nevertheless, so far the vast majority of studies of OA have been one-sidedly focused on cartilage changes and conducted on patients with end-stage disease. Only a limited number of studies have assessed OA radiographic prevalence and its progression in subjects younger than 50 years [61;74;75;124]. Thus, the knowledge of the early phases of OA is rather limited.

In recent years, emphasis has been placed on the development of molecular markers that can provide an early warning of the initiation of articular matrix breakdown and be used as a method for earlier diagnosis of OA. In previous biomarker studies on elderly patients, only single markers originating from cartilage or bone tissue have been used separately, which does not offer a full overview of the complex metabolic changes in different joint tissues. The investigation of OA calls for a simultaneous usage of a whole set of radiographic and non-radiographic means, among them biomarkers originating from different joint tissues. The present study is designed largely on the assumption of the above understanding.

In order to achieve a systematic overview of the complex processes of early knee OA, a cross-sectional and longitudinal study was performed using radiographic and ultrasonographic methods and a full set of molecular markers originating from several joint tissues.

The present study is the first to evaluate the value of an extensive set of joint tissue biomarkers in middle-aged subjects with early radiographic knee OA.

Biochemical investigation

In the present study, we sought to assess whether a set of joint tissue biomarkers could differentiate progressive and non-progressive cases of early-stage knee OA in middle-aged subjects. To achieve this goal, we simultaneously investigated several aspects of bone and cartilage metabolism cross-sectionally, as well as longitudinally, in early stage progressive and non-progressive knee OA. We assayed *en bloc* specific markers of type I collagen synthesis (PINP) and resorption (CTx-I), specific markers of type II collagen synthesis (PIIANP) and degradation (CTx-II), non-collagenous markers of bone (OC) and a mainly cartilage (COMP), as well as a novel non-collagenous, marker of bone resorption: urinary MidOC.

Specification of the role of the two biomarkers COMP and MidOC

Although initially considered to be an entirely cartilage-specific molecule reflecting cartilage breakdown [89–90], COMP has now been identified in several other articular structures, such as synovial capsule, ligaments and tendons. However, the concentration of COMP in these tissues is substantially lower than that in cartilage and menisci [92–93]. There is even evidence suggesting that COMP is also secreted by osteoblasts and vascular smooth muscle cells [91]. Our first attempts were therefore devoted to gathering more detailed information on the associations between COMP and radiographically assessed cartilage damage expressed by JSN, osteophytes and changes in US-detected soft tissues in early-stage knee OA.

We found that serum COMP values were significantly higher in subjects with TF osteophytes compared to those without any radiographic disease. In addition, the levels of COMP turned out to be substantially affected by the changes in knee-joint soft tissues, especially meniscal changes. Therefore, compared to other more specific biomarkers, COMP seems to be an even better reflector of the changes in the joint, as it consists of the contributions of different articular tissues. These results, which were based on subjects about 20 years younger than those investigated in earlier studies [113;114;117;120], indicate that in early-stage OA, the appearance of osteophytes is accompanied by increased serum levels of COMP. Recently, Kraus *et al.* have even postulated that COMP is a marker of osteophytes [130]. Our study has therefore contributed to the evaluation of the wider role of COMP among other biomarkers and has clarified its essence.

A noncollagenous marker OC is synthesized by osteoblasts and has therefore been widely used as a biomarker of bone formation [79;111;120]. Its biological function is considered to be related to the inhibition of bone matrix mineralization [108]. However, total serum OC is a complex marker that embraces multiple molecular fragments, some of them associated not only with bone synthesis but also with resorption [106–107]. OC fragments accumulating in the urine might give qualitatively different information concerning bone turnover [107]. It has been previously shown that the urinary mid-molecule fragments of OC were suppressed in post-menopausal women with osteoporosis who received bisphosphonate alendronate therapy [106;110]. Based on the above data, we assumed that molecular forms of OC in urine might not show the same characteristics as its mother molecule in serum (total OC). To clarify the role of urinary OC, we studied a newly developed marker – MidOC.

Before implementing this brand new marker in the OA study, we assayed MidOC in a small group of post-menopausal women with osteoporosis receiving bisphosphonate – risedronate treatment. We assayed MidOC along with three other bone markers: serum total OC, PINP and CTx-I. Indeed, we observed that the behaviour of MidOC was entirely different from the well known bone formation markers total OC and PINP. In fact, quite the opposite was found: a parallel decrease was seen in the levels of MidOC and the well-characterized bone resorption marker sCTx-I after even the first month of

risedronate treatment, while the levels of bone formation markers sOC and sPINP did not differ from the pre-treatment values at that time point. Moreover, during therapy, the strongest correlations were found between the changes in MidOC and CTx-I. These results serve as important evidence of MidOC behaving similarly to a marker of bone degradation. However, the association between MidOC and the bone resorption marker CTx-I was not strong enough to assume a complete analogy between these markers. Therefore, in OA, MidOC as a noncollagenous marker of bone resorption reflects a different aspect of bone degradation. Assuming that MidOC reflects a different aspect of bone resorption, we used this new marker in the present early knee OA study. To the best of my knowledge, this study is the first to evaluate the role of MidOC in knee OA.

Radiographic investigation

There is limited data on the prevalence of radiographic knee OA in middle-aged subjects. According to the few previous studies advanced radiographic knee OA (OA grade ≥ 2 by KL or Nagaosa) has been found in 1.5 -14.3% of people aged 35–54 [3;72;73].

In the present study, we observed that altogether 56% of the middle-aged subjects with chronic knee complaints had radiographic signs of knee OA, the majority of them (90%) being diagnosed with knee OA grade 1. It is important to mention that in our study as many as 38% of patients with grade 1 OA had PF joint involvement alone, mainly due to osteophytosis, highlighting the importance of PF joint assessment. The high prevalence of PFOA among middle-aged subjects shows the importance of separate assessment of TF and PF joints; otherwise, the subjects with early isolated PF joint involvement could easily be missed as subjects with no OA or, even worse, could be classified as controls.

The diagnosis of OA was in more than half of the cases based on the presence of osteophytes alone and in one-third of the cases on osteophytes and JSN in combination. So, methodically we investigated osteophytosis and JSN as different entities of OA. Cross-sectional and longitudinal studies have shown that, at least in the knee joint, osteophytes may be a more reliable indicator of early disease than a grade 1 JSN [55;58]. In a recent review, V.B. Kraus stated that, as osteophytes and JSN involve different pathophysiologies, in biomarker studies, analyses of these features should ideally be looked at individually [6]. Thus, global KL OA grades may easily dilute the important information of OA processes, especially in the subtle early phase.

In the few previous studies available on middle-aged subjects, the radiographic OA progression has been assessed in different ways, based on either TF JSN [75;124] or KL grade [74;124], making it difficult to compare the results of different study groups. Thorstensson *et al*. documented that 86% of middle-aged subjects with knee pain developed incident TFOA (KL ≥1) over 12 years, and incident PFOA occurred in 31% over five years [74]. Single studies conducted in subjects as young as in our study have confirmed knee OA progression over four and 12 years [75;124]. In the present study, the overall

progression rate of early knee OA over six years was 56% (71 out of 128), with the majority of cases progressing from grade 0 to 1. The overall OA progression rate seemed to be a little lower but compatible with the five-year follow-up data by Thorstensson *et al.* [74]. In almost half of the cases (44%), the radiographic progression of OA was based on the development or growth of osteophytes alone vs. only in 20% of cases on JSN alone. The separate assessment of osteophytes and JSN in OA prevalence and progression confirmed the importance of osteophytosis as the earliest radiographic sign of OA.

Due to the lack of systematic follow-up data, the natural course of knee OA progression has been assumed to be continuous. The study by Sharif *et al.* was the first to indicate the non-linear pattern of knee OA progression over five years that was associated with different levels of sCOMP [64]. Indeed, following subjects over six years, we observed a radiographically non-consistent course of OA progression, with periods of progression and stabilization. Thus, in the majority of OA patients, the radiographic status of knee OA is constant or progresses with intermittent stops, being therefore slow, except for a few cases with continuous progression. Through longitudinal investigation, we observed five different radiographic groups of OA progression, indicating the remarkable heterogeneity of the disease. The above findings were largely confirmed by the recent study by Bartlett *et al.* [131], who followed a large cohort of OA patients aged 39–80 over two years by JSN and even differentiated seven distinct groups of different OA trajectories. In conclusion, both of these studies demonstrate that plenty of knee OA patients do not progress over two to six years, representing the most common pattern, while the rest develop a progressive course at different speeds: slow, moderate or rapid.

Associations between biomarkers and US-detected articular soft tissues

Although it is now widely accepted that the OA process can be initiated in any articular tissue, the importance of soft tissue changes has been clearly underestimated. For that reason, there is also a remarkable lack of knowledge concerning the potential value of articular biomarkers in the case of OA-related soft tissue changes. Jung *et al.* were the first to investigate the relationship between biomarkers and US-detected soft tissue changes in patients with knee OA [127]. Their study, which employed relatively few patients, demonstrated associations between the levels of general joint tissue biomarkers (HA and COMP) and US-detected effusion, synovial proliferation, medial osteophytes and capsular distension [127]. To fill the above-described knowledge gap, we examined the possible associations between a large set of biomarkers and US-detected soft tissue changes in early-stage knee OA. To the best of our knowledge, this study is the first to investigate associations between US findings and biomarkers in middle-aged knee OA subjects. Beside such common US-detected features as suprapatellar effusion, synovial thickening, meniscal changes and Baker`s cysts, the presence of calcifications in the quadriceps and patellar tendons were documented – an aspect that seems to have been overlooked.

We succeeded in demonstrating several significant associations between biomarkers and US-detected soft tissue changes. Our results confirmed that in patients with early-stage knee OA US-detected soft tissue changes play a major role in the overall variability of bone and cartilage biomarkers. At the same time, the observed diverse links between biomarkers and soft tissue changes indicated the remarkable complexity of the OA metabolic processes in knee joint soft tissues. Moreover, the findings turned out to be substantially influenced by gender differences and in females also by menopausal status. In pre-menopausal women, US-detected synovitis was related to the increased synthesis of type I collagen (as shown by PINP) and the decreased expression of type II collagen (by PIIANP). The latter shifts seem to indicate that the presence of synovitis probably retards the synthesis of articular cartilage. Some features of synovitis, such as thickening of the synovial layer and the presence of Baker's cysts, predicted up to 20% of the variability of bone marker sPINP. As a considerable part of synovial tissue consists of type I collagen, the activation of synthetic processes in synovium might be responsible for the higher serum levels of PINP [132]. Surprisingly, several associations were observed between biomarkers and the presence of tendon calcifications. In women, all three bone markers – PINP, CTx-I and OC – were associated with quadriceps tendon calcifications. As type I collagen is the major collagen in tendons [133], these metabolic markers may indicate ongoing active metabolic changes in tendons. On the other hand, in post-menopausal women, patellar tendon calcifications were strongly correlated with cartilage degradation (by uCTx-II) but not with cartilage synthesis (by sPIIANP). These findings, however, suggest that tendon calcifications accompany the active degradation processes in articular cartilage. The presence of tendon calcification seemed to signal a phase of knee OA with simultaneous activation of metabolic changes in bone and soft tissue metabolism but degradation in articular cartilage. These diverse findings have never been described before. In men, quite the opposite was observed: tendon calcifications were related to cartilage synthesis (based on PIIANP) but not to cartilage degradation (based on CTx-II), suggesting that, in the case of tendon calcification, there is a strong predilection for the production of type II collagen. Hence, in early-stage knee OA the presence of tendon calcifications might indicate the predominance of cartilage reparation in men and cartilage degradation in women. Tendon calcifications made it possible to predict up to 35% of the variability in CTx-II and up to 38% of the variability of PIIANP, in women and men, respectively.

