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IRINA KERNA

The contribution of *ADAM12* and *CILP* genes to the development of knee osteoarthritis

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The contribution of *ADAM12* and *CILP* genes to the development of knee osteoarthritis

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TABLE OF CONTENT

1. LIST OF PUBLICATIONS 7
2. ABBREVIATIONS 8
3. INTRODUCTION 10
4. REVIEW OF THE LITERATURE 13
   - 4.1. Osteoarthritis (OA) 13
     - 4.1.1. OA consensus definition and modern conception 13
     - 4.1.2. Main aspects of OA diagnosis: clinical and radiological 13
     - 4.1.3. General aspects of the pathogenesis of OA as a whole-joint disease 14
       - 4.1.3.1. Narrowing of joint space 14
       - 4.1.3.2. Development of osteophytes 15
       - 4.1.3.3. Synovitis in OA 15
       - 4.1.3.4. Biochemical markers in OA 17
     - 4.1.4. Systemic risk factors 18
       - 4.1.4.1. Age, gender and body weight 18
       - 4.1.4.2. The role of genetic factors in OA development 19
         - 4.1.4.2.1. Heritability of OA and genetic studies 19
         - 4.1.4.2.2. Candidate gene association studies 20
   - 4.2. ADAM12: characteristics and relation to OA 22
     - 4.2.1. ADAM12 biochemical structure and association with human diseases 22
     - 4.2.2. Association studies of *ADAM12* gene variants with knee OA 24
     - 4.2.3. Functional role of ADAM12 in knee OA development 25
   - 4.3. CILP: characteristics and relation to OA 25
     - 4.3.1. Biochemical structure of CILP 25
     - 4.3.2. The association of the *CILP* gene and protein with knee OA . 26
5. AIMS OF THE STUDY 28
6. PATIENTS AND METHODS 29
   - 6.1. Study subjects 29
   - 6.2. Methods 30
     - 6.2.1. Radiographic examination 30
     - 6.2.2. Genotyping of selected SNPs in *ADAM12* and *CILP* genes ... 32
     - 6.2.3. Assay of the ADAM12-S protein and other OA biochemical markers 34
     - 6.2.4. Assays of *ADAM12* and *CILP* mRNA expression in synovial and cartilage samples 36
     - 6.2.5. Evaluation of histological synovitis 37
     - 6.2.6. Evaluation of protein expression in the synovial membrane... 38
     - 6.2.7. Statistical analysis 39

7. RESULTS 41
7.1. Associations of the *ADAM12* gene and protein with radiographic knee OA and OA-associated synovitis 41
7.1.1. An association study for *ADAM12* gene SNPs (Papers I and II) 41
7.1.2. The association of ADAM12-S protein with radiographic knee OA and OA biomarkers (Paper III) 45
7.1.3. The expression of *ADAM12* mRNA in synovial tissue and its association with knee OA and histological synovitis (Papers IV and V) 46
7.1.4. The synovial expression of ADAM12 protein (Paper IV) 47
7.2. Association of *CILP* gene with knee OA and OA-associated synovial membrane inflammation (Paper VI) 48
7.2.1. An association study for *CILP* gene SNP 48
7.2.2. The expression of *CILP* mRNA in synovial tissue and its association with knee OA and histological synovitis 48
7.2.3. The synovial expression of CILP1 protein 49
8. DISCUSSION 52
8.1. ADAM12 contribution to OA processes at the gene, protein and mRNA levels 52
8.1.1. The association of *ADAM12* gene SNPs with radiographic knee OA 52
8.1.2. The role of ADAM12 protein in the development of radiographic knee OA features 54
8.1.3. The role of the *ADAM12* gene and protein in synovial inflammation 56
8.2. The implication of CILP in synovial inflammation and its relation to radiographic knee OA 58
8.2.1. The association of *CILP* gene polymorphism with radiographic knee OA 58
8.2.2. The synovial membrane as an additional source of CILP production 59
8.2.3. The relation of synovial expression of *CILP* gene and protein to synovial fibrosis and radiographic knee OA 60
8.3. Limitations of the study 61
9. CONCLUSIONS 63
10. SUMMARY IN ESTONIAN 64
ACKNOWLEDGMENTS 69
REFERENCES 71
PUBLICATIONS 83
CURRICULUM VITAE 139

1. LIST OF PUBLICATIONS

I. Kerna I, Kisand K, Tamm AE, Lintrop M, Veske K, Tamm AO. Missense single nucleotide polymorphism of the ADAM12 gene is associated with radiographic knee osteoarthritis in middle-aged Estonian cohort. Osteoarthritis Cartilage 2009;7:1093−8.

II. Kerna I, Kisand K, Tamm AE, Kumm J, Tamm AO. Two single-nucleotide polymorphisms in ADAM12 gene are associated with early and late radiographic knee osteoarthritis in Estonian population. Arthritis 2013; 2013:878126. doi: 10.1155/2013/878126.

III. Kerna I, Kisand K, Laitinen P, Tamm AE, Kumm J, Lintrop M, Tamm AO. Association of ADAM12-S protein with radiographic features of knee osteoarthritis and bone and cartilage markers. Rheumatol Int 2012; 32:519−23.

IV. Kerna I, Kisand K, Suutre S, Murde M, Tamm A, Kumm J, Tamm A. The ADAM12 is upregulated in synovitis and postinflammatory fibrosis of the synovial membrane in patients with early radiographic osteoarthritis. Joint Bone Spine 2014;81:51−6.

V. Kerna I, Kisand K, Tamm A, Tamm A. Synonymous SNP influences Adam12 mRNA expression level in synovial tissue. J Mol Genet Med 2013;7:321−2.

VI. Kerna I, Kisand K, Suutre S, Tamm A, Tamm A. CILP1 down-regulation in the synovial membrane of patients with advanced radiographic knee osteoarthritis and up-regulation in fibrosis-associated synovia remodelling. (Submitted).

Author's contributions to the original publications:

Papers I and II: author involved in planning the study, subject recruitment, obtaining of questionnaire data and collection of samples. She performed all laboratory work, including DNA extraction and the gene association study, the statistical analysis, and wrote the first drafts of the manuscripts.

Paper III: study planning, subject and sample selection, performing of statistical analysis and writing the first draft of the paper.

Papers IV, V and VI: study planning, subject and sample selection, performing the extraction of RNA from cartilage and synovial biopsies, measurement of mRNA expression, evaluation of immunohistochemical staining, performing of statistical analysis and writing the first drafts of the papers.

2. ABBREVIATIONS

ACR	– American College of Rheumatology
ADAM12	– disintegrin and metalloproteinase domain-containing protein 12
ADAM12-L	– long (transmembrane) isoform of ADAM12
ADAM12-S	– short (secreted) isoform of ADAM12
BMI	– body mass index
CI	– comorbidity index
CILP	– cartilage intermediate layer protein
DNA	– deoxyribonucleic acid
ECM	– extracellular matrix
EGF	– epidermal growth factor
EULAR	– European League Against Rheumatism
FD	– family doctor
GDF5	– growth differentiation factor 5
GWAS	– genome-wide association study
HWE	– Hardy-Weinberg equilibrium
IGF1	– insulin-like growth factor 1
IGFBP	– insulin-like growth factor-binding protein
IHC	– immunohistochemistry
IL	– interleukin
JSN	– joint space narrowing
K&L	– Kellgren-Lawrence grading system
K2-EDTA	– dipotassium ethylenediaminetetraacetic acid
KOA	– knee osteoarthritis
KOOS	– Knee injury and Osteoarthritis Outcome Score
LA	– linkage analysis
LD	– linkage disequilibrium
MMP	– matrix metalloproteinase
mRNA	– messenger ribonucleic acid
MSC	– mesenchymal stem cells
OA	– osteoarthritis
OARSI	– Osteoarthritis Research Society International
OPH	– osteophytes
PCR	– polymerase chain reaction
PFJ	– patellofemoral joint
RFLP	– restriction fragment length polymorphism
rKOA	– radiographic knee osteoarthritis
RNA	– ribonucleic acid
s-CTX-I	– serum C-terminal cross-linked telopeptides of type I collagen
SNP	– single nucleotide polymorphism
s-PINP	– serum procollagen type I amino-terminal propeptide
SRC	– Spearman rank correlation test
TFJ	– tibiofemoral joint

TGF – transforming growth factor
TNF – tumor necrosis factor
u-CTX-II – urinary C-telopeptide fragments of type II collagen
UTR – untranslated region
WET – Wilcoxon rank sum exact test

3. INTRODUCTION

Osteoarthritis (OA) is the most common arthritic disorder and a leading cause of musculoskeletal disability. The most commonly affected joints in OA are the spine, hip, knee and hand joints (Brandt et al., 2003). OA is estimated to affect almost 40% of subjects over the age of 70 (Lawrence et al., 2008). The economic burden of arthritis is estimated to be 1−2.5% of the gross national product in the Western world (Bitton, 2009). The ageing of the population, rising problem of obesity and lack of definitive treatment for the prevention of the disease, or reducing its progression, ensure that the impact of OA on public health will continuously increase.

Epidemiological studies have demonstrated that OA is a complex disorder with a large number of environmental and genetic risk factors (Figure 1). Genetic factors have been shown to influence the dynamics and outcomes of OA at various stages of the disease, as have other OA risk factors, such as obesity, skeletal shape, bone mass and synovial inflammation (Valdes et al., 2011a). The general purpose of genetic research in the OA field is 1) to identify underlying causative genes and pathways, and increase the understanding of molecular mechanisms of the disease, with potential implications for novel therapeutic approaches, and 2) increasing the ability to predict the risk of the disease through identification of risk geno- and haplotypes (van Meurs et al., 2012).

In OA, the knee is the most significant clinically affected site (Peat et al., 2001; Figure 2). In fact, since 2002 the genetic pattern of knee OA (KOA) has been investigated by genome-wide linkage analysis, numerous associated studies of candidate genes have been performed, and several genes are now known to be consistently associated with the risk of KOA (Valdes et al., 2011a). However, considerable progress still needs to be achieved in the understanding of the pathogenic OA pathways associated with genetic susceptibility. The identification of novel unknown members and the elucidating of the mechanisms of OA susceptibility genes are possible through functional genomic research into affected joint tissue (Meulenbelt, 2011). Moreover, the complex consideration of promising candidate genes on the mRNA and protein levels, in cartilage and other joint tissues, could significantly improve the current knowledge of disease pathogenesis and even provide hypotheses for novel treatment research in OA fields.

The identification of a novel gene associated with OA risk is challenging due to the large amount of heterogeneity within the clinical subset of the disease, heterogeneity between different ethnic groups and the appearance of small effects of OA at many loci, requiring a large number of study participants to establish genetic risk (Valdes et al., 2011a). Nowadays, there are only a few genes that have been shown to have a strong association to OA, and there have been many association studies showing undefined significance (Chapman et al., 2012; Reynard et al., 2012). Of the described novel genes that are potentially

implicated in OA pathogenesis, *ADAM12* (disintegrin and metalloproteinase domain-containing protein 12) and *CILP* (cartilage intermediate layer protein) seems to be promising and little investigated OA contributors.

ADAM12 is a member of the ADAM family, which belongs to the main proteolytic enzymes that regulate extracellular matrix turnover in cartilage (Aigner et al., 2006). Several associated studies have implicated *ADAM12* as a potential OA candidate gene (Valdes et al., 2004; Loughlin, 2005). Moreover, an over-expression of *ADAM12* mRNA was found in OA cartilage (Okada et al., 2008), suggesting disease-associated altered behaviour in OA, at least in joint cartilage. However, there is a lack of systematic data on the actual relation of *ADAM12* to OA genetic risk and its possible OA-associated pathogenetic pathway in human joints.

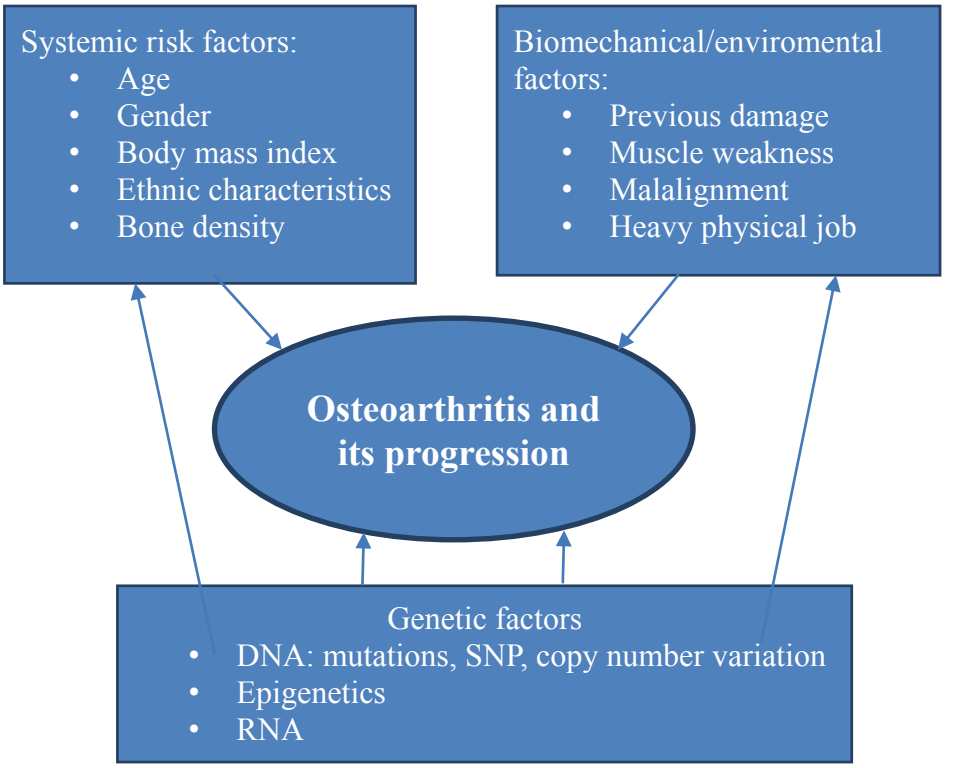

Figure 1. The complexity and interaction between risk factors involved in osteoarthritis.

SNP: single nucleotide polymorphism

Another novel promising OA marker is *CILP* (also known as *CILP1*). CILP1 is an extracellular matrix glycoprotein, which is chiefly expressed in cartilage tissue (Lorenzo et al., 1998a). The amount of CILP1 protein increases with the ageing of human articular cartilage and up-regulation of *CILP* expression has

been described in early and late stages of OA (Lorenzo et al., 2004). However, there is increasing evidence that CILP may be produced in non-cartilaginous tissue, as well as in cartilage (Cardenal et al., 1996; Bernardo et al., 2011). Similarly to ADAM12, there is only limited information on the behaviour of CILP in OA-altered tissue other than cartilage. Therefore, the advancement of our knowledge of the contribution of these two genes to knee OA pathophysiologic processes can provide new insights into disease mechanisms and provide direction in the development of new treatment strategies.

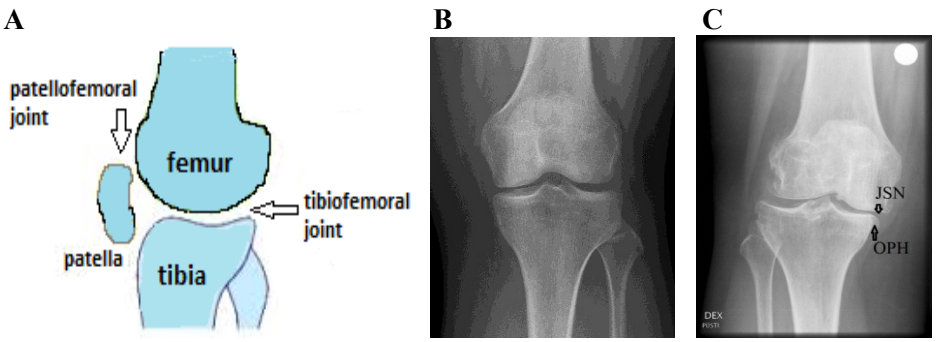

Figure 2. The structure of knee joints and their radiographic imaging. A) The knee joint is composed of three functional compartments: the medial and lateral tibiofemoral compartment and patellofemoral compartment. B) X-ray radiographs of the knee (no radiographic OA features). C) Radiographic knee OA−presence of osteophytes (OPH) and narrowing of joint space (JSN) in the knee joint (courtesy of Department of Radiology of Tartu University Hospital).

4. REVIEW OF LITERATURE

4.1. Osteoarthritis (OA)

4.1.1. OA consensus definition and modern conception

In the second half of the twentieth century, the definition of OA changed. OA is no longer considered to be a degenerative disease of ageing cartilage and a form of hypertrophic arthritis. According to new concept, in addition to articular cartilage, OA involves all of the tissues of diarthrodial joints, including subcondral bone, ligaments, capsules, synovial membrane and periarticular muscles (Flores et al., 2003). Therefore, recognizing OA as a whole-joint disease emphasises the importance of the evaluation of all tissues of synovial joints in the understanding of OA pathogenesis. Moreover, in the past decade the concept of OA as a degenerative disease of ageing cartilage has been giving way to the concept of OA as a form of arthritis with an inflammatory component (Attur et al., 2002). Due to the aneural and avascular nature of cartilage, OA seems not to comply with the criteria of the four cardinal signs of Celsius (*rubor et tumor cum calore et dolor)*. However, the inflammatory process can alternatively be characterized as the release and activation of toxic cellular mediators which promote tissue injury (Attur et al., 2002). Therefore, nowadays OA is considered to be an inflammatory form of arthritis, with focal synovitis and signs of inflammation at the molecular level within joint cartilage (Attur et al., 2002; Loeser, 2008).

4.1.2. Main aspects of OA diagnosis: clinical and radiological

Clinical diagnosis of KOA

Clinical OA refers to a syndrome of joint pain accompanied by varying degrees of functional limitation and reduced quality of life. The classic diagnostic KOA criteria were published by the Subcommittee on Osteoarthritis of the American College of Rheumatology (ACR) (Altman et al., 1986). According to ACR criteria, the diagnosis of KOA relies on knee pain accompanied by the following criteria: age over 50 years, stiffness lasting less than 30 minutes, crepitus, bony tenderness, bony enlargement, absence of acute inflammation, radiographically detected osteophytes and laboratory findings of synovial fluid changes, excluding inflammatory arthropathy (Altman et al., 1986). In 2010 the European League Against Rheumatism (EULAR) published its recommendations for the diagnosis of KOA (Zhang et al., 2010). They include three main symptoms − knee pain, brief morning stiffness, and functional limitation − in combination with three indications revealed by physical examination: crepitus, restricted movement and bony enlargement. However, the correlation of this clinical OA with early radiographic OA features at the individual level is rather poor and debatable (Creamer et al., 1997; Creamer, 2000).

