Proteomic characterization of the normal human medial meniscus body using data-independent acquisition mass spectrometry

Elin Folkesson1,2 | Aleksandra Turkiewicz1 | Martin Rydén1,2 | Harini Velocity Hughes1 | Nesperin Ali1 | Jon Tjörnstrand1,3 | Patrik Önnerfjord2 | Martin Englund1,4

1Faculty of Medicine, Department of Clinical Sciences Lund, Orthopaedics, Clinical Epidemiology Unit, Lund University, Lund, Sweden
2Faculty of Medicine, Department of Clinical Sciences Lund, Rheumatology and Molecular Skeletal Biology, Lund University, Lund, Sweden
3Department of Orthopaedics, Skåne University Hospital, Lund, Sweden
4Clinical Epidemiology Research and Training Unit, Boston University School of Medicine, Boston, Massachusetts

Correspondence
Elin Folkesson, Department of Clinical Sciences Lund, Lund University, Molecular Skeletal Biology-BMC-C12, Klinikgatan 28, 221 00 Lund, Sweden.
Email: elin.folkesson@med.lu.se

Funding information
Vetenskapsrådet, Grant/Award Numbers: 2014-2348, 2014-3303; Foundation for Research in Rheumatology (FOREUM), Grant/Award Number: 018EnglundPreCI; Krapperup Foundation; IngaBritt och Arne Lundbergs Forskningsstiftelse; Swedish Rheumatology Association; Anna-Greta Crafoord Foundation; Crafoordstiftelsen; Alfred Österlunds Stiftelse; Greta och Johan Kocks stiftelse; H2020 European Research Council, Grant/Award Number: 771121; Governmental Funding of Clinical Research within the National Health Service (ALF)

Abstract
Recent research suggests an important role of the meniscus in the development of knee osteoarthritis. We, therefore, aimed to analyze the proteome of the normal human meniscus body, and specifically to gain new knowledge on global protein expression in the different radial zones. Medial menisci were retrieved from the right knees of 10 human cadaveric donors, from which we cut a 2 mm radial slice from the mid-portion of the meniscal body. This slice was further divided into three zones: inner, middle, and peripheral. Proteins were extracted and prepared for mass spectrometric analysis using data-independent acquisition. We performed subsequent data searches using Spectronaut Pulsar and used fixed-effect linear regression models for statistical analysis. We identified 638 proteins and after statistical analysis, we observed the greatest number of differentially expressed proteins between the inner and peripheral zones (163 proteins) and the peripheral and middle zones (136 proteins), with myocilin being the protein with the largest fold-change in both comparisons. Chondroadherin was one of eight proteins that differed between the inner and middle zones. Functional enrichment analyses showed that the peripheral one-third of the medial meniscus body differed substantially from the two more centrally located zones, which were more similar to each other. This is probably related to the higher content of cells and vascularization in the peripheral zone, whereas the middle and inner zones of the meniscal body appear to be more similar to hyaline cartilage, with high levels of extracellular matrix proteins such as aggrecan and collagen type II.

Keywords
data-independent acquisition, meniscus, proteomics
1 | INTRODUCTION

Osteoarthritis (OA) is a chronic joint disease traditionally characterized by loss of articular cartilage and changes in the underlying bone. However, recent research in knee OA highlights an important role for the knee meniscus in the disease’s etiology and pathogenesis. The menisci are two fibrocartilage discs located between the femoral and tibial condyles in the knee, and their main function is load transmission. Damage to the meniscus, which can occur due to acute knee trauma or as a result of degenerative changes, is associated with increased risk for knee OA.

Little is known of the molecular processes involved in the slow degradation of meniscal tissue implicated in the early stages of OA. There is also very limited knowledge of the molecular composition of the normal meniscus. Thus, more knowledge is needed about the meniscus, both in health and OA. Mass spectrometry (MS) coupled with liquid chromatography (LC) has become one of the most powerful methods to analyze protein content in complex samples. MS-based proteomics allows for a comprehensive analysis of a wide variety of tissues, including cartilage tissues. With non-targeted MS it is possible to identify several hundreds of proteins, even thousands, in a single analysis. Therefore, as a first step to gain new knowledge on the role of the meniscus in OA pathogenesis, our aim was to investigate the proteome of the normal human medial meniscus body, and to study how protein expression varies in the peripheral (vascularized), middle and inner (avascular) zones of the meniscus body, using a global MS approach.

