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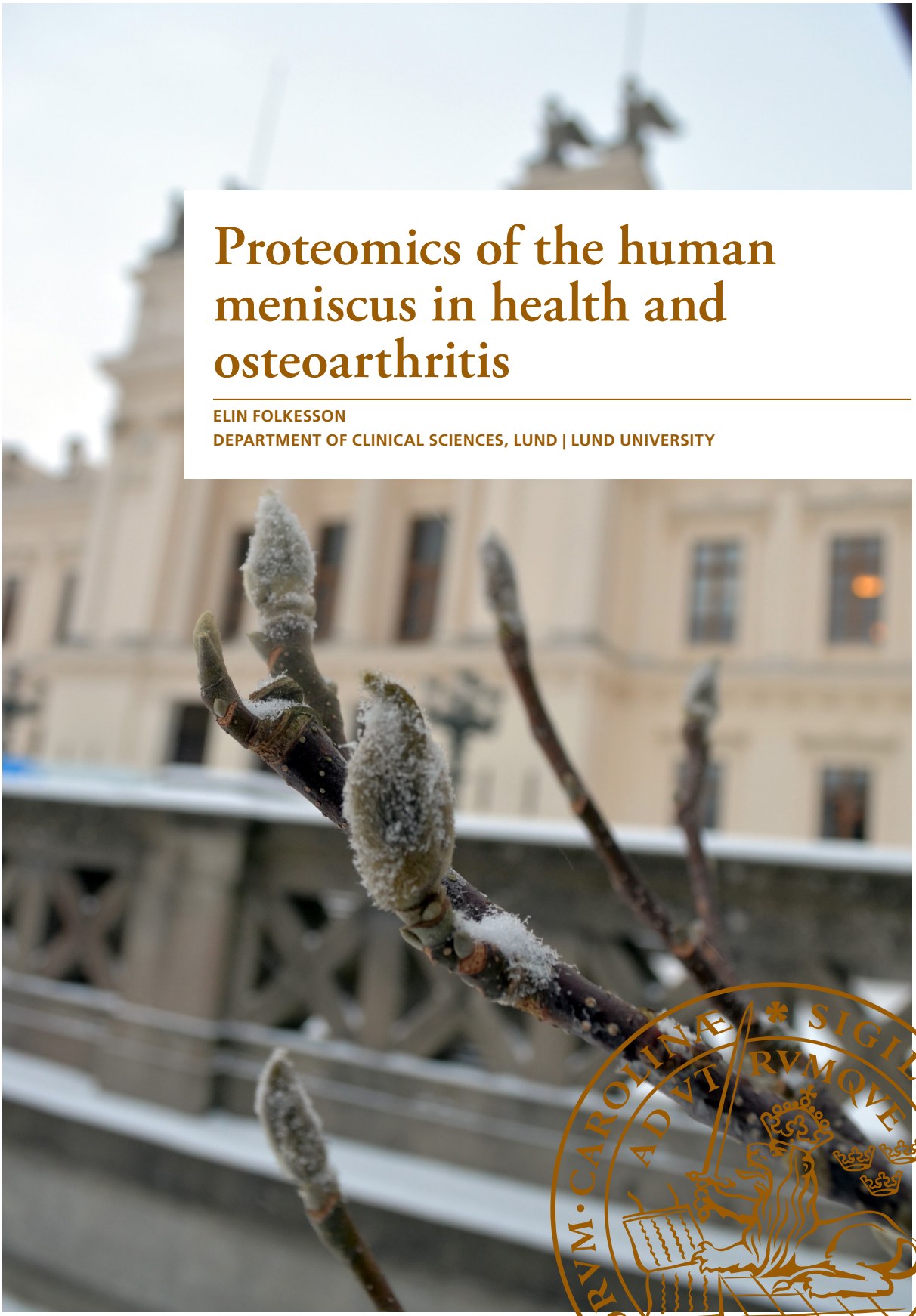

Proteomics of the human meniscus in health and osteoarthritis

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LUND
UNIVERSITY

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Abstract

Introduction: Osteoarthritis (OA) is one of the most common causes of knee pain, and the most common form of arthritis. It causes pain and disability, and there is currently no cure. Recent research in knee OA highlights the role of the meniscus in OA pathology. In this thesis, the overall aim was to study the proteome and microstructure of the human meniscus, both in healthy subjects and OA patients, in order to increase our knowledge about the human meniscus and the processes that occur during degeneration and OA.

Design: In the first part of this thesis, posterior horns of medial and lateral menisci from deceased donors and OA patients were analysed with histology and micro-computed tomography (μCT), in order to compare the microstructure of healthy and OA menisci (paper I). In the second, and major part of this thesis, human menisci were analysed with mass spectrometry (MS)-based proteomics. First, meniscus body samples and articular cartilage samples from deceased donors were compared using two different MS-based methods – data-independent acquisition (DIA) and data-dependent acquisition (DDA) (paper II). Next, three zones (peripheral, middle and inner zone) of the body of the medial meniscus from deceased donors were compared using DIA (paper III). Lastly, meniscal plugs from the same meniscal posterior horns that had been used in paper I, were analysed with DIA.

Results: In paper I, we found that higher histopathological scores were associated with more degeneration, and that medial menisci from OA patients had the highest degree of degeneration. We also observed that the degree of degeneration varied between the different sections of the meniscus. In paper II, we showed that the majority of the proteins were similar between articular cartilage and meniscus, but that there were nonetheless some proteomic differences. Furthermore, we found that DIA identified more differentially expressed proteins, with less missing values. In paper III, we observed that many proteins were common to all zones of the meniscus, and that the majority of meniscal proteins were associated with ECM organisation. The largest zonal differences were between the peripheral and inner zones. In paper IV, where we compared menisci from OA patients and deceased donors, we showed that the largest differences could be seen between the medial menisci from the two groups, and that there was an increase of proteoglycans and proteins such as MMP3 and TIMP1.

Conclusions: Due to the many advantages of DIA, we considered it superior to DDA and used it in subsequent MS analyses. Although articular cartilage and meniscus had similar proteomic compositions, we detected several differences by MS. We also detected large differences between the peripheral and inner zones of the healthy meniscus body, probably related to the higher degree of cellularity and vascularisation in the peripheral zone. On the other hand, the middle and inner zones of the meniscus contained more extracellular proteins and therefore appeared to be more similar to articular cartilage. Comparison of healthy and OA menisci revealed increased degeneration of the OA menisci, with more disorganised collagen networks and the presence of cysts and calcifications. The OA menisci also had an increase of proteins such as MMP3 and TIMP1, which suggests simultaneous activation of both catabolic and anabolic processes. The increased degeneration in OA menisci suggests a strong association between OA and meniscal degeneration.

Key words

Osteoarthritis, meniscus, cartilage, proteomics, mass spectrometry, knee joint

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II. **Folkesson, E**, Turkiewicz, A, Englund, M, Önnerfjord P. Differences in protein expression using data-dependent and data-independent acquisition mass spectrometry in the analysis of human knee articular cartilage and menisci: a pilot analysis. *BMC Musculoskeletal Disorders*, 2018; 19(1): 416.

III. **Folkesson, E**, Turkiewicz, A, Rydén, M, Hughes, H.V, Ali, N, Tjörnstrand, J, Önnerfjord, P, Englund, M. Proteomic characterization of the normal human medial meniscus body using data-independent acquisition mass spectrometry. *In revision.*

IV. **Folkesson, E**, Turkiewicz, A, Ali, N, Rydén, M, Hughes, H.V, Tjörnstrand, J, Önnerfjord, P, Englund, M. Proteomic comparison of osteoarthritic and reference human menisci using data-independent acquisition mass spectrometry.
In progress.

Abbreviations

ACL	anterior cruciate ligament
BMI	body mass index
CI	confidence interval
DIGE	differential in-gel electrophoresis
ECM	extracellular matrix
FDR	false discovery rate
GO	gene ontology
HE	haematoxylin and eosin
HMDS	hexamethyldisilazane
lateral^{OA}	lateral menisci from medial compartment knee osteoarthritis patients
lateral^{ref}	lateral menisci from reference subjects (deceased donors)
LC	liquid chromatography
LXR/RXR	liver X receptor/retinoid X receptor
µCT	micro-computed tomography
medial^{OA}	medial menisci from medial compartment knee osteoarthritis patients
medial^{ref}	medial menisci from reference subjects (deceased donors)
MMP	matrix metalloprotease
MRI	magnetic resonance imaging
MS	mass spectrometry
OA	osteoarthritis
PBS	phosphate-buffered saline
PCL	posterior cruciate ligament
PPI	protein-protein interactions
RA	rheumatoid arthritis
RhoGDI	Rho guanosine diphosphate (GDP) dissociation inhibitor
SafO-FG	safranin-O–Fast Green
SLRP	small leucine-rich proteoglycan
TIMP	metalloproteinase inhibitor
TKR	total knee replacement

Abstract

Introduction

Osteoarthritis (OA) is one of the most common causes of knee pain, and the most common form of arthritis. It causes pain and disability, and there is currently no cure. Recent research in knee OA highlights the role of the meniscus in OA pathology. In this thesis, the overall aim was to study the proteome and microstructure of the human meniscus, both in healthy subjects and OA patients, in order to increase our knowledge about the human meniscus and the processes that occur during degeneration and OA.

Design

In the first part of this thesis, posterior horns of medial and lateral menisci from deceased donors and OA patients were analysed with histology and micro-computed tomography (μCT), in order to compare the microstructure of healthy and OA menisci (**paper I**). In the second, and major part of this thesis, human menisci were analysed with mass spectrometry (MS)-based proteomics. First, meniscus body samples and articular cartilage samples from deceased donors were compared using two different MS-based methods – data-independent acquisition (DIA) and data-dependent acquisition (DDA) (**paper II**). Next, three zones (peripheral, middle and inner zone) of the body of the medial meniscus from deceased donors were compared using DIA (**paper III**). Lastly, meniscal plugs from the same meniscal posterior horns that had been used in **paper I**, were analysed with DIA.

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Due to the many advantages of DIA, we considered it superior to DDA and used it in subsequent MS analyses. Although articular cartilage and meniscus had similar proteomic compositions, we detected several differences by MS. We also detected large differences between the peripheral and inner zones of the healthy meniscus body, probably related to the higher degree of cellularity and vascularisation in the peripheral zone. On the other hand, the middle and inner zones of the meniscus contained more extracellular proteins and therefore appeared to be more similar to articular cartilage. Comparison of healthy and OA menisci revealed increased degeneration of the OA menisci, with more disorganised collagen networks and the presence of cysts and calcifications. The OA menisci also had an increase of proteins such as MMP3 and TIMP1, which suggests simultaneous activation of both catabolic and anabolic processes. The increased degeneration in OA menisci suggests a strong association between OA and meniscal degeneration.

Introduction

Knee osteoarthritis

Prevalence

The prevalence of knee pain has increased considerably during the last 20 years, and approximately 1 in 4 adults suffer from frequent knee pain[1,2]. One of the most common causes of knee pain in the older population is osteoarthritis (OA)[3]. OA is also one of the top causes of years lived with disability in the world[4,5]. In Sweden, approximately 26% of all adults over 45 years of age have doctor-diagnosed OA, with the knee being the most affected joint[6].

Risk factors and symptoms

The major risk factors for knee OA include overweight and obesity, previous knee injury, increasing age, female gender and occupational activities such as kneeling or heavy lifting[7]. In addition, more than 20 genetic loci associated with OA have been identified, indicating a genetic component in OA aetiology[8]. The most common symptoms in knee OA are pain and stiffness of the knee joint, which can lead to loss of function and problems in daily life[9]. In OA, there is a large discordance between the symptoms that patients experience and the presence of radiographic changes, which means that a patient with no symptoms can show changes in the joint by imaging, such as magnetic resonance imaging (MRI) or X-ray, and vice versa.

Affected tissues in the knee

Generally, OA is described as the loss of homeostasis between synthesis and degradation of extracellular matrix (ECM) components, although the reason for this imbalance is still unknown[10]. OA is traditionally characterised by loss of cartilage and changes to the underlying subchondral bone, such as the formation of bone cysts and osteophytes[11]. It has also been established that there is some synovitis and inflammation in OA[12,13]. Additionally, recent research in knee OA has highlighted an important role for the meniscus in OA aetiology and pathogenesis[14,15].

Diagnosis and treatment

The most common way of diagnosing OA today is through a combination of medical history, symptoms and a medical examination. Sometimes, further tests such as a radiographic examination may be necessary, but the findings may be non-specific. There are no laboratory tests or biochemical markers that are being used in the clinic, although there is a lot of research in this area[9].

One way of visually assessing the severity of tissue degradation in the knee joint during OA is using the Outerbridge classification system. The system was originally developed to classify chondromalacia patella, but has since been used to classify other chondral lesions in the body[16]. The system assigns a grade of 0 to IV to the chondral area of interest, where 0 is normal cartilage and IV includes erosion of the cartilage and exposed subchondral bone (Table 1)[16–18].

Table 1.
Features of the Modified Outerbridge classification system of chondral lesions[16,17].

Grade	Features
0	Normal articular cartilage
I	Softening and swelling of articular cartilage
II	Fibrillation and/or superficial fissuring of articular cartilage
III	Deep fissuring of articular cartilage (without exposed subchondral bone)
IV	Erosion and full-thickness loss of articular cartilage which exposed subchondral bone

There is currently no cure for OA, and the majority of treatment options are focused on relieving symptoms, by reducing pain and disability. This is mainly done by pharmacological treatment with analgesics and anti-inflammatory drugs, and rehabilitative methods such as physiotherapy, exercise and weight-loss. For severe, end-stage OA, the gold standard is total knee replacement (TKR), where the damaged joint is replaced by a prosthesis. Today, TKR is one of the most common surgical procedures, and the number of surgeries is expected to rise[19]. During the last 25 years, research has also increasingly targeted regenerative treatments and biological reconstructions of the joint[20], where for example Brittberg *et al.* were among the first to present data on autologous transplantation of chondrocytes[21]. Stem cells have also been proposed as potential disease-modifying treatments in musculoskeletal disorders, although more research is needed to fully elucidate the potential of stem cells in OA[22].

The meniscus

The normal meniscus

Etymology and function

The term *meniscus* comes from the Greek word μηνίσκος (meniskos*)* which means "crescent". It is the diminutive of μήνη (mene*)* which means "moon"[23]. They are so named due to their crescent shape, although the lateral meniscus is more circular in shape compared to the medial. There are two menisci in each knee, one medial and one lateral, where they reside as two fibrocartilaginous wedges between the medial and lateral femoral condyles and the tibial plateau (Fig. 1). They are generally divided into three zones; the posterior horn, central body and anterior horn (Fig. 2). First believed to be a vestigial remnant from embryonal development without function, we now know that the menisci have important roles in the knee, such as in shock absorption, load transmission and joint stabilisation[24–26]. In addition, the menisci have also been proposed to be involved in joint lubrication, nutrition and proprioception of the knee[27].

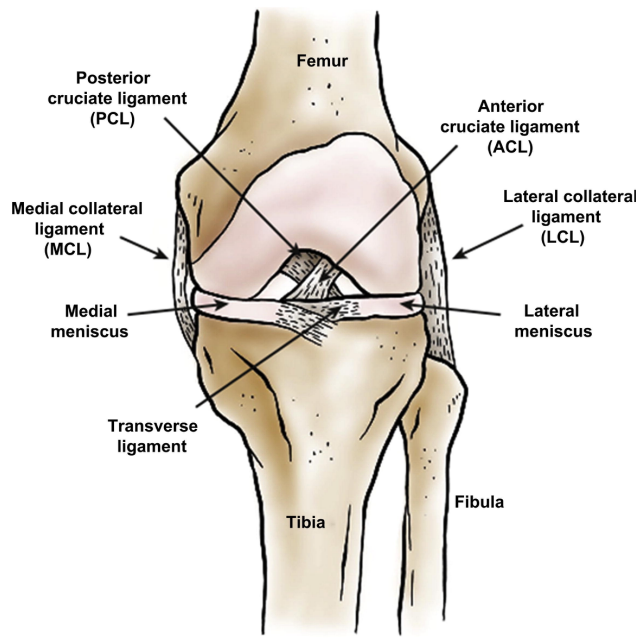

Figure 1.
Anterior view of the anatomy of the knee joint. Reproduced with permission from Makris *et al*., *Biomaterials*, 2011[28].