Radiological diagnosis

Traditionally, the standardized radiographic protocols for measuring joint space width in the medial tibiofemoral compartment have become accepted for measurement of OA-related structural change in the knee joint (Thomas et al., 1975). The presence and severity of disease typically are determined using the Kellgren and Lawrence (K&L) grading system (Kellgren et al., 1963). The main limitations of the K&L system involve the invalid interpretation of the osteophyte (OPH) status for the classification of the disease, and other invalid assumptions by mixing distinct constructs and conflating different OA radiographic features (OPH and joint space narrowing) into one scale (Hunter et al., 2012). The significance of the detailed evaluation of OA radiographic features has been confirmed by genetic (Uitterlinden et al., 2000) and biomarker studies (Kraus, 2011). According to modern considerations, OPH and narrowing of joint space (JSN) appear to involve different pathways, and different pathophysiologic steps. This suggests that these two radiographic KOA features should not be consolidated and ideally should be looked at individually (Kraus, 2011).

Because of the inherent limitations of the K&L system, other individual grading systems for the various disease features at the different knee compartments have been developed. *Nagaosa et al.* have offered grading systems which meet the requirements of a separate investigation of the appearance of OPH and JSN, as well as permitting the evaluation of OA features for tibio- and patellofemoral knee joints (Nagaosa et al., 2000). This system provides separate instructions for JSN grading in both genders and demonstrates good reproducibility with other OA atlases (e. g. the atlas of Osteoarthritis Research Society International (OARSI) (Nagaosa et al., 2000).

4.1.3. General aspects of the pathogenesis of OA as a whole-joint disease

4.1.3.1. Narrowing of joint space

Articular cartilage is avascular and aneural connective tissue providing cover for the osseous components of the joints and serving as a load-bearing material with absorbing impact.

In OA, alterations in the normal biochemical activity of chondrocytes are mostly caused by abnormal biochemical factors (e.g. trauma and repetitive mechanical injury) or genetic factors (e.g. the mutation of matrix molecules) (Fukui et al., 2001; Goldring et al., 2007). During the early OA stage, the anabolic activities in cartilage are up-regulated through an increased synthesis of extracellular matrix (ECM) proteins (Martel-Pelletier et al., 2010), which is followed by amplified catabolic activity (Goldring et al., 2009; Kapoor et al., 2010). The catabolic phase is reflected by the increased production of matrix metalloproteinases (MMP), aggrecanases, higher expressions of regulatory

proteins, transcription factors, and stress and apoptotic markers (Goldring et al., 2008; Bertrand et al., 2010). The synthesis of metalloproteinase tissue inhibitors in the OA joint is decreased, leading to an imbalance between MMPs and their inhibitors (Kanyama et al., 2000). Furthermore, chondrocytes produce a large number of inflammatory mediators, including cytokines and chemokines, as well as reactive oxygen species, which promote OA-associated cartilage destruction (Loeser, 2008). All these alterations lead to a progressive loss of cartilage, beginning with fibrillation of the superficial zone and followed by matrix loss and the development of fissures (Goldring et al., 2010).

4.1.3.2. Development of osteophytes

Osteophytosis is one of the main radiographic features of OA and can be a source of pain and loss of function. It is unclear whether OPH develop because of pathological joint alterations or during physiological remodelling processes secondary to joint changes. Human and animal studies have demonstrated that OPH formation can occur without cartilage damage, suggesting that osteophytosis can be triggered in the undamaged joint as a result of mechanical stimuli or ageing (van der Kraan et al., 2007). In OA, cartilage damage confirmed by JSN is reported to be highly associated with the presence of OPH (Boegard et al., 1998a).

Mesenchymal stem cells (MSC) present in the periosteum or synovium are thought to be the precursors of OPH (Shirasawa et al., 2006), suggesting that activated synovium can contribute to OPH formation. In addition to MSC in the periostium and synovial membrane, macrophage-like cells also contribute to OPH formation (van der Kraan et al., 2007). The growth factors produced by synovial macrophages seem to be a major factor in this process (Blom et al., 2004; van Lent et al., 2004). Of those, the most potent factor appears to be transforming growth factor beta (TGF-β), which has been shown to be extensively expressed by human OPH (Uchino et al., 2000). Additionally, insulin-like growth factor 1 (IGF-1) has been shown to be involved in the development of OPH (van der Kraan et al., 2007). Therefore, in osteoarthrotic joints, the formation of OPH and degradation of articular cartilage represent two different processes characterized by various pathophysiological mechanisms, triggers, metabolic activity and many undiscovered factors. The investigation of both processes as separate mechanisms and the elaboration of novel agents involved in OA pathophysiological pathways will expand our current knowledge and help to resolve several unclear aspects of OA pathology.

4.1.3.3. Synovitis in OA

Over the past decade, more attention has been paid to the importance of synovial inflammation in OA. Historically OA was traditionally considered a non-inflammatory arthropathy due to the relatively low count of neutrophils in

the synovial fluid and the absence of systemic signs of inflammation (Wenham et al., 2010). Currently, the re-interpretation of OA as a whole-joint disease provides greater interest in understanding the pathophysiological role of synovial membrane inflammation in OA development.

In OA, chondrocyte and synovial cells become activated due to exposure to environmental events, such as mechanical stress, inflammatory cytokines, and altered organization of matrix proteins, including degradation products (Heinegård et al., 2011). The synoviocytes produce a large number of inflammatory cytokines, chemokines and other inflammatory mediators (e.g. tumor necrosis factor alpha, interleukin (IL)-1β, IL-6 and IL-8), which diffuse into the synovial fluid and act on the cartilage matrix to promote cartilage destruction (Golgring et al., 2011). It is clear that in the affected OA joint a vicious positive feedback loop takes place, involving cartilage breakdown and synovial inflammation.

Synovitis is often considered to be a major factor associated with the risk of the progression of cartilage loss and signs and symptoms of disease, including joint pain, swelling and stiffness (Sellam et al., 2010). The pathological features of synovial inflammation are found in at least half of the patients with OA, in both early and late OA (Ayral et al., 2005), being more aggressive in early OA (Benito et al., 2005).

Histologically, OA-associated synovia changes demonstrate chronic synovitis features (Ayral et al., 2005). Four patterns of OA-associated synovial changes have been described: (1) hyperplastic, (2) fibrotic, (3) detritus-rich, and (4) inflammatory (Oehler et al., 2002). The hypertrophic pattern (synovial lining and villous hyperplasia) has been predominantly diagnosed in patients with early knee OA, and the inflammatory pattern has been observed equally in both early and late OA (Oehler et al., 2002). The detritus-rich (macromolecular cartilage and bone debris) and fibrotic (capsular fibrosis) patterns have most often been observed in patients with late-stage disease (Oehler et al., 2002).

Indeed, synovial fibrosis is often seen in the end stage of the disease (Revell et al., 1998). In OA the fibrosis of synovial membrane can be characterized as an abnormal healing process with excessive deposition of extracellular matrix proteins, resulting in the alteration of the structure and remodelling of synovia. Fibrogenesis is a response to a variety of insults, such as inflammation, trauma and infection, resulting in an inflammatory reaction (Bastiaansen-Jenniskens et al., 2013). Blood monocytes and tissue macrophages, rapidly recruited into fibrotic tissue, are an important source of fibrotic cytokines (Gharaee-Kermani et al., 2001), of which TGF-β seems to be the most potent (Gruel et al., 2009). The latter, along with other functions, recruit fibroblasts to the site of an injury and stimulate them to proliferate (Gharaee-Kermani et al., 2001). Fibrosis in the OA joint seems to be an important contributor to joint stiffness and a source of pain (Hill et al., 2007). Thus synovial fibrosis and synovial inflammation represent good targets for improvement in current OA treatment strategies and the relief of pain.

Therefore, inflammation of synovial membrane cannot be ignored as an important factor associated with OA clinical symptoms and disease progression. Nevertheless, even today, the role of synovitis is often overlooked. There are multiple pathways and mediators that can directly contribute to the development and persistence of synovitis, whose molecular pattern is currently under-investigated. Further efforts to understand the molecular variability of mechanisms associated with OA synovitis will help to provide insight into the nature of the disease and its heterogeneity.

4.1.3.4. Biochemical markers in OA

Despite the focus in recent years on the development of OA imaging, the identification of patients at risk for incident early disease or disease progression remains challenging. Therefore, there is still a need for the development of biochemical markers that have the potential for the early identification of impaired articular tissue metabolism (e.g. matrix degradation), and that can lead to earlier treatment to prevent cartilage and bone degradation. Metabolic changes in OA involve all joint tissues, as well as different pathophysiological pathways, and therefore are most adequately reflected by complex biomarker assays. In terms of the investigation of new players in OA, investigative markers should be evaluated simultaneously with biomarkers, reflecting the metabolism of different joint tissues, for further understanding of OA patho-physiologic steps.

In the past decade, a large number of possible OA biomarkers have been studied, and several of them have demonstrated promising results for potential diagnostic and predictive purposes in the OA field. One of the most extensively investigated biomarkers is a fragment of carboxy-terminal telopeptide of type II collagen (CTX-II). The collagen type II is the major part of the articular extracellular matrix. CTX-II reflects the intensity of collagen II degradation by proteolytic enzymes, such as MMP, collagenases and cysteine proteases (Yasuda et al., 2005; Burrage et al., 2006). Owing to its small size, it is freely filtered by the renal system and concentrated and measured in the urine (Elsaid et al., 2006). Urinary CTX-II (u-CTX-II) correlates well with progressive JSN, as well as with osteophytosis, and reflects the burden of disease as well has prognostic, efficacy of intervention and diagnosic value (Kraus, 2011).

The molecular markers of bone turnover have been significantly less studied than cartilage markers. Degradation of type I collagen, which is a major collagen in bone during subchondral bone resorption, can be detected by increased levels of N- and C-terminal cross-linked telopeptides (NTX-I and CTX-I) (Garnero, 2007). Of the markers reflecting bone synthesis, a greater diagnostic value has been suggested for the amino-terminal peptide of type I procollagen (PINP) (von der Mark, 1999). Recent data have confirmed that PINP has diagnostic value for progressive osteophytosis and predictive value for extensive OA progression (Kumm et al., 2013).

4.1.4. Systemic risk factors

OA is a multi-factor disorder, in whose development the complexity of systemic risk factors plays a crucial role. Therefore, in research in the OA field attention must be paid to all OA risk factors as important covariates.

4.1.4.1. Age, gender and body weight

Age

Age is recognized as a strong OA risk factor. An increase in OA incidence and prevalence has been reported for all joints in the elderly population (Felson et al., 2000; Lawrence et al., 2008). The biological changes associated with ageing seem to contribute to an imbalance between catabolic and anabolic activity in the joint. According to published data, older chondrocytes are characterized by a loss in normal mitogenic activity and respond poorly to growth factor stimulation, as they are unable to maintain homeostasis in the articular cartilage (Guerne et al., 1995; Shane Anderson et al., 2010).

Gender

Women are more likely to suffer from OA than men, and also in more severe forms (Srikanth et al., 2005). Genetic association studies have reported gender-dependent differences in susceptibility to OA (Valdes et al., 2004; Valdes et al., 2006), and several epidemiological studies have shown sex-specific differences in both prevalence and severity of disease (Felson et al., 1998; Richette et al., 2003). Therefore, there is evidence that innate sex-specific differences may exist at the molecular level that contribute to disease severity. A clear increase in OA in women during menopause suggests that hormonal factors may play a role in the development of OA.

Body weight

Obesity is a well-known risk factor for OA development in such weight-bearing joints as knees and hips. Numerous studies have demonstrated a strong connection between obesity-associated joint overload and OA, being first reported by Fletcher (Fletcher et al., 1945). Mechanical overload seems to activate chondrocytes and accelerate cartilage degeneration (Sellam et al., 2012). Additionally, an increase in body mass index (BMI) has a negative effect on muscle strength, balance and gait (Messier, 2010), which can also contribute to OA development.

4.1.4.2. The role of genetic factors in OA development

4.1.4.2.1. Heritability of OA and genetic studies

OA has a complex pathology, with numerous environmental and genetic risk factors. Generally, OA is thought to be caused by a complex interaction between environmental and genetic factors (Valdes et al., 2009). A variety of epidemiological studies have demonstrated that genetic susceptibility is a key regulator of OA aetiology (Peach et al., 2005). Genetic variations may influence several OA risk factors, including obesity, skeletal shape, bone mass and synovitis (Valdes et al., 2011a). Furthermore, it has been shown that generalized OA phenotype, sensitivity to pain and disease progression may be also determined by genetic susceptibility (Valdes et al., 2010a; van Meurs et al., 2009; Kerkhof et al., 2010).

In the 1940s familial clustering of OA was first formally studied by Stecher, who performed a study of Heberden's nodes of the fingers within affected families (Stecher et al., 1941). Later, OA inheritance has been reported in multiple subsequent studies on other common forms of OA affecting the knee (Chitnavis et al., 1997; Felson et al., 1998; Hirsch et al., 1998; Neame et al. 2004), hip (Lindberg et al., 1986, Chitnavis et al., 1997; Lanyon et al., 2000) and spine (Bijkerk et al., 1999; Sambrook et al., 1999). According to published genetic studies, there is a clear difference between sub-populations of OA and different OA phenotypes, such as distinct genetic traits being linked to OA of the knee, hands or hip (Madry et al., 2012). For knee OA, in classic twin studies heritability has been calculated to be 39% in woman after controlling for other OA risk factors, such as age, sex and BMI (Spector et al., 1996). Additionally, a magnetic resonance imaging (MRI)-based twin study provided evidence for the importance of genetic factors in determining cartilage volume, showing the heritability for total knee cartilage volume to be 73% (95% CI 51−85%) (Hunter et al., 2003).

Human genetic studies focusing on the identification of important molecular pathways associated with the development of OA pathological changes may help to unravel the mechanisms responsible for the appearance of joint damage and its progression. OA is generally recognized as a complex multi-factor disease that has mostly small and modest effect susceptibility loci (Reynard et al., 2012). Over the past decade efforts have been focused on the search for loci that predispose to OA. The following approaches most often have been attempts to provide insight into the complexity of OA genetics: genetic linkage studies (LA), genome-wide association studies (GWAS) and candidate gene studies.

LA exploits the fact that genes have a tendency to be inherited together because they are located close to each other. After identification of a linkage, all genes in the linked region need to be identified and in this manner candidate genes of interest for a disease can be established. LA has been successful in localizing chromosomal regions containing highly penetrant genetic variants. However, in a common disease, such as OA, the success of LA is very limited

for a number of reasons, including the low power of identification of genes with modest effects and the large impact of environmental influences (Risch et al., 1996).

After the completion of the Human Genome Sequencing project, which provided the opportunity to systematically search across the genome and to test large numbers of common genetic variants for association with disease, GWAS have been successfully applied to the study of many complex diseases. GWAS make it possible to examine the associations of thousands of common genetic variants with disease. Recently, two reasonably large GWAS studies for OA were published: the Rotterdam and arcOGEN (arc Osteoarthritis Genetics) GWAS. Both studies revealed loci with genome-wide significance ($p<5x10^{-8}$) for association with knee and hand OA (Kerkhof et al., 2010), and knee and hip OA (arcOgen consortium, 2012).

While the implementation of GWAS has been highly successful for several traits, a number of challenges and problems remain. The acceptable genome-wide significance level is thresholds below $p<5x10^{-8}$**.** The detection of these robust signals are possible only with very large sample sizes (thousands of cases and controls), or in the case of genetic variants with large size effects, which are rather rare in common diseases, such an OA (Chapman et al., 2012). So it is very likely that many associations with smaller p-values may in fact also be true positives, hidden by their heterogeneity or lack of power. Moreover, there has been growing criticism of the huge resources needed for GWAS, and of the "missed heritability" problem (Chapman et al., 2012). Thus the combined analysis of a large number of studies and international collaboration is a possible direction for further OA studies.

4.1.4.2.2. Candidate gene association studies

Candidate gene association studies are hypothesis-based studies offering a relevant approach for the investigation of genetic variations in complex diseases. Candidate studies focusing directly on a single gene and frequently on functionally significant polymorphisms have played an important role in discovering genes involved in complex diseases. The main limitation of candidate gene studies is the very small region of the genome under investigation (Chapman et al., 2012). Over the past decade, more than 80 genes have been subjected to OA candidate gene association analysis, including genes encoding ECM components, genes affecting such bone characteristics as bone mineral density, and genes encoding enzymes and catabolic and anabolic cytokines (Dai et al., 2010; Valdes et al., 2011a).

The strongest association with KOA was reported for rs143383 of the growth differentiation factor 5 gene (*GDF5*) in the European population (including Estonians) (Valdes et al., 2011b; Table 1). The GDF5 protein is an extracellular signaling molecule that is a member of the TGF-β super-family, which participates in the development, repair and maintenance of joint tissue,

including bone and cartilage (Khan et al., 2007). The rs143383 is one of a minority of variants that demonstrate a connection with KOA in different ethnic populations (Miyamoto et al., 2007). Moreover, rs143383 is reported as having the strongest association with hip OA (Miyamoto et al., 2007), but this relation seems to be more controversial than for knee OA.

Another remarkable association with knee and hip OA in the European population (including Estonians) was reported for rs12901499, which is located within the first intron of *SMAD3* (Valdes et al., 2010b; Table 1). SMAD3 is an intracellular protein involved in cell signaling that links the TGF-β signal with variations in gene transcription. TGF-β-SMAD3 signaling is essential for maintaining articular cartilage; SMAD3 mutant mice homozygous developed degenerative joint disease similar to human OA (Yang et al., 2001). However, since the association of rs12901499 to OA has been reported only by one research, further genetic and functional studies should be performed for understanding of its role in OA susceptibility.

Another gene, encoding TGF-β-interacting–asporin (*ASPN*), was also reported to be associated with KOA. A variable number of tandem repeats in the *ASPN* gene were associated with decreased TGF-β1-mediated chondrogenesis *in vitro* and were reported to be strongly associated with an increased risk of KOA in an Asian population with no overall association in a European cohort (Nakumura et al., 2007; Atif et al., 2008; Table 1). Additionally, the importance of inflammation in OA pathogenesis confirms a strong association of variants in the gene encoding the IL1 receptor antagonist with the severity of KOA (Attur et al., 2010; Table 1).

Table 1. List of candidate genes demonstrating the most significant associations with knee OA in different populations.

SNP	Gene	OR	p-value	Pathway	Ethnic group
rs143383	*GDF5*	1.16	$8x10^{-9}$	Bone morphogenetic protein, joint development	European (Valdes et al., 2011b)
Asp14 in VNTR allele	*ASPN*	1.95	$1.3x10^{-6}$	TGF-β signaling	Asian (Nakamura et al, 2007)
rs12901499	*SMAD3*	1.22	$7x10^{-6}$	TGF-β signaling	European (Valdes et al., 2010b)
Haplotype: rs419598-rs315952-rs9005	*IL1Ra*	0.15	$1x10^{-4}$	Inflammation	European (Attur et al., 2010)

GDF5− growth differentiation factor 5
ASPN−asporine
TGF-β−transforming growth factor beta
IL1Ra−interleukin1 receptor antagonist

Furthermore, some smaller studies have suggested the role of genes encoding cartilage degradation enzymes and proteases, including disintegrin and metalloproteinase domain protein 12 *(ADAM12),* as well as cartilage glycoproteins (*CILP*), in increased KOA risk (Valdes et al., 2004; Valdes et al., 2006). Both genes encode multifunctional proteins, which potentially might be implemented in molecular pathways associated with OA development.

The potential contribution of those genes to KOA was described in a British cohort (Valdes et al., 2004), but so far there are no data for other European regions, including Estonia. Moreover, no meta-analysis involving the elucidation of the role of *ADAM12* or *CILP* genes in OA susceptibility has been performed.

4.2. ADAM12: characteristics and relation to OA

4.2.1. ADAM12 biochemical structure and association with human diseases

ADAM12 is a member of the ADAM family, secreted glycoproteins that are related to snake venom metalloproteases and matrix metalloproteases. ADAM12 is an active metalloproteinase that has cell-binding and cell-signaling properties (Kveiborg et al., 2008; for review, see the UniProt and GeneCards databases). *ADAM12* expression is increased mainly in remodelling and fast growing tissue, characterized by cell fusion and growth (Wewer et al., 2005). *ADAM12* is expressed in most mesenchymal cell types, such as osteoblasts (Verrier et al., 2004), chondroblasts (Kveiborg et al., 2006), adipoblasts (Kawaguchi et al., 2003) and such fusogenic cells as osteoblasts (Abe et al., 1999) and placental trophoblasts (Gilpin et al., 1998). Additionally, the *ADAM12* transcript is found to increase during muscle regeneration in mice, suggesting a role of *ADAM12* in myogenesis (Bornemann et al., 2000). Some studies have indicated that TGF-β is implemented in the transcriptional regulation of *ADAM12* and is able to induce the expression of *ADAM12* in several differential pathways (Le Pabic et al., 2003; Le Pabic et al., 2005).

The human *ADAM12* gene contains over 8,800 single nucleotide polymorphisms (SNP; NCBI home database) and encodes two alternatively spliced transcripts: the long transmembrane protein (ADAM12-L) and the short soluble form (ADAM12-S). Both isoforms contain pro, catalytic, disintegrin-like, cysteine-rich and epidermal growth factor (EGF)-like domains. The ADAM12-S isoform lacks the transmembrane and cytoplasmic domains, but contains a unique stretch of 33 amino acids at the C-terminus (Jacobsen et al., 2009; Figure 3).

Both ADAM12 forms (long and short) are active sheddases. ADAM12-L has been shown to cleave various pro-forms of membrane-bound growth factors and cytokines, such as pro-forms of EGF (Horiuchi et al., 2007), heparin binding EGF (Asakura et al., 2002) and the insulin-like growth factor binding protein-5

(IGFBP-5) (Okada et al., 2008). Similarly to the long isoform, ADAM12-S also mediates the cleavage of IGFBP-3 and -5 (Loechel et al., 2000), suggesting a role for ADAM12 in IGF-system regulation during embryonic growth and development (Jacobsen et al., 2009).

The functions of ADAM12, in addition to catalytic events, include cell-binding activities, which are linked to disintegrin-like, cysteine-rich, and EGF-like domains. In an *in vitro* attachment assay, the disintegrin domain of ADAM12 binds to integrin, which provides a link between the actin cytoskeleton and the extracellular matrix (Eto et al., 2000). The over-expression of *ADAM12* has been shown to be associated with the induction of the reorganization of the actin cytoskeleton, reduced cell adhesion and changes in cell survival (Kawaguchi et al., 2003). The cytoplasmic tail of ADAM12-L could potentially communicate signals between the intra- and extracellular space *via* the transmembrane domain (Jacobsen et al., 2009).

Numerous investigations have suggested *ADAM12* as an important regulator in both the normal development of tissues and a variety of pathological states (Wewer et al., 2005), making it a potential drug target. High expression levels of both forms of ADAM12 have been observed in several types of carcinomas, including breast, liver, gastric, bladder and prostate cancers, as well as glioblastomas (for review see Arribas et al., 2006). The possible contribution of the ADAM family to cancerogenesis probably can be explained by the activation of EGF receptor signaling, via the release of growth factors from the cell surface (Kenny et al., 2007; Blobel, 2005). Similarly, both isoforms of ADAM12 are highly expressed by trophoblasts during placental growth (Gilpin et al., 1998; Ito et al., 2004). Significantly reduced levels of ADAM12 in maternal serum have been found to be associated with such chromosomal disorders as Down syndrome and Edward syndrome (Laigaard et al., 2003; Laigaard et al., 2005a), as well as preeclampsia (Laigaard et al., 2005b), making ADAM12-S a potential biomarker of pregnancy abnormality.

In contrast, the contribution of ADAM12 to inflammatory responses is largely unknown. According to recent studies, *ADAM12* is up-regulated in the interface tissue surrounding implants during inflammatory responses and aseptic osteolysis associated with hip replacement implants (Ma et al., 2005). ADAM12 levels are also reported to be significantly increased in T-cells infiltrating the spinal cord with experimental autoimmune encephalomyelitis in mice (Toft-Hansen et al., 2004). Additionally, several studies suggest the implementation of ADAM12 in the development of inflammation-induced fibrosis in skin and muscle models (Peduto et al., patent WO/2011/024146).

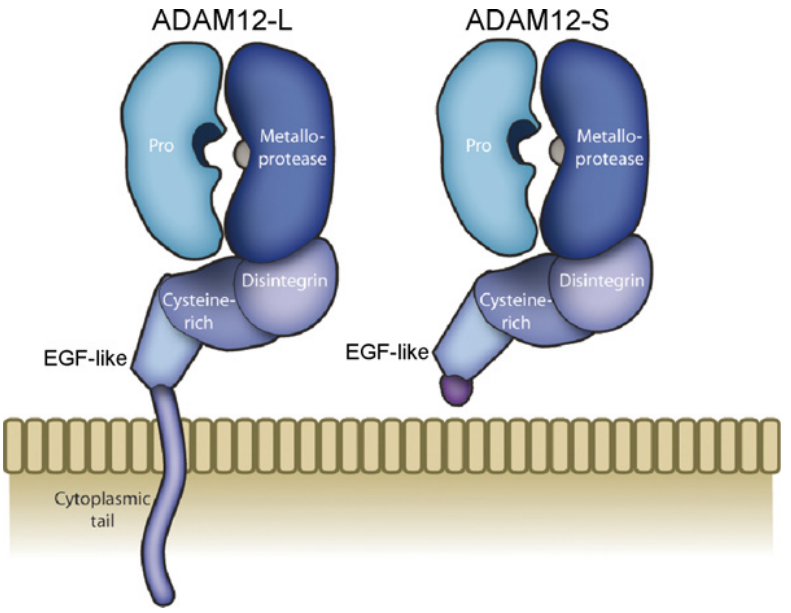

Figure 3. Schematic illustration of human ADAM12 isoforms. The long transmembrane ADAM12-L and the alternative shorter ADAM12-S that is released from the cell, are both shown as globular molecules with the prodomain noncovalently associated with the metalloprotease domain (Kveiborg et al., 2008).

4.2.2. Association studies of ADAM12 gene variants with knee OA

Several reports have indicated that *ADAM12* might also contribute to OA, but this relationship has only been sparsely studied. Four single nucleotide polymorphisms in *ADAM12*–rs3740199 (c.142G>C, p.Gly48Arg), rs1871054 (c.1154+145G>A), rs1278279 (c.1515G >A, p.Asn505Asn), and rs1044122 (c.2475T>C, p.Ala825Ala)–have so far been investigated in relation to OA in the European population (Valdes et al., 2004; Valdes et al., 2006; Limer et al., 2009). Up to now, the missense variant rs3740199 in the ADAM12 gene was reported to be associated with increased risk of OPH development and progression in the Chingford study (UK), which included only female subjects (Valdes et al., 2004). Later, an extensive GOAL study (UK) was unable to replicate this association (Limer et al., 2009); however, the authors of the replication study focused on global radiographic KOA and did not perform a separate analysis for different KOA radiographic features (JSN and OPH). As previously mentioned, OPH and JSN appear to involve different pathways and pathophysiologies, and should ideally be looked at individually (Kraus, 2011). Therefore, several important associations could hypothetically be missed because of a confluenting of radiographic parameters, as well as the lack of a separate analysis of both genders.

In another British case-control study, the associations between all four mentioned *ADAM12* SNPs and KOA were insignificant, but one haplotype in the *ADAM12* gene significantly increased the risk of KOA in men, as well as in women (Valdes et al., 2006). However, similarly to the GOAL study, in this research no stratification by OA ascertainment criteria was performed. Taken together, the impact of *ADAM12* on OA pathogenesis remains controversial and the possible association should be investigated in more detail and in different populations. Moreover, since in the discovery of association signals detailed investigation is important, there is a need for systemic research with stratification by sex and by KOA radiographic features.

4.2.3. Functional role of ADAM12 in knee OA development

The identification of compelling genetic associations should be followed by functional analyses having a task to assess how the associated alleles modulate gene or protein function. *In vitro*, ADAM12-L is found to be over-expressed in OA chondrocytes, and its immunoreactivity correlates with chondrocyte proliferation (Okada et al., 2008). Functionally, ADAM12-L may contribute to OA-associated chondrocyte proliferation through the mediation of the cleavage of IGFBP-5 (Okada et al., 2008). Moreover ADAM12-S has been found to stimulate bone growth by modulation of chondrocyte proliferation and maturation in murine models (Kveiborg et al., 2006), which support the functional importance of ADAM12 in chondrocyte metabolism and, in this way, possibly contribute to OA.

Nevertheless, there are no systematic data reflecting the possible role and importance of ADAM12 in OA pathophysiologic processes in different joint tissues. No extensive genetic research or studies for elaboration of ADAM12's functional impact in the OA field have been performed. The relation of ADAM12 to OA still remains unclear and controversial, and specific pathways contributing to OA need to be elucidated. Since separate evaluation either of genetic susceptibility or functional consequence have provided only ambiguous information, the possible contribution to OA ideally should be investigated extensively at the gene, RNA and protein levels.

4.3. CILP: characteristics and relation to OA

4.3.1. Biochemical structure of CILP

Cartilage intermediate layer protein (CILP) is a monomeric glycoprotein of the extracellular matrix that plays a role in cartilage scaffolding (Tsuruha et al., 2001; see for review UniProt and GeneCards databases). There are two isoforms of CILP–CILP1 and CILP2–with homology of over 50% (Johnson et al., 2003; Figure 4). Both CILPs are post-translationally processed into amino (CILP1-1 and CILP2-1) and carboxyl forms (CILP1-2 and CILP2-2) (Johnson et al., 2003).

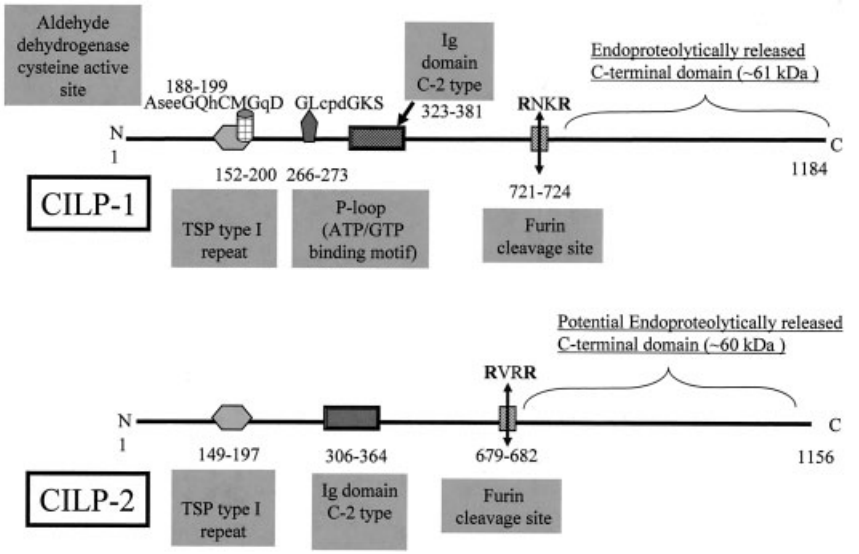

Figure 4. Schematic representation of CILP1 and CILP2 sequences. CILP1 and CILP2 demonstrate 50% of homology, sharing trombospondin motif I, Ig type C2 domains and furin cleavage sites. The aldehyde dehydrogenase cystein active site is unique to CILP1 (Johnson et al., 2003).

Each of the N-terminal domains of CILP-1 and CILP-2 contains a substantially conserved immunoglobulin C-2 type domain and thrombospondin type I repeat domain (Johnson et al., 2003; Figure 4). The latter could potentially modulate the anchoring ability to other ECM components, including glycosaminoglycans and TGF-β1 (Johnson et al., 2003; Barallobre-Barreiro et al., 2012). *In vitro* studies have demonstrated that the N-terminal domain of CILP binds TGF-β1 directly and antagonizes TGF-β1 in rabbit chondrocytes (Seki et al., 2005). The carboxyl-terminal half of CILP1 corresponds to a protein homologous to porcine nucleotide pyrophosphohydrolase (NPP) (Lorenzo et al., 1998b) and was initially claimed to have an NPP function (Masuda et al., 1997a), but later research failed to confirm this enzymatic activity (Johnson et al., 2003; Seki et al., 2005).

4.3.2. The association of the CILP gene and protein with knee OA

Genetic association studies have reported significant associations of the rs1561888 (c.-106–412C>T) SNP in the three prime untranslated region (3`-UTR) of the CILP gene with radiographic KOA (rKOA) in male subjects (Valdes et al., 2006). So far, no replication studies for this variant have been performed in European or other populations.

The amount of CILP1 protein increases with the ageing of human articular cartilage (Lorenzo et al., 1998a; Lorenzo et al., 1998b). Functionally, CILP1 might play a role in cartilage repair (Lorenzo et al., 1998a). According to published data, CILP is important in maintaining cartilage homeostasis in humans, and its dysfunction contributes to several diseases affecting cartilage, including lumbar disc disease and OA (Seki et al., 2005; Min et al., 2009; Min et al., 2010).

In mice, the injection of recombinant CILP1 has been shown to induce arthropathy (Tsuruha et al., 2001; Yao et al., 2004). Moreover, autoantibodies to the CILP1 fusion protein have been detected in about one-tenth of the patients with OA, suggesting the involvement of CILP1 in the immune-mediated process of joint destruction (Tsuruha et al., 2001). In OA cartilage, the expression of *CILP* has been shown to be up-regulated in early and late stages of the disease (Lorenzo et al 1998b; Lorenzo et al., 2004), suggesting the specificity of CILP1 in OA and providing a basis for considering CILP1 as a potential OA biomarker. Currently, there are only limited data on *CILP* gene regulation (Mori et al., 2006), and no functional research has been published identifying OA susceptibility pathways; therefore, the impact of *CILP* in OA pathogenesis is currently unclear and data regarding its involvement in specific pathophysiological steps are rather limited.

In fact, CILP1 is produced by chrondrocytes and has been found to be specifically expressed in cartilage, localizing in the intermediate layer of articular cartilage, but not in the superficial or in the deepest regions (Lorenzo et al., 1998a). Additionally, CILP1 has been reported to be abundantly over-expressed in lumbar disc tissue (Hirose et al., 2002). At present, little is known about *CILP* mRNA and protein expression in non-cartilaginous tissues, but there are suggestions that CILP may not be exclusively produced by chondrocytes. In particular, CILP1 and CILP2 have been detected in mouse connective tissue (Bernardo et al., 2011). Thus, the cartilage-specific origin of CILP is currently unclear, suggesting the possibility of production in other tissue of the synovial joint. However, the original source and type of cells responsible for CILP production in non-cartilaginous tissue is currently unknown. Furthermore, similarly to ADAM12, there are no identifiable data on *CILP* mRNA and protein expression pattern, or on the CILP function in synovial joint tissues, other than cartilage. Since the evaluation of gene/protein expression in different tissue may be able to assess which particular pathways are implicated in disease initiation and progression (Reynard et al., 2013), there is a need for further survey of CILP in osteoarthrotic joints. The intensive investigation of CILP in different tissues of the OA joint might reveal unknown aspects of its biological nature, as well as provide insight into the mechanisms involved in OA pathophysiological pathways.

5. AIMS OF THE STUDY

To perform an analysis of *ADAM12* and *CILP* genes on DNA−RNA−protein levels in radiographic knee osteoarthritis on the basis of an Estonian cohort.

Specific aims:

I. To investigate possible associations of selected SNP of *ADAM12* (rs3740199, rs1871054, rs1278279 and rs1044122) and *CILP* (rs1561888) with rKOA.

II. To investigate the relationship of the serum level of ADAM12 protein with the radiographic features of KOA and OA molecular markers.

III. To investigate the association between histological synovitis, features of rKOA and levels of *ADAM12* and *CILP* mRNA in bioptic samples.

IV. To clarify the possible origin of ADAM12 and CILP1 proteins in the synovial tissue and the relationship between the local production of proteins and the level of mRNA expression.

6. PATIENTS AND METHODS

6.1. Study subjects

The study consisted of two different study groups: a population-based cohort recruited in 2002−2006 from southern Estonia and subjects undergoing arthroscopy in Tartu University clinics in 2007−2010. All of the recruited subjects participated in in-depth clinical examinations, including inquiry, performance tests, X-ray radiography of the knee joints, donating blood samples for DNA extraction (dipotassium ethylenediaminetetraacetic acid (K2-EDTA) containing tubes) and biomarkers assays (tubes containing blood activators), as well urine samples. Subjects with previous medical histories or radiographic features of rheumatoid arthritis and other inflammatory arthropathies in the knees were excluded from the study.

A population-based cohort was selected from three Family Doctor (FD) registers of the small towns of Elva and Võru, in southern Estonia. The first questionnaire was mailed to all 1793 individuals aged 35–57 years registered with these FDs. Altogether, 964 individuals responded (54%). Of these, 506 confirmed the presence of KOA complaints (knee pain: 65%, other problems e.g. crepitus and stiffness: 35%), and the remaining 458 said they had no KOA problems. A further 475 (among them, 67% female) of the 964 responders agreed to in-depth clinical examinations, and, of these, 308 subjects reported knee problems (pain: 70%, other symptoms: 30%). Thus the study group included 26.5% of the subjects of the selected age group. In 33 subjects (14 women) blood DNA samples were not available and in four subjects (three women) data on knee joint radiographic examinations were missing. So, the final population-based study group consisted of 437 subjects (302 women, 135 men), with a mean age of 45.2 years.

The arthroscopy group consisted of 91 subjects (including 49 women and 42 men) aged 32−60, with a mean age of 47.4, and they had undergone knee arthroscopy due to chronic knee problems (several months to years prior) in Tartu University clinics in 2007−2010. Of those, in 44 subjects (24 women, 20 men), aged 32−60, with a mean age of 46.7, synovial and cartilage tissue biopsies were available for histological examination and total RNA extraction.

The current study was conducted as part of a large interdisciplinary project, having the task of a multifaceted inquiry into osteoarthritis in an Estonian cohort. All participants completed short-form health surveys (SF36), questionnaires about knee problems (Knee injury and Osteoarthritis Outcome Score: KOOS) and previous medical history forms. In all of the participants, radiographic evaluation of KOA, as well as assessment of bone and cartilage OA biomarkers, had previously been done (Kumm et al., 2009).

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 allocation of study subjects and study design is presented in Table 2

Table 2. Design of undertaken study and allocation of study subjects.

Part of original study	Population-based group N=437	Arthroscopy group N=91	Publications
Genetic association study of *ADAM12* and *CILP* SNPs with rKOA (N=437)	437	–	I, II
ADAM12-S protein assay * (N=276)	185	91	III
Evaluation of histological synovitis in rKOA (N=44)	–	44	IV
Evaluation of synovial *ADAM12* and *CILP* mRNA and protein expression in rKOA (N=44)	–	44	IV, V, VI

SNP: single nucleotide polymorphism
rKOA: radiographic knee osteoarthritis
*–for selection of samples, see methods chapter

6.2. Methods

6.2.1. Radiographic examination

For radiographic assessment in the tibiofemoral joint (TFJ), standardised weight-bearing antero-posterior radiographs of both knees were performed. Axial radiographs of the patellofemoral joint (PFJ) were taken in the standing position, with 60°flexion of knee joints, according to the Boegård technique (Boegård et al., 1998a). The two main features of KOA, the presence of JSN and OPH development, were estimated independently in both TFJ and PFJ, according to the grading system (grades 0–3) of *Nagaosa et al.* (Nagaosa et al., 2000). The highest grade of JSN or OPH was regarded as the stage of KOA in the corresponding joint (TFJ or PFJ). The highest grade of OA for each subject in TFJ or PFJ was regarded as the global stage of KOA. The distributions of rKOA, age and BMI in the genetic investigation group, the biomarkers assay group and the mRNA expression and immunostaining group are presented in Tables 3a and 3b.

Table 3a. A summary of radiographic knee osteoarthritis (OA) evaluation and characteristics in subjects with genotyped *ADAM12* and *CILP* gene polymorphisms and in the ADAM12-S protein assay group.

	OA in TFJ			**OA in PFJ**			**OPH**		
	Grade 0	**Grade 1**	**Grade 2-3**	**Grade 0**	**Grade 1**	**Grade 2-3**	**Grade 0**	**Grade 1**	**Grade 2-3**
Subjects with genotyped *ADAM12* and *CILP* gene SNPs	N=211	N=187	N=39	N=225	N=177	N=35	N=184	N=205	N=48
Females, %	68	71	67	68	73	62	67	73	63
Age (yr) Mean (±SD)	44.3(5.9)	46.1(5.5)	48.2(5.5)*	44.3(6.0)	46.5(5.3)	47.6(5.4)*	44.4(5.9)	44.9(5.5)	47.9(5.6)*
BMI Mean (±SD)	25.9(4.6)	28.6(4.8)	30.1(5.7)*	26.3(4.6)	28.2(5.2)	29.3(5.5)*	25.6(4.5)	28.2(4.5)	29.8(5.3)*
Subjects with evaluated ADAM12-S level	N=110	N=119	N=47	N=133[1]	N=99	N=43	N=102	N=118	N=56
Females, %	58	60	63	67	61	63	59	58	64
Age (yr) Mean (±SD)	46.2(6.3)	47.5(6.7)	50.3(6.7)*	46.1(6.6)	47.9(6.6)	50.2(6.3)*	45.7(6.2)	47.6(6.7)	50.5(6.6)*
BMI Mean (±SD)	26.6(5.1)	28.7(4.4)	30.6(5.8)*	26.5(4.6)	29.4(4.9)	30.3(5.8)*	26.1(4.7)	28.9(4.8)	30.5(6.1)*

OA in TFJ − radiographic osteoarthritis in tibiofemoral joint; OA in PFJ − radiographic osteoarthritis in patellofemoral joint; OPH − osteophytes in TFJ and PFJ; BMI − body mass index.

* – p-value <0.001 (compared to group with radiological stage 0, t-test). [1]In one case of the 276, the radiographs from PFJ were considered ineligible for precise evaluation of OA stages.

Table 3b. The distribution of radiographic knee osteoarthritis (KOA) and main characteristics of subjects with investigated synovial expression of *ADAM1*2 and *CILP* mRNA.

Radiographic KOA grade	All	Men	Women
0	5	2	3
1	29	14	15
2	9	4	5
3	1	0	1
Joint space narrowing			
0	25	13	12
1	12	4	8
2	7	3	4
Age (years, mean±SD)	46.7 ± 6.0	47.2 ± 5.4	46.3 ± 6.5
BMI (mean±SD)	28.5 ± 5.0	29.3 ± 4.5	27.9 ± 5.2

BMI: body mass index

6.2.2. Genotyping of selected SNPs in ADAM12 and CILP genes

Four SNPs in *ADAM12*–rs3740199 (c.142G>C, p.Gly48Arg), rs1871054 (c.1154+145G>A), rs1278279 (c.1515G>A, p.Asn505Asn) and rs1044122 (c.2475T>C, p.Ala825Ala)–and one SNP in *CILP* genes–rs1561888 (c.-106–412C>T)–were genotyped in all participants. Rs3740199 in the second exon is a non-synonymous polymorphism, which results in substitution of amino acid glycine to arginine at position 48 in the ADAM12 protein. Recently, a rare new single nucleotide variation at the same position was revealed by sequencing (c.142G>T, p.Gly48Trp), with a frequency of 0.2% in the European population (Database of Single Nucleotide Polymorphisms). Since our study was performed before this rare variant was revealed, the used methodology did not allow us to identify this allele. The rs1278279 and rs1044122 lying in exon 14 and exon 21, respectively, were synonymous substitutions, which did not change the encoded amino acid, and rs1871054 was intronic SNP in 11. intron of the *ADAM12* gene. The SNP rs1561888 was located in the 3`-UTR of the *CILP* gene. The selection of *ADAM1*2 and *CILP* genetic variants for the current study was performed based on previously published data of genetic association studies in different cohorts (Loughlin, 2005; Valdes et al., 2004; Valdes et al., 2006). The list of laboratory techniques used is presented in Table 4.

DNA was extracted from whole EDTA-blood by phenol-chloroform extraction (John et al., 1991) or by salting (Aljanabi et al., 1997) (Table 4). In 189 subjects of the population-based cohort, rs3740199 and rs1871054 SNPs were amplified by polymerase chain reaction (PCR) and analysed by restriction fragment length polymorphism (RFLP) (Figure 5 and Table 4). The primers and restrictases used are shown in Table 5. In another 248 participants, rs3740199 and rs1871054 were genotyped using TaqMan® SNP Genotyping Assays

(C_1419869_1 and C_12049599_10). The rs1278279 and rs1044122 in the *ADAM12* gene and rs1561888 in *CILP* were detected by TaqMan® SNP Genotyping Assays (C_3077142_1, C_3077192_10 and C_1839361_1, Applied Biosystems, Foster City, CA) in all subjects (Table 4). Each sample of extracted DNA was normalized to a concentration of 10 ng/µl using DNAse-free water. The quantitative PCR assay was performed using an ABI PRISM 7000 SDS

Table 4. Brief characteristics of techniques used in the current study.

Laboratory work	Used techniques
DNA extraction	Salting, phenol-cholophorm method
Genotyping: SNP in *ADAM12* gene	 RFLP: rs3740199, rs1871054 TaqMan ®SNP genotyping assay: rs3740199, rs1871054, rs1044122, rs1278279
CILP gene	RFLP; TaqMan ®SNP genotyping assay: rs1561888
ADAM12-S protein measurement	DELFIA1/AutoDELFIA® ADAM12 research kit: sandwich-type DELFIA assay
RNA extraction	Qiagen Rneasy® Mini kit Qiagen Rneasy® Fibrous Tissue kit
mRNA synovial expression of *ADAM12* and *CILP*	TaqMan® gene expression assay: ADAM12: ADAM12-L, ADAM12-S, ADAM12-B (primers identical for both) CILP: CILP1
ADAM12 protein synovial expression	Immunochistochemistry: Abcam Ltd, ab56366
CILP1 protein synovial expression	Immunochistochemistry: Ab CILP1-1, Ab CILP1-2 (courtesy of Prof. Dick Heinegård, Lund University, Sweden)
Synovitis examination	Formalin-fixed paraffin-embedded sections stained by haematoxylin-eosin and by Van Gieson method

RFLP − restriction fragment length polymorphism

analyser. Each assay well had a 5-µl reaction volume, consisting of 2.5 µl of 20X TaqMan Universal PCR master mix (Applied Biosystems, Foster City, CA), 0.06 µl of 40x TaqMan primers, 1.5 µl of DNAse-free water and 1 µl of DNA. The amplification was performed according to the standard protocol of the TaqMan® Allelic Discrimination Protocol. The regenotyping of rs1561888 was performed with RFLP (Tables 4 and 5).

6.2.3. Assay of ADAM12-S protein and other OA biochemical markers

For the assays, the sera from 276 subjects (165 women, 111 men) were used: 185 samples (116 women, 69 men) from the southern Estonian population-based cohort and 91 samples (49 women, 42 men) from the arthroscopy group. Serum samples from the population-based group, based on previously known radiographic diagnoses of KOA for stage 0, stage 1 and stages 2−3, were randomly selected from available blood specimens. The extracted sera were stored at −20Cº until measurement. The concentrations of ADAM12-S protein in the serum samples were measured using the DELFIA1/AutoDELFIA® ADAM12 research kit (Perkin Elmer Life and Analytical Sciences, Turku, Finland; Table 4), according to the producer's manual (Valinen et al., 2009). The sensitivity of the assay was 0.19 μg/l, defined as two standard deviations above the single at zero dose, measured over 10 replications.

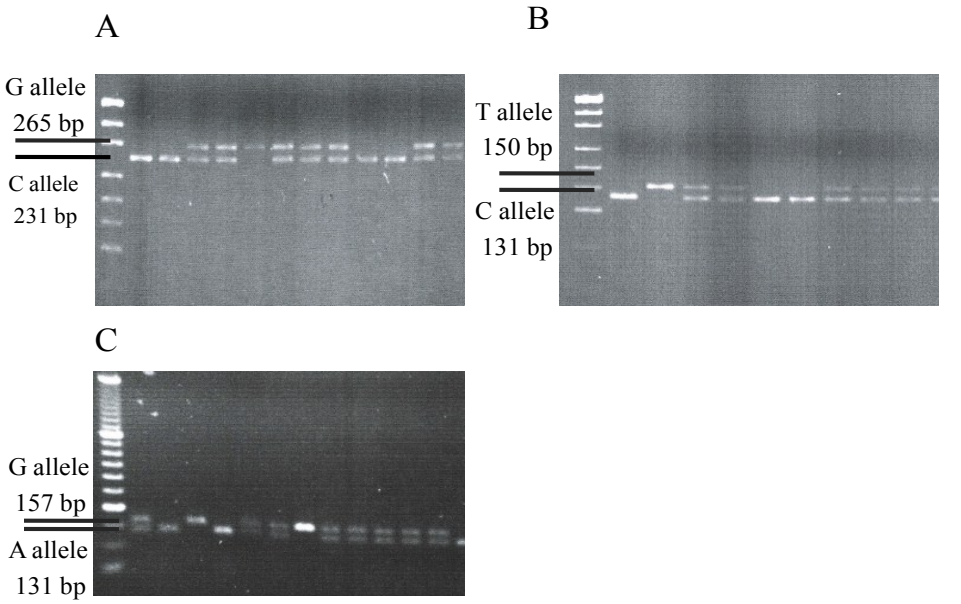

Figure 5. The genotyping of rs3740199, rs1871054 (*ADAM12* gene) and rs1561888 (*CILP* gene) by restriction length polymorphism (A) rs3740199 (B) rs1871054 (C) rs1561888.

Table 5. Primers and restrictases used for the genotyping of rs3740199 and rs1871054 in the *ADAM12* gene and rs1561888 in the *CILP* gene by restriction fragment length polymorphism technique.

rs3740199 (*ADAM12*)		
	Forward primer	5` GGA TCC CTC ATC AGC ACT GTC AC 3´
	Reverse primer	5`GCT CAT GAA GTT AGA GCC T 3´
	Restrictase	PpiI
rs1871054 (*ADAM12*)		
	Forward primer	5`TCT GCT TTG ACA GTG TGC ATG GCT 3’
	Reverse primer	5` GCT CTC CAG AGT ACA GGT CAC 3`
	Restrictase	Eco91I
rs1561888 (*CILP*)		
	Forward primer	5` TCA CAC AGG CGG GTA CAT ATT AAG GTC 3’
	Reverse primer	5` AGA AAT GTG TTC CAG CTG CAG CC 3´
	Restrictase	PagI

In all subjects in this group, the following markers had previously been measured: marker of cartilage collagen resorption−u-CTX-II and bone markers s-PINP and s-CTX-I (Kumm et al., 2009). U-CTX-II was assayed using the U-CartiLaps®ELISA kit (Immunodiagnostic Systems, Herlev, Denmark). The values of u-CTX-II were corrected for urinary creatinine, which was measured by Jaffe’s kinetic method. Bone markers were measured on the Elecsys 2010 automated analyser (electrochemiluminescent sandwich immunoassay, Roche).

Calculation of comorbidity index

Based on the questionnaire responses, previous medical history information, medications used and other lifestyle factors were analysed. A comorbidity index (CI) was calculated based on the Functional Comorbidity Index (Groll et al., 2005), relying on self-reported available medical histories. Finally, CIs were created by adding one point to the index for the presence of each of the following: hypertension, diabetes, history of cancer, lung disease (chronic obstructive pulmonary diseases and asthma), gastric or duodenal ulcers, depression, and liver or cardiovascular disease (angina, myocardial infarction or stroke).

6.2.4. Assays of ADAM12 and CILP mRNA expression in synovial and cartilage samples

Synovial tissue samples (weight 10−50 mg) were harvested from synovial capsules (from macroscopic lesions) using biopsy forceps (Piranha Rongeur, Atlantech) for the histological evaluations and total RNA extractions. In 10 of the 44 patients, second samples of the tissue were obtained from a macroscopically unchanged synovium (suprapatellar recessus). Articular cartilage biopsies (weight 10−45 mg) were obtained from the medial and lateral condyle of the femur.

RNA extraction from the biopsies of cartilage and synovial membrane

The obtained biopsies were immediately stabilized in 300 µl of RNAlater™ (Ambion, Inc., Austin, TX) solution overnight at 4 ºC and stored at −80 ºC until RNA extraction. All synovial tissue samples were ground after thawing using a T10 basic ULTRATURRAX ® dispenser (IKA® Werke GmbH & Co. KG, Staufen, Germany) and additionally homogenized with a syringe (needle G20). Total RNA was isolated from the homogenized synovial tissue samples using a Qiagen Rneasy® Mini kit or Qiagen Rneasy® Fibrous Tissue Mini kit (QIAGEN, Valencia, CA, Table 4), according to the provided manufacturer's manuals.

The yield of total RNA was detected by NanoDrop® NT-1000 spectrophotometer (NanoDrop Technologies, Inc., Montchanin, DE, USA), and RNA integrity was analysed by an Agilent RNA 6000 Nano Chip on an Agilent 2100 Bioanalyser (Agilent Technologies, Santa Clara, CA, USA). The total RNA extracted from cartilage tissue showed insufficient quality (RNA integrity index–RIN−from undetectable to 3.1) and therefore cartilage samples were excluded from further study. The RIN of total RNA from synovial samples was in the range 6.1−10 (a mean of 8.2). All samples with RIN over 6 were included in the study.

Synthesis of cDNA

Reverse transcription was performed using oligo-dT(18) primers and a Superscript III reverse transcriptase (Invitrogen Ltd, Paisley, UK), according to the manufacturer's manual. A RiboLock RNase Inhibitor (Fermentas) was used to inhibit the activity of RNases. cDNA was stored at −80°C until used in quantitative PCR.

TaqMan real-time PCR

TaqMan quantitative real time PCR (RT-PCR) was conducted to measure the expression of *ADAM12* and *CILP*. Three different TaqMan® gene expression assays (Hs00185774_m1, Hs00222216_m1 and Hs01106104_m1; Applied Bio-

systems, Foster City, CA) were selected for the detection of the expression of *ADAM12*−isoform I (ADAM12-Long or *ADAM12-L*), isoform II (ADAM-Short or *ADAM-S*), and total *ADAM12* (*ADAM12-B,* assay for identical regions of both isoforms), respectively. A TaqMan primer probe Hs001736147_m1 was used for the quantification of *CILP* expression (Table 4). Human β-actin endogenous control (Primer-Limited) (Applied Biosystems) was used as a housekeeping gene.

The quantitative RT-PCR assays were performed by an ABI PRISM 7900 HT SDS (Applied Biosystems) in 384-well plate format, according to the standard protocol (TaqMan® Gene Expression Assays Protocol). The PCR reaction was performed in three parallels. The data were analysed using the Sequence Detection Systems software, version 2.2.2 (Applied Biosystems). The RT-PCR reactions were performed as a multiplex (the housekeeper and gene of interest in the same well). The relative quantification levels of the gene expression were calculated using the $2^{-\Delta\Delta CT}$ method (CT=crossing points, cycle number where the fluorescence crossed the threshold 0.1): ΔCT=CT (target gene)-CT (reference gene); ΔΔCT=ΔCT (patients)-ΔCT (reference) (Livak et al., 2001). An acceptable standard deviation between the parallels was assigned as 0.2 and in the case of mean CT over 36 cycles as 0.4. The cDNA of a macroscopically non-pathological synovial specimen was used as a reference sample for all other synovial biopsies.

