**Identification of tissue-dependent proteins in knee OA synovial fluid**

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**ABSTRACT**

**Objective:** For many proteins from osteoarthritic synovial fluid, their intra-articular tissue of origin remains unknown. In this study we performed comparative proteomics to identify osteoarthritis-specific and joint tissue-dependent secreted proteins that may serve as candidates for osteoarthritis biomarker development on a tissue-specific basis.

**Design:** Protein secretomes of cartilage, synovium, Hoffa’s fat pad and meniscus from knee osteoarthritis patients were determined using liquid chromatography tandem mass spectrometry, followed by label-free quantification. Validation of tissue-dependent protein species was conducted by ELISA on independent samples. Differential proteomes of osteoarthritic and non-osteoarthritic knee synovial fluids were obtained via similar proteomics approach, followed by ELISA validation.

**Results:** Proteomics revealed 64 proteins highly secreted from cartilage, 94 from synovium, 37 from Hoffa’s fat pad and 21 from meniscus. Proteomic analyses of osteoarthritic *versus* non-osteoarthritic knee synovial fluid revealed 70 proteins with a relatively higher abundance and 264 proteins with a relatively lower abundance in osteoarthritic synovial fluid. Of the 70 higher abundance proteins, 23 were amongst the most highly expressed in the secretomes of a specific intra-articular tissue measured. Tissue-dependent release was validated for SLPI, C8, CLU, FN1, RARRES2, MATN3, MMP3 and TNC. Abundance in synovial fluid of tissue-dependent proteins was validated for IGF2, AHSG, FN1, CFB, KNG and C8.

**Conclusions:** We identified proteins with a tissue-dependent release from intra-articular human knee OA tissues. A number of these proteins also had an osteoarthritis-specific abundance in knee synovial fluid. These proteins may serve as novel candidates for osteoarthritis biomarker development on a tissue-specific basis.

**INTRODUCTION**

Knee osteoarthritis (OA) is an increasingly prevalent disabling condition with serious socioeconomic impact on a global scale ([1](#_ENREF_1), [2](#_ENREF_2)). Currently, no effective treatment exists to attenuate OA progression, and the current standard of care involves conservative pain-killing drug therapies, physiotherapy and visco-supplementation of the affected joint. These therapies are all aimed at postponing total joint arthroplasty, which is an invasive surgical approach to alleviate the patient’s OA-related pain and regain mobility when the condition reaches end-stage. Knee OA is currently diagnosed radiographically by significant joint space narrowing, osteophyte formation and subchondral bone lesioning ([3](#_ENREF_3)). However, these macroscopic changes are indicative of the disease progressing towards its end-stage, and often the patient presents with symptoms such as pain, joint stiffness and debilitating mobility limitations prior to diagnosis ([4](#_ENREF_4)).

The development of OA is multifactorial, comprising genetic, biomechanical, ageing, environmental, epigenetic, cellular, and metabolic factors contributing to the intra-articular disease process and ultimately leading to a final common pathway of articular cartilage degeneration ([4-8](#_ENREF_4)). Despite the fact that OA is recognized as a total-joint disease, intra-articular pathobiological processes occur in a tissue-specific manner ([5](#_ENREF_5)). Since the synovial fluid is in contact with many of the important intra-articular tissues, it represents a promising body fluid for developing OA biomolecular diagnostics to access the condition of the joint as whole, as well as with a potential for joint tissue-specific diagnostics ([9](#_ENREF_9), [10](#_ENREF_10)).

In the search of diagnostic targets that are capable of identifying biomolecular changes in knee joint homeostasis, efforts have been made to understand the biomolecular composition of synovial fluid and to identify OA-related changes in its composition. Research has previously characterised OA-dependent proteomic, transcriptomic, metabolomic and lipidomic changes in the synovial fluid of the human knee joint ([10-13](#_ENREF_10)). From these studies it became evident that the knee joint synovial fluid composition undergoes a great number of detectable biomolecular changes specific for OA, and depending on distinct OA subtypes, but also related to the severity of cartilage damage ([14-17](#_ENREF_14)). However, the origin of OA-driven alterations in the biomolecular composition of synovial fluid is poorly documented.

In this study, we aimed to delineate potential tissue-dependent proteins in the synovial fluid of knee OA patients. To this end, we performed a comparative proteomic analysis of the secretomes of Hoffa’s fat pad, synovium, meniscus and articular cartilage of OA knees. Proteins with a tissue-dependent amount present in the secretomes were identified and the OA-specificity of their abundance was determined in knee synovial fluid. We expect that the identification of tissue-dependent and OA-related protein species from specific intra-articular tissues implicated in knee OA pathogenesis will fuel the development of novel synovial fluid-based diagnostic approaches aiming at grading and subtyping knee OA.