In females, suprapatellar effusion, meniscal changes and subchondral contour defects together predicted up to 29% of the variability of sCOMP. This finding served as additional evidence for the point of view that, besides cartilage degradation, the level of sCOMP is substantially influenced by changes in soft tissues.

The above findings enable us to conclude that a wide-ranging set of articular biomarkers, such as COMP, CTx-II, PIIANP and PINP, is certainly necessary to have an overview of the ongoing complicated processes in articular tissues

during OA. US seemed to offer definite, unique and clinically easily available information on the complex processes in articular soft tissues in early-stage knee OA.

Associations between cartilage biomarkers and radiographic knee OA progression

The vast majority of cartilage biomarker studies on OA progression have focused on elderly subjects with advanced-stage disease [112;114;117;119–121]. The limited number of studies conducted on middle-aged subjects has shown that cartilage biomarkers seem to have both diagnostic [122] and prognostic [123–124] roles in early-stage knee OA. However, the above studies have investigated only a single aspect of OA cartilage metabolism by IGF-1 [123], COMP [122] or PIICP [124], whereas a simultaneous assessment of the synthesis and degradation of cartilage has not been addressed. In these studies, the follow-up period was long enough – three to 12 years – but only two time points were registered: baseline and endpoint. The above-described approach does not make it possible to explore the non-linear course of OA, which is present according to recent studies.

It is clear, that the complexity of the early OA processes calls for more extensive studies and a systematic approach. To gain a more comprehensive overview of early-stage OA, we assayed cartilage biomarkers reflecting both sides of the metabolism: synthesis and resorption of cartilage (type II), collagen (PIIANP and CTx-II, respectively) and a noncollagenous marker of articular tissues that is presumed to signal mainly cartilage degradation, COMP.

As described above, following each subject radiographically over six years, a non-consistent course of OA radiographic progression was observed. Therefore, the duration of the present biomarker study was divided into two consecutive three-year periods – the first three years and the last three years of follow-up (i.e. 2002 to 2005 and 2005 to 2008).

The associations between cartilage biomarkers and the presence of radiographic knee OA features examined at three different time points (at baseline, three- and six-year follow-ups) were different over the course of six years and largely dependent on gender, age, the phase of OA development and OA radiographic features (osteophytes or JSN). The presence of TF osteophytes was reflected by increased cartilage degradation (by COMP and CTx-II). At the same time, the thinning of articular cartilage in TF as well as in PF joints was associated with a decrease in cartilage synthesis (by PIIANP). A similar finding was observed by Garnero *et al.* in elderly patients with advanced TF JSN [116]. Thus, at the group level, lower values of PIIANP signal the presence of cartilage damage both in early and advanced cases of knee OA.

So far, radiographic knee OA progression has been diagnosed based on the thinning of JSW or on the increase in global KL grade. These criteria, however, overlook the first radiographic sign of early OA – osteophytosis. Separate assessment of osteophytes and JSN enabled us to reveal a certain pattern of metabolic shifts in the course of progressive knee OA. Over the first three years

of follow-up, we observed a significant correlation between COMP and progressive osteophytosis, whereas over the subsequent three years the correlation was between CTx-II and progressive JSN. These results indicate that these two markers of cartilage degradation (COMP and CTx-II) reflect different aspects of the early OA. COMP, reflecting a noncollagenous part of cartilage degradation, seems to respond to the disease earlier in the period, predominantly expressed as progressive osteophytosis. The phase of progressive osteophytosis seems to be followed by a more advanced disease, expressed by progressive cartilage damage, reflected by the higher output of uCTx-II. Thus, the described biochemical processes in OA-affected articular tissues serve as more evidence of the complex course of the disease.

We observed that the values of all studied cartilage markers – COMP, CTx-II and PIIANP – were higher among the subgroup of subjects with progressive osteophytosis when compared to subjects without OA. According to the detailed analysis, serum COMP values were higher in the case of subtle OA changes: isolated progressive osteophytosis only in the TF compartment (cases without JSN and PF involvement). On the other hand, the uCTx-II values were higher in more extensive cases of progression, such as progression in the TF compartment (progressive TF osteophytosis + TF JSN in combination) or progressive OA (osteophytosis + JSN) simultaneously in several knee compartments. The latter association was also true for sPIIANP. Several associations between the values of sPIIANP and radiographic knee OA progression, however, did not remain significant after adjustment for age, gender and BMI, as sPIIANP had a strong correlation with BMI, especially in females. To the best of our knowledge, this is the first attempt to describe the activation of cartilage metabolism in early progressive knee OA.

It is known that osteophytes represent areas of new cartilage and bone formation [134]. Osteophyte formation starts with chondrogenesis within fibrous mesenchymal tissue, which then results in a cartilage-like structure called a chondrophyte (fibrocartilaginous cells). The core of the chondrophyte undergoes hypertrophy, followed by ossification (chondro-osteophyte). Eventually, the entire structure turns into bone (osteophyte) [36]. As described above, in the present cohort, progressive osteophytosis was associated with enhanced turnover in both the collagenous and noncollagenous components of cartilage (based on uCTx-II, sCOMP and sPIIANP). We propose several possible explanations for the associations between progressive osteophytosis and cartilage biomarkers (Paper V). First, during the formation of a stage III osteophyte, it consists of aggrecan, collagen type II and even chondrocytes [35]. Therefore, during progressive osteophytosis, the products of cartilage metabolism (uCTx-II, sPIIANP and sCOMP) may be released into the systemic circulation in higher concentrations. The serum pool of COMP might also indicate changes in soft tissues (meniscal changes) or reflect enhanced osteoblastic activity in the chondro-osteophytic stage of osteophyte development [135]. As a more direct explanation, according to some MRI studies, knee cartilage defects may accompany radiographically detected osteophytes of the

same joint, either indirectly [136] or causally [63]. Therefore, the increase in cartilage degradation (by uCTx-II and sCOMP) in progressive osteophytosis might indicate accompanying defects in articular cartilage not yet visible through plain radiography.

According to the BIPED criteria, biomarkers are presumed to have five different categories of performance, among them a diagnostic and prognostic one [82]. Following the above criteria, all three cartilage biomarkers used in the present study have been shown to be prognostic markers of knee OA progression in elderly patients [117–119]. In the present study, serum COMP and urinary CTx-II concentrations, assayed at the three-year follow-up, had some predictive value for OA progression during the subsequent three years. However, the association was limited only to the subset of patients who demonstrated a more extensive knee OA progression (osteophytosis+JSN) simultaneously in different knee joint compartments. As a result, sCOMP and uCTx-II had some predictive value, although only in subjects who subsequently developed more severely expressed radiographic features of OA.

The results of the present study enable us to conclude that all three studied cartilage biomarkers, CTx-II, PIIANP and COMP, had a diagnostic role for OA progression (mainly expressed by progressive osteophytosis) and two of them, COMP and CTx-II, also had some predictive value for subsequent OA progression if there was more extensive knee OA progression (osteophytosis+JSN) simultaneously in different knee compartments. The predictive potential of cartilage markers was therefore clearly expressed only among some radiographic subgroups. This study confirms the view that separate assessment of osteophytosis and JSN as different entities of OA is crucially important in biomarker studies. During early phases of OA, distinct metabolic pathways of articular tissues were activated, especially those associated with the presence and progression of osteophytosis.

Associations between bone biomarkers and radiographic knee OA progression

Two decades ago it was suggested that changes in bone rather than cartilage might be responsible for the initial pathophysiological events in OA [30–31;33]. Nevertheless, so far, bone involvement has received inadequate attention in human OA. Studies on bone turnover markers have been focused on elderly patients with advanced-stage knee OA and provided rather conflicting data. Cross-sectional studies have revealed a decrease in bone resorption (by sCTx-I and uCTx-I) and mineralization (by sOC) [79], or no change at all [111;115; 120]. Thus, so far, it is unclear whether OA is characterized by increased [62;94;122,137] or decreased [79;119] bone turnover. In a longitudinal study by Bettica *et al.,* bone resorption was higher in post-menopausal women with progressive knee OA when compared to controls [62], indicating the possible prognostic value of these markers.

Previous studies have mainly focused on bone resorption markers reflecting degradation of type I collagen (CTx-I and NTx-I). At the same time, the other

aspect of bone turnover – synthesis of bone collagen (by the levels of PINP) – has not been routinely assessed. Instead of PINP, serum levels of a non-collagenous marker, OC, have been assayed as a measure of bone formation [79;111;120]. However, as OC is a complex marker that embraces molecular fragments also reflecting bone resorption and inhibition of bone matrix mineralization [108], it should not be regarded as a marker of merely bone formation (i.e. as an equivalent to PINP).

In the present study, we investigated simultaneously the synthetic, resorptive and mineralization aspects of bone metabolism in early-stage progressive and non-progressive knee OA. To achieve this, we assayed *en bloc* specific markers of type I collagen synthesis (PINP) and resorption (CTx-I), as well as a non-collagenous marker, OC, and a novel noncollagenous marker of bone resorption, MidOC.

Our results on the associations between bone marker values and progressive radiographic knee OA again supported the view that global knee OA grades fail to reveal important information about the OA process. Indeed, when we used global radiographic OA grades, only a single positive correlation was observed between six-year progression in TFOA+PFOA grade and higher serum values of PINP. However, when different OA features – JSN and osteophytes – were analyzed separately and some radiographic forms of progressive knee OA were differentiated, we were able to demonstrate several diagnostic and prognostic values of the bone markers for progressive knee OA. We established that progressive TF osteophytosis was accompanied by the activation of bone formation (by PINP), bone resorption (by MidOC), and by changes in bone mineralization (by OC), providing evidence of the diagnostic value of these biomarkers. It is important to note that the association between progressive osteophytosis and bone resorption was reflected only by the novel noncollagenous marker MidOC, and not by the well-known collagen type I degradation marker CTx-I. This discrepancy between the two bone resorption markers indicates that these biomarkers represent qualitatively different aspects of bone resorption. Based on these results, we can speculate that the course of knee OA is comprised of a certain sequence of events. Changes in bone formation and mineralization, as well as in the degradation of the noncollagenous part of bone, precede the collagenous side of bone degradation. However, this hypothesis needs further clarification through larger experimental and clinical material.

Interestingly, three years from baseline, no diagnostic association was observed between the above bone markers and progressive OA, indicating a metabolically non-continuous course of knee OA. This finding is in accordance with our six-year radiographic data from the same cohort, which revealed a non-continuous course of knee OA, with periods of progression and stabilization. Moreover, radiographically, markedly fewer cases of OA progression were found over the first three years of follow-up when compared to the last three years. Thus, we established a metabolically and radiographically non-continuous course of knee OA. In the second period of follow-up, we observed fewer

cases of radiographic progression, as well as lower metabolic activity of bone tissue.

In our material, progressive osteophytosis, as well as more extensive knee OA progression (i.e. combined progression in several knee compartments, including both osteophytes and JSN), was preceded by the activation of the synthesis of type I collagen expressed by serum PINP. This finding provides evidence of the prognostic value of PINP for progressive knee OA. At the same time, no prognostic value was found for the other bone markers: OC, CTx-I and MidOC. The enhanced synthesis of type I collagen (by PINP) possibly reflects the activation of reparative processes in early-stage knee OA. At the same time, the resorptive activity was only modest and there was no prognostic association between the bone resorption markers and progressive knee OA. Later, when the disease had progressed, enhanced bone resorption became evident.

As already observed for cartilage markers, several associations between bone markers and progressive osteophytosis were found. There are at least two possible explanations for the associations between progressive osteophytosis and higher levels of bone markers. Firstly, the higher levels of bone markers, especially bone formation markers, presumably reflect the ossification stage of osteophyte formation. Secondly, higher levels of PINP, a marker of type I collagen that is present also in soft tissues, e.g. mensici, ligaments and tendons, might also reflect changes in soft tissues (meniscal changes) that might accompany progressive osteophytosis.

Thus, our findings from patients with early radiographic knee OA demonstrate the activation of bone metabolism in the case of progressive disease. Prior to radiographic knee OA progression, we observed an activation of reparative processes in bone (bone formation by PINP), which eventually turned out to be inefficient. Later, we documented radiographic OA progression, which was accompanied by an activation of all aspects of bone turnover (by PINP, OC and MidOC). Resorption of the noncollagenous bone (by MidOC) seems to be enhanced before the degradation of collagen type I (by CTx-I).

Three of the studied bone markers (PINP, OC and MidOC) had diagnostic value for progressive osteophytosis, and one of them, PINP, had prognostic value for progressive osteophytosis, as well as for more extensive cases of OA progression (simultaneous progressive osteophytosis + JSN in several knee compartments).

Surprisingly, there is no previous knee OA study available that has systemically evaluated the possible value of cartilage and bone biomarkers simultaneously on the same material, except for a single study by Petersson *et al.* conducted in 1998 [122]. In this study, it was found that the cartilage marker COMP and bone marker BSP were both associated with incident radiographic knee OA.

Based on the results of logistic regression analysis, we established that increasing values of the cartilage marker COMP, as well as the bone markers OC and MidOC, were significantly associated with an increased risk for progressive TF osteophytosis when compared to subjects without radiographic evi-

dence of knee OA. It seems reasonable to assume that OA is a disease affecting mainly articular cartilage and may be better reflected by changes in cartilage biomarkers. However, a comparison of the odd ratios of COMP and MidOC for predicting progressive osteophytosis (OR 1.41 vs. 5.32, respectively) allowed us to conclude that in early-stage knee OA changes in bone metabolism are not only present but can even dominate over changes in cartilage. To the best of our knowledge, the present study was the first to investigate the value of the novel bone resorption marker MidOC in knee OA – a marker that, among all other studied biomarkers, turned out to be the strongest risk predictor for progressive osteophytosis.

9. SUMMARY

The present study offered several new insights into the complex early phases of knee OA and proposed a possible sequence of metabolic events in joint tissues during the early phases.

We demonstrated that more than half of the middle-aged subjects with knee joint complaints had radiographically evident knee OA. The longitudinal study revealed that the overall progression rate of early-stage knee OA was 56%, with the majority of cases progressing from grade 0 to grade 1, and they were largely based on osteophytosis.

We established that, during early phases of OA, distinct metabolic pathways of articular tissues were activated, especially those associated with the presence and progression of osteophytosis. Separate assessment of osteophytes and JSN enabled us to reveal a certain pattern of shifts in cartilage and bone metabolism in the course of progressive knee OA.

A noncollagenous cartilage degradation (reflected by COMP) seems to develop earlier in the disease, in a period predominantly expressed as progressive osteophytosis. The phase of progressive osteophytosis seems to be followed by a more advanced disease, expressed by progressive cartilage damage, reflected by the higher output of uCTx-II. Thus, these two markers of cartilage degradation (COMP and CTx-II) signal different aspects of the early OA process.

Prior to radiographic knee OA progression, we observed an activation of reparative processes in bone (by PINP) that eventually turned out to be inefficient. The phase of inefficient bone reparation was followed by shifts in all aspects of bone turnover: activation of synthesis and degradation (by PINP and MidOC), as well as by changes in bone mineralization (by OC). However, the resorption of nonocollagenous bone (by MidOC) seems to be enhanced before the degradation of collagen type I (by CTx-I).

We observed a radiographically and metabolically non-continuous course of knee OA over six years. In the second period of follow-up (the last three years of follow-up), we observed fewer cases of radiographic progression, as well as lower metabolic activity in cartilage and bone tissue.

The present study is the first to demonstrate that, in early-stage knee OA, the metabolism of bone, cartilage and soft tissues was simultaneously activated. Although initially considered to be a disease affecting mainly articular cartilage, our results indicate that, at least in early-stage knee OA, the changes in bone metabolism may even dominate over that of cartilage. Moreover, we observed a surprisingly important contribution of ultrasonographically detected soft tissue changes to the systemic value of all studied cartilage markers (COMP, CTx-II and PIIANP) and of the bone marker PINP.

We discovered that all three studied cartilage markers (COMP, CTx-II and PIIANP) had diagnostic value for progressive osteophytosis, and two of them (COMP and CTx-II) also had prognostic roles for progressive osteophytosis and

JSN. At the same time, three of the studied bone markers (PINP, OC and MidOC) had diagnostic value, again for progressive osteophytosis, and one of them, PINP, demonstrated a predictive value for more extensive cases of OA progression (osteophytosis + JSN simultaneously in several knee compartments). We specified the resorptive origin of the novel bone marker MidOC, which among all other studied biomarkers turned out to be the strongest risk predictor for progressive osteophytosis.

Based on the findings of the present study, we can offer some recommendations for future OA investigations: (i) the complex nature of knee OA calls for a multifaceted methodical approach, including simultaneous biochemical and radiologic assessment of the changes in bone, cartilage and soft tissues; (ii) the status of osteophytes and JSN must be examined separately, as they indicate different phases of OA, and the presence/progression of which are likely to be reflected by different biomarkers; (iii) the impact of gender should be carefully evaluated; (iv) some bone and cartilage biomarkers may be used in early knee OA to evaluate the diagnostic or prognostic value for knee OA, mainly for progressive osteophytosis; (v) as early knee OA revealed its non-continuous course radiographically, as well as biochemically, more than two time points should be evaluated when following the disease longitudinally.

10. CONCLUSIONS

- The present study is the first to demonstrate the simultaneous activation of cartilage, bone and soft tissue metabolism in early-phase knee OA.
- At least in early-stage knee OA, the changes in bone metabolism may even dominate over that of cartilage. The latter conclusion is based on the finding that, among all studied biomarkers, a novel marker of bone resorption – MidOC – appeared to be the strongest risk predictor of progressive osteophytosis.
- COMP, as a predominantly cartilage-specific biomarker, is substantially reflected by the presence and growth of osteophytes and changes in knee joint soft tissues, especially in menisci. Therefore, we confirm the view that COMP reflects the systemic contribution of different articular tissues.
- More than half (56%) of the middle-aged subjects with chronic knee complaints had radiographic knee OA, and in the majority of cases OA grade 1. The progression rate of radiographic knee OA over six years was 56%, with the majority of cases based on osteophytosis. The radiographic course of early knee OA turned out to be heterogeneous and non-continuous, with intermittent periods of progression and stabilization.
- Ultrasonographically detected soft tissue changes were common in early-stage knee OA, and among these the most prevalent findings were calcification in the patellar and quadriceps tendons, synovial thickening, suprapatellar effusion, Baker's cysts and meniscal changes. The variability of articular biomarker values was substantially influenced by the accompanying US-detectable changes in joint soft tissues:
  - The presence of tendon calcifications was accompanied by cartilage reparation (by PIIANP) in men and cartilage degradation (by CTx-II) in women.
  - In women, US-detected findings of synovitis were simultaneously related to the increased synthesis of type I collagen (by PINP) and the decreased expression of type II collagen (by PIIANP), indicating that the presence of synovitis probably retards the synthesis of articular cartilage.
- The present study revealed a certain pattern of shifts in cartilage and bone metabolism over the course of progressive knee OA:
  - Higher serum values of the noncollagenous cartilage degradation marker COMP reflected the earlier disease period predominantly expressed by progressive osteophytosis, which was followed by more advanced disease, expressed by JSN, reflected by a higher level of uCTx-II.
  - Radiographic knee OA progression was preceded by activated bone reparation (by PINP) and followed by activation of all aspects of bone turnover: synthesis of collagen type I (by PINP), degradation of non-collagenous bone (by MidOC) and changes in bone mineralization (by OC).
- All three studied cartilage biomarkers, COMP, CTx-II and PIIANP, had diagnostic value for progressive osteophytosis and two of them, COMP and

CTx-II, also had some predictive role for subsequent more extensive OA progression (osteophytosis and JSN in combination).

- Three of the studied bone markers, PINP, OC and MidOC, had diagnostic value for progressive osteophytosis, and one of them, PINP, also had predictive role for subsequent more extensive OA progression subsequent more extensive OA progression.
- The present study demonstrated that the complex nature of knee OA calls for a multifaceted methodical approach including: (i) biochemical and radiologic assessment of the simultaneous changes in bone, cartilage and soft tissues; (ii) evaluation of the impact of gender and different phases of the disease; (iii) separate assessment of OA radiographic features, osteophytes and JSN, the presence/ progression of which are likely to be reflected by different biomarkers; and (iv) the assessment of more than two time points in longitudinal studies, as early OA has a non-consistent course radiographically as well as biochemically.

11. SUMMARY IN ESTONIAN

Liigeskudede molekulaarsed markerid põlveliigese varase osteoartroosi korral: rahvastikupõhine longitudinaalne uuring keskealistel isikutel

Uuringu eesmärgid

1. Uurida, milliseid metaboolseid protsesse ja muutusi põlveliigese kudedes peegeldavad seerumist määratav kõhre oligomeetriline maatriksproteiin (COMP) ja uus luukoe päritoluga biomarker – uriini ostekaltsiini keskfragment (MidOC).
2. Hinnata põlveliigese röntgenoloogilise osteoartroosi (OA) levimust ja progressiooni keskealistel põlveliigese kaebustega isikutel.
3. Selgitada võimalikke seoseid kõhre- ja luukoe päritoluga molekulaarsete markerite ja ultraheli uuringul hinnatavate liigeskudede muutuste vahel varases staadiumis OA haigetel.
4. Analüüsida kõhrekoe molekulaarsete markerite diagnostilist ja ennustavat väärtust põlveliigese OA progresseeruvatel juhtudel.
5. Analüüsida luukoe molekulaarsete markerite diagnostilist ja ennustavat väärtust põlveliigese OA progresseeruvatel juhtudel.

Uuritavad ja meetodid

Krooniliste põlvekaebustega (kestusega > 3 kuu) uuritavad vanuses 34–55 aastat leiti Elva perearsti nimistu alusel. Ankeedile vastanud 348-st inimesest osales lõplikus longitudinaalses uuringus 161 isikut (101 naist ja 60 meest, keskmine vanus 45,0 ± 6,2 a.). Uuringud teostati kolmel järjestikusel ajapunktil, uuringu algul (2002 a.), keskel (2005 a.) ja lõpus (2008 a.).

Uuringus nõustus osalema 73 liigeskaebusteta isikut. Neist 40 isikut, kel puudusid röntgenoloogilised põlveliigese OA tunnused (25 naist ja 15 meest), hõlmati kontrollgrupina.