Embryonic development

During embryonic development, the menisci are formed as a result of condensation of the intermediate layer of the mesenchymal tissue surrounding the joint capsule, and they reach their characteristic shape between the 8th and 10th week of gestation[29,30]. During development, the menisci contain a high number of cells and are completely vascularised. As the foetus becomes more developed, the degree of vascularisation and the number of cells start to decrease, and the collagen content increases[27].

Attachment

The medial and lateral menisci are attached to several other structures in the knee, including to each other (through the anterior horns). The posterior and anterior horns of both the medial and lateral menisci are also attached to the tibia. Further, the peripheral zone and the central body of the medial meniscus are attached to the joint capsule and the deep medial collateral ligament, making it less mobile than the lateral meniscus[31,32]. Another difference is that, since the lateral meniscus has a more circular shape, it covers a larger area of the tibial surface than the medial meniscus[32].

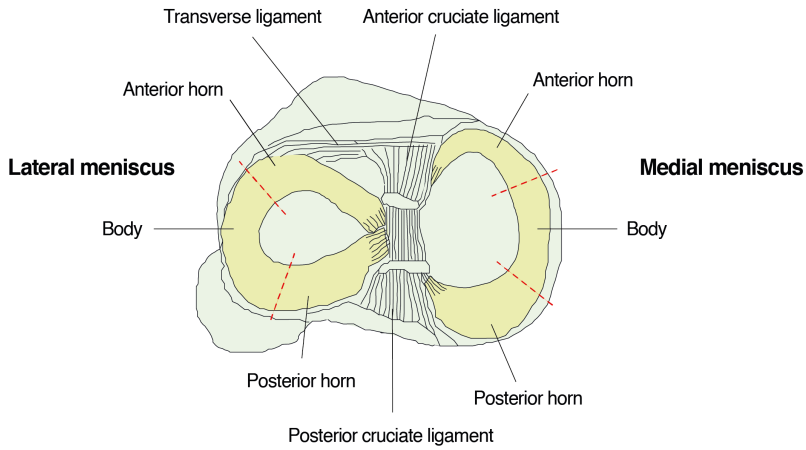

Figure 2.
Anatomy of the tibial plateau with menisci and ligaments, as seen from above.

Vascularisation and innervation

The meniscus, in contrast to articular cartilage, is both vascularised and innervated[30,33–36]. In the adult meniscus, the outer and middle zones (also called red-red zone and white-red zone) are vascularised (Fig. 3)[34]. The inner zone (also called white-white zone) of the meniscus has very low blood supply, if any. Blood supply affects the healing capacity of the meniscus, which appears to be better in the vascularised zones[34]. The main part of the blood supply originates from the medial, lateral and middle geniculate

arteries[37]. The vascularised zones of the meniscus rely on blood for their supply of nutrients, while the avascular zone is dependent on other methods of nourishment. For example, nutrients may be received from the synovial fluid via diffusion[23]. Furthermore, one study of human and bovine menisci reported that the menisci have canal-like structures that penetrate from the surface deeper into the tissue, which might also be a way of providing the avascular parts of the meniscus with nutrients from the surrounding synovial fluid[38].

The menisci are also innervated, and the nerve supply follows roughly the same pattern as the vascularisation, penetrating the joint capsule to reach the menisci[30,35,36]. Three types of mechanoreceptors have been identified in human menisci – Ruffini endings, Pacinian corpuscles and Golgi tendon organs – of which all appear to be more prominent in the horns of the meniscus, and in the posterior horn in particular[27].

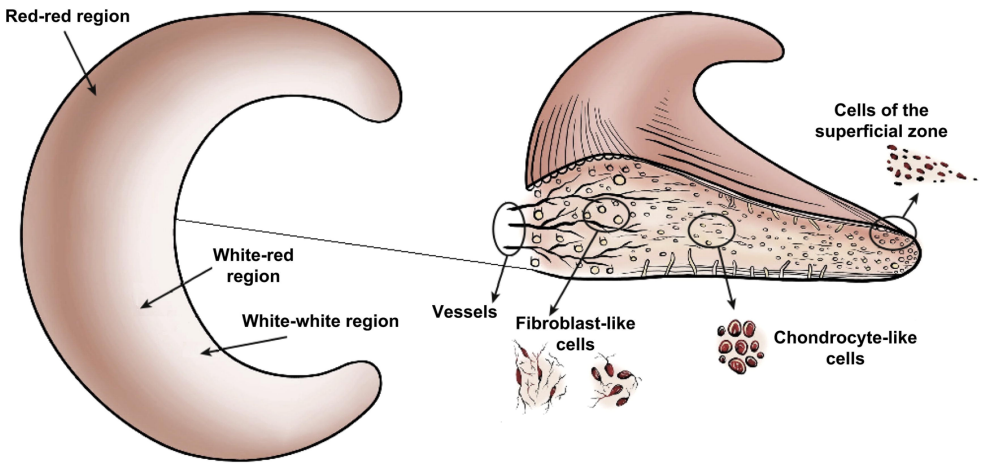

Figure 3.
Regional variations in vascularisation and cell types of the meniscus. *Left:* During development, the menisci are completely vascularised and contain a large number of cells. At later stages of gestation, both vascularisation and cellularity decrease. In adulthood, the red-red zone contains the majority of blood vessels, although some can be found in the white-red zone, whereas the white-white zone is completely avascular. *Right:* Cells in the peripheral, vascularised zone of the meniscus are elongated and fibroblast-like in appearance, while cells in the middle and inner zones are more round and chondrocyte-like. The superficial layer of the meniscus has been reported to host progenitor cells. Reproduced with permission from Makris *et al*., *Biomaterials*, 2011[28].

Cells of the meniscus

Unlike articular cartilage, which only has one cell-type (chondrocytes)[33], the meniscus contains several different cell types in its different regions[39,40]. In the peripheral and middle zones of the meniscus, the cells are mostly elongated and fibroblast-like, whereas in the inner zone, the cells are mostly round and chondrocyte-like (Fig. 3)[39]. In addition, the superficial regions have been reported to host progenitor cells, which have been suggested to play a role in the meniscus´ response to injury[41]. The meniscus also contains less cells than articular cartilage, where the cell density is approximately 14 000

cells/mm^3 [42]. The cell density of the meniscus is approximately 200 – 2 800 cells/mm^3, depending on the zone, where the peripheral zone is suggested to have the highest density[43].

Meniscus extracellular matrix

Just like articular cartilage, the meniscus primarily consists of dense ECM, where the main components are water and collagens, together with lower levels of proteoglycans, glycoproteins and non-collagenous proteins[23].

In a healthy meniscus, water constitutes 65-70% of the tissue's weight, and the posterior horn appears to contain slightly more water[44,45]. Most of the water is bound to the proteoglycans of the meniscus[23]. Proteoglycans are large, negatively charged, hydrophilic and heavily glycosylated proteins that consist of a core protein with one or more glycosaminoglycan (GAG) chains attached[46,47]. By binding water, proteoglycans support the meniscus under compression and greatly contribute to the viscoelastic properties of the meniscus[48]. Proteoglycans are much more prominent in articular cartilage than meniscal tissue, and contribute to approximately 1-2% of the dry weight of menisci[23,49]. Some of the major proteoglycans in the meniscus are aggrecan, biglycan and decorin[28,50], while the main GAG chains of meniscal tissue are chondroitin-6-sulphate and chondroitin-4-sulphate, and to a smaller extent, chondroitin and dermatan sulphate[49].

Collagens make up around 20% of the meniscus tissue. In contrast to articular cartilage, where type II collagen is the predominant collagen, the most common collagen in meniscus is type I collagen, particularly in the peripheral and middle zones[27,33]. However, in the inner zone of the meniscus, type II collagen is more common than type I collagen[51].

Collagens contribute to the meniscus' ability to withstand load, by being responsible for the tissue's tensile strength. The majority of the type I collagen fibers in the deeper zones are circumferentially oriented, parallel to the peripheral border (Fig. 4). They combine with the meniscotibial ligaments to attach the meniscal horns to the tibia, which during weightbearing produces axial forces across the knee that in turn compress the menisci, resulting in distributed circumferential stresses (or hoop stresses)[23,26]. The circumferential collagen fibers are interweaved with radially oriented "tie" fibers, which further contribute to the meniscus´ load bearing functions[52]. There are also radially oriented collagen fibers in the superficial zones of the meniscus[27].

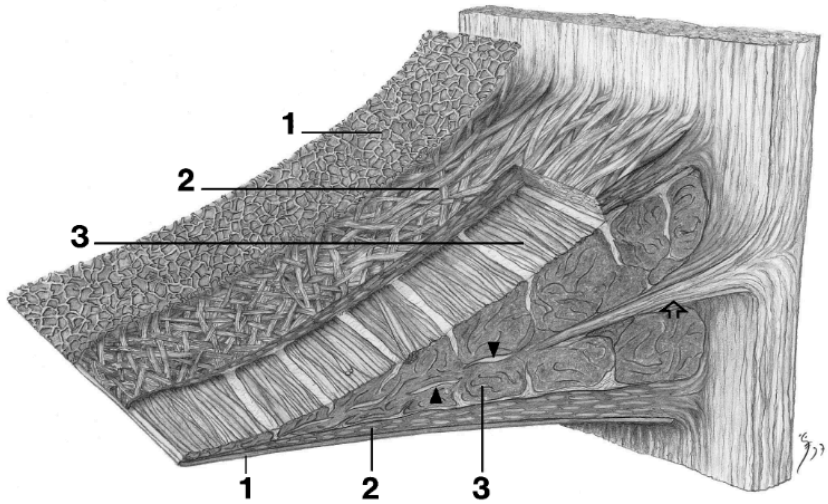

Figure 4.
Drawing of collagen organisation in the meniscus, based on scanning electron microscopy. *(1)* The superficial network: a meshwork of thin fibrils covering the surfaces of the meniscus. *(2)* Lamellar layer: a layer of lamellae of collagen fibrils. In the area of the external circumference of the anterior and posterior horns, the bundles of collagen fibrils are arranged in a radial direction. In all other parts of this layer, the collagen fibril bundles intersect at various angles. *(3)* Central main layer: the layer where the majority of the collagen fibrils of the meniscus are located. The collagen fibrils are circularly oriented, and occasionally interwoven with radial collagen fibrils (arrowheads). In the peripheral border of the meniscus, loose connective tissue radially penetrates the circular fibril bundles from the joint capsule (arrow). Reproduced with permission from Petersen and Tillmann, *Anat Embryol*, 1988[53].

Other groups of matrix proteins found in the ECM of human menisci are non-collagenous proteins and glycoproteins, such as the two adhesive glycoproteins fibronectin and thrombospondin, which are both essential for cell-matrix connections, through interactions with integrins and other receptors[54–57]. Other non-collagenous proteins found in the meniscus ECM are the matrilins, which are important for matrix assembly[58]. Hyaluronan and proteoglycan link proteins (or link proteins), which stabilise proteoglycan aggregates with hyaluronic acid, have also been identified in human menisci[59–62].

Meniscus pathology

Meniscus pathology in the general public

Meniscal pathologies are very common, and the pathology that most people associate with the meniscus is a traumatic tear, usually in the context of a sports injury. This type of tear is most common among young, active individuals with previously healthy knees. During acute knee trauma, usually in combination with twisting of the knee, the meniscus comes under enormous pressure and force between the femoral condyle and tibial plateau, causing a longitudinal tear, where the meniscus ruptures vertically, and

parallel to the circumferentially oriented collagen fibers[63]. Less common is the radial tear where the meniscus ruptures perpendicular to the circumferential fibers[63].

Another type of tear is the degenerative tear, in which there are horizontal cleavage lesions or flap tears of the meniscus, usually in the body or posterior horn of the medial meniscus[64,65]. These types of tears often develop over time, are more rarely associated with an acute knee trauma, and are commonly accompanied by a general destruction of the meniscus[64–66]. Importantly, Englund *et al.* has reported that degenerative tears appear to be frequent in the general population (with or without symptoms), and increase with age, ranging from 19% among women 50 to 59 years of age to 56% among men 70 to 90 years of age[64]. This shows a remarkably high incidence of meniscus tears in the general population, which could be considered as a normal part of ageing. The same study also showed that most of these tears do not directly cause knee symptoms, since 60% of the individuals with meniscal tears did not experience any pain, stiffness or aching of the knee[64].

As previously mentioned, the medial meniscus is more firmly attached in the joint, with attachments to both the tibia and joint capsule[31,32]. This makes the medial meniscus less mobile than the lateral meniscus, which probably explains why the medial meniscus has been reported to be more prone to tears compared to the lateral meniscus[64].

However, it is not only the integrity of the meniscal tissue that is important; the position of the meniscus within the joint is also very important. It has been reported that meniscal tears are often accompanied by meniscal extrusion, which is when the meniscus is partially or totally displaced from the tibial articular cartilage, usually due to a disruption of the circumferential collagen fibers[67,68].

Meniscectomy and knee OA

Meniscectomy is a surgery in which the whole (total) or the injured part (partial) of the meniscus is removed. Total meniscectomy was first described by Sir Thomas Annandale in 1885[69], and four years later Annandale published another report in which he, encouraged by the positive results and good recovery made by the patients, advocated for complete removal of a damaged meniscus, instead of repair[70]. This strategy continued to be the preferred strategy for a very long time, until several studies were published from the 1960s to the 1980s, which reported high incidences of knee symptoms and reduced function of the knee joint, in addition to a high incidence of late degenerative changes and radiographic knee OA, among patients who had undergone a total meniscectomy[71–75].

Today, it is known that the meniscus is important for a functional knee[76]. Therefore, partial meniscectomy using arthroscopy, first described in the 1960s, is now the preferred type of meniscectomy for symptomatic meniscal injuries. It has also been shown to be superior to total meniscectomy, enabling faster recovery and return to sports, for example[77,78]. However, studies have also shown that the long-term effects of

partial meniscectomy are similar to those of total meniscectomy[79–84]. Furthermore, even though many patients experience an improvement after a meniscectomy, it is not clear if it is the procedure itself that is responsible for the effect. Indeed, several randomised clinical trials have reported no additional benefit of arthroscopic meniscectomy compared to sham-surgery or only physical therapy in patients with a meniscal tear, both with and without OA[85–87]. In the case of sham-surgery, the improvement was sustained even two years after surgery[88].

Meniscal pathology and OA

It has been suggested that the biomechanics of the knee play an important role in the development of knee OA, i.e. factors leading to increased biomechanical loading in the joint lead to a pathological response of the joint tissues[89]. There are multiple risk factors for overloading of the knee, including obesity, occupational hazards such as kneeling, knee malalignment, and knee trauma, all of which could lead to meniscal damage or extrusion[90,91]. Other factors, such as ageing, can also cause meniscal damage and degradation of the meniscal ECM[64,92], and hence loss of meniscal function. Since the meniscus has a critical function in the knee to maintain normal biomechanics, loss of its function, either due to damage or resection, can increase the load on other structures of the knee[26]. Thus, meniscal damage can consequently result in loss of articular cartilage[93,94], changes of the trabecular bone[95,96], increased mineral bone density[97,98], and development of subchondral bone marrow lesions[99]. These changes further increase the malalignment of the joint and alter its loading, which can lead to even more degeneration and pathological changes in the joint, and by definition OA. This has been referred to as the "meniscal pathway" to OA, proposed by Englund *et al.* in 2012 (Fig. 5)[15]. Moreover, in the same way that a damaged or injured meniscus can lead to altered biomechanics in the joint, which in turn can result in the degenerative changes seen in OA, partial removal of the meniscus by meniscectomy has also been shown to increase the risk of OA[100,101].