**Materials and Methods**

**OA knee tissue secretomes**

Full thickness cartilage explants from medial and lateral femoral condyles, a synovial explant, an explant from Hoffa’s fat pad (obtained from the core of the fat pad and avoiding synovial tissue adjacent to the fat pad) and explants from the medial and lateral menisci (avoiding synovial tissue present at the outer margins of the meniscus) were obtained as waste material following total knee arthroplasty for each of the four donors with end-stage knee OA (MEC approval 2017-0183) (Supplementary Table 1). These samples were designated as the dependent cohort. The same tissue explants were also collected from an additional 12 knee OA patients who underwent total knee arthroplasty and were designated as an independent cohort (Supplementary Table 2). Tissue explants were rinsed thoroughly in 0.9% NaCl and then pre-incubated at 37⁰C, 5% CO2 for 24 hours in a tissue/media ratio of 150 mg tissue/ml medium (Dulbecco’s Modified Eagle’s Medium-F12 without phenol red (Thermofisher, Massaschussets, USA) supplemented with 1% Glutamax (Thermofisher, Massaschussets, USA) and 1% antibiotic/antimycotic (Invitrogen, Carlsbad, US)). After 24-hours of pre-incubation, medium was fully refreshed at identical tissue/media ratio. Secretomes were then obtained following 48 hours conditioning of these cultures. Secretomes were freshly processed by differential centrifugation (5 minutes at 1200 RPM, 10 minutes 15000 RPM, room temperature). To inhibit proteolytic degradation of supernatants protease inhibitors were added (Complete Mini, EDTA-free Protease Inhibitor Cocktail, Sigma Aldrich, Saint Louis, USA). Secretomes samples were aliquoted and stored at -80⁰C until further analysis.

**Synovial fluid collection and processing**

Knee OA synovial fluid was used from three out of the four patients from the dependent cohort and 10 out of the 12 patients from the independent cohort (MEC approval 2017-0183) (Supplementary Table 1, patient 2, 3 and 4; Supplementary Table 2, patient 6, 7, 8, 9, 11, 12, 13, 14, 15 and 16. K&L scores are indicated in these tables.). Knee OA synovial fluids were processed within half an hour of collection. Samples were processed by differential centrifugation (5 minutes at 1200 RPM, 10 minutes 15000 RPM, room temperature). Non-OA knee synovial fluid from 10 individuals was purchased from Articular Engineering, Illinois, USA. Synovial fluid was collected directly from post mortem macroscopically normal joints and stored in aliquots at -80oC until shipping. Details including race, age, sex and cause of death are included in Supplementary Table 3. To inhibit proteolytic degradation protease inhibitors were added (Complete Mini, EDTA-free Protease Inhibitor Cocktail, Sigma Aldrich, Saint Louis, USA). Synovial fluid samples were aliquoted and stored at -80⁰C until further analysis.

**Total protein content measurements**

Total protein content was measured in samples using the Nano Orange Protein Quantification kit according to manufacturer’s instructions (Invitrogen, Carlsbad, USA). Fluorescence was measured using a Tristar LB 942 (Berthold, Bald Wildbad, Germany) at 485 nm excitation and 590 nm emission.

**Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and silver staining**

Secretome samples (containing 1.25 μg of total protein) of the dependent cohort were loaded on a 13% SDS-PAGE gel (Protean II xi cell, Bio-Rad, Hercules, USA) and proteins were resolved by electrophoresis. The gel was silver stained to visualize protein bands (Pierce Silver Stain Kit, Thermofisher, Massaschussets, USA) according to the manufacturer’s instructions.

**Enzyme-linked immune sorbent assay (ELISA) validation**

Differential abundance of selected protein species in secretomes or synovial fluids was validated by ELISAs from various manufacturers (Supplementary Table 4). Measurements were performed according to the manufacturer’s instructions.