Uue biomarkeri – osteokaltsiini keskfragmendi (MidOC) olemuse uurimiseks hõlmati 19 postmenopausis osteoporoosiga naist vanuses 49–66 a., kes said 12 kuu jooksul ravi bisfosfonaat- risedronaadiga. Luukoe päritoluga molekulaarsete markerite käitumist (sh. MidOC korral) uuriti seoses luu mineraalse tiheduse muutustega vastuseks ravile.

OA röntgenoloogilisi tunnuseid (osteofüüte ja liigesvahemiku kitsenemist) uuriti kõigis kolmes ajapunktis nii põlveliigese tibiofemoraalses (TF) kui patellofemoraalses (PF) osas. Liigesvahemiku kitsenemist (LVK) ning osteofütoosi hinnati nelja-astmelisel skaalal vastavalt Nagaosa ja tema kaastöötajate hindamissüsteemile [56]. Röntgenoloogilist progressiooni defineeriti kui: (i) osteofüütide või LVK kujunemist isikutel, kel eelneval uuringul OA tunnuseid puudusid, või (ii) olemasolevate osteofüütide ja LVK astme ja/või arvu suurenemist jälgimisperioodi jooksul.

Ultraheli (UH) uuringud teostati kasutades mitmesageduslikku 7,5 MHz lineaarandurit. Osteofüütide ja *Baker*`i tsüstide olemasolu, kõõluste ja kõhrekoe läbimõõtu, meniskide muutusi, sünoviaalset hüperperfusiooni ja liigesefusiooni registreeriti vastavalt EULAR juhistele ning hinnati kaheastmelisel skaalal [128]. Lisaks eelnevatele dokumenteeriti uue lähenemisena ka kõõluste lubjastumiste ning subkondraalse luu kontuuri defektide olemasolu. Ultraheli uuringud teostati kokku 106 isikul (73 naisel ja 33 mehel).

Materjal (seerum ja uriin) laboratoorseteks määramisteks koguti kõigis kolmes ajapunktis.

Kõhrekoe sünteesi hinnati seerumi II A tüüpi prokollageeni amino-terminaalse propeptiidi (sPIIANP), lammutamist – uriini II tüüpi kollageeni C-terminaalse telopeptiidi fragmentide (uCTx-II) ning seerumi kõhre oligomeetrilise maatriksproteiini (sCOMP) väärtuste alusel ELISA meetodil.

Luukoe sünteesi hinnati seerumi I tüüpi prokollageeni amino-terminaalse propetiidi (sPINP), mineralisatsiooni – osteokaltsiini (sOC) ning lammutamist I tüüpi kollageeni C-terminaalse telopeptiidi (sCTx-I) väärtuste alusel rakendades elektrokemiluminestsentsmetoodikat.

Uut luukoe päritoluga biomarkerit – uriini osteokaltsiini keskfragmenti (uMidOC) määrati ELISA meetodil.

Statistiliseks andmetöötluseks kasutati mitteparameetrilisi meetodeid (Spearman'i astakkorrelatsiooni, Mann-Whitney U-testi). Tulemused kohandati vanuse, KMI, menopausist tulenevate mõjude suhtes kasutades osakorrelatsiooni. Kuna biomarkerite väärtused on suures osas soost sõltuvad, hinnati mehi ning naisi enamikel juhtudel eraldi. Seoseid biomarkerite väärtuste ja OA röntgenoloogiliste tunnuste vahel hinnati Spearmani astakkorrelatsioonide alusel. Biomarkerite diagnostilist ja prognostilist väärtust röntgenoloogilise progresseerumise juhtudel võrreldes mitte-progresseerujatega hinnati Mann-Whitney U-testi alusel. Biomarkeri seerumi- või uriinikontsentratsiooni muutuse seost OA röntgenoloogilise progresseerumisega võrreldes mitteprogresseeruvate juhtudega (šansside suhe) hinnati rakendades logistilist regressioonanalüüsi. Mitmene regressioonanalüüs oli kasutusel hindamaks ulraheli alusel leitud pehmete kudede muutuste rolli biomarkerite väärtuste variaabelsuses.

Tulemused ja järeldused

1. Käesoleva uurimusega õnnestus esmakordselt näidata, et OA varases faasis on samaaegselt aktiveeritud nii kõhre-, luu- kui ka pehmete kudede ainevahetus.
2. Vähemalt OA algfaasis võivad luukoe ainevahetuse muutused isegi domineerida kõhrekoe haaratuse üle. Uuritud biomarkerite seas osutus luukoe lammutamise uus marker, MidOC, tugevaimaks riski ennustajaks osteofüütide progressiooni suhtes.
3. COMP`i kui valdavalt kõhrespetsiifilise biomarkeri kontsentratsioon seerumis oli oluliselt mõjutatud osteofüütide kujunemise ja kasvu ning pehmete

kudede (eeskätt meniskide) muutuste poolt. Käesolev töö kinnitas, et COMP on üldine liigeskudede biomarker, mis on üheaegselt mõjutatud mitmete erinevate liigeskudede muutuste poolt.

4. Põlveliigeste ultraheli-uuringutel hinnatavate pehmete kudede muutused olid levinud OA varases staadiumis, sagedamini leiti kõõluste lubjastumist, sünoviaalkoe paksenemist, suprapatellaarset efusiooni, *Baker*'i tsüste ja meniskide muutusi. Liigese pehmete kudede ainevahetuse muutused mõjutavad märkimisväärselt biomarkerite kontsentrat-sioone vereseerumis ja uriinis.
   – Kõõluste lubjastumisega kaasnes meestel kõhrekoe sünteesi aktiveerumine (PIIANP alusel) ning naistel selle lammutamine (CTx-II alusel).
   – Naistel kaasnes sünoviidiga I tüüpi kollageeni sünteesi aktivatsioon (PINP alusel) ja II tüüpi kollageeni sünteesi vähenemine (PIIANP alusel), mis viitab sünoviidi pärssivale mõjule kõhre sünteesil.
5. Rohkem kui pooltel (56%) põlvevaevustega keskealistest inimestest esinesid OA röntgenoloogilised tunnused. Kuue jälgimisaasta jooksul süvenes haigus 56% uuritutest, enamikel juhtudel progresseeruva osteofütoosina. Põlveliigese varase OA röntgenoloogiline kulg oli heterogeenne ja mittepidev, hõlmates vahelduvalt haiguse stabiliseerumise ja süvenemise perioode.
6. Käesoleva uurimusega õnnestus selgitada liigeskudede ainevahetuse muutuste iseloomulikku mustrit OA varase progresseerumise juhtudel:
   – Mittekollageense kõhrekoe lammutamine (COMP alusel) oli aktiveeritud just OA varases staadiumis, mida valdavalt iseloomustas süvenev osteofütoos. Sellele järgnes II tüüpi kollageeni lammutamine (CTx-II alusel), mida röntgenoloogiliselt iseloomustas süvenev LVK. Need kõhrekoe biomarkerid esindavad seega haiguse erinevaid staadiume.
   – OA röntgenoloogilisele progressioonile eelnes luu sünteesi aktivatsioon (PINP alusel) ja järgnes luukoe sünteesi (PINP alusel) ja mittekollageense luukoe lammutamise (MidOC alusel) aktivatsioon ning mineralisatsiooni muutused (OC alusel).
7. Osteofütoosi süvenemisega kaasnes kõhrekoe sünteesi (PIIANP alusel) ja lammutamise (CTx-II ja COMP alusel) aktivatsioon, samas kui OA laialdasemale progressioonile (osteofütoos ja LVK kombinatsioonis) eelnes kõhrekoe lammutamise suurenemine (CTx-II ja COMP alusel). Seega, kõik kolm uuritud kõhrekoe biomarkerit omasid diagnostilist väärtust progresseeruva osteofütoosi suhtes, ning kaks neist, COMP ja CTx-II, samuti ennustavat rolli laialdasema OA progressiooni suhtes (osteofütoos ja LVK samaaegselt mitmes põlveliigese lokalisatsioonis).
8. Osteofütoosi süvenemisega kaasnes luukoe sünteesi aktiveerumine (PINP alusel), mittekollageense luukoe lammutamine (MidOC alusel) ja muutused luukoe mineralisatsioonis (OC alusel), samas kui OA laialdasemale progressioonile (osteofütoos ja LVK kombinatsioonis) eelnes I tüüpi kollageeni sünteesi kiirenemine (PINP alusel). Kolm uuritud luumarkerit neljast, PINP, OC ja MidOC, omasid diagnostilist väärtust progresseeruva osteofütoosi suhtes, ja üks neist, PINP, samuti ennustavat rolli OA laialdasema progressiooni juhtudel.

9. Käesoleva uuringu alusel võib järeldada, et põlveliigese OA keerukas olemus nõuab mitmetahulist metoodilist lähenemist, sealhulgas: (i) liigeskõhre, luu- ja pehmete kudede biokeemiliste ja radioloogiliste muutuste samaaegset hindamist; (ii) soo, vanuse, KMI ja haiguse faasi arvestamist; (iii) OA röntgenoloogiliste tunnuste – osteofüütide ja LVK – eraldi käsitlemist kuna patofüsioloogiliselt iseloomustavad need haiguse erinevaid staadiume ning on peegeldatavad erinevate biomarkerite poolt; ning (iv) rohkem kui kahe jälgimispunkti kasutamist longitudinaalsetes uuringutes kuna haigus omab nii biokeemiliselt kui radioloogiliselt mittepidevat kulgu.

12. ACKNOWLEDGEMENTS

I express my sincere gratitude to all my colleagues, my family and friends who have helped, supported and encouraged the publishing of my papers and completing the final thesis.

First and foremost I express my deepest and warmest gratitude to my supervisor, the head of the department of laboratory medicine, Professor Agu Tamm, whose endless enthusiasm aroused my interest in research already during my residency course in laboratory medicine, who introduced me the fascinating world of biomarkers and kindly provided me the opportunity to work in his research group. I am sincerely grateful for his constant guidance and encouragement at every step of my PhD studies and for the excellent discussions that have immensely improved my scientific thinking. I express my sincere gratitude to my other supervisor Professor Kalervo Väänänen from University of Turku for his valuable advice that has improved my thesis. I also thank Prof. Väänänen for providing excellent opportunities for laboratory work in the Institute of Biomedicine, University of Turku.

I am thankful to dr. Riina Kallikorm and dr. Kai Kisand for critically reviewing and improving my final thesis.

I thank my opponent prof. Ingemar Petersson for agreeing to provide objective critisicm of my thesis at the commencement.

I am grateful to the head of Clinic of Internal Medicine, Professor Margus Lember for his help and valuable comments throughout my PhD studies.

I am immensely thankful to the co-author of all my papers – Dr. Ann Tamm – for all our thought-provoking discussions and support at every step of my studies and for the profound contribution to the collection of clinical material.

I am most grateful to Dr. Mare Lintrop – a co-author of all my articles – for precise and thorough radiographic evaluation of the study subjects and for valuable discussions which in the time of my PhD studies also provoke my deep interest for the world of radiology and has led me to become radiologist.

I am grateful to Dr. Kaisa Ivaska for her help and excellent expertize in carrying out biomarker, osteocalcin midfragments, immunoassay and for her valuable advice, comments and contribution in improving my second paper. I thank Dr. Kadri Rohtla for her companionship in the research of bone markers and for her contribution to my second paper. I thank Dr. Kalle Kisand for the excellent guidance and expertize in carrying out immunoassays.

I express my deep gratitude to the head of Clinic of Radiology, Dr. Pilvi Ilves, for the constant encouragment and support during my PhD studies and for providing excellent opportunities to complete my final thesis.

I am grateful to Dr. Ülle Hansen and Mrs.Veera Raudsepp for remarkable consistency and success with subject recruitment and study material acquisition. I am grateful to Dr. Karin Veske for precise and dedicated radiographic and ultrasonographic examinations.

I express my gratitude to all my colleagues in the Department of Laboratory Medicine who all have participated in the study. I express my gratitude to Mrs. Anne Krips for her continuous enthusiasm, smooth organization and for her warm personality that has created a friendly atmosphere in the whole department. I am most grateful to Dr. Urmo Kööbi for our fruitful discussions, and his valuable comments and kind support. I thank Ms. Maret Vija, and Mrs. Helja Karo for technical assistance.

I gratefully thank Dr. Katrin Reimand for the smooth co-ordination of laboratory work. I am indebted to Mrs. Mare Vähi for statistical supervision. I am also thankful to Mrs. Ester Jaigma and Mr. Richard Adang for the profound and thorough linguistic revision of my manuscripts and final thesis.

I am obliged to all the study participants for their agreement to provide data about their health.

Last, but definitely not least, I would specially like to thank my family and my friends for their constant support, patience and encouragement throughout my studies.

This study was funded by the European Calcified Tissue Society/ Czech Society for Metabolic Diseases Eastern Europe Young Investigator Grant and by the Estonian Science Foundation Grant No 5308.

REFERENCES

1. Felson DT. Developments in the clinical understanding of osteoarthritis. Arthritis Research Ther 2009;11:203
2. Felson DT. Epidemiology of osteoarthritis. In: Osteoarthritis, Brandt KD, Doherty M, Lohmander LS, Ed. New York: Oxford University Press 2003:9–16
3. Tamm A, Lintrop M, Veske K, Hansen U, Tamm A. Prevalence of patello – and tibiofemoral osteoarthritis in Elva, Southern Estonia. J Rheumatol 2008;35(3): 543–44
4. Brandt KD, Doherty M, Lohmander LS. Definition and classification of osteo-arthritis. In: Osteoarthritis, Brandt KD, Doherty M, Lohmander LS, Ed. New York: Oxford University Press 2003:1–8
5. Golightly YM, Marshall SW, Kraus VB, Renner JB, Villaveces A, Casteel C, *et al.* Biomarkers of incident radiographic knee osteoarthritis. Arthritis Rheum 2011; 63(8):2276–83
6. Kraus VB. Osteoarthritis year 2010 in review: biochemical markers. Osteoarthritis Cartilage 2011;19:346–53
7. Kiss I, Morocz I, Herczeg L. Localization and frequency of degenerative changes in the knee joint: evaluation of 200 necropsies. Acta Morphol Hung 1984;32:155–63
8. Brandt KD, Doherty M, Lohmander LS. Introduction: the concept of osteoarthritis as failure of the diarthrodial joint. In: Osteoarthritis, Brandt KD, Doherty M, Lohmander LS, Ed. New York: Oxford University Press 2003:69–71
9. Kraus VB. Waiting for action on the osteoarthritis front. Curr Drug Targets 2010; 11(5):518–20
10. Spector TD, Cicuttini F, Baker J, Loughlin J, Hart D. Genetic influences on osteoarthritis in women: a twin study. Br Med J 1996;312:940–3
11. Zhai G, Hart DJ, Kato BS, MacGregor A, Spector TD. Genetic influence on the progression of radiographic knee osteoarthritis: a longitudinal twin study. Osteoarthritis Cartilage 2007;15:222–225
12. Richmond RS, Carlson CS, Register TC, Shanker G, Loeser RF. Functional estrogen receptors in adult articular cartilage: estrogen replacement therapy increases chondrocyte synthesis of proteoglycans and insulin-like growth factor binding protein 2. Arthritis Rheum 2000; 43:2081–90
13. Martin JA, Buckwalter JA. Aging, articular cartilage chondrocyte senescence and osteoarthritis. Biogerontology 2002;3:257–64
14. Todd Allen R, Robertson CM, Harwood FL, Sasho T, Williams SK, Pomerleau AC, Amiel D. Characterization of mature vs aged rabbit articular cartilage: analysis of cell density, apoptosis-related gene expression and mechanisms controlling chondrocyte apoptosis. Osteoarthritis Cartilage 2004; 12:917–923
15. Robertson CM, Pennock AT, Harwood FL, Pomerleau AC, Allen RT, Amiel D.Characterization of pro-apoptotic and matrix-degradative gene expression following induction of osteoarthritis in mature and aged rabbits. Osteoarthritis Cartilage 2006;14:471–76
16. Abramson SB, Attur M. Developments in the scientific understanding of osteoarthritis. Arthritis Res Ther 2009;11:227
17. Leach RE, Baumgard S, Broom J. Obesity: its relationship to osteoarthritis of the knee. Clin Orthop Relat Res 1973;93:271–73
18. Poole AR, Guilak F, Abramson SB. Etiopathogenesis of osteoarthritis. In: Osteo-arthritis. Diagnosis and medical/surgical management 4th ed., Moskowitz RW,

Altman RD, Hochberg MC, Buckwalter JA, Goldberg V., Ed. Philadelphia. Lippincott Williams & Wilkins 2007:27–49
19. Bay-Jensen A-C, Andersen TL, Charni-Ben Tabassi N, Kristensen PW, Kjaersgaard-Andersen P, Sandell L, Garnero P, Delaisse J-M. Biochemical markers of type II collagen breakdown and synthesis are positioned at specific sites in human osteoarthritis knee cartilage. Osteoarthritis Cartilage 2008;16:615–23
20. Kiani C, Chen L, Wu YJ, Yee AJ, Yang BB. Structure and function of aggrecan. Cell Res 2002;12:19–32
21. Sandell LJ. Modern molecular analysis of a traditional disease: progression in osteoarthritis. Arthritis Rheum 2007;56(8):2474–77
22. Squires GR, Okouneff S, Ionescu M, Poole AR. The pathobiology of focal lesion development in aging human articular cartilage and molecular matrix changes characteristic of osteoarthritis. Arthritis Rheum 2003;48(5):1261–70
23. Mankin HJ, Brandt KD. Biochemistry and metabolism of articular cartilage in osteoarthritis. In: Moskowitz RW, Howell DS, Goldberg VM, Mankin HJ, editors. Osteoarthritis: diagnosis and medical/surgical management. 2nd ed. Philadelphia: WB Saunders; 1992: 109–54
24. Hollander AP, Pidoux I, Reiner A, Rorabeck C, Bourne R, Poole AR. Damage to type II collagen in aging and osteoarthritis starts at the articular surface, originates around chondrocytes, and extends into the cartilage with progressive degeneration. J Clin Invest 1995;96(6):2859–69
25. Burrage PS, Mix KS, Brinckerhoff CE. Matrix metalloproteinases: role in arthritis. Front Biosci 2006;11:529–43
26. Yasuda Y, Kaleta J, Brömme D. The role of cathepsins in osteoporosis and arthritis: rationale for the design of new therapeutics. Adv Drug Deliv Rev 2005;57(7):973–93
27. Kozaci LD, Buttle DJ, Hollander AP. Degradation of type II collagen, but not proteoglycan, correlates with matrix metalloproteinase activity in cartilage explant cultures. Arthritis Rheum 1997;40:164–74
28. Parks WC, Wilson CL, Lopez-Boado YS. Matrix metalloproteinases as modulators of inflammation and innate immunity. Nat Rev Immunol 2004;4:617–29
29. Sondergaard BC, Henriksen K, Wulf H, Oestergaard S, Schurigt U, Bräuer R, Danielsen I, Christiansen C, Qvist P, Karsdal MA. Relative contribution of matrix metalloprotease and cysteine protease activities to cytokine-stimulated articular cartilage degradation. Osteoarthritis Cartilage 2006;14:738–48
30. Anderson-MacKenzie JM, Quasnichka HL, Starr RL, Lewis EJ, Billingham ME, Bailey AJ. Fundamental subchondral bone changes in spontaneous knee osteoarthritis. Int J Biochem Cell Biol 2005;37(1):224–36
31. Hayami T, Pickarski M, Zhuo Y, Wesolowski GA, Rodan GA, Duong le T. Characterization of articular cartilage and subchondral bone changes in the rat anterior cruciate ligament transection and meniscectomized models of osteoarthritis. Bone 2006; 38(2):234–43
32. Reichenbach S, Guermazi A, Niu J, Neogi T, Hunter DJ, Roemer FW, McLennan CE, Hernandez-Molina G, Felson DT. Prevalence of bone attrition on knee radiographs and MRI in a community-based cohort. Osteoarthritis Cartilage 2008;16(9):1005–10
33. Bailey AJ, Buckland-Wright C, Metz D. The role of bone in osteoarthritis. Age Ageing 2001; 30(5): 374–78
34. Mastbergen SC, Lafeber FP. Changes in subchondral bone early in the development of osteoarthritis. Arthritis Rheum 2011; 63(9):2561–63

35. Gelse K, Söder S, Eger W, Diemtar T, Aigner T. Osteophyte development – molecular characterization of differentiation stages. Osteoarthritis Cartilage 2003;11:141–48
36. Blaney Davidson EN, Vitters EL, van Beuningen HM, van de Loo FAJ, van den Berg WB, van der Kraan PM. Resemblance of osteophytes in experimental osteoarthritis to transforming growth factor beta-induced osteophytes: limited role of bone morphogenetic protein in early osteoarthritic osteophyte formation. Arthritis Rheum 2007; 56:4065–73
37. Buckland-Wright JC, Lynch JA, Dave B. Early radiographic features in patients with anterior cruciate ligament rupture. Ann Rheum Dis 2000;59(8):641–46
38. Dieppe P, Cushnaghan J, Young P, Kirwan J. Prediction of the progression of joint space narrowing in osteoarthritis of the knee by bone scintigraphy. Ann Rheum Dis 1993;52(8):557–63
39. Mansell JP, Tarlton JF, Bailey AJ. Biochemical evidence for altered subchondral bone collagen metabolism in osteoarthritis of the hip. Br J Rheumatol 1997; 36(1):16–19
40. Mansell JP, Bailey AJ. Abnormal cancellous bone collagen metabolism in osteoarthritis. J Clin Invest 1998;101(8):1596–603
41. Sniekers YH, Intema F, Lafeber FP, van Osch GJ, van Leeuwen JP, Weinans H, Mastbergen SC. A role for subchondral bone changes in the process of osteoarthritis; a micro-CT study of two canine models. BMC Musculoskelet Disord 2008;9:20
42. Bolbos RI, Zuo J, Banerjee S, Link TM, Ma CB, Li X, Majumdar S. Relationship between trabecular bone structure and articular cartilage morphology and relaxation times in early OA of the knee joint using parallel MRI at 3 T. Osteoarthritis Cartilage 2008;16(10):1150–59
43. Attur MG, Dave M, Akamatsu M, Katoh M, Amin AR. Osteoarthritis or osteoarthrosis: the definition of inflammation becomes a semantic issue in the genomic era of molecular medicine. Osteoarthritis Cartilage 2002;10:1–4
44. van den Berg WB. Osteoarthritis year 2010 in review: pathomechanisms. Osteoarthritis Cartilage 2011;19:338–41
45. Aigner TV. Osteoarthritis and inflammation – inflammatory changes in osteoarthritic synoviopathy. In Osteoarthritis, Inflammation and Degradation: A Continuum. Edited by Buckwater J. Amsterdam: IOS Press 2007:219–235
46. Ayral X, Pickering EH, Woodworth TG, Mackillop N, Dougados M. Synovitis: a potential predictive factor of structural progression of medial tibiofemoral knee osteoarthritis – results of a 1 year longitudinal arthroscopic study in 422 patients. Osteoarthritis Cartilage 2005;13(5):361–67
47. Altman R, Asch E, Bloch D, Bole G, Borenstein D, Brandt K, Christy W, Cooke TD, Greenwald R, Hochberg M et al. Development of criteria for the classification and reporting of osteoarthritis: classification of osteoarthritis of the knee. Diagnostic and therapeutic criteria committee of the American Rheumatism Association. Arthritis Rheum 1986;29:1039–49
48. Altman RD. Classification of disease: osteoarthritis. Semin Arthritis Rheum 1991;20(6,Suppl 2):40–7
49. Kraus VB, Burnett B, Coindreau J, Cottrell S, Eyre D, Gendreau M, Gardiner J, *et al.* Application of biomarkers in the development of drugs intended for the treatment of osteoarthritis. Osteoarthritis Cartilage 2011;19(5):515–42
50. Creamer P, Hochberg MC. Why does osteoarthritis of the knee hurt – sometimes ? Br J Rheumatol 1997;36(7):726–28

51. Aspelund G, Gunnarsdottir S, Jonsson P, Jonsson H. Hand osteoarthritis in the elderly. Application of clinical criteria. Scand J Rheumatol. 1996;25(1):34–6
52. Ornetti P, Brandt K, Hellio-Le Graverand M-P, Hochberg M, Hunter DJ, Kloppenburg M, *et al.* OARSI–OMERACT definition of relevant radiological progression in hip/knee osteoarthritis. Osteoarthritis Cartilage 2009;17(7):856–863.
53. Conaghan PG, Hunter DJ, Maillefert JF, Reichmann WM, Losina E. Summary and recommendations of the OARSI FDA osteoarthritis Assessment of Structural Change Working Group. Osteoarthritis Cartilage 2011;19:606–610.
54. Kellgren JH, Lawrence JS. Radiological assessment of osteoarthrosis. Ann Rheum Dis 1957;16(4):494–502
55. Spector TD, Hart DJ, Byrne J, Harris PA, Dacre JE, Doyle DV. Definition of osteoarthritis of the knee for epidemiological studies. Ann Rheum Dis 1993; 52(11):790–94
56. Nagaosa Y, Mateus M, Hassan B, Lanyon B, Doherty M. Development of logically devised line drawing atlas for grading of knee osteoarthritis. Ann Rheum Dis 2000; 59:587–95
57. Altman RD. Gold GE. Atlas of individual radiographic features in osteoarthritis, revised. Osteoarthritis Cartilage 2007;15 Suppl A:A1–56
58. La Valley MP, McAlindon TE, Chaisson CE, Levy D, Felson DT. The validity of different definitions of radiographic worsening for longitudinal studies of knee osteoarthritis. J Clin Epidemiol 2001;54:30–39
59. Hart DJ, Spector T. Kellgren & Lawrence grade 1 osteophytes in the knee – doubtful or definite? Osteoarthritis Cartilage 2003;11:149–50
60. Felson DT, Nevitt MC, Yang M, Clancy M, Niu J *et al*. A new approach yields high rates of radiographic progression in knee osteoarthritis. J Rheumatol 2008;35 (10):2047–54.
61. Boegard TL, Rudling O, Petersson IF, Jonsson K. Joint space width of the tibiofemoral and of the patellofemoral joint in chronic knee pain with or without radiographic osteoarthritis: a 2-year follow-up. Osteoarthritis Cartilage 2003;11: 370–76
62. Bettica P, Cline G, Hart DJ, Meyer J, Spector TD. Evidence for Increased Bone Resorption in Patients With Progressive Knee Osteoarthritis. Longitudinal Results From the Chingford Study. Arthritis Rheum 2002;46(12):3178–84
63. Ding C, Garnero P, Cicuttini F, Scott F, Cooley H, Jones G. Knee cartilage defects: association with early radiographic osteoarthritis, decreased cartilage volume, increased joint surface area and type II collagen breakdown. Osteoarthritis Cartilage 2005; 13:198–205.
64. Sharif M, Kirwan JR, Elson CJ, Granell R, Clarke S. Suggestion of nonlinear or phasic progression of knee osteoarthritis based on measurements of serum cartilage oligomeric matrix protein levels over five years. Arthritis Rheum 2004;50:2479–88
65. Lane NE, Brandt K, Hawker G, Peeva E, Schreyer E, Tsuji W, Hochberg MC. OARSI-FDA initiative: defining the disease state of osteoarthritis. Osteoarthritis Cartilage 2011; 19(5):478–82
66. deMiguel Mendieta E., Cobo Ibanez T., Uson Jaeger J. *et al.* Clinical and ultrasonographic findings related to knee pain in osteoarthritis. Osteoarthritis Cartilage 2006;14:540–44
67. Naredo E., Cabero F., Palop M.J. A comparative study with clinical and radiographic assessment. Osteoarthritis Cartilage 2005;13:568–74

68. Chatzopoulos D., Moralidis E., Markou P. Baker`s cysts in knees with chronic osteoarthritis pain: a clinical, ultrasonographic, radiographic and scintigraphic evaluation. Rheumatol Int 2008;29:141–46
69. Eckstein F, Mosher T, Hunter D. Imaging of knee osteoarthritis: data beyond the beauty. Curr Opin Rheumatol 2007;19:435–43
70. Davis CR, Karl J, Granell R, Kirwan JR, Fasham J, Johansen J, Garnero P, Sharif M. Can biochemical markers serve as surrogates for imaging in knee osteoarthritis ? Arthritis Rheum 2007;56(12):4038–47
71. Guermazi A, Hunter DJ, Roemer FW. Plain radiography and magnetic resonance imaging diagnostics in osteoarthritis: validated staging and scoring. J Bone Joint Surg Am 2009;91 Suppl 1:54–62
72. van Saase JL, van Romunde LK, Cats A, Vandenbroucke JP, Valkenburg HA. Epidemiology of osteoarthritis: Zoetermeer survey. Comparison of radiological osteoarthritis in a Dutch population with that in 10 other populations. Ann Rheum Dis 1989;48:271–80
73. Petersson IF, Boegard T, Saxne T, Silman AJ, Svensson B. Radiographic osteoarthritis of the knee classified by the Ahlback and Kellgren & Lawrence systems for the tibiofemoral joint in people aged 35–54 years with chronic knee pain. Ann Rheum Dis 1997;56:493–96
74. Thorstensson CA, Andersson ML, Jönsson H, Saxne T, Petersson IF. Natural course of knee osteoarthritis in middle-aged subjects with knee pain: 12-year follow-up using clinical and radiographic criteria. Ann Rheum Dis 2009;68:1890–93
75. Schouten JS, van den Ouweland FA, Valkenburg HA. A 12 year follow-up study in the general population on prognostic factors of cartilage loss in osteoarthritis of the knee. Ann Rheum Dis 1992;51:932–37
76. Osborne D, Woodhouse S, Meacock R. Early changes in the sulfation of chondroitin in guinea-pig articular cartilage, a possible predictor of osteoarthritis. Osteoarthritis Cartilage 1994; 2(3):215–23
77. Huebner JL, Hanes MA, Beekman B, TeKoppele JM, Kraus VB. A comparative analysis of bone and cartilage metabolism in two strains of guinea-pig with varying degrees of naturally occurring osteoarthritis. Osteoarthritis Cartilage 2002;10(10):758–67
78. Reijman M, Hazes JM, Bierma-Zeinstra SM, Koes BW, Christgau S, Christiansen C, Uitterlinden AG, Pols HA. A new marker for osteoarthritis: cross-sectional and longitudinal approach. Arthritis Rheum 2004;50(8):2471–78
79. Garnero P, Piperno M, Gineyts E, Christgau S, Delmas PD, Vignon E. Cross sectional evaluation of biochemical markers of bone, cartilage, and synovial tissue metabolism in patients with knee osteoarthritis: relations with disease activity and joint damage. Ann Rheum Dis 2001; 60(6): 619–26
80. Vignon E, Garnero P, Delmas P, Avouac B, Bettica P, Boers M, et al. Recommendations for the registration of drugs used in the treatment of osteoarthritis: an update on biochemical markers. Osteoarthritis Cartilage 2001;9:289–93
81. Garnero P, Ayral X, Rousseau JC, Christgau S, Sandell LJ, Dougados M, *et al.* Uncoupling of type II collagen synthesis and degradation predicts progression of joint damage in patients with knee osteoarthritis. Arthritis Rheum 2002;46:2613–24
82. van Spil WE, DeGroot J, Lems WF, Oostveen JC, Lafeber FP. Serum and urinary biochemical markers for knee and hip-osteoarthritis: a systemic review applying the consensus BIPED criteria. Osteoarthritis Cartilage 2010; 18:605–12

83. Garnero P. Noninvasive biochemical markers in osteoarthritis. In: Osteoarthritis. Diagnosis and medical/surgical management 4th ed., Moskowitz RW, Altman RD, Hochberg MC, Buckwalter JA, Goldberg V., Ed. Philadelphia. Lippincott Williams & Wilkins 2007:215–32
84. Aigner T, Zhu Y, Chansky HH, Matsen FA, Maloney WJ, Sandell LJ. Re-expression of type II A procollagen by adult articular chondrocytes in osteo-arthritic cartilage. Arthritis Rheum 1999;42:1443–50
85. Hayes CW, Jamadar DA, Welch GW, Jannausch ML, Lachance LL, Capul DC, Sowers MR. Osteoarthritis of the knee: comparison of MR imaging findings with radiographic severity measurements and pain in middle-aged women. Radiology 2005;237(3):998–1007
86. Dahlberg L, Billinghurst RC, Manner P, Nelson F, Webb G, Ionescu M *et al.* Selective enhancement of collagenase-mediated cleavage of resident type II collagen in cultured osteoarthritic cartilage and arrest with a synthetic inhibitor that spares collagenase 1 (matrix metalloproteinase 1). Arthritis Rheum 2000;43(3):673–82
87. Chu Q, Lopez M, Hayashi K, Ionescu M, Billinghurst RC, Johnson KA et al. Elevation of a collagenase generated type II collagen neoepitope and proteoglycan epitopes in synovial fluid following induction of joint instability in the dog. Osteoarthritis Cartilage 2002;10(8):662–69
88. Heinegard D, Bayliss M, Lorenzo P. Pathogenesis of structural changes in the osteoarthritic joint: Articular cartilage. In: Osteoarthritis, Brandt KD, Doherty M, Lohmander LS, Ed. New York: Oxford University Press 2003:73–82
89. Hedhom E, Antonsson P, Hjerpe A, Aeschlimann D, Paulsson M, Rosa-Pimentel E *et al.* Cartilage matrix proteins: an acidic oligomeric protein (COMP) detected only in cartilage J Biol Chem 1992; 267(9):6132–36
90. Oldberg A, Antonsson P, Lindblom K, Heinegard D. COMP (cartilage oligomeric matrix protein) is structurally related to the thrombospondins. J Biol Chem 1992;267(31):22346–50
91. Neidhart M, Hauser N, Paulsson M, DiCesare PE, Michel BA, Häuselmann HJ. Small fragments of cartilage oligomeric matrix protein in synovial fluid and serum as markers for cartilage degradation. Br J Rheumatol 1997;36(11):1151–60
92. Müller G, Michel A, Altenburg E. COMP (cartilage oligomeric matrix protein) is synthesized in ligament, tendon, mensicus, and articular cartilage. Connect Tissue Res 1998;39(4):233–44
93. Petersson IF, Boegard T, Dahlström J, Svensson B, Heinegard D, Saxne T. Bone scan and serum markers of bone and cartilage in patients with knee pain and osteoarthritis. Osteoarthritis Cartilage 1998;6(1):33–9
94. Otterness IG, Swindell AC, Zimmerer RO, Poole AR, Ionescu M, Weiner E. An analysis of 14 molecular markers for monitoring osteoarthritis: segregation of the markers into clusters and distinguishing osteoarthritis at baseline. Osteoarthritis Cartilage 2000;8(3):180–85
95. Risteli J, Risteli L. Chapter 19: Products of bone collagen metabolism. In: Dynamics of bone and cartilage metabolism. Seibel MJ, Robins SP, Bilezikian JP eds. Academic Press, San Diego; 1999: 275–287
96. Liu, SH, Yang RS, al-Shaikh R, Lane JM. Collagen in tendon, ligament, and bone healing. A current review. Clin Orthop Relat Res 1995;318: 265–78
97. Robins SP. Fibrillogenesis and maturation of collagens. In: Dynamics of Bone and Cartilage Metabolism, Seibel MJ, Robins SP, Bilezikian JP eds. Academic Press, San Diego; 1999: 31–42

98. von der Mark K. Structure and Biosynthesis of Collagens. In: Dynamics of Bone and Cartilage Metabolism, Seibel M, Robins S, Bilezikian J eds. Academic Press, San Diego; 1999: 3–30
99. Nishimoto SK, Price PA. Secretion of the vitamin K-dependent protein of bone by rat osteosarcoma cells. Evidence for an inracellular precursor. J Biol Chem 1980;255(14):6579–83
100. Price PA, Williamson MK, Lothringer JW. Origin of the vitamin K-dependent bone protein found in plasma and its clearance by kidney and bone. J Biol Chem 1981;256(24):12760–66
101. Farrugia W, Melick RA. Metabolism of osteocalcin. Calcif Tissue Int 1986; 39(4):234–38
102. Taylor AK, Linkhart S, Mohan S, Christenson RA, Singer FR, Baylink DJ. Multiple osteocalcin fragments in human urine and serum as detected by a midmolecule osteocalcin radioimmunoassay. J Clin Endocrinol Metab 1990; 70(2): 467–72
103. Baumgrass R, Williamson MK, Price PA. Identification of peptide fragments generated by digestion of bovine and human osteocalcin with the lysosomal proteinases cathepsin B,D, L, H and S. J Bone Miner Res 1997; 12(3):447–55
104. Gundberg CM, Weinstein RS. Multiple immunoreactive forms of osteocalcin in uremic serum. J Clin Invest 1986; 77(6):1762–67
105. Ivaska KK, Hentunen TA, Vääräniemi J, Ylipahkala H, Pettersson K, Väänänen HK. Release of intact and fragmented osteocalcin molecules from bone matrix during bone resorption in vitro. J biol Chem 2004; 279(18):18361–9
106. Srivastava AK, Mohan S, Singer FR, Baylink DJ. A urine midmolecule ostecalcin assay shows higher discriminatory power than a serum midmolecule ostecalcin assay during short-term alendronate treastment of osteoporotic patients. Bone 2002; 31(1):62–69
107. Ivaska KK, Käkönen SM, Gerdhem P, Obrant KJ, Pettersson K, Väänänen HK. Urinary osteocalcin as a marker of bone metabolism. Clin Chem 2005;51(3):618–28
108. Robey PG, Boskey AL. The composition of bone. In: Primer on the metabolic bone diseases and disorders of mineral metabolism 7th ed. American Society for Bone and Mineral Research, Rosen CJ, Ed. Washington, DC, The Sheridan Press 2008:32–38
109. Ivaska KK, Hellman J, Likojärvi J *et al.* Identification of novel proteolytic forms of osteocalcin in human urine. Biochem Biophys Res Commun 2003; 306(4):973–80
110. Ivaska KK, Pettersson K, Nenonen A, Uusi-Rasi K, Heinonen A, Kannus P, Väänänen K. Urinary osteocalcin is a useful marker for monitoring the effect of alendronate therapy. Clin Chem 2005;51:2362–65
111. Jordan KM, Syddall HE, Garnero P, Gineyts E, Dennison EM, Sayer AA, Delmas PD, Cooper C, Arden NK. Urinary CTX-II and glucosyl-galactosyl-pyridinoline are associated with the presence and severity of radiographic knee osteoarthritis in men. Ann Rheum Dis 2006;65(7):871–7
112. Dam EB, Byrjalsen I, Karsdal MA, Qvist P, Christiansen C. Increased urinary excretion of C-telopeptides of type II collagen (CTX-II) predicts cartilage loss over 21 months by MRI**.** Osteoarthritis Cartilage 2009; 17: 384–389.
113. Sharif M, Granell R, Johansen J, Clarke S, Elson C, Kirwan JR. Serum cartilage oligomeric matrix protein and other biomarker profiles in tibiofemoral and

patellofemoral osteoarthritis of the knee. Rheumatology (oxford) 2006;45(5):522–26
114. Vilim V, Olejarova M, Machacek S, Gatterova J, Kraus VB, Pavelka K. Serum levels of cartilage oligomeric matrix protein (COMP) correlate with radiographic progression of knee osteoarthritis. Osteoarthritis Cartilage 2002; 10:707–13
115. Cibere J, Zhang H, Garnero P, Poole AR, Lobanok T, Saxne T *et al.* Association of biomarkers with pre-radiographically defined and radiographically defined knee osteoarthritis in a population-based study. Arthritis Rheum 2009; 60:1372–80
116. Rousseau JC, Zhu Y, Miossec P, Vignon E, Sandell LJ, Garnero P, Delmas PD. Serum levels of type IIA procollagen amino terminal propeptide (PIIANP) are decreased in patients with knee osteoarthritis and rheumatoid arthritis. Osteoarthritis Cartilage 2004;12(6):440–47
117. Sharif M, Saxne T, Shepstone L, Kirwan JR, Elson CJ, Heinegard D *et al.* Relationship between serum cartilage oligomeric matrix protein levels and disease progression in osteoarthritis of the knee joint. Br J Rheumatol 1995; 34:306–10
118. Sharif M, Kirwan J, Charni N, Sandell LJ, Whittles C, Garnero P. A 5-yr longitudinal study of type IIA collagen synthesis and total type II collagen degradation in patients with knee osteoarthritis – associations with disease progression. Rheumatology 2007;46:938–43
119. Sowers MF, Karvonen-Gutierrez CA, Yosef M, Jannausch M, Jiang Y, Garnero P *et al.* Longitudinal changes of serum COMP and urinary CTX-II predict X-ray defined knee osteoarthritis severity and stiffness in women. Osteoarthritis Cartilage 2009; 17:1609–14
120. Bruyere O, Collette J, Kothari M, Zaim S, White D, Genant H *et al.* Osteoarthritis, magnetic resonance imaging, and biochemical markers: a one year prospective study. Ann Rheum Dis 2006; 65:1050–54
121. Hunter DJ, Jiang Li, LaValley M, Bauer DC, Nevitt M, DeGroot J et al. Cartilage markers and their association with cartilage loss on magnetic resonance imaging in knee osteoarthritis: the Boston Osteoarthritis Knee Study. Arthritis Res Ther 2007;9R108
122. Petersson IF, Boegard T, Svensson B, Heinegard D, Saxne T. Changes in cartilage and bone metabolism identified by serum markers in early osteoarthritis of the knee joint. Br J Rheumatol 1998; 37:46–50
123. Schouten JS, Van den Ouweland FA, Valkenburg HA, Lamberts SW. Insulin-like growth factor-1: a prognostic factor of knee osteoarthritis. Br J Rheumatol 1993; 32:274–80
124. Sugiyama S, Itokazu M, Suzuki Y, Shimizu K. Procollagen II C propeptide level in the synovial fluid as a predictor of radiographic progression in early knee osteoarthritis. Ann Rheum Dis. 2003; 62:27–32
125. Sharif M, George E, Dieppe PA. Correlation between synovial fluid markers of cartilage and bone turnover and scintigraphic scan abnormalities in osteoarthritis of the knee. Arthritis Rheum 1995;38(1):78–81
126. Berry PA, Maciewicz RA, Cicuttini FM, Jones MD, Hellawell CJ, Wluka AE. Markers of bone formation and resorption identify subgroups of patients with clinical knee osteoarthritis who have reduced rates of cartilage loss. J Rheumatol 2010;37(6):1252–59
127. Jung YO, Do JH, Kang HJ, *et al.* Correlation of sonographic severity with biochemical markers of synovium and cartilage in knee osteoarthritis patients. Clin Exp Rheumatol 2006;24:253–59

128. Boegard T, Rudling O, Petersson IF, Sanfridsson J, Saxne T, Svensson B *et al.* Joint-space width in the axial view of the patello-femoral joint. Definitions and comparison with MR imaging. Acta Radiol 1998;39:24–31
129. Backhaus M., Burmester G., Gerber T. *et al.* Guidelines for musculoskeletal ultrasound in rheumatology. Ann Rheum Dis 2001;60:641–49
130. Kraus VB, Kepler TB, Stabler T, Renner J, Jordan J. First qualification study of serum biomarkers as indicators of total body burden of osteoarthritis. PloS One 2010;5(3):e9739
131. Bartlett SJ, Ling SM, Mayo NE, Scott SC, Bingham CO. Identifying common trajectories of joint space narrowing over two years in knee osteoarthritis. Arthritis Care Res 2011;63(12):1722–28
132. Levick JR, Price FM, Mason RM. Synovial matrix-synovial fluid system of joints. In: Wayne D. Comper (ed) Extracellular Matrix Volume 1. Tissue Function. Harwood Academic Publisher,1996:328–377
133. Gelse K, Pöschl E, Aigner T. Collagens-structure, function, and biosynthesis. Adv Drug Deliv Rev 2003;55:1531–46
134. Hashimoto S, Creighton-Achermann L, Takahashi K, Amiel D, Coutts RD, Lotz M. Development and regulation of osteophyte formation during experimental osteoarthritis. Osteoarthritis Cartilage 2002; 10:180–7
135. Di Cesare PE, Fang C, Leslie MP, Tulli H, Perris R, Carlson CS. Expression of cartilage oligomeric matrix protein (COMP) by embryonic and adult osteoblasts. J Orthop Res. 2000;18:713–20
136. Boegard T, Rudling O, Petersson IF, Jonsson K. Correlation between radiographically diagnosed osteophytes and magnetic resonance detected cartilage defects in the tibiofemoral joint. Ann Rheum Dis. 1998; 57:401–7
137. Naitou K, Kushida K, Takahashi M, Ohishi T, Inoue T. Bone mineral density and bone turnover in patients with knee osteoarthritis compared with generalized osteoarthritis. Calcif Tissue Int 2000;66:325–29

PUBLICATIONS

CURRICULUM VITAE

Name: Jaanika Kumm
Date of Birth: 24.06.1978
Citizenship: Estonian
Address: University of Tartu, Clinic of Internal Medicine,
L. Puusepa 6–222, 51014, Tartu, Estonia
Phone: + 372 731 9342
+ 372 5819 4902
e-mail: jaanika.kumm@kliinikum.ee

Education and employment

1985–1996	Pärnu Co-educational Gymnasium
1997–2003	University of Tartu, medical faculty
2003–2007	Residency training in laboratory medicine, University of Tartu
2006–2012	PhD student, University of Tartu, Clinic of Internal Medicine
2007–2011	Residency training in radiology, University of Tartu
2011–	Radiologist in Tartu University Hospital, Clinic of Radiology
2011–	Assistant in radiology, University of Tartu

Membership in professional societies

Estonian Society of Laboratory Medicine
Estonian Society of Radiology
European Society of Calcified Tissues

Scientific work

Main areas of research include the metabolic changes in early-stage knee osteoarthritis, molecular markers of articular cartilage and bone metabolism, the diagnostic and prognostic value of biomarkers for progressive knee joint osteoarthritis, the prevalence of early radiographic knee osteoarthritis in middle-aged subjects and radiographic changes in the early stage of the disease.