2 | MATERIALS AND METHODS

2.1 | Materials

N-Ethylmaleimide, 6-aminocaproic acid, benzamidine hydrochloride hydrate, dithiothreitol (DTT), iodoacetamide, ammonium bicarbonate (AMBIC), formic acid, and high-performance liquid chromatography (HPLC) grade acetonitrile and Solvents A (0.1% formic acid in water) and B (0.1% formic acid in acetonitrile) for LC-MS were purchased from Sigma-Aldrich (St. Louis). Guanidine hydrochloride (GdnHCl) and anhydrous sodium acetate (NaAc) were purchased from Merck (Darmstadt, Germany). Trypsin Gold (MS grade) was purchased from Promega (Madison, WI). The water used in this study was purified using a MilliQ apparatus (Millipore, Billerica, MA). The Pierce Quantitative Colorimetric Peptide Assay was purchased from Thermo Fisher Scientific (Rockford) and Nanosep 30K Omega Centrifugal Devices were purchased from Pall Life Sciences (Ann Arbor). Reversed-phase C18 columns were purchased from Nest Group (Southborough, MA).

2.2 | Donor menisci

We sampled meniscal tissue from a biobank of human knee tissues at Skåne University Hospital, Lund, Sweden. For the biobank, complete menisci were obtained within 48 hours post-mortem from donors at the hospital, and the specimens were frozen at −80°C within 2 hours of extraction. Donors had to be 18 years or older, and consent was required from either the deceased or from the deceased’s closest relatives, for use of tissue for general medical research purposes after death, via the Swedish donor register. Persons with known diagnoses of rheumatoid arthritis (RA) or knee OA were excluded. Further, donors who were known drug addicts or had positive serology of HIV or hepatitis were excluded. The biobank and this study have passed ethical review at the regional ethics committee in Lund (2015/39 and 2016/865).

For the purposes of this study, we selected the medial menisci from the right knees of 10 donors (five women and five men). The menisci were thawed in phosphate-buffered saline, and to be eligible for the study, we required them to be macroscopically intact (minor calcifications were ignored) (Figure 1A). Further, we also inspected the medial compartment of the femoral cartilage (the load-bearing region) from the same individuals (also in the biobank) and required the cartilage to be macroscopically intact.
To prepare the tissue samples for MS analysis, the meniscus was dissected free of synovium and a 2 mm thick radial slice was cut from the middle part of the body of each meniscus (Figure 1B). The slices (n = 10) were further divided into three parts approximately corresponding to (a) the inner (avascular) zone, that is, the free edge, (b) the middle (partly vascularized) zone, and (c) the outer (vascularized) zone, that is, the edge that is attached to the synovium (Figure 1B). These samples were then stored at −80°C until further analysis.

2.3 | Preparation of meniscal tissue for MS analysis

The tissue was pulverized in liquid nitrogen using a pestle and mortar technique, after which the pulverized tissue was weighed. Proteins were then extracted using 15 volumes (15 µL buffer/mg dry powder) of chaotropic buffer (4 M GdnHCl, 50 mM NaAc, 100 mM 6-aminocaproic acid, 5 mM benzamidine, 5 mM N-ethylmaleimide, pH 5.8), incubated for 24 hours on an orbital shaker at +4°C, and then centrifuged at 13 200×g at +4°C for 30 minutes. The resulting supernatant was the protein extract used for further analysis.

Fifty microliters of each sample extract were reduced using 4 mM DTT, shaking at +56°C for 30 minutes. Extracts were then alkylated using 16 mM iodoacetamide for 1 hour at room temperature in the dark. In order to remove residual salts, extracts were precipitated with nine volumes of ethanol incubated for 4 hours at −20°C, after which the precipitate was dried in a SpeedVac and suspended in 100 µL of 0.1 M AMBIC buffer, pH 8.5. All samples were then digested using 2 µg Trypsin Gold, by incubating on a shaker at +37°C for approximately 16 hours. The peptide concentrations of the digests were determined using the Pierce Quantitative Colorimetric Peptide Assay according to the manufacturer’s instructions. Samples (10 µg) were diluted to 200 µL in 50 mM AMBIC buffer with 0.5 M sodium chloride (to minimize ionic interactions). In order to remove peptides with glycosaminoglycan (GAG) chains from the samples, they were centrifuged through Nanosep 30K Omega Centrifugal Devices. Samples were subsequently desalted in reversed-phase C18 columns with 2% acetonitrile in 0.2% formic acid and eluted using 50% acetonitrile in 0.1% formic acid.

2.4 | Mass spectrometry analysis—data-independent acquisition

The digested samples (n = 30) were analyzed using a Q-Exactive quadrupole Orbitrap benchtop mass spectrometer (Thermo Fisher Scientific). 1 µg of the protein digest was injected into an Easy nLC 1000 HPLC system (Thermo Fisher Scientific) equipped with an Acclaim PepMap 100 nanoViper pre-column (C18, 3 µm particles, 75 µm i.d. 2 cm long; Thermo Scientific) and an Acclaim PepMap RSLC nanoViper analytical column (C18, 2 µm particles, 75 µm i.d. 25 cm long; Thermo Scientific). A heated ion transfer setting of 260°C was used for desolvation together with a spray voltage of +2000 V. On-line reversed-phase separation was performed using a flow rate of 300 nL/min. A binary gradient of 125 minutes was used, starting from 5% B to 7% B in 5 minutes, then increasing to 20% B in 85 minutes, further increasing to 30% B in 20 minutes, and with a final increase to 90% B in 5 minutes, after which it ended with 90% B isocratic for 10 minutes. For the MS settings, the MS1 scan (390–1010 m/z) was set to have a resolution of 70 000, 1 × 106 automatic gain control (AGC), and 100 ms maximum ion injection time. This was followed by data-independent acquisition (DIA) collision-induced dissociation MS2 scans at a resolution of 35 000, 1 × 106 automatic gain control (AGC), and 120 ms maximum ion injection time. A loop count of 24 was used in the range of 400 to 1200 m/z. The isolation windows were 26.0 m/z wide, including 0.5 Da overlaps.

2.5 | Data analysis

The 30 DIA raw files of the meniscal samples were converted to HTRMS format using HTRMS Converter (Biognosys AG, Switzerland) in order to decrease data analysis search time. A subsequent protein search was conducted in Spectronaut Pulsar (Biognosys AG, Switzerland) with the human protein database (150612_UP5640_n20200) as background proteome, using a library-free workflow, DirectDIA. Default settings were used for the search, with the following modifications: cysteine carbamoyldimethylation as a fixed modification, and deamination, pyro-glutamic acid (N-terminal Glu to pyroglutamic acid), methionine oxidation, and hydroxyproline as variable modifications. Precursor quantitation was performed at the MS2 level, and the area under the curve was used as a quantitation type. The top three peptides (proteotypic) for each protein were averaged to calculate protein abundance.

2.6 | Statistical analysis

Proteins with a maximum of 10% missing values per zone were included in the statistical analysis (i.e., one missing value per protein allowed among the ten donor samples analyzed 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). The statistical analysis was performed in the open-source software R (version 3.5.1; https://www.R-project.org) using the limma package available from Bioconductor. Protein intensity was transformed using logarithm with base 2. Within limma, we fitted a series of fixed effects linear regression models to estimate the differences between the three zones. The estimates of standard errors were moderated using a simple empirical Bayes model, and confidence intervals for a given confidence level were derived for the paired comparisons of interest. The Benjamini and Hochberg method was used to control the false discovery rate (FDR) at 1%. In this procedure, we included all overall F-tests, which tested if there was at least one statistically significant difference between the three zones. Further, for all statistically significant proteins, paired posthoc tests were performed to make paired comparisons between all zones. Thus 510 comparisons were
included in the FDR control. We estimated confidence intervals consistent with the applied FDR control procedure as described by Benjamini and Yekutieli\textsuperscript{13} and the obtained nominal confidence level for the intervals was 0.994. All estimates in the results and discussion sections are presented with 99% confidence intervals in brackets.

### 2.7 Functional enrichment analysis

We investigated the overrepresentation of protein-protein interactions (PPI) in our data by conducting protein network analysis. Additionally, functional enrichment analysis was performed in order to determine if any gene ontology (GO) terms were overrepresented in the network. First, all protein accessions were mapped to updated Swiss-Prot accessions, yielding a total of 634 protein accessions for further analysis. All identified proteins, as well as proteins differentially expressed between the zones, were searched in the publicly available STRING database version 11.0.\textsuperscript{14} In the resulting network, each node represents a protein and each edge represents a PPI. The line thickness of the edge denotes the confidence for the particular interaction. The whole genome was used as the statistical background, and a minimum interaction score of 0.4 was required (medium confidence). The whole genome was used as the statistical background, and a minimum interaction score of 0.4 was required (medium confidence). The whole genome was used as the statistical background, and a minimum interaction score of 0.4 was required (medium confidence).

#### 2.8 SDS-page and Western blot

In order to validate our MS results, SDS-page and Western blots were performed on meniscus tissue extracts. For experimental procedures, please see Supporting Information Material S1.

### 3 RESULTS

#### 3.1 Donor characteristics

The 10 donors had a median age of 51 years (range 18-77) and a median body mass index of 26.2 (range 16-42) (Table 1).

#### 3.2 Differential expression of proteins

We identified a total of 638 proteins in the MS analysis of all three meniscal zones, of which 405 had a maximum of one missing value per zone (Table S2). Out of these, 170 proteins differed in at least one of the comparisons between the zones. We were only interested in these 170 proteins in further comparisons. Among these, 163 proteins, the vast majority, differed between the peripheral and inner zones (Figure 2). Of these, 129 proteins had a higher intensity in the peripheral zone, including versican and hyaluronan and proteoglycan link protein 3 (HPLN3) with fold-changes of 4.9 (1.5, 14.9) and 5.3 (1.6, 18.4) respectively, and 34 proteins had a higher intensity in the inner zone, including collagen alpha-1 (II) 0.10 [0.03, 0.29] and serine protease HTRA1 (0.29 [0.12, 0.66]). Myocilin was the protein with the highest fold-change between the peripheral and inner zones (42.2 [16.0, 119.4]). Furthermore, 136 proteins had different abundances between the middle and peripheral zones. Of these, 101 had a higher expression in the peripheral zone, for example, myocilin (18.4 [6.5, 48.5]), and 35 had a higher expression in the middle zone, for example, fibromodulin (0.33 [0.15, 0.70]) and collagen alpha-1 (II) chain (0.16 [0.06, 0.50]). Only eight proteins had a difference in abundance between the middle and inner zones large enough to pass the FDR control procedure, of which seven had a higher expression in the inner zone, for example, chondroadherin (3.7 [1.4, 10.6]) and HPLN3 (3.2 [1.0, 11.3]). Lists of the differentially expressed proteins from each of these comparisons between the three zones are detailed in Tables S3, S4, and S5.

#### 3.3 The most abundant proteins were similar between the zones

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-oxoglutara dehydrogenase, mitochondrial, had the highest abundance values in the inner zone (Table 2). Many proteins were common to all zones and among the top 20 proteins in each of the three zones, 15 were the same, however in varying orders in the different zones. Several other

### Table 1 Characteristics of the study subjects

| Subject number | Sex  | Age, y (at death) | BMI, kg/m\(^2\) |
|----------------|------|------------------|-----------------|
| 1              | Female | 18     | 16.4              |
| 2              | Female | 32     | 22.8              |
| 3              | Female | 61     | 23.3              |
| 4              | Female | 74     | 25.5              |
| 5              | Female | 77     | 22.3              |
| Mean (SD) (female) | ... | 52.4 (26.2) | 22.1 (3.4) |
| 6              | Male  | 49     | 33.0              |
| 7              | Male  | 50     | 34.2              |
| 8              | Male  | 52     | 26.8              |
| 9              | Male  | 58     | 33.2              |
| 10             | Male  | 43     | 42.4              |
| Mean (SD) (male) | ... | 50.4 (5.4) | 33.9 (5.6) |
| Mean (SD) (all) | ... | 51.4 (17.9) | 28.0 (7.6) |
SLRPs, such as mimecan, decorin, and lumican, were also among the 10 proteins with the highest abundance values in all zones (Table 2).

3.4 PPI and functional enrichment analysis (biological differences)

The PPI network of all identified proteins in this study, retrieved from STRING, contained 596 nodes and 5228 edges, compared to the STRING algorithm’s expected number of 2187 edges for an equivalent sample of random nodes, that is, without PPI enrichment. Similarly, the PPI network of proteins upregulated in the peripheral zone compared to the inner zone contained 129 nodes and 700 edges (compared to 224 edges expected), and the corresponding numbers for proteins upregulated in the inner zone compared to the peripheral zone were 35 nodes and 173 edges (compared to 10 edges expected). All networks showed significant enrichment of PPI, with a $P$-value of $<1.0 \times 10^{-16}$. The functional
enrichment analysis of all identified meniscal proteins in this study showed extracellular matrix (ECM) organization and exocytosis as biological processes that were enriched in this network (Table 3). Further, the majority of proteins upregulated in the peripheral zone were intracellular proteins present in the cytosol (Figure 3A, Table 4), whereas 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 organization (Figure 3B, Table 5).

3.5 Validation experiment of MS results

A Western blot (WB) analysis was performed in order to validate the MS results for two of the identified proteins - COMP and fibromodulin. COMP was not differentially expressed between the zones, and the WB shows very similar results between the zones (Figure S1). Fibromodulin was differentially expressed between the middle and peripheral zones, and the WB analysis reports similar results (Figure S1).

4 DISCUSSION

In this study, we aimed to characterize the proteome of the human medial meniscus body and investigate possible zonal differences within this region. We observed large differences in protein abundances between the different zones of the medial meniscus body, with the peripheral zone appearing most different compared to the middle and inner zones.

Two chondroitin sulfate proteoglycans were identified in our study - aggrecan and versican, and both were highly abundant in all three
TABLE 3  Top five enriched biological process and cellular component GO terms in all identified proteins in the study, based on the STRING search, together with a FDR-adjusted P-value

| GO term     | Biological process                        | Count in data set | Adjusted P-value |
|-------------|-------------------------------------------|-------------------|------------------|
| GO:0043062  | Extracellular structure organization       | 95 of 339         | 1.39 × 10⁻⁵¹     |
| GO:0030198  | Extracellular matrix organization          | 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     |

Abbreviations: FDR, false discovery rate; GO, gene ontology.

zones of the meniscus. Aggrecan was not among the differentially expressed proteins identified between the zones in this study, however, we found that versican was more abundant in the peripheral zone compared to the inner zone (Figure 2). This is consistent with previous reports that versican mRNA levels are higher in the peripheral parts of the meniscus. Versican has been reported to be involved in many processes, including cell adhesion, proliferation, and migration, as well as ECM assembly. It has also been reported to bind to hyaluronan and proteoglycan link proteins, also just called link proteins, which are important constituents of the ECM in cartilage tissue, and which stabilize proteoglycan aggregates with hyaluronic acid. In our analysis, the link protein HPLN3 was more abundant in the peripheral zone compared to the inner zone (Figure 2), as well as in the middle zone compared to the inner zone. This is consistent with the expression pattern of versican, which supports previous reports of a relationship between the two.

Approximately 10% to 30% of the medial meniscus has been reported to be penetrated by blood vessels, which originate from the meniscus’ peripheral attachment to the joint capsule and synovium. In our analysis, we could identify several proteins associated with blood circulation, for example, the two subunits of hemoglobin (HBA and HBB), which were more abundant in the peripheral zone compared to the middle zone and the inner zone (Figure 2, Table S3, S4). Other circulating blood proteins, for example, serum albumin and serotransferrin, were among the proteins with the highest abundance values in all three zones in our analysis. Blood supply is likely the key source of nutrients in the meniscus, especially in the vascularized peripheral zone. However, nutrients may also be received from the synovial fluid via diffusion, or through canal-like structures that have been observed to penetrate from the surface into deeper meniscus tissue, which may be especially important for nutrient uptake in the avascular parts of the meniscus.

The protein with the highest fold-change between the peripheral and inner zones was myocilin (Figure 2). Myocilin is a glycoprotein that is found mainly in the trabecular meshwork of the eye, but also in other ocular and non-ocular tissues. It has been observed to mediate myelination in the peripheral nervous system, and it may promote cell-matrix adhesion and cell migration. In a previous study by our group, myocilin was found to be more abundant in the meniscus than articular cartilage. In this study, myocilin expression was higher in the peripheral zone than the middle and inner zones, which supports the hypothesis that the middle and inner zones are more similar to articular cartilage than the peripheral zone. Previously, it was reported that myocilin had a higher expression in synovial fluid from healthy controls than from individuals with meniscal injuries or pathologies, indicating that myocilin might be involved in pathological processes of the meniscus. However, the exact function of myocilin in the meniscus needs to be investigated further.

A large portion of the ECM in the menisci consists of collagens, which are important for maintaining the tensile strength of the meniscus. Several different types of collagen could be quantified in this study, and 16 of these were differentially expressed between the zones. Overall, collagen type I and VI had the highest abundance levels (Table 2), in line with a previous report that type I collagen was the most prevalent collagen in the meniscus. This also differentiates the meniscus from articular cartilage, in which type II collagen is more common. In our study, several different types of collagen differed in abundance between the zones. Several studies have reported that type II collagen is enriched in the inner zone of the meniscus, making it more similar to articular cartilage. This was confirmed in our study, where the collagen alpha-1 (II) chain was 6 to
10 times higher in the middle and inner zones (Table S4, Figure 2), compared to the peripheral zone.

Finally, in order to achieve an even wider understanding of the biology of the normal meniscus, we performed functional enrichment and network analyses on all proteins identified in the study. As expected, the proteins of the meniscus are mainly extracellular proteins associated with ECM organization, and proteins involved in exocytosis (Table 3). When we performed the same analyses on proteins that were more abundant in the peripheral zone compared to the inner zone, we found that all proteins but one were intracellular and the majority had biological roles in intracellular processes and organization of cellular components (Table 4). Furthermore, we also identified a cluster of ribosomal proteins (e.g., RPL8, RPS9, and RPL6) that is enriched in the peripheral zone (Figure 3A), as well as a small cluster of collagens. Taken together, these results support the conclusion that there are more cells in the peripheral zone of the meniscus, compared to the inner zone.