**Liquid chromatography tandem mass spectrometry and label-free quantification of tissue secretome proteomes**

Proteolytic digestion was performed on 10 μg protein of each individual sample using 10 μl Strataclean (Agilent, Genomics, UK). In-solution tryptic digestion of protein samples was carried out following sequential reduction and alkylation in 3 mM DTT (60 °C for 10 min) and then 9 mM iodoacetamide (30 min in the dark at room temperature) with trypsin at a ratio of 1:50 protein: trypsin ratio overnight at 37 °C ([18](#_ENREF_18)). Samples were randomized for loading onto the instrument using Excel (Microsoft, USA). All samples were run in a single batch. 500 ng of each tryptic digest was subjected to liquid chromatography tandem mass spectrometry (LC-MS/MS), using a 2 h gradient. Data-dependent analyses were conducted on a QExactive HF quadrupole-Orbitrap mass spectrometer coupled to a Dionex Ultimate 3000 RSLC nano-liquid chromatograph (Hemel Hempstead, UK). Sample digests were loaded onto a trapping column (Acclaim PepMap 100 C18, 75 µm x 2 cm, 3 µm packing material, 100 Å) using a loading buffer of 0.1% (v/v) TFA, 2 % (v/v) acetonitrile in water for 7 minutes at a flow rate of 12 µL min-1. The trapping column was then set in-line with an analytical column (EASY-Spray PepMap RSLC C18, 75 µm x 50 cm, 2 µm packing material, 100 Å) and the peptides eluted using a linear gradient of 96.2 % A (0.1 % [v/v] formic acid):3.8 % B (0.1 % [v/v[ formic acid in water/acetonitrile [80/20] [v/v]) to 50 % A:50 % B over 90 minutes at a flow rate of 300 nL min-1, followed by washing at 1% A:99 % B for 5 minutes and re-equilibration of the column to starting conditions. The column was maintained at 40oC, and the effluent introduced directly into the integrated nano-electrospray ionisation source operating in positive ion mode. The mass spectrometer was operated in data dependent acquisition mode with survey scans between m/z 350-2000 acquired at a mass resolution of 60,000 (FWHM) at m/z 200. The maximum injection time was 100 ms, and the automatic gain control was set to 3e6. The 12 most intense precursor ions with charges states of between 2+ and 5+ were selected for MS/MS with an isolation window of 2 m/z units. The maximum injection time was 100 ms, and the automatic gain control was set to 1e5. Fragmentation of the peptides was by higher-energy collisional dissociation using normalized collision energy of 30%. Dynamic exclusion of mass/charge values to prevent repeated fragmentation of the same peptide was used with an exclusion time of 20 sec. For label-free quantification, the raw files of the acquired spectra were analyzed by the Progenesis QI software (Waters, Manchester, UK) ([19](#_ENREF_19)) which aligns the files and then peak picks for quantification by peptide abundance. Briefly, the top five spectra for each feature were exported from Progenesis QI and utilised for peptide identification with our local Mascot server (Version 2.6.2), searching against the Unihuman Reviewed database with carbamidomethyl cysteine as a fixed modification and methionine oxidation as a variable modification, peptide mass tolerance of 10 ppm and fragment tolerance of 0.01 Da.

**Liquid chromatography tandem mass spectrometry and label-free quantification of synovial fluid samples**

We utilized our previously optimised method for synovial fluid proteomics using Proteominer beads ([20](#_ENREF_20)). Briefly, individual synovial fluid samples were thawed and treated with 1 µg/ml hyaluronidase as previously described ([19](#_ENREF_19)) and centrifuged at 10000g for 10 minutes to remove any particulates. Protein concentrations of synovial fluid were determined by Bradford assay (Thermofisher, Massaschussets, USA). A volume of sample equivalent to 5 mg of protein was added to 10 µL of ProteoMiner beads (BioRad, UK) according to the manufacturer’s instructions. Beads were re-suspended in 80 µL of 25 mM ammonium bicarbonate and 5 µL of 1% (w/v) Rapigest SF (Waters, UK) added and the sample heated at 80˚C for 10 minutes. On bead trypsin digestion was undertaken ([19](#_ENREF_19)). Tryptic digests were subjected to LC-MS/MS, using a 2 hour gradient using methods described above. Label-free quantification was undertaken using methods described above.

**Mass spectrometry proteomics data**

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD014321 and 10.6019/PXD014321 ([21](#_ENREF_21)).

**Statistical Analyses**

Statistical analysis was undertaken in Progenesis QI software (Waters, Manchester, UK). Proteins identified following label-free quantification were retained if they had at least two unique peptides. Protein abundances were logarithm (base 10) transformed and normalised relative to a selected reference sample.

To assess differences in normalised abundance in the dependent cohort between cartilage, Hoffa's fat pad, synovium and meniscus secretomes, we implemented repeated measures one-way analysis of variance (RM-ANOVA). For the RM-ANOVA, we specified our factor variable to be tissue and our within-subject dependent variable as the donors. No shrinkage methods were applied. Differentially expressed proteins between tissue-specific secretomes were defined as those with a false discovery rate (FDR) q value < 0.05 and fold-change that exceeded 2 for any tissue ([22](#_ENREF_22)).