6.2.5. Evaluation of histological synovitis

Paraffin blocks for microscopical investigation were available for 42 of the 44 patients. The biopsy samples obtained from two subjects were determined to be unsuitable for histological evaluation and were therefore excluded from the study. Histological evaluation was performed by a clinical pathologist on formalin-fixed paraffin-embedded sections stained by haematoxylin-eosin and by Van Gieson method (Table 4). The microscopic features of synovitis were evaluated according to the system proposed by *Loeuille et al*. (Loeuille et al., 2005). Six parameters were evaluated separately: (1) number of synovial lining cells, (2) subsynovial infiltration by lymphocytes and plasma cells, (3) surface fibrin deposition, (4) congestion related to blood vessel vasodilatation and/or proliferation, (5) fibrosis, and (6) perivascular edema. Each parameter was scored on a range of 0−3. The sum of scores based on the six described parameters provided the final grade of histological synovitis, which was classified as absent (*grade 0*), mild (*grade 1*), moderate (*grade 2*), or severe (*grade 3).*

6.2.6. Evaluation of protein expression in the synovial membrane

Immunohistochemistry of the ADAM12 protein

Deparaffinised sections were treated with 3% H_2O_2 to inactivate endogenous peroxidase and then with Dako REAL Antibody Diluent (S2022; Dako Denmark A/S, Glostrup, Denmark) to block non-specific binding. After blocking, sections were incubated with mouse monoclonal antibody to ADAM12 (ab56366; Abcam Ltd, Table 4) overnight at 4°C. The primary antibody concentration was 3 µg/ml. Visualization of the primary antibodies was performed using the commercial kit "Dako REAL™ EnVision™ Detection System, Peroxidase/DAB+, Rabbit/Mouse" (K5007; Dako Denmark A/S). The washing steps in between were done in phosphate-buffered saline (PBS), which contained 0.07% of Tween 20 as a detergent. Toluidine blue (Applichem, Darmstadt, Germany) was used for background staining.

Samples were evaluated in a semi-quantitative manner by two examiners as follows: no staining=0, mild staining=1 (a few stained cells in the field; magnification 40x), moderate staining=2 (more than five stained cells), and strong staining=3 (more than 10 stained cells). No immunohistochemical staining was noted in negative control samples where the primary antibody was omitted.

Immunohistochemistry of CILP1-1 and CILP1-2 proteins

Deparaffinised sections were treated with 3% H_2O_2 to inactivate endogenous peroxidase. The sections were then treated with hyaluronidase solution at a concentration of 80 IU/ml (HYA01; FertiPro N.V., Belgium) for 60 minutes at 37°C and proteinase K solution at a concentration of 30 µg/ml (AppliChem GmbH, Germany) for 30 minutes at 37°C, to improve antibody penetration of the tissue section. Then the sections were treated with Dako REAL Antibody Diluent (S2022; Dako Denmark A/S, Glostrup, Denmark) to block non-specific binding. After blocking, sections were incubated with rabbit polyclonal antibody to CILP1-1 or CILP1-2 (courtesy of Prof. Dick Heinegård, Lund University, Sweden; Table 4) overnight at 4°C. The primary antibody concentration was 500 ng/ml for both antibodies. Visualization of the primary antibodies was performed using the commercial kit "Dako REAL™ EnVision™ Detection System, Peroxidase/DAB+, Rabbit/Mouse" (K5007; Dako Denmark A/S, Glostrup, Denmark). The washing steps in between were done with PBS which contained 0.07% of Tween 20 as a detergent. Toluidine blue (Applichem, Darmstadt, Germany) was used for background staining.

To control immunostaining specificity with CILP antibodies, additional blocking with CILP1 peptides was performed. Peptides CILP1-1 and CILP1-2 (courtesy of Prof. Dick Heinegård, Lund University, Sweden) were resuspended in DSMO with a final concentration of 10 mg/ml in blocking solution. Rabbit anti-CILP1-1 or anti-CILP1-2 polyclonal antibody was added

to the solution and the mixture was incubated for one hour at room temperature. After incubating, the immunostaining was continued according to the protocol described in the previous chapter.

Samples were evaluated in a semi-quantitative manner by two examiners as follows: no staining=0, mild staining=1 (less than five blood vessels with stained cells in the field (magnification 40x)), moderate staining=2 (more than five blood vessels with stained cells). No immunohistochemical staining was noted in negative controls where the primary antibody was omitted.

6.2.7. Statistical analysis

All the analyses were carried out in an R-environment ver. 2.4.0 (The R Foundation for Statistical Computing, Boston, MA).

SNPs associations study

For the estimation of associations between haplotypes of selected SNPs and radiological traits of OA, a haplo.stats (R-project, haplo.cc) package was used. The Hardy-Weinberg equilibrium (HWE) was evaluated using the genetics package (R-project, HWE. test). A p-value of 0.05 was designated as statistically significant.

The allele frequencies of genotyped SNPs were compared to those reported previously for the European population within the HapMap project (HapMap Population Diversity) and the Nottingham cohort (Valdes et al., 2006), using the Yates chi-square test.

Power estimates were performed using the Quanto ver. 1.2.4 software with the following options: population risk of 12%, significance level 0.05, log additive inheritance model (Quanto software). The results of the linkage disequilibrium (LD) mapping were generated using Haploview software (Barret et al., 2005).

The association between the SNPs and different radiographic features of KOA was evaluated using the logistic regression model. The ages, body mass indices and genders of the subjects were used as the covariates in all statistical calculations. To correct for the effects of multiple comparisons, we used the false discovery rate control of Benjamini and Yekutieli (the B-Y method), which provides an acceptable balance of type I and II errors.

Biomarkers analysis

Spearman's rank correlation (SRC) analysis was applied to investigate the associations between the level of ADAM12-S protein and other biomarkers or radiographic features of KOA. Owing to the asymmetric distribution and tied values of ADAM12-S, a permutation test (100000 simulations) was used to correct Spearman's rho test p-values. Additionally, the Bonferroni correction

was applied to avoid an inflated risk of type I error, and a p-value of 0.006 was designated as significant. The Wilcoxon rank sum exact test (WET) was used for comparison of the ADAM12-S values in subjects with and without radiographic traits of KOA.

ADAM12 and *CILP* mRNA expression analysis

SRC test was used to determine the association between mRNA relative expression level ($2^{-\Delta\Delta CT}$), the grade of histological synovitis, and protein expression in the synovial membrane. As the data did not fit normal distribution, WET was used to compare gene expression in patients' groups with different grades of rKOA. The Kruskal-Walls test or WET (if only two genotypes were detected in the study group) was applied for the evaluation of the association between mRNA expression level and genotyped polymorphisms.

Immunohistochemical staining (IHC)

SRC test was used to determine the correlation between IHC and gene expression level. The multiple linear regression was applied for evaluation of the association between IHC, histological synovitis and KOA radiographic features (age and BMI were used as covariates). The CILP1-1 IHC associations with *CILP* mRNA expression were additionally evaluated by a logistic linear model (with age and BMI as covariates).

7. RESULTS

7.1. Associations of *ADAM12* gene and protein with radiographic knee OA and OA-associated synovitis

7.1.1. An association study for ADAM12 gene SNPs (Papers I and II)

The distribution of genotypes and minor allele frequencies (MAF) of the investigated SNPs in the *ADAM12* gene are shown in Table 6. All genotypes in our study groups were found to be in HWE. The MAF of all genotyped SNPs were similar to those reported by other studies of subjects of European descent (HapMap Project; Valdes et al., 2006). The degree of linkage disequilibrium (LD) within the *ADAM12* gene was found to be predominantly weak (D`0.01−0.53); a strong LD (D'>0.8) was found only between rs1044122 and rs1278279, showing that these two polymorphisms belonged to one haploblock (see Paper II, Figure 1).

ADAM12 and early radiographic KOA (grade 0 versus grade 1)

The T allele of rs1044122 carried the increased risk of early rKOA in TFJ in the women's group (OR 1.63, cor.p=0.038; Table 7), whereas no such association was observed for men or for the group as a whole. Of the KOA radiographic features, rs1044122 was associated only with the presence of OPH in TFJ (OR 1.57, cor.p=0.038; Table 7).

ADAM12 and advanced radiographic KOA (grade 0 versus grade ≥2)

Differently from early rKOA, the genetic impact of the *ADAM12* gene on more advanced (grade≥2) rKOA development was noticed only in male subjects. In our study group, the C allele of rs1871054 carried the highest risk for the appearance of advanced rKOA features (Table 8), mostly related to radiological changes in TFJ (OR 3.82, cor.p=0.023; Table 8). Again, of the rKOA features, the genetic risk was associated with osteophytosis in TFJ (OR 3.03, cor.p=0.038; Table 8).

The study power for investigated SNPs varied from 88−91% at minimum OR 1.3. For sr3740199 and rs1278279, no statistically significant associations with rKOA were found.

Taken together, in our group two *ADAM12* gene SNPs demonstrates associations with rKOA, whereas the most significant relations were observed for osteophytosis.

Association of *ADAM12* gene haplotypes with radiographic KOA

Haplotype analysis revealed that two haplotypes of *ADAM12* gene were associated with increased risk of rKOA (see Paper II, Table 4). The most significant association was observed for haplotype CCAT (rs3740199, rs1871054, rs1278279 and rs1044122) in male subjects, which was found to be related to increased risk of OA in TFJ (p=0.014). Of the two main rKOA features, risk was predominantly associated with the occurrence of OPH (p=0.014).

Table 6. The distribution of minor alleles and genotypes of investigated SNPs in *ADAM12* and *CILP* genes.

***ADAM12* gene**				
rs3740199		All	Men	Women
MAF	C allele	35%	37%	33%
Genotypes	GG	189	56	133
	GC	194	58	136
	CC	54	21	33
rs1871054				
MAF	C allele	50%	47%	50%
Genotypes	CC	102	30	72
	CT	228	68	160
	TT	107	37	70
rs1278279				
MAF	A allele	16%	17%	15%
Genotypes	GG	310	92	218
	AG	116	40	76
	AA	11	3	8
rs1044122				
MAF	C allele	36%	36%	35%
Genotypes	TT	181	56	125
	CT	200	60	140
	CC	56	19	37
***CILP* gene**				
rs1561888				
MAF	G allel	45%	48%	43%
Genotypes	AA	123	31	92
	AG	238	78	160
	GG	76	26	50

MAF – the frequency of minor allele

Table 7. The associations of rs1044122 SNP in the *ADAM12* gene with early radiographic knee osteoarthritis (OA).

Rs1044122		Grade 0	Grade 1	OR (95%CI)	p-value	corrected p-value**
	OA in TFJ					
All	T allele	61%	68%	1.37 (1.01−1.87)	0.042	NS
	TT	82 (39%)	79 (43%)	2.43 (1.17−5.05)	0.017	**0.036**
	CT	93 (45%)	92 (50%)	3.26 (1.10−4.62)	0.026	NS
	CC	34 (16%)	14 (7%)	1*	1*	
Women	T allele	60%	70%	1.63 (1.12−2.37)	0.018	**0.038**
	TT	53 (37%)	58 (44%)	3.78 (1.5−9.54)	0.005	**0.010**
	CT	66 (46%)	65 (50%)	2.92 (1.18−7.23)	0.021	**0.043**
	CC	25(17%)	8 (6%)	1*	1*	
Men	T allele	65%	63%	0.90 (0.51−1.59)	NS	NS
	TT	30 (45%)	21 (38%)	0.99 (0.28−3.47)	NS	NS
	CT	27 (40%)	28 (51%)	1.38 (0.40−4.78)	NS	NS
	CC	10 (15%)	6 (11%)	1*	1*	
	OPH in TFJ					
All	T allele	62%	68%	1.33 (0.97−1.81)	NS	NS
	TT	95 (40%)	67 (42%)	2.44 (1.22−4.88)	0.021	**0.044**
	CT	107 (45%)	81 (51%)	2.32 (1.17−4.60)	0.021	**0.044**
	CC	38 (16%)	11 (7%)	1*	1*	
Women	T allele	60%	70%	1.57 (1.08−2.29)	0.018	**0.038**
	TT	61 (37%)	50 (45%)	3.81 (1.42−10.23)	0.008	**0.017**
	CT	76 (47%)	56 (50%)	3.09 (1.17−8.16)	0.023	**0.048**
	CC	27 (16%)	6 (5%)	1*	1*	
Men	T allele	65%	63%	0.90 (0.51−1.59)	NS	NS
	TT	34 (45%)	17 (37%)	1.09 (0.30−3.91)	NS	NS
	CT	31 (41%)	24 (52%)	1.70 (0.49−5.99)	NS	NS
	CC	11 (14%)	5 (11%)	1*	1*	

OA in TFJ−radiographic osteoarthritis in tibiofemoral joint; OPH−osteophytes; *CC genotypes were analysed as the base; NS−non-significant.

** For multiple comparison correction, the Benjamini & Yekutieli method was used. Statistically significant p-values are indicated in bold.

Table 8. The associations of rs1871054 in the *ADAM12* gene with advanced (grade≥2) knee osteoarthritis (OA).

Rs1871054		Grade 0	Grade 2-3	OR (95%CI)	p-value	corrected p-value**
	OA in TFJ					
All	C allele	50%	55%	1.37 (0.80−2.36)	NS	NS
	CC	47 (22%)	14 (36%)	1.87 (0.66−5.32)	NS	NS
	CT	119 (57%)	15 (38%)	0.66 (0.25−1.70)	NS	NS
	TT	45 (21%)	10 (26%)	1*	1*	
Men	C allele	45%	73%	3.82 (1.36−10.73)	0.011	**0.023**
	CC	12 (18%)	8 (62%)	10.11 (1.29−79.45)	0.028	NS
	CT	35 (53%)	3 (23%)	1.26 (0.17− 9.54)	NS	NS
	TT	19 (29%)	2 (15%)	1*	1*	
Women	C allele	53%	46%	0.84 (0.43−1.65)	NS	NS
	CC	35 (24%)	6 (23%)	0.49 (0.15−1.56)	NS	NS
	CT	84 (58%)	12 (46%)	0.70 (0.18−2.77)	NS	NS
	TT	25 (17%)	8 (31%)	1*	1*	
	OPH in TFJ					
All	C allele	51%	55%	1.30 (0.76−2.21)	NS	NS
	CC	56 (23%)	14 (37%)	1.64 (0.60−4.49)	NS	NS
	CT	134 (56%)	14 (37%)	0.59 (0.23−1.51)	NS	NS
	TT	50 (21%)	10 (26%)	1	1	
Men	C allele	47%	73 %	3.03 (1.11−7.53)	0.018	**0.038**
	CC	16 (21%)	8 (62%)	6.63 (1.15−38.17)	0.034	NS
	CT	39 (51%)	3(23%)	1.66 (0.30−9.40)	NS	NS
	TT	21 (28%)	2 (15%)	1*	1*	
Women	C allele	53%	46%	0.84 (0.43−1.64)	NS	NS
	CC	40 (24%)	6 (24%)	0.72 (0.19−2.68)	NS	NS
	CT	95 (58%)	11 (44%)	0.46 (0.15−1.43)	NS	NS
	TT	29 (18%)	8 (32%)	1*	1*	

TFJ − tibiofemoral joint; OPH−osteophytes; * TT genotypes were analysed as the base; NS − non-significant.
** For multiple comparison correction, the Benjamini & Yekutieli method was used. Statistically significant p-values are indicated in bold.

7.1.2. The association of ADAM12-S protein with radiographic knee OA and OA biomarkers (Paper III)

The ADAM12-S protein in the study group and correlation with rKOA

The ADAM12-S protein was found in detectable values in 43 subjects (15.6% of the total) in the range of 0.03–14.66 μg/l, with no statistical difference between the genders (p=0.41, WET). In another 233 participants, the level of ADAM12-S was below detection limits (Table 9, Figure 6).The level of ADAM12-S protein did not correlate with age or BMI in the investigated subjects.

Table 9. The characteristics of observed ADAM12-S protein values in the study group.

	Range	Median	Mean
ADAM12-S,μg/l			
All	0−14.66	0.0	0.448
Men	0−13.70	0.0	0.297
Women	0−14.66	0.0	0.543

The level of ADAM12-S protein was significantly higher in the sera of patients with advanced grades (grades 2 and 3) of OPH (W=3427.5, p=0.0008, WET), as well as the late stage of rKOA in TFJ (W =3060.5, p=0.0015, WET) or PFJ (W=3476.5, p=0.0004, WET), when compared with the subjects without rKOA traits (grade 0).

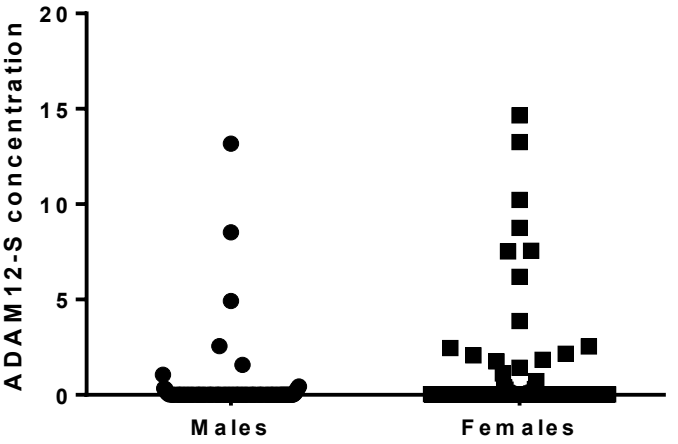

Figure 6. The distribution of ADAM12-S protein values in the study group.

The correlation between ADAM12-S protein and other OA biomarkers.

No correlation was observed between the u-CTX-II levels and the ADAM12-S values in the whole group (see Paper III, Table 2). However, there was a trend toward a positive correlation between ADAM12-S protein and u-CTX-II in female subjects (ρ=0.213, p=0.019, SRC). No statistically significant correlations between levels were observed for s-CTX-I or s-PINP and ADAM12-S protein.

Briefly, the ADAM12-S protein in our study was found in significantly higher concentrations in subjects with advanced rKOA than in participants without rKOA features, which may suggest up-regulation of ADAM12 production in advanced diseases.

7.1.3. The expression of ADAM12 mRNA in synovial tissue and its association with knee OA and histological synovitis (Papers IV and V)

The expression level of *ADAM12* mRNA in synovial tissue

All three isoforms of *ADAM12* were expressed in synovial tissue. Of the two *ADAM12* isoforms, synovial expression was much higher for *ADAM12-L* (see Paper IV, Figure 1). The proportion of secretable *ADAM12-S* represents only 4% of the overall expression of *ADAM12* in the synovium. The *ADAM12* mRNA expression was not related to the ages or BMIs of subjects and was similar in men and women. There was no difference in *ADAM12* mRNA expression in samples of macroscopically pathological and non-damaged synovial membrane collected from the same person.

Association of *ADAM12* mRNA expression with histological synovitis and rKOA

In our study group, the histological synovitis features was diagnosed in 85% of subjects. We found a weak but significant correlation between the expression of *ADAM12-B* and the grade of histological synovitis (ρ=0.28, p=0.039, SRC; see Paper IV, Figure 2). This association was significant only in males (ρ=0.49, p=0.02, SRC). From synovitis features the expression level of both splice variant−*ADAM12-L* (ρ=0.30, p=0.028, SRC) and *ADAM12-S* (ρ=0.33, p=0.029, SRC)−weakly but significantly correlated with the grade of fibrosis of the synovia (see Paper IV, Figure 2).

