Pain in Osteoarthritic Joints:

Biological Signaling and 3D Models based on Imaging

Birgitta Gatenholm

Department of Orthopaedics
Institute of Clinical Sciences at
Sahlgrenska Academy
University of Gothenburg

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bgatenholm@gmail.com

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To Ellinor

“We choose to go to the moon in this decade, not because that will be easy, but because it will be hard”

– John F. Kennedy

Abstract

Osteoarthritis (OA) is the most common joint disease, causing disability in middle-aged and elderly patients worldwide and imposing a huge socioeconomic burden. OA of the knee joint is a major cause of joint pain and, along with back pain, it accounts for the two most-reported causes of chronic pain. Despite intensive research in the field, knowledge of the genesis of pain and the pathological processes involved in the development of OA is still very limited. In addition to this, the diagnostic tools used today have low sensitivity and are unable to visualize early disease signs. There is a need for a better understanding of the pain genesis in OA and better early diagnostic tools.

The overall aim of this thesis was to elucidate the mechanism of the pain in osteoarthritic knee joints, with the emphasis on the role of biological signalling and detailed mapping of cartilage damage and the subchondral region. We furthermore investigated future potential diagnostic tools and treatment exploring different imaging techniques and 3D bioprinting.

Study I provided an overview of the field of joint pain with special emphasis on neuropeptides. The review concluded that neuropeptides play not only an important role in nociception but also a regulatory role in many biologic processes, such as bone turnover, inflammation and angiogenesis. **Study II** was a pilot study including tissue samples from six patients with OA undergoing total knee arthroplasty (TKA). We developed a method for analyzing endogenous peptides using Liquid chromatography–mass spectrometry (LC-MS) in cartilage and subchondral bone. In **Study III,** we performed 3D imaging and modeling of morphologic changes in the cartilage and subchondral bone of OA patients using equilibrium partitioning of an ionic contrast agent (EPIC) micro computed tomography (CT). We developed a reproducible and semi-automatic method to visualize structural changes in the cartilage and subchondral bone. In **Study IV,** we compared different imaging techniques and used the 3D images rendered to produce a computer-aided design (CAD) model for visualizing the osteochondral lesion and repairing cartilage damage by 3D bioprinting with chondrocytes. The following study (**Study V**) further investigated cartilage damage repair by forming micro-mass pellets using chondrocytes or chondrocyte-derived induced pluripotent stem cells (iPSCs) with or without OA extracellular matrix (ECM). Our last study (**Study VI**) was a clinical prospective study of 47 patients with knee OA undergoing TKA. Patients were pre- and post-operatively monitored with the knee injury and osteoarthritis outcome score (KOOS) and their experienced pain and quality of life were then correlated to morphologic changes in cartilage and subchondral bone determined with EPIC microCT.

This dissertation presents two new methods for the early diagnosis of OA and possible intervention with 3D bioprinting. The interplay between cartilage and subchondral bone, as well as cartilage damage, neuropeptide signaling and pain, was made apparent. These studies may lead to the development of early diagnostic tools for OA with the potential to make a great contribution to the reduction of suffering and health costs associated with OA.

Keywords: Osteoarthritis, pain in knee joints, cartilage lesions, micro-computed tomography, 3D CAD models, neuropeptides

Sammanfattning på svenska

Artros är den vanligaste ledsjukdomen som orsakar invalidiserande rörelseinskränkning hos medelålders- och äldre patienter världen över med skenande socioekonomiska kostnader.

Knäartros är en av de vanligaste orsakerna till ledvärk och tillsammans med ryggsmärta utgör den de två största orsakerna till kronisk smärta. Trots intensiv forskning inom artrosfältet är kunskapen om smärtmekanismerna och patologin bakom utvecklingen av artossjukdomen relativt okänd. De diagnostiska verktygen som används idag har låg sensitivitet och är dåliga på att visualisera tidiga sjukdomstecken. Det finns ett behov av bättre diagnostiska verktyg och kunskap rörande smärtmekanismerna bakom artros.

Målsättningen med detta avhandlingsarbete var att förbättra kunskaperna om orsaken till smärta vid knäartros med ett fokus på biologisk signalering och detaljerad kartläggning av broskskador och det underliggande subkondrala benet. I avhandlingsarbetet har vi även studerat olika möjliga framtida diagnostiska verktyg samt möjligheten till nya behandlingsmetoder med 3D bioprinting.

Delarbete I handlade om en fördjupning i orsakerna till ledvärk med fokus på neuropeptider. Slutsatsen var att neuropeptider spelar en viktig roll inte enbart i smärtsignaleringen utan även i många andra biologiska processer så som den skeletala ombyggnaden, inflammation och angiogenes. **Delarbete II** var en pilotstudie som inkluderade vävnad från 6 patienter som genomgick knäartroplastik. I denna studie presenterade vi en ny metod för att analysera och identifiera endogena petider i brosk och subkondralt ben med hjälp av Liquid chromatography–mass spectrometry (LC-MS). I **Delarbete III** utvecklade vi en reproducerbar och delvis automatisk metod för att visualisera strukturella förändringar i brosk och subkondralt ben vid artrossjukdom genom undersökning med equilibrium partitioning of an ionic contrast agent (EPIC) micro computed tomography (CT). I **Delarbete IV** studerade vi en tibiaplatå med artrosförändringar och jämförde olika diagnostiska verktyg för att skapa 3D bilder och en Computer-Aided Design (CAD) modell av de morfologiska förändringarna i brosk och ben vid artrossjukdom. Den 3D modell som skapades användes sedan för att med hjälp av 3D bioprinting fylla broskskada med kondrocyter. I uppföljande **Delarbete V** studerade vi effekten av extracellulärmatrix (ECM) från artrospatienter i form av micropellets på kondrocyter och kondrocyter från pluripotenta stamceller(iPSCs). **Delarbete VI** var en klinisk prospektiv studie av 47 patienter med knäartros som följdes pre- och postoperativt efter knäartroplastik. Patienterna rapporterade den självupplevda knäfunktionen genom att fylla i knee injury and osteoarthritis outcome score (KOOS) enkäter och deras upplevda smärta och livskvalitet korrelerades därefter till deras morfologiska förändringar i brosk och subkondralt ben studerat med EPIC microCT.

I denna avhandling har vi presenterat två nyutvecklade metoder som skulle kunna användas för tidig diagnostisering av knäartros samt en möjlig behandlingsprincip med 3D bioprinting. Vidare presenterar vi ett samband mellan broskskada, neuropeptider och smärta. Förhoppningen är att de nya metoderna kommer kunna hjälpa patienter till tidig diagnos och genom detta både förhindra lidande och minska de idag skenande hälsoekonomiska kostnaderna av artrossjukdomen.

List of papers

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. **Neuropeptides: important regulators of joint homeostasis**

Gatenholm.B, Brittberg.M

Knee Surgery Sports Traumatology Arthroscopy. 2019 Mar;27(3):942-949

II. **Peptidomic analysis of cartilage and subchondral bone in OA patients**

Gatenholm.B, Gobom.J, Skillback.T, Blennow.K, Zetterberg.H, Brittberg.M

European Journal of Clinical Investigation. 2019 May;49(5):e13082

III. **Spatially matching morphometric assessment of cartilage and subchondral bone in osteoarthritic human knee joint with micro-computed tomography**

Gatenholm.B, Lindahl.C, Brittberg.M, Stadelmann VA

Bone. 2019 Mar;120:393-402

IV. **3D bioprinting with chondrocytes into an osteoarthritic chondral lesion based on patient specific 3D CAD model**

Gatenholm.B, Lindahl.C, Brittberg.M, Simonsson.S

Manuscript under revision for Cartilage

V. **Cartilage tissue formation was degraded by osteoarthritic extracellular matrix micro particles and prevented by 3D bioprinting**

Gatenholm.B, Lindahl.C, Forsholm.A, Ekholm.J, Brantsing.C, Brittberg.M, Lindahl.A, Simonsson.S

In Manuscript

VI. **Study of osteoarthritic knee joints: correlation between pain, quality of life and 3D cartilage morphology**

Gatenholm.B, Lindahl.C, StadelmannVA, Brittberg.M

In Manuscript

Contents

Abbreviation

ACAN	Aggrecan
ACI	Autologous chondrocyte implantation
AM	Additive manufacturing
ACL	Anterior cruciate ligament
BLOCKS	Boston Leeds Osteoarthritis Knee Score
BME	Bone marrow edema
CAD	Computer-aided design
CGRP	Calcitonin gene-related peptide
CNS	Central nervous system
CT	Computed tomography
Dicom	Digital imaging and communications in medicine
ECM	Extracellular matrix
EPIC	Equilibrium partitioning of an ionic contrast agent
GAG	Glycosaminoglycans
HR-pQCT	High resolution peripheral quantitative computed tomography
ICRS	International Cartilage Research Society
iPSCs	Induced pluripotent stem cells
JSN	Joint space narrowing
JSW	Joint space width
KL	Kellgren and Lawrence
KOOS	Knee injury and Osteoarthritis Outcome Score
KOSS	Knee Osteoarthritis Scoring System
LC-MS	Liquid chromatography mass spectrometry
microCT	Micro-computed tomography
MOAKS	MRI Osteoarthritis Knee Score
MRI	Magnetic resonance imaging
MWCO	Molecular weight cut-off
NPY	Neuropeptide Y
OA	Osteoarthritis
OARSI	Osteoarthritis Research Society International
PBS	Phosphate buffer saline
PCL	Poster cruciate ligament
PROM	Patient-reported outcome measurement
PG	Proteoglycans
QOL	Quality of life

ROI	Region of interest
SP	Substance P
Stl	Stereolithography
TKA	Total knee arthroplasty
TMT	Tandem mass tag
UOA	Unwounded osteoarthritis
VIP	Vasoactive intestinal peptide
WOA	Wounded osteoarthritis
WORMS	Whole Organ MRI Score

1. Introduction

Osteoarthritis (OA) is the most common joint disease with a prevalence that increases exponentially with age. It is predicted that, with the aging population, the prevalence of OA will steadily increase. Globally, it has been estimated that about 10 percent of the world's population at age 60 or above have symptomatic problems associated with OA [1]. For the individual patient, the main issue is the increase in pain and the lowered activity levels leading to a decrease in quality of life. However, the disease also carries a high socioeconomic burden and the cost of treating OA in Sweden has been calculated to be around 12 billion of SEK a year [2].

OA is divided into two classes: idiopathic or posttraumatic OA. Both types of OA are characterized by changes in the biochemical and mechanical properties of the cartilage, which leads to articular cartilage degradation. The most common sites for developing OA are the hands, knees, hips and spine. Little is yet known about the mechanisms underlying the development of OA but certain risk factors such as age, heredity, gender, obesity, long exposure to physical loads, mechanical factors (such as malalignment) and trauma have been identified [3]. Patients with OA experience symptoms of pain, joint stiffness, reduced movement and swelling especially after activity. In the diseased joint pathological changes such as cartilage degradation, subchondral sclerosis, osteophyte formation, synovitis and joint capsule swelling can be observed [4].

OA was initially thought to be a disease of the articular cartilage but today it is regarded as a disease affecting all components of the joint. One critical element in the development of OA is the imbalance in the cross talk between cartilage and subchondral bone [5-8]. Subchondral bone changes were previously thought to be secondary to the development of OA but recent studies suggests that subchondral bone changes may be important for OA disease progression and may sometimes even precede cartilage damage [9]. There is a close interaction between the different components in the joint such as the synovia, cartilage and the subchondral bone, which interact together to adapt to load and catabolic factors during injury and inflammation. These theories reflect a somewhat new approach to treating the joint as an organ and initiating studies on interactions between tissues such as cartilage and subchondral bone in the process of OA development.

2. Background

2.1 The knee joint

In order to perform the accurate diagnostics and treatment of injuries to the knee it is important to understand the normal anatomy and biomechanics of the knee. The knee joint is a hinge joint consisting of two joints namely the femorotibial joint and the patellofemoral joint. It is the largest joint in the human body, with a large range of motion allowing both flexion and rotation under heavy loading conditions [10]. In the femorotibial joint, the medial and lateral condyles articulate with the tibial plateau. The medial femoral condyle is responsible for greater weight bearing and is larger compared with the lateral condyle. The structure of the condyles allows the movement of the femur to the tibia on three axes [11]. Between the femoral condyles and the tibial plateau are the medial and lateral menisci which are anatomically crescent-shaped consisting of fibrocartilage with the function of reducing friction and assisting in rotational motion. The patellofemoral joint is a saddle joint between patella and the femoral trochlea and it is extremely important for knee stability through its transmission of extensor forces across the knee by increasing the moment arm of the quadriceps.

The knee joint is further stabilized by the ligament structures surrounding the bones. The main ligaments are the collateral ligaments located extracapsularly and the cruciate ligaments located intracapsularly. The cruciate ligaments consist of an anterior and posterior structure which crosses intracapsularly within the knee. The anterior cruciate ligament (ACL) plays a crucial role in stabilizing flexion and rotation in the knee, hindering the anterior translation of the tibia relative to the femur while the posterior cruciate ligament (PCL) hinders the posterior translation of the tibia relative to the femur and is the only ligament which provides stability posteriorly during flexion. The medial and lateral collateral ligaments are located extracapsularly. Their main purpose is to restrain from valgus and varus rotation and translation of the tibia in the medial and lateral direction. Figure 1 illustrates the knee joint anatomy.

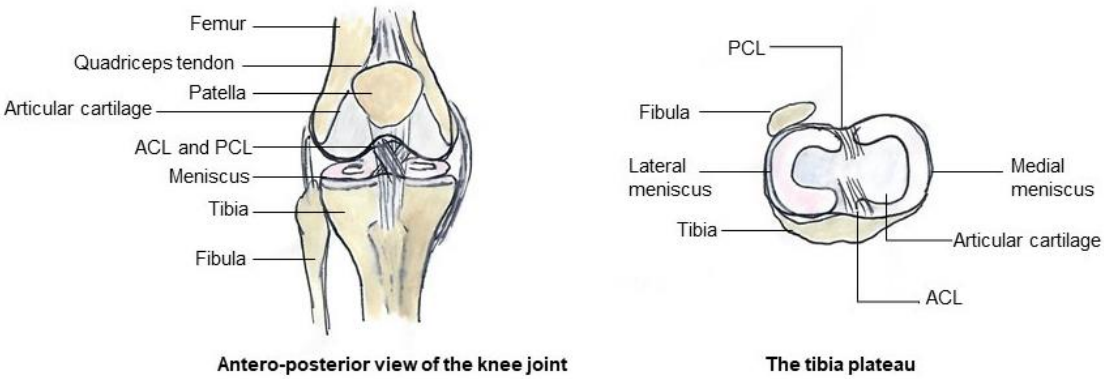

FIGURE 1. Knee joint anatomy

2.2 Articular cartilage

Articular cartilage is a unique and highly specialized connective tissue. Its main function is to facilitate load transmission, lubricate and reduce friction during articulation. Unlike many other tissues articular cartilage lacks neuronal innervation and vascularization, properties which enable cartilage to withstand challenging biomechanical conditions, but they also make the tissue susceptible to damage and reduce healing capacity.

Genesis of cartilage

While much is known today about the structural organization and biomechanical properties of the articular cartilage, strikingly little is still known about the genesis of cartilage in the developmental stages. The complex organization seen in the mature articular cartilage is not found in any other tissue counting the epiphyseal region of the growing individual. Articular cartilage formation is, however, known to be strongly related to joint development. During the first stage in the future joint site, mesenchymal condensation, which differentiates into cartilage-like structures, occurs. The condensation process involves the activation of both cell adhesion molecules, such as cadherins and N-CAM and matrix receptor molecules [12, 13]. An interzone is then formed in what will become the joint space, with an intermediate layer and two

prechondrogenic layers [14]. The interzone is important for the whole of further skeletal development, working as a signaling center and controlling chondrocyte proliferation and differentiation. Research indicates that the intermediate layer later develops into the synovium, ligaments and the capsule. The prechondrogenic layer develops into the epiphysis and forms the growth plate. It is still unclear when and how the articular chondrocytes form, but some research indicates that they originate not from mesenchymal condensation but from the mesenchymal interzone cells from a subpopulation of cells which do not activate the Matrilin 1 gene [15]. The last stage of joint development is the process of joint cavitation.

Biologic and structural composition

The major components of articular cartilage are; a dense extracellular matrix (ECM) consisting of water, collagen and proteoglycans and specialized cells called chondrocytes. The major purpose of the ECM is to hold water which is essential for the biomechanical properties of cartilage. The tissue is macroscopically smooth and homogenous with an approximate thickness of between 1 and 5mm.

Zonal division

The articular cartilage is subdivided into zones which are organized differently, in both their biological and structural composition. The zones are the superficial zone, the middle zone, the deep zone and the calcified zone as illustrated in Figure 2. The superficial zone, which makes up 10-20% of the total cartilage volume, is composed of a dense collagen layer organized parallel to the articular surface [16]. The superficial layer works mainly as a protective layer for the deeper layers. In the superficial layer the chondrocytes are fairly numerous and are flattened in their structure [17].

Below the superficial layer is the middle zone, sometimes called the transitional zone, taking up 40-60% of total cartilage volume. The chondrocytes in this layer are less numerous and are spherical in shape, while the collagen fibers, which are thick in this zone, are organized obliquely. The middle zone has a proteoglycan rich ECM and the main purpose of this layer is to withstand compression forces [18].

The deep zone is the least cell- and water-dense zone. It makes up 30-40% of the total cartilage volume. In the deep zone, the proteoglycan content is the highest and the collagen fibril thickness is the largest. The collagen

fibrils are arranged perpendicular to the articular surface, withstanding compression forces best of all layers [19]. The chondrocytes in the deep layer are spherical and arranged in columns perpendicular to the articular surface.

The calcified zone attaches the cartilage to the bone. In this zone, there is a very limited number of chondrocytes. The calcified zone plays an important role in integrating cartilage and its collagen fibrils with the subchondral bone.

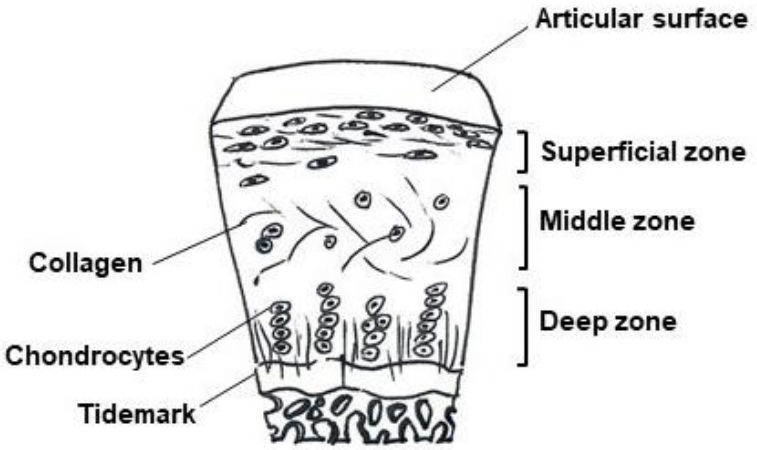

FIGURE 2 The basic science of articular cartilage

Modified image from Ondrésik M., Oliveira J.M., Reis R.L. (2017) Knee Articular Cartilage. In: Oliveira J., Reis R. (eds) Regenerative Strategies for the Treatment of Knee Joint Disabilities. Studies in Mechanobiology, Tissue Engineering and Biomaterials, vol 21. Springer, Cham

Chondrocytes

Chondrocytes are the specialized cells found in the articular cartilage which develop from mesenchymal stem cells [20, 21]. The chondrocytes vary in shape and density in the articular cartilage depending on the zones in which they are situated, and only make up around 2% of the total cartilage volume, but their important purpose is to produce and maintain

the ECM, mainly aggrecan and collagen II [17]. As the articular cartilage is avascular, the chondrocytes depend on the diffusion of the synovial fluid for oxygen and nutrient supply. The chondrocytes are isolated in their surrounding matrix and rarely form cell-to-cell contact. The cells do however respond to both mechanical and biochemical stimuli [22]. Due to the limited ability of chondrocytes to replicate, the cartilage has a limited ability to heal and is consequently sensitive to injuries.

Collagen

Multiple types of collagen are present the articular cartilage, but the most abundant one is type II collagen, which accounts for 90% of the collagen found in the articular cartilage. Collagen distribution varies through the different zones where the highest concentration can be found in the superficial zone, after which the concentration declines down the zones [19]. Type II collagen is made up of three alpha chains which are coiled to form a triple helix. The helixes bind covalently and assembles into collagen fibrils. The helical structure of collagen provides the cartilage with shear and tensile strength [23, 24].

Proteoglycans

Proteoglycans (PG) are the second most abundant macromolecules in the ECM. They are built up by a protein core covalently bonded to glycosaminoglycans (GAG) which forms side chains. GAGs are chains of polysaccharide which are negatively charged due to attached carboxylate or sulfate groups [25]. The most abundant PG in articular cartilage is aggrecan, which is a proteoglycan aggregated with hyaluronic acid and link protein [26]. Aggrecan is important for maintaining the unique biomechanical properties of articular cartilage by attracting water into the tissue through hydrostatic pressure. An early sign of cartilage deterioration is aggrecan degradation either through the proteolytic cleavage of aggrecan by aggrecanases or the cleavage of hyaluronic acid [27].

Water

The largest component of the articular cartilage ECM is water, which makes up 80% of the wet weight of cartilage. The water concentration decreases down the zones from about 80% in the superficial zone to 65% in the deep zone [28]. Joint movement makes water continuously move in

and out of the articular cartilage and contributes to the lubrication and transportation of nutrients through diffusion.

Biomechanics

Articular cartilage is a unique specialized tissue withstanding high repetitive loads and providing a low-friction surface. The different zonal arrangements give the cartilage its biomechanical properties such as resistance to tensile, shear and compressive forces. In the superficial zone, where the collagen levels are high, there is high fluid flow due to compressive forces and a resistance to tensile and shear forces due to the parallel arrangement of the collagen fibrils. In the middle zone, the collagen levels are moderate and obliquely arranged, resisting shear compression. The high PG content in this zone creates a low fluid flow, a resistance to compression and moderate matrix compression. The deep zone consists of a low level of collagen fibrils, but the highest level of PG compared with all zones. This produces strong resistance to compressive forces by creating hydrostatic pressure and allowing only low fluid flow [29, 30].

The biomechanical behavior of articular cartilage consists of two phases, the fluid phase and the solid phase. The fluid phase consists of mainly water and inorganic ions such as sodium, calcium, chloride and potassium while the solid phase consists of the ECM [31-33].

The viscoelastic properties and strength to withstand compressive loading that articular cartilage exhibits are controlled by both flow-dependent and flow-independent mechanisms. The flow-dependent viscoelasticity is a result of the interstitial fluid and the drag it causes known as biphasic viscoelastic behavior [30, 34]. The flow-independent viscoelasticity is caused by the motion of collagen and proteoglycan matrix.

To maintain matrix homeostasis at a normal level, the articular cartilage is also dependent on a certain mechanical stress caused by movement. Type II collagen, aggrecan and matrix synthesis are downregulated at gene expression level, when cartilage experiences static compression or immobilization [35, 36]. However, dynamic loading does the contrary, by increasing type II collagen and aggrecan synthesis, activating matrix metalloproteinase inhibitors and increasing levels of PGs [37].

2.3 Cartilage lesions

Articular cartilage has a poor regeneration and healing capacity, mainly due to its avascular nature and the limited ability of chondrocyte progenitor cells to initiate an on-site repair. This makes the tissue susceptible to both mechanical and biochemical injuries.

The initiation of OA has been observed when the synthesis of the extracellular matrix (ECM) is incomplete, for instance, through the mutation of collagen II. The erosion of the cartilage, followed by bone contact, synovitis and bone marrow lesions, leads to severe pain and swelling in the patient's joint. Disturbances of the proprioceptive, kinesthetic and vasoregulatory nerves can represent the primary pathogenic events in joint degeneration seen in OA. Cartilage is, however, an aneural tissue and there is a lack of understanding of how structural changes in cartilage can cause pain.

Classification of cartilage lesions

Cartilage lesions and injuries are commonly found during arthroscopy and radiographic imaging. Arthroscopic studies have reported an estimated incidence of full-thickness cartilage lesions of between 5-10% [38, 39]. Today, many different techniques are used and discussed for articular cartilage treatment, but there is still limited knowledge about the damage that needs to be treated and the genesis of pain. Furthermore, there are limitations in the consensus on cartilage injury classifications which makes assessments of injuries and repair difficult to evaluate.

Outerbridge classification system

One commonly used arthroscopic classification system for grading cartilage lesions in the knees, hips and shoulders is the Outerbridge classification system (see Figure 3). Developed in 1961, the system was initially thought to be a descriptive system of chondromalacia of the patella [40]. The Outerbridge classification system grades the cartilage area from visual inspection by arthroscopic or open surgery [41]. The cartilage area is graded from 0 to IV. Grade 0 represents normal cartilage. Grade I is represented by softening and swelling of the cartilage. In order to evaluate this, there is a need to touch the cartilage surface to make an instrumental assessment. Grade II is a partial-thickness defect which does not exceed 1cm in diameter and does not reach the subchondral bone. Grade III is

represented by a full-thickness lesion exceeding 1cm in diameter and reaching the subchondral bone. Grade IV is the most serious defect, with cartilage erosion and subchondral bone exposure. Although the Outerbridge classification system is widely used both in research and at clinics, it has weak to moderate interobserver reliability and is not well developed for treatment guidance [42].

FIGURE 3 Outerbridge classification system

International Cartilage Research Society (ICRS) cartilage lesion classification system

The ICRS cartilage lesion classification system classifies cartilage lesions by their depth and area (see Figure 4). The depth is graded 0-4 and the area is graded from normal to severely abnormal [43, 44]. ICRS 0 is macroscopically normal cartilage. ICRS 1a is cartilage with an intact surface but softness of the surface or fibrillation while ICRS 1b also includes lacerations and fissures in the surface. ICRS scores of 1a and 1b are classified as nearly normal cartilage. An ICRS score of 2 is considered abnormal cartilage. The cartilage defect in ICRS 2 goes deeper than the surface but is less than 50% of the total cartilage thickness. ICRS 3 is a cartilage lesion that goes deeper than 50% of the total cartilage thickness and is classified as severely abnormal. All ICRS 3 subgroups are lesions that break through different depths of cartilage layer down to the subchondral bone plate. Deep cartilage lesions caused by severe joint trauma can extend down through the subchondral bone and are then classified as severely abnormal and ICRS 4. The arthroscopic ICRS classification system has been proven to show good interobserver and intraobserver reliability [45].

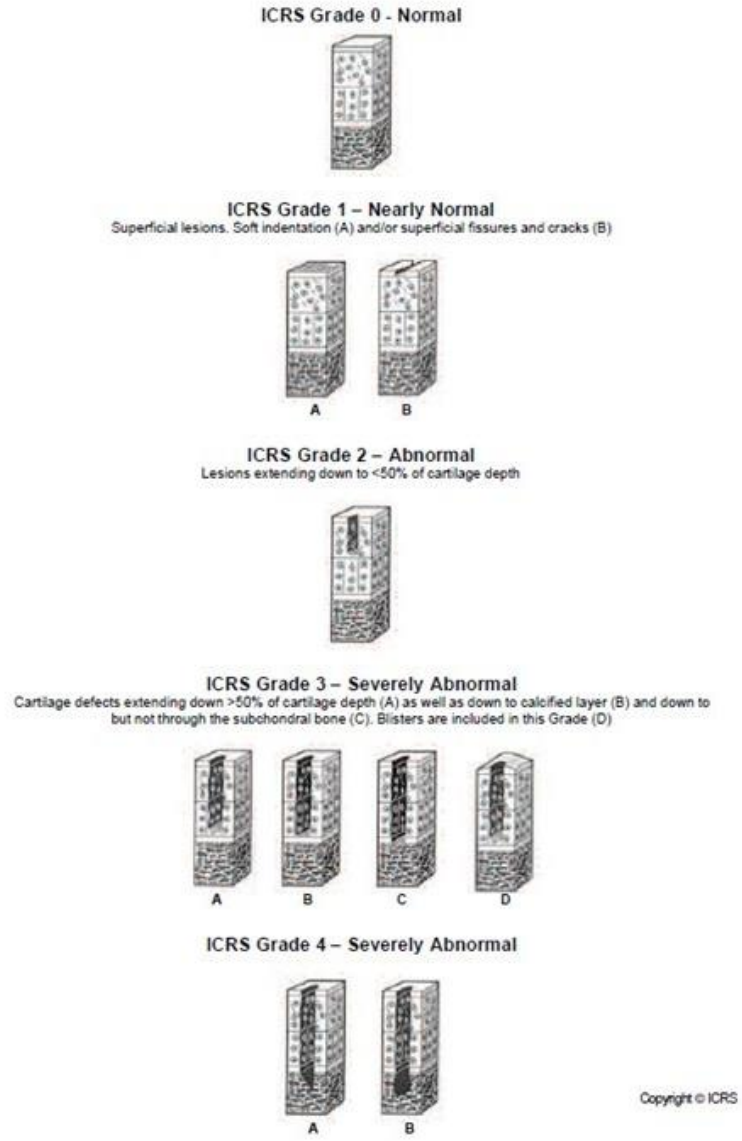

FIGURE 4 ICRS Classification system

Reprinted with permission from ICRS

2.4 Osteoarthritis

Development of osteoarthritis

Osteoarthritis has long been regarded as a degenerative disease of the articular cartilage caused by wear and tear of the cartilage surface of the joint. However, research has shown that OA is not exclusively a disorder of articular cartilage but a disease affecting the whole joint with multiple components that are involved in the development of OA such as inflammation with synovitis, changes in the peri-articular and subchondral bone, as well as the connective tissue [46]. There is an interplay between multiple risk factors that initiates and affects the progression of OA. These risk factors are increasing age, obesity, female gender and joint instability caused by either ligament injury or muscle weakness.

Cartilage degradation

Cartilage degradation can arise from the tissue's own inability to withstand stress, for example if the supply of nutrients and oxygen to the cartilage is limited. The initiation of OA has also been observed when the synthesis of the extracellular matrix (ECM) is incomplete, by the mutation of collagen II, for example [47]. The cartilage and chondrocytes undergo certain biological changes which result in the development of OA. The chondrocytes are activated, cluster and make a phenotypical shift, leading to surface fibrillation, cartilage matrix degradation and the calcification of cartilage, as well as subchondral bone plate thickening and the vascular invasion of the subchondral bone [48, 49]. Parallel to this process, cartilage-degrading proteinases are upregulated, stimulating catabolic activity and eventually apoptosis. There is also an enzymatic degradation of proteoglycans and collagen which causes irreversible damage, and the loss of elastic properties and load-bearing capacity.

Inflammatory response

While OA is not regarded as a classic inflammatory disease with systematic signs of inflammation, the inflammatory response plays an important role in both the disease progression and pain signaling. The infiltration of the synovium by inflammatory cells and signaling molecules leads to excess synovial fluid, joint swelling and stiffness also affecting the peripheral nervous system and nociceptive signaling from the joint [50]. Mechanical stress and inflammation induce the expression of

inflammatory-related and catabolic genes such as nitric oxide synthase-2, cyclooxygenase-2, MMP-1,3 and 13 and ADAMTS-4 and 5 [51-53]. Recent experiments with transgenic mice have demonstrated disease progression by cartilage deterioration via MMP-13 overexpression [54]. Cytokines which are upregulated, produced and accumulated by chondrocytes in clonal clusters play a destructive role in OA. Cytokines which have been seen to be expressed in articular cartilage degradation are IL-1, TNF, IL-17 and IL-18. These cytokines also induce other proinflammatory cytokines and proteinases which all contribute to matrix degradation.

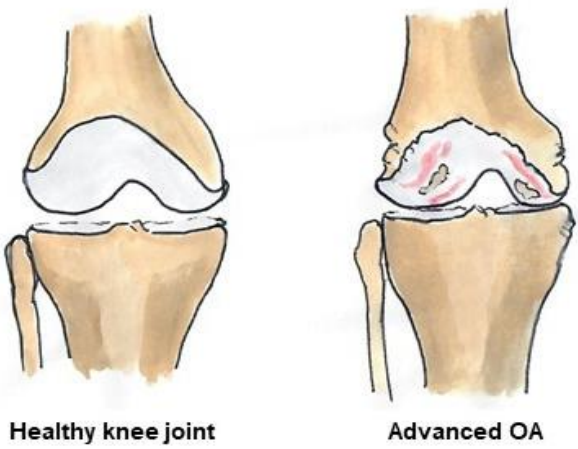

FIGURE 5 OA disease progression

Periarticular and subchondral bone changes

During the disease development, the subchondral bone is also affected. Typical signs of OA progression are subchondral bone thickening and the formation of new bone sprouts called osteophytes, and, in the late stages, a progression to direct bone-to-bone contact which is illustrated in Figure 5 [55, 56]. The erosion of the cartilage, followed by bone contact, synovitis and bone marrow lesions, leads to severe pain and swelling in the patient's joint. Disturbances of the proprioceptive, kinesthetic and vasoregulatory nerves can represent the primary pathogenic events in the joint degeneration seen in OA.

2.5 Diagnostics

The main diagnostic tool for identifying OA is conventional radiography which shows the presence of osteophytes and joint space narrowing (JSN) [57-60]. With conventional radiography, it is also possible quantitatively to measure joint space width (JSW). Semi quantitative scoring systems have been developed to classify stages of OA. The Kellgren and Lawrence (KL) scale is widely used to measure the presence and absence of marginal osteophytes and JSN [61]. The golden standard in OA diagnostics is recording weight-bearing antero-posteriorly (AP) and lateral X-rays of knee joints and grading according to the KL scale using grades 0 to 4 (0 = normal, 1 = doubtful pathology, 2 = minimal osteophytes, possible narrowing, cysts, and sclerosis, 3 = moderate, such as definite osteophytes and joint space narrowing, 4 = severe, with large osteophytes and definite joint space narrowing) .

The Osteoarthritis Research Society International (OARSI) instead grades tibiofemoral JSN and osteophytes separately for each compartment of the knee. This grading system is based on six grades which take account of the depth of the lesion and the progression of OA [62].

There have been several studies that have aimed to develop OA diagnostics using radiography [63, 64]. There is a correlation between radiographic findings and OA disease, but patients with painful OA early in the disease process in particular might not have any radiographic changes and studies show that the correlations between radiographic evidences of OA and pain are weak [65, 66].

Magnetic resonance imaging (MRI) offers several advantages as an imaging tool compared with conventional radiography because it is able to visualize cartilage and its pathologic changes. The progress of OA can be followed using MRI and semi-quantitative scoring approaches; the Whole Organ MRI Score (WORMS), the Knee Osteoarthritis Scoring System (KOSS), the Boston Leeds Osteoarthritis Knee Score (BLOCKS) and the MRI Osteoarthritis Knee Score (MOAKS) [67]. A number of MRI techniques have been developed to characterize cartilage qualitatively and to quantify cartilage volume [68-71]. MRI is, however, seldom used for the diagnosis of OA patients because of the high costs and long waiting times for examination.

There have been few publications suggesting ultrasonography examination as an alternative tool for the diagnosis of OA [72, 73]. There are multiple advantages to using ultrasonography. Ultrasonography produces a multi-planar image, involves no radiation and is a cost-effective diagnostic tool.

Scintigraphy has been shown to predict loss of joint space in patients with established OA of the knee joint. This finding could be used to detect increased activity of the subchondral bone that may indicate a future fast OA development. The simple scintigraphy technique has been the base for further development of PET- and SPECT-scans to be used in OA diagnostics [74, 75].

2.6 Sources of pain in the OA joint

Joint pain is mediated through many different signaling pathways involving local pain signaling through neuropeptides and nociceptors. Peripheral signaling occurs through mostly unmyelinated C-fibers innervating the joint capsule, ligaments, periosteum, subchondral bone and partly the meniscus to the central signaling involving the spinal dorsal horn [76, 77]. Figure 6 illustrates the innervation of the knee joint.

To further analyze the origin of pain in joints, the origin can be defined as either peripheral or central sites. Many studies have shown that OA patients have lower pain thresholds at multiple body sites [78, 79]. Other studies have focused on finding the correlations between join space narrowing, and assessments of cartilage loss by radiographic studies with clinical measurements of pain [80].

The main question is where the pain arises from, since the cartilage is a non-innervated tissue. There have been studies showing that peripheral sources of pain are correlated to nerves in the synovium and bone. Torres *et al.* correlated the flattening of articular surfaces to pain severity [81]. The subchondral bone, a highly vascularized bone lamellar region immediately underlying the articular cartilage, is also involved in OA. Unmyelinated free nerve endings are present in the subchondral bone and could presumably be involved in pain signaling in OA.

There are also multiple mechanisms of pain sensitization modifying the pain that is experienced involving not only the peripheral and central nervous system but also the brain. Cognitive and psychological factors

have been shown to play an important role in the way pain is experienced. Prognostic factors for higher levels of pain in the future were a higher level of knee pain at base line, bilateral knee symptoms, female gender and depressive symptoms [82-87]. Another strong prognostic factor for OA development is obesity [88-91]

Possible sources of pain in patients with OA include [79]

- Synovial membrane
- Joint capsule
- Periarticular ligament
- Periosteum
- Subchondral bone

The subchondral bone related causes of pain include

- Osteophyte formation
- Subchondral micro fractures
- Bone angina due to decreased blood flow + elevated intra medullary pressure
- Bone marrow edema (BME).

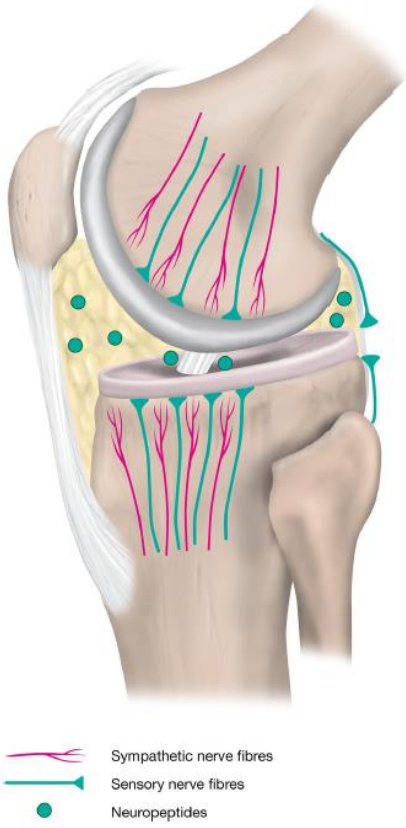

FIGURE 6 Schematic illustration of innervation and vascularization in knee joint pain

An illustration of proposed innervation and vascularization, as well as neuropeptide distribution in joint pain development.

Modified illustration from Grassel S, Muschter D (2017) Peripheral nerve fibers and their neurotransmitters in osteoarthritis pathology. Int J Mol Sci 18:931

Figure from Knee Surgery Sports Traumatology Arthroscopy. 2019 Mar;27(3):942-949.

2.7 Biological signaling and neuropeptides

Neuropeptides are a large and very diverse group of neurosignaling molecules. Definition wise, neuropeptides should be produced and genetically coded by neurons, released by the depolarization of sensory and autonomic nerve terminals and bound to neuronal receptors [92]. In the mammalian genome, it has been found that there are more than 70 genes coding for neuropeptides and their precursors [93].

Neuropeptide-containing nerves could be considered to play an important role in pain genesis in OA due to their both sensory and regulatory role. Neuropeptides of interest are calcitonin gene-related peptide (CGRP), substance P (SP), vasoactive intestinal peptide (VIP) and neuropeptide Y (NPY). Neural transmitters that are thought to be involved in the development and maintenance of pain have been suggested to be involved in these regulatory loops [94-97]. In addition to this, it is known that chondrocytes have receptors for substance P [98, 99].

Neuropeptides	**Biological functions**
SP	Stimulates MMP13 and matrix degradation [100]
	Exercise-induced modulation of the immune system [101-103]
CGRP	Exercise-induced modulation of the immune system [101-103]
	Proinflammatory (TNF alpha) [104]
	Increase in bone mass, stimulation of osteoblast differentiation [105-107]
VIP	Exercise-induced modulation of the immune system [101-103]
	Immunomodulator, drives macrophages towards anti-inflammatory polarization [108, 109]
NPY	Decrease in bone mass, reduced bone mineral density [110, 111]

2.8 Role of subchondral bone

OA was initially thought to be isolated to morphological changes in cartilage, but it is now regarded as a whole joint disease where the subchondral bone plays an important role in pain and disease development [112-117].

The subchondral bone involvement in the cartilage damage and repair process might be much wider than currently believed and its involvement could be part of the development of OA and the following pain genesis. Numerous researchers have demonstrated the existence of several channels between the subchondral region and cartilage [118, 119]. When the fine homeostatic balance is disturbed, due to a cartilage lesion for example, the local secretion of neuropeptides in the subchondral region may occur and the neuropeptides could be transported via the channels to the cartilage and subsequently directly influence receptors on the chondrocytes [120-122]. A similar scenario may be involved in osteoarthritic pain [123-127].

2.9 Treatment alternatives for early OA

Young patients with early OA signs and large osteochondral defects are today a clinical challenge much due to the limited treatment possibilities. Isolated traumatic cartilage lesions have been treated successfully with biological treatments such as microfracturing, autologous chondrocyte implantation (ACI) and osteochondral transplants. Microfracturing is the surgical technique in which the bone marrow is stimulated by drilling down into subchondral bone, forming a clot and stimulating the migration of stem cells [128, 129]. ACI is a two-step surgical process which involves arthroscopic biopsy collection and then after a process of cell culturing implantation of chondrocytes [130]. Reconstruction with an osteochondral plug is a single step surgical process in which an osteochondral plug is harvested and placed into the lesion site [131]. Large cartilage lesions are, however, commonly more painful, many times proceeding to manifest OA and research so far shows failure in biological treatments [132]. Early biological or regenerative treatment with the possibility of hindering progression towards manifest OA would have huge socio-economic benefits.

There has been research driven towards finding new biological surgical approaches towards treating early OA. Osteochondral cell-free scaffolds

have been tested on femoral defects showing pain relief and a stable five-year follow-up [133]. ACI has also been suggested as a treatment for early OA lesions, clinical studies have shown promising results [134].

3. Aims

The primary aim of this thesis has been to investigate the role of neuropeptides in articular joint pain and osteoarthritis. Furthermore, the thesis investigates 3D modeling and different imaging techniques for the diagnosis of early cartilage and subchondral bone changes in OA.

The specific aims of this thesis were:

- To develop a method for measuring neuropeptides in cartilage and subchondral bone using peptidomics
- To map morphologic changes in OA using imaging techniques
- To improve non-invasive diagnostic imaging by developing a method for the 3D modeling of chondral and subchondral changes in OA
- To compare different imaging techniques in rendering 3D models
- To use 3D models to develop potential future treatments for pre-OA, early OA and osteochondral lesions
- To correlate patient-reported outcome using the KOOS with morphologic cartilage and subchondral bone changes

4. Methods

4.1 Peptidomics

The ability to identify and quantify large numbers of proteins from biological samples by mass spectrometry (MS) in combination with and liquid chromatography (LC) has initiated the field of proteomics, enabling an unbiased, hypothesis-generating approach to studying disease [135-137].

In the most commonly used workflow for proteomics, proteins are extracted from the biological sample, followed by reduction and alkylation of cysteine disulfides, and proteolytic digestion of the proteins, often using trypsin, to peptides that are analyzed by LC-MS [138]. The mass spectrometer is operated in the data-dependent mode, recording fragment ion (MS/MS) spectra of as many detected peptides as possible. Using bioinformatics software, these MS/MS spectra can then be used to identify the peptides in protein sequence databases and map them to their protein precursors. Many MS-based identification and quantitation tools have been developed in recent years [139-141].

Peptidomics differs from proteomics in two aspects: the preparation process in peptidomics targets at enriching small naturally occurring peptides and no proteolytic digestion is used in the sample preparation. In peptidomics, it is crucial that the selected MS method has both an accurate mass measurement and provide tandem mass fragmentation to give additional information on peptide sequencing [142].

Peptidomic research in the field of OA has been conducted predominantly on synovial fluid and synovium. Studying bone and cartilage has been challenging due to the high mineral composition of the tissue and the dominance of collagen in cartilage extracellular matrix. This makes it difficult to find suitable protocols for peptide extraction.

In Study II, we developed a protocol for peptide extraction from cartilage and subchondral bone. Tissue samples were de-identified and collected as left-over tissue from total knee replacements (TKA). The sampling process was approved by the patients and followed guidelines approved by the Ethics Committee at the University of Gothenburg, Sweden. Tissue

samples were brought directly from surgery to the laboratory and an osteochondral plug was drilled out of the femoral condyle using a T-Lok bone marrow biopsy needle (Argon Medical Devices, Frisco, TX, USA).

Three osteochondral plugs were taken from areas with unwounded cartilage (UOA) and three samples were taken from wounded areas with severe OA (WOA), illustrated in Figure 7. In order to extract endogenous peptides, the osteochondral plugs needed to be demineralized and treated with a high concentration (1.2mol/L) of HCl overnight. The demineralized samples and supernatant were collected and isotopically labeled with tandem mass tag (TMT) reagents for multiplex quantification, and analyzed by LC-MS/MS on a high-resolution Orbitrap mass spectrometer. Figure 8 shows a typical fragment ion mass spectrum of samples analyzed in Study II.

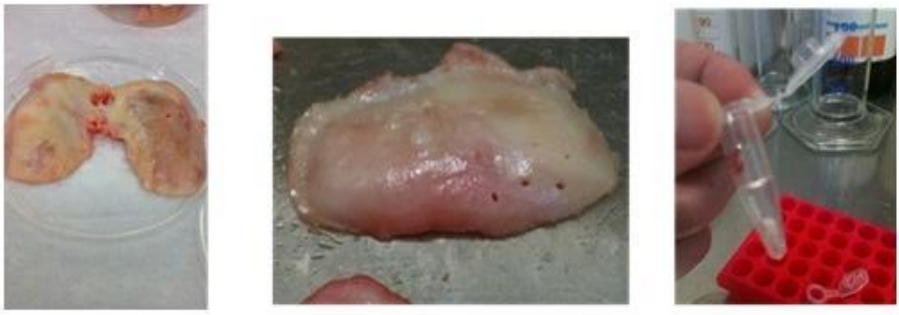

FIGURE 7 Osteochondral plug drilling

Plugs were taken from, on the left, wounded OA areas (WOA) and, in the middle, from unwounded cartilage (UOA). Picture to the right shows the size of an osteochondral plug.

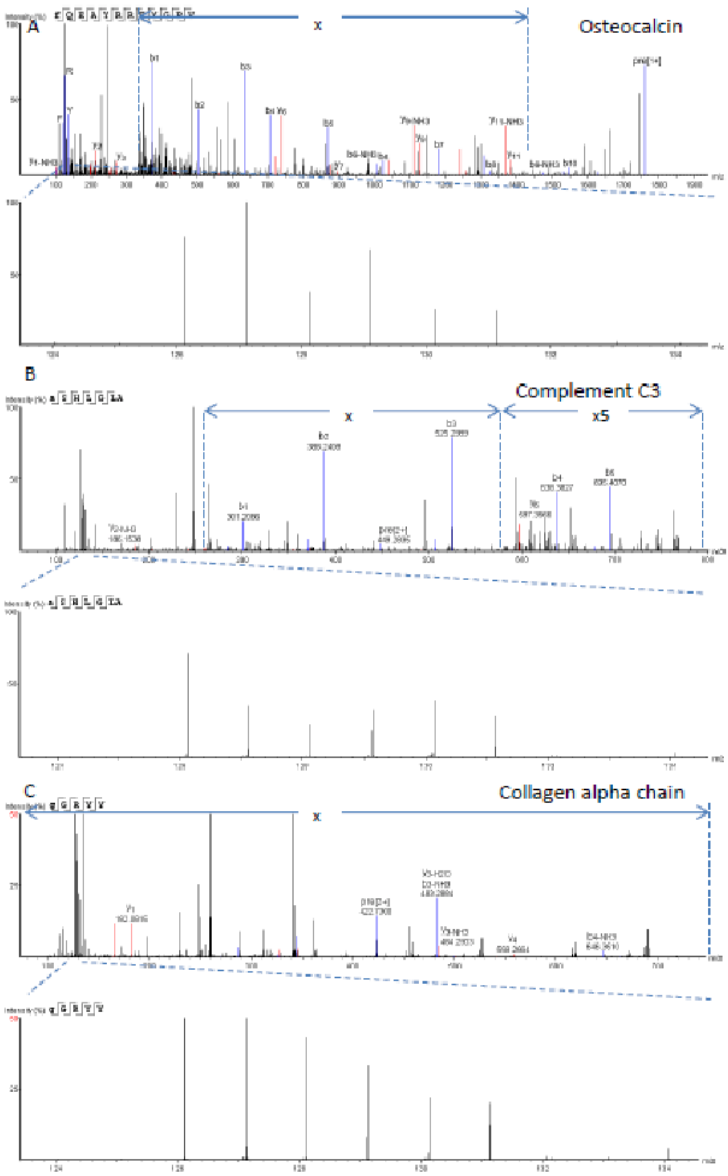

FIGURE 8 Fragment ion mass spectra
Identification of endogenous peptides (A) Osteocalcin 89-100, (B) Complement C3 742-747 and (C) Collagen alpha chain 1212-1216.
Figure from European Journal of Clinical Investigation.
2019 May; 49(5):e 13082.

Data analysis

In Study II, peptide identification was performed using PEAKS Studio (Bioinformatics Solutions Inc, Canada). The following settings were used: database: UniProt/SwissProt; taxonomy: homo sapiens; parent mass error tolerance: 20 ppm; enzyme: none; fixed modifications: carbamidomethylation, TMT6-plex; variable modifications: oxidation of methionine. Peptide identification was validated by using a target-decoy approach with a target false discovery rate (FDR) of 5% [143]. Software Proteome Discoverer 2 (Thermo Fisher Scientific, USA) was used to determine TMT reporter ion intensities. MSCluster was used to match spectra of the same peptide in different TMT sets. Peptide identification results from PEAKS were used to annotate the cluster list [144].

4.2 Micro-computed tomography

Micro-computed tomography (microCT or µCT) is a medical imaging technique but it has many applications beyond the medical field. Similar to computed tomography (CT), microCT uses radiation to scan layer by layer (slices) through the objects and collect pixels or voxels which are volumetric data points in the micrometer range. Microtomography scanners offer isotropic resolution which means that the display of images does not need to be restricted to the conventional axial images. It is possible for a software program to build a volume by 'stacking' the individual slices, one on top of the other. Different volume rendering techniques have been developed to create 3D images from many scans which can then be converted by a computer into 3D models.

MicroCT is able to provide 3D quantitative analyses of the morphology of tissues at micron-level and it has therefore been used extensively for analyzing the microstructure of bone. Soft tissues such as cartilage are, however, undetectable by microCT due to its low X-ray attenuation, and it is impossible to segment cartilage from other soft tissues in these images. One possible solution for imaging cartilage using microCT is to use contrast agents. The idea of using contrast agents to visualize cartilage has developed from the magnetic resonance imaging field. Back in 1992, Kusaka *et al.* reported that MRI could be used to show the uptake of manganese or gadolinium in cartilage dependent on proteoglycan distribution [145]. This technique has been developed by several scientists who were able to visualize proteoglycan depletion using a gadolinium probe and MRI [146, 147]. A gadolinium contrast agent was successfully

used for the quantitative imaging of proteoglycans with microCT in bovine cartilage [148]. Palmer *et al.* showed that the analysis of cartilage matrix can be performed by detecting the equilibrium partitioning of an ionic contrast agent via microCT (EPIC-microCT) [149]. The negatively charged contrast agent used in the study was ioxaglate (Hexabrix), which was shown to diffuse where GAG depletion had occurred. The distribution of the contrast agent was shown to be inversely related to the density of negatively charged GAGs after equilibrium was achieved [149, 150]. Negatively charged GAGs such as sulfated GAGs (sGAG) are important for maintaining the lubricant properties of the cartilage and the loss of sGAGs is an early indication of changes in cartilage related to OA [151].

MicroCT with an ionic negatively charged contrast agent (EPIC-microCT) is a potentially very powerful tool not only for imaging GAG distribution in cartilage but also for determining cartilage 3D morphology. In recent years, the EPIC technique has been evaluated for the analysis of human cartilage in preclinical and clinical settings. Research has shown that there is a difference in the diffusion and X-ray attenuation of Hexabrix in healthy and mechanically damaged cartilage [152]. There are several recent reports on the use of contrast agent combined with CT in clinical settings for the analysis of cartilage. This technique is defined as enhanced computed tomography (CECT) [153-156] . In a recent paper, the simultaneous quantitation of cationic and non-ionic contrast agents in articular cartilage was accomplished using synchrotron microCT imaging [157].

In Studies III and VI, we used an µCT 100 instrument from Scanco Medical, Switzerland (see Figure 9) to analyze the joints. This instrument is designed for handling larger samples and offers a very wide field of view while maintaining high imaging resolution. The maximum scan size is 100 x 140 mm (ØxL) and the maximum specimen size is 100 x 160 mm (ØxL). Variable filter and X-ray power settings greatly increase the range of materials that can be scanned.

FIGURE 9 The Micro-CT (µCT 100) instrument from Scanco Medical, Switzerland used in the studies

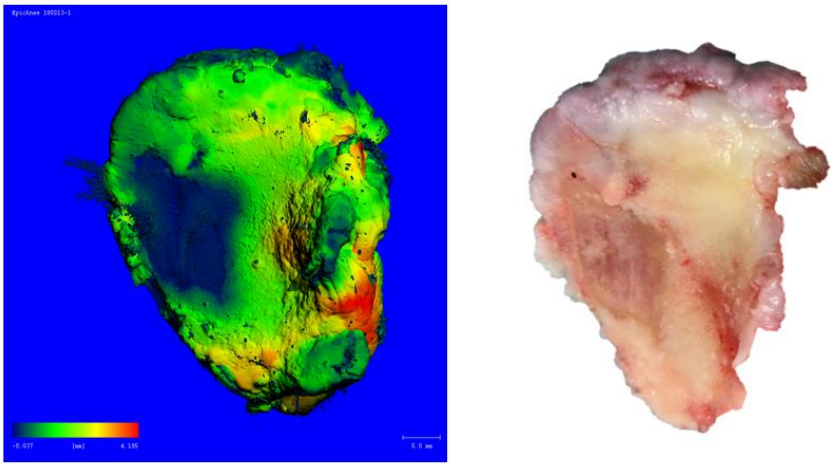

FIGURE 10 EPIC microCT Imaging

Thickness map of cartilage (left image) obtained by EPIC microCT of a medial condyle from a tibia plateau with severe OA (right image).

4.3 3D Imaging of an OA defect site

MicroCT is a powerful imaging tool when it comes to providing 3D geometry and investigating the morphology of objects with micron scale resolution. The scanning process takes a long time, however, and scans contain an enormous amount of data which need to be filtered in order to convert the data into a 3D model. EPIC microCT imaging of the joint provides information on bone volume, bone porosity, the amount of hydroxyapatite in bone tissue and cartilage volume and thickness. A typical OA joint exhibits substantial cartilage loss all the way down to the bone (see figure 10).

In Studies III and IV, tibial plateaus were retrieved from patients with OA who underwent total knee arthroplasty surgery (TKA) at Sahlgrenska University Hospital, Gothenburg, Sweden. De-identified tissue sampling followed a procedure approved by the ethical committee in Gothenburg.

In Study IV one tibia plateau was then scanned using imaging tools which are relevant in the clinical setting. The tibia plateau was scanned using MRI, CT and a handheld 3D scanner.

The following parameters were used for the MRI scan (Philips Ingenia 3 Tesla): coil: wrist coil, scan type: 3D, technique: SE (Spin Echo), TE: shortest, flip angle: 90, TR: 1500 ms FOV FH: 180 mm; AP: 180 mm; RL: 100mm.

The following parameters were used for the CT scan (Siemens SOMATOM Force): total: 1781 mAs; total DLP: 56 mGycm; scan 1: 120kV, mAs/ref: 19 mA; CTDiVol (mGy): 0.07L; DLP (mGycm): 1.5; Ti: 2.3 s; cSL: 0.6mm; Scan 2: 100 kV, mAs/ref: 19 mA; CTDiVol (mGy): 0.04L; DLP (mGycm): 1.0; Ti: 2.5 s; cSL: 0.6mm; Scan 3A: 70 kV; mAs/ref: 200 mA; Scan 3B: Sn150 kV; mAs/ref: 50 mA. CTDiVol (mGy): 4.66L; DLP (mGycm): 53.9; Ti: 2.5 s; cSL: 0.6 mm.

For the 3D scanner, the following apparatus was used: TRIOS 3 wireless, 3Shape A/S, Copenhagen, Denmark.

4.4 3D model

MRI and CT scans provided 3D data of the cartilage in a manner similar to microCT, but with a lower resolution. The scans of cartilage using both imaging tools still contained an extremely large amount of data. Data were saved in a Digital Imaging and Communications in Medicine (dicom) format, which is the global standard for providing medical imaging information data.

MicroCT and other 3D imaging tools collect point cloud which can be used to generate 3D models. Computer aided design (CAD) involves the use of computer systems with the aim of generating, modifying and designing 3D models. The process of capturing the 3D object and transforming it into a 3D virtual model is called the reverse-engineering process since the traditional engineering process converts a 3D model into a 3D object.

Figure 11 shows the work flow in the reverse-engineering process for creating a CAD model of an OA tibia. Once the model is created, it can then be used for the fabrication of a replica using additive manufacturing (AM)

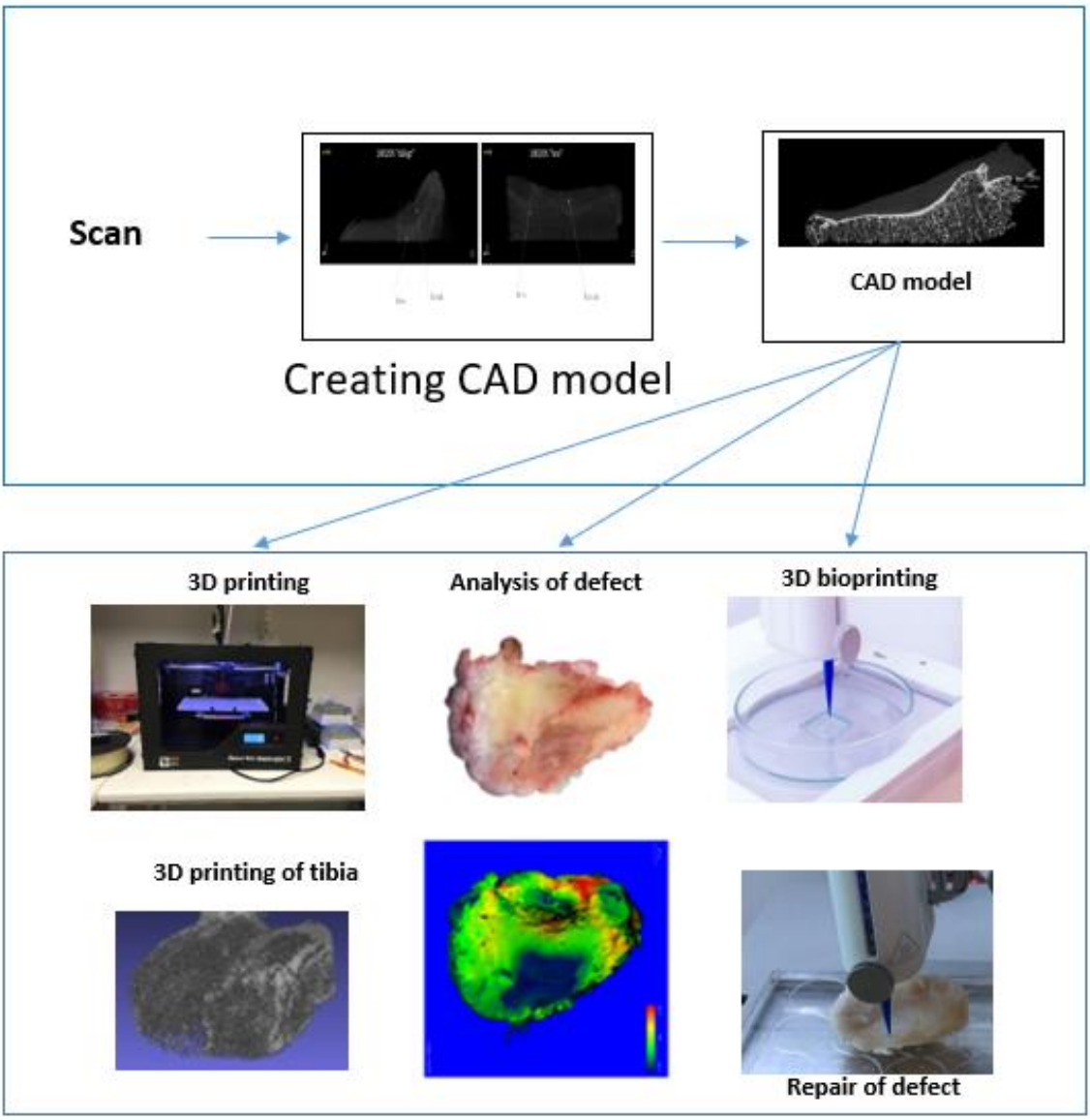

FIGURE11 Creating a CAD model

Work flow of reverse-engineering process for creating a tibia CAD model and using a CAD model.

tools such as 3D printing. A CAD model can also be used for biomechanical simulation. A recent development in AM technology, 3D bioprinting, also makes it possible to repair the cartilage using human cells and supporting biomaterial.

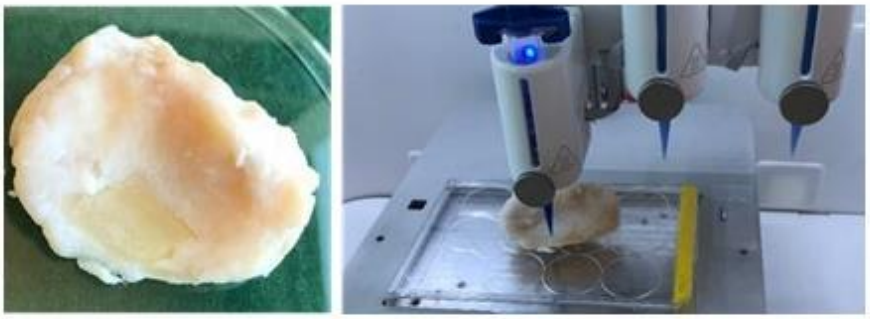

FIGURE 12 3D bioprinting

Tibial plateau with OA defect (left) and 3D bioprinter repairing cartilage with human chondrocytes and supporting biomaterial (right).

4.5 3D bioprinting

3D bioprinting technology enables the biofabrication of living tissues and organs. The principles resemble those in 3D printing, but, instead of fusing or melting material layer by layer into a 3D object, the liquid bio-ink is deposited in a predetermined position. Bio-inks are typically hydrogels, synthetic or natural, and can be mixed with human cells. A typical 3D bioprinter can contain several printing heads enabling the deposition of several cell types. The motion of bioprinting is controlled by a computer language called G-code. The first step in the preparation of G-code was the conversion of the point cloud in dicom files into stereolithography (stl) files which represent triangular-faced mesh.

For the reverse-engineering process, the stl file obtained from the 3D portable scanner was used. The digital repair of the OA defect in the model of the tibial plateau was generated after 3D scanning and using CATIA V5 (Dassault Systèmes, France) software. The OA defect with surrounding

area was focused on and from this a surface model was created. The OA defect was then sketched out of the surface model. A copy of the OA defect surface was translated to level out with the healthy cartilage. By defining the boundaries of the OA defect surface and the translated OA defect surface the final surface for creating a closed solid volume was found. Figure 12 shows the tibial plateau with an OA defect (left) and a 3D bioprinter repairing cartilage with human chondrocytes and supporting biomaterial (right).

4.6 Patients

Study VI

Patients with advanced OA scheduled for total knee arthroplasty (TKA) surgery from April 2019 to July 2019 at Sahlgrenska University Hospital, Sweden were offered the chance to participate in the study. The patients were interviewed preoperatively about their knee function, earlier injuries and surgeries and were asked to fill out Knee injury and Osteoarthritis Outcome Score (KOOS) questionnaire preoperatively and 8-10 weeks postoperatively. Data on age and gender were collected. The exclusion criteria were anything that made the patient un-able to fill out the KOOS questionnaire, such as reduced eyesight, dementia, and limited linguistic skills in the Swedish language. In addition to this, patients with a diagnosis of rheumatic Arthritis were excluded. Fourty-seven patients were included in the study. The patients were aged 46 to 83 years with a mean age of 71.2 (SD 8.9).

4.7 Knee injury and Osteoarthritis Outcome Score (KOOS)

The KOOS is a well-established patient-reported outcome measurement (PROM) questionnaire used in both research and clinical settings. The KOOS is used to evaluate patients with both traumatic knee injuries and OA. The questionnaire evaluates the patients' function both in daily life and during recreation and sport activities. The KOOS is divided into five different subscales, namely pain, other symptoms, function in daily living (ADL), function in sport and recreation (Sport/Rec) and knee-related quality of life (QOL). For each subscale a normalized score is calculated (100 equaling no symptoms and 0 equaling extreme symptoms).

4.8 Ethics

Study I No ethical approval is needed in a narrative review.

Study II used patient tissues left over after TKR surgery. Tissue sampling was approved by patients and followed a procedure approved by the Regional Ethics Review Board Gothenburg, Sweden. Since samples were de-identified, the need for ethical approval was waived.

Study III used twelve tibia plateaus which were retrieved from patients with advanced OA who underwent TKR surgery at Sahlgrenska University Hospital, Sweden following a procedure approved by the Regional Ethics Review Board Gothenburg, Sweden. Since samples were de-identified, the need for ethical approval was waived.

Study IV used the tibial plateau retrieved from a patient with advanced OA who underwent TKR surgery at Sahlgrenska University Hospital, Sweden, following a procedure approved by the Regional Ethics Review Board Gothenburg, Sweden. Since sample was de-identified, the need for ethical approval was waived.

Study V used cartilage from the tibia plateau which was retrieved from a patient with advanced OA who underwent TKR surgery at Sahlgrenska University Hospital, Sweden, following a procedure approved by the Regional Ethics Review Board Gothenburg, Sweden. Since sample was de-identified, the need for ethical approval was waived.

Study VI is a clinical prospective cohort study of patients diagnosed with advanced OA who underwent TKR surgery at Sahlgrenska University Hospital, Sweden. The study was approved by the Regional Ethics Review Board Gothenburg, Sweden (Dnr: 713-17). All the patients were informed orally and with written forms preoperatively. All the patients gave their informed consent prior to their inclusion in the study.

4.9 Statistical methods

Study II

The data were log transformed to be normally distributed. Statistical significance for the difference between mean values for the peptide abundance from unwounded (UOA) and wounded (WOA) zones (90%

significance level $p \leq 0.1$) was tested using a two-tailed paired t-test. The peptidomic scan generated a large amount of data and due to this and multiple hypothesis testing, a multiple testing adjustment analysis was made with Holm's and Hochberg's method.

Study III

The correlation between photographically examined surface fraction and microCT analysis was evaluated by fitting a linear model with the lm function. Staining dynamics was evaluated by a sigmoid function that was fitted using nonlinear least squares. Morphometric data were analyzed with one-way ANOVA performed using the aov function. Confidence levels of $p < 0.05$ and $p < 0.01$ were considered statistically significant and highly significant respectively. All analyses were performed in the R programming language (version 3.3.3).

Study VI

For correlation testing between KOOS pain, defect surface fraction (Def.S/Cond.S) and cartilage surface fraction (Cart.S/Cond.S) as well as between KOOS QoL, defect surface fraction (Def.S/Cond.S) and cartilage surface fraction (Cart.S/Cond.S) Spearman's correlation test was used. The data were analyzed using version 9 of the SAS system for Windows. For the morphometric microCT data one-way ANOVA were performed using the aov function. All microCT data analyses were carried out using the R programming language (version 3.3.3)

5. Summary of Studies

Study I is a review which provides an overview of the field of joint pain and potential mechanisms for joint pain development with special emphasis on neuropeptides.

Study II is a pilot study describing the development of a method for analyzing endogenous peptides using LC-MS/MS in cartilage and subchondral bone. The pilot study used tissues from six patients with OA undergoing TKA.

Study III describes the 3D imaging and modeling of the morphologic changes in the cartilage and subchondral bone of OA patients using EPIC microCT by developing a method that is reproducible and semi-automatic.

Study IV (manuscript accepted for publication after major revision) compares different imaging techniques and uses the 3D images to produce a CAD model for visualizing the osteochondral lesion and performing a cartilage damage repair by 3D bioprinting with chondrocytes.

Study V (in manuscript) further investigated cartilage damage repair. We created a micromass of pellets using chondrocytes or chondrocyte-derived iPSCs with or without OA extracellular matrix (ECM) derived from OA patients to investigate the effect of OA ECM on chondroinductivity.

Study VI (in manuscript) is a clinical prospective study of 47 patients with knee OA. It includes the pre- and post-operative monitoring of patients' experienced pain with the KOOS and their correlation to morphological changes in cartilage and subchondral bone using EPIC microCT.

5.1 Study I

Aim: The aim of this study was to investigate what is known about the mechanism of joint pain with special emphasis on the role of neuropeptides in pain transmission and their potential role in the progression of OA.

Introduction:

Joint pain is one of the first symptoms of OA. There are several possible sources including: the synovial membrane, joint capsule, periarticular ligaments, periosteum and subchondral bone [121]. The pathologic changes in the subchondral bone and synovial lining induce pain by irritating sensory nerve endings. This results in the release of cytokines, leukotrienes and prostaglandins [97, 158]. The neuropeptide-containing nerves in the joint tissue represent an important regulatory pathway. Disturbing joint homeostasis results in the release of local auto/paracrine factors. Neuropeptides are small proteins which are used by neurons for communication. They function as neurotransmitters and are found in terminals of sensory and autonomic nerves. The depolarization of these nerves results in the release of neuropeptides. Neuropeptides bind to free nerve endings and change the afferent sensitivity[159].

Methods:

Papers published between January 1990 and September 2017 were searched using the following databases: Web of Science Core, MEDLINE/PubMed and Scopus. The following keywords were used in the search: joint pain and neuropeptides, neuropeptides and inflammation and osteoarthritis, neuropeptides and osteopenia and osteoarthritis, neuropeptides and bone formation and osteoarthritis and neuropeptides and vascularization and osteoarthritis.

Study selection: Existing neuropeptides were evaluated and grouped into six categories; neuropeptides in general, in inflammation, in physical activity, in bone involvement, in vascularization and, finally, in the central nervous system (CNS). The quality of literature on pain in relation to neuropeptides was evaluated by assessing the level of evidence of the selected scientific papers.

Results:

Figure 13 illustrates pain signaling pathways involving neuropeptide release in the wounded tissue to signaling through peripheral sensory fibers, dorsal root ganglion and through the dorsal horn communication and activation of pain centers in the brain. One important finding in this review was that what is seen in the subchondral bone and synovia mirrors actions in the CNS. Furthermore, there is no single neuropeptide that could be used as a marker of the degree of pain in the joint. However, SP, CGRP, VIP and NPY are the major peptides involved in the generation of pain. The interplay between them and other neuropeptides and cytokines influences the way noxious stimuli are transduced, transmitted and modulated for a final pain perception as part of a complex cascade of events. The immune system is also involved in these signaling cascades and might subsequently stimulate the release of endogenous opioids by opioid-containing immune cells, which might become a means of treating joint pain.

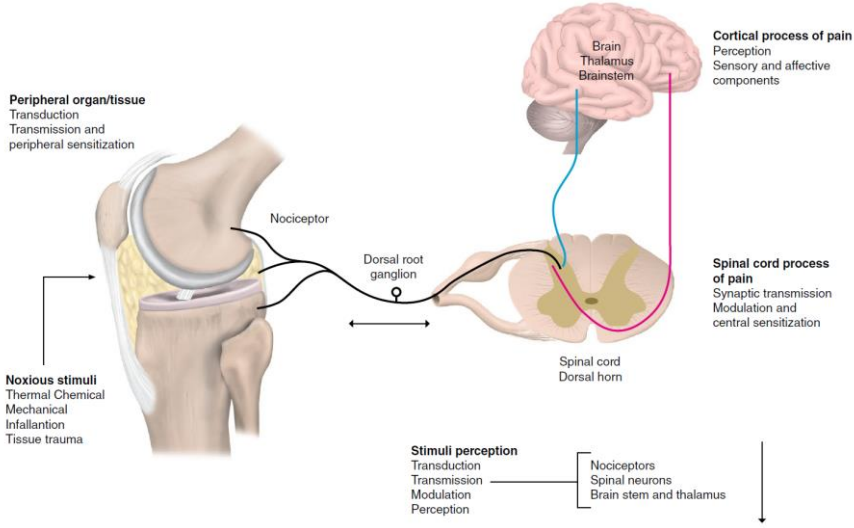

FIGURE 13 Pain signaling pathways

Illustrating peripheral signalling pathways in the joint and central signalling pathways through dorsal root ganglion, dorsal horn communication and activation of pain centra in the brain.

Figure from Knee Surgery Sports Traumatology Arthroscopy. 2019 Mar;27(3):942-949.

Conclusions:

The articular joint should be seen as an organ where local joint pain development and maintenance is influenced by interplay between the local transmitters in the joints and their dependence on the CNS.

5.2 Study II

Aim: The aim of this study was to develop a new method for directly analyzing osteochondral samples which could be performed in the operating room without cell culture. A method of this kind would enable the identification of potential peptide biomarkers to elucidate the mechanisms involved in the development of osteoarthritis and pain.

Introduction:

Due to the low sensitivity of current diagnostic methods, there has been increasing interest in findings biomarkers to detect pathologic developments in the osteoarthritic joint, by analyzing synovial fluid, serum and plasma [160-163]. The high mineral composition of bone and the dominance of collagen in the extracellular matrix of cartilage mean that traditional extraction protocols are not useful. Solid bone and cartilage tissue have therefore been difficult to study using protein and peptide analysis methods. The biomarkers in the fields of cancer and neurodegenerative diseases have been successfully characterized and quantified with peptidomics [142, 164]. Peptidomics enable the analysis of protein fragments by using mass spectrometry. Peptides play a key role in many regulatory processes. They are both hormones and signaling molecules that are active as endogenous peptides, where the largest group is neuropeptides.

This pilot study explored the potential for performing peptidomic analysis directly on knee joint tissue samples in order to identify peptides that may be involved in pain signaling and can also serve as biomarkers of OA. In order to be able to conduct an analysis, we developed a method for peptide extraction. The endogenous peptides extracted from osteochondral biopsies taken from wounded and macroscopically non-wounded cartilage areas in osteoarthritic knee tissue samples were analyzed by liquid chromatography mass spectrometry (LC-MS) using the tandem mass tag (TMT) technique for quantification. The method introduces the potential for broadening the search and identification of biomarkers, as well as extending our knowledge of pain and pathologic mechanisms involved in OA.

Methods:

Osteochondral plugs from wounded and macroscopically unwounded zones of the femoral condyle were collected from six patients with manifest osteoarthritis (OA) who were undergoing TKA. Figure 14 shows the schematic workflow of the steps in the analysis of cartilage and subchondral biopsies from OA patients. The samples were demineralized and supernatant was collected and isotopically marked with tandem mass tag (TMT) labeling and analyzed using liquid chromatography coupled with tandem mass spectrometry LC-MS/MS.

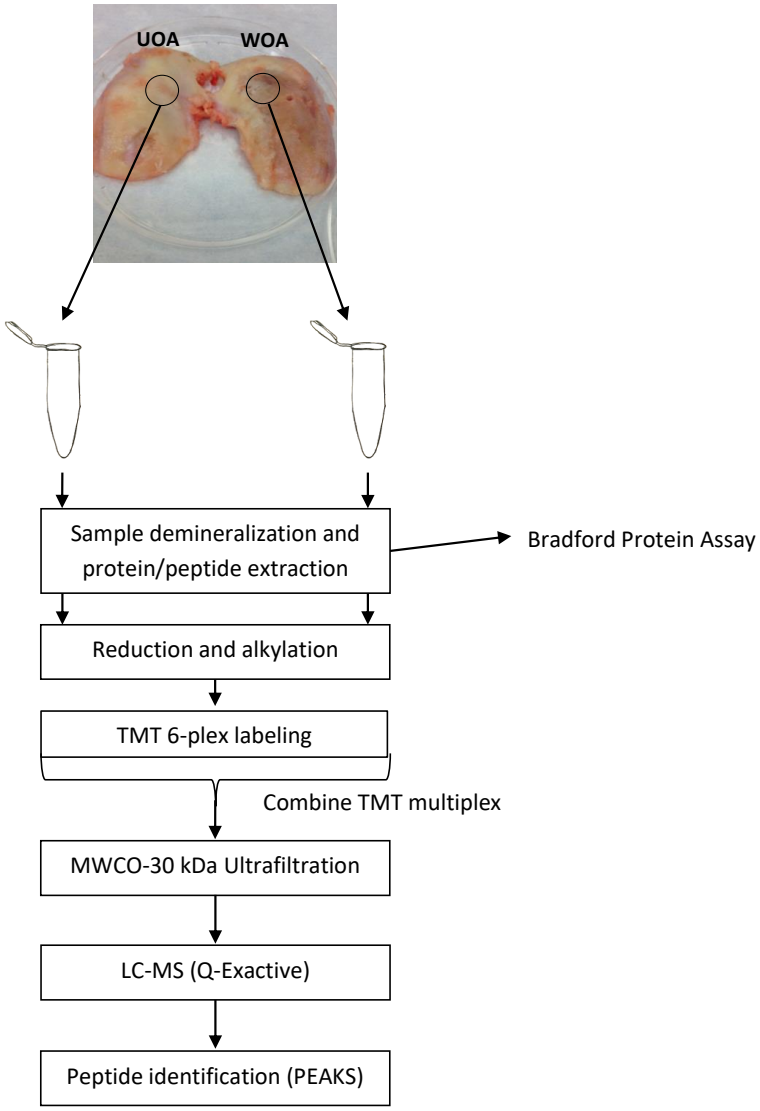

FIGURE 14 Schematic workflow of peptidomic analysis of cartilage and subchondral biopsies from OA patients

Samples from six patients were included in the study. Three biopsies were taken from wounded (WOA) and unwounded (UOA) zones. Peptides were extracted and iTRAQ labeled. Labeled samples were then fractioned using ultrafiltration and analyzed on an LC-MS (Q-Exactive). Figure from European Journal of Clinical Investigation. 2019 May;49(5):e 13082.

Results:

Extracts of endogenous peptides, which were collected after molecular weight cut-off (MWCO) ultrafiltration, were analyzed by LC-MS in the data-dependent mode. The peptide identification was then performed by database searching.

A total of 6,292 endogenous peptides were identified, derived from 915 proteins (889 protein groups). Of these, 601 peptides (derived from 156 proteins) carried a TMT label and 462 of the identified peptide chains could be matched with the database used for identification.

A total of 566 endogenous peptides, in the six patients included in the study, were found to differ significantly with a P-value of ≤ 0.1 in unwounded zones compared with wounded osteoarthritic zones. Of the significantly differing findings, only eight proteins and endogenous peptides were identified in the database search. After the multiple testing adjustments there was no significant difference in peptides expressed in wounded and unwounded zones (Table I).

Table I. Identified proteins found in three or more patients with a p-value of ≤0.1.

Gene	Identification Uniprot	Peptide se-quence	Present in # patients	Sample mean relative dif-ference (WOA-UOA)	p-value
HBB	Hemoglobin subunit beta	HLTPE	6	-0.5	0.009
C3	Complement C3	ASHLG LA	5	-0.6	0.009
COL1A1	Collagen al-pha-1(I) chain	GGRY Y	6	-0.5	0.026
BGLAP	Osteocalcin	EAYRR FYGPV	5	-0.5	0.046
COL1A2	Collagen al-pha-2(I) chain	GGGY DFGYD GDFY	3	-0.4	0.069
FGA	Fibrinogen al-pha chain	PGFFSP MLGEF VSETE SRG	5	-0.3	0.073
COL1A1	Collagen al-pha-1(I) chain	HDGG RYYR	6	-0.3	0.082
FGA	Fibrinogen al-pha chain	RHPDE AAF	4	-0.4	0.096

Conclusions:

This pilot study shows promising results for enabling the peptidomic analysis of cartilage and bone straight out of the operating room. With further refinement, peptidomics can potentially become a diagnostic tool for OA, and improve our knowledge of disease progression and the genesis of pain. It is also possible that peptidomic data may constitute the basis for the development of a panel of OA biomarkers using immunochemical measurement tools.

5.3 Study III

Aim: The objective of this study was to develop a reproducible, semi-automatic method for analyzing spatially matching cartilage and bone morphology in human osteoarthritic knee joints using microCT.

Introduction:

Diagnostics of OA is most frequently carried out using conventional X-rays [165-167]. This method does not correlate well with patients symptoms, is not sensitive enough to visualize early signs of changes in cartilage morphology and is strictly a two-dimensional (2D) analysis [168, 169]. In the past, OA was thought to be isolated to changes in the morphology of cartilage, but it is now regarded a whole joint disease. The subchondral bone plays an important role in pain and in the development of disease [112-114, 116].

In this study, we evaluated morphological changes in both cartilage and subchondral bone in OA patients using microCT and EPIC microCT. To the best of our knowledge, this has not been done previously. The reason is that while both methods use the same imaging device, the resulting images are different enough to introduce the challenges usually associated with multimodal imaging, such as the co-registration and co-localization of regions of interest (ROI). In the OA context, spatially matching ROIs in the articular surface and the subchondral bone have to be identified in order to study their interactions. In ordinary microCT image processing, ROIs are drawn (semi-) manually in axial or sagittal slices. For articular cartilage, this is tedious and imprecise, because cartilage has X-ray absorption similar to neighboring tissues and, furthermore, it is not in a single plane.

Methods:

Sample selection:

Twelve tibia plateaus were retrieved from patients with severe OA undergoing TKA surgery at Sahlgrenska University Hospital, Sweden following a procedure approved by the Ethical committee in Gothenburg, Sweden. The tibial plateaus where stored in phosphate buffer saline (PBS, Sigma Aldrich) containing protease inhibitors (1% Protease Inhibitor Cocktail Set I, CalBiochem, San Diego, CA) prior to analysis.

Macroscopic observation

The tissue was taken from the operating room and processed, photographing it and then washing it multiple times in PBS and removing soft tissue. The plateau was then split into two condyles through the eminentia. Visual inspection was carried out by an experienced surgeon and the cartilage defects were contoured on the photos. Photos were imported into Fiji where the total condyle surface (Cond.S) and defect surface (Def.S) were extracted using measurement tools, and the defect surface fraction (Def.S/Cond.S) was computed.

MicroCT analysis

Condyles were scanned in air with a cabinet cone-beam microCT scanner (µCT100 Scanco Medical, Bruttisellen, Switzerland) operated at 70kVp, 200uA, 500ms; 1,000 projections/180°. The slices were reconstructed across an image matrix size of 2,048 x 2,048 voxels, with a nominal voxel size of 36 µm, resulting in a scan volume 20 mm long and 75.3 × 75.3mm in the axial plane (scan time 40min).

EPIC microCT

A baseline scan was performed. The condyles were then immersed in a solution of 40% ionic CT contrast agent Hexabrix (Hexabrix 320, Guerbet AG, Zürich, Switzerland) and 60% PBS at room temperature under slow agitation. Three condyles were immersed for 4, 8, 16, 24 and 33 hours and then re-scanned after each period to determine the diffusion dynamics of the contrast agent. 20h was an optimal staining time and the following specimens were immersed in the solution once for 20h and scanned. The second scan, referred to as "EPIC- scan" with reference to Palmer *et al.*[149], was performed with the same settings as the baseline scan. Finally, three specimens were immersed in PBS for 48h after the EPIC-scan to desorb Hexabrix before histological processing. Figure 15 shows the workflow of the imaging process.

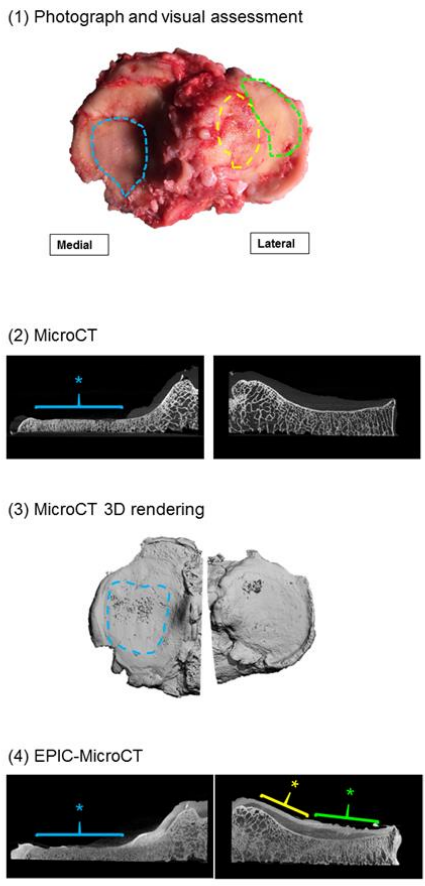

FIGURE 15 Work flow of the imaging process

(1) Photograph and visual assessment: immediately post-op the samples were photographed and evaluated visually by an expert. Large deep defect down to bone is indicated in blue, while areas indicated in yellow and green, suggests areas of healthier region and shallow cartilage defects.
(2) The specimen is then microCT scanned. In the microCT scan the deep defect can be identified (blue star).
(3) In a 3D rendering of the segmented scan the deep defect can be detected from excessive porosity on the condyle surface (blue dashed zone).
(4) The specimen is finally EPIC-scanned. The defect can be observed (blue star) and variations in the Hexabrix staining can be observed (yellow and green stars) confirming visual assessment.
Figure from Bone. 2019 Mar;120:393-402.

Results:

The method for identifying defects was successfully validated against photographic examinations and verified by conventional histopathology (see Figure 16). Cartilage thickness and volume were significantly lower for the OA condyle compared with the healthy condyle. Bone fraction, bone tissue mineral density, cortical density and trabecular thickness differed significantly depending on the level of cartilage damage.

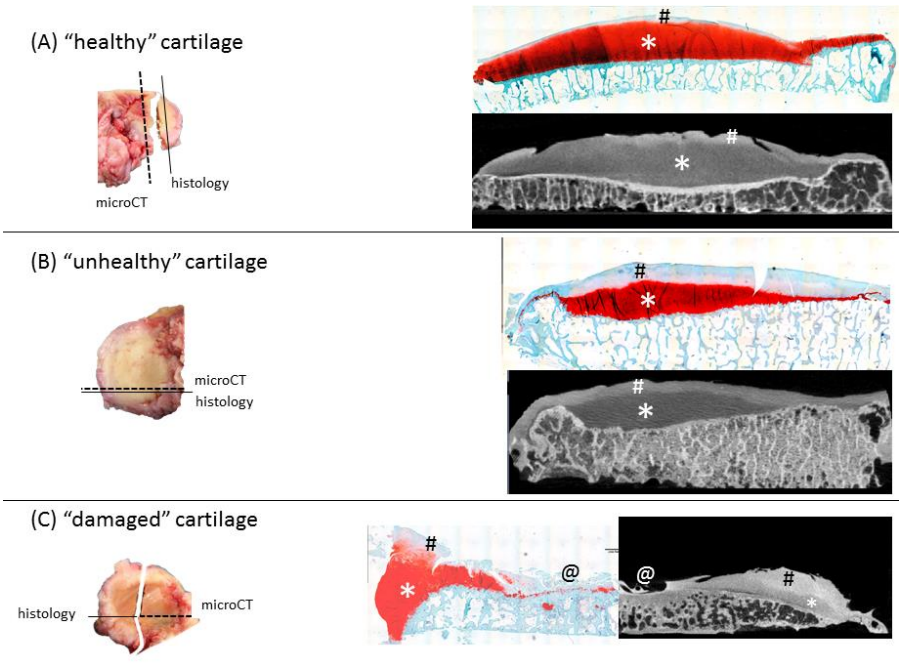

FIGURE 16 Comparison between histology and EPIC microCT

(A) Macroscopically "healthy" tibia condyle was separated in two parts after surgery, one part was used for EPIC microCT, one was used for histology (Saffranin-O). (B) A condyle was first EPIC-scanned then desorbed and used for histology. (C) like (A) in two pieces. * shows healthy cartilage with high sGAG content and # low sGAG content and @ shows defect. The comparison shows a good match between histology and microCT.

Figure from Bone. 2019 Mar;120:393-402.

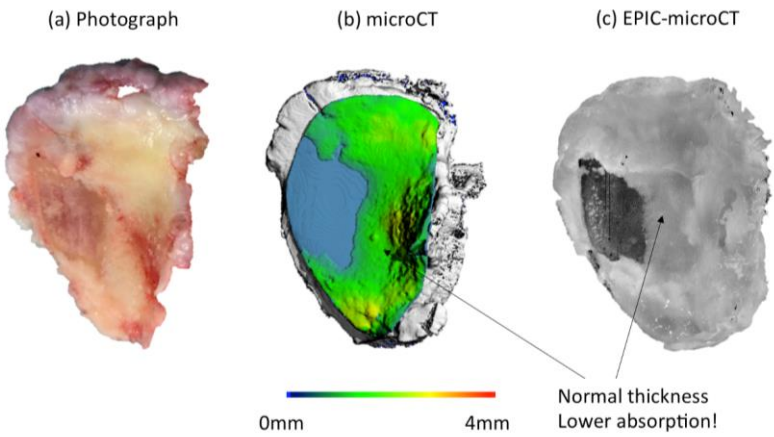

FIGURE 17 Overview of the imaging methods:
(a) post-op photograph (b) microCT with thickness map overlay (c) EPIC microCT

Conclusions:

In this paper we reported the development of a semi-automatic workflow to analyze whole tibia condyles with automatic ROI generation, defect identification, and mapping of damage. With this new methodology presented here, cartilage and the underlying subchondral bone can be analyzed simultaneously due to spatial matching. The findings with EPIC microCT were verified with histological analysis with safranin O-stained sections.

5.4 Study IV

Aim: The objective of this study was to compare different imaging techniques for rendering a 3D CAD model of a tibial plateau from a patient with end-stage OA. The model was then used to 3D bioprint into the cartilage lesion with chondrocytes and bio-ink.

Introduction:

Articular joint injuries and the degeneration of articular cartilage are associated with pain and disability with huge costs to society.

It is possible to treat local chondral and osteochondral lesions successfully with cell therapies and microfracturing but large defects and OA still remain as challenging areas and are therefore a subject for cartilage tissue engineering. Tissue engineering, where scaffold materials are used in combination with cells, has been proposed as an approach to repair bone and cartilage defects. Combining tissue engineering with additive manufacturing (three-dimensional (3D) printing) makes it possible to construct a patient specific implantation tailored to the patient from medical imaging data. For this purpose, different medical imaging techniques such as MRI, CT and other 3D scanning techniques, can be used for the 3D reconstruction of the defect site. To achieve an anatomic 3D reconstruction of the defect site in high resolution, obtaining the exact shape that fits the damaged area is critical for treatment.

In this study, we scanned a tibial plateau OA defect site using various imaging tools and created a 3D model of the tibia for 3D bioprinting with surplus allograft chondrocytes from a planned ACI procedure.

Methods:

3D imaging

One tibial plateau was selected for this study. For preservation, the sample was then fixated in 10% formaldehyde for 24h, decalcified in 2.5% formic acid for 10 days and finally washed and stored in phosphate buffer saline (PBS) solution prior to further analysis. The sample was photographed with a high-resolution 3dMD camera (3dMD Limited, UK) using 360 torsos, photography using seven pods each with three cameras, making a total of 21 cameras which were used for imaging. The sample was then

scanned using various clinical 3D imaging tools: magnetic resonance imaging (MRI), computed tomography (CT) and a 3D portable scanner.

CAD model

To create a CAD model of the OA defect, the stl. file was loaded into CATIA V5 (Dassault Systèmes, France). The tibial plateau image was subdivided into a focus area which included the OA defect. From this area, a surface model was created. Within this surface model the OA defect was sketched out and a new surface was created. This surface was copied and translated 0.25mm in an appropriate direction to level out approximately with the healthy cartilage. By creating boundaries of the OA defect and the translated surface, a surface joining the OA defect and translated surface was created. By joining these three surfaces (OA defect, translated surface and joining surface), a closed solid volume was created. The volume is then saved as a stl file which was used to 3D print model using PA12 material and an EOS P760 printer from EOS.

In situ bioprinting

A BioX 3D bioprinter (Cellink AB, Sweden) along with the generated CAD file of the cartilage filling was used. The precision in xy and z was good and to calibrate the printing of the repair on the damaged knee, the stl file of the repair was flattened and remarkable points of the repair were placed on the 3D plastic printed of the damaged knee. Without turning it, remarkable points on the 3D plastic printed of the damaged knee were then placed on a 96 well plate lid and marked with a pen. While printing, the remarkable points of the real damage were placed on the prepared marks of the 96-well plate. Z calibration was then performed in the middle of the damaged construct.

Results:

In this study, we demonstrate how imaging tools available in the clinic can be used to create 3D models. With clinical tools like CT, MRI and other 3D imaging, an exact reconstruction of cartilage defects can be produced in patient-specific 3D models. With a template of this kind, the healthy twin cartilage copies of the diseased and surgically debrided area can be produced by 3D bioprinting with chondrogenic cells in bio-ink. These cells could be genetically modified to produce specific growth factors and could be primary chondrocytes or iPS cells. As proof of concept, we were able to use the 3D scanning of a tibial plateau to make final CAD models of OA

defects using special CAD software. Furthermore, the 3D scanning portable instrument (3DScanner), which has been recently introduced for odontology, was found to be superior with regard to time and resolution with 3D imaging tools such as MRI and CT. The visualization is improved with a high resolution and this is why we suggest that a 3D scanner of this kind is the best choice for future arthroscopic tissue engineering procedures.

In addition, we show that the 3D bioprinted chondrocytes produced extracellular matrix after being 3D bioprinted into a cartilage defect site obtained using 3D imaging tools. The 3D bioprinted chondrocytes produced aggrecan (ACAN) and collagen type 2 (ColII typeB) characteristic of native cartilage after two weeks of differentiation in cell culture media.

Conclusions:

This study demonstrates that, to obtain a 3D model of the OA defect to be used as a template for the 3D printing of chondrogenic cells in bio-ink, the defect area was best visualized by a handheld 3D scanner, while CT and MRI were not as precise. We also show that it is feasible, using the 3D modeling of an osteoarthritic cartilage lesion, to fill the defect via the 3D bioprinting of human primary chondrocytes containing bio-ink. The cell viability remained high after bio-ink printing, with a slight decrease on day 14 (72%), as seen following differentiation. At that time, high levels of a mature version of collagen type 2 (ColII typeB) and aggrecan (ACAN) could also be found, indicating that workflow supports differentiation towards native articular cartilage.

5.5 Study V

Aim: The aim of this study was to investigate the effect of OA extracellular matrix on 3D bioprinted chondrocytes

Introduction:

ECM-containing matrices are attractive for regenerative medicine due to their prospective ability to aid in stem cell recruitment, differentiation and healing. However, the progression of OA disease causes the cartilage extracellular matrix (ECM) to be gradually destroyed, with a subsequent loss of tissue that leads to impaired joint function, pain and disability [170]. For local cartilage repair surgery using the autologous chondrocyte implantation (ACI) technique [130], chondrocytes seeded in a scaffold need to mature and produce extra cellular matrix (ECM) first *in vitro* and finally *in vivo* after implantation. The ECM is a complex of self-assembled macromolecules and is mainly composed of a collagenous network and proteoglycans [171]. As an initial response to the loss of ECM components in OA, tissue repair increases through secretions of anabolic factors, increased cell proliferation, and matrix remodeling [172, 173]. The repair response is not strong enough to restore the loss of cartilage volume and this results in the production of a repair matrix that has inferior properties compared with the original hyaline articular cartilage [174]. The cells to be printed need scaffold material.

The most natural scaffold is the extracellular matrix surrounding the chondrocytes in a developed cartilage. A matrix of this kind can be utilized by using cartilage extracellular matrix (ECM)-derived particles developed through processes involving physical pulverization [175, 176]. However, to our knowledge, the effect of ECM from an OA donor (OA-ECM) during the 3D bioprinting of chondrocytes and its effect on chondrocyte-derived iPSCs has not yet been investigated.

Methods:

Human cartilage was retrieved during total knee arthroplasty from a patient with advanced OA, frozen on dry ice and stored at -80 ° C prior to experiment. The cartilage was cut into fine pieces and then placed in tissue lyser at 50Hz until the cartilage had been simultaneously disrupted and homogenized to fine particles. Between the tissue lyser procedure, the

cartilage was cooled on dry ice. The cartilage samples were placed in the tissue lyser for two to five minutes per route and 16 total routes were used.

Two different bio-inks were tested: 60/40 nanofibrillated cellulose with alginate (NFC/A) with or without ECM, which were prepared according to the following protocol: 60/40: 60% nanocellulose and 40% alginate mixed under sterile and aseptic conditions. The ECM bio-ink was prepared by mixing ECM microparticles with the bio-ink. The mixing was performed at room temperature, the mixing tools were sterile and mixing was performed in LAF benches to avoid the risk of contamination. The concentration of chondrocytes was 10 million per ml in 60/40 bio-ink. The 3D bioprinting was performed at room temperature in a clean room with a 3D bioprinter from Cellink AB (Sweden).

To compare the effect of ECM microparticles from an OA donor (OA-ECM) on the cartilage micro-tissue formation of chondrocyte-derived iPSCs, the samples were divided into two groups: **1.** 96 microtissue pellets and **2.** 96 microtissue pellets with added ECM powder.

Results:

The iPSCs showed normal cartilage microtissue formation in the absence of OA-ECM. On the other hand, the addition of OA-ECM micro-particles had a degenerative impact on the microtissue cartilage and caused substantial cartilage degradation after five weeks. Since this was observed in 96 samples and compared with 96 samples without OA-ECM, we conclude that this result is statistically significant.

The 3D bioprinted chondrocytes differentiated for two weeks produced extra cellular matrix containing proteoglycans (stained blue). For the 3D bioprinted chondrocytes, cultured in the presence of OA-ECM particles and differentiated for two weeks, a larger area of extracellular matrix (blue) was observed, indicating that the ability of the 3D bioprinted chondrocytes to produce extracellular matrix is stimulated by the presence of OA-ECM. Further, the cell nuclei (stained purple) appeared larger in 3D bioprinted chondrocytes with OA-ECM compared with without OA-ECM. Moreover, more cells in the clusters were observed in the 3D bioprints in the presence of OA-ECM.

Conclusion:

We conclude that the pathological tissue used herein has a deteriorating effect, despite the fact that the OA-ECM particles lacked cells that had probably been destroyed by the processing of the cartilage into ECM microparticles. The microtissue degeneration caused by OA-ECM appears to be protected by the enclosure in the ink-material after 3D bioprinting, although we still observed differences that might be similar to characteristic features of OA cartilage. So, although the 3D bioprinting process appears to have a protective function, we conclude that this is not fully protected.

5.6 Study VI

Aim: To investigate whether there is a correlation between morphological changes in cartilage and subchondral bone and patient-experienced pain and PROMs.

Introduction:

Pain is the main reason for patients to seek medical care, but our knowledge of pain mechanisms in OA is very limited. The aim of this study is to explore the correlation between patient-experienced pain, radiological findings, mapping of the cartilage and subchondral bone, as well as how the patient recovers after TKA surgery.

Methods:

50 patients, 25 male and 25 females were included in the study, but 3 patients were excluded for different reasons, resulting in 47 patients for the final analysis. All the patients were interviewed preoperatively about previous knee injuries and previous knee surgeries. Patients also filled out KOOS questionnaires pre- and 8-10 weeks postoperatively. During surgery, the tibial plateaus were photographed and prepared for analysis. All the tibial plateaus were analyzed with EPIC microCT to map the extent of morphological changes in the cartilage and subchondral bone.

Results:

Cartilage and bone morphometric data

- Cartilage thickness was statistically significantly lower in condyles with defects compared with condyles with no defect.
- Bone volume, trabecular thickness, trabecular TMD and cortical TMD were all significantly affected by overlying cartilage status.

Correlation analysis

No statistically significant correlation was found when comparing:

- the sum of defect surface analyzed with microCT and KOOS Pain preoperatively

- the sum of cartilage surface fraction analyzed with microCT and KOOS Pain preoperatively
- the sum of defect surface analyzed with microCT and KOOS Quality of life (QOL) preoperatively
- the sum of cartilage surface fraction analyzed with microCT and KOOS Quality of life (QOL) preoperatively.

Conclusion:

This was the first time the recently developed semi-automatic EPIC microCT analysis of spatially matching cartilage and subchondral bone had been performed on a larger scale. The morphometric data showed a statistically significant correlation with cartilage thickness and defect, as well as the fact that underlying bone was affected by the status of the overlying cartilage. We were unable to find any correlation between the morphometric data obtained and KOOS Pain or KOOS QOL.

6. Discussion

This doctoral thesis is based on both preclinical and clinical studies with the emphasis on investigating the genesis of joint pain, biological signaling, imaging and self-scored experience of knee function in patients with manifest OA.

Joint pain and biomarkers

Joint pain is the key symptom in OA and is one of the main reason patients visit their general practitioner [177-179]. The diagnostic tools used today are poor when it comes to visualizing early disease onset and there is a great deal of disagreement between patient-experienced pain and radiographic findings [180-183]. The pathophysiology behind joint pain is complex and involves pain signaling at nociceptor level but also the process of both peripheral and central sensitization. In addition to this, psychological factors such as depression and anxiety also affect the pain sensation [64, 184]. Even though pain is such a predominant symptom, there is still very limited knowledge and data relating to the underlying pain mechanisms.

In this thesis, the mechanism of joint pain has been investigated, with special emphasis on the role of neuropeptides in pain transmission and their potential role in the progression of OA. The literature search in Study I indicates that there is still a knowledge gap and a limited number of studies, especially studies with a high level of evidence. One reason for this could be that, in the last few decades, much of the research on OA has focused on the disease progression and it is only recently that the focus has shifted to understanding joint pain and patient-reported outcomes. Another reason could be that pain is highly subjective, affected not only by biological processes but also by the patients' mental and health status [185]. Large studies often conclude that there is high heterogeneity within studied populations. A systematic review and meta-analysis concluded that strong risk factors for developing severe pain in the knee were higher knee pain intensity at baseline, bilateral knee symptoms and depression [82]. In that meta-analysis, it was surprising that obesity and female gender did not emerge as strong risk factors. Other population studies have, however, identified obesity and female gender as prognostic factors for escalating pain development [186-189]. In addition to this, there are multiple

challenges in finding biomarkers and developments in the technological advances have so far been slow.

Given the limitations of radiography, there is a growing interest in finding better diagnostic tools, especially for patients with early OA symptoms. In recent years, the research field of biomarkers in OA diagnostics has rapidly developed. Proteomics, the identification of proteins by mass spectrometry, has been used to identify potential biomarkers by analyzing synovium, synovial fluid and serum [160, 190-192]. Many interesting proteins have been identified and the most promising correlate to inflammation, cartilage turnover and synovium [193]. The potential of biomarkers is not only to diagnose early disease but also to find targeting drugs and hopefully early treatment to stop disease progression.

Identifying endogenous peptides

Cartilage may be involved in the pain signaling process, but it is not able to generate pain on its own, due to its aneural structure. The surrounding tissues, such as the subchondral bone, synovia and periosteum, are, however, heavily innervated and studies have shown a correlation between pain and subchondral bone changes [194-196]. Since we believe that early disease changes can be visualized in cartilage and the subchondral bone, we wanted to develop a method to investigate neuropeptide levels in this tissue. We based our study design in Study II on earlier experiments with protein extraction from bone and research conducted in the field of neurodegenerative diseases [142]. Our aim was to attempt to identify the most pain-related neuropeptides, namely SP, CGRP, VIP and NPY. We were unable to identify these specific neuropeptides, but we identified numerous endogenous peptides involved on different levels in neurogenic signaling.

Another interesting finding was endogenous peptides associated with complement C3. The degree to which inflammation plays a role in OA is still heavily debated. Inflammation is, however, a part of disease progression with regard to synovitis [197, 198]. Genetic studies have shown increased activation of C3 in synovium from patients with both early and late stages of OA [199]. There is a theory that complement activation might trigger uncontrolled inflammation which then leads to disease progression and joint deterioration. Recent proteomic research on synovial fluid and synovium from patients with OA shows high activation and expression of complement, indicating that complement can play a key

role in OA pathogenesis. Furthermore, the same research using knock-out and wild type mice, confirmed that complement activation is crucial for arthritis development [200].

Clinical relevance of peptidomics

Peptidomics, the method of identifying endogenous peptides quantitatively and qualitatively using mass spectrometry, has already been used in many clinical settings in finding new and important biomarkers [142]. The method has emerged from proteomics but instead of examining proteins it examines endogenous peptide fragments.

In clinics, peptide biomarkers in cerebrospinal fluid have been used to predict progression to Alzheimer disease and urinary peptides have been used in both chronic kidney disease and prostate cancer diagnostics [164, 201, 202]. The new method developed in Study II for the peptidomic analysis of cartilage and subchondral bone is useful because it is applicable in the clinical setting. Instead of analyzing biomarkers expressed in cell culture the diseased tissue is analyzed straight away. The new method has the potential for finding important biomarkers for early disease detection by taking biopsies. There is an ongoing pilot project called The Human Plasma Proteome Project, which shows promising results when analyzing human blood samples [203]. Ideally, findings from the peptidomic analysis of cartilage and subchondral bone could lead to measurable biochemical markers in blood samples. One major challenge with biomarkers in OA is that the disease itself is somewhat complex, with large scale heterogeneity in clinical disease onset and progression and the joint tissues involved. Another challenge is that current radiographic diagnostics of OA are not sensitive enough to evaluate the relevance of identified biomarkers. We need better diagnostic tools in order to develop diagnostics with biomarkers [204]. There is, however, a real incentive to find relevant biomarkers and potential treatment, as this will have a tremendous positive effect not only on the individual patient's quality of life but also for society as a whole from a socioeconomic point of view.

Pain, PROMs and morphologic changes in osteoarthritis

OA is a degenerative disease leading to eventual cartilage loss, affecting up to one-third of the population older than 50 years, with significant costs worldwide. Rather than considering OA as an isolated cartilage disease,

recent research has focused on a more whole-joint perspective, where several tissues are thought to be involved in the disease progression [205].

The currently used radiography and pain assessments lack precision and are based on subjective evaluations, which leads to discordance between clinical and radiographic findings [206]. Many studies assessing the relationship between radiography and pain have not, however, taken account of confounding factors and, in knee matching studies comparing the painful and normal knee in the same individual, there is a strong correlation between radiographic findings and knee pain [207].

In Study VI, for the first time, we investigated a larger group of patients with a newly developed semi-automatic method for evaluating cartilage and bone morphology using EPIC microCT. The data found in this study confirmed the findings in our pilot study (Study III) that cartilage thickness was significantly lower in condyles with an OA defect. In addition to this the bone tissue density was found to be lower, but the bone volume fraction was significantly larger in condyles with an OA defect suggesting new bone formation below the cartilage defect. It is well established that in OA the subchondral bone becomes sclerotic and undergoes structural changes [208, 209]. Histological analysis of OA tissue supports these findings showing increased bone formation and volume in the subchondral bone [116]. Guinea pig and primate animal models of OA also show increased density and reduced porosity in the subchondral bone [210, 211]. Recent research indicates that subchondral bone changes are coupled with articular cartilage degeneration and can be seen early in the disease process [212]. The subchondral bone may change its structural properties as a response to increased load due to cartilage deterioration.

Multiple studies have shown a correlation between bone marrow lesions, subchondral bone erosion and pain [213-215]. When we correlated the morphometric cartilage and bone data with PROMs and reported pain, we did not, however, find any statistically significant correlation. The most apparent reason for this is that the group that was studied was too small to find any significant differences. Another reason could be that there is large heterogeneity in OA patients, especially when it comes to patient-reported outcome and pain. We did, however, find an interesting trend between quality of life and cartilage defects suggesting that a smaller defect would have a larger negative effect on patient reported quality of life. One possible explanation for this could be that smaller defects may trigger pain signaling between cartilage and subchondral bone causing more

discomfort and consequently affecting the patient-reported quality of life. A full-size defect with less remaining cartilage could then lack this interaction and have a less negative effect on patient-reported quality of life. Another possible explanation could be that patients with more exposed bone have had OA for a longer time and have adjusted to the discomfort and pain to which the disease leads.

In recent years, patient-reported outcomes have become more valued and studied, as the health care system has shifted towards a more value-based approach. Patient-reported outcome measurements also provide important information about the effectiveness of treatment and information about patients who may not benefit from treatment. Even though we did not find any statistically significant correlation between morphometric data and PROMs in our study, we believe this kind of research linking biological changes with patient-experienced symptoms is the key to improving and developing the treatment of OA [216].

Imaging Osteoarthritis

The imaging technique used today for OA diagnostics is conventional X-ray visualizing joint space narrowing, subchondral sclerosis and osteophytes. To assess the knee joint bilateral anteroposterior weight-bearing with full extensions is the golden standard [217]. This protocol has been used since the 1970s, even though many new imaging techniques have been developed. Conventional X-ray has a low sensitivity to detect cartilage loss and in a study comparing MRI and conventional X-ray, a significant number of patients were lacking disease signs on conventional X-ray but cartilage loss was visualized by MRI [218].

MRI offers several advantages as it is able to visualize bone and cartilage pathology. Bone marrow edema-like lesions (BMLs) are especially well visualized with MRI and multiple studies have shown a correlation between BMLs and cartilage lesions [194, 219, 220]. Changes in the subchondral bone are also well visualized with MRI and are seen long before they appear on a conventional X-ray. Moreover, recent developments in MRI technology have enabled for quantitative measurements of important macromolecules such as proteoglycans and collagen [221].

OA is now regarded as a whole joint disease and because of this there is a need to develop imaging techniques that are able to assess all the different

tissues in the joint. As of today, no imaging technique alone has good enough sensitivity and specificity to visualize all intra-articular structures [222].

3D bioprinting-future cartilage repair technique

When cartilage lesions are detected, then it is crucial to repair them in order to prevent the development of OA. For smaller isolated cartilage lesions, there are multiple treatment alternatives, but large chondral and osteochondral defects remain a clinical challenge. Tissue engineering can create solutions when it comes to where the tissue itself lacks regenerative capability, like, for example, when it comes to articular cartilage defects. Despite many years of effort, tissue engineering has not yet provided any clinical solution to the repair of damaged cartilage. We think that creating the CAD model based on the imaging of damaged cartilage is a prerequisite for any further repair.

3D bioprinting is the emerging new technology for the 3D biofabrication of tissue by layering cells with a biomaterial. 3D bioprinting is a part of additive manufacturing (AM) technology, which is often called "bottom-up fabrication". It is ideal for patient-specific treatment because the movements of the 3D bioprinter's printing head are controlled by G-code, which is prepared from a CAD file. In this way, 3D printing complement what the imaging technique provides. 3D imaging tools scan the organ/tissue layer by layer and the 3D printer reconstructs the organ/tissue by depositing layer upon layer of cells and supporting biomaterial (bio-ink). The outcome has the potential to mimics the normal tissue both structurally and biologically.

Cartilage is, however, a complex tissue in both its structural and biological composition, giving it unique biomechanical properties to withstand shear stress and a high load. This is probably the reason why it has been so difficult to find tissue engineering solutions for cartilage repair. The advancement in the 3D bioprinting field has allowed for the development of both printing methods for chondrocytes and the use of different scaffold materials with the aim of creating biocompatible hydrogels. The resolution of the 3D bioprinters is high enough to place a single cell in the exact 3D position. The zonal architecture of cartilage tissue can be potentially reproduced using different chondrocytes originating from the respective zones. The number of cells can be varied through different zones. 3D bioprinting can be used to repair not only cartilage but also subchondral

bone. Each layer which is imaged by, for example, microCT provides information on the exact microarchitecture and composition of bone tissue. When this information is transformed to G-code, the 3D bioprinter is able to deposit the scaffold with an exact composition matching the mechanical properties of bone and then provide the gradient of material properties going from bone to cartilage.

If 3D bioprinting were to be regarded as a future cartilage repair technique, one should compare it with currently available repair techniques. In symptomatic chondral defects the cartilage is abnormal and mechanical overloading leads to high matrix metalloproteinase production which has a damaging effect on surrounding cartilage [223, 224]. The debridement technique is based on removal of all unstable cartilage and the abrasion of calcified zones. The microfracture technique combines debridement with the drilling of holes, which results in the filling of the defects with a super-clot, which is ideal environment for the differentiation of pluripotent stem cells from bone marrow [128, 225, 226]. Mosaicplasty is a cartilage repair technique in which osteochondral plugs are taken from healthy area and placed to fill the defect [227]. ACI uses the injection of culture-expanded autologous chondrocytes into a chondral defect beneath a periosteum patch [130, 228]. The field of cartilage repair is full of reports comparing the efficacy of the different cartilage repair techniques [229]. There are, however, very few procedures in which the different techniques are combined. Here, 3D bioprinting could fill the gap in the field of the cartilage repair techniques. A 3D bioprinter is a robotic device which is able precisely to control the motion of the holder in the xyz direction. A typical bioprinter is equipped with printing heads which are holders of syringes filled with hydrogels and cells. But the holder can be easily equipped with a drilling device and a punching device with tweezers in addition to syringes. After imaging the damaged joint, the data can be converted into CAD file and the repair process can be programmed in G-code. The printer is able to place cultured chondrocytes in exact positions in different zones, and surround them with small pieces of autologous cartilage removed with tweezers from healthy zones. When reconstruction is finished, the microdrills can be applied to bone to fill the repaired area with a blood clot and stem cells and then periosteum patch can then be placed on top.

There are multiple challenges that remain before 3D bioprinting is able to manage cartilage repair in the clinic. The printing of hydrogel laden cells (cells which are mixed with hydrogel) affects the cell survival rate and cell

interaction within the printed tissue [230, 231]. This makes the tissue relatively unstable over a long period of time. Matching the biomechanical properties of cartilage tissue is another challenge. Chondrocytes first produce extracellular matrix after several weeks of culturing. This can be addressed by selecting suitable mechanical properties of the hydrogel used as bio-ink. The bio-ink we used in Study IV is a hydrogel which can be tuned to match the mechanical properties of cartilage.

To date, bioprinters have not been adjusted for use in the operating room lacking instrumental sterility and printing on plane surfaces mostly suitable for laboratory environments. It is also important that future 3D bioprinters are able to be operated arthroscopically. Interestingly, in Australia, the BioPen, a handheld device using 3D printing principle, is currently being evaluated by orthopedic surgeons [232]. The device ejects the cells and the matrix which is solidified with a UV-lamp, a similar procedure to that used by dentist when repairing a tooth cavity.

Another challenge is that the bioprinted tissue does not perfectly match the defect. In Study IV, we created an exact print by the process of reverse engineering to create a CAD model from CT, MRI and 3D handheld scanner images of an OA lesion. 3D bioprinting has great potential in the treatment of early OA lesions and hindering their advancement towards manifest OA disease with cartilage degeneration.

3D bioprinting with autologous chondrocytes combined with stem cells or in the future with iPSC-derived chondrocytes directly in the injured area should be evaluated in the near future. The topological placement of cells with surrounding biomaterial can be performed with great precision using 3D bioprinting technology.

6.1 Limitations

The role of neuropeptides in pain signaling in OA is still largely unexplored. As concluded in Study I, of all the published papers between January 1990 and September 2017, only three papers were Level 1A-B. The majority of the published papers were Level 5. With such a limited high level of evidence studies, it is difficult to perform systematic reviews and much of the knowledge in the field is instead based on expert opinions.

The obvious limitation in Study II was that we analyzed tissues from only six patients. We did not find any significant differences in wounded OA regions compared with unwounded regions when we adjusted for multiple testing. Due to the large amount of data a peptidomic scan is able to generate, there is a need to study a larger population in order possibly to find any statistically significant variations. Furthermore, TMT labeling turned out to be incomplete and follow-up experiments on control tissue showed that the TMT markers were very thermosensitive. When carrying out the database search, the vast majority of the identified peptides were unable to be identified. Advances in peptidomic research can hopefully broaden the database.

One limitation in Study III was that there were a limited number of patients and the group studied was fairly homogeneous; all the patients had medial severe OA. This makes it difficult to draw any conclusions that are applicable to the whole OA population. Another limitation is related to the tibial plateaus that were studied. Due to variations in tibia cutting, there was a variation in tissue thickness and this consequently affected the thickness of the subchondral bone being analyzed. Moreover, the use of contrast agent Hexabrix was another study limitation, as this contrast agent is no longer accepted for human *in-vivo* studies due to its nephrotoxicity. Finally, the articular surface needed to be drawn manually, which affects the precision of the measurements.

In Study IV, only one tibial plateau from a patient with OA was analyzed. With OA, there is a large variation in the depth of the lesion and the three-dimensional shape of the cartilage defect. To evaluate the imaging protocol further, a wider variety of tibial plateaus with different degrees of disease progression should be tested. In both Study IV and V, cell culturing took place over a short period of time and one limitation is that we are unable to conclude how the 3D-printed tissue would look when it comes to biomechanical properties and cell viability over a longer period of time.

Furthermore, the mechanical properties of the chondrocyte prints have not been tested to determine how they withstand the compression forces of the femur in a joint.

Study VI included a limited number of patients and the follow-up post TKA was only eight weeks. It is difficult to draw any conclusions from data from such a small, heterogeneous population. All the studied patients showed severe signs of OA and we can only hypothesize that the EPIC microCT works well on detecting early OA changes as well. The scanning time was very long, which resulted in a huge number of data points for further analysis. Consequently, the data processing time was long and a powerful computer was needed. After evaluating the data, we realized that it was not necessary to collect that many data points. The resolution can be much lower and, as a result, the data processing time can be shorter.

7. Conclusion

Study I investigated the function of neuropeptides in the knee joint. Several neuropeptides are collectively involved in nociception, bone turnover, inflammation and angiogenesis. We concluded that, with the current knowledge substance P is perhaps the most interesting neuropeptide involved in the joint homeostasis.

In Study II a novel protocol for the analysis of neuropeptides was developed using peptidomic analysis which can be performed on bone and cartilage straight from the operating room. With further work, peptidomics can be developed as a diagnostic tool for the detection of biomarkers for early disease diagnostics, biological signaling and quantitative analysis and thereby the objective measurement of pain.

Study III focused on the determination of the 3D morphology of cartilage and subchondral bone in OA joints using microCT. This new method allows for the spatial matching analysis of cartilage and underlying subchondral bone. There was a significant difference in subchondral bone morphometry under cartilage defects compared with areas without cartilage defects.

In Study IV, MRI, CT and a 3D scanner were used to create 3D images of damaged cartilage. All these imaging tools can be used to create CAD models of OA joints which will help clinicians to select intervention tools. In addition, we demonstrated that a 3D model of the OA defect generated with handheld a 3D scanner can be used as a blue-print for the 3D bioprinting of chondrogenic cells in bio-ink directly into the defect. High levels of a mature version of collagen II (ColII type B) and aggrecan (ACAN) were found, indicating that this procedure supports chondrocyte differentiation towards native articular cartilage and could thus be used for early intervention.

In Study V we investigated the effect that ECM from an OA donor had on the 3D printing of chondrocytes and chondrocyte-derived iPSCs. Even though 3D printing had a somewhat protective effect, despite lacking viable cells, OA-ECM had a deteriorating effect on printed cartilage microtissue.

Study VI was a prospective clinical study comprising 47 OA patients, correlating pain, quality of life and morphologic cartilage and

subchondral bone changes analyzed with EPIC microCT. We did not find any statistically significant correlation between patient-experienced pain, quality of life and morphologic changes in the OA joint. However, the results indicate that there is a trend towards smaller cartilage defects having a greater impact on quality of life.

8. Future perspectives

The degree of joint pain is most often the discriminating factor when it comes to deciding whether a patient is ready for a surgical intervention. Today's imaging techniques are not good enough to identify more exactly which part of the joint is the main cause of pain. A better knowledge of the exact location of joint pain early in a degenerative process may help us to target the area of pain and degeneration with more precise surgical tools.

With the research presented in this thesis we may in the future be able to measure the concentration of certain pain mediating neuropeptides in relation to the joint location. The most dominant neuropeptides could be used as markers in SPECT and/or PET camera analysis. By combining imaging with neuropeptide markers, it would then be possible to correlate the neuropeptide concentration in a certain location with the degree of tissue degeneration.

It should, however, be noted that the peptidomic method developed in this thesis requires a biopsy from bone and, at the current stage also requires substantial processing in the laboratory. Current peptidomic development shows promising results using serum for endogenous peptide identification. The health-care system today has well-established routines for serum analysis and it is to be hoped that biomarkers identified in cartilage and bone can also be identified in blood samples which would enable simpler implementation of this technique in the clinical setting. Additionally, data processing requires long period of time and access to different data-bases. With the future development of artificial intelligence, the current limitations of data processing can hopefully be overcome. A peptidomic profile related to OA could potentially be translated into a simpler biomarker panel using standard immunochemical techniques.

The semi-automatic microCT imaging we have developed should be tested and used in the future for the early diagnosis of cartilage damage. The new image processing workflow may be combined with *in-vivo* high-resolution peripheral quantitative computed tomography HR-pQCT scans to identify early changes in the cartilage and subchondral bone morphology prior to OA development. This will require large-scale optimization since the defect position needs to be first determined in 3D space by performing a rapid scan, after which a smaller area should be selected for further analysis.

When cartilage lesions are detected, it might be important to repair them at an early stage in order to prevent further development into OA. For small to medium-sized lesions ACI has shown very good outcomes. For larger traumatic and early OA lesions finding a secure repair methodology is still challenging. Despite many years of effort, tissue engineering has still not provided satisfactory solutions for large cartilage restorations. Creating the CAD model based on the imaging of damaged cartilage is a prerequisite for any further repair. Early interventions may then be possible; future joint preservation.

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11. Appendix