To assess differences in normalised protein abundance between the synovial fluid of OA and non-OA donors, we implemented a one-way analysis of variance (ANOVA). The condition factor was set according to whether the protein originated from OA or non-OA synovial fluid. OA and non-OA synovial fluid donors were independent donors. Differentially expressed proteins between OA and non-OA synovial fluid were defined as those with an FDR q value < 0.05 and a fold change that exceeded 2 between conditions ([22](#_ENREF_22)).

95% confidence intervals for pairwise differences between means were generated for any statistically significant proteins as determined by the above criteria (R, Version 4.0.1). The 95% confidence intervals were adjusted based on parameters of selection to allow for control of false coverage rate (FCR) ([23](#_ENREF_23), [24](#_ENREF_24)).

Statistical analysis of tissue secretome ELISA data was performed using Graphpad Prism (version 7, La Jolla, California, USA). Data were log-transformed and protein abundances between cartilage, Hoffa’s fat pad, synovium and meniscus were compared using a repeated measures ANOVA both for the dependent and the independent cohort. Multiple comparisons between tissues were performed using a Fisher’s LSD test. Significance was accepted at a p value < 0.05. For the synovial fluid ELISA data, protein abundances in synovial fluid samples of OA and non-OA donors were compared using a Mann-Whitney U test. Significance was defined as a p-value of <0.05.

All principal component analysis calculations (PCA) were performed in R (Version 4.0.1). Log transformed normalised protein abundances were centered and scaled. For OA vs non-OA synovial fluid we used the prcomp function within the stats package to extract principal components ([25](#_ENREF_25)). For the multiple tissue secretomes dataset, due to the repeated measures design, we used the PCA function within the *mixOmics* package to generate a multilevel PCA plot that first calculates within subject variance prior to dimensionality reduction ([26](#_ENREF_26)).

**RESULTS**

**Preparation of OA knee tissue-derived secretomes**

Mean total protein concentration of cartilage secretomes samples was 233.4 μg/mL (range 191-285 μg/mL), of Hoffa’s fat pad secretomes 298.7 μg/mL (range 203-444 μg/mL, of synovium secretomes 326.5 μg/mL (range 253-404 μg/mL) and of meniscus secretomes samples 275.0 μg/mL (range 167-362) μg/mL. To evaluate sample quality and potential gross differences in protein profiles in the different secretomes a silver stained SDS-PAGE demonstrated sharp, distinct bands, confirming sample quality (Figure 1). In addition, tissue-dependent enrichment of specific protein bands was observed. This indicates the presence of tissue-dependent proteins in the OA knee tissue secretomes.

**Identification of protein species in OA knee tissue secretomes**

To identify the protein species in the OA knee tissue secretomes, label-free mass spectrometry proteomic analysis was undertaken. Principal component analysis of the acquired proteomes demonstrated tissue-dependent protein clustering (Figure 2A). A total of 216 proteins were identified with highest mean significant detection in one of the tissue type-dependent secretomes (Supplementary Table 5). From these 216 protein species, 64 were highest secreted from cartilage, 94 from synovium, 37 from Hoffa’s fat pad and 21 from meniscus. Four examples of proteins with a tissue-dependent amount detected in the secretomes are shown in Figure 2B-E. The amount of Antileukoproteinase (SLPI) was highest in the secretome of cartilage and lowest in the secretome of synovium (Figure 2B). The lowest amount of Matrix metalloproteinase 3 (MMP3) was found in the secretome of Hoffa’s fat pad and highest of cartilage (Figure 2C). The amount of Complement C8 alpha chain (C8A) was highest in the meniscus secretome and lowest in secretome of synovium (Figure 2D). Finally, the amount of Retinoic acid receptor responder protein 2 (RARRES2) was highest in the secretome of cartilage and lowest in synovium secretome (Figure 2E). The OA knee tissue-dependent levels of these four protein species in the acquired secretomes was validated by means of ELISA (Figure 2F-I). A full list of proteins with highest significant amount per tissue-specific secretome is provided in Supplementary Table 5, and a full list of differentially expressed proteins with the corresponding confidence intervals for each tissue is in Supplementary Table 6.

**Validation of tissue-dependent secretome proteins in an independent cohort**

We next investigated whether our mass spectrometry data is applicable within a general knee OA population. To this end, the proteins measured in Figure 2, and an additional selection of proteins with a highest amount in the secretomes of the tissues from Supplementary Table 5 was measured by ELISA in the tissue secretomes of 12 independent knee OA patients (the independent cohort; Supplementary Table 2). ELISA measurements were performed for SLPI, C8 complex, CLU, FN1, RARRES2, MATN3, MMP3 and TNC (Figure 3). The amount of SLPI in the secretome was highest from cartilage and lowