Knee injuries and their consequences – the impact of impact

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Per Swärd

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In this work it was found that subjects with post-traumatic OA secondary to an ACL injury have more joint space narrowing and more osteophytes in the lateral compartment than in the medial compartment, compared with subjects with non-traumatic OA. Furthermore, it was found that an acute knee injury is associated with instant and sustained synovial fluid biochemical alterations within the first month of knee injury, suggestive of increased cartilage turnover and severe joint inflammation. Those subjects who sustained an osteochondral fracture with disrupted cortical bone in association with the soft tissue knee injury had increased joint inflammation. In an in vitro bovine cartilage study, mechanical injury to cartilage increased the matrix metalloproteinase-induced cleavage of cartilage aggrecan. Moreover, findings from this model suggest that the aggrecan degradation may differ between cytokine-stimulated cartilage explants compared with cartilage explants mechanically injured and (or) co-incubated with joint capsule.

Conclusively, the findings in this work underline the fact that the initial impact associated with an ACL appears to be important in terms of the risk of developing post-traumatic OA. In addition, this work emphasizes how the acute biological response to injury could be involved in cartilage degradation. A greater understanding of these processes could lead to the improved management of knee-injured patients and possibly delay, or even prevent, OA development.

Key words: Anterior cruciate ligament, knee injury, post-traumatic osteoarthritis, cartilage, bone, synovium, inflammation, proteases, aggrecanase, matrix metalloproteinase, aggrecan.

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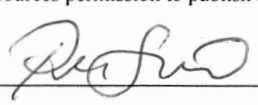
Signature  Date 14/4 - 2014

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Cover illustration: Mouse knee joint. Bone, hyaline cartilage and meniscus visualised by toluidine blue staining (40 x magnification). Published with the kind permission of Dr. André Struglics.

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Contents

Abstract

Anterior cruciate ligament (ACL) injuries are common, severe knee injuries that result in a high risk of developing knee osteoarthritis (OA) in the affected individuals. As proof of high impact forces applied to cartilage and bone at the time of injury, traumatic bone marrow lesions and osteochondral fractures, located predominantly in the lateral tibiofemoral compartment, are commonly associated with an ACL injury. The subsequent risk of OA may be closely associated with the knee injury mechanism and the panorama of injuries in the knee sustained at the onset of injury. The purpose of this work was to acquire a better understanding of how the initial impact, related to the trauma mechanism of acute knee injuries, may influence acute and chronic knee pathology.

In this work it was found that subjects with post-traumatic OA secondary to an ACL injury have more joint space narrowing and more osteophytes in the lateral compartment than in the medial compartment, compared with subjects with non-traumatic OA. Furthermore, it was found that an acute knee injury is associated with instant and sustained synovial fluid biochemical alterations within the first month of knee injury, suggestive of increased cartilage turnover and severe joint inflammation. Those subjects who sustained an osteochondral fracture with disrupted cortical bone in association with the soft tissue knee injury had increased joint inflammation. In an *in vitro* bovine cartilage study, mechanical injury to cartilage increased the matrix metalloproteinase-induced cleavage of cartilage aggrecan. Moreover, findings from this model suggest that the aggrecan degradation may differ between cytokine-stimulated cartilage explants compared with cartilage explants mechanically injured and (or) co-incubated with joint capsule.

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Populärvetenskaplig sammanfattning

Främre korsbandsskador är vanliga, allvarliga och traumatiska knäskador. I kombination med associerade meniskskador och ändrad ledbelastning leder skadan på sikt till att en stor andel individer utvecklar artros i ung ålder. Detta benämns post-traumatisk artros. På kort sikt kan skadan leda till oförmåga att fortsätta sin aktivetet på samma nivå som tidigare. De kraftiga kompressionskrafter över brosk och ben då skenben och lårben kolliderar i skadeögonblicket leder till skador och celldöd i vävnaderna Detta bidrar sannolikt till risken för att utveckla post-traumatisk artros. Hos fler än hälften av de skadade individerna kan man med MR visualisera en fraktur i kortikalt ben på skenben och/eller lårben. Skadorna som uppstår i samband med traumat är framförallt lokaliserade lateralt i knäleden och kan tillsammans med blödning i knäleden initiera ett inflammationssvar. Dessutom initieras en läkningsprocess som åtföljs av en ökad omsättning (nysyntes och nedbrytning) av olika broskproteiner. Det broskprotein som framförallt har undersökts under utförandet av detta avhandlingsarbete är aggrekan. Molekylen aggrekan består av ett protein på vilket det sitter ett stort antal sockerkedjor. Dessa är kraftigt negativt laddade och bidrar till broskets funktion genom att attrahera positiva motjoner, vilka genom diffusion drar till sig vatten. Detta leder till ett svullnadstryck och medför att brosket kan motstå de krafter som uppstår när man belastar knäleden. Under artrosprocessen bryts aggrekanet ned av olika enzym som finns i broskmiljön. Framförallt antas så kallade aggrekanaser och matrix metalloproteaser (MMP) ha stor betydelse. Dessa enzym klyver aggrekanmolekylen på olika ställen och genererar olika långa aggrekanfragment som kan mätas i ledväska och i broskmedium. Ett fragment som bildas efter klyvning av aggrekanaser och som analyseras i både arbete III och V är ARGS-aggrekan. Ett fragment som bildas efter klyvning av MMP är FFGV-aggrecan (arbete V).

Avhandlingen utgår från frågeställningen av vad ett vridvåld i knäleden (knäledsdistorsion) innebär i det akuta skedet och på längre sikt. I första delarbetet gjordes en jämförelse av lokalisationen av artrosförändringar mellan knäleder som har ett definierat trauma (främre korsbandsskada) mot de som inte har detta. Vi fann mer lateralt lokaliserad ledspringesänkning och osteofyter i knäleder där det funnits ett definierat trauma vilket indikerar att det initiala traumat har en viktig roll i den post-traumatiska artrosprocessen. Dessutom kan det vara en viktig

klinisk implikation, då det verkar som att efter en främre korsbandsskada utvecklas artros både medialt och lateralt i knäleden. Artros hos individer utan tidigare trauma är oftast lokaliserad medialt i knäleden.

I andra delarbetet belyses vilken inverkan knäledens ställning har i samband med artrosutveckling efter en korsbandsskada. Vi fann att individer med varusställning (hjulbenthet) hade mer artros i sitt skadade knä jämfört med de med normal eller valgusställning (kobenthet). Även om skillnaderna mellan grupperna var stora, var de inte statistiskt säkerställda. Mer forskning på området för att utreda om varusställning ökar risken att utveckla artros efter en främre korsbandsskada är indicerad.

Arbete III och IV utgår från mätning av ledvätskekoncentrationen av olika brosk-, ben- och inflammationsmarkörer. Vid jämförelse mellan individer med akut knäskada och knäfriska kontroller, visade sig ledvätskekoncentrationer i knäleden av total aggrekan och ARGS-aggrekan vara förhöjda från 1-3 dagar efter skadan upp till 23 dagar efter skadan. Ett annat broskprotein, COMP var förhöjt från 2-3 dagar efter skadan upp till 23 dagar efter skadan. Den nya kunskap som dessa fynd indikerar är att omsättningen av både aggrekan och COMP ökar nästan omedelbart efter skadan. Även flera pro-inflammatoriska proteiner och benassocierade proteiner återfanns i högre koncentrationer i knäleden hos de knäskadade individerna från 0-23 dagar efter knäskadan. I arbete IV undersöktes hur ovan nämnda biomarkörer relaterade till förekomst av MR-visualiserade osteokondrala frakturer där skenben och lårben kolliderade i samband med den främre korsbandsskadan. De inflammatoriska cytokinerna interleukin-8 och tumour necrosis factor-α var förhöjda i ledvätskan hos de individer som ådragit sig en osteokondral fraktur med åtföljande avbrott i det kortikala benet. Sådana frakturer skulle således kunna vara viktiga för det initiala inflammationssvaret på skadan, men även för risken att utveckla artros på lång sikt. Om så är fallet får utvärderas i framtida studier.

I arbete V studerades effekterna på aggrekannedbrytning av ett trubbigt våld mot ungt kalvbrosk i laboratoriemiljö samt effekterna av att odla ungt kalvbrosk tillsammans med ledkapsel. Mekanisk skada av brosket ledde till ökad MMP-aktivitet, medan en antydan till ökad aggrekanasaktivitet sågs i mekaniskt skadat brosk som odlades tillsammans med ledkapsel. I mekaniskt skadat brosk som behandlades med cytokin sågs mycket kraftig aggrekanasaktivitet, men ingen MMP-aktivitet.

Denna avhandling belyser en del processer som skulle kunna ha betydelse för utvecklingen av artros efter en allvarlig knäskada och speciellt efter en främre korsbandsskada. Sammantaget stärker den antagandet att traumat över ben och brosk som sker i samband med att en främre korsbandsskada uppkommer kan ha stor betydelse för risken att utveckla post-traumatisk artros.

Introduction

Preface

Like many diseases, the manifest disease and pathogenesis of osteoarthritis (OA) are multidimensional. The dimensions of OA range from clinical symptoms and macroscopic features of disease through biomechanics to microscopic events at cell, extracellular matrix and molecular level. An increasing amount of knowledge is starting to bridge these dimensions, but there are still many large holes to fill, in order better to understand the disease.

The main advantage of post-traumatic OA from the scientist's perspective is that the time of disease onset is known and the progression of the disease is relatively rapid compared with non-traumatic OA. One of the major drawbacks from the patients' perspective is that they are affected by the disease at a young age.

During my time as a PhD student, I have tried better to understand why and through which processes a severe knee injury can lead to post-traumatic OA. This knowledge is crucial in order to understand the disease and to be able to construct viable treatment options and advice for subjects with severe knee injuries. The main hypothesis underlying the present thesis was that the impact on cartilage, bone and soft tissues of the knee, inflicted at the time of injury, plays an essential role when it comes to the future risk of developing post-traumatic OA. The strength of the present thesis is that, via the papers in the study, it links different clinical parameters and molecular mechanisms that may be important for post-traumatic OA development.

List of studies

I. **Swärd P**, Kostogiannis I, Neuman P, Von Porat A, Boegard T, Roos H. Differences in the radiological characteristics between post-traumatic and non-traumatic knee osteoarthritis. *Scand J Med Sci Sports.* 2010;20(5):731-739.

II. **Swärd P**, Fridén T, Boegard T, Kostogiannis I, Neuman P, Roos H. Association between varus alignment and post-traumatic osteoarthritis after anterior cruciate ligament injury. *Knee Surg Sports Traumatol Arthrosc.* 2013;21(9):2040-2047.

III. **Swärd P**, Frobell R, Englund M, Roos H, Struglics A. Cartilage and bone markers and inflammatory cytokines are increased in synovial fluid in the acute phase of knee injury (hemarthrosis)--a cross-sectional analysis. *Osteoarthritis Cartilage.* 2012;20(11):1302-1308.

IV. **Swärd P**, Struglics A, Englund M, Roos H, Frobell R. Soft tissue knee injury with concomitant osteochondral fracture is associated with higher degree of acute joint inflammation. *Am J Sports Med.* Published online March 24, 2014. DOI: 10.1177/0363546514524924.

V. **Swärd P**, Hansson M, Lohmander SL, Wang Y, Grodzinsky A, Struglics A. Evidence of increased protease activity in mechanically injured cartilage co-cultured with joint capsule. *Manuscript*.

Abbreviations

ACL	Anterior cruciate ligament
ADAMTS	A disintegrin and metalloproteinase with thrombospondin motifs
BMI	Body mass index
BSP	Bone sialoprotein
C2C	Newly formed epitope after cleavage of collagen at the type II collagen primary cleavage site
COMP	Cartilage oligomeric matrix protein
CRP	C-reactive protein
CS	Chondrotin sulphate
DAMP	Damage-associated molecular patterns
ECM	Extracellular matrix
HA	Hyaluronan
KS	Keratan sulphate
MMP	Matrix metalloproteinase
MRI	Magnetic resonance imaging
OA	Osteoarthritis
OCL	Osteocalcin
OPN	Osteopontin
PF	Patellofemoral
sGAG	Sulphated glycosaminoglycan
SPARC	secreted protein acidic and rich in cysteine
TGF-β	Tumour growth factor-β
TIMP	Tissue inhibitor of matrix metalloprotease
TNF-α	Tumour necrosis factor-α
TF	Tibiofemoral

Background

The knee joint

The knee joint is located between the two other joints of the lower limb; the hip and the ankle. The proximal end of the tibia and the distal end of the femur form the medial and lateral tibiofemoral compartments. The patella and the anterior part of the distal femur form the patellofemoral joint. Together, these joints form the knee joint. High demands are imposed on the knee joint and it has several functions which are essential for human beings to walk, run and jump. Primarily, it enables flexion-extension of the lower limb in the sagittal plane. In extension, full or close to full, the knee must be able to withstand the strong forces imposed on the knee by gravity. In flexion, rotation at the knee enables the leg to position the foot before placement. Small movements in the varus/valgus direction are also possible at the knee joint but only when the knee is flexed. Joint stability during movement is attained by the shape of the articular surfaces, the collateral and cruciate ligaments, the menisci and tendons and muscles crossing the knee joint (Figure 1) [1].

The anterior cruciate ligament

The anterior cruciate ligament (ACL) has two functional bundles which connect the femur and the tibia; the anteromedial and the posterolateral bundles, named after their insertion sites on the tibia (Figure 2). These bundles bridge the posteromedial aspect of the lateral condyle and the medial tibial plateau where they insert next to and anterior to the tibial spines. The ACL has an intra-articular location, but it is separated from the synovial fluid by a synovial lining. The main blood supply originates from the femur and specifically from the central geniculate artery [2, 3]. Pacinian corpuscles, Golgi tendon organs and Ruffini endings are mechanoreceptors present in the ACL which contribute to the proprioceptive sense [4]. The extracellular matrix (ECM) of the ACL contains collagen types I, II, III and V, elastin and proteoglycans. The tensile properties of the ACL are mainly related to bundles of collagen type I and cross-linking of these [5, 6]. In the normal

ACL, fibroblasts reside along collagen bundles and are important for normal ligament turnover. Moreover, cells with progenitor potential are present [6]. The function of the ACL is to provide tibiofemoral joint stability in anterior-posterior translation and in internal-external rotation [7]. The ACL also restrains movements in the varus-valgus direction. The anteromedial and posterolateral bundles of the ACL act in synergy to stabilise the knee joint through its entire range of motion. In flexion, the anteriomedial bundle is tauter; in extension, the posterolateral bundle is tauter [3]. The combination of valgus and internal rotation of the tibia has been shown to increase ACL strain more than either motion alone [8].

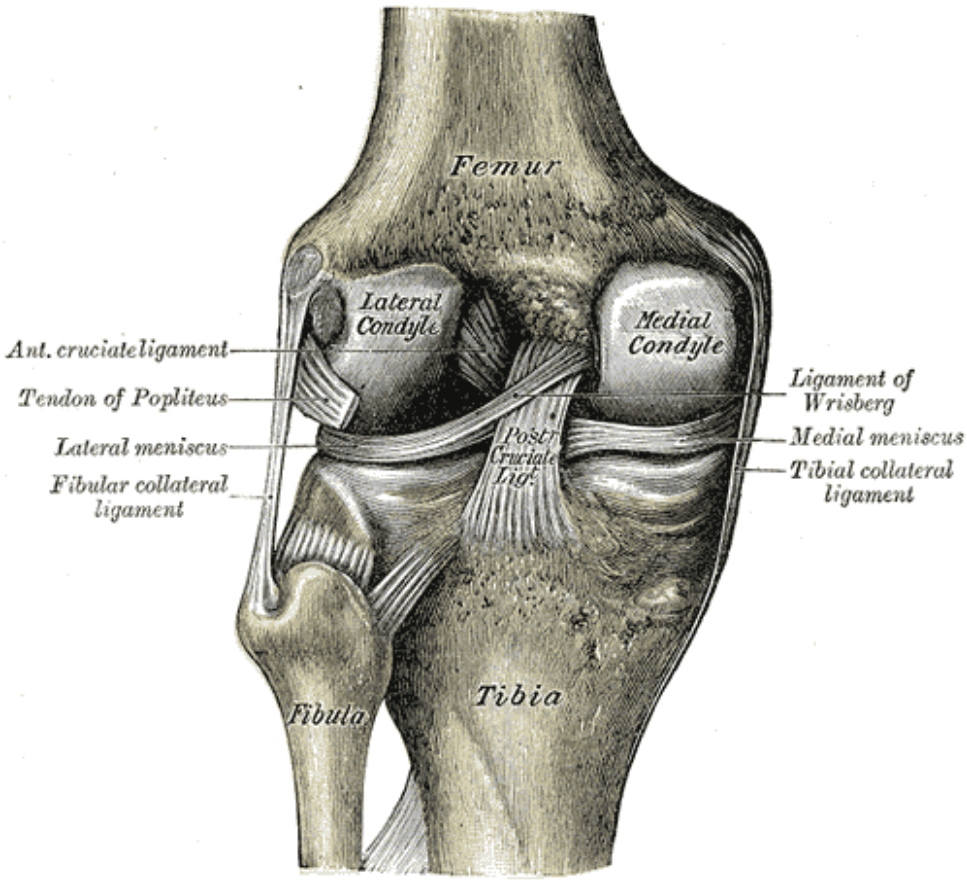

Figure 1. Posterior view of the left knee showing the anterior and posterior cruciate ligaments and the menisci. The image which is from the 20th US edition of Gray's Anatomy of the Human Body was originally published in 1918 and has been transferred into the public domain.

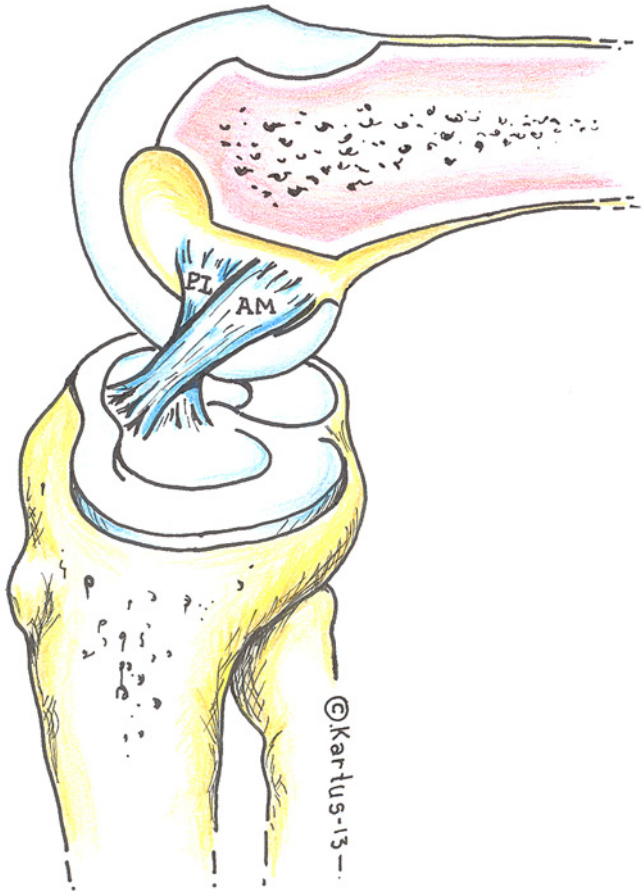

Figure 2. Schematic drawing of the double-bundle ACL anatomy. AM, anteromedial bundle; PL, posterolateral bundle. Published with kind permission. © C. Kartus.

Anterior cruciate ligament tears

In Sweden, football (soccer) is the most common activity associated with ACL injury for both men and women. The second most common activity associated with ACL injury is downhill skiing for women and floorball for men (www.aclregister.nu). The incidence of ACL tears was shown to be 81 per 100,000 subjects aged between 10 and 64 years [9]. Based on these numbers, some 6,000 ACL injuries occur in the Swedish population every year, of which ~3,000 ACLs are surgically reconstructed. The indications for ACL reconstruction in Sweden are symptoms of instability and the failure of conservative treatment (www.aclregister.nu). In the USA, approximately 200,000 ACL reconstructions

are performed every year [10]. Women are known to be more susceptible to ACL rupture compared with men, and are injured at a younger age [11, 12]. Anatomic, neuromuscular and hormonal variations between men and women have been proposed to explain this observed difference [13-16]. From the individual's perspective, the injury may, in the short term, lead to knee dysfunction and an inability to continue sports participation at the same level as pre-injury. In a recent review, it was summarised that, although normal to nearly normal knee function was regained in most ACL-reconstructed individuals, a relatively small number of individuals returned to their pre-injury activity level and competitive sports [17]. Psychological factors, including fear, lifestyle changes and personality, have an impact on why subjects do not return to their pre-injury sports activity after ACL reconstruction [18]. In professional sports, the return to play after ACL injury is much greater and Waldén et al. [12] reported that 94% of elite level football players returned to training within 10 months and that 89% participated in a match within 12 months after ACL reconstruction.

An ACL tear also leads to a high risk of developing OA of the injured knee at a young age [19-22]. ACL re-injury/graft rupture and the high risk of contralateral ACL injury are other important issues that require consideration when advising an individual to return to sport [23]. In a reasonably sized study, in terms of the queries tested, it was reported that 4.5% sustained a graft rupture and 7.5% sustained a contralateral ACL injury during the five-year follow-up after ACL reconstruction. Importantly, 29% of individuals younger than 20 at the time of the first ACL injury sustained an ACL injury to either knee during follow-up [24]. Taken as a whole, the high risk of ACL injury, specifically in young women, and future complications related to the injury are regarded as one of the major problems in sports medicine [25].

Anterior cruciate ligament injury mechanism

In the literature, the ACL injury mechanism is most often described as contact or non-contact. This definition may be misleading, as most ACL injuries that occur in contact sports are associated with a "non-contact" mechanism [26, 27]. A contact ACL injury mechanism typically occurs in American football, when the subject plants his foot and, at the same time, is tackled at the knee from the lateral side, resulting in a valgus collapse of the knee joint. The injury panorama of this injury mechanism often results in lesions of the ACL, medial collateral ligament and medial meniscus, referred to as the O'Donoghue triad [28]. Several mechanisms that may result in a non-contact ACL injury have been proposed. Most typically, the injury occurs as the athlete plants the foot with the knee in slight flexion and

during landing, side-cutting or deceleration. In this position, knee valgus motion, internal rotation of the tibia towards the femur or external rotation of the femur towards the tibia, combined with anterior translation of the tibia, ensue. This leads to high strain in and the rupture of the ACL [26, 29, 30]. This ACL injury mechanism, also known as the pivot shift injury, induces simultaneous lateral tibiofemoral compartment subluxation and combined compressive force, explaining the typical location of bone marrow lesions, on the posterolateral tibial plateau and the midportion of the lateral femoral condyle [2]. The impact site on the lateral femur is related to the degree of knee flexion at the time of injury. With the knee in a high degree of flexion or extension at the time of injury, the lateral tibia impacts the posterior or the anterior part of the lateral femur respectively [2].

Differences in the ACL injury mechanism between the sexes have been described. Investigating basketball players, Krosshaug et al. [31] reported that a valgus collapse in association with the injury was five times more common in women. A lower incidence of meniscal tears associated with the ACL injury has been indicated in women [32], which could be related to differences in injury mechanism between men and women. Furthermore, besides from showing that young age was associated with more traumatic bone marrow lesions on the lateral femoral condyle, it was in a study by Bisson et al. [33] demonstrated that male gender associated with mild traumatic bone marrow lesions on the lateral femoral condyle and tibial plateau. Male gender also associated with moderate and severe traumatic bone marrow lesions on the lateral femoral condyle. These findings may further highlight gender-specific differences in ACL injury mechanism and as a result, factors other than valgus collapse may be of greater importance for compressive injuries to soft tissues and bone associated with ACL injury. Even if they were not investigating bone-bruise patterns, Fridén et al. [27] proposed that the degree of compression between the tibia and femur was related to weight-bearing or non-weight-bearing at the time of injury. A non-weight-bearing ACL injury mechanism, which typically occurs when skiing, was associated with larger numbers of intact menisci, indicating a lower degree of joint compression in this type of injury [27]. In a large-scale investigation of 525 subjects, younger age and not jumping at the time of ACL injury were associated with a bone bruise [34]. Jumping at the time of injury may indicate a non-weight-bearing ACL injury and these findings are thus in line with the hypothesis put forward by Fridén et al. [27].

Knee injury panorama

An acute knee injury with joint effusion and intra-articular bleeding (hemarthrosis) suggests significant intra-articular pathology. Anterior cruciate ligament tears, meniscal lesions and lesions of the medial collateral ligament are common after rotational knee injury [9]. In subjects (n=1,145) with an acute knee injury in whom MRI (magnetic resonance imaging) was performed a median of eight days after the trauma, 52% had sustained an ACL injury, 17% had transient patellar dislocation and 28% a medial collateral ligament tear. Among the ACL-injured subjects, 55% also sustained an associated meniscal tear [35]. These findings are in line with previous investigations of smaller study samples, regarding both the panorama of knee injuries [9] and the high prevalence of meniscal tears concomitant to the ACL tear [36, 37]. The majority of studies and the historical view indicate that lateral meniscal tears are more common than medial tears after ACL injury [36, 38-40]. However, recent studies have described a similar prevalence or even more medial than lateral meniscal tears in association with ACL injury [9, 37]. In a recent MRI-based study, the most common meniscal tear associated with the ACL injury was a longitudinal tear of the posterior horn of the medial meniscus [9]. The authors speculate that these tears may be difficult to detect during routine arthroscopy and could progress and produce symptoms in an unstable knee [9]. Isolated or multiple articular cartilage lesions are also a frequent finding after ACL injury [32, 41]. Posterolateral knee injury, which is relatively uncommon, is an important diagnosis which, if left untreated, can lead to severe knee disability [42, 43].

In almost all individuals, an acute knee injury also leads to a collision between the tibial plateau and the femoral condyle, as visualised using MRI by traumatic bone marrow lesions at the site of impact. These have been described as “fingerprints of injury mechanism”, or “kissing lesions” and have been detected in almost all subjects who have suffered an ACL tear [2, 44, 45]. Typically, the collision occurs in the lateral tibiofemoral compartment between the non-articular posterolateral tibial plateau and the articular midportion of the lateral femoral condyle [44, 46, 47]. Depending on the injury mechanism, other locations of traumatic bone marrow lesions may be present (see above). Traumatic bone marrow lesions in the medial tibiofemoral compartment have been ascribed to the contre-coup mechanism, but they are less common and are mostly associated with bone marrow lesions of the lateral compartment [48]. It should be noted that medial bone marrow lesions are commonly found in subjects with a combined ACL and posterolateral knee injury [42].

At the site of impact between tibia and femur, more than half of ACL-injured subjects also sustain a traumatic osteochondral fracture [39, 44]. These fractures

are prevalent in the lateral tibiofemoral compartment overlying the traumatic bone marrow lesions and present with or without the disruption of cortical bone (Figure 3). They indicate strong impact forces between the tibia and femur at the time of injury and correlate with the size of bone marrow lesions [39, 44] and the likelihood of associated meniscal tears [39]. The traumatic bone marrow lesions represent trabecular fractures, bleeding or oedema. In one study, human biopsy samples of cartilage and subchondral bone overlying MRI-detected bone bruises were obtained at a median of 4.5 weeks after ACL injury. Glycosaminoglycan loss and chondrocyte necrosis were observed in the overlying cartilage. In the subchondral bone, osteocyte necrosis was indicated by empty lacunae [49].

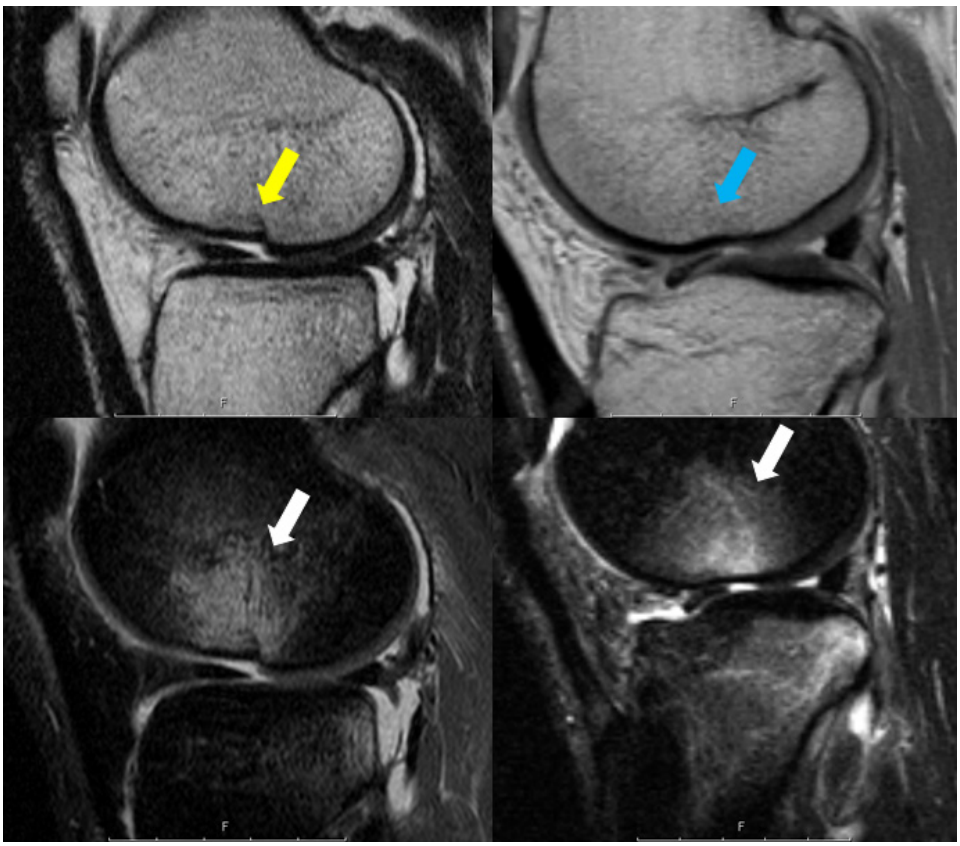

Figure 3. Magnetic resonance images of knees used in **Study IV**. Upper row: proton density T2-weighted sequence and bottom row: short-tau inversion recovery (STIR) sequence of knees with osteochondral fractures with disrupted cortical bone in the femur (yellow arrow) and without disrupted cortical bone in the femur (blue arrow). The STIR sequence images (bottom row) clearly show the surrounding post-traumatic bone marrow lesion of the osteochondral fracture above, indicating traumatic impact forces (white arrows).

Osteoarthritis

Knee osteoarthritis

Knee OA is more common in women and knee OA incidence and prevalence increase with increasing age. Knee OA may be isolated, or may be part of generalised OA, defined as affecting the hands and at least one large joint, or affecting three or more joints [50]. Obesity has been shown to be a highly important and modifiable risk factor for OA development and progression [51]. Adding to the complexity of the disease, the metabolic syndrome and two of its components, central obesity and hypertension, are also associated with the incidence of severe knee OA requiring total knee replacement, independent of body mass index (BMI) [52]. Systemically increased factors derived from visceral adipose tissue have been shown to increase the risk of hand OA and may also be involved in the pathogenesis of knee OA [53]. Today, knee OA is a leading cause of global disability. The ageing population and increasing rates of obesity worldwide forecast an increasing need for health care related to knee OA in the future [54]. Symptomatic knee OA affects almost 7% of the US population 50-84 years of age, of which approximately 50% are obese [55]. Petersson et al. [56] reported that 1.5% of individuals 35-54 years of age, living in a district in the southwest of Sweden, had symptomatic non-traumatic knee OA. The rates of radiographic knee OA are much higher. In the elderly (over the age of 60 to 75), the prevalence of radiographic OA was shown to be between 31-45% [57-59]. There may, furthermore, be ethnic differences regarding knee OA location. For example, in the Chinese, the lateral tibiofemoral compartment is affected more often than in Caucasians [60]. Importantly, OA has a large impact on quality of life and was estimated to reduce the remaining quality-adjusted life expectancy in persons with knee OA by 10-13%, with the higher rates applying to younger individuals with knee OA [55]. As indication of the increasing incidence of symptomatic knee OA, the numbers of total knee replacements performed in the USA more than doubled from 1999-2008. In younger individuals (45- to 64-year-olds), the numbers more than tripled. In addition to increasing obesity rates, this increase may be related to wider indications of surgery and increasing numbers of severe knee injuries in young individuals [61]. An increased understanding of knee

OA risk factors, how best to treat symptoms and find treatments that can prevent or stop OA progression is needed.

Apart from age, gender and obesity, also genetics, knee injuries and abnormal joint loading influence the risk of developing knee OA. Seven genetic variants associated with knee OA or total knee replacement have been identified [62] and 39% of the risk of developing knee OA has been attributed to genetic variation [63]. The possibility that abnormal, or overly high loads can lead to progressive cartilage degradation was indicated by the increased risk of knee OA in occupations where there is frequent heavy lifting, kneeling or squatting [64]. Moreover, malalignment has been shown to increase the risk of OA [65, 66] and elderly male former elite athletes engaged in non-impact sports have an increased knee OA prevalence after adjustment for previous knee injury, age, gender, BMI and occupational load [67].

Post-traumatic knee osteoarthritis

As things stand, some 12% of the total OA burden has been ascribed to post-traumatic OA [68]. It has been proposed that the incidence of post-traumatic OA is increasing in relation to increased numbers of individuals engaged in sports and over the last few decades, the increase in sports participation has been substantial. In the USA, the number of women participating in high-school sports has roughly doubled every decade [46]. Instability is as a major cause of disability after ACL injury [69]. This has influenced the treatment of ACL injuries and ACL reconstruction has been a preferred treatment, with the aim of re-establishing knee stability [70]. However, no differences in the long-term risk of OA development have been shown between ACL-injured subjects treated with or without primary ACL reconstruction in recent systematic literature analyses and from the early results of a randomised controlled trial [19, 21, 71, 72]. Associated injuries may be of greater significance for the long-term prognosis. In a review by Øiestad et al. [21], it was concluded that the prevalence of knee OA after an isolated ACL injury was 0-13%. The prevalence of knee OA after an ACL tear with an associated meniscal tear was 21-48% [21].

Differences in the classifications relating to the radiographic grading of OA between different studies have led to difficulties juxtaposing the current knowledge [21, 71]. In a recent meta-analysis only including studies using the Kellgren & Lawrence classification and with a minimum follow-up time of 10 years, it was shown that non-ACL-reconstructed knees had an increased relative risk of developing any grade of OA. However, the relative risk of progression to moderate or severe OA tended to be higher in ACL-reconstructed knees [71].

Cartilage

The proximal tibia, the distal femur and the patella are covered by a thin layer of hyaline cartilage, forming the articular surfaces of the knee joint. Normal cartilage is avascular, aneural, with no lymphatic vessels and the only cell type found in cartilage, the chondrocyte, obtains nutrition mainly from passive diffusion from synovial capillaries (see below). In normal articular knee cartilage, only 1-3% of the wet weight tissue consists of chondrocytes [51]. Some 70% of the tissue consists of water, whereas 20% of the wet weight is collagen (mainly type II; the fibril-forming collagen of cartilage) and approximately 5% is aggrecan [73]. The main function of cartilage is to distribute the load applied to the underlying bone and to allow movements of low friction at the knee joint [74]. These functional properties are preserved by the chondrocytes which maintain cartilage hemostasis by inducing proteolysis and the production of non-fibrillar collagens, proteoglycans and other non-collagenous molecules, as a reaction to biomechanical and biochemical stimuli [75].

The capacity of cartilage to absorb and distribute high loads at a specific site is related to the composition of cartilage ECM and specifically to the integrity of the fibrillar collagen and aggrecan networks that are present. Aggrecan forms large aggregates by binding to hyluronan and is substituted by negatively charged sulphated glycosaminoglycans (sGAGs) [76]. The high negative charge attracts counter-ions and, by diffusion, water is attracted to the aggrecan molecule. This results in a swelling pressure in cartilage which is retained by the tensile strength of the collagen fibril network and gives cartilage the ability to withstand compressive loads [76]. The fibrillar collagen and aggrecan networks, on the other hand, are dependent on other molecules present in the cartilage ECM, such as cartilage oligomeric matrix protein (COMP), non-fibrillar type IX collagen and members of the small leucin-rich repeat protein family. Important functions of these molecules include the regulation of collagen fibril formation and other collagen networks in the cartilage ECM. They also enable interactions between different ECM molecules and interactions with the chondrocytes [76-78].

Type II collagen has a long half-life (about 100 years) and its degradation is believed to be practically irreversible [79]. Several collagenases are able to cleave fibrillar type II collagen, of which the matrix metalloproteinase (MMP)-13 is believed to be the most important [51, 77]. Cleavage at the primary cleavage site in type II collagen by these collagenases generates two fragments, of which one can be detected by a neo-epitope antibody (against the newly formed epitope; named C2C). Interestingly, increased synovial fluid concentrations of C2C are associated with pre-radiographic cartilage lesions in the knee joint of ACL-injured subjects [80]. Aggrecan has a more rapid turnover. The cleavage of aggrecan can

be mediated by several proteolytic enzymes (Figure 4). The aggrecanases-1 and -2 (ADAMTS-4 and -5; a disintegrin and metalloproteinase with thrombospondin motifs), and, secondly, the MMPs are believed to be most important [81-84]. Another somewhat abundant protein in cartilage is COMP, which is believed to be important for the fibrillation of type II collagen in cartilage and for stabilising the collagen network in the adult cartilage. Its turnover is increased during the early progression of OA [77]. The turnover of COMP is also increased after an acute knee injury, as observed by increased synovial fluid concentrations from within one week of injury up to several years later [85].

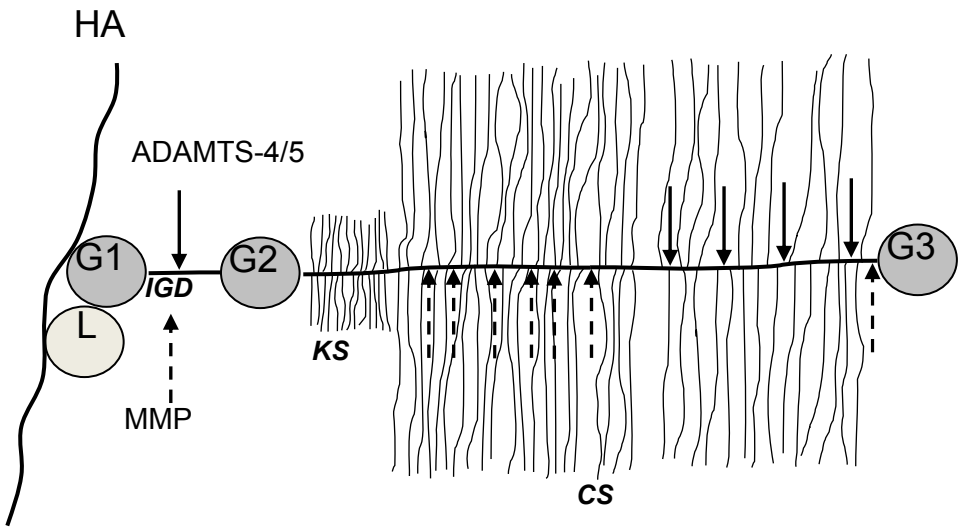

Figure 4. Aggrecan is bound to hyaluronan (HA); an interaction stabilised by the link protein (L). Aggrecan is composed of three globular domains; G1 (N-terminal side), G2 and G3, an inter-globular domain (IGD), one region rich in keratan sulphate (KS) and two regions rich in chondroitin sulphate (CS1 and CS2). Several enzymes have been shown *in vitro* to cleave aggrecan in the IGD domain [81, 83, 84, 86]. Aggrecanase cleavage at the TEGE373↓374ARGS site and MMP cleavage at the IPEN341↓342FFGV site have been demonstrated in humans [81, 83, 84, 87-89]. Suggestive of rapid aggrecan turnover by increased aggrecanase activity, increased concentrations of ARGS-SELE and ARGS-CS1 fragments have been shown in the synovial fluid of subjects with acute knee injury. Moreover, increased aggrecanase cleavage in the aggrecan CS2 domain generates GRGT-G3, GLGS-G3 and AGEG-G3 fragments [88]. Figure reproduced with the kind permission of Dr. André Struglics.

Cartilage can be divided into regions of different ECM organisation and molecular content, as well as chondrocyte organisation, shape and function throughout the depth of cartilage: the superficial (tangential), central (intermediate) and deep (radial) layers of cartilage [90]. The transitional zone between the cartilage and subchondral bone is called the calcified cartilage layer and it creates a barrier and

attachment site between cartilage and subchondral bone [91]. Detected differences in cell appearance and function between the different layers of cartilage include the following. (1) The number of chondrocytes per cartilage volume is higher in the superficial zone compared with deeper layers and the chondrocytes appear flattened and are aligned horizontally parallel to the joint surface [92, 93]. The superficial zone chondrocytes, furthermore, produce lubricin, as opposed to the deeper layers [94]. There is also a high content of collagen fibres running parallel to the cartilage surface, although there may be differences between weight- and non-weight-bearing regions (see below) [7]. (2) In the central layer, the chondrocytes are rounder and the proteoglycan content is higher compared with the superficial or deep zones [92, 95]. (3) In the deep layer, the collagen fibres are thicker and round chondrocytes are aligned in columns [90, 92]. In addition to these depth-related differences, the cartilage ECM also differs depending on the proximity to the chondrocytes. With an increasing distance from the cells, ECM is classified as pericellular, territorial and interterritorial, with apparent differences in ECM organisation and function [77]. Mesenchymal progenitor cells, which could have the potential to regenerate focal cartilage defects, have been found in cartilage [96].

Different cartilage regions in the knee joint, furthermore, show differences in cell and ECM organisation attributed to the different mechanical loads between regions. Rolauffs et al. [93] detected four distinct superficial zone chondrocyte patterns; strings, clusters, pairs and singles in the knee joint. Different joint surfaces of the knee were typically dominated by only one of these four patterns. The predominant pattern of the femoral condyles, meniscus-covered medial tibial plateau and patellofemoral grove were strings, pairs and clusters respectively. Central regions of the tibial plateau are associated with a less organised collagen fibre orientation, which could be a consequence of high compressive loads over this region. On the other hand, peripheral regions of the tibial plateau (i.e. beneath the menisci) are associated with a more organised collagen fibre orientation more parallel to the surface. This could be a consequence of the high tensile stresses to which these regions are exposed [7, 97].

Cartilage and knee osteoarthritis

One of the main features of knee OA is the loss of articular cartilage. Over the years, much of the research on OA has therefore focused on the events involved in cartilage degradation. At cartilage level, OA development is associated with cartilage fissures, swelling, chondrocyte hypertrophy and phenotypic changes in the chondrocytes. As the disease progresses, cartilage thinning and the exposure of subchondral bone occur [51]. Several important clues to the way cartilage is affected during the different stages of OA development and progression have been

identified during the past few decades. Proteases able to degrade cartilage constituents have been highlighted and MMP-13 and ADAMTS-5 in particular [79]. In models using genetically modified mice, deleting the catalytic domain of ADAMTS -5 protects the cartilage from degradation [98]. Furthermore, knock-in-induced resistance to cleavage at the wild type ARGS↓TEGE-aggrecan site significantly protects the cartilage from degradation [99]. Induced MMP-13 deficiency also inhibits cartilage degradation [100]. Underlining the complexity of the post-traumatic OA mouse model, at least 29 different genetic modifications have been shown to be protective of cartilage erosion and 19 have been shown to increase cartilage erosion in different OA models [101]. Human OA is characterised by a whole joint disease and inducing MMP-13 deficiency did not lead to reduced osteophyte formation in a mouse OA model [100]. Many other molecular mechanisms have been implicated in OA pathogenesis and recent studies have, for example, indicated important roles for tumour growth factor (TGF)-β and complement activation in OA pathogenesis [51, 102, 103].

Cartilage and post-traumatic knee osteoarthritis

Acute knee injuries are associated with radiographic OA progression and joint space narrowing indicative of cartilage erosion (see above). One major limitation of using radiographic signs to detect cartilage injury is the relatively long time from injury until these changes occur and the fact that changes indicating cartilage loss, such as joint space narrowing, may be related to other factors, such as the integrity of the menisci [104]. Recent advances in MRI have led to an increase in our understanding of early post-traumatic changes in cartilage which may precede post-traumatic OA development [105, 106]. Compositional cartilage MRI (delayed gadolinium-enhanced imaging of cartilage (dGEMRIC), T1-rho and T2 mapping) can depict information on the composition of cartilage. Whereas dGEMRIC and T1rho may distinguish changes in cartilage proteoglycan content, T2 mapping relates to the cartilage water content and indirectly to type II collagen content and orientation [105]. Before the development of these techniques, cartilage biopsies revealed important information on changes in cartilage composition after ACL injury. Suggestive of collagenase activity, biopsy samples acquired from the non-weight-bearing articular cartilage of the intercondylar notch show collagen denaturation and the cleavage of type II collagen less than and more than one year post-ACL rupture [107, 108]. Interestingly, an increase in cartilage GAG content was also observed [107]. At time points after one year, lower type II collagen content in the cartilage of the intercondylar notch was observed [108]. Biopsy of the cartilage adjacent to bone marrow lesions on the lateral femur showed a decrease in GAG in median 4.5 weeks after ACL injury [49]. In line with these findings, Li et al. [109] showed elevated T1rho values (indicating GAG loss), of

the posterolateral tibial cartilage in ACL-injured knees at baseline. Tiderius et al. [110] showed that the estimated GAG content (assessed by dGEMRIC) was lower in both the medial and lateral femoral cartilage within mean three weeks after ACL injury. At two-year follow-up, recovery but not normalization, was observed in the lateral tibifemoral compartment whereas the estimated GAG decrease in the medial tibiofemoral compartment seemed to remain at a constant low level [111]. Potter et al. [41] demonstrated MRI-detectable cartilage lesions at the time of ACL injury in 100% of patients, primarily affecting the lateral tibial plateau and secondly the lateral femur. A progressive increase in cartilage lesion severity was observed with time lapsed after injury [41].

Synovium

The non-bony cavity of the knee joint is enclosed by the synovium. It consists of the intima, a continuous layer of macrophages and specialised synovial fibroblasts (synoviocytes), and the subintima, which is the underlying tissue. The subintima is a fibrous ECM where blood and lymph vessels, nerves, stationary fibroblasts and immune cells reside. The synovial fibroblasts have a distinct phenotype. Of particular importance to the joint environment, they experience high activity from an enzyme which converts UDP-glucose to UDP-glucoronate, an essential component for hyaluronan synthesis [112]. Furthermore, synovial fibroblasts, along with superficial zone chondrocytes, produce lubricin, which is particularly essential for the boundary lubrication of cartilage [113].

The main route for nutritional access to the avascular cartilage is passive diffusion from capillaries of the synovium. They are located in the subintima, just beneath the intima. The capillary endothelial cells form a size-selective barrier allowing the flux of water, nutrients and proteins into the joint cavity. The synovial fluid-to-plasma ratio of plasma proteins decreases as the molecular radius of the protein increases [114]. Importantly, the cells of the intima are loosely connected and there is no basement membrane. As a result, the interstitial fluid of the synovium and the synovial fluid form an unbroken continuum [115]. The efflux of molecules from the knee joint occurs through drainage via the lymph vessels for molecules ~2 to ~10 nm in size. Smaller molecules, for example, some cytokines, can also access the circulation via diffusion into capillaries. Larger molecules, such as hyaluronan and large aggrecan fragments, may be restricted from efflux via the lymph vessels. At high intra-joint pressures, these large molecules could create a filter cake, increasing the outflow resistance across the synovium and limiting fluid escape from the joint [115, 116]. In the event of synovitis (see below), the

size selectivity of the endothelial barrier is decreased, leading to an increase in the influx of large proteins and joint effusion [117].

The meniscus

The meniscus is a crescent-shaped fibro-cartilaginous tissue rich in collagen type I. The molecular constituents of the meniscus are quite different from those of cartilage. For example, compared with knee-joint hyaline cartilage, the collagen fibrils are thin, the amount of aggrecan and chondroadherin is low, whereas the amount of asporin is high [118]. The tensile strength and function of the menisci are related to the circumferentially oriented collagen fibres woven together with radial fibres [119]. In principle, the menisci play an important role in reducing cartilage loads by distributing the load between the femur and tibia over a larger area. Compromising this function, such as in the case of meniscus tear or meniscectomy, leads to increased peak and average loads over the tibiofemoral cartilage [120] and a high risk of OA [19, 121, 122]. The increased loading of tibial cartilage is closely related to both the amount and type of medial meniscectomy (anterior, posterior or longitudinal) [120].

The long-term outcome of ACL injury is closely related to the integrity of the menisci (see above). However, subjects with an ACL tear (isolated or combined) present with the first radiographic signs (joint space narrowing) of cartilage degradation approximately ten years prior to subjects with an isolated meniscal tear [123]. Not only the status of the menisci at index injury but also the risk of secondary meniscus injury are important for the risk of future tibiofemoral OA after ACL injury [124]. Meniscus injury also appears to be important when it comes to developing patellofemoral OA [125, 126]. The menisci and specifically the medial meniscus may contribute to joint stability and restrain anterior translation in the ACL-deficient knee but not in knees with an intact ACL [127, 128]. This renders the medial meniscus susceptible to tears in the ACL-deficient knee and this has been advocated as an important reason to perform ACL reconstructive surgery [129].

Bone

The bony parts of the knee joint, the femur, tibia and patella, have a dense and compact outer structure, the cortical bone, and a porous inner structure, the trabecular bone. In the knee, trabecular bone and cartilage are separated by the

osteochondral plate; a thin layer of cortical bone (the subchondral plate) and the calcified cartilage layer [130].

Bone is a metabolically active tissue. After the peak bone mass has been reached at the age of 20-30 years, bone remodelling is characterised by a higher bone resorption rate compared with the corresponding bone formation. This results in a net loss of bone mass [131].

Bone and knee osteoarthritis

Bone-related alterations associated with OA include bone marrow lesions, osteophytes, subchondral bone sclerosis, cyst formation, tidemark duplication and thickening of the calcified cartilage layer [130]. Microcracks through the osteochondral plate, ingrowth of nerves, vessels and endothelial proliferation within the non-calcified cartilage are associated with OA and could increase cross-talk between cartilage and bone [132, 133]. These changes, in combination with changes in bone homeostasis, may induce cartilage erosion [103, 133, 134]. An important role for increased TGF-β signalling in the subchondral bone has been suggested [103].

It is unknown whether pathological changes in bone precede or follow osteoarthritic changes at molecular level in cartilage. The parallel progression of disease in both tissues is, however, a likely scenario. This was illustrated by the increase in both serum bone sialoprotein (BSP) and COMP in subjects with chronic knee pain who went on to develop early radiographic knee OA [135]. The serum concentrations of these proteins were, furthermore, higher in subjects with bone scan abnormalities [136]. Even though these studies indicate parallel alterations in cartilage and bone homeostasis during the course of early OA development, abnormal alterations in bone typically precede those in cartilage, as visualised by different imaging techniques. For example, osteophyte formation and scintigraphic changes indicative of increased bone turnover typically occur before joint space narrowing [137]. Changes in bone shape, furthermore, predict the onset of radiographic OA [138]. These differences could, however, be related to the higher metabolic rate in bone compared with that in cartilage and the more rapid response of bone to changes in joint load.

Bone and post-traumatic knee osteoarthritis

In line with OA seen in subjects without previous knee joint trauma, early signs of bone disturbance are also evident after severe knee injury. Bone mineral loss in the knee of ACL-injured subjects was demonstrated after ACL reconstruction [139].

In a study population of 121 subjects followed prospectively after ACL injury, it was recently shown that bone shape changes of femur, tibia and patella could be detected by advanced analyses methods applied to sequential MR images already after two years. Preliminary analyses suggest that these changes could relate to osteophyte formation. [140]. In the same study population, changes in articular bone curvature were observed within three months of the injury [141]. Buckland-Wright et al. [142] demonstrated thickening of horizontal trabeculae and a high prevalence of osteophytes in the medial tibial compartment within four years of ACL injury. Taken as a whole, these findings indicate changes in bone metabolism and remodelling at an early stage after ACL injury.

In subjects with an acute knee injury, cartilage and bone involvement occurs instantly, as the tibia and femur collide, resulting in cartilage injury, bone marrow lesions and osteochondral fractures [41, 44, 49, 143]. Traumatic bone marrow lesions gradually decrease in size, but fewer than half resolve within one year after the injury [144]. In most studies, their presence has not been shown to correlate to clinical outcome [34, 145]. Anterior cruciate ligament-injured subjects with a bone marrow lesion of the medial tibia, a trabecular fracture of the lateral femur or a more severe intra-articular lesion, i.e. osteochondral fracture, may, however, have poorer clinical outcome scores [39, 146, 147]. Johnson et al. [146] observed that a trabecular fracture, by the authors termed a geographical bone bruise [47], on the lateral femur was associated with the increased size and duration of effusion, a time-lag in time until the normal range of motion was regained and increased pain in the acute phase of ACL injury.

Inflammation

Inflammation may play an important role in the pathogenesis of OA [148]. Indicative of this, synovitis, which has been shown to be reflected by increased plasma C-reactive protein (CRP) concentrations, is a prevalent finding in OA [149]. More importantly and underlining the importance of inflammation in OA, synovitis has been shown to be prognostic of MRI detected cartilage loss in subjects without radiographic OA [150]. Increased numbers of several inflammatory cell populations have been detected in the OA joint capsule, of which macrophages, T cells and mast cells are most abundant [151]. Of particular interest, mast cells have been found at higher cell numbers in OA compared to RA joint capsule and their mediators (histamine and tryptase) at higher concentrations in the synovial fluid in subjects with OA compared to those with RA [151, 152]. Inflammatory and synovial cells may contribute to joint synovial and cartilage

inflammation by producing cytokines, prostaglandins and proteases, such as neutrophil elastase, aggrecanases and different MMPs [153-157].

Proteases, which are essential for the degradation of cartilage molecules, can be induced and activated by inflammatory molecules. Numerous *in vitro* studies have demonstrated aggrecanase and MMP activity (including collagenase activity) and injurious effects on cartilage structure and chondrocyte function (survival) in the presence of pro-inflammatory cytokines [158-161]. In fact, adding tumour necrosis factor (TNF)-α to a cartilage explant *in vitro* can lead to the total dissolution of the explant mediated through the upregulation of proteases in the chondrocytes [74]. *In vivo*, in a mouse model, Malfait et al. [162] showed that the intra-articular administration of TNF-α led to the rapid degradation of the cartilage aggrecan by the induction of aggrecanase activity. The aggrecan degradation was reversible and these findings are in line with the work of others [163]. Typically, collagen degradation, which is irreversible in *in vitro* cartilage explant systems, does not begin until after several days of inflammatory stimuli and not until most aggrecan molecules have been degraded [79]. Further emphasising the protective role of intact aggrecan in the *in vitro* cartilage explant system, aggrecanase inhibition not only prevented interleukin (IL)-1-induced aggrecan degradation but also prevented collagen degradation [160]. However, in more complex systems, increased collagen degradation may parallel that of aggrecan. In a horse model, increased synovial fluid concentrations of the C2C epitope were detected within 24 hours of lipopolysaccharide-induced joint inflammation, suggestive of increased collagenase activity [164]. Also suggesting increased protease activity, cross-linked peptides of type II collagen were detected in the synovial fluid early in the acute phase of knee injury [165].

The fact that a "soup" of several pro-inflammatory cytokines or molecules leads to more rapid cartilage degradation was furthermore observed when human and bovine cartilage explants were exposed to both interleukin (IL)-1 and plasminogen compared with when explants were exposed to IL-1 alone [166]. Moreover, the combined effects of TNF-α, IL-6 and sIL-6r on human and bovine knee cartilage explants caused more GAG release than the individual cytokines alone [161].

Inflammation may also mediate pain in OA. In a recent meta-analysis, it was concluded that subjects with OA have modestly higher circulating CRP levels than controls and that there is great variation in the observed associations between different studies [167]. Circulating CRP levels appeared to be associated more with symptoms than radiographic OA changes [167]. Moreover, synovitis is associated with symptoms including pain [168]. The fact that intra-articular corticosteroids supplied to osteoarthritic joints lead to a rapid reduction in joint pain further implicates inflammation as an important mediator of pain in OA [169]. Interestingly, this effect may be related to a reduction in synovial

inflammation [169]. It has, however, not been demonstrated that, by inhibiting knee joint inflammation, corticosteroids are able to reduce cartilage degradation. In a randomised controlled trial, investigating intra-articular hyaluronan treatment and intra-articular hyaluronan + corticosteroid treatment, no significant MRI progression of OA was observed in either study group during the one-year course of the study [170].

Prostaglandins may contribute to sustained joint inflammation and pain. As proof of this, subjects waiting for total knee replacement displayed a dose-responsive decrease in knee synovial fluid concentrations of TNF-α, vascular endothelial growth factor and IL-6 after two weeks of non-steroidal anti-inflammatory drug treatment [171]. Pain and knee function improvements were also observed.

Molecules (intact or cleavage products) of cartilage ECM or yielded from chondrocytes are able to activate toll-like receptors and complement and thereby stimulate inflammation. It has been suggested that the inflammation associated with OA is mediated primarily through this mechanism [74, 102]. In subjects with OA who undergo total knee replacement, the symptoms are reduced after the operation, which could be related to the stopped release of cartilage molecules into the synovial fluid, leading to a reduced degree of synovial inflammation subsequent to the operation [74]. However, subjects with higher synovial fluid concentrations of inflammatory markers at the time of total knee replacement experience fewer improvements in pain outcomes following surgery, underpinning the importance of pathological hemostasis in joint tissues other than cartilage for the clinical symptoms associated with OA (for example, synovitis) [172].

Inflammation and post-traumatic knee OA

The trauma inflicted on joint tissues at the time of an acute knee injury initiates an immune response. This response may be exaggerated by bleeding into the joint and the degree of acute hemarthrosis may be important for both acute and chronic cartilage degradation by activating inflammatory pathways [173]. Typically, acute tissue injury leads to the release of damage-associated molecular patterns (DAMPs). These molecules, intact or proteolytically processed ECM molecules like biglycan, tenacin-C and hyaluronic acid fragments, and cell-associated molecules, such as high-mobility group box 1 (HMGB1) and uric acid are able to activate immune cells by binding to pattern recognition receptors, including toll-like receptors [174-176]. In response to this activation, the immune cells release a variety of different cytokines and chemokines that notify other cells, including macrophages and neutrophils, of ongoing tissue injury [177, 178]. In the acute phase of knee injury, these events can be monitored by analysing the synovial fluid. Rapid increases in synovial fluid concentrations of pro-inflammatory

cytokines and proteases such as elastase and MMP-3 have been observed [153, 179, 180]. At a later stage, T-cells that have been recruited to the site of injury, together with macrophages, may induce a state of chronic inflammation, depending on the present cytokine environment [178]. These courses of events after acute knee injury have not been extensively studied. However, clinical joint effusion and investigations of synovial fluid from acutely knee-injured subjects suggest an initial hyper-inflammatory state [146, 179-181]. Importantly, these events are controlled by anti-inflammatory cytokines and protease inhibitors which may maintain homeostasis during the pro-inflammatory stage and thus reduce the proteolytic activity in cartilage [166]. The fact that both IL-1Ra and the protease inhibitor, tissue inhibitor of matrix metalloprotease (TIMP)-1 are increased in the synovial fluid after acute knee injury indicates that this could be the case [179, 180, 182].

Underpinning the importance of inflammation in post-traumatic OA development, injections of corticosteroids into the joint of ACL-transected dogs during surgery and at different time points after surgery led to reduced osteophyte size and reduced histological severity of cartilage lesions [183]. IL-1ra administered intra-articularly to subjects with recent ACL injury, furthermore, led to a reduction in pain and knee functional limitations, but it is not known whether such treatment can inhibit OA progression after ACL injury [184].

Objectives

The overall objective of this thesis was to acquire a better understanding of how the initial impact, related to the trauma mechanism of acute knee injuries, may influence acute and chronic knee pathology.

Specific objectives

Study I

To investigate differences in the location of radiographic joint space narrowing and osteophytes of the knee in patients with and without a previous knee trauma

Study II

To investigate whether alignment of the contralateral uninjured knee is associated with post-traumatic OA of the injured knee 15 years after an ACL injury

Study III

To investigate differences in synovial fluid concentrations of cartilage and bone markers and pro-inflammatory cytokines between acutely injured knees (within one month after acute knee injury) and knees of healthy reference subjects

To study differences in concentrations with regard to time from injury to aspiration

Study IV

To investigate whether knees, after an acute soft tissue injury with an osteochondral fracture and specifically if also associated with disrupted cortical bone, have higher concentrations of cartilage and bone markers and pro-inflammatory cytokines than acutely injured knees without an osteochondral fracture

Study V

To investigate differences in the proteolytic degradation of aggrecan in bovine cartilage explants exposed to mechanical trauma, co-incubation with joint capsule or exogenous TNF-α treatment

Subjects

To address the questions in the present thesis, subjects from four distinctly different cohorts were included. Three of these cohorts have been used in previous investigations (**Studies I** and **II**) [20, 22, 27, 56, 124, 126]. In **Studies III** and **IV**, a convenience patient cohort was used. This cohort was related to an ongoing randomised controlled trial (RCT) (ISRCTN 84752559, http://www.controlled-trials.com) [185]. Seventy-nine of the 111 patients included in **Studies III** and **IV** were not included in the RCT for various reasons. In **Studies III** and **IV**, synovial fluid samples from ten knee-healthy individuals were used as reference. These samples were acquired and have been used in previous cross-sectional investigations [81, 165, 186, 187]. Details on all subjects used in the thesis can be found in **Studies I-IV** and an overview on the subjects is presented in Figures 5-8.

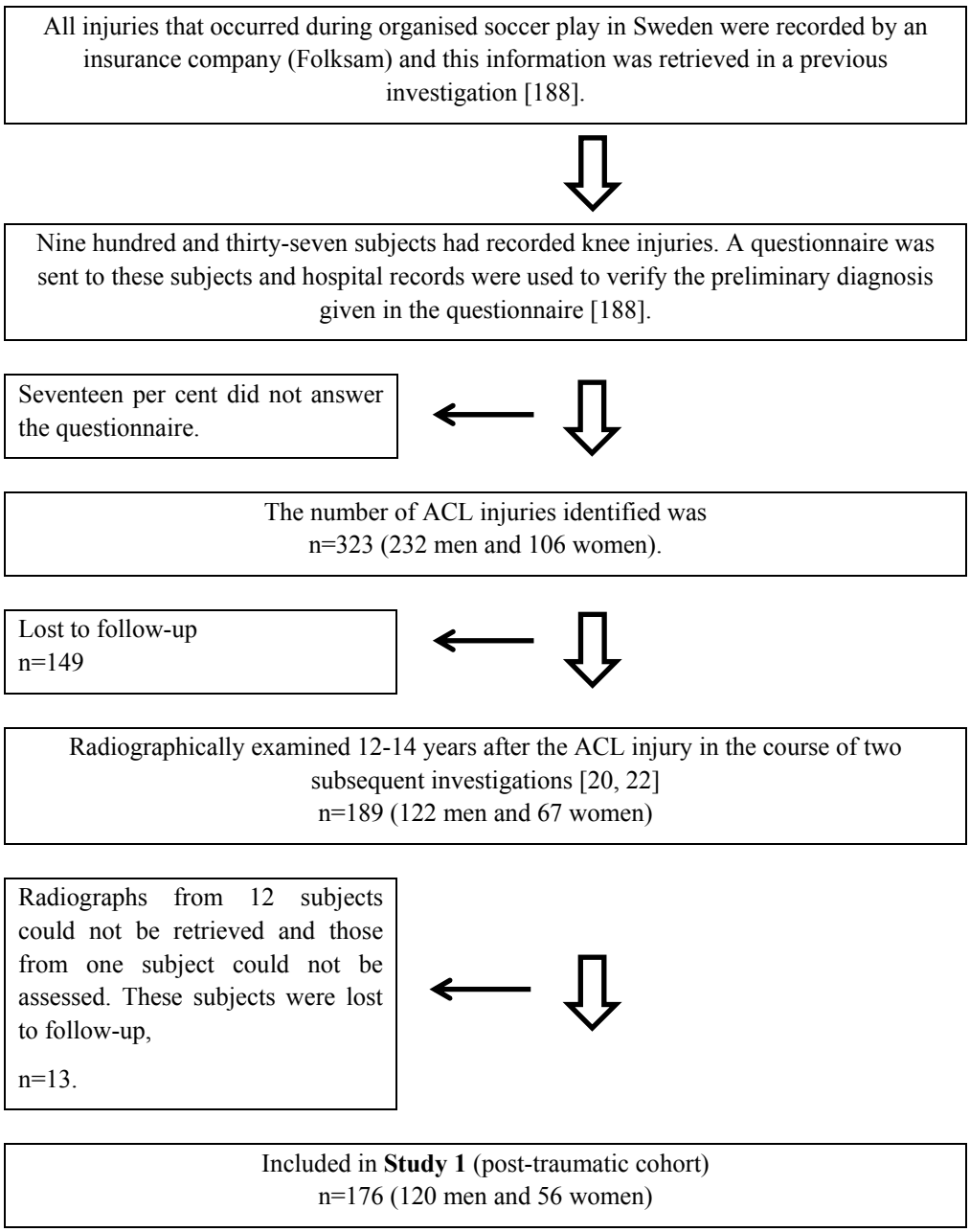

Figure 5. Flowchart detailing the inclusion of subjects and loss to follow-up in the post-traumatic cohort (**Study I**).

In 1990, 5,254 subjects between the ages of 35-54 were identified in a region in the south-west of Sweden using the central population register as part of a previous study. A questionnaire was sent to a randomly selected sample from this population (n=2,000), evenly distributed regarding age and gender [56].

147 subjects did not answer the questionnaire.

Of the 1,853 subjects who answered, chronic knee pain with a duration of > 3 months was reported in: n=279.

Lost to follow-up

n=75

Agreed to undergo clinical, biochemical and radiographic examination in 1990-1991: n=204

Excluded because of any current or former evidence of arthritis or trauma,

n=19

The subjects with chronic "unexplained" knee pain were followed up in 1995-1997,

n=185.

Lost to follow-up

n=30

Radiographic examination of the most painful knee at inclusion (index knee) was performed at follow-up (1995-1997) in those subjects who agreed [56]. These subjects constitute the non-traumatic cohort in **Study I**

n=155.

Figure 6. Flowchart detailing the inclusion of subjects and loss to follow-up in the non-traumatic cohort (**Study I**).

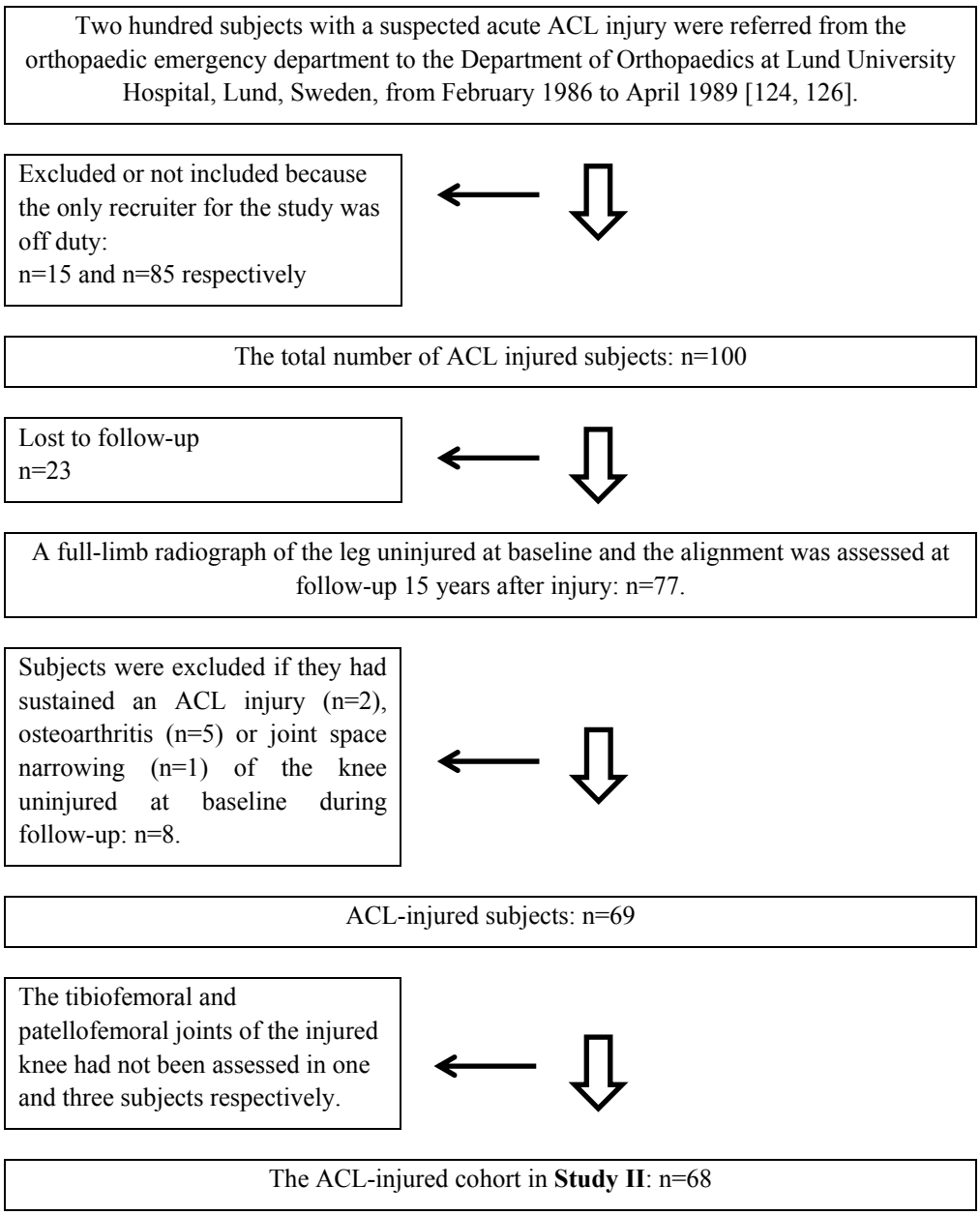

Figure 7. Flowchart detailing the inclusion of subjects and loss to follow-up in the ACL-injured cohort in **Study II**.

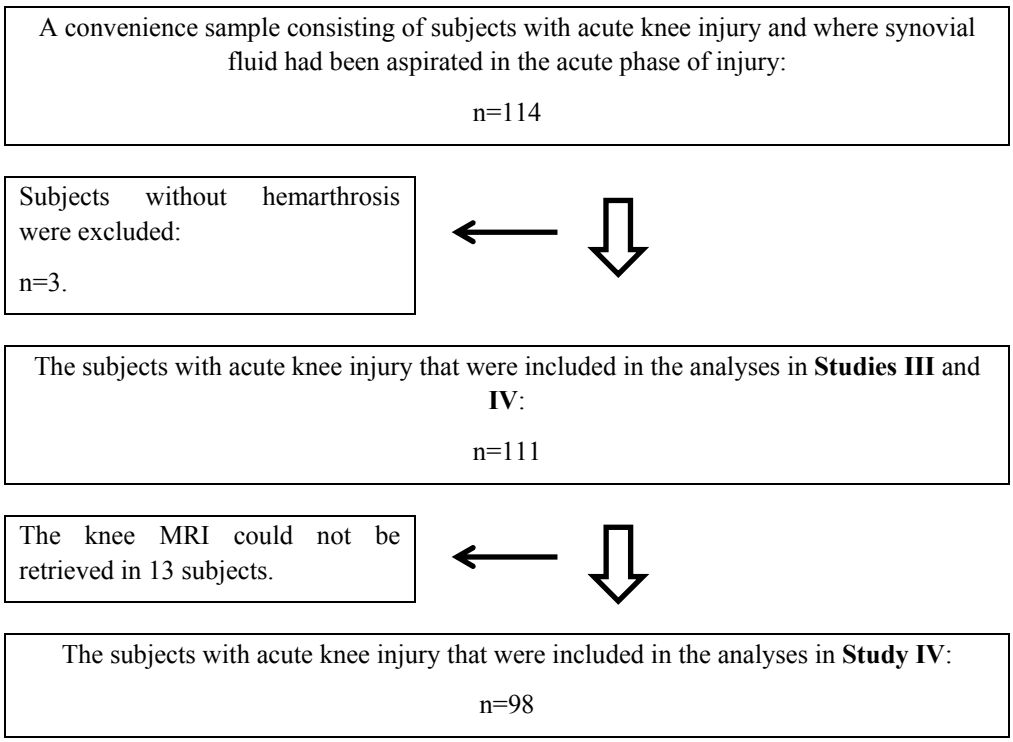

Figure 8. Flowchart detailing the inclusion of subjects in the acute knee-injured cohorts in **Studies III** and **IV**.

Methods

An overview of the main methods used in the papers in the thesis is presented in Table 1.

Table 1. The main methods used in the course of the thesis.

Methods	Study I	Study II	Study III	Study IV	Study V
Radiography of the tibiofemoral joint	X	X			
Radiography of the patellofemoral joint		X			
Full-limb radiography		X			
Knee MRI	X			X	
Alcian blue precipitation			X		
ARGS-aggrecan ECL immunoassay			X		
Human bone panel II ECL immunoassay			X		
Human pro-inflammatory II ECL immunoassay			X		
COMP sandwich ELISA				X	
Western blot					X

ECL: electrochemiluminescence, MRI: magnetic resonance imaging, COMP: cartilage oligomeric matrix protein, ELISA; enzyme-linked immunosorbent assay

Radiography of the knee (Studies I & II)

In **Study I**, the ACL-injured knee of the post-traumatic cohort and the index knee of the non-traumatic cohort was radiographed and classified according to OA features. In **Study II**, the ACL-injured knee and the contralateral knee uninjured at baseline were radiographed to enable classification according to OA features. In the uninjured leg of this cohort, full-limb radiography was used to assess knee

alignment. The different techniques of knee and full-limb radiography are presented in Table 2.

Table 2. Knee and full-limb radiography techniques

	Knee position	Body weight distribution	Fluoroscopic positioning of the X-ray beam
Study I			
Post-traumatic cohort (TF joint)	15° of knee flexion	Equally distributed over both legs	No
Non-traumatic cohort (TF joint)	30–50° of knee flexion. The medial aspect of the foot was parallel to the central X-ray beam.	Almost the whole weight was on the leg of the examined knee.	Yes, the central beam was adjusted to be tangential to the anterior and posterior aspects of the medial tibial plateau.
Study II			
TF joint	20° of knee flexion	Equally distributed over both legs	Yes, fluoroscopic guidance was used to depict an optimal view of the medial compartment
PF joint	50° of knee flexion	Equally distributed over both legs	No
Full-limb	Knee in full extension	Equally distributed over both legs	No

TF: tibiofemoral, PF: patellofemoral

In **Study I**, the tibiofemoral joint of the post-traumatic and non-traumatic cohorts was classified according to the recommendations of the Osteoarthritis Research Society International (OARSI), using the atlas of individual radiographic features in OA, regarding joint space narrowing and osteophytes [189]. The tibiofemoral joint was also classified according to the Kellgren-Lawrence (K-L) scale and radiographic OA was defined as a K-L grade ≥ 2 [190].

In **Study II**, the tibiofemoral joint of the injured and contralateral knee uninjured at baseline and the patellofemoral joint of the injured knee were classified according to the recommendations of the OARSI. Radiographic OA was defined as joint space narrowing ≥ 2 in the medial or lateral tibiofemoral or patellofemoral compartments, a sum of marginal osteophyte grades ≥ 2 in the same tibiofemoral

compartment or grade 1 joint space narrowing combined with a grade 1 osteophyte in the same tibiofemoral compartment. Radiographic OA was also defined as the sum of osteophyte grades ≥ 2 in the patellofemoral joint or grade 1 joint space narrowing, combined with a grade 1 osteophyte in the patellofemoral joint. The definition amended for tibiofemoral OA approximates a Kellgren-Lawrence grade of ≥ 2, widely used as a cut-off for radiographic OA [190].

MRI of the knee (Studies I & IV)

The MR images obtained and used in **Studies I** and **IV** were taken with one of three 1.5T machines (Philips Intera, Eindhoven, the Netherlands; Siemens Impact, Erlangen, Germany; or Siemens Avanto). Proton density T2-weighted sequences (PDT2) and short-tau inversion recovery (STIR) sequences were obtained in the coronal and sagittal planes using a circular polarised surface coil. The MRI sequences were selected for diagnostic purposes in the clinical setting and complement each other well in the classification of both soft tissue and bone defects. The slice thickness was between 3 or 4 mm for the STIR sequence and 3 mm for the PDT2 sequence. In both **Studies I** and **IV,** MR images were assessed by an experienced musculoskeletal radiologist (TB) who classified the images for primary diagnosis (**Studies I** and **IV**), secondary soft tissue injuries, traumatic bone marrow lesions and osteochondral fractures (**Study IV**). Meniscal tears and traumatic bone marrow lesions were classified as present or absent for the entire knee. A trabecular fracture was defined as a line with a low signal parallel to the cortex, visualised on the PDT2 sequences, combined with a surrounding traumatic bone marrow lesion visualised on the STIR sequences. An osteochondral fracture was defined as a trabecular fracture combined with depressed cortical bone with or without disruption of the cortical bone [44].

Alcian blue precipitation (Study III)

In **Study III**, Alcian blue precipitation was used to measure sulphated glycosaminoglycan (sGAG), as described by Björnsson [191]. Positive charges of Alcian blue interact with the negatively charged sGAGs and precipitate when exposed to an acid environment. The amount of sGAG can thereafter be quantified using a spectrophotometer. As aggrecan is by far the most sGAG-substituted proteoglycan in cartilage [192], the sGAG concentration is a valid measurement of the total amount of aggrecan in synovial fluid.

Electrochemiluminescence (Study III)

In **Study III,** we used electrochemiluminescence immunoassays (Meso Scale Discovery, Gaithersburg, MD, USA) to measure ARGS-aggrecan, bone markers and pro-inflammatory cytokines in the synovial fluid of acutely injured knees and the knees of healthy reference subjects. The measurement of ARGS-aggrecan was as previously described [87] and the measurement of bone markers and pro-inflammatory cytokines was made according to the manufacturer's instructions.

ELISA (Study IV)

In **Study IV**, we measured COMP concentrations in the synovial fluid of acutely injured knees and the knees of healthy reference subjects using a sandwich enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (BioVendor R&D, Brno, Czech Republic).

Western blot (Study V)

In **Study V**, we used Western blotting to identify and quantify different aggrecan fragments in medium and left in bovine cartilage explants cultured under different conditions. Samples were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred onto polyvinylidene fluoride (PVDF) membranes. The membranes were incubated with primary and secondary antibodies and the proteins were detected with enhanced chemiluminescence Plus (GE Healthcare) using film (Amersham Hyperfilm enhanced chemiluminescence) or with a luminescence image analyser (Fuji Film LAS-1000) and Multi Gauge v3.2 (Fuji Film) software 18. A set of well-characterised primary antibodies directed at different sites in the aggrecan molecule were used and the specificity of the primary antibodies was confirmed by Western blot-blocking experiments using immunogen peptides.

To be able to quantify aggrecan fragments using Western blotting, G3-, FFGV- and ARGS-aggrecan standards were prepared *in vitro* from purified aggrecan (A1D1). The standards were deglycosylated and five to six standard points were loaded on the gels. The standards and samples were then quantified using the luminescence image analyser (Fuji Film LAS-1000) and Multi Gauge v3.2 (Fuji Film) software. The quantity in μg of each fragment was calculated, within the linear range of the standard curve [89].

Statistical methods

All tests were two-tailed with $p < 0.05$ indicating statistical significance. Because of small study samples in **Study V** (n=3), we presumed that large differences were needed to reveal statistically significant differences between conditions. We therefore considered $p < 0.1$ as a statistical trend. In **Study I,** logistic regression analysis adjusted for gender was used to analyse differences in radiographic features between post- and non-traumatic OA. In **Study II**, odds ratios were calculated to estimate the association between knee OA and knee alignment (varus vs normal/valgus alignment). Because of the small number of subjects in this investigation, multivariate analysis was not performed due to the risk of unstable estimates. In **Studies III** and **IV**, skewed data were log10 transformed before analysis to ascertain normal distribution. Normality distribution was tested using the Shapiro-Wilk test and graphically. Two-group comparisons for non-normally distributed data were performed with the Mann-Whitney test (**Study IV**). In **Study V,** all tests were performed using the paired samples t-test of statistical significance and normal distribution was assumed. When analysing medium samples, these were normalised against the total glycosaminoglycan content in the explant system before statistical testing to account for *de novo* aggrecan production (**Study V**). In **Study IV,** two-group comparisons were made using analysis of covariance (ANCOVA) with adjustment for days between injury and synovial fluid aspiration, gender and age.

Methodological considerations

Subjects

There are several important advantages and shortcomings relating to the cohorts investigated in the course of the present thesis. In **Study I**, both the post-traumatic and non-traumatic cohorts were well characterised and the sample sizes were probably sufficient to detect important differences between the populations. The time to follow-up of the post-traumatic cohort was adequate to allow radiographic OA development (12-14 years). Moreover, to ascertain that the non-traumatic cohort was in fact non-traumatic, 59 randomly selected individuals underwent MRI and only one subject had suffered an ACL tear. However, the fact that a large number of individuals were lost to follow-up, especially in the post-traumatic cohort (48%), creates a risk of selection bias. The lack of information on meniscal tears among the ACL-injured women and some of the men and unawareness of when the meniscal tear was sustained among the men must be regarded as a limitation of **Study I**.

As in **Study I**, the time from ACL rupture to follow-up in the subjects in **Study II** was sufficient for radiographic OA to develop (15 years). Although **Study II** has several limitations, the most important is that the study was conducted on a prospectively recruited cohort, but the hypothesis and measurements relevant to the hypothesis were made subsequently, at the 15-year follow-up. Only 68 subjects were included and this somewhat low number was insufficient to draw any firm conclusions regarding the findings of the study. The study design was, however, justified by the hypothesis-generating approach of **Study II**. The detailed characterisation of the subjects included in **Study II** is an advantage of the study.

Regarding **Studies III** and **IV**, which were conducted using the same cohort, the sample sizes were probably large enough for the questions that were being addressed. **Study III** comprised 111 subjects and **Study IV** 98 subjects. The MRI images of 13 subjects could not be retrieved, thus explaining this discrepancy. It is important to note that the subjects in this cohort constitute a convenience sample, where 79 of the 111 patients included were not included during an ongoing RCT [185]. Additional subjects were recruited just before or after the RCT recruitment process. The inclusion and exclusion criteria of the RCT influenced the

heterogeneity of the included subjects, which is a limitation. In particular, the age distribution and the number of subjects with a previous knee injury were probably different from those of a consecutively recruited cohort.

Imaging

Several different radiographic techniques were used to detect joint space narrowing and osteophytes during the course of the thesis. In **Study I**, the non-traumatic cohort was radiographed using fluoroscopy guidance aligning the X-ray beam tangentially to the medial tibial plateau, with almost the full weight on the examined knee and knee flexion of 30-50°. In the post-traumatic cohort, a fixed knee angle was used and the knee was flexed about 15°. Although the prior technique is superior in visualising both the joint space and marginal osteophytes, these differences will not have biased our results. The approach amended in **Study I** does not reflect differences in the prevalence of specific radiographic features between the two cohorts but rather the relationship between joint space narrowing and osteophytes in the lateral and medial compartments and osteophytes on the tibia and femur within the same cohort. In **Study II**, the contralateral uninjured knee was visualised with the knee in full extension. This view is not optimal for visualising the joint space and some subjects with early OA may have been overlooked and included in further analyses.

Regarding the MRI classification, assessment was made by a single musculoskeletal radiologist (TB). No reproducibility measurements were made, which is a limitation of **Study IV**.

Results

Radiological characteristics of post- and non-traumatic knee osteoarthritis (Study 1)

According to the Kellgren-Lawrence classification [190], 77% and 39% of subjects had radiographic tibiofemoral OA in the post-traumatic and non-traumatic cohorts respectively. The distribution of radiographic joint space narrowing and osteophytes (Altman grade ≥ 1) in the two cohorts is shown in Figure 9 [189]. Joint space narrowing in the lateral tibiofemoral compartment and osteophytes on the medial and lateral femur and the lateral tibia were more common in the post-traumatic cohort.

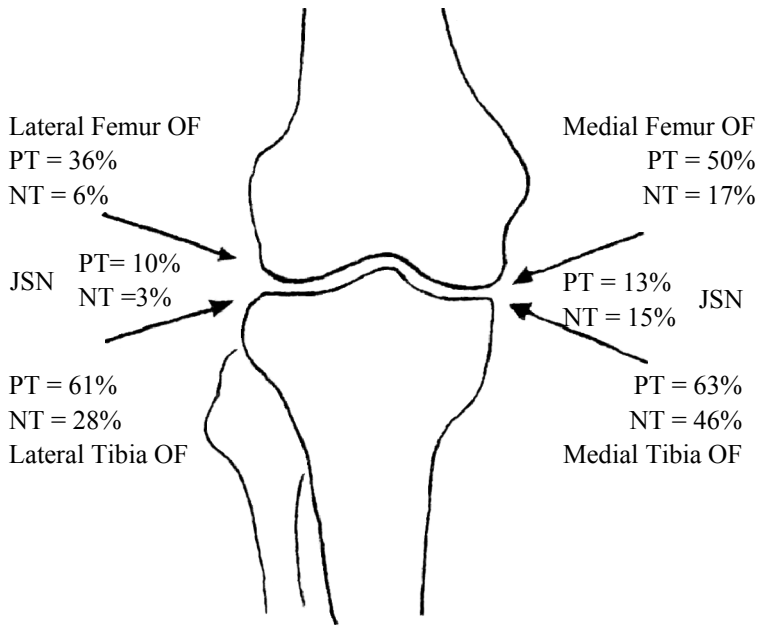

Figure 9. The distribution of osteophytes (OF)s and joint space narrowing (JSN) in the knees of the post-traumatic (PT) and non-traumatic (NT) cohorts.

The 95% confidence intervals for the odds ratios of belonging to the post-traumatic cohort compared with the non-traumatic cohort revealed differences in the radiological characteristics between the two cohorts. When compared with the non-traumatic cohort, subjects in the post-traumatic cohort had an increased risk of lateral tibiofemoral joint space narrowing compared with medial tibiofemoral joint space narrowing (borderline significance). A higher risk of osteophytes on the medial and lateral femur and the lateral tibia (borderline significance) compared with the medial tibia was also observed (Table 3).

Table 3. Risk of joint space narrowing and osteophyte formation in the post-traumatic and non-traumatic cohorts*

	Post-traumatic vs non-traumatic OR (95% CI)
Joint space narrowing	
Medial	0.8 (0.5-1.6)
Lateral	4.4 (1.5-13.6)
Osteophytes	
Medial tibia	0.7 (0.4-1.2)
Medial femur	3.3 (1.7-6.5)
Lateral tibia	2.1 (1.2-3.6)
Lateral femur	4.1 (1.9-8.9)

*The relative risk was expressed as OR and adjusted for gender. All patients in the post-traumatic cohort had sustained an anterior cruciate ligament tear. Patients in the non-traumatic cohort had no known knee injuries. OR, odds ratio; CI, confidence interval

Alignment and the risk of post-traumatic osteoarthritis (Study II)

In **Study II**, the hip-knee-ankle angle was assessed in the contralateral uninjured knee at follow-up 15 years after ACL injury. The contralateral uninjured knee was used as a proxy for the alignment of the injured knee at baseline. Varus alignment of the contralateral knee was associated with an increased risk of knee OA, defined as tibiofemoral and/or patellofemoral knee OA, of the ACL-injured knee. These findings did not, however, reach statistical significance (odds ratio: 3.9 (1.0-15.8), p value: 0.052. The distribution of knee OA in relation to knee alignment is shown in Figure 10.

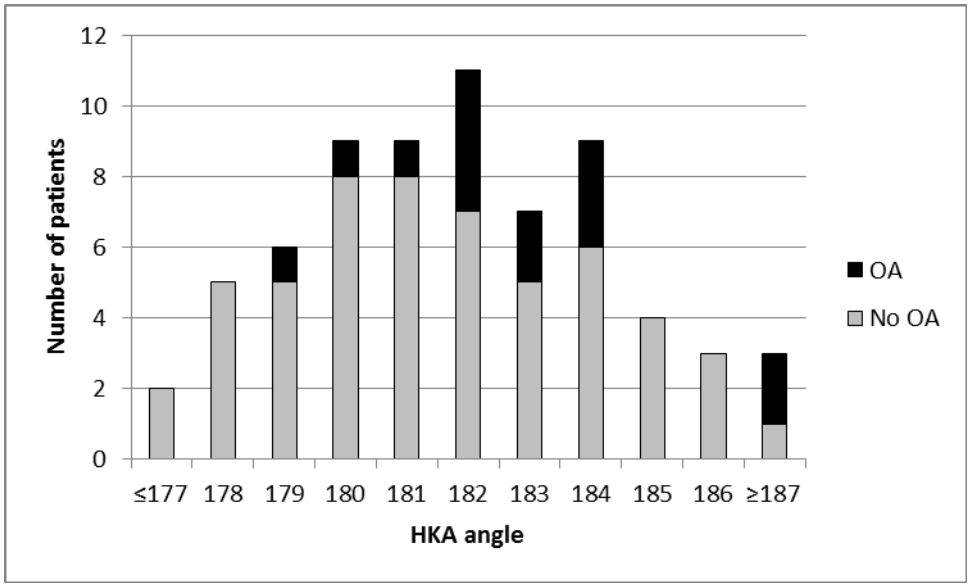

Figure 10. The distribution of alignment, expressed as the hip-knee-ankle (HKA) angle, among subjects with and without knee osteoarthritis (OA)

Cartilage and bone markers and inflammatory cytokines in the acute phase of knee injury (Studies III & IV)

In **Studies III** and **IV,** we analysed the differences in synovial fluid concentrations of several cartilage (ARGS, sGAG, COMP) and bone markers (osteocalcin, secreted protein acidic and rich in cysteine (SPARC), osteopontin) and inflammatory cytokines (IL-1β, IL-6, IL-8, TNF-α) in acutely injured knees (n=111) compared with reference knees (n=10). The synovial fluid concentrations of ARGS, COMP, SPARC, osteopontin and all cytokines, but not sGAG or osteocalcin, were higher in injured knees compared with knees of age- and gender-matched reference subjects. We also found that the synovial fluid concentrations of ARGS, sGAG and COMP were higher in knees aspirated one to three days after injury and thereafter but not in those injured and aspirated the same day (ARGS), or the same day and day one after injury (sGAG and COMP). Biomarkers of bone formation (SPARC), bone resorption (osteopontin) and inflammation were higher in acutely injured knees at all investigated time points (0-23 days after acute knee injury). A depiction of the relative median biomarker levels in synovial fluid samples from acutely injured knees related to biomarker levels in the knees of age- and gender-matched reference subjects is shown in Figure 11.

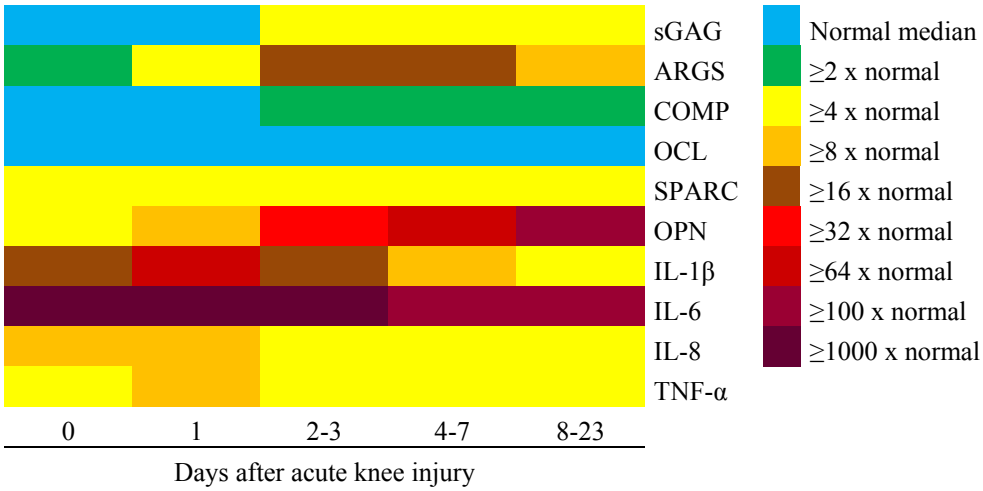

Figure 11. Relative increase in median biomarker levels in synovial fluid samples from acutely injured knees related to median biomarker levels in knees of age- and gender-matched reference subjects. These are listed above in a heatmap. A normal median was defined as a median concentration in the knee injured subjects between 0.5 and 2 times that of the median concentration in reference subjects. COMP, cartilage oligomeric matrix protein; IL, interleukin; OCL, osteocalcin; OPN, osteopontin; sGAG, sulphated glycosaminoglycan; SPARC, secreted protein acidic and rich in cysteine; TNF, tumour necrosis factor.

Osteochondral fractures and joint inflammation (Study IV)

Acute knee injury leads to impact on the bone and cartilage, resulting in traumatic bone marrow lesions and osteochondral fractures [44]. In the present study, we used the presence of an osteochondral fracture as a sign of stronger impact trauma in knees with soft tissue knee injuries. We hypothesised that a disruption of the cortical bone would lead to a higher degree of joint inflammation and increased levels of bone markers in synovial fluid. We found that acutely injured knees with any osteochondral fracture had higher synovial fluid concentrations of TNF-α. Moreover, knees with disrupted cortical bone had higher synovial fluid concentrations of SPARC, IL-8, and TNF-α, compared with knees without osteochondral fractures, and higher synovial fluid concentrations of IL-8 and TNF-α, compared with knees with osteochondral fractures but intact cortical bone (Table 4).

Table 4. Differences in biomarker concentrations in the synovial fluid between knees with or without osteochondral fractures

	Any OC fracture (n=67)	**vs.**	**No OC fracture** (n=31)	**vs.**	**OC fracture with disrupted cortical bone** (n=38)	**vs.**	**OC fracture without disrupted cortical bone** (n=29)
	Median (IQR)	Adjusted p-values	Median (IQR)	Adjusted p-values	Median (IQR)	Adjusted p-values	Median (IQR)
sGAG (µg/ml)	168 (78-306)		135 (76-331)	0.917	171 (62-354)	0.703	162 (79-303)
ARGS (pmol/ml)	73 (32-171)	0.472	59 (18-185)	0.239	70 (34-173)	0.612	73 (29-155)
OCL (ng/ml)	35 (23-51)	0.624	42 (26-56)	0.682	29 (18-51)	0.917	37 (31-52)
SPARC (ng/ml)	471 (322-767)	0.061	407 (140-685)	**0.030**	492 (328-754)	0.569	456 (267-773)
OPN (ng/ml)	75 (20-160)	0.110	29 (6-79)	0.110	84 (20-157)	0.569	56 (19-191)
IL1-β (pg/ml)	11 (4-30)	0.537	12 (5-28)	0.419	11 (4-37)	0.501	11 (4-24)
IL-6 (pg/ml)	3386 (740-14425)	0.996	3602 (1254-14896)	0.842	2833 (455-16868)	0.767	4092 (845-10706)
IL-8 (pg/ml)	211 (89-417)	0.223	138 (67-413)	**0.028**	278 (148-628)	**0.004**	124 (78-265)
TNF-α (pg/ml)	9 (7-12)	**0.013**	7 (5-14)	**0.004**	11 (7-15)	**0.012**	7 (6-11)
COMP (µg/ml)	30 (21-36)	0.814	28 (21-47)	0.557	29 (20-33)	0.553	30 (23-48)

Between-group statistical testing was performed using analysis of covariance with adjustment for days between the injury and synovial fluid aspiration, age at injury and gender. Skewed data were log10 transformed before analysis to ascertain normal distribution. Significant differences ($P < 0.05$) are in bold. COMP, cartilage oligomeric matrix protein; IL, interleukin; IQR, interquartile range; OC, osteochondral; OCL, osteocalcin; OPN, osteopontin; sGAG, sulphated glycosaminoglycan; SPARC, secreted protein acidic and rich in cysteine; TNF, tumour necrosis factor

Protease activity in bovine cartilage explants co-incubated with joint capsule and/or mechanically injured (Study V)

In **Study V**, we investigated how and by which proteases aggrecan is degraded in cartilage explants exposed to mechanical trauma, co-incubated with joint capsule or treated with exogenous TNF-α. The main findings were that: After normalising for total GAG: (1) the release of FFGV-aggrecan into medium was increased in mechanically injured cartilage, regardless of co-incubation with joint capsule (p<0.5, Figure 12). This suggests increased MMP activity in both these conditions. (2) In mechanically injured cartilage co-incubated with joint capsule, there tended to be an increased release of ARGS-aggrecan (p<0.1, Figure 13). This suggests increased aggrecanase activity in this condition. We also found that, in the mechanically injured and TNF-α stimulated cartilage, only aggrecanase activity was detected. No MMP activity was observed as measured by Western blotting in cartilage (Figure 14) or FFGV-aggrecan in medium in this condition (data not shown).

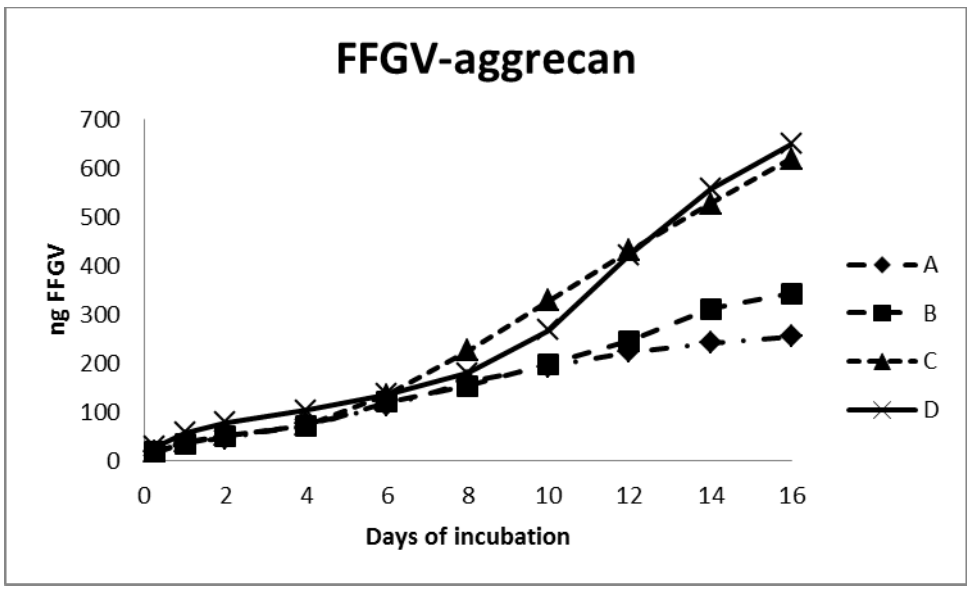

Figure 12. The mean accumulated amount of FFGV fragments detected in the medium over the 16-day incubation period. The conditions are: (A) uninjured cartilage; (B) mechanically injured cartilage; (C) uninjured cartilage co-cultured with joint capsule and (D) mechanically injured cartilage co-incubated with joint capsule.

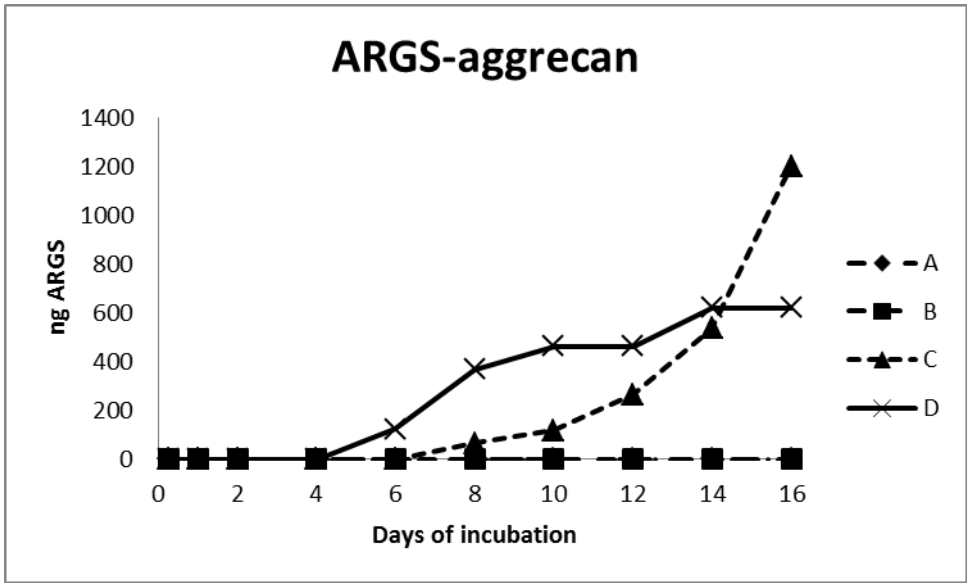

Figure 13. The mean accumulated amount of ARGS fragments detected in the medium over the 16-day incubation period. The conditions are: (A) uninjured cartilage; (B) mechanically injured cartilage; (C) uninjured cartilage co-cultured with joint capsule and (D) mechanically injured cartilage co-incubated with joint capsule.

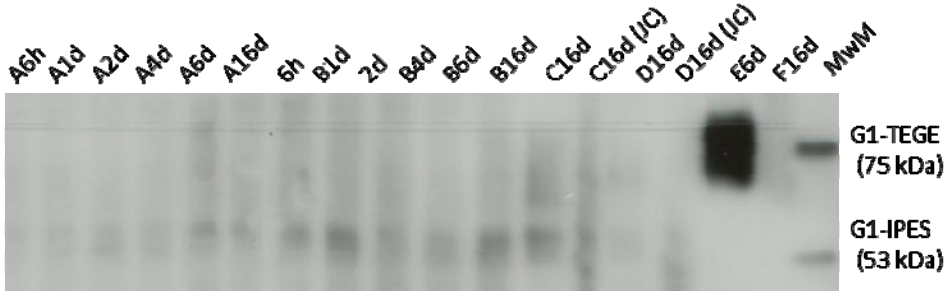

Figure 14. Western blot of the cartilage conditions: A (uninjured cartilage), B (mechanically injured cartilage), C (uninjured cartilage co-cultured with joint capsule (JC), D (mechanically injured cartilage co-incubated with joint capsule, E (mechanically injured cartilage treated with exogenous tumour necrosis factor (TNF)-α and F (joint capsule). The cartilage and joint capsule explants were cultured from six hours (h) up to 16 days (d). The G1-TEGE and G1-DIPES fragments were detected using an antibody against the G1 domain of the aggrecan molecule. No G1-DIPES fragment was detected in the mechanically injured cartilage treated with exogenous TNF-α. MwM: molecular weight marker.

Conclusions

- Subjects with post-traumatic knee OA secondary to an ACL injury have more joint space narrowing and more osteophytes in the lateral tibiofemoral compartment than in the medial compartment compared with subjects with non-traumatic OA.
- Subjects with post-traumatic OA also have more osteophytes on the medial femur compared with the medial tibia.
- Varus alignment may be a risk factor for OA 15 years after an ACL injury, although the observed difference could not be verified statistically.
- Acute knee injury is associated with instant and sustained synovial fluid biochemical alterations within the first month of knee injury, suggestive of increased aggrecan degradation and severe joint inflammation.
- In subjects with soft tissue knee injuries, an osteochondral fracture with disrupted cortical bone is associated with increased joint inflammation in the acute phase of knee injury.
- Mechanical injury of bovine cartilage explants increases MMP activity in cartilage.
- In mechanically injured cartilage, co-incubated with joint capsule, aggrecanase activity appears to be increased.
- Cytokine stimulation may induce aggrecan degradation by a different pathway compared with when cartilage explants are mechanically injured and (or) co-incubated with joint capsule.

Discussion and future perspectives

Post-traumatic and non-traumatic knee osteoarthritis

There are indications that post-traumatic and non-traumatic knee OA should not be regarded as one disease. When comparing age-associated and instability-induced mouse knee OA models, the effects of genetic modification led to different outcomes in approximately one-third of models [101]. Valdes et al. [62], showed that subjects who undergo total knee replacement due to post-traumatic OA had a non-significantly larger proportion of genetic risk variants compared with non-traumatic subjects. Moreover, it has been suggested that the inflammatory pathways between post-traumatic OA and non-traumatic OA, such as metabolic syndrome OA, crystal-induced OA and OA related to ageing, may differ. Berenbaum [148] speculated that the origin and types of inflammatory molecules involved in cartilage degradation could vary between post- and non-traumatic OA. Furthermore, the development of subchondral bone alterations may differ between non-traumatic and post-traumatic knee OA. Buckland-Wright et al. [142] showed that thickening of the horizontal subchondral trabeculae and osteophytosis were early findings in ACL-injured subjects, whereas subchondral cortical plate thickening was not. Subchondral cortical plate thickening was, however, found in patients with early non-traumatic knee OA [193]. In **Study I,** we showed that, the post-traumatic cohort (ACL-injured subjects) had proportionally more joint space narrowing in the lateral tibiofemoral compartment than in the medial compartment (borderline significance) compared with the non-traumatic cohort. Moreover, compared with the non-traumatic cohort, the post-traumatic cohort had more osteophytes in the lateral tibiofemoral compartment and more osteophytes on the femur compared with the medial tibia. Although there are many similarities in the presentation and risk factors of post- and non-traumatic knee OA [62, 121], these findings may further support the separation of non- and post-traumatic OA and that these entities should be considered using different approaches.

Future investigations that clarify potential differences in the biological response leading to post- and non-traumatic OA could be important. This knowledge could lead to the improved management of knee-injured subjects, as well as subjects with already established post-and non-traumatic OA.

The knee injury and the impact

As proof of the high impact forces applied to cartilage at the time of injury, traumatic bone marrow lesions and osteochondral fractures, located predominantly in the lateral tibiofemoral compartment, are commonly associated with an ACL injury [39, 44]. Osteochondral fractures are associated with meniscal tears [39] and increased size of the adjacent traumatic bone marrow lesions [39, 44]. This underlines the fact that their presence represents a proxy for the strong impact forces applied to the knee at the time of acute knee injury. The long-term consequences of osteochondral fractures and whether these injuries increase the risk of developing OA have not yet been evaluated. In the short term, however, their presence may be associated with a poorer clinical outcome [39, 146].

Importantly, the impact between the tibia and femur at the time of ACL injury leads to chondrocyte and osteocyte death at the site of impact [49, 194, 195]. Chondrocyte death has also been shown in cartilage explants where supra-physiological loads were applied over the cartilage. This also leads to the disruption of the collagen fibrillar network [196]. Chondrocytes left in the cartilage may adopt a catabolic phenotype in an attempt to repair the injured ECM by increasing the expression of aggrecanases (ADAMTS 4 and 5), MMPs and growth factors [197, 198]. During the present thesis, we have provided some evidence of increased activity by both aggrecanases and MMPs after mechanical impact on cartilage. The increased ARGS-aggrecan concentrations in synovial fluid as early as one day after an acute knee injury in **Study III** is indicative of an increased aggrecanase activity. In **Study V**, we showed that the mechanical injury of cartilage explants increases MMP but not aggrecanase activity in cartilage. The combination of mechanical injury and the co-incubation of cartilage with joint capsule induced increased MMP and a trend towards increased aggrecanase activity. These events could have implications for human disease and the increase in protease activity most probably leads to progressive cartilage erosion.

Articular fractures have been shown to induce synovitis. With increasing fracture severity, increased synovial inflammation and reduced peri-articular bone density were observed [194]. This indicates that fractures of cortical bone, including those associated with soft tissue knee injuries, could increase cross-talk between chondrocytes, bone and synovial cells. In **Study IV**, we found elevated concentrations of SPARC, IL-8 and TNF-α in those knees with an identified osteochondral fracture, with disrupted cortical bone in association with soft tissue knee injuries. These differences could be attributed to the breakage of the cortical bone and the concurrent initiation of fracture healing [199]. Other factors related to the occurrence of osteochondral fractures, such as the degree of impact forces

applied over the articular surfaces, cell death and the degree of hemarthrosis [49, 173, 200], could also be important for the inflammatory response to trauma.

In a recent *in vitro* articular fracture model, chondrocyte death was found mostly along fracture lines and, interestingly, a rapid decrease in chondrocyte viability was shown over the first two days after injury [201]. The production of inflammatory mediators and reactive oxygen species as a response to the injury could induce chondrocyte stress and progressive chondrocyte apoptosis and necrosis [202, 203]. Taken as a whole, these findings suggest that, in the very acute phase of knee injury, there may be a window of treatment opportunity where measures to salvage chondrocytes could improve long-term outcomes after intra-articular fracture and also after ACL injury [204].

Mechanical impact and its role in osteoarthritis initiation after ACL injury

An acute knee injury, exemplified by the ACL injury, induces events that may lead to knee OA in the near future [19, 21]. The subsequent risk of OA may be closely associated with the knee injury mechanism and the panorama of injuries in the knee sustained at the onset of injury [27]. An ACL injury is associated with hemarthrosis, a high degree of joint inflammation, large numbers of meniscal tears, cartilage lesions and osteochondral fractures and all these factors may be important for the risk of OA [32, 44, 173]. The most important risk factor for post-traumatic OA after ACL injury appears to be an associated meniscus injury [21]. The likely explanation of this is that, depending on the type of meniscal tear, or the degree of meniscectomy, the peak loads on specific cartilage areas may be increased several times [120, 205]. Consequently, could associated meniscal injuries be the sole answer to why ACL-injured subjects develop post-traumatic OA? Probably not; the findings in **Study IV** suggest that the initial impaction forces applied over the joint surfaces may also be important. Others have shown that patients with ACL injuries (isolated or combined with injury to the meniscus or collateral ligaments) showed the first signs of OA (radiologic joint space narrowing) at an average age of about 40 years. Patients with isolated meniscus injuries had the same stage of disease ten years later on average [123]. Furthermore, significant cartilage erosion visualised using MR imaging is also evident after "isolated" ACL injury [41]. In animal models of OA, post-traumatic OA development results from many of the intra-articular injuries associated with an ACL tear. Anterior cruciate ligament transection, meniscal destabilisation and resection and fracture of the articular surface all induce OA in these models [101, 194].

In **Study I**, we showed that lateral compared with medial tibiofemoral joint space narrowing and osteophytosis increased in ACL-injured subjects compared with subjects with non-traumatic OA. There were also more osteophytes on the femur compared with the medial tibia. With this in mind, there was still more medial compared with lateral OA features, such as joint space narrowing and osteophytes, in the ACL-injured group, in line with the findings of others [142, 206, 207]. Importantly, our findings on joint space narrowing and osteophyte locations in the post-traumatic cohort may largely mirror the locations of acute and chronic cartilage lesions in the ACL-injured knee. Of particular interest to our findings, in a seven- to 11-year prospective investigation of ACL-injured subjects, Potter et al. [41] consequently (at several time points after ACL injury) reported that MRI-detected cartilage lesions were found with the highest severity grade on the lateral tibia. With decreasing severity grade, lesions were detected on the lateral femur, patella, medial femur, trochlea and medial tibia [41].

Our findings have, furthermore, been confirmed in two subsequent studies showing that early non-traumatic OA predominantly affects the medial tibiofemoral compartment, whereas post-traumatic OA (after ACL injury) affects both the medial and lateral tibiofemoral compartments [208, 209]. Huétink et al. [208] followed subjects who underwent MRI because of subacute (≤ 4 weeks) knee problems for 10 years. Subjects with ACL injury at baseline had an increased risk of radiographic joint space narrowing and osteophytes medially. In the same study as visualised on MRI, ACL injury increased the risk of diffuse cartilage lesions and osteophytes medially, whereas an increased risk of focal cartilage defects, osteophytes, bone marrow lesions and subchondral cysts was observed in the lateral compartment.

In overall terms, our and subsequent findings suggest that early post-traumatic OA after ACL injury often affects both the lateral and the medial tibiofemoral compartments. This suggests that the initial trauma which predominantly affects the lateral tibiofemoral compartment at the time of injury is important for future OA risk in that very compartment.

Immunity vs biomechanics in driving the OA process after anterior cruciate ligament rupture

In recent years, there has been an intense debate on the relative contributions of inflammation and biomechanics in initiating and driving the OA process. With the knowledge acquired during the present thesis and from the findings of others, this view should perhaps be broadened and also include the magnitude of the impact at injury as an important factor in post-traumatic OA. It is likely that mechanical

damage to joint tissues at the time of ACL rupture, the degree of acute and chronic inflammation and the abnormal joint loading over time potentiate one another. To conclude, the initial impact applied over the articular surfaces may be a determinant of how the cartilage responds to inflammatory activation and withstands long-term abnormal loading [204].

Inflammation

The inflammatory component of non-traumatic and post-traumatic OA may be considerable [102, 148, 175, 210, 211]. However, even though there are substantial data suggesting that joint inflammation plays an important role in OA progression, viable treatment options limiting joint inflammation and thereby OA progression in humans are not available. The inhibition of specific cytokines has not provided convincing evidence that the OA process could be stopped or slowed down [148].

After ACL injury, the severity of arthroscopically detected post-traumatic cartilage lesions has been shown to correlate with synovial fluid concentrations of IL1-β, TNF-α and osteopontin and negatively with IL-1Ra [212, 213], indicating that the inflammation may play an important role in post-traumatic OA. It is also well understood that an acute knee injury induces a high degree of inflammation which is associated with clinical symptoms such as effusion and pain [146, 153, 179-181, 214]. In **Study III**, we demonstrated highly elevated concentrations of the pro-inflammatory cytokines IL-1β, IL-6, IL-8 and TNF-α in the acute phase of knee injury (0-23 days after injury). These findings are compatible with those of previous studies [153, 179, 180]. We also demonstrated elevated concentrations of the bone markers osteopontin and SPARC but not of osteocalcin. Osteocalcin is considered to be a specific marker of osteoblast activity [215]. Osteopontin and SPARC, however, in addition to regulating bone remodelling, may be released by several cell types and be involved in tissue remodelling and inflammation in conjunction with tissue injury [216, 217]. We suggest that this at least partly explains the increased concentrations of these markers in synovial fluid in the acute phase of knee injury. Interestingly, although the synovial fluid concentrations of pro-inflammatory cytokines appear to decrease with time from injury, SPARC concentrations may remain elevated at a constant level and osteopontin concentrations appear to further increase as the time from injury increases, at least within the first 23 days after injury. Based on these findings and given the pro-inflammatory abilities of SPARC and osteopontin [216-220], these molecules could cause sustained inflammation and cartilage degradation in the knee after the acute injury [213, 221, 222].

The acute increase in pro-inflammatory cytokines and bone markers in synovial fluid was accompanied by increased ARGS-aggrecan, sGAG and COMP, suggesting increased cleavage and turnover of aggrecan and COMP as early as one to three days after injury (**Studies III** and **IV**). Increased synovial fluid concentrations of these and other potential markers of progressive cartilage ECM erosion, such as crosslinked peptides from type II collagen, have been shown in previous investigations many years after acute knee injury [81, 85, 88, 165], suggesting that the ACL injury induces processes that permanently change the biochemical joint environment. Typically, pro-inflammatory concentrations in synovial fluid appear to normalise within a few months. However, in subsets of subjects, inflammation may persist several years after an ACL injury and influence the production of molecules important for cartilage knee joint function such as lubricin [153]. Cameron et al. [179] showed that, while IL-1ra concentrations increased in the acute phase, they were markedly reduced in the chronic phase (≥3 months). As a result, not only increased joint inflammation, as measured by synovial fluid TNF-α or IL-1β, for example, but also the relationship between pro-inflammatory and chondroprotective agents, such as IL-1ra, IL-10 and lubricin, are essential when considering the potential effect of inflammation on the risk of post-traumatic OA. Although these studies do not prove that a high degree of joint inflammation after ACL injury leads to cartilage erosion, this is highly plausible. General cartilage erosion also observed at sites not affected by the initial impact suggests that the biochemical joint environment and perhaps the degree of inflammation are essential for progressive cartilage erosion after an ACL injury. Cartilage lesions and collagen cleavage in the non-weight-bearing cartilage of the intercondylar notch early after ACL injury support this [41, 107, 108]. An equal distribution of radiographic features between the medial and lateral tibiofemoral compartments (findings from **Study I**) and the fact that patellofemoral and tibiofemoral OA are often found concurrently after ACL injury may also support this [126, 223].

Biomechamics

It has been emphasised that knee OA is caused by increased physical forces across a confined cartilage region or by too high a general joint load, as in the case of obesity [7, 224]. The increased forces acting on the cartilage could be related to the shape of the joint or could be acquired, after an ACL or meniscal tear, for example [7, 65, 66, 225]. In most cases, both inherent factors and acquired factors may, however, interact to induce increased cartilage stress [19, 121]. The age of a subject inevitably also influences the ability of cartilage to withstand a change in loads and to induce reparative measures [123]. In a large-scale study (n=5,086), Sri-Ram et al. [129] showed that subjects aged > 30 years ran a significantly

increased risk of chondral lesions and medial but not lateral meniscal tears at the time of ACL reconstruction. Disruption of the ACL, furthermore, leads to abnormal gait patterns and changes in the load on cartilage, observed in both non-reconstructed and ACL-reconstructed subjects [226]. A common understanding is that increased tibial translation and tibiofemoral rotation due to the loss of ACL function leads to a change in cartilage load, increasing the risk of knee OA [7]. Moreover, quadriceps strength may not recover fully after ACL injury, leading to excessive loads on cartilage during gait and increasing the risk of post-traumatic OA [227]. One intriguing hypothesis is that cartilage regions which, over a lifetime have adapted to withstand tensile rather than compressive loads (see above), may suddenly, after ACL rupture and due to changes in joint motion, be exposed to high compressive loads [7]. In combination with other risk factors for post-traumatic OA, this could induce loads that are too high for cartilage to withstand and lead to post-traumatic OA.

One such risk factor may be varus malalignment, which is known to predispose to future knee OA [65, 66]. Malalignment (either varus or valgus) is also a risk factor for both tibiofemoral and patellofemoral OA progression [228, 229]. The risk of tibiofemoral OA is probably mediated by the adduction moment at the knee and increased medial-to-lateral knee cartilage loads [230]. Increased loading of the medial tibiofemoral compartment in subjects with varus alignment could explain why, in **Study II**, we found indications that varus alignment was associated with an increased risk of OA. Neuman et al. [111] showed that GAG content as measured by dGEMRIC approached normal values in the lateral femoral cartilage but remained at a low value in medial femoral cartilage from baseline to two-year follow-up. In general, the medial compartment is affected by higher loads [230] which could be important for these observed differences between the medial and lateral tibiofemoral cartilage. Altered loading patterns of cartilage secondary to the ACL injury could also have influenced on these differences [7].

More knowledge of the way the magnitude of the impact compared with chronic instability and the degree of cartilage loading influences acute and chronic joint inflammation and cartilage degradation after ACL injury is essential in order better to define the factors that are most important in OA initiation and progression.

Concluding remarks

The present thesis highlights processes that may be relevant to the risk of developing post-traumatic OA. In overall terms, the acquired knowledge suggests that, in addition to meniscus injuries, the impact forces imposed on cartilage and subchondral bone at the time of ACL injury may predispose to OA progression at the site of impact (**Study I**). Disruption of the cortical bone where the femur and tibia collide at the time of injury lead to increased inflammation in the acute phase of knee injury, which could influence the long-term risk of developing OA (**Study IV**). This, however, needs to be tested in trials appropriately designed to test this theory. Furthermore, synovial fluid concentrations of sGAG, ARGS-aggrecan and COMP were increased one to three days after knee injury, suggesting an almost immediate increase in the turnover of several cartilage ECM molecules (**Study III** and **IV**). Combining these findings with those of others, which have indicated a similarly early increase in collagen type II cleavage after acute knee injury (personal communication: Dr. Nobuyuki Kumahashi), suggests an almost immediate turnover of the entire cartilage ECM. These findings may challenge the predominant belief that the initiating event in pathological cartilage degradation is the cleavage of aggrecan.

Underlying this hypothesis, high concentrations of catabolic agents such as IL-1 and TNF-α induce rapid aggrecan cleavage in cartilage explants. Collagen cleavage is not initiated until most aggrecan has been degraded and released into medium [79]. In a more complex explant system, where mechanically injured cartilage explants were co-incubated with joint capsule, we detected increased MMP activity and a trend towards increased aggrecanase activity. In mechanically injured cartilage explants treated with exogenous TNF-α, a high degree of aggrecanase activity was detected but no MMP activity at all, either in medium or in cartilage (**Study V**). Taken as a whole, this could underline the non-physiological approach of using single or multiple catabolic agents to induce cartilage degradation and indicates that the cytokine-stimulated cartilage explant system may be overly simplistic. This may further challenge the theory that the kinetics of cartilage erosion involves early aggrecanase activity, whereas MMP activity only occurs later in the disease process.

In overall terms, the findings in this work underline the fact that the initial impact associated with an ACL injury appears to be important for the risk of developing

post-traumatic OA. The strong forces acting over the cartilage and other tissues at the time of injury lead to severe joint inflammation and blood and marrow content makes its way into the joint. Moreover, rapidly progressing chondrocyte death occurs [201]. From a clinical perspective, the first few hours after an ACL tear could be essential when attempting to stop ongoing cartilage degradation which was initiated at the time of injury. These measures could reduce the risk of OA and we therefore propose that research delineating the importance of the acute and sustained biological response to injury should be undertaken. Ongoing longitudinal trials in which the authors may be able to link biochemical processes in synovial fluid or blood to the risk of cartilage degradation using MRI or radiography offer a promise of increased knowledge of the processes that are most important. This could lead to the improved management of knee-injured patients and possibly delay, or even prevent, post-traumatic OA development.

Some findings in the present thesis could have clinical applications. Based on the findings in **Study I,** showing that early post-traumatic OA is often located in both the lateral and the medial tibiofemoral compartments, we recommend detailed pre-operative planning before surgery such as high tibial osteotomy. It is possible that the outcome of transferring the load on the knee in a lateral direction, in these subjects, leads to lateral tibiofemoral OA progression.

In **Study II**, we showed that subjects with varus alignment ran an increased risk of post-traumatic knee OA after ACL injury (borderline significance). We suggest that this hypothesis should be tested in a larger study population to evaluate the impact of alignment on post-traumatic OA. Speculatively, alignment could influence the ACL injury mechanism and the forces applied over the knee joint at the time of ACL injury, with higher impaction forces applied on the lateral joint surfaces. We therefore suggest that the relationship between alignment and injuries to cartilage and bone sustained at the time of ACL injury should be investigated.

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