Conversely, functional enrichment analysis of proteins more abundant in the inner zone compared to the peripheral zone revealed that most proteins upregulated in the inner zone are associated with ECM organization and collagen trimer formation and organization (Table 5). Network analysis of these proteins reveals a very distinguished cluster of collagens, with which serine protease HTRA1 appears to associate (Figure 3B). HTRA1 has previously been observed to degrade cartilage ECM proteins, and was found to be the most abundant protease in articular cartilage. Taken together, these results suggest that the inner zone of the meniscus is mainly comprised of ECM, and thereby similar to articular cartilage in composition. Similar results were obtained in the functional enrichment analysis of proteins that were differentially expressed between the peripheral and middle zones.

Furthermore, in order to validate our MS results we performed a Western blot analysis for two of the identified proteins; COMP and fibromodulin. WBs of both proteins confirmed the relative expression patterns we observed between the meniscal zones using MS. Although protein levels are difficult to quantify precisely with WB, the analysis supports the validity of our approach to protein identifications and quantifications using MS, through confirmation using an independent method.

In this study, the results were based on a library-free data analysis approach, however, we also performed a data search against a spectral library in order to see if we could identify more proteins. The details about the data search can be found in Supporting Information Material S2. Indeed, we did identify more proteins (n = 881), however, we also observed more missing values. After excluding proteins with more than one missing value in each zone, some 50 fewer proteins remained compared to the library-free approach. We prioritized to include as many proteins as possible in the statistical analysis, hence we chose to use the library-free approach.

We would like to highlight some limitations of this study. First, the sample size is unfortunately relatively small. However, this is a pilot study and we acknowledge the need of future replication studies. Second, even though we use menisci from healthy donors, we appreciate there are some variations among the donors. In a previous study by our group together with collaborators which included the same menisci, we reported that the donor menisci varied quite a lot in histopathological score, which appeared to increase with age. Third, the age and BMI range of this study are
rather large. This increases the generalizability of the results, however, our matched analytical approach helped to control the variability between donors. Furthermore, it would have been desirable to include lateral menisci in addition to medial menisci in our analysis. However, due to overall constraints in the number of donor samples that were feasible to analyze in a single MS series, we chose to focus on the medial meniscus, which is more often affected by pathological changes. Moreover, our protein extraction method based on guanidine hydrochloride is suboptimal for collagen extraction, and may produce varying results based on extent of collagen cross-linking or subtype. This, unfortunately, makes interpretation about overall collagen expression difficult in our study. Finally, a few proteins in this study could not be included in the functional enrichment analysis because they were not found in the STRING database. However, the majority of these were variants of the variable domain of immunoglobulin kappa (Table S1), and their exclusion is not expected to impact the final results of the analysis.

A strength of this study is our use of DIA-based mass spectrometry, instead of the more widely used data-dependent acquisition.

### Table 4
Top five 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 a FDR-adjusted P-value

| GO term | Biological process                                      | Count in data set | Adjusted P-value |
|---------|---------------------------------------------------------|-------------------|------------------|
| GO:0071840 | Cellular component organization or biogenesis         | 83 of 5342        | 8.22 × 10^{-15}  |
| GO:0016043 | Cellular component organization                        | 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}  |

### Table 5
Top five 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 a FDR-adjusted P-value

| GO term | Biological process                                      | Count in data set | Adjusted P-value |
|---------|---------------------------------------------------------|-------------------|------------------|
| GO:0030198 | Extracellular matrix organization                      | 13 of 296         | 2.50 × 10^{-13}  |
| GO:0030199 | Collagen fibril organization                            | 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}   |

### Table 4
Top five 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 a FDR-adjusted P-value

| GO term | Biological process                                      | Count in data set | Adjusted P-value |
|---------|---------------------------------------------------------|-------------------|------------------|
| GO:0005581 | Collagen trimer                                         | 19 of 88          | 1.29 × 10^{-14}  |
| GO:0098644 | Complex of collagen trimers                            | 10 of 19          | 1.70 × 10^{-10}  |
| 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}  |

Abbreviations: FDR, false discovery rate; GO, gene ontology.
(DDA) method. DIA is generally considered to be more precise in peptide quantification, and to have higher reproducibility of peptide identification than DDA. In a previous study, we compared DDA and DIA using human samples of articular cartilage and meniscus, in which we observed that DIA yielded more precise estimates, less missing values, and less variation than DDA.28 lendings increased confidence to the results of this study.

5 | CONCLUSION

In total, 170 proteins differed between the different zones of the medial meniscus body, with the peripheral one-third of the medial meniscus body differing substantially from the more internal zones, which were more similar. This can probably be explained by the fact that the peripheral zone is vascularized and innervated and appears to host a higher content of cells, whereas the middle and inner zones appeared similar to hyaline cartilage, with high levels of extracellular matrix proteins such as aggrecan and collagen type II. We believe that this study has contributed to an increased understanding of the basic biology and proteomic constitution of the normal human meniscus body, which may be useful when trying to understand processes in the degenerated meniscus during OA.

ACKNOWLEDGMENTS

We would like to thank the personnel at the Tissue Bank (Vävnadsbanken) at Skåne University Hospital in Lund and the Division of Forensic Medicine in Lund for the collection of biobank tissue. Furthermore, we would like to acknowledge Karin Lindblom and Anders Aspberg for valuable experimental and scientific input regarding the Western blot analyses.

FUNDING INFORMATION

This project has received funding from the European Research Council (ERC) under the European Union’s Horizon 2020 research and innovation programme (grant agreement No 771121) (ME) and the Foundation for Research in Rheumatology (FOREUM) (018EnglundPreCl, ME). The study was also funded by grants from the Swedish Research Council (2014-2348 ME and 2014-3303 PÖ), Alfred Österlund Foundation (ME, PÖ), the Crafoord Foundation (ME, PÖ), the Anna-Greta Crafoord Foundation (PÖ), the Swedish Rheumatology Association (ME, PÖ), the IngaBritt and Arne Lundberg Research Foundation (PÖ, MS-instrument), the Krapperup Foundation (PÖ), the Greta and Johan Kock Foundation (PÖ, ME), and Governmental Funding of Clinical Research within the National Health Service (ALF) (ME).

CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

AUTHOR CONTRIBUTIONS

Conception and design: EF, PÖ, and ME. Provision of study materials and tissue preparation: ME, EF, VH, PÖ, JT, and NA. Statistical analysis: AT, EF, and MR. Drafting of the article: EF. Critical revision of the article for important intellectual content: AT, PÖ, VH, MR, JT, NA, and ME. Interpretation of results and final approval of the article: All coauthors.

REFERENCES

1. Englund M, Haugen IK, Guermazi A, et al. Evidence that meniscus damage may be a component of osteoarthritis: the Framingham study. Osteoarthr Cartilage. 2016;24(2):270-273.
2. Cook JL, Kuroki K, Stoker AM, et al. Meniscal biology in health and disease. Connect Tissue Res. 2016.
3. Fox AJ, Wanivenhaus F, Burge AJ, Warren RF, Rodeo SA. The human meniscus: a review of anatomy, function, injury, and advances in treatment. Clin Anat. 2015;28(2):269-287.
4. Melrose J, Fuller ES, Little CB. The biology of meniscal pathology in osteoarthritis and its contribution to joint disease: beyond simple mechanics. Connect Tissue Res. 2017;58:282-294.
5. Kumm J, Roemer FW, Guermazi A, Turkiewicz A, Englund M. Natural history of intrameniscal signal intensity on knee MR images: six years of data from the osteoarthritis initiative. Radiology. 2016;278(1):164-171.
6. Englund M, Guermazi A, Roemer FW, et al. Meniscal tear in knees without surgery and the development of radiographic osteoarthritis among middle-aged and elderly persons: the multicenter osteoarthritis study. Arthritis Rheum. 2009;60(3):831-839.
7. Rai M, Brophy RH, Sandell LJ. Osteoarthrits following meniscus and ligament injury. Curr Opin Rheumatol. 2019;31(1):70-79.
8. Ruiz-Romero C, Rego-Perez I, Blanco FJ. What did we learn from ‘omics’ studies in osteoarthritis. Curr Opin Rheumatol. 2018;30(1):114-120.
9. Önnerfjord P, Khubat A, Reinhold FP, Svensson O, Heinegård D. Quantitative proteomic analysis of eight cartilaginous tissues reveals characteristic differences as well as similarities between subgroups. J Biol Chem. 2012;287(23):18913-18924.
10. Schubert OT, Röst HL, Collins BC, Rosenberger G, Aebersold R. Quantitative proteomics: challenges and opportunities in basic and applied research. Nat Protoc. 2017;12(7):1289-1294.
11. Old WM, Meyer-Arendt K, Aveline-Wolf L, et al. Comparison of label-free methods for quantifying human proteins by shotgun proteomics. Mol Cell Proteomics. 2005;4(10):1487-1502.
12. Silva JC, Gorenstein MV, Li G-Z, Vissers JPC, Geromanos SJ. Absolute quantification of proteins by LCMSE a virtue of parallel ms acquisition. Mol Cell Proteomics. 2006;5(1):144-156.
13. Benjamini Y, Yekutieli D. False discovery rate confidence intervals for selected parameters. J Am Stat Assoc. 2005;100(469):71-81.
14. Szklarczyk D, Gable AL, Lyon D, et al. STRING v11: protein-protein association networks with increased coverage, supporting functional discovery in genome-wide experimental datasets. Nucleic Acids Res. 2018.
15. Fuller ES, Smith MM, Little CB, Melrose J. Zonal differences in meniscus matrix turnover and cytokine response. Osteoarthr Cartilage. 2012;20(1):49-59.
16. Wight TN. Versican: a versatile extracellular matrix proteoglycan in cell biology. Curr Opin Cell Biol. 2002;14(5):617-623.
17. Matsumoto K, Shionyu M, Go M, et al. Distinct interaction of versican/PG-M with hyaluronan and link protein. J Biol Chem. 2003;278(42):41205-41212.
18. Kiani C, Chen L, Wu YJ, Yee AJ, Yang BB. Structure and function of aggrecan. Cell Res. 2002;12(1):7290106-7290132.
19. Hascall V, Heinegård D. Aggregation of cartilage proteoglycans. I. The role of hyaluronic acid. J Biological Chem. 1974;249(13):4232-4241.