ELULOOKIRJELDUS

Nimi: Jaanika Kumm
Sünniaeg: 24.06.1978
Kodakondsus: eesti
Aadress: Tartu Ülikooli Sisekliinik,
L. Puusepa 6–222, 51014, Tartu, Eesti
Telefon: + 3727319342
+ 37258194902
e-post: jaanika.kumm@kliinikum.ee

Haridus- ja ametikäik

1985–1996	Pärnu Ühisgümnaasium
1997–2003	Tartu Ülikooli arstiteaduskond
2003–2007	Arst-resident laboratoorse meditsiini erialal, SA Tartu Ülikooli Kliinikum
2006–2012	Doktorant, Tartu Ülikooli Sisekliinik
2007–2011	Arst-resident radioloogia erialal, Tartu Ülikooli Radioloogiakliinik
2011–	Radioloog SA Tartu Ülikooli Kliinikumi Radioloogiakliinik
2011–	Assistent radioloogia erialal, Tartu Ülikool

Ühiskondlik tegevus
Eesti Laborimeditsiini Ühingu liige
Eesti Radioloogia Ühingu liige
European Society of Calcified Tissues liige

Teaduslik tegevus
Peamised uurimisvaldkonnad hõlmavad põlveliigese osteartroosi (OA) varase staadiumi ainevahetuslikke muutusi, liigeskõhre ja luukoe päritolu molekulaarseid markereid, biomarkerite diagnostilist ja ennustavat väärtust põlveliigese OA progresseerumise juhtudel, põlveliigse OA varase staadiumi levimust keskealistel ning selle radioloogilist kulgu.

DISSERTATIONES MEDICINAE UNIVERSITATIS TARTUENSIS

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

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

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

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

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

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

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

138. **Priit Kampus.** Impact of inflammation, oxidative stress and age on arterial stiffness and carotid artery intima-media thickness. Tartu, 2007.
139. **Margus Punab.** Male fertility and its risk factors in Estonia. Tartu, 2007.
140. **Alar Toom**. Heterotopic ossification after total hip arthroplasty: clinical and pathogenetic investigation. Tartu, 2007.
141. **Lea Pehme.** Epidemiology of tuberculosis in Estonia 1991–2003 with special regard to extrapulmonary tuberculosis and delay in diagnosis of pulmonary tuberculosis. Tartu, 2007.
142. **Juri Karjagin.** The pharmacokinetics of metronidazole and meropenem in septic shock. Tartu, 2007.
143. **Inga Talvik.** Inflicted traumatic brain injury shaken baby syndrome in Estonia – epidemiology and outcome. Tartu, 2007.
144. **Tarvo Rajasalu.** Autoimmune diabetes: an immunological study of type 1 diabetes in humans and in a model of experimental diabetes (in RIP-B7.1 mice). Tartu, 2007.
145. **Inga Karu.** Ischaemia-reperfusion injury of the heart during coronary surgery: a clinical study investigating the effect of hyperoxia. Tartu, 2007.
146. **Peeter Padrik.** Renal cell carcinoma: Changes in natural history and treatment of metastatic disease. Tartu, 2007.
147. **Neve Vendt**. Iron deficiency and iron deficiency anaemia in infants aged 9 to 12 months in Estonia. Tartu, 2008.
148. **Lenne-Triin Heidmets.** The effects of neurotoxins on brain plasticity: focus on neural Cell Adhesion Molecule. Tartu, 2008.
149. **Paul Korrovits.** Asymptomatic inflammatory prostatitis: prevalence, etiological factors, diagnostic tools. Tartu, 2008.
150. **Annika Reintam.** Gastrointestinal failure in intensive care patients. Tartu, 2008.
151. **Kristiina Roots.** Cationic regulation of Na-pump in the normal, Alzheimer's and CCK_2 receptor-deficient brain. Tartu, 2008.
152. **Helen Puusepp.** The genetic causes of mental retardation in Estonia: fragile X syndrome and creatine transporter defect. Tartu, 2009.
153. **Kristiina Rull.** Human chorionic gonadotropin beta genes and recurrent miscarriage: expression and variation study. Tartu, 2009.
154. **Margus Eimre.** Organization of energy transfer and feedback regulation in oxidative muscle cells. Tartu, 2009.
155. **Maire Link.** Transcription factors FoxP3 and AIRE: autoantibody associations. Tartu, 2009.
156. **Kai Haldre.** Sexual health and behaviour of young women in Estonia. Tartu, 2009.
157. **Kaur Liivak.** Classical form of congenital adrenal hyperplasia due to 21-hydroxylase deficiency in Estonia: incidence, genotype and phenotype with special attention to short-term growth and 24-hour blood pressure. Tartu, 2009.

158. **Kersti Ehrlich.** Antioxidative glutathione analogues (UPF peptides) – molecular design, structure-activity relationships and testing the protective properties. Tartu, 2009.
159. **Anneli Rätsep.** Type 2 diabetes care in family medicine. Tartu, 2009.
160. **Silver Türk.** Etiopathogenetic aspects of chronic prostatitis: role of mycoplasmas, coryneform bacteria and oxidative stress. Tartu, 2009.
161. **Kaire Heilman.** Risk markers for cardiovascular disease and low bone mineral density in children with type 1 diabetes. Tartu, 2009.
162. **Kristi Rüütel.** HIV-epidemic in Estonia: injecting drug use and quality of life of people living with HIV. Tartu, 2009.
163. **Triin Eller.** Immune markers in major depression and in antidepressive treatment. Tartu, 2009.
164. **Siim Suutre.** The role of TGF-β isoforms and osteoprogenitor cells in the pathogenesis of heterotopic ossification. An experimental and clinical study of hip arthroplasty. Tartu, 2010.
165. **Kai Kliiman.** Highly drug-resistant tuberculosis in Estonia: Risk factors and predictors of poor treatment outcome. Tartu, 2010.
166. **Inga Villa.** Cardiovascular health-related nutrition, physical activity and fitness in Estonia. Tartu, 2010.
167. **Tõnis Org.** Molecular function of the first PHD finger domain of Autoimmune Regulator protein. Tartu, 2010.
168. **Tuuli Metsvaht.** Optimal antibacterial therapy of neonates at risk of early onset sepsis. Tartu, 2010.
169. **Jaanus Kahu.** Kidney transplantation: Studies on donor risk factors and mycophenolate mofetil. Tartu, 2010.
170. **Koit Reimand.** Autoimmunity in reproductive failure: A study on associated autoantibodies and autoantigens. Tartu, 2010.
171. **Mart Kull.** Impact of vitamin D and hypolactasia on bone mineral density: a population based study in Estonia. Tartu, 2010.
172. **Rael Laugesaar.** Stroke in children – epidemiology and risk factors. Tartu, 2010.
173. **Mark Braschinsky.** Epidemiology and quality of life issues of hereditary spastic paraplegia in Estonia and implemention of genetic analysis in everyday neurologic practice. Tartu, 2010.
174. **Kadri Suija.** Major depression in family medicine: associated factors, recurrence and possible intervention. Tartu, 2010.
175. **Jarno Habicht.** Health care utilisation in Estonia: socioeconomic determinants and financial burden of out-of-pocket payments. Tartu, 2010.
176. **Kristi Abram.** The prevalence and risk factors of rosacea. Subjective disease perception of rosacea patients. Tartu, 2010.
177. **Malle Kuum.** Mitochondrial and endoplasmic reticulum cation fluxes: Novel roles in cellular physiology. Tartu, 2010.
178. **Rita Teek.** The genetic causes of early onset hearing loss in Estonian children. Tartu, 2010.

179. **Daisy Volmer.** The development of community pharmacy services in Estonia – public and professional perceptions 1993–2006. Tartu, 2010.
180. **Jelena Lissitsina.** Cytogenetic causes in male infertility. Tartu, 2011.
181. **Delia Lepik.** Comparison of gunshot injuries caused from Tokarev, Makarov and Glock 19 pistols at different firing distances. Tartu, 2011.
182. **Ene-Renate Pähkla**. Factors related to the efficiency of treatment of advanced periodontitis. Tartu, 2011.
183. **Maarja Krass.** L-Arginine pathways and antidepressant action. Tartu, 2011.
184. **Taavi Lai.** Population health measures to support evidence-based health policy in Estonia. Tartu, 2011.
185. **Tiit Salum.** Similarity and difference of temperature-dependence of the brain sodium pump in normal, different neuropathological, and aberrant conditions and its possible reasons. Tartu, 2011.
186. **Tõnu Vooder**. Molecular differences and similarities between histological subtypes of non-small cell lung cancer. Tartu, 2011.
187. **Jelena Štšepetova.** The characterisation of intestinal lactic acid bacteria using bacteriological, biochemical and molecular approaches. Tartu, 2011.
188. **Radko Avi.** Natural polymorphisms and transmitted drug resistance in Estonian HIV-1 CRF06_cpx and its recombinant viruses. Tartu, 2011, 116 p.
189. **Edward Laane**. Multiparameter flow cytometry in haematological malignancies. Tartu, 2011, 152 p.
190. **Triin Jagomägi.** A study of the genetic etiology of nonsyndromic cleft lip and palate. Tartu, 2011, 158 p.
191. **Ivo Laidmäe.** Fibrin glue of fish (*Salmo salar*) origin: immunological study and development of new pharmaceutical preparation. Tartu, 2012, 150 p.
192. **Ülle Parm.** Early mucosal colonisation and its role in prediction of invasive infection in neonates at risk of early onset sepsis. Tartu, 2012, 168 p.
193. **Kaupo Teesalu.** Autoantibodies against desmin and transglutaminase 2 in celiac disease: diagnostic and functional significance. Tartu, 2012, 142 p.
194. **Maksim Zagura**. Biochemical, functional and structural profiling of arterial damage in atherosclerosis. Tartu, 2012, 162 p.
195. **Vivian Kont.** Autoimmune regulator: characterization of thymic gene regulation and promoter methylation. Tartu, 2012, 134 p.
196. **Pirje Hütt.** Functional properties, persistence, safety and efficacy of potential probiotic lactobacilli. Tartu, 2012, 246 p.
197. **Innar Tõru.** Serotonergic modulation of CCK-4- induced panic. Tartu, 2012, 132 p.
198. **Sigrid Vorobjov.** Drug use, related risk behaviour and harm reduction interventions utilization among injecting drug users in Estonia: implications for drug policy. Tartu, 2012, 120 p.
199. **Martin Serg.** Therapeutic aspects of central haemodynamics, arterial stiffness and oxidative stress in hypertension. Tartu, 2012, 156 p.