The relative expression values of *ADAM12* mRNA in subjects with and without radiographic features of KOA were similar.

The influence of polymorphisms of *ADAM12* on mRNA expression level

Our data showed that the GG homozygotes (n=36) of rs1278279 in *ADAM12* had lower relative expression levels of *ADAM12-B* in the synovium, compared to subjects with AG genotype (n=8; no AA homozygotes were found in study group) (W=36, p=0.03, WET). This suggests that rs1278279 could have a regulatory impact on *ADAM12* mRNA expression.

7.1.4. The synovial expression of ADAM12 protein (Paper IV)

IHC staining revealed ADAM12 protein expression predominantly in macrophage and fibroblast cells in 80% of the investigated synovial samples (Figure 7). The ADAM12 protein expression was related to the global grade of histological synovitis (R^2=0.11, p=0.024, linear regression) and correlated with the *ADAM12-B* mRNA expression (ρ=0.30, p=0.034, SRC). Of the two splice variants of ADAM12, mRNA correlation with IHC staining was noticed only for the short isoform (ρ=0.38; p=0.014, SRC).

Briefly, the above results demonstrate up-regulation of ADAM12 in histological synovitis, and particularly in synovial fibrosis on the mRNA and protein levels, suggesting its possible involvement in inflammation-associated fibrosis development.

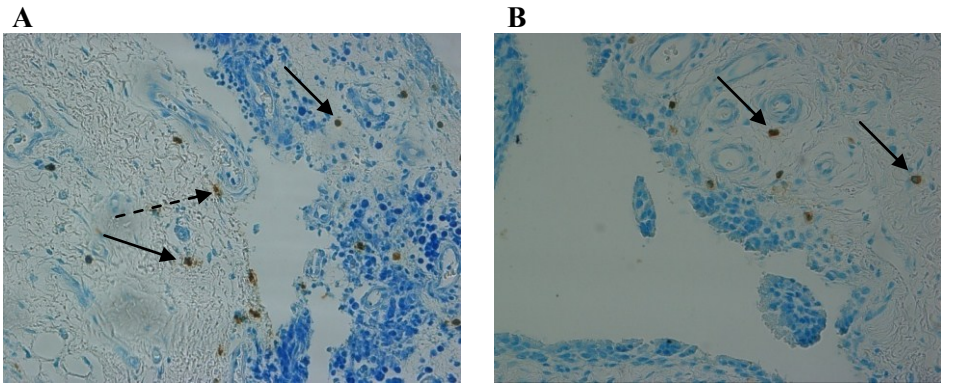

Figure 7. The expression of the ADAM12 protein in investigated synovial samples. ADAM12 was mainly expressed in synovial macrophages (A and B-black arrow) and fibroblasts (A-dashed arrow, magnification 40x).

7.2. Association of *CILP* gene with knee OA and OA-associated synovial membrane inflammation (Paper VI)

7.2.1. An association study for CILP gene SNP

After genotyping by two methods (RFLP and TaqMan® allele discrimination assay), the rs1561888 of the *CILP* gene in our study group was not in HWE (p=0.036), owing to the too large proportion of heterozygotes (Table 6). The regenotyping results of RFLP were similar, with overall discordance from the TaqMan® technique of 0.9 %.

In our material, the G allele of rs1561888 carried the risk of early (grade 1) rKOA in PFJ (OR 1.45, 95%CI 1.08−1.94, p=0.014, cor. p=0.031). Of the two genders, this association was important only in female subjects (OR1.52, 95%CI 1.07−2.17, p=0.020, cor. p=0.045). Of the two main radiographic KOA features, the rs1561888 was associated only with the occurrence of OPH in PFJ (1.41, 95%CI 1.05−1.90, p=0.022, cor. p=0.05). Again, the risk was found in females (1.51, 95%1.06−2.15, p=0.022, cor. p=0.052) and not in males.

7.2.2. The expression of CILP mRNA in synovial tissue and its association with knee OA and histological synovitis

CILP mRNA expression in synovial tissue

In 42 of the 44 samples, the *CILP* mRNA calculated relative expression was in the range 0.01−13.99 (median=0.33) and in two samples it was calculated as zero. *CILP* expression was not related to the ages, BMIs, genders or reported physical activities of the investigated subjects. No difference in *CILP* mRNA expression was noticed in inflamed and macroscopically intact synovial membrane from the same subject.

CILP mRNA expression in rKOA

The expression of *CILP* mRNA in the synovial tissue was significantly lower in patients with advanced stages (grade≥2) of JSN in TFJ, compared to subjects without changes in the joints (W=104, p=0.0006, WET). This difference was significant only in male patients (W=33, p=0.004, WET; Figure 8).

CILP mRNA expression in synovitis

No associations were found between *CILP* mRNA expression and the final grade of histological synovitis. But there was significant correlation between *CILP* mRNA and severity of fibrosis of the synovium (ρ=0.31, p=0.026, SRC).

These data suggest that synovial membrane can be an additional source of *CILP* production in the synovial joint and *CILP* synovial expression seems to be

down-regulated in subjects with advanced cartilage degradation (narrowing of joint space).

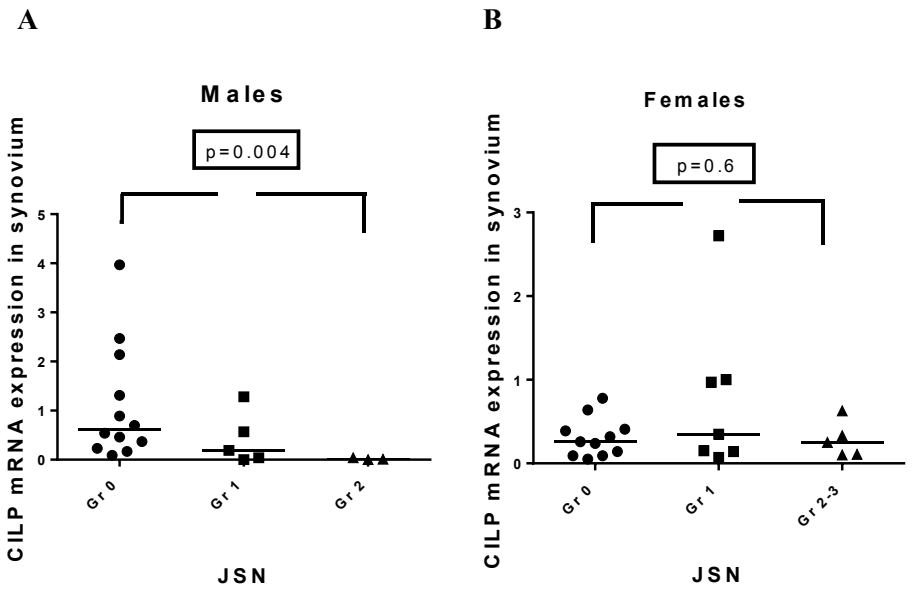

Figure 8. The association of *CILP* mRNA expression in synovia with joint space narrowing (JSN) in the study group. The expression level of *CILP* mRNA was significantly lower in male patients with late stages of radiographic diseases (A). No such differences were observed in females (B).

7.2.3. The synovial expression of CILP1 protein

Immunostaining with CILP1-1 antibody detected protein in 22 of the 42 subjects (52%). The CILP1-1 protein in synovial membrane localized mainly in the media and adventitia layers of blood vessels, but also extracellularly in connective tissue, mainly in macrophage-like and fibroblast-like synoviocytes (Figure 9).

Surprisingly, the *CILP* mRNA expression correlated negatively with CILP1-1 IHC (ρ= –0.33, p=0.018, SRC; Figure 10). Additionally, CILP1-1 protein expression in synovial tissue was in negative correlation with the ages of the investigated subjects (ρ= –0.38, p=0.013). No specific IHC staining was observed for CILP1-2 in synovial tissue (Figure 9).

Taken together, these results support the suggestion that synovia seem to be an additional source of CILP1 production.

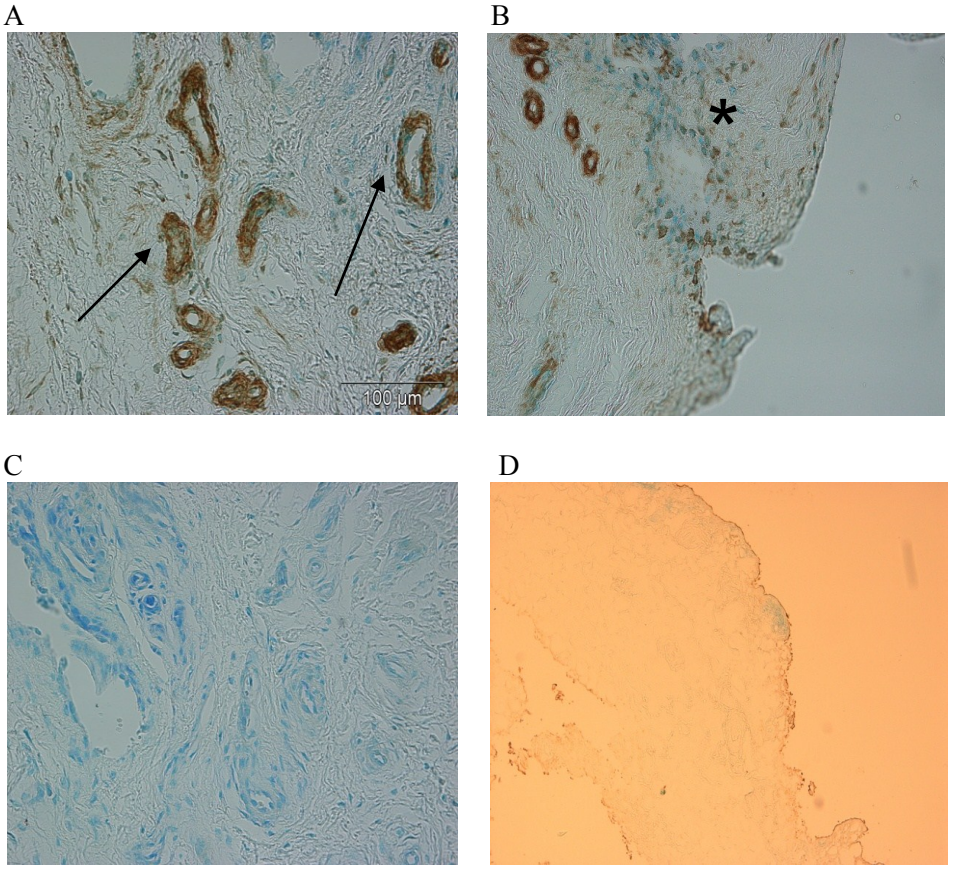

Figure 9. The immunohistochemical (IHC) staining of synovial membrane with CILP1-1/2 antibody. The CILP1-1 protein in synovial membrane localized mainly in adventitia and the media of blood vessels (A, arrows, magnification 40x), and in the extracellular matrix of connective tissue (B, asterisks, magnification 20x). No IHC staining was observed after blocking with CILP1-1 peptide (C, magnification 40x). No specific staining was observed for the CILP1-2 protein (D, magnification 10x).

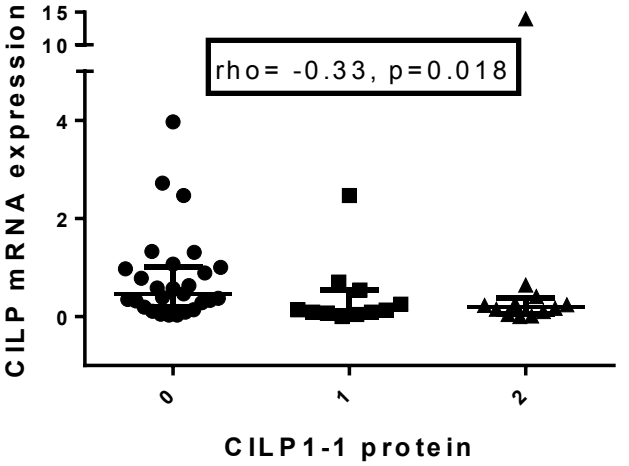

Figure 10. Results of CILP1-1 immunostaining and its correlation with *CILP* mRNA. *CILP* mRNA expression in synovial tissue correlated negatively with CILP1-1 protein synovial expression (Spearman rank correlation). CILP1-1 immunohistochemical staining was measured as follows: no staining (0), mild (1) and moderate staining (2); see methods for details.

8. DISCUSSION

The current research focused on the implication of *ADAM12* and *CILP* genes on KOA development, based on an association study of the SNPs of both genes, an evaluation of the protein product of the *ADAM12* gene, and *ADAM12* and *CILP* mRNA expression assays in synovial tissue of subjects with knee OA. In the current study, we demonstrated that ADAM12 is associated with rKOA processes at different levels (gene, protein and mRNA). We first reported synovial expression of *CILP* mRNA and protein, and revealed the similar behaviour of both investigated genes in synovia, which were involved in inflammation-induced fibrogenesis and had a greater impact on male subjects.

8.1. ADAM12 contribution to OA processes on the gene, protein and mRNA levels

8.1.1. The association of ADAM12 gene SNPs with radiographic knee OA

Genetic background is an important determinant of OA. In particular, the identification of OA-susceptibility genes makes it possible to predict disease phenotypes, construct OA-prediction models and implement novel treatment strategies in the future.

Our study was designed to investigate the possible contribution of selected genetic variants of the *ADAM12* gene to rKOA in a middle-aged population cohort, where early rKOA was expected to be predominant. Moreover, the evaluation of the *ADAM12* genetic contribution to different pathophysiological processes of OA (OPH formation and JSN) was one of the main tasks of our study.

The *ADAM12* gene has been only sparsely investigated in associations with knee and hip OA. *ADAM12* was reported to be associated with an increased risk of the development and progression of KOA in females by the Chingford study (UK), whereas genetic risk has mostly been associated with the appearance of osteophytes (Valdes et al., 2004). Later, an extensive GOAL study (UK) was unable to replicate this association (Limer et al., 2009). However, a study by *Limer et al.* used conflated rKOA scores without separation of different rKOA features.

The results of our study demonstrated that two *ADAM12* SNPs were related to increased risk of rKOA in the Estonian cohort. Notably, a higher risk was observed for early (grade 1) as well for more advanced (grades 2−3) radiographic changes, suggesting continuous involvement of *ADAM12* in OA processes from the early radiographic phase. Furthermore, it is notable that of the two main rKOA features SNPs in *ADAM12* genes carried the highest risk only for the appearance of OPH in both early and late rKOA. Moreover, the

haplotype containing risk alleles of both SNPs also carried the risk for osteophytosis. This indicates the impact of *ADAM12* on OPH formation and supports the finding that in radiographic KOA studies the two main radiographic features (JSN and OPH) should be evaluated separately, and the conflating of those into global OA can cloud the analysed information.

Interestingly, from genotyped *ADAM12* polymorphisms the statistically significant associations with KOA were found for synonymous SNP (sSNP) rs1044122 (c.2475T>C, p.Ala825Ala) and intronic variant rs1871054 (c.1154+145G>A), suggesting the functionality of those variants in the KOA. Moreover, the other sSNP of *ADAM12,* rs1278279 (c.1515G>A, p.Asn505Asn), in our material influences the level of gene expression in synovial tissue, possibly due to changes in secondary structures of mRNA. The sSNP is characterized by substitution of the nucleotide in triplet without amino acid change in protein content. In the past decade many studies reported associations between synonymous SNPs and the risk of common diseases (Wellcome Trust Case Control Consortium, 2007). Indeed, according to recent broad survey, sSNPs are likely to be associated with disease as non-synonymous SNPs and also shared very similar effect size of disease association (Chen et al., 2010). The mechanisms through which sSNPs might be causal include influencing promoter activity and the conformation and stability of pre-mRNAs (Capon et al., 2004), or changing the rate of protein folding (Kimchi-Sarfaty et al., 2007). In the case of intronic SNP, the common molecular mechanisms for alteration of mRNA levels affect transcription, RNA elongation, splicing or maturation (Wang et al., 2011).

Therefore, our data support the opinion that in common diseases like OA, in addition to amino-acid causing variants, other polymorphism like intronic and sSNPs are likely to be involved in the disease mechanisms. These variants should be included in the functional studies and under-investigation of such variants could lead to missing a significant number of disease-associated gene changes.

Previous genetic OA studies suggest that innate sex-specific differences may exist at the molecular level, which may contribute to disease appearance and severity (Loughlin et al., 2004; Rodriguez-Fontenla et al., 2012). In addition, the results of our study demonstrated different contributions of *ADAM12* to rKOA in both genders (with a higher risk of rs1044122 only in women and of rs1871054 only in men). This supports the putative gender-specific impact of the *ADAM12* gene on incident OA, whose molecular basis is currently unclear. Since our study was not designed to determine the probable reason for this phenomenon, we can't explain the observed sex-specific differences. However, our data, along with the results of other similar surveys, indicate that in genetic studies associations for both genders should be evaluated separately.

It is well known that sample size may have an impact on results. The final analysis of genetic associations (Paper II) did not reveal associations for rs3740199 in *ADAM12,* with rKOA being observed in the significantly smaller

group (Paper I). This missense SNP results in substitution of amino acid glycine to arginine at position 48 in ADAM12 protein, which is expected to be relatively neutral for extracellular proteins (Betts et al., 2003). The reason for this discrepancy is probably related to the putative controversial contribution of this variant to KOA in men and women. For example, in our study, we found that a haplotype containing the C allele of rs3740199 carried the risk of OA in men, and a haplotype containing the G allele increased the risk in women. Also, it has been hypothesized that this SNP can act as a modulator of genetic susceptibility only in the presence of other alleles (Valdes et al., 2006).

Moreover, in 2012 an additional variant of this SNP–the non-synonymous change of glycine to tryptophan (g.128019025C>A, p.Gly48Trp)–was described by sequencing and published in the ClinSeq project (Database of Single Nucleotide Polymorphisms). This variant of rs3740199 is not widely studied at present and seems to be very rare in the European population (0.2%). The genotyping of selected SNPs in our study was performed before this rare variant was revealed and the methodology used did not allow us to identify subjects with the A allele. It seems possible that if subjects with this genotype were present in our group they could be misdiagnosed as CC genotype. However, in view of the low frequency of the A allele, it is improbable that the presence of this variant in our study group influenced the results obtained.

In conclusion, our data support the consideration of the *ADAM12* gene as potentially associated with increased risk of KOA during early and late stages of the disease, and it seems to be predominantly related to osteophytosis (bone remodelling and neochondrogenesis).

8.1.2. The role of ADAM12 protein in the development of radiographic knee OA features

In the current study, we evaluated the relation of ADAM12-S, a protein product of an OA candidate gene, to rKOA and several bone and cartilage biomarkers. According to our results, the level of ADAM12-S increased in advanced stages of KOA and this elevation correlated with the severity of the disease. As with genetic associations for *ADAM12* gene SNPs, the elevation of ADAM12-S was related only to one of the two main radiographic features of rKOA: osteophytosis in both tibio- and patellofemoral joints. This finding, along with previous data from genetic studies, suggests the involvement of ADAM12 in pathophysiologic pathways associated with OPH formation.

Basically, osteophyte formation indicates repair attempts and seems to be primarily a process of neochondrogenesis of mesenchymal stem cells present in the periosteum. The main physiological functions of ADAM12 include three categories: catalytic function (mainly the processing of growth factors), cytoskeletal reorganization and cellular signaling; both splice variants of ADAM12−ADAM12-L and ADAM12-S−are active shedases (Jacobsen et al., 2009). Experimental data demonstrate that the expression of the membrane-

anchored form of *ADAM12* (*ADAM12-L*) is up-regulated in chondrocytes and correlated with chondrocyte proliferation (Okada et al., 2008). Moreover, the expression of *ADAM12* mRNA and protein was selectively enhanced by TGF-β, which in turn is a potent inducer of chondrocyte growth (Okada et al., 2008). The notable contribution of ADAM12 to chondrocyte proliferation seems to be linked to its proteolytic activity in relation to IGFBP-5, whereas the latter is a substrate for both ADAM12 isoforms (Loechel et al., 2000). ADAM12-mediated cleavage of IGFBP-5 leads to the enhanced bioavailability of IGF-1 from the IGF-1-IGFBP-5 complex, resulting in chondrocyte proliferation. The results of our study, demonstrating the relation of ADAM12 to osteophytosis on the gene and protein levels, support the importance of ADAM12 in the process of neochondrogenesis and bone remodelling during OA.

Often in OA joints the development of OPH is accompanied by notable degradation of cartilage and can even pre-date this process. *Boegård* and his co-workers reported that the appearance of OPH at both TFJ and PFJ is associated with MRI-detected cartilage defects, whether joint space narrowing is present or not (Boegård et al., 1998b; Boegård et al., 1998c). Hypothetically, owing to its multifunctional domain structure, ADAM12 could contribute to both main OA processes: the development of osteophytes and cartilage degradation. Both ADAM12 isoforms share pro- and catalytic domains linked to proteolytic activity, as well as desintegrin-like, cysteine-rich and EGF-like domains in the body of the protein involved in cell-binding events (Jacobsen et al., 2009). Indeed, several studies have found that ADAM12 may cleave ECM components, including collagen IV, fibronectin and gelatin (Roy et al., 2004). A relevant study did not confirm this catalytic activity of recombinant ADAM12 on ECM substrates (Jacobsen et al., 2009), so the role of ADAM12 as an ECM protease remains unresolved. The trend observed in our study of a correlation between ADAM12-S and the marker of cartilage degradation u-CTX-II seems to support the possible catalytic activity of ADAM12 related to cartilage ECM components and may suggest the putative relation of ADAM12 to cartilage matrix deterioration.

Taking into account the putative relation of ADAM12-S to osteophytosis, our failure to detect a correlation between ADAM12-S and bone biomarkers (s-CTX-I for resorption and s-PINP for formation) may be explained by the biological nature of the selected biomarkers. Both s-PINP and s-CTX-I are markers of the turnover of type I collagen, whose strongest expression was experimentally detected in bone tissue. Although the expression of type I collagen can be found in superficial fibrous layer of osteophytes, the strong expression of type II collagen was demonstrated in all of the cartilaginous and fibrocartilaginous areas, being a hallmark of chondrogenic differentiation in the cartilaginous zone of osteophytes (Aligner et al., 1995). Moreover possible, that in process of osteophyte formation the blood level of both biomarkers will be changed in very small ranges, which is not sufficient for reaching statistical significance.

Taken together, our data suggest that ADAM12 may contribute to both KOA pathogenetic processes: the development of osteophytes (neochondrogenesis and bone remodelling) and cartilage degradation. Of those processes, ADAM12 seems to have a stronger impact on OPH formation, which was observed in the genetic association study and on the protein level.

8.1.3. The role of ADAM12 gene and protein in synovial inflammation

Our results demonstrate clearly that *ADAM12* is expressed in the synovial membrane and that the expression of this gene is up-regulated during synovial inflammation. Notably, this association was found not only at the gene level (as mRNA expression) but also in the presence of a large amount of ADAM12 protein in inflamed synovia. In addition, our results underline the importance of synovial inflammation in the course of OA, which is consistent with the increasing body of knowledge showing that inflammatory synovitis can be an early feature of OA and is not restricted to patients with end-stage disease (Sellam et al., 2010; Attur et al., 2010).

The involvement of ADAM12 in synovial membrane inflammation has not yet been investigated and only one study has reported the relatively low expression of *ADAM12* (13% of synovial tissue biopsies) in OA samples (Komiya et al., 2005). In contrast, we found a much higher expression of both *ADAM12* mRNA (100%) and protein (80%) in the investigated synovial membrane biopsies. This discrepancy may be related to differences in the primers used for the detection of expression rate: only the *ADAM12-L* isoform was measured in Komiya`s study, while both splice variants were measured in our research.

The contribution of ADAM12 to inflammatory responses and tissue remodelling is largely unknown. Animal studies have shown that the long-term over-expression of *ADAM12* is associated with inflammation, but also with accelerated fibrosis and adipogenesis in the skeletal muscle of *mdx*-mice (Jørgensen et al., 2007). Additionally, the TGF-β induced expression of *ADAM12* has been reported to be associated with liver fibrogenesis in cultured human hepatic stellate cells (Le Pabic et al., 2003). The up-regulation of *ADAM12* mRNA on fibrotic samples of the synovium observed in our study also suggests the possible involvement of ADAM12 in fibrosis-associated remodelling of the synovial membrane. Moreover, our findings are in agreement with a recent patent description suggesting the use of ADAM12 as a new regulator for inflammation-induced fibrosis for the prevention or even treatment of fibrosis-related complications (Peduto et al., patent WO/2011/024146). In the light of our study and other results, it seems reasonable to propose the hypothesis that inhibitors of ADAM12 could serve as drug candidates for modulating the course of OA.

TGF-β is a very potent profibrotic cytokine (Gruel et al., 2009) and strongly regulates the synthesis of extracellular matrix components and many proteins involved in their turnover, including MMPs and their inhibitors (Le Pabic et al., 2005). Some studies have indicated that ADAM12 transcription is regulated by endocrine factors, including TGF-β, which has been shown to induce ADAM12 expression via two different pathways (Le Pabic et al., 2003; Le Pabic et al., 2005). In turn, ADAM12 is found to facilitate TGF-β signaling at the membrane via interaction with a type II receptor, modulating TGF-β receptor trafficking and controlling the localization of TGF-β receptors to early endosomes (Atfi et al., 2007). This suggests that ADAM12 may have a function as an important component in TGF-β signaling, and that ADAM12 and TGF-β may form a functional feedback loop in the processes of fibrogenesis.

In our study, an association with synovial fibrosis was observed for both splice variants of ADAM12 (long and short). Therefore, we suggest that both proteins share domains functionally associated with the development of fibrosis. According to published data, it is probable that, in fibrogenesis, the desintegrin domain of ADAM12 is involved. This domain binds integrin, which is an important signal transducer between intra- and extracellular space (Eto et al., 2000; Zhao et al., 2004; Thodetti et al., 2005). Furthermore, the experimental data suggest that surface over-expression of ADAM12 alters the activity of cell surface integrins, leading to cytoskeletal reorganization (Kawaguchi et al., 2003).

In summary, the results of our study enhance the understanding that synovitis is an integral component of knee OA and that the expression of *ADAM12* is associated with synovitis even in the early phases of the disease. Moreover, we propose that ADAM12 may be associated with the development of synovial fibrosis and thereby may impair the normal function of the synovial lining. A better understanding of the biological and molecular mechanisms of synovial inflammation and of the role of ADAM12 in it could potentially lead to the development of new therapeutic approaches, thereby potentially slowing cartilage degradation.

Considering the results of our ADAM12 study, we can state that in an Estonian population ADAM12 was related to incident rKOA, leading to a higher genetic risk, showing an increase in the patient's serum on the protein level, and playing a role in OA-associated synovitis development.

8.2. The implication of CILP in synovial inflammation and its relation to radiographic knee OA

8.2.1. The association of CILP gene polymorphism with radiographic knee OA

The rs1561888, located in the 3-`UTR region of the *CILP* gene, deviated from HWE in our material. Generally, a departure from HWE can be explained by natural selection, population admixture, inbreeding, experimental errors or duplication (Lee et al., 2008). Clearly, genotyping errors are considered the most common reason for HWE imbalance. However, since our results are confirmed by two techniques, with a minimal difference of less than 1%, we believe that the genotyping of rs1561888 is correct and that the deviation from HWE in the investigated cohort is true. Inbreeding and population admixture seem not to be very reliable reasons to explain the HWE imbalance, as the population investigated from south-east Estonian towns is considered an open population that has remained relatively stable for several centuries. The distribution of rs1561888 genotypes in our study group revealed an excessively large proportion of heterozygotes. As is known, in several disorders (e.g. sickle-cell anaemia allele carriers in malaria outbreaks, and cystic fibrosis mutation carriers in cholera) the heterozygotes have a higher relative fitness than either the dominant or recessive homozygote genotype (Gabriel et al., 1994; Bridges, 2002). It is not excluded that this SNP may have importance in several disorders associated with over-dominance.

In the case of KOA, for rs1561888 we observed associations with early disease. Similarly to the risk associated with rs1044122 of *ADAM12* variants, the *CILP* polymorphism carried the higher risk only for OPH formation in women. Previously, the sex-specific effect of *CILP* variants in musculoskeletal disorders (OA and lumbar disc degeneration) has been reported in different ethnic cohorts (Valdes et al., 2006; Min et al., 2010). Our results support the gender-dependent impact of rs1561888 in KOA, whereas its functionality is currently unclear. Regulatory elements within the 3'-UTR region can influence post-transcriptional gene expression, localisation and stability of the mRNA, translation control as well as polyadenylation (Barret et al., 2012; Pichon et al., 2012). A growing number of diseases stand to become linked with such elements and correlation between different disorders (including OA) with polymorphisms within the 3`-UTR has been reported (Egli et al., 2009; Pichon et al., 2012; Friese et al., 2013). Hypothetically, rs1561888 could be this kind of variant and further investigation could help to evaluate the functional importance of this polymorphism.

8.2.2. The synovial membrane as an additional source of CILP production

The current study revealed detectable *CILP* mRNA expression in synovial tissue, supporting the possibility of non-cartilaginous CILP1 production. Notably, this data was supported by immunochistochemical staining, which detected immunoreactivity with the CILP1-1 antibody in more than half of the investigated synovial samples. CILP1 has been detected in the intermediate zone of articular cartilage (Lorenzo et al., 1998a) and is thought to be highly specific only to this tissue. The amount of CILP1 was shown to increase in aged and osteoarthritic cartilage, giving rise to the suggestion of CILP1 as a promising OA biomarker. Recent studies have also discovered its expression in other tissues of synovial joints, including the meniscus (Johnson et al., 2003) and intervertebral disc (Seki et al., 2005). A recent study also localized CILP in non-cartilaginous tissue, particularly in the heart and skeletal muscle (Bernardo et al., 2011). Furthermore, the porcine homologue of human CILP1 was detected in synovial fluid (Masuda et al., 1997b), indicating the possibility that CILP1 could be also produced in synovia. Thus, our results support the suggestion that CILP1 can be considered to be not only a marker of cartilage homeostasis, but also a possible indicator of whole joint metabolism.

In cartilage, CILP1 is produced by chondrocytes (Lorenzo et al., 1998a). Synovial tissue contains various cells, such as the pseudoepitelial lining, and type A macrophage-like and secretory fibroblast-like type B synoviocytes (Revell et al., 1998). In our study, the immunoreactivity with the CILP1-1 antibody in synovia was predominantly observed in the media and adventitia of synovial blood vessels and, in smaller proportions, diffused extracellularly in connective tissue (macrophage-like and fibroblast-like cells). Based on this observation, we suggest that the main candidates responsible for CILP1 production in synovia could be smooth muscle cells, macrophage-like and fibroblast-like synoviocytes. Although it has not been confirmed experimentally, our data suggest that CILP1 in synovial membrane, similarly to cartilage tissue, could be secreted and deposited in the synovial extracellular matrix by synoviocytes.

The observed immunoreactivity with the CILP1-1 protein confirms the deposition of CILP1 in synovial membrane. However, the observed negative correlation of *CILP* mRNA and CILP1-1 protein is a bit confusing. Similarly, the discrepancies in *CILP* mRNA and protein expression in cartilage and in the intervertebral disc have also been reported by other researchers, which has been explained by possible alternative splicing, rapid turnover of protein or repressed translation (Masuda et al., 2001). Moreover, there is a suggestion that the expression of mRNA can capture at most 40% of the variation of protein expression in cultured mammalian cells, emphasizing the importance of post-transcriptional regulatory mechanisms in cellular development (Tian et al., 2004). The negative correlation between *CILP* mRNA and protein observed in our study may have been caused by a regulatory mechanism acting at the post-

transcriptional level (translational repression, alternative splicing, decreased protein stability or reduced translation efficiency, for example through a blocking of the protein translation machinery).

In conclusion, our data suggest that synovial membrane could be an additional source of CILP1 production in synovial joints, although the proportion of CILP1 synovial production is presently unclear.

8.2.3. The relation of synovial expression of CILP gene and protein to synovial fibrosis and radiographic knee OA

CILP in synovial fibrosis

Similarly to *ADAM12*, *CILP* in our study was associated with the severity of synovial fibrosis. The function of CILP in synovial tissue is currently unknown. In cartilage, CILP is thought to modulate the architecture of the cartilage matrix (Tsuruha et al., 2001) and may act as a TGF-β antagonist. *In vitro* studies have demonstrated that in bovine cartilage *CILP* expression is enhanced by TGF-β and inhibited by IGF-1 (Johnson et al., 2003). The TGF-β-dependent induction of *CILP* mRNA has been reported in rabbit nucleus pulposus cells (Seki et al., 2005) and human cell lines (HeLa and HuH-7) (Mori et al., 2006), which seems to be basically mediated by SMAD3, acting directly through cis-elements in the *CILP* promoter region (Mori et al., 2006). Conversely, CILP1 acts as a negative regulator of TGF-β1, and CILP1 protein binds TGF-β1 directly *in vitro* (Seki et al., 2005). This suggests that CILP1 and TGF-β1 may form a functional negative feedback loop (Mori et al., 2006) that could control chondrocyte metabolism and the production of ECM proteins. There are no experimental data regarding the regulation of *CILP* in fibrogenesis. TGF-β1 plays a central role in tissue repair, and excessive TGF-β1 contributes to the development of tissue fibrosis (Branton et al., 1999). Hypothetically, in synovial fibrosis TGF-β1-CILP feedback might act as follows: over-expression of TGF-β1 induce the production of *CILP* mRNA, which in turn acts as a TGF-β1 antagonist, partially modulating TGF-β1 activity and playing a role in tissue remodelling.

The observed *CILP* mRNA over-expression in our study suggests its involvement in the fibrogenesis of the synovial membrane, theoretically antagonizing TGF-β1 activity. Since recent research has also detected an enhanced level of *CILP* mRNA in cardiac post-ischemic fibrosis (Barallobre-Barreiro et al., 2012), the impact of *CILP* in the development of fibrosis does not seem to be exclusively associated with OA changes.

Synovial _CILP_ expression in rKOA

However, an OA process still seems to influence the synovial expression of *CILP*. Our results showing significant down-regulation of *CILP* mRNA in severe cartilage destruction (JSN grade≥2) suggests a possible reduction of

CILP synovial production in advanced disease. In contrast, in cartilage *CILP* expression was significantly up-regulated during ageing and the development of OA, as has been reported by previous studies (Lorenzo et al., 1998a). We are not yet able to explain the different expression pattern of *CILP* in different tissues of the same joint during OA. The regulation of *CILP* has not been widely studied. In addition to TGF-β1, another member of the TGF super-family–bone morphogenetic protein 2–has been shown to induce *CILP* expression (Wang et al., 2012). Our study was not focused on the detection of *CILP* regulatory mechanisms in different tissue, so we can't be sure which mechanisms are involved in the various expressions of *CILP* mRNA in joint tissues. The possible reasons could include a decrease in synovial CILP1 production via a negative feedback mechanism (due to high simultaneous up-regulation in cartilage), changes in the cellular composition of the synovial membrane or different activations of *CILP* regulatory pathways in synovium and cartilage.

Similarly to *ADAM12, CILP* demonstrates a notable gender-dependent contribution in rKOA. In our study group, the down-regulation of *CILP* mRNA expression in subjects with joint space narrowing was observed only in male subjects. Notably, sport and physical activity do not influence *CILP* expression, which could explain the existing difference between genders *a priori.* Moreover, the gender specific impact of *CILP* in cartilage disease, including KOA, which seems to be more prominent in males, has been reported in genetic studies with different ethnic cohorts (Valdes et al., 2006; Min et al., 2009). Our results also suggest that the most drastic changes in *CILP* mRNA expression during KOA occur in males, although this result needs to be replicated with larger cohorts.

In conclusion, our data from the *CILP* study demonstrate that, in addition to chondrocytes, CILP might be produced in synovial joints, probably by smooth cells of blood vessels and synoviocytes, and it plays a role in the fibrogenesis of the synovial membrane. The behaviour of *CILP* in the synovia of OA joints seems to be distinct from that reported in cartilage, and the molecular basis of this variation is currently unclear.

8.3. Limitations of the study

Our study has several limitations. First of all, part of the CILP study failed, as happens quite often in scientific work. Despite the fact that the planned genetic association study of rs1561888 *CILP* SNP generally failed in terms of Hardy-Weinberg disequilibrium, our data may have importance in exploring other disorders (e.g over-domination) potentially associated with this SNP. Our failure in RNA extraction from cartilage biopsy samples is probably related to the small sample size and the extraction techniques used (manual grinding and extraction kits). Indeed, in most similar studies, sample tissue was obtained

during large surgical intervention, such as joint replacement. Joint replacement surgery definitely provides more material with better quality, but for an investigation of the early phase of the disease arthroscopic biopsy was the only available approach. However, our failed attempt could provide additional data for other researchers working in this field, suggesting the preference of automatic sample processing over manual for very small cartilage samples, and the benefit of certain RNA extraction kits.

Secondly, for a genetic study 437 subjects is a relatively small number for detecting weak genetic associations. Indeed, single population-based studies often provide an insufficient number of participants of required ages. However, we had an adequate power for both *ADAM12* SNPs demonstrated associations with rKOA. Moreover, the population-based design was the main advantage in this case. In large genome-wide linkage studies, as well as in smaller case-control studies, it is quite difficult to ensure homogeneity in radiographic/ clinical diagnosis (e.g. heterogeneity in imaging techniques, insufficient radiographic evaluation of controls, and a tendency to selection and recall bias). In general, the Estonian population can be considered to be an open population. It should be noted that selecting a southern Estonia cohort was the strength of the current study, as the population of this region has been stable in terms of residence for two centuries. Thus, we investigated a small but homogeneous cohort and our study provides data for precise evaluation and identical clinical/ diagnostic testing of all recruited participants. Additionally, the recruitment of middle-aged subjects and focus on early rKOA could potentially reduce the effect of confounding co-morbidites observed in older cohorts.

Finally, the main limitation of the ADAM12-S assay study was the small number of subjects with detectable protein values in their sera. In fact, the ADAM12 protein is expressed mainly during the development and differentiation stages in fast-growing tissues, such as the placenta and malignant tumours (Wewer et al., 2005). Furthermore, the research kit used for the detection of the ADAM12-S protein in this study was mainly developed to measure the ADAM12 protein in the sera of pregnant women, in whom the median concentration is higher than 100 µg/l even in the first trimester of pregnancy (Laigaard et al., 2003). However, despite the essential difference in the prospective by the manufacturer and our real study group, we were able to detect ADAM12-S concentrations in more than 15% of the subjects. Obviously, a more sensitive ADAM12 measuring kit with lower detection limits would improve the assay precision of ADAM12-S in OA studies.

9. CONCLUSIONS

1) The present study is the first to demonstrate the association of ADAM12 with the pathophysiological processes of knee KOA at the gene, protein and mRNA levels.
2) The *ADAM12* gene carried the genetic risk of KOA in an Estonian population-based cohort. Two SNPs, belonging to different haploblocks, were associated with the features of radiographic KOA: the T allele of rs1044122 in early osteophytosis (grade 1) in women, and the C allele of rs1871054 in more advanced (grade≥2) osteophytosis in men. This suggests the potential gender-specific impact of *ADAM12* on the onset of the development of osteophytes, determining the early or more advanced disease phenotype.
3) More advanced rKOA was characterized by the increased production of serum ADAM12-S protein, which was found at higher concentrations in the late stages of the disease.
   - This indicates the possibility that the proteolytic activity of ADAM12-S could be involved in the regulation of the growth and proliferation of chondrocytes, and contribute to osteophyte development.
   - The observed correlation between ADAM12-S and the marker of cartilage degradation u-CTX-II may suggest the putative relationship of ADAM12 with cartilage deterioration.
4) The expression of *ADAM12* mRNA and protein in synovia was up-regulated during synovial inflammation, especially in fibrosis, suggesting the implication of ADAM12 in fibrogenesis, most likely facilitating TGF-β signaling by TGF-β receptor.
5) Cartilage intermediate layer protein (CILP1), a marker of cartilage metabolism and a new OA biomarker, was found in synovial tissue at the mRNA and protein levels. Hence, our results demonstrate that synovial membrane may be an additional source of CILP1 production in joints.
   - According to immunohistochemical investigation, the main sources of CILP1 in synovial tissue seem to be smooth muscle cells in the adventitia and media of synovial blood vessels, as well as macrophage-like and fibroblast-like cells.
6) Similarly to *ADAM12*, the synovial expression of *CILP* mRNA was found to be up-regulated in fibrotic synovial samples, suggesting its involvement in the development of post-inflammatory fibrosis.
7) Unlike *ADAM12*, the synovial expression of *CILP* mRNA was down-regulated in the advanced stages of cartilage degradation (JSN), suggesting a decrease in CILP1 production during the advanced stages of knee OA, at least in synovial tissue.

10. SUMMARY IN ESTONIAN

ADAM12 ja CILP geenide roll põlve osteoartiidi arengus

Taust

Osteoartriit ehk osteoartroos (OA) on kõige levinum multifaktoriaalne ja polügeenne liigeshaigus, mis võib haarata kõiki liigeseid. Kõige sagedamini esineb põlve-, puusa- ja käeliigeste OA. Kliiniliselt kõige olulisemaks avaldumisekohaks on põlveliigesed (Peat et al., 2001). Viimasel dekaadil on OA käsitlus ja definitsioon muutunud. Kui varem on käsitletud OA üksnes kõhre degeneratiivse haigusena, siis nüüdseks on selgunud, et OA haarab kõiki liigeskudesid, kaasa arvatud kõhr, subkondraalne luu, sünoviaalmembraan, sidemed ja lihased (Flores et al., 2003). Üha enam on ka andmeid, et vaatamata klassikalise põletiku tunnuste puudumisele kõhres, on põletiku komponent OA kujunemise puhul oluline. Nii kondrotsüüdid kui ka sünoviaalkoe rakud on võimsad põletiku mediaatorite allikad (Attur et al., 2002; Loeser, 2008). Sünoviiti esineb rohkem kui pooltel põlve OA (POA) haigetest ja seda peetakse üheks faktoriks, mis seostub nii haiguse sümptomite ja progressiooniga kui ka võimendab kõhre lammutamist (Sellam et al., 2010).

OA on klassikaline multifaktoriaalne ehk komplekshaigus, mille kujunemisel on oluline roll nii väliskeskkonna faktoritel kui geneetilisel eelsoodumusel (Goldring et al., 2007). Kaksikute uuringute andmetel on POA päritavus naistel vähemalt 40% (Spector et al., 1996). Geneetilised faktorid võivad mõjutada OA kulgu ja raskust, kuid samuti ka teisi OA riskifaktoreid nagu ülekaal, luustiku kuju ja sünoviit (Valdes et al., 2011a). Nagu teiste komplekshaiguste puhul, on POA kulgu mõjustavad geenid väikese efektiga ja etniliste erinevustega, mistõttu nende kindlakstegemine ei ole lihtne (Reynard et al., 2012; Valdes et al., 2011a). POA kandidaatgeenidena on nimetatud kokku üle 80 geeni, sealhulgas mitmeid liigeskõhre metabolismi anaboolselt ja kataboolselt mõjutavaid geene (Chapman et al., 2012). Geneetiliste uuringute eesmärgiks on esiteks, geneetiliste seoste kaudu haiguse patogeneetiliste radade leidmine, mida tulevikus oleks võimalik potentsiaalselt mõjutada medikamentidega. Teiseks, haiguse riski geno- ja haplotüüpide identifitseerimine (van Meurs et al., 2012). POA kandidaatgeenidest väärivad tähelepanu *ADAM12* (desintegriin ja metallopeptidaasi domään 12) ja *CILP* (kõhre keskmise kihi proteiin) geenid (Valdes et al. 2004), mille seost OA-ga on suhteliselt vähe uuritud. Mõlemas geenis on kirjeldatud riskialleele, mis on seotud POA esinemise ja progressiooniga UK kohordis (Valdes et al., 2004; Valdes et al., 2006). Eesti populatsioonis nende kahe geeni seost põlve OA-ga siiani ei ole uuritud. Samas on mõlemal geenil potentsiaali saada tulevase POA ravi sihtmärkideks. Selleks aga on vaja selgitada haiguse patogeneetilisi radasid üksikasjalikumalt, s.t näidata kandidaatgeenide seoste olemasolu nii geeni, vastava valgu kui ka RNA tasemel.

Uurimuse eesmärgid

Käesoleva uuringu eesmärgiks oli uurida *ADAM12* ja *CILP* geenide seotust põlve OA patogeneesi teatud aspektidega DNA, mRNA ning valgu tasemel Eesti populatsioonis.

Spetsiifilised eesmärgid:

1. Hinnata *ADAM12* (rs3740199, rs1871054, rs1278279, rs1044122) ja *CILP* (rs1561888) geenide ühenukleotiidsete polümorfismide (SNP-de) riski POA tekkes Eesti populatsiooni kohordis.
2. Analüüsida veres mõõdetava ADAM12 valgu kontsentratsiooni seoseid POA peamiste radioloogilise tunnustega ja teiste OA biomarkeritega.
3. Uurida *ADAM12* ja *CILP* seoses sünoviidi ning radioloogilise POA-ga, määrates RNA ekspressiooni POA liigese sünoviaalmembraanis ning leides nende näitajate seoseid sünoviidi histoloogiliste tunnustega.
4. Selgitada ADAM12 ja CILP1 valgu võimaliku produktsiooniallikat sünoviaalmembraanis ning analüüsida seost valgu ja mRNA ekspressiooni vahel.

Uuritavate grupid ja metoodika

Uurimus hõlmas kaht erinevat uuritavate gruppi–populatsioonil baseeruv kohort Lõuna-Eestist (N=437, keskmine vanus 45,2 a., 302 naist ja 135 meest) ning artroskoopiliselt ravitud patsientide grupp (N=91, keskmine vanus 47,4 a., 49 naist ja 42 meest). Artroskoopia grupis koguti kõhre ja sünoviaalbiopsia proovitükke RNA eraldamiseks ja histoloogilise sünoviidi hindamiseks 44 haigel (keskmine vanus 46,7 a., 24 naist ja 20 meest). Kõik uuritavad andsid vereproovi DNA eraldamiseks ning vere ja uriiniproovi biomarkerite määramiseks.

DNA eraldati vereproovidest soolamise või fenool-kloroform metoodikaga. SNP-d *ADAM12* geenis (rs3740199, rs1871054, rs1278279, rs1044122) ja *CILP* geenis (rs1561888) määrati TaqMan® SNP genotüpiseerimise meetodiga ja restriktsiooni meetodiga.

ADAM12-S valku määrati seerumist kokku 276 isikul (185 populatsiooni grupis ja 91 artroskoopia grupis), kasutades kommertsiaalset reaktiivide komplekti (kitti) DELFIA/AutoDELFIA® ADAM12 (Perkin Elmer Life and Analytical Sciences). Kõhre ja sünoviaalmembraani biopsiatest eraldati RNA, kasutades Qiageni kommertsiaalseid kitte Rneasy® Mini kit või Rneasy® FibrousTissue. Kõhre proovidest eraldatud RNA kvaliteet osutus edasiseks uuringuteks ebapiisavaks. *ADAM12* ja *CILP* mRNA ekspressioon määrati kvantitatiivse PCR abil kasutades TaqMan® sonde.

Sünoviidi histoloogiliste tunnuste hindamiseks värviti biopsiatükkidest valmistatud koelõigud hematoksüliin-eosiini ja van Gieson meetodil. Sünoovia preparaatides hinnati 6 erinevat parameetrit: (1) epiteelirakkude proliferatsiooni, (2) infiltreeritust lümfotsüütide ja plasmarakkudega, (3) pindmisi fibriini ladestusi, (4) hüpereemiat, (5) fibroosi ja (6) perivaskulaarset turset. Kuue tunnuse skooride summeerimisega saadi histoloogilise sünoviidi aste (0−3).

ADAM12 ja CILP1 valgu ekspressiooni sünoviaalmembraanis hinnati immunohistokeemilise värvimise teel.

Andmete statistilistel töötlemisel kasutati vabavara R. Geeni polümorfismide seoseid POA-ga uuriti logistilise regressiooni mudelite abil, kus lisateguritena võeti arvesse uuritavate vanus, sugu ja kehamassi indeks. Mitmiktestimise korrektsiooniks valiti Benjamini-Yekutieli meetod.

Haplotüüpide geneetilise riski hindamiseks kasutati tarkvara paketti „haplo.stats". Valgu ja mRNA ekspressiooni seost POA tunnustega hinnati mitteparameetriliste meetoditega (Spearmani astakkorrelatsioon, Wilcoxoni test) ja mitmikkorrektsiooniks kasutati permutatsiooni testi või Bonferroni korrektsiooni.

Käesolev uurimus on osa suurest kompleksuuringust, milles osalenud patsientidel oli röntgenülesvõtteid põlveliigestest hinnatud Nagaosa-Doherty süsteemi järgi (Nagaosa et al., 2000), s.t. eraldi osteofüütide esinemist ja liigespilu kitsenemist. Samuti oli määratud luu-kõhrekoe biomarkerid u-CTX-II (II tüüpi kollageeni C-terminaalse telopeptiidi fragmendid uriinis), s-PINP (I tüüpi prokollageeni amino-terminaalne propeptiid seerumis) ning s-CTX-I (I tüüpi kollageeni C-terminaalse telopeptiid), et hinnata nende kudede sünteesi ja lammutamise intensiivsust.

Tulemused

1. Eesti kohordis oli naistel varajase radioloogilise POA risk seotud *ADAM12* geeni SNP-ga rs1044122 (OR 1,63, 95%CI 1,12−2,37; cor. p=0,038) ja meestel hilise POA risk SNP-ga rs1871054 (OR 3,82, 95%CI 1,36−10,73; cor. p=0,023).
2. Radioloogilistest tunnustest määrasid *ADAM12* geeni variandid ainult osteofüütide tekke riski (OR 1,57; p=0,038 rs1044122 (T alleel) ning OR 3,03; p=0,038 rs1871054 (C alleel) jaoks), kusjuures osteofütoosi riskiga oli samuti seotud kahte riskialleeli sisaldav haplotüüp (p=0,014).
3. ADAM12-S valgu kontsentratsioon seerumis oli kõrgem väljendunud radioloogilise POA-ga (aste 2−3) isikutel võrreldes isikutega, kelle puudusid POA radioloogilised tunnused tibiofemoraalses (p=0,0015) ning patellofemoraalses liigestes (p=0,0004). Sarnaselt geeniuuringuga, ADAM12-S valgu väärtused korreleerusid ainult osteofüütide astmega (p=0,003).
4. Esines seos ADAM12-S ja kõhre lammutamise marker u-CTX-II vahel naiste grupis (ρ=0,213; p=0,019).
5. *ADAM12* mRNA ekspresseerus kõikides uuritud sünoviaalbiopsia proovides ja immunohistokeemilise värvimisega kinnitus ka ADAM12 valgu süntees sünoovias.
6. *ADAM12* mRNA ekspressioon sünoviaalses membraanis korreleerus histoloogilise sünoviidi astmega (ρ=0,28; p=0,039).

7. Sünoviiti iseloomustavatest tunnustest esines seos sünoviaalse fibroosi ja *ADAM1*2 mRNA mõlema vormi ekspressiooni vahel (*ADAM12-L* ρ=0,30; p=0,028 ning *ADAM12-S* ρ=0,33; p=0,029 puhul).
8. *CILP* mRNA ja vastav valk ekspresseerusid enamikes uuritud sünoovia proovides.
9. Immuunhistokeemiline uuring: CILP1-1 antikehad värvisid sünoviaalkoe veresoonte adventiitsia ja meedia kihte ning makrofaagide- ja fibroblastide-taolisi sünoviotsüüte. Immunoreaktiivsust CILP1-2 antikehaga sünoviaalses membraanis ei leitud.
10. Kui patsientidel oli liigespilu ilmsesti kitsenenud (röntgenoloogiline aste 2−3), oli *CILP* mRNA sünoviaalne ekspressioon oluliselt madalam võrreldes POA radioloogiliste tunnusteta isikutega (p=0,0006). Antud seos esines ainult meeste grupis (p=0,004).
11. Sarnaselt *ADAM12* geeniga, leiti korrelatsioon *CILP* mRNA sünoviaalne ekspressiooni ning sünoovia fibroosi raskusastmega (ρ=0,31; p=0,026).

Järeldused

1. Käesolev uurimus näitab esmakordselt olulisi seoseid põlve OA ja ADAM12 vahel nii geeni (DNA, mRNA) kui ka valgu tasemel.
2. Kahte erinevasse haplobloki kuuluvad *ADAM12* SNP-d seostusid radioloogilise POA riskiga: rs1044122 seostus algava osteofütoosiga (aste 1) naistel ja rs1871054 väljendunud osteofütoosiga (aste 2−3) meestel. See viitab võimalikule soo-spetsiifilisele *ADAM12* rollile osteofüütide formeerumisel haiguse varajases või kaugelearenenud staadiumis.
3. Kaugelearenenud POA-le (aste 2−3) on iseloomulik sekreteeritud ADAM12-S kõrgenenud kontsentratsioon vereseerumis:
   - mis viitab võimalusele, et ADAM12-S moduleerib kondrotsüütide proliferatsiooni ning soodustab osteofüütide arengut eeldatavasti insuliinisarnase kasvufaktori kättesaadavuse regulatsiooni kaudu.
   - leitud korrelatsioon ADAM12-S valgu ja kõhre lammutamise markeri u-CTX-II vahel lubab oletada, et ADAM12-S katalüütiline aktiivsus võib soodustada ka II tüüpi kollageeni lõhustamist.
4. *ADAM12* mRNA ja valk sünoviaalses koes olid üleekspresseeritud sünoviidiga patsientidel, selgemini oli üleekspressioon väljendunud fibroosi faasis. See muutus viitab *ADAM12* võimalikule osalemisele koe fibroseerumisel, mis võiks toimuda TGF-β signaalraja moduleerimise kaudu.
5. Uut OA biomarkerit CILP1, mida seostatakse kõhrega ja selle metabolismiga, leiti sünoviaalses koes nii mRNA kui ka valgu tasemel. Leid osutab võimalusele, et sünoviaalmembraan võib olla täiendavaks CILP1 produktsiooni allikaks sünoviaalsetes liigestes. Histokeemilise uuringuga täpsustati, et CILP1 võivad toota sünoovia veresoonte silelihasrakud ning makrofaagi- ja fibroblasti-taolised sünoviotsüüdid.

6. Sarnaselt *ADAM12*-ga, leiti *CILP* mRNA üleekspressioon fibrootilistes sünoviaalmembraani proovides. See viitab CILP1 võimalikule osalemisele põletikujärgse fibroosi formeerumisel, arvatavasti TGF-β aktivatsiooni kaudu.
7. Erinevalt *ADAM12*-st oli *CILP* mRNA sünoviaalne ekspressioon meestel oluliselt madalam kõhre kaugelearenenud degradatsioon faasis (patsientide põlve liigespilu ahenemine astmes 2−3), viidates *CILP* sünoviaalse produktsiooni vähenemisele haiguse hilises faasis.

Käesolev uurimus näitab olulisi seoseid põlve OA ja *ADAM12* vahel nii geeni (DNA, mRNA) kui ka valgu tasemel. Tulemused viitavad võimalusele, et *ADAM12* osaleb liigeskudede remodelleerumisprotsessides−osteofüütide tekkes ja sünoovia fibroseerumises. Sarnaselt *ADAM12*-ga, leiti *CILP* seos osteoartriitiliste protsessidega liigeses. Seni on seda valku seostatud liigeskõhre metabolismiga, kuid käesolevas töös näidatakse *CILP* ekspressiooni ka liigese sünoviaalkoes. Uuring demonstreerib mõlema geeni sarnast käitumist OA sünoviaalses membraanis, viitades nende rollile sünoviaalkoe fibroseerumisel.

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