ORCID

Elin Folkesson http://orcid.org/0000-0002-6119-5804
20. Collier S, Ghosh P. Effects of transforming growth factor beta on proteoglycan synthesis by cell and explant cultures derived from the knee joint meniscus. *Osteoarthr Cartilage*. 1995;3(2):127-138.

21. Arnoczky SP, Warren RF. Microvasculature of the human meniscus. *Am J Sports Medicine*. 1982;10(2):90-95.

22. Fox AJ, Bedi A, Rodeo SA. The basic science of human knee menisci. *Sports Heal Multidiscip Approach*. 2012;4(4):340-351.

23. Bird Sweet M. A system of canals in semilunar menisci. *Ann Rheum Dis*. 1987;46(9):670-673.

24. Ueda J, Yue BY. Distribution of myocilin and extracellular matrix components in the corneoscleral meshwork of human eyes. *Invest Ophth Vis Sci*. 2003;44(11):4772-4779.

25. Kwon HS, Johnson TV, Joe MK, et al. Myocilin mediates myelination in the peripheral nervous system through ErbB2/3 signaling. *J Biol Chem*. 2013;288(37):26357-26371.

26. Goldwich A, Scholz M, Tamm ER. Myocilin promotes substrate adhesion, spreading and formation of focal contacts in podocytes and mesangial cells. *Histochem Cell Biol*. 2009;131(2):167-180.

27. Kwon H, Tomarev SI. Myocilin, a glaucoma-associated protein, promotes cell migration through activation of integrin-focal adhesion kinase-serine/threonine kinase signaling pathway. *J Cell Physiol*. 2011;226(12):3392-3402.

28. Folkesson E, Turkiewicz A, Englund M, Önnnerfjord P. Differential protein expression in human knee articular cartilage and medial meniscus using two different proteomic methods: a pilot analysis. *BMC Musculoskelet Disord*. 2018;19(1):416.

29. Roller B, Monibi F, Stoker A, Bal B, Cook J. Identification of novel synovial fluid biomarkers associated with meniscal pathology. *J Knee Surg*. 2014;29(01):047-062.

30. Chen S, Fu P, Wu H, Pei M. Meniscus, articular cartilage and nucleus pulposus: a comparative review of cartilage-like tissues in anatomy, development and function. *Cell Tissue Res*. 2017;370(1):53-70.

31. Cheung HS. Distribution of type I, II, III and V in the pepsin solubilized collagens in bovine menisci. *Connect Tissue Res*. 2009;16(4):343-356.

32. Melrose J, Smith S, Cake M, Read R, Whitelock J. Comparative spatial and temporal localisation of perlecan, aggrecan and type I, II and IV collagen in the ovine meniscus: an ageing study. *Histochem Cell Biol*. 2005;124(3-4):225-235.

33. Troeberg L, Nagase H. Proteases involved in cartilage matrix degradation in osteoarthritis. *Biochim Biophys Acta*. 2012;1824(1):133-145.

34. Wu J, Liu W, Bemis A, et al. Comparative proteomic characterization of articular cartilage tissue from normal donors and patients with osteoarthritis. *Arthritis Rheum*. 2007;56(11):3675-3684.

35. Kestilä I, Folkesson E, Finnilä MA, et al. Three-dimensional microstructure of human meniscus posterior horn in health and osteoarthritis. *Osteoarthr Cartilage*. 2019;27:1790-1799.

36. Hu A, Noble WS, Wolf-Yadlin A 2016. Technical advances in proteomics: new developments in data-independent acquisition. *F1000research* 5:F1000 Faculty Rev-419.

**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section.

---

**How to cite this article:** Folkesson E, Turkiewicz A, Rydén M, et al. Proteomic characterization of the normal human medial meniscus body using data-independent acquisition mass spectrometry. *J Orthop Res*. 2020;38:1735-1745. https://doi.org/10.1002/jor.24602