There is further support for an important role of the meniscus in OA from several studies[14,102–105]. One longitudinal study observed that meniscal damage and extrusion at baseline were more frequent in knees that developed radiographic OA compared to control knees[103]. Chan *et al.* reported a high prevalence of meniscal degeneration in OA patients using MR imaging[102]. Another study found that the frequency of meniscal tears was higher in symptomatic OA patients compared to asymptomatic individuals[104]. These results clearly indicate an association between the meniscus and OA, albeit a complex association. It appears as though a meniscal lesion can lead to the development of OA, but also that OA can cause meniscal damage, which can drive the disease further. More knowledge is therefore needed about the human meniscus in health and OA.

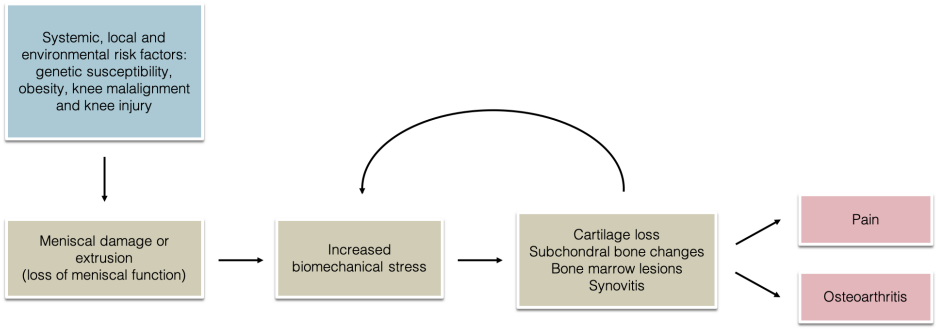

Figure 5.
The "meniscal" pathway to knee osteoarthritis. Loss of meniscal function leads to increased biomechanical stress within the joint, which results in further destruction of the joint through cartilage loss, changes in the subchondral bone, bone marrow lesions, and synovitis. These changes further increase biomechanical stress in the joint, eventually leading to pain and osteoarthritis. Reproduced and adjusted with permission from Englund *et al.*, *Nat Rev Rheumatol*, 2012[15].

Proteomics

Proteomics is the large-scale study of proteomes, which are sets of proteins expressed in a certain organism or tissue at a certain time. The term proteome may refer to all proteins expressed in a species, e.g. humans, or in a specific organ, e.g. the heart. Proteomics allows us to study where and when proteins are expressed, their response to environmental stimuli, and differences in protein expression between biological systems, e.g. healthy and diseased[106]. Since the proteome is dynamic and can change over time, it will reflect the current environment of a cell, organ or organism. Thus, proteomics may be regarded as taking a "snap-shot" of the protein expression in a particular biological system at a particular time point[107].

Protein expression ultimately decides the phenotype of a cell or tissue. Therefore, proteomic analysis is necessary to obtain a full picture of any biological state, complementary to studies of genes through e.g. genomics (all genes present in an organism or tissue) or transcriptomics (genes expressed in a given tissue or biological state). Only by studying the proteome is it possible to investigate the mechanisms of action, protein-protein interactions, and the functions, modifications and localisations of different proteins[107].

Techniques

There are several available methods that can be used to analyse the proteome. The most common ones are mass spectrometry (MS)-based techniques or gel-based techniques,

such as differential in-gel electrophoresis (DIGE). Sometimes, they are also used in combination[107,108].

Discovery-based MS

In this thesis, a label-free, discovery-based MS method was used in three of the studies. In the case of discovery-based MS, the term discovery refers to the fact that we are not targeting any specific proteins in the analysis. Instead, we try to identify as many proteins as possible. During the last decades, this has become one of the most powerful methods to analyse the proteome of complex samples, which has made it possible to identify several hundreds of proteins, even thousands, in a single analysis[109,110]. It allows for comprehensive analyses of a wide variety of tissues, including cartilage tissues[111]. The most common approach of performing discovery-based MS methods is called bottom-up, in which proteins are digested with trypsin into short peptides, which in turn are separated, usually by a method called liquid chromatography (LC)[112]. Before the peptides enter the mass spectrometer, they need to be ionised, often done using electrospray ionisation, a method that was awarded the Nobel Prize in Chemistry in 2002[113,114]. In the mass spectrometer, two MS measurements are then performed in tandem (Fig. 6). In the first step, called MS1, a mass analyser separates the so-called precursor ions by their mass-to-charge ratios (m/z) to determine what is to be fragmented. In tandem MS mode, the precursor ions are isolated to enter a collision cell, where they are fragmented (usually by collision with a gas), which results in product ions. The product ions in turn undergo another separation in the second mass analyser, called MS2, and are subsequently detected in order to generate a tandem mass spectrum (MS2 spectrum). These spectra, or patterns, are specific for each ion, and can together with its m/z value, be matched against *in silico* spectra in protein sequence databases in order to identify the peptides present in the sample[112]. The identified peptides can then be mapped to proteins, and the signal intensities of either the precursor or product ions can be used to estimate relative changes in protein abundance across samples[109].

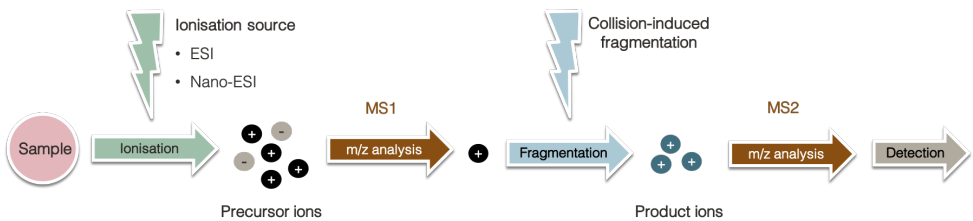

Figure 6.
Schematic representation of tandem mass spectrometry.

Different modes of acquisition

Depending on the type of acquisition method that is used, either all or a selection of precursor ions will be isolated to undergo fragmentation. In data-dependent acquisition (DDA) mode (also known as shotgun proteomics), only a selection of precursor ions are chosen, based on predefined criteria, e.g. the top *n* most intense ions identified in MS1[115]. This is in contrast to data-independent acquisition (DIA) where all precursor ions within a certain mass-to-charge ratio (m/z) range (isolation windows) are selected for fragmentation and MS2[109,116]. The size of the isolation windows in DIA can either be fixed, i.e. constantly the same size, usually around 20-25 Da in width, or variable, where the windows are more narrow in the centre of the mass range, where the density of the precursor ions tends to be the highest[117]. The resulting analysis generates very complex MS2 spectra composed of fragments from multiple precursors in each spectrum, in contrast to the more specific precursor selection achieved through narrow windows in DDA (usually around 1 Da wide). The complexity of the resulting DIA data puts higher demands on computerised data analysis, and several software solutions have therefore been developed. However, even though DIA has become increasingly utilised, DDA is still the preferred MS method in many proteomic studies.

Aims of the thesis

The overall aim of this thesis was to study the proteomic constitution and microstructure of the human meniscus in both healthy subjects and OA patients. More knowledge about the human meniscus and the processes that occur during degeneration is crucial in order to aid earlier diagnosis or identify potential therapeutic targets for OA.

The specific aims were:

Paper I: To detail the microstructural differences between osteoarthritic and normal human menisci using histopathological methods.

Paper II: To identify proteomic similarities and differences between normal human articular cartilage and menisci, as well as to compare two different types of mass spectrometry acquisition methods.

Paper III: To elucidate the proteomic composition of normal human menisci and potential regional differences using data-independent acquisition mass spectrometry.

Paper IV: To map proteomic differences between osteoarthritic and normal human menisci using data-independent acquisition mass spectrometry.

Material and Methods

Study designs

A summary of the study designs for each paper included in this thesis is presented in Table 2.

Table 2.
Overview of study designs and methodology in the papers included in this thesis.

	Paper I	Paper II	Paper III	Paper IV
Study design	Retrospective cohort study	Retrospective cohort study	Retrospective cohort study	Retrospective cohort study
Study population	Healthy donors and OA patients	Healthy donors	Healthy donors	Healthy donors and OA patients (same as paper I)
Gender distribution (% females)	50%	30%	50%	50%
Tissue	Meniscus	Meniscus and articular cartilage	Meniscus	Meniscus
Sample size (per group)	n = 10	n = 3	n = 10	n = 7 - 9
Laboratory method	Histopathology and µCT	DIA/DDA LC-MS/MS	DIA LC-MS/MS	DIA LC-MS/MS
Aim	Two comparisons: (1) Healthy vs OA (2) Medial vs lateral meniscus	Two comparisons: (1) DDA vs DIA (2) Meniscus vs articular cartilage	Regional comparisons	Two comparisons: (1) Healthy vs OA (2) Medial vs lateral meniscus

DIA=data-independent acquisition, DDA=data-dependent acquisition, LC-MS/MS=liquid chromatography tandem mass spectrometry, OA=osteoarthritis

Study populations

In **papers I, III** and **IV** of this thesis, tissue material was obtained from a local biobank (the MENIX biobank) at Skåne University Hospital in Lund, managed by our research group. This biobank includes samples from patients undergoing arthroscopic meniscectomy or TKR, at Trelleborg hospital in Trelleborg, Sweden. It also includes samples from deceased donors, either from the Tissue Bank (Vävnadsbanken) in Lund or from forensic medicine cases at Rättsmedicinalverket in Lund. **Paper II** also included deceased donors, but not from the MENIX biobank.

Meniscectomy and TKR patients

From the meniscectomy patients, who are primarily younger individuals with sports injuries, e.g. ruptured menisci or anterior/posterior cruciate ligaments (ACL/PCL), only smaller pieces of resected tissue are collected, as well as blood samples. There are also some samples from surgeries in middle-aged patients in whom meniscal tears were believed to cause mechanical symptoms or pain in the knee. In this thesis, however, only tissues collected from the TKR patients were used (**papers I** and **IV**). TKR patients contributing to the biobank are predominantly older, female individuals with OA, from whom entire menisci are collected. Additionally, blood, tibial and femoral plateaus, and synovial fluid are also collected from these patients. All obtained samples are frozen (at -80°C) within 2 hours after extraction. Informed consent is required before the surgery and all tissue collections are performed with ethical permission from the local ethics committee.

Furthermore, during TKR surgery, the surgeon is required to classify the knee joint articular cartilage of all patients according to the Outerbridge classification system (Table 1). In order to be classified as a medial compartment knee OA patient (which was the criterion to be included as an OA patient in our studies), the Outerbridge grade were required to be IV in the medial compartment, and 0 or I in the lateral compartment. In addition, the surgeons are also required to sketch the patients' menisci (on a standardised form). In order for the patient menisci to be included in our studies, these sketches needed to indicate that some of the posterior horns remained for both the medial and lateral compartments.

In **papers I** and **IV**, 50% of the included patients were females.

Deceased donors

The biobank also contains tissue from deceased donors, whose samples were used in **papers I, III** and **IV**. The following inclusion criteria were applied: over 18 years of age, having no known OA or rheumatoid arthritis (RA) diagnoses, and proof that the deceased person wished to donate their organs to medical research, or consent from a relative of the deceased for the same. In addition, the following exclusion criteria were also applied: no HIV or hepatitis infection or drug addiction. From the deceased donors, entire menisci, articular cartilage/bone plugs and synovial fluid are obtained within 48 hours post-mortem and frozen (at -80°C) within 2 hours after extraction. All collection is performed with ethical permission from the local ethics committee.

In **paper II** of this thesis, meniscus and articular cartilage from deceased donors were used, but not from the MENIX biobank. Instead, these tissues were collected at the University Hospital in Oslo, Norway, with similar inclusion and exclusion criteria as for the MENIX biobank. Collection procedures were approved by the local ethics

committee in Oslo. From these donors, smaller full-depth articular cartilage pieces (approximately 1x1 cm) were taken from the medial tibial condyle. Full-depth meniscus pieces were taken from the central body of the medial meniscus, including all three zones (inner, middle and peripheral).

In **papers I, III** and **IV**, 50% of the included donors were females, while in **paper II** it was 30%.

Terminology

In **papers I** and **IV**, where medial and lateral menisci from both deceased donors and OA patients were included, a special terminology was applied. Medial and lateral menisci from deceased donors were referred to as medial^{ref} and lateral^{ref} menisci, respectively, and medial and lateral menisci from OA patients were referred to as medial^{OA} and lateral^{OA} menisci, respectively.

Proteomic analyses (papers II, III and IV)

Sample preparation

Before tissue dissection, the menisci were thawed and rinsed in phosphate-buffered saline (PBS). The menisci were also visually inspected. The menisci from OA patients were required to have at least two thirds of the substance of the posterior horn remaining. It was not uncommon for the inner third to be missing, due to degeneration. Furthermore, the donor menisci were required to be macroscopically intact, but some minor calcifications were allowed. The tibial and femoral cartilage from the medial compartment of the same donors were also inspected and were required to be macroscopically intact.

An overview of the sample preparation process can be viewed in figure 7. In **paper III**, medial menisci from ten deceased donors were included, from which slices were cut from the mid portion of the meniscus body and further cut into three parts, corresponding to the three vascularisation zones of the meniscus; inner (avascular white-white zone), middle (slightly vascularised white-red zone) and peripheral (vascularised red-red zone). In **paper IV**, medial and lateral menisci from ten deceased donors and ten OA patients were included, from which plugs were punched from the middle part of the posterior horn of each meniscus. The top and bottom ends (1/4) of the plugs were cut off and only the middle parts of the plugs were used in this paper. Tissue pieces used in **paper II** were already dissected when sent to our group.

Tissue pieces to be analysed with MS (**papers II-IV**), were pulverised in liquid nitrogen using a pestle and mortal technique, after which proteins were extracted using a guanidine hydrochloride (GdnHCl) medium. GdnHCl is a chaotropic agent that denatures proteins by disrupting hydrogen bonds. Samples were subsequently reduced, alkylated (blocking disulphide bonds) and precipitated with ethanol to remove excess salts from the samples. Since a bottom-up MS technique was used in the proteomics papers, the proteins needed to be digested into peptides, which was performed using the protease trypsin. Just prior to the MS analysis, samples were filtered on centrifugal devices to remove GAG chains, after which samples were further cleaned and desalted on reversed-phase C18 columns (**paper III**) or solid phase extraction plates (**paper IV**). In **paper II**, instead of desalting on columns or plates, samples were desalted and fractionated into two fractions (eluted with 10% and 50% acetonitrile respectively) using a high pH reversed-phase peptide fractionation kit.

MS platforms

Two different mass spectrometry set-ups have been used in this thesis. In each case, the same LC system, an Easy nano-LC 1000 HPLC system was used, but, in combination with two different mass spectrometers – a Q-Exactive™ (**paper II** and **III**) or a Q-Exactive™ HF-X (**paper IV**), both from Thermo Fisher Scientific. The same types of pre-columns (C18, 3 µm particles, 75 µm inner diameter, and 2 cm long) and analytical columns (C18, 2 µm particles, 75 µm inner diameter, and 25 cm long) were used in all three papers. Ion transfer was performed under heat and high voltage. In **papers II** and **III** the temperature was 260°C and the voltage was +2000 V, while in **paper IV** they were 280°C and +1850V, respectively. The system was, in both set-ups, controlled by Xcalibur™ Software (Thermo Fisher Scientific). In addition, in **paper IV**, we used a column heater (EASY-Spray source, Thermo Fisher Scientific), and nano-LC peptide separation was performed at 45°C to enable better reproducibility of retention times and for reduced back-pressure.

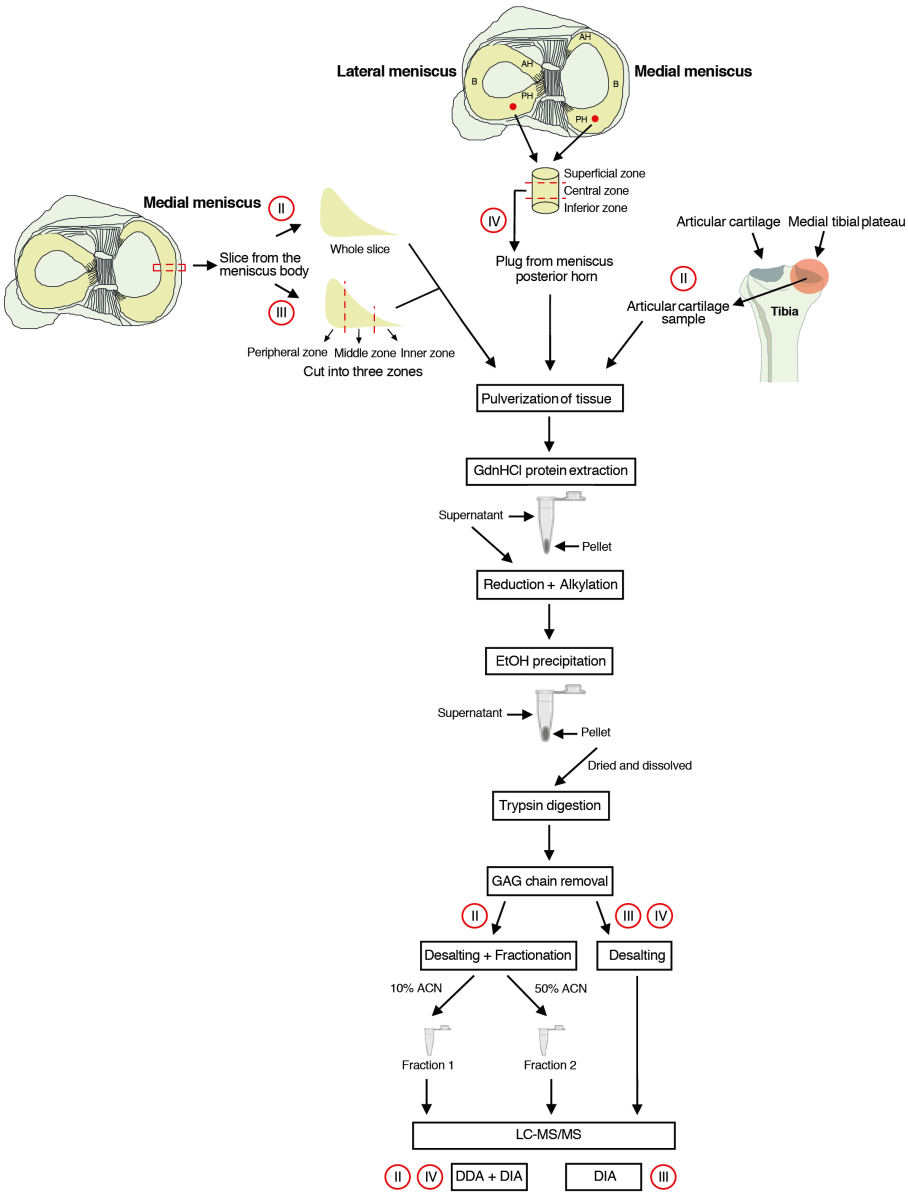

Figure 7.
Overview of sample preparation for proteomic analyses. In paper II, whole slices (II) from the body of medial menisci were used, in addition to articular cartilage samples (II). In paper III, slices from the body part of medial menisci were cut into three parts, corresponding to the three zones of the meniscus – the peripheral, middle and inner zones (III). In paper IV, tissue plugs from the posterior horn of lateral and medial menisci were used (IV). In all three papers, the tissue pieces were prepared in the same manner until the desalting step (please refer to "Sample preparation" in "Material and methods" for details) . All samples were analysed with LC-MS/MS, but in different modes; DDA and DIA were used in papers II and IV (II, IV), whereas only DIA was used in paper III (III). *GdnHCl* = Guanidine hydrochloride, *EtOH* = Ethanol, *GAG* = Glycosaminoglycan, *ACN* = Acetonitrile, *LC-MS/MS* = Liquid chromatography coupled with tandem mass spectrometry, *DDA* = Data-dependent acquisition, *DIA* = Data-independent acquisition.

Discovery MS

In this thesis, only discovery MS was performed, but two different acquisition methods were applied; DDA and DIA. In **paper II**, a comparison of DDA and DIA was carried out in order to determine the method that yielded the best results for cartilaginous tissues. Subsequently, in **paper III** only DIA was used, and in **paper IV**, DIA was the main acquisition method, but some samples were run with DDA in order to create a spectral library for data analysis. A summary of the various MS settings used in **papers II-IV** can be seen in Table 3.

Table 3.
The different MS settings used in **papers II**, **III** and **IV**.

	Settings	Paper II (DIA)	Paper II (DDA)	Paper III (DIA)	Paper IV (DIA)	Paper IV (DDA)
	Gradient length (min)	135	85/135*	125	125	125
MS1	**m/z range**	390-1210	400-1200	390-1010	350-1650	375-1400
	Resolution	70,000	70,000	70,000	120,000	120,000
	ACG	$1x10^6$	$1x10^6$	$1x10^6$	$3x10^6$	$3x10^6$
	Maximum injection time (ms)	100	100	100	100	50
MS2	**m/z range**	400-1200	-	400-1200	350-1650	-
	Resolution	35,000	17,500	35,000	45,000	15,000
	ACG	$1x10^6$	$1x10^6$	$1x10^6$	$3x10^6$	$1x10^5$
	Maximum injection time (ms)	120	60	120	Automatic	30
	DIA loop count	32	-	24	26	-
	DIA isolation window size (m/z)	26	-	26	Variable	-
	DIA isolation window overlap (Da)	0.5	-	0.5	1	-

*Four DDA runs were run with the same gradient as the DIA runs in order to create a spectral library. ACG=automatic gain control

MS data analysis

In **paper II**, the raw DDA data files were searched against a human Swiss-Prot database using Proteome Discoverer™ 2.1 (Thermo Fisher Scientific). For each protein, peak intensities based on the average of their top 3 peptides, represented the abundance of the protein in the tissue extracts[118]. A spectral library was created in Proteome Discoverer using four DDA runs (which were run using the same gradient as the DIA runs) and subsequently imported into Skyline Daily (MacCoss Laboratories), where the DIA data were analysed and matched against the generated spectral library. Since the aim of this study was to compare the DDA and DIA protocols to select the best approach for further studies of cartilaginous samples, we chose to narrow down the number of proteins included in the comparison. Therefore, only a subset of the identified proteins was selected. This subset of proteins was based on three certain criteria: (1) proteins had to have at least two unique peptides per protein, (2) proteins

had to be classified as an ECM protein and (3) proteins had to be identified in both tissue types. Manual peak selection of the peptides was performed in Skyline and based on this selection of proteins. The proteins and peptides remaining after peak selection (n=103) were the basis of the statistical analysis, where only MS1 data was included. In order to receive peak area values from the DIA analysis that were comparable to those from the DDA analysis, a manual approach, similar to that performed in Proteome Discoverer, was performed on the DIA data. All peak area intensity values for each precursor ion were summed up, resulting in one peak area value for each protein in each fraction. In order to get one peak area value for each sample, values from the two fractions from each sample were added together, and this value was used for further analysis. Only proteins with quantitative information in all donors from both tissues (n=90 proteins) were included in the statistical analysis.

In **papers III** and **IV**, the data analysis was performed in Spectronaut™ Pulsar (Biognosys AG, Switzerland). In **paper III**, the same human protein database from Swiss-Prot was used as the background proteome, and a library-free workflow called directDIA™ was implemented. In **paper IV**, a more updated version of the human Swiss-Prot database was applied, and the DIA data were searched against a spectral library based on all the DIA runs as well as 14 DDA runs. In both papers, the top 3 peptides for each protein were averaged to calculate protein abundance, and only proteotypic peptides were used[119]. Proteotypic peptides are peptides that are solely found in one single known protein and therefore uniquely represent that protein[119]. Precursor quantitation was performed at the MS2 level, and area under the curve was used as quantitation type.

Microstructural analyses (paper I)

Sample preparation for microstructural analyses

Papers I and **IV** used the same menisci for their respective analyses, and hence the same visual inspection of the menisci applied to both studies. After the plug was punched out from the posterior horn (for analysis in **paper IV**), the remaining part of the posterior horn, in addition to a small portion of the body, was cut off and fixed in 4% saline-buffered formaldehyde. These samples were then sent to collaborators at the University of Oulu, Finland, for histopathology and *ex vivo* micro-computed tomography (µCT), reported in **paper I**.

Preparation for µCT

A slice from the body part of the meniscus was cut (Fig. 8 A) and prepared for µCT by being dehydrated in ethanol, then treated with hexamethyldisilazane (HMDS) and air-

dried. These steps were performed according to a recently published protocol by Kestilä *et al.*[120].

Preparation for histopathology

From the remaining pieces after excision of the µCT section, slices were obtained for histopathological analyses. Prior to slicing, the fixed samples were further dehydrated with alcohol, cleared with xylene, and embedded in paraffin. Vertical, wedge-shaped slices were cut from three different locations, perpendicular to the circumferentially oriented collagen bundles. The remaining paraffin blocks were melted, and the tissues were reoriented and re-embedded in paraffin, followed by the cutting of horizontal slices (from the inner to the peripheral zone, at an approximately 30° angle to the tibial plateau) from two different locations (Fig. 8 B and C).

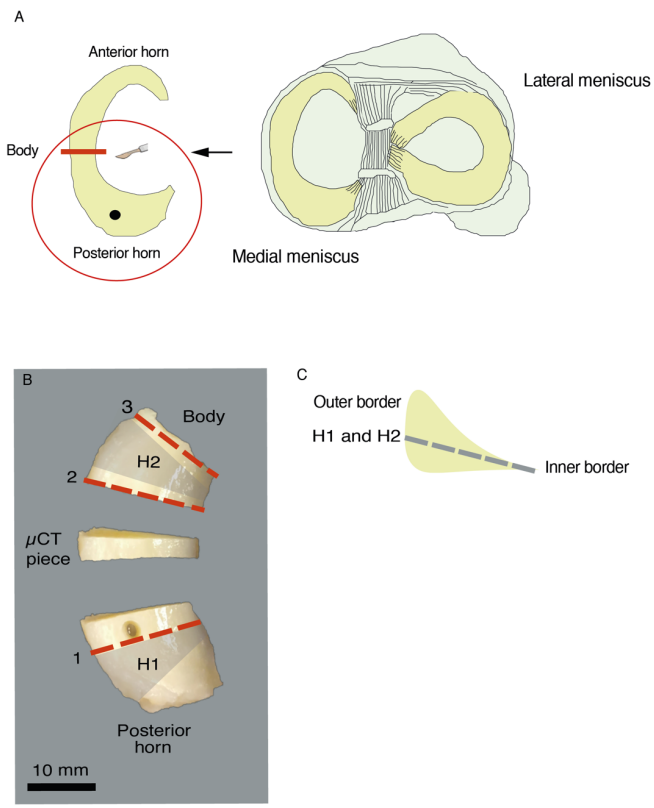

Figure 8.
An overview of the sample preparation for the microstructural analyses. *A)* A schematic illustration of dissection of the meniscus posterior horn. *B)* The locations from where the vertical (1, 2, 3) and horizontal (H1 and H2) slices were taken for histopathological analysis, as well as from where the µCT piece was cut from the meniscus posterior horn. The tissue extracted from the hole in the posterior horn (adjacent to slice 1) was reserved for paper IV in this thesis. C: A schematic illustration of the plane from which the horizontal histological slices (H1 and H2) were taken. Reproduced with permission from Kestilä *et al.*, *Osteoarthritis Cartilage*, 2019[121].

Histopathology

All slices were stained with haematoxylin and eosin (HE), and Safranin-O–Fast Green (SafO–FG), and subsequently imaged with a digital pathology slide scanner. HE staining is one of the most common types of staining for cells and tissues[122]. Haematoxylin stains nucleic acids with a blue-purple colour, while eosin stains proteins pink in a non-specific manner[122]. Typically, a tissue stained with HE will have blue nuclei and pink cytoplasm and ECM. Safranin-O is a cationic dye that stains proteoglycans and GAG chains in a dark pinkish-red colour[123].

Sample processing, staining, and scoring were performed according to Pauli *et al.*[92] with some minor modifications. The following features were scored: (1) tissue surface characteristics (smooth, or degree of fibrillation, clefts or undulation) of the tibial, femoral and outer borders, (2) cellularity (normal, hypercellular, hypocellular, and acellular regions), (3) matrix and collagen organisation (degeneration, calcification, cyst formation, oedema, fraying and tears) and (4) SafO-FG staining intensity of the matrix. Each feature was individually scored (referred to as a sub-score) and added up to a total score. The scoring was first performed independently by two blinded graders, after which consensus scoring was done for each sub-score, from which an overall consensus score was calculated for each meniscus section. Each total consensus score was translated into a grade from 1 to 4, which represented different degrees of degeneration (Table 4).

Table 4.

The scoring system used in paper 1, based on Pauli *et al.*[92].

Total score	Grade	Phenotype
0-4	1	Normal tissue
5-9	2	Mild degeneration
10-14	3	Moderate degeneration
15-18	4	Severe degeneration

Ex vivo micro-computed tomography (µCT)

Image acquisition was performed using a SkyScan desktop µCT from Bruker. The following settings were applied: tube voltage of 40 kV, tube current of 250 µA, no additional filtering, isotropic voxel size of 2.0 µm, number of projections – 2400, averaging 5 frames/projection, and exposure time of 1815 ms. NRecon software (Bruker) was used for image reconstruction, during which beam-hardening and ring-artefact corrections were applied. Image rendering was performed with CTVox software provided by the manufacturer.

Statistical analyses

In **paper I**, the statistical analyses consisted of descriptive statistics of the histopathological scores, as well as linear regression models where the histopathology scores for each meniscus section were treated as a continuous variable. All analyses were first unadjusted, then adjusted for age, and finally adjusted for age and body mass index (BMI). For comparisons between the groups (medial^{ref}, lateral^{ref}, medial^{OA} and lateral^{OA}), a mixed linear regression model was used with the histopathology score as outcome. The Satterwhite method was used for estimation of degrees of freedom and the *lincom* command in Stata was used to derive the comparisons of interest from the linear model. The mean difference between the four groups was calculated with 95% confidence intervals (CI). Stata (Release 15, StataCorp) was used for all statistical analyses.

In **paper II**, the data were analysed using a linear mixed-effect model fitted through restricted maximum likelihood using the ANOVA method for computing degrees of freedom. The tissue donor was included as a random effect and the tissue (meniscus or articular cartilage) was included as a fixed effect. The data were $\log_2$-transformed before the analysis. To mimic a typical analysis of data in proteome studies, we also performed an analysis with a control of the false discovery rate (FDR), using the method of Benjamini and Hochberg[124]. We considered proteins with a two-tailed p-value less or equal to 0.05 to be differentially expressed. Stata (Release 14, StataCorp) was used for all statistical analyses.

In **paper III**, only proteins with a maximum of 10% missing values per condition were included in the statistical analysis, i.e. one missing value per zone (peripheral, middle or inner) was allowed among the ten donor samples analysed for each zone. This resulted in a maximum of three missing values per protein in the whole data set (i.e. from all ten donors, for all three zones). Protein intensity was transformed using logarithm with base 2. Using the *limma* (Bioconductor) package in R (https://www.R-project.org), a series of fixed effects linear regression models were fitted to estimate the differences between the zones. The 99% CIs were estimated, consistent with the applied FDR control procedure as described by Benjamini and Yekutieli[125].

In **paper IV**, only proteins with a maximum of one missing value per group (i.e. medial^{ref}, lateral^{ref}, medial^{OA} and lateral^{OA}) were included. This resulted in a maximum of four missing values per protein in the whole dataset (i.e. from all four groups). All protein intensities were transformed using log function with base 2 before the analysis. A linear mixed effects model was applied, with transformed intensities as outcome, and group, protein type and their interaction as independent variables. Subject identifier was included as a random effect to account for correlation between measurements from the same individual. Furthermore, the model was adjusted for age and BMI. All statistical analyses were performed in Stata (Release 15, StataCorp).

Additionally, in **papers III** and **IV**, functional enrichment and protein network analyses were performed, in order to investigate the biological relevance of the results. In **paper III**, this was performed by searching the data in the publicly available STRING database, version 11.0[126], where a protein network analysis was performed in order to investigate the overrepresentation of protein-protein interactions (PPI) in our data. Additionally, functional enrichment analysis was also performed in order to determine if any gene ontology (GO) terms were overrepresented in the network. In **paper IV**, this analysis was performed in Ingenuity® Pathway Analysis (IPA®, QIAGEN Bioinformatics, Fall release, 2019). The proteins were mapped and compared to known pathways, diseases, functions, and connecting regulators. A z-score for each pathway was calculated as a statistical measure of the match between the expected direction of the relationship and the observed protein expression, and was used to determine how strong the overrepresentation was; positive for regulators with activating capacity and negative for those with inhibitory capacity. It was also investigated whether any protein networks were overrepresented in our data.

Results

Paper I

Higher histopathological scores are associated with more degeneration

When comparing meniscus slices of a higher histopathological grade with those of a lower histopathological grade, by studying virtual light microscopy images, it appears that the higher grades are associated with more degeneration of the meniscus borders, i.e. the tibial and femoral surfaces of the meniscus as well as the outer border, together with more intense SafO-FG staining and disorganised collagen networks (Fig. 9).

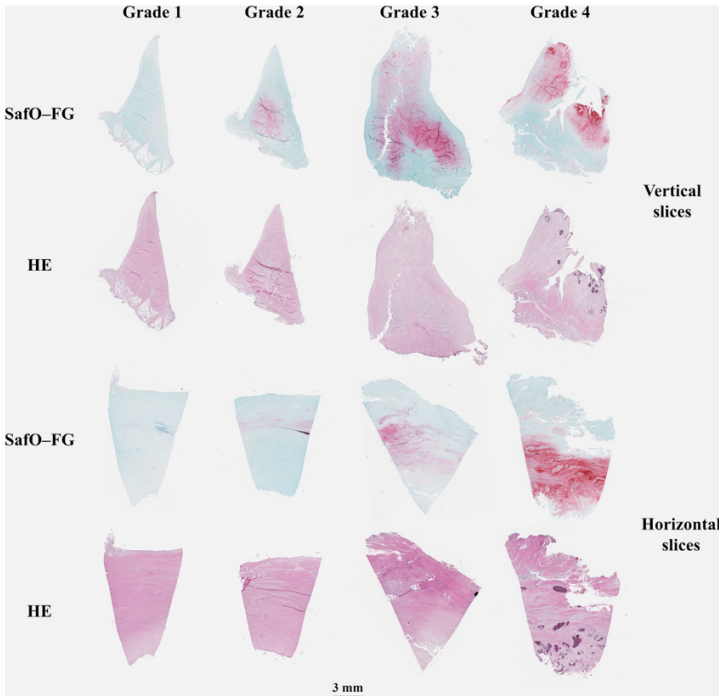

Figure 9.
Representative histological images from SafO–FG and HE-stained vertical and horizontal meniscal slices for Pauli's meniscus histopathological grades 1-4[92]. Slices were imaged with a digital pathology slide scanner. Reproduced with permission from Kestilä *et al.*, *Osteoarthritis Cartilage*, 2019[121].

Several structural features were visible in both the three-dimensional (3D) µCT and histological images, including surface integrity and collagen network organisation. Both these structural elements appeared to show similar results in the µCT analysis and the histology analysis, with greater surface degeneration and more collagen disorganisation in menisci with higher histopathological grades compared to those with lower grades (Fig. 10). Furthermore, the µCT analysis also showed calcifications (predominately in the menisci with higher histopathological grades) and the channel-like structures that were observed to penetrate the meniscus from its outer border (predominately in the menisci with lower histopathological grades) (Fig. 10).

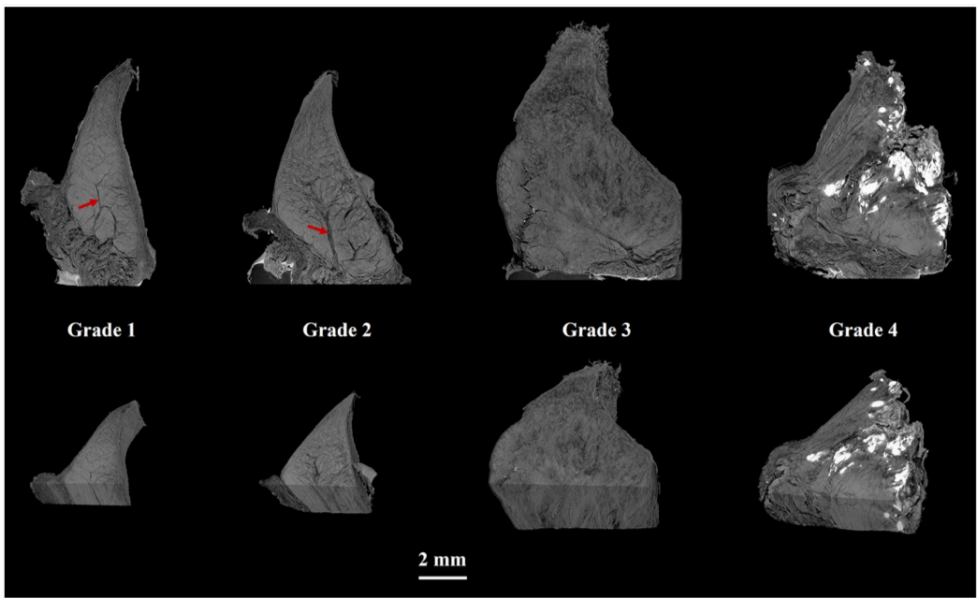

Figure 10.
Volumetric visualisations of µCT images from meniscal samples representing Pauli's meniscus histopathological grades 1–4[92]. µCT images appear to contain similar microstructural information as the histology images. Channel-like structures were clearly visible, especially in the samples with lower grades (red arrows). Ectopic calcification appeared as white areas in the µCT images, and was especially prominent in grade 4 samples. Reproduced with permission from Kestilä *et al.*, *Osteoarthritis Cartilage*, 2019[121].

Medial^{OA} menisci had the highest degree of degeneration

Of all groups, medial^{OA} menisci had the highest total histopathological consensus scores, while the medial^{ref}, lateral^{ref} and lateral^{OA} menisci had similar scores. After adjusting for age, and for age and BMI, the medial^{OA} menisci had a 3.9 points higher total score (95% CI 2.6, 5.3) compared to the medial^{ref} menisci. Medial^{OA} menisci also had higher scores than lateral^{OA} menisci (3.9 [2.8, 5.0]). No such differences could be observed between the lateral^{OA} and lateral^{ref} menisci (0.8 [-0.6, 2.2]), or between the medial^{ref} and lateral^{ref} menisci (0.8 [-0.3, 1.9]) (Fig. 11 A).

The degree of degeneration differs between the different sections of the meniscus

The medial^{OA} menisci were found to have higher histopathological scores in all three sections compared to the medial^{ref} menisci. The medial^{OA} menisci also had higher total scores in sections 2 and 3 compared to the lateral^{OA} menisci (Fig. 11 B). While the largest difference in total score was observed between these two groups in section 3 (closest to the body region, 5.5 [3.8, 7.2]), the smallest difference was observed in section 1 (closest to the posterior horn) with a difference of 1.7 (-0.04, 3.4) (Fig. 11 B). Although, the medial^{ref}, lateral^{ref} and lateral^{OA} menisci had similar scores overall, in section 1, the lateral^{OA} menisci had higher scores than the lateral^{ref} menisci (3.7 [1.8, 5.7]) and the medial^{ref} had higher scores than the lateral^{ref} menisci (2.1 [0.4, 3.8]).

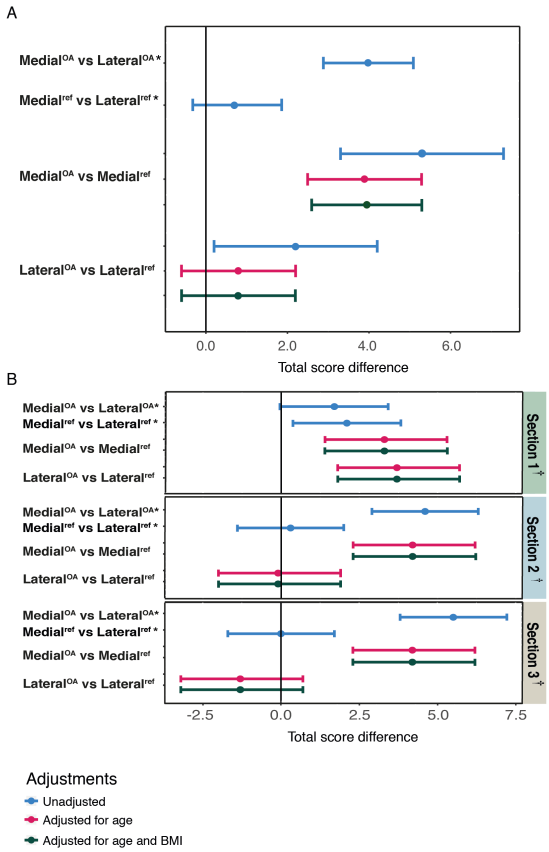

Figure 11.
Comparisons of the posterior horns from medial and lateral reference menisci with posterior horns of medial and lateral menisci from patients with medial compartment knee OA, where *(A)* shows the mean difference in total histopathological scores (Pauli) with 95% confidence intervals, and *(B)* shows the mean difference in total histopathological (Pauli) scores displayed with 95% confidence intervals in three different sections† (referring to histological slices 1,2 and 3 from the meniscus). The model was adjusted for age, and then for age and BMI. *This comparison was performed between medial and lateral menisci from the same knee, and thus adjusted for all person- and knee level confounding through use of a fixed effects model. Reproduced and adjusted with permission from Kestilä *et al.*, *Osteoarthritis Cartilage*, 2019[121].

Paper II

The majority of proteins are similar between articular cartilage and meniscus

A total of 673 proteins were identified in the DDA analysis. Out of these, 358 proteins had at least two unique peptides and could be identified in at least three of the samples, and were used for further analysis. Among these, the majority of proteins (n=307) were common between meniscus and articular cartilage, while 40 proteins were unique to the meniscus, and 11 proteins were unique to the articular cartilage (Fig. 12). The majority of the ECM proteins included in the statistical analysis also had similar abundance in meniscus and articular cartilage, both in the DDA and DIA analyses (Fig. 13 A). Among the proteins with higher levels in articular cartilage, membrane-associated phospholipase A and lysozyme C yielded the largest differences in the DIA analysis and DDA analysis, respectively. Among the proteins with higher levels in meniscus, dermatopontin showed the largest difference in both analyses (Fig. 13 B, Table 5). Aggrecan was the protein with the highest intensity in articular cartilage, whereas it was serum albumin in the meniscus.

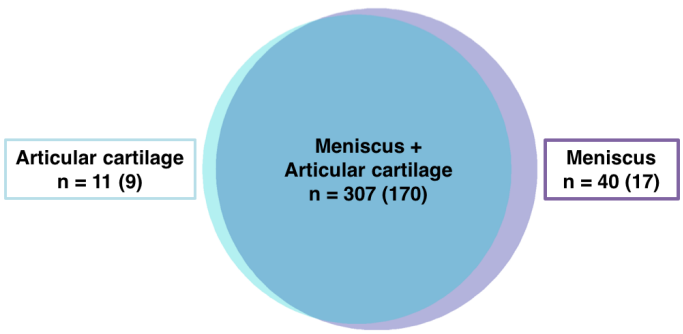

Figure 12.
Venn diagram displaying the distribution of proteins in the meniscus and articular cartilage. Only proteins that had at least two unique peptides and could be identified in at least three of the samples were included in the figure. The majority of proteins were common between the two tissues. The corresponding numbers for proteins classified as extracellular are shown in brackets. Reproduced with permission from Folkesson et al., *BMC Musculoskelet Disord*, 2018[127].

DIA exhibits more differentially expressed proteins and narrower confidence intervals than DDA

21 proteins were differentially expressed between articular cartilage and the meniscus in the DIA analysis, and 19 remained different after FDR control (Fig. 13 B, Table 5). However, in the DDA analysis, only 11 proteins were differentially expressed, and only four of these remained after FDR control (Fig. 13 B). On average, the ratios of protein abundance between articular cartilage and the meniscus were similar even if numerical differences were found. In addition, the DDA approach appeared to yield higher estimated intensity values than DIA.

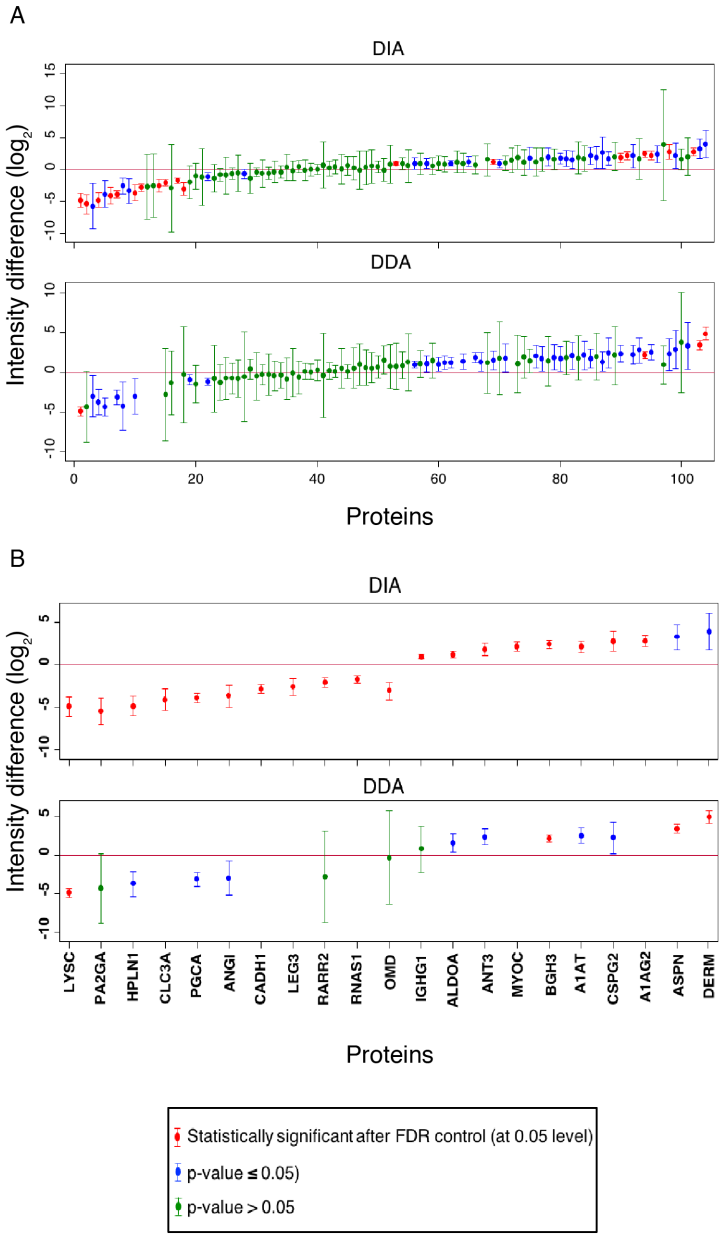

Figure 13.

Intensity differences on a $\log_2$ scale, displayed with 95% confidence intervals, between meniscus and articular cartilage in DDA and DIA, for *(A)* all 90 proteins included in the statistical analysis and *(B)* for the proteins that were statistically significantly different in at least one of the methods. Reproduced with permission from Folkesson et al., *BMC Musculoskelet Disord*, 2018[127].

Table 5.
Proteins that were differentially expressed in at least one of the methods (DDA or DIA), displayed with meniscus (M) vs articular cartilage (C) ratios together with 95% confidence intervals. Repoduced with permission from Folkesson *et al.*, *BMC Musculoskelet Disord*, 2018[127].

Protein		DIA		DDA	
Protein name	**Entry name**	**M vs C Ratio**	**95% CI**	**M vs C Ratio**	**95% CI**
Phospholipase A2, membrane associated	PA2GA	**0.02**	0.008, 0.07	0.05	0.002, 1.08
Lysozyme C	LYSC	**0.03**	0.02, 0.08	**0.03**	0.02, 0.05
Link protein 1	HPLN1	**0.03**	0.02, 0.08	**0.07**	0.02, 0.23
C-type lectin domain family 3 member A	CLC3A	**0.06**	0.02, 0.14	-	-
Aggrecan core protein	PGCA	**0.07**	0.04, 0.10	**0.11**	0.06, 0.21
Angiogenin	ANGI	**0.08**	0.03, 0.19	**0.12**	0.03, 0.57
Osteoadherin	OMD	**0.12**	0.06, 0.24	0.81	0.01, 53.12
Cadherin-1	CADH1	**0.14**	0.10, 0.20	-	-
Galectin-3	LEG3	**0.17**	0.09, 0.34	-	-
Retinoic acid receptor responder protein 2	RARR2	**0.24**	0.16, 0.35	0.14	0.002, 8.30
Ribonuclease pancreatic	RNAS1	**0.31**	0.23, 0.42	-	-
Immunoglobulin heavy constant gamma 1	IGHG1	**1.90**	1.59 2.27	1.67	0.22, 12.63
Fructose-bisphosphate aldolase A	ALDOA	**2.31**	1.77, 3.01	**2.87**	1.27, 6.50
Antithrombin-III	ANT3	**3.52**	2.10, 5.92	**5.07**	2.45, 10.48
Alpha-1-antitrypsin	A1AT	**4.37**	2.76, 6.93	**5.66**	2.91, 11.01
Myocilin	MYOC	**4.40**	2.92, 6.65	-	-
Transforming growth factor-beta-induced protein ig-h3	BGH3	**5.32**	3.75, 7.54	**4.43**	3.29, 5.97
Versican core protein	CSPG2	**6.64**	3.02, 14.57	**4.71**	1.16, 19.16
Alpha-1-acid glycoprotein 2	A1AG2	**6.83**	4.39, 10.64	-	-
Asporin	ASPN	**9.40**	3.32, 26.63	**10.79**	7.39, 15.7771
Dermatopontin	DERM	**14.97**	3.34, 66.99	**29.25**	16.356, 52.28

Statistically significant ratios (p-value ≤ 0.05) are marked in **bold**.

Paper III

The largest zonal differences in human menisci can be found between the peripheral and inner zones

A total of 638 proteins were identified in the MS analysis of all three meniscal zones. Out of the 405 proteins included in the statistical analysis, 170 proteins differed in at least one of the comparisons between the zones. Among these, 163 differed between the peripheral and inner zones (Fig. 14), of which 129 proteins had a higher intensity in the peripheral zone, and 34 proteins had a higher intensity in the inner zone. Myocilin was the protein with the highest fold-change overall, with a fold-change between the peripheral and inner zones of 42.2 (95%CI 16.0, 119.4).

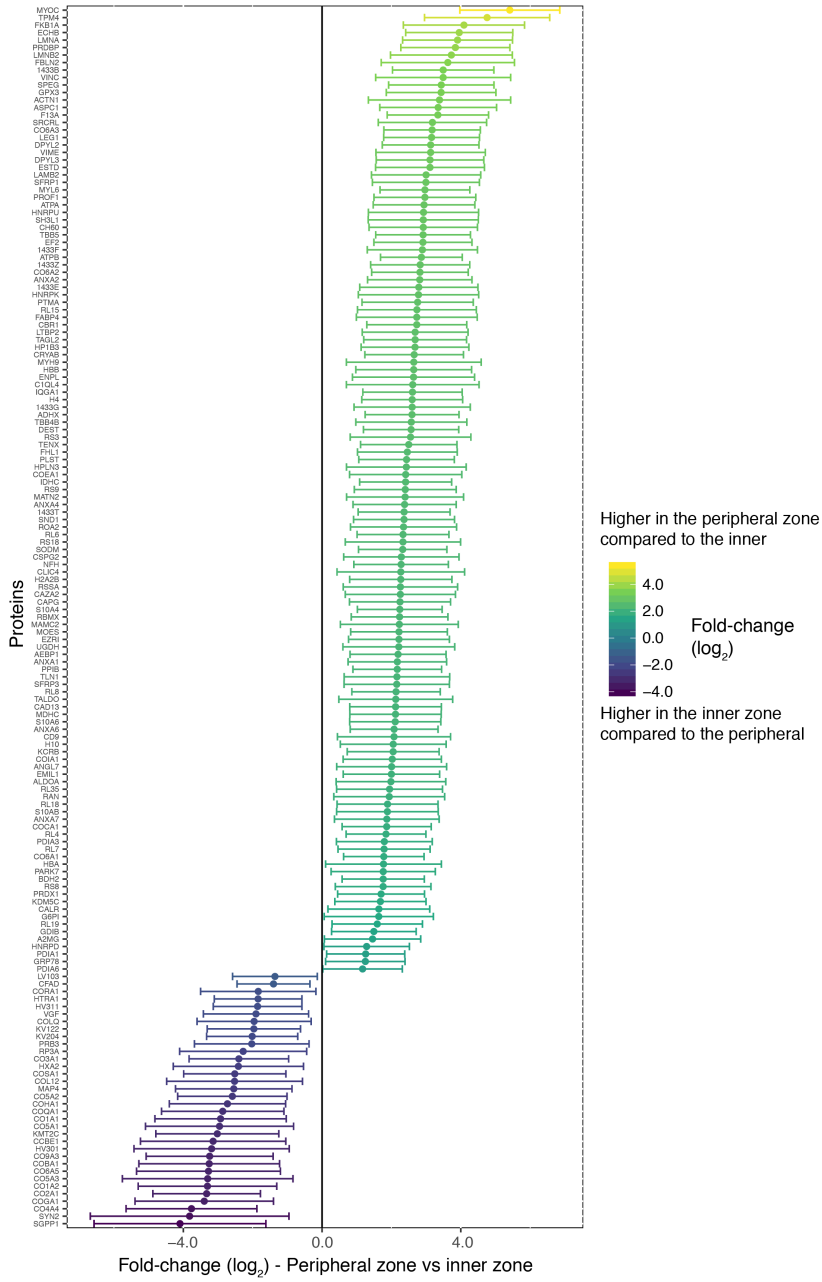

Figure 14.

Visualisation of all differentially expressed proteins between the peripheral and inner zone (y-axis). Log_2-transformed fold-changes are displayed as point estimates, with 99% confidence intervals as error bars (x-axis). The colour of the point estimates and error bars represents the log_2 fold-change, according to the scale bar on the right.

136 proteins had different abundances between the middle and peripheral zones. Of these, 101 had a higher expression in the peripheral zone, and 35 had a higher expression in the middle zone. Only 8 proteins were differentially expressed between the middle and inner zones, of which 7 had a higher expression in the inner zone.

PRELP is one of the most abundant proteins in human menisci

The small leucine-rich proteoglycan (SLRP) PRELP had the highest abundance values among the proteins in the middle and peripheral zones in this analysis, while 2-oxoglutarate dehydrogenase, mitochondrial, had the highest abundance values in the inner zone. Many proteins were common to all zones, and among the top 20 proteins in each of the three zones, 15 were the same, although in varying orders of abundance in the different zones.

The majority of meniscal proteins are associated with ECM organisation

The functional enrichment analysis of all identified proteins, in all zones, in this study showed ECM organisation and exocytosis as enriched biological processes (Table 6). Indeed, the proteins upregulated in the inner zone compared to the peripheral zone were mainly collagens or proteins related to collagens, and therefore involved in ECM organisation (Table 7). Conversely, the majority of proteins upregulated in the peripheral zone were intracellular proteins present in the cytosol (Table 8).

Table 6.
Top 5 enriched biological process and cellular component GO terms in all identified proteins in the study, based on the STRING search, together with FDR-adjusted p-values.

GO term	Biological process	Count in data set	Adjusted p-value
GO:0043062	Extracellular structure organisation	95 of 339	1.39×10^{-51}
GO:0030198	Extracellular matrix organisation	83 of 296	8.16×10^{-45}
GO:0045055	Regulated exocytosis	102 of 691	4.90×10^{-34}
GO:0006887	Exocytosis	103 of 774	4.80×10^{-31}
GO:0032940	Secretion by cell	114 of 959	8.75×10^{-31}
GO term	**Cellular component**	**Count in data set**	**Adjusted p-value**
GO:0005576	Extracellular region	310 of 2505	1.15×10^{-108}
GO:0044421	Extracellular region part	208 of 1375	7.41×10^{-80}
GO:0031012	Extracellular matrix	89 of 283	6.46×10^{-53}
GO:0005615	Extracellular space	155 of 1134	8.26×10^{-52}
GO:0060205	Cytoplasmic vesicle lumen	87 of 340	9.73×10^{-46}

Table 7.
Top 5 enriched biological process and cellular component GO terms in proteins more abundant in the inner zone compared to the peripheral zone, based on the STRING search, together with FDR-adjusted p-values.

GO term	Biological process	Count in data set	Adjusted p-value
GO:0030198	Extracellular matrix organisation	13 of 296	2.50×10^{-13}
GO:0030199	Collagen fibril organisation	8 of 39	1.00×10^{-12}
GO:0071230	Cellular response to amino acid stimulus	5 of 60	1.05×10^{-5}
GO:0009887	Animal organ morphogenesis	10 of 865	5.35×10^{-5}
GO:0001501	Skeletal system development	8 of 457	5.35×10^{-5}
GO term	**Cellular component**	**Count in data set**	**Adjusted p-value**
GO:0005581	Collagen trimer	19 of 88	1.29×10^{-34}
GO:0098644	Complex of collagen trimers	10 of 19	1.70×10^{-20}
GO:0005788	Endoplasmic reticulum lumen	16 of 299	7.09×10^{-20}
GO:0005583	Fibrillar collagen trimer	9 of 11	7.09×10^{-20}
GO:0044420	Extracellular matrix component	11 of 59	6.04×10^{-19}

Table 8.
Top 5 enriched biological process and cellular component GO terms in proteins more abundant in the peripheral zone compared to the inner zone, based on the STRING search, together with FDR-adjusted p-values.

GO term	Biological process	Count in data set	Adjusted p-value
GO:0071840	Cellular component organisation or biogenesis	83 of 5342	8.22×10^{-15}
GO:0016043	Cellular component organisation	78 of 5163	8.95×10^{-13}
GO:0048519	Negative regulation of biological process	73 of 4953	6.04×10^{-11}
GO:0006614	SRP-dependent co-translational protein targeting to membrane	13 of 92	1.43×10^{-10}
GO:0070972	Protein localization to endoplasmic reticulum	14 of 123	1.94×10^{-10}
GO term	**Cellular component**	**Count in data set**	**Adjusted p-value**
GO:0044444	Cytoplasmic part	109 of 9377	3.10×10^{-15}
GO:0005829	Cytosol	76 of 4958	2.12×10^{-13}
GO:0042470	Melanosome	15 of 105	2.99×10^{-13}
GO:0005737	Cytoplasm	113 of 11238	9.74×10^{-12}
GO:0043209	Myelin sheath	15 of 157	3.31×10^{-11}

Paper IV

There are larger differences between medial menisci from reference subjects and OA patients, compared to medial and lateral menisci from the same individuals

In this study, we identified a total of 835 proteins, of which 331 had a maximum of one missing value per sample group and were therefore included in the statistical analysis. The biggest differences in protein intensities were found between the medial^{OA} and medial^{ref} menisci, with a majority of proteins showing a higher mean $\log_2$ intensity in the medial^{OA} menisci (Fig. 15). One of these proteins was haemoglobin subunit alpha (HBA), with a fold-change of 25.63 (with 95% CI 10.27, 64.0). In contrast, very few

differences were found between the lateral^{ref} and lateral^{OA} menisci. Myocilin was one of the few proteins that showed a difference in this comparison, with a fold-change of 0.23 (0.09, 0.61). In within-person comparisons of lateral and medial menisci from the same knees, lateral^{REF} menisci generally had higher log_2 protein intensities than medial^{ref} menisci, whereas most proteins had similar intensities in lateral^{OA} and medial^{OA} menisci.

The proteoglycan content is increased in menisci from OA patients

Several proteoglycans were identified in this study, including several major SLRPs, such as asporin and lumican, and other proteoglycans, such as aggrecan and versican. The majority of the identified proteoglycans had higher intensities in the medial^{OA} menisci compared to medial^{ref} menisci. These differences could not be seen between lateral^{OA} and lateral^{ref} menisci, and they were not as prominent between lateral^{OA} and medial^{OA} menisci. Three proteoglycans – chondroadherin, versican and perlecan – exhibited higher intensities in the lateral^{ref} and medial^{ref} menisci.

Several canonical pathways are increased in menisci from OA patients

In this study, several canonical pathways were found to be increased in medial^{OA} menisci compared to medial^{ref} menisci (Fig. 16 A). LXR/RXR (liver X receptor/retinoid X receptor) activation had the highest z-score among these pathways (z-score 2.83). RhoGDI (Rho-GDP dissociation inhibitor) signalling was the only pathway that was decreased in the medial^{OA} menisci (z-score -2.45). One of the other major protein networks found among the proteins that differed between medial^{OA} and medial^{ref} menisci was a network of proteins associated with connective tissue disorders, organismal injury and abnormalities, and tissue development, including proteins such as matrix metalloprotease 3 (MMP3), versican and several collagens (Fig. 16 B).

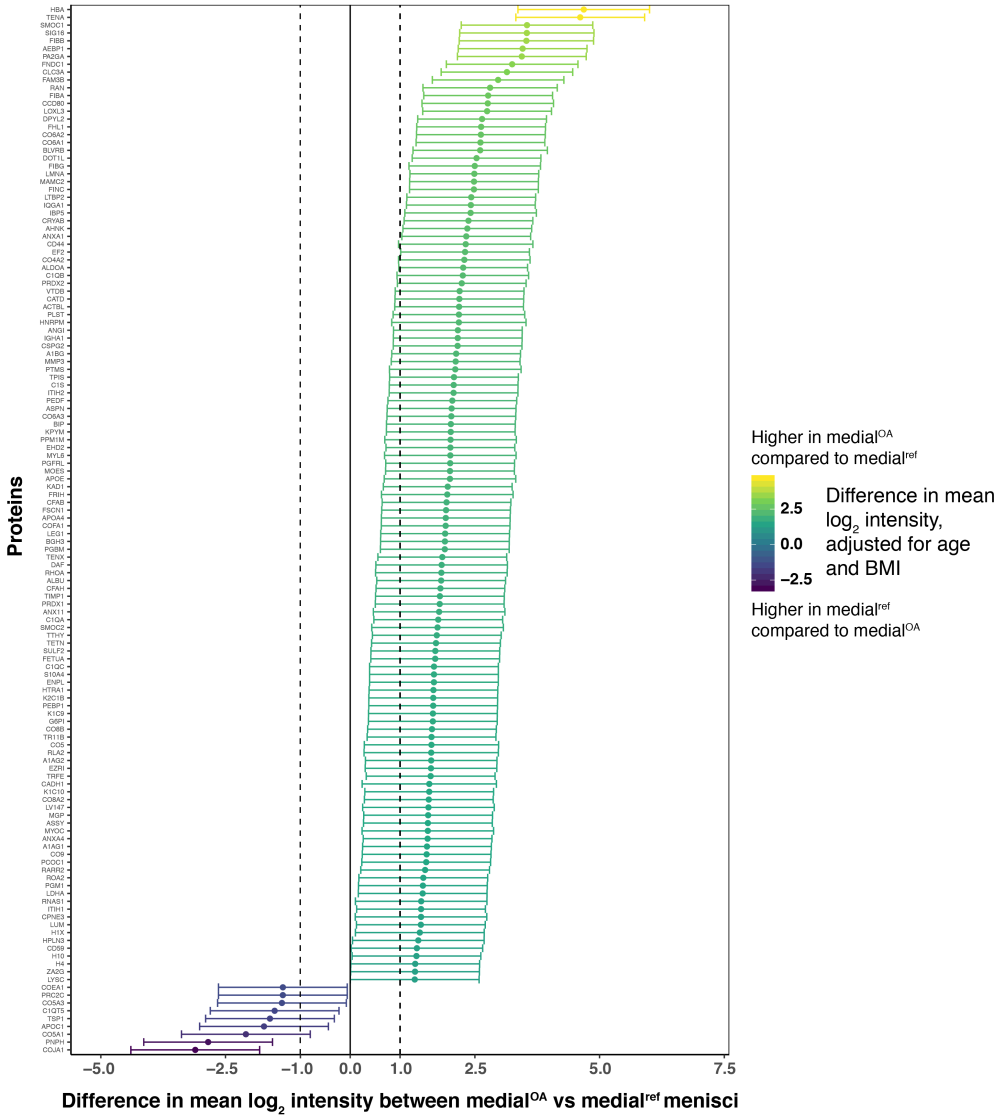

Figure 15.
Visual representation of differentially expressed proteins (y-axis) in medial^{OA} vs medial^{REF} menisci. Log_2-transformed mean intensity differences are displayed as point estimates with 95% confidence intervals as error bars (x-axis). The colour of the point estimates and error bars represents the difference in log_2 mean intensity between the groups, according to the scale bar on the right.

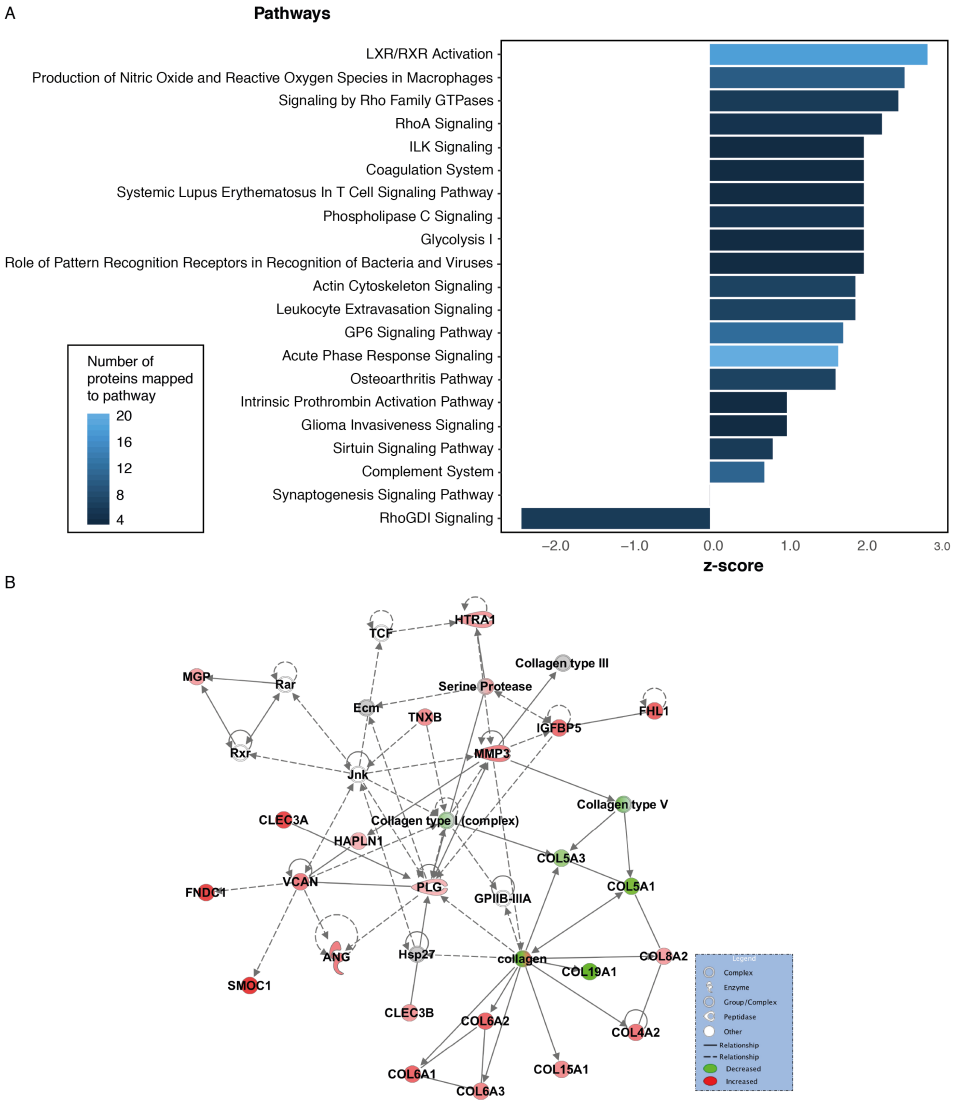

Figure 16.
Results from the pathway analysis of the proteins differing between the medial^{OA} and medial^{REF} menisci, which was performed in IPA®. *(A)* Bar plot displaying the most increased or decreased pathways mapped to the proteins. The z-score is a statistical measure of the match between the expected direction of the relationship and the observed protein expression, and a higher z-score corresponds to a stronger overrepresentation of a given pathway. The colour of the bars represents the number of proteins that could be mapped to that specific pathway. *(B)* One of the most prominent networks mapped to the differing proteins was a network of proteins associated with connective tissue disorders, organismal injury and abnormalities, and tissue development.

Discussion

The main focus of this thesis was to increase our knowledge about the meniscus, both in health and in OA. The first part of this thesis was about the three-dimensional microstructure of human menisci, where we could observe large differences in microstructure between menisci from deceased donors and OA patients. The second, and major part of the thesis, was about proteomic analyses of human menisci, where we compared the proteome of reference menisci with human articular cartilage, investigated zonal differences in reference menisci and compared the proteome between menisci from deceased donors and OA patients.

Despite proteomic similarities between articular cartilage and meniscus, there are significant differences

As expected, the majority of the identified proteins in **paper II** were common between articular cartilage and the meniscus. Only 14% of the proteins were unique to either of the tissues. In spite of this, there were some key proteomic differences, which could be attributed to differences in biology, such as the differences in vascularisation. Moreover, several proteins were found to differ between the tissues, e.g. asporin and versican, of which both were more abundant in the meniscus samples. Versican was first described as a fibroblast proteoglycan[128], and versican mRNA levels have previously been reported to be higher in fibroblasts compared to chondrocytes[129]. Therefore, the higher of levels of versican in the meniscus could be explained by the presence of both chondrocyte-like and fibroblast-like cells in the meniscal tissue[39]. However, the exact function of versican in the meniscus is still not clear.

Aggrecan was the most abundant proteoglycan in the cartilage samples, and indeed also the most abundant protein overall in that tissue. This is not surprising since aggrecan is the major proteoglycan of articular cartilage[58,61]. It was also approximately ten times more abundant in articular cartilage than in the meniscus, which is consistent with previous studies, and could be due to differences in proteoglycan synthesis between the different cell types of articular cartilage and the meniscus[111,130,131].

The protein with the largest differential expression between the meniscus and articular cartilage was dermatopontin, with a higher intensity in the meniscus. Dermatopontin is an ECM protein previously associated with promotion of cell attachment and spreading of dermal fibroblasts[132]. The presence of fibroblast-like cells in the outer

regions of menisci could explain the increased intensity of dermatopontin in our meniscus samples[40].

The lateral side of the knee does not appear to be as affected during medial compartment OA

The histological analyses of **paper I** showed that the medial^{OA} menisci had significantly higher histopathological scores compared to the lateral^{OA} and medial^{ref} menisci. It was also observed that the lateral^{OA} and lateral^{ref} menisci had similar scores. The same pattern could also be observed in the proteomics analysis of these meniscal samples in **paper IV**, where the largest differences were between the medial^{OA} and medial^{ref} menisci, whereas very few differences were seen between the lateral^{OA} and lateral^{ref} menisci. This suggests that the degenerative changes that occur in the posterior horn during medial compartment knee OA are indeed mainly localised to the medial side of the joint, and that the lateral side remains largely unaffected. Interestingly, another study from our group, where the same menisci were analysed with MRI, showed similar results[133]. In this MRI study, it was suggested that the results potentially imply that the lateral meniscus could be considered as a within knee reference when studying medial compartment OA, since the comparison would eliminate differences between knees or individuals such as knee alignment, age, sex, BMI etc., that otherwise could influence the results[133].

The most posterior part of the posterior horn of the menisci is most prone to degeneration

The medial^{OA} menisci showed higher histopathological scores in all three sections of the posterior horn compared to the medial^{ref} menisci, and had higher scores than the lateral^{OA} menisci in sections 2 and 3. However, in section 1, which is the most posterior section of the posterior horn, the difference between medial^{OA} and lateral^{OA} menisci was smaller than in the other sections. Additionally, in this section, the histopathological scores were higher in the lateral^{OA} and medial^{ref} menisci compared to the lateral^{ref} menisci, and this section was also the one with the highest histopathological score among the lateral^{OA} menisci. This would indicate that even though the lateral menisci are not as degenerated in medial compartment knee OA, the posterior tip of the meniscus (both lateral and medial) is more prone to degenerate, and is perhaps where the degeneration initiates. This is supported by previous reports that have shown that degeneration is most commonly found in the posterior horn of both medial and lateral menisci, both among OA patients and the general population[64,92,134].

There are zonal differences in proteomic composition in menisci from healthy donors

Although many proteins could be identified in all three zones of the meniscus, we observed large differences in protein abundance between the zones. Some of these differences could be ascribed to differences in vascularisation between the zones, but there were also differences in proteoglycan expression. Two of the proteoglycans

identified in **paper III**, aggrecan and versican, were highly abundant in all zones, however only versican was differentially expressed, with a higher intensity in the peripheral zone compared to the inner zone. This is consistent with a previous study which reported higher levels of versican mRNA in the peripheral parts of the meniscus[135]. Versican has been reported to be involved in many processes, including cell adhesion, proliferation, and migration, as well as ECM assembly[136], but the exact function of versican in the meniscus and the reason it is increased in the peripheral zone, are still unknown and require more research to elucidate.

Furthermore, the functional enrichment analysis on all proteins identified in **paper III** showed that the proteins in the meniscus, in all zones, are mainly extracellular proteins associated with ECM organisation, or proteins involved in exocytosis and cell secretion. This is expected since the meniscus primarily consists of ECM interposed with cells[23,39]. Moreover, the proteins that were more abundant in the peripheral zone compared to the inner zone were mainly intracellular, and involved in intracellular processes and organisation of cellular components. This suggests that the peripheral zone contains more cells than the inner zone. Indeed, a previous study has suggested that the peripheral zone has a higher cell density compared to the other zones[43]. In contrast, the proteins that were more abundant in the inner zone compared to the peripheral zone were found to be associated with ECM organisation and collagen trimer formation and organisation. The network analysis also revealed a distinguished cluster of collagens associated with the serine protease HTRA1, which has been found to be the most abundant protease in articular cartilage[137]. Therefore, these results suggest that the inner zone of the meniscus is more similar to articular cartilage in its composition, which is consistent with the presence of chondrocyte-like cells in that zone[39]. Similar results were found comparing the peripheral zone with the middle zone.

Vascularisation of the meniscus – and differences between articular cartilage and meniscus

Several proteins associated with blood circulation were identified in **paper III**, and several of them, e.g. the two subunits of haemoglobin (HBA and HBB), were more abundant in the peripheral zone compared to the middle and inner zones of the meniscus. This corresponds well with previous reports of the peripheral third of the meniscus being vascularised[34]. However, several blood proteins, such as serum albumin and serotransferrin, were among the most abundant proteins overall, in all three zones. HBB and serotransferrin were also among the top 10 proteins in the meniscus samples in **paper II**. Furthermore, since articular cartilage is known to be avascular[33], it was not surprising that one of the most abundant proteins in human plasma, alpha-1-antitrypsin (A1AT), was differentially expressed between the meniscus and articular cartilage samples, being on average five times more abundant in the meniscus samples.

However, in **paper II**, several plasma proteins could also be identified in the articular cartilage samples. The presence of plasma proteins in an avascular tissue, and the presence of plasma proteins in the avascular parts of the meniscus, might be explained

by the ability of cartilaginous tissues to absorb molecules, in this case plasma proteins, from their surroundings, e.g. synovial fluid or the synovium[138]. Both articular cartilage and all zones of the meniscus are in contact with the synovial fluid of the joint, and synovial fluid is an ultra-filtrate of plasma and therefore contains plasma proteins.

Furthermore, angiogenesis, or the growth of new blood vessels from pre-existing vasculature, has been reported to be associated with OA[138–141]. In OA, angiogenesis has been seen to occur in the osteochondral junction, the synovium and the menisci, accompanied by increased growth of sensory nerves, which has been hypothesised to contribute to the pain of OA[139,141,142]. Angiogenin is an angiogenic protein[143,144], which in **paper IV** of this thesis, was one of the proteins with higher intensity in the medial^{OA} menisci compared to the medial^{ref} menisci. This corresponds well with the observations of increased angiogenesis in OA. Interestingly, HBA was also one of the proteins that showed a higher mean intensity in the OA menisci in **paper IV**, which could be a result of increased angiogenesis. It might also be due to internal haemorrhages within the menisci of OA patients, caused by tissue damage and degeneration. Another potentially contributing factor could be intra-articular bleeding in the OA patients during TKR surgery, which may cause the menisci to be contaminated by blood, but since all menisci were rinsed with PBS prior to dissection, surface contamination by blood is a less likely explanation for the observed differences.

Several interesting features were observed in histology and μCT analyses of the meniscus, including channel-like structures that penetrated from the peripheral border, deeper into the meniscal tissue. These channel-like structures do not significantly attenuate X-rays, and therefore might represent vascularisation. However, they appear to penetrate deeper than what has been reported in the literature. Similar structures have previously been described as loose connective tissue by Petersen and Tillman[53]. Another study reported radially oriented tie-fibres that form a branching and sheet-like network wrapping around the circumferentially oriented collagen bundles in the meniscus[52], which could explain our observed structures. In addition, the same study reported that blood vessels, surrounded by proteoglycan-rich regions, are enclosed by these tie-fibre sheets[52]. Indeed, we observed similar branch-like proteoglycan-rich structures in the SafO-FG-stained menisci, which appear to coincide with the channel-like structures we observe in μCT. However, in order to determine what these structures are, and to rule out that they simply are blood vessels, we need to perform further histological evaluations with specific stains for vascular and perhaps neurological structures, in the future.

Proteoglycan content is increased in menisci from OA patients

In **paper I**, we observed increased matrix staining using SafO-FG in the medial^{OA} menisci, indicating an increase in proteoglycan content with increasing degeneration. The same pattern could be observed in **paper IV**, where the same menisci were analysed with MS. In this study, several proteoglycans showed higher intensities in the medial^{OA}

menisci compared to the medial^{ref} menisci. In articular cartilage, OA is associated with loss of proteoglycans[145], but the opposite has been observed in menisci during OA[45,146]. This has been hypothesised to be an attempt at repair or regeneration[45,146]. In order to understand this interesting difference between articular cartilage and menisci, and to investigate the role of the increased proteoglycan content in degenerating menisci, more studies are needed. Perhaps the difference is a result of the different cell types found in articular cartilage and the meniscus.

There is increased activation of both catabolic and anabolic processes in menisci from OA patients

In **paper IV**, proteomic comparison of menisci from OA patients and reference subjects was performed, along with pathway analysis of the proteins differing between the two groups. In this pathway analysis, the pathway with the highest z-score was LXR/RXR activation, which was increased in medial^{OA} compared to medial^{ref} menisci. The LXR/RXR pathway consists of receptors that, when dimerised, can function as transcription factors[147,148]. Interestingly, studies have reported the involvement or dysregulation of LXR/RXR pathways in OA tissue[149–152]. For example, treatment with RXR agonists decreased aggrecan and fibrillin-2 gene expression and increased MMP13 gene expression in chondrocytes, which are changes associated with tissue degradation[150]. Interestingly, the same study also reported an opposite effect for treatment with LXR agonists, which decreased the expression of MMP13 and MMP2, genes associated with cartilage breakdown. This indicates that LXR activation might act protectively on articular cartilage. In **paper IV** of this thesis, we observed an increase of both LXR and RXR activation in medial^{OA} menisci compared to medial^{ref} menisci. Similarly, we also observed a simultaneous increase of both the proteinase MMP3, which induces breakdown of cartilage, as well as its inhibitor metalloproteinase inhibitor 1 (TIMP1), in the medial^{OA} menisci compared to the medial^{ref} menisci. Together, these results may indicate that there is an on-going degradation process in the OA menisci, but also a simultaneous attempt to stop the degradation. In addition, MMP3 has been suggested as a potential biomarker for OA with high levels in serum, synovium, synovial fluid and articular cartilage from OA patients[153–156]. We can now show that this also appears to apply to the human meniscus.

All tissue samples were frozen and thawed prior to processing and analysis

In this thesis, all tissue specimens used were frozen and then thawed prior to sample processing and analysis, which could have an effect on tissue integrity, with for example enlarged extracellular spaces and shrunken cells[157]. Unfortunately, the biobanking procedure necessitates the freezing step, and it would be a large logistical challenge to avoid this, especially with patient samples obtained from the hospital in Trelleborg. Nevertheless, all samples in the studies underwent the same sample preparation processes, and the results in each study should therefore be comparable to each other.

On a positive note, storage of tissue at -80°C has been shown to preserve proteins well for many years[158].

A HMDS-based µCT method allows 3D visualisation of meniscal tissue

As a first step to investigate the biology of the human meniscus, a histology and µCT analysis of reference and OA menisci was performed in **paper I**, in order to increase our knowledge about the three-dimensional microstructure of the meniscus. In addition to histological tissue staining, a HMDS-based µCT methodology was used. This method had previously been used in articular cartilage with a successful outcome[120,159], and was therefore predicted to work well with meniscal tissue.

HMDS-based µCT offers several advantages over existing tissue imaging methods such as conventional histology and clinical or *ex vivo* MRI. It enables volumetric visualisation and evaluation of the tissue's 3D organisation, better resolution, and the possibility of an analysis free of the use of an external contrast agent, since drying the samples using HMDS allows contrast to arise from the tissue itself, and not from the distribution of any specific contrast agent. In this thesis, we show that this method enables unique 3D visualisation of several features such as collagen organisation, surface morphology, and tissue calcification that can otherwise only be seen using 2D histology techniques, which indeed we observed in our histology analysis.

Despite all the advantages with the µCT analysis, there are some drawbacks. Even though HMDS has been shown to be a suitable drying agent that preserves the microstructure of osteochondral samples[120], it should be noted that the tissue structure in HMDS-dried meniscus samples may not resemble its intact state *in vivo*. Furthermore, µCT, like other imaging techniques, has artefacts. In **paper I**, the most significant artefacts were streaking artefacts caused by calcifications in the menisci. These occur due to substantial differences in X-ray attenuation between calcified and non-calcified meniscus regions, and could be prevented by decalcifying the samples prior to analysis. However this treatment would make calcifications less visible in the µCT images, and would not be a true representation of the structural state of the menisci.

DIA is superior to DDA in regards to variation and missing values

In the next step of our analysis of the meniscus, we wanted to use proteomics to further increase our knowledge of meniscal biology. MS-based proteomics offers more detailed molecular information about the tissue compared to microstructural methods such as histology and µCT, and allows the simultaneous analysis of several hundreds of proteins. In the second paper of this thesis, we aimed to compare two different MS methods, DDA and DIA, in order to decide which method to use in future studies. We found that the DIA analysis found more differentially expressed proteins, even after FDR control, which can be explained by the more precise estimates yielded by the DIA analysis, compared to DDA. This, however, could be explained by the fact that the

peptides were manually selected in DIA, resulting in the removal of peptides with worse chromatographic performance.

Nonetheless, one of the biggest advantages with DIA was the lower number of missing values. This was probably due to differences in how data are acquired between the methods. DIA measures everything within a certain isolation window[109,116,160], whereas DDA only selects the most abundant ions to measure. If too many peptides elute at the same time in one DDA MS1 scan, this could result in missing quantitation of lower abundance peptides. This also makes DDA less reproducible compared to DIA. On the other hand, even though DIA seemed superior to DDA in several ways, both methods, as expected, gave on average similar point estimates of the differences between articular cartilage and the meniscus (the two tissues analysed in **paper II**). But, all in all, the advantages of DIA were several, which prompted us to use this method in the subsequent **papers III** and **IV**.

Protein extraction using GdnHCl is suboptimal for collagen analysis

In all three of the proteomic studies of this thesis, several collagens were identified. However, in all these studies, the protein extraction method was based on GdnHCl, which is suboptimal for collagen extraction; depending on the extent of collagen cross-linking or the subtypes present in different samples, the extraction results may vary[111]. Therefore, it might be difficult and premature to draw extensive conclusions about the identified collagens, without further validation.

Data analysis is a challenge when conducting MS experiments

Apart from sample preparation, one of the largest challenges with MS is the large amount of raw data that is produced, which requires advanced software to analyse. In **paper II** of this thesis, analysis of the DIA data was performed in Skyline, which required tedious, time-consuming manual peak selection. Skyline also required a spectral library, consisting of DDA runs, to be used for the search, which also increased the instrument time necessary for analysis. Development and implementation of new software, such as Spectronaut™ Pulsar, has greatly compensated for these drawbacks of DIA. First of all, with Spectronaut, which was used for data analysis in **papers III** and **IV**, it is possible to search your DIA data without a spectral library, by a process called directDIA™. This is done by the software by first converting the DIA spectra into pseudo DDA spectra, and by subsequently searching them with the search engine Pulsar. Next, a targeted re-extraction on the DIA data is performed, which results in less missing values and good retrieval of low abundance peptides. This data analysis method was used in **paper III**. In **paper IV**, however, a spectral library was created using a combination of both DDA and DIA runs. An additional advantage of Spectronaut is that it contains an algorithm for automatic peak selection, making it less time-consuming than Skyline, which was used in **paper II**.

In **papers III** and **IV**, functional enrichment and pathway analyses were also implemented. These analyses are based on current knowledge and published data about different proteins and their interactions with other proteins, and their involvement in various pathways[161]. It is a way to summarise the biological relevance of the results in a more comprehensive way. However, the human body is much more complex than what can be included in a single pathway, and in reality, it can be several thousands of interactions between different pathways that are responsible for a certain phenotype. Still, pathway analysis can be a useful tool when trying to interpret and understand the biological relevance of complex proteomic data.

Conclusions

- HMDS-based μCT successfully allows unique 3D visualisation of meniscus microstructures.
- The lateral side of the knee does not appear to be as affected as the medial side during medial compartment knee OA.
- The most posterior part of the posterior horn of the menisci is most prone to degeneration.
- Proteoglycan content is increased in menisci from OA patients.
- Despite similarities in proteomic constitution between articular cartilage and the meniscus, there are significant differences between the two tissue types, such as higher levels of vascularisation and asporin and versican content in meniscus samples.
- When comparing two MS methods, DIA data yielded more precise estimates and more differentially expressed proteins, with fewer missing values compared to DDA.
- There were large zonal differences in proteomic composition in menisci from healthy donors, where the peripheral zone was more cellular and vascularised, whereas the middle and inner zones were more similar to articular cartilage, with high levels of ECM proteins such as aggrecan and collagen type II.
- OA menisci appear to have higher levels of angiogenin and HBB, which could suggest increased angiogenesis or internal bleedings in these menisci.
- There appears to be increased activation of both catabolic and anabolic processes in menisci from OA patients, with increased levels of MMP3 and TIMP1.
- Both microstructural and proteomic analyses showed degeneration of OA menisci, suggesting a strong association between OA and meniscal degeneration.

Future perspectives

The papers in this thesis have provided new knowledge about the proteome and microstructure of the human meniscus, both in health and disease. Nevertheless, to increase our knowledge even further and to improve our understanding of the role of the meniscus in OA, more research is needed.

First of all, it would be desirable to repeat the studies in new cohorts and other individuals, preferably with larger sample sizes.

Another interesting topic concerning the human meniscus would be to explore what may be considered normal ageing and what may be considered disease. Several studies, including **paper I** in this thesis, have suggested that the meniscus, like any other tissue in the body, deteriorates with increasing age[92,121,162]. Therefore, it would be interesting to study a larger number of reference menisci without OA, from donors of varying ages, in order to investigate how the proteome changes with age.

In several of the papers in this thesis, variations in vascularisation were observed, both between the different zones of the meniscus, and between menisci from OA patients and references. It has been suggested that vascularisation affects the healing ability of the meniscus, and that angiogenesis is a part of OA pathogenesis[138–141]. It would be interesting to further investigate the association of angiogenesis with OA, particularly in the meniscus, and to see if the same angiogenic changes can be seen in age-related degeneration. Furthermore, in the histology and μCT analyses in **paper I**, we observed channel-like structures, although we could not determine what they are. Are they blood vessels? If they indeed are blood vessels, why do we predominately observe them in menisci with lower histopathological grades? To answer the question of the origin of these structures, histological staining for vascular structures would be a suitable analysis in the future.

In **paper III**, we investigate the proteomic differences between the three zones of the human healthy meniscus. We are also planning to do this in OA menisci, since it would be interesting to see if the same zonal differences can be seen, and if some zones are more prone to degeneration. Several studies have reported that the posterior horn is the part of the meniscus that most predominantly degenerates, which is why a comparison of the different regions of the meniscus, i.e. the posterior horn, body and anterior horn, would be of interest as well.

In this thesis, the main focus has been on the reference menisci and menisci from OA patients, however the biobank also contains meniscus pieces from meniscectomy patients. These patients are mainly younger subjects who have sustained a traumatic knee injury, which required a small piece of the meniscus to be removed. According to previous research, these patients suffer a higher risk of developing knee OA[15,100,101], and would therefore be truly interesting to study. How does their meniscal proteome differ from the end-stage knee OA patients, and indeed reference menisci? Would it be possible to identify any activated disease processes in these menisci? It would also be interesting to see if we would observe any of the changes that we observe in OA menisci, already in the menisci from knees with traumatic injuries. This would substantially increase our knowledge about the OA disease process and how a meniscal injury could lead to knee OA.

Furthermore, as we continue to explore the proteome of the human meniscus, and as our knowledge of the processes and mechanisms that occur in the meniscus during OA increases, we will be able to identify a panel of potential OA biomarkers based on meniscus degeneration. Since the meniscus itself is quite inaccessible and inappropriate to be used for screening purposes, it would be interesting, and desirable, to see if we would be able to identify such markers in synovial fluid, or even more desirably in plasma or serum, using targeted analyses. The long-term goal would be to identify early diagnostic markers for meniscal degeneration and/or OA that could aid earlier diagnosis, which would make it possible to start potential treatment earlier. However, since there is no cure for OA today, new treatments are needed, and in addition to the potential identification of OA biomarkers, an increased knowledge of the biology of the meniscus in health and OA could also help us identify new potential treatment targets.

Populärvetenskaplig sammanfattning (Summary in Swedish)

Artros är den vanligaste reumatiska ledsjukdomen i Sverige och drabbar ungefär var fjärde person över 45 år. Även globalt är artros vanligt och år 2010 uppskattades ungefär 250 miljoner människor världen över lida av knäledsartros. Ålder, övervikt och en historia av ledskador eller överbelastning, exempelvis p.g.a. av tungt arbete, är de vanligaste riskfaktorerna, men man har också sett att det är vanligare att kvinnor drabbas. Smärta och stelhet i leden är de vanligaste symptomen, och den drabbade leden kan ibland svullna upp.

I en frisk led täcks benytan av en stark och glatt vävnad som kallas ledbrosk. Brosket underlättar för leden att röra sig mer friktionsfritt. I knäleden finns det också en annan typ av halvmåneformade, broskliknande vävnader som kallas menisker. Det finns två i varje knä, och deras främsta funktion är att vara stötdämpande, stabiliserande och jämna ut krafterna i knät. Meniskerna består främst av en vävnad som kallas extracellulärmatrix, som är ett nätverk av olika molekyler och som omger de celler som finns i vävnaden.

Traditionellt sett har artros karakteriserats av en förhöjd nedbrytning av ledbrosk tillsammans med förändringar i det underliggande benet, men studier under senare år har visat att även meniskerna drabbas vid artros. Man har också sett att personer som drabbas av meniskskador i högre utsträckning drabbas av artros. Idag finns inga botemedel mot artros, utan endast behandling av symptomen, såsom smärtlindring eller fysioterapi. Därför behövs mer kunskap om vad händer i leden för att kunna utveckla nya behandlingar.

Målet med den här doktorsavhandlingen har varit att studera mänskliga menisker och undersöka vilken betydelse meniskerna har vid artros. Detta har vi gjort genom att undersöka friska menisker från avlidna vävnadsdonatorer och sjuka menisker från artrospatienter. Vi har studerat proteiner, vilket är stora biomolekyler som ibland kallas för kroppens byggstenar, och hur uttrycket av dessa proteiner varierar mellan olika delar av menisken samt mellan sjuka och friska menisker. Vi har också studerat hur extracellulärmatrixet i menisken förändras vid artros.

Vi kan visa att menisker från artrospatienter i större utsträckning har en aktiv nedbrytningsprocess där vävnaden är oorganiserad med förkalkningar och

vätskeansamlingar. Vi har också sett att uttrycket av olika proteiner skiljer sig mellan sjuk och frisk meniskvävnad där sjuka artrosmenisker har en ökning av proteiner som bryter ned andra proteiner, s.k. proteaser, samtidigt som vävnaden svarar med att öka produktionen av proteiner kan motverka den här nedbrytningen.

Den här avhandlingen har ökat vår kunskap om meniskens biologi och de förändringar som sker i menisken vid artros. Fler studier är dock nödvändiga för att fullt klargöra vad som startar dessa sjukdomsprocesser. Förhoppningen är att i framtiden kunna hitta proteiner som kan hjälpa oss att tidigare upptäcka artros och urskilja vilka personer som har högre risk att drabbas, samt att bidra till utvecklingen av nya läkemedel mot artros.

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Proteomics of the human meniscus in health and osteoarthritis

Osteoarthritis the most common joint disease in Sweden, and causes pain and dysfunction of the affected joint. It has a large impact on the daily life for those suffering from it. Today, there is no cure for osteoarthritis, hence more knowledge about the disease mechanisms is needed. Traditionally, knee osteoarthritis has been characterised by a degradation of the articular cartilage of the knee joint, but recent research suggest a role of the meniscus in osteoarthritis pathology. This thesis presents several methods to analyse human menisci that have increased our knowledge about the three-dimensional microstructure and proteome of healthy and osteoarthritic human menisci.

Elin Folkesson was born in 1991 in Kalmar in Småland. She moved to Lund in 2010 to study biomedicine at Lund University, and decided to stay to pursue a PhD. In her spare time she enjoys watching and participating in all kinds of sports, watching movies and tv series, and spending time with her friends and family. She also enjoys to travel, where Greece and Japan are two favourite countries to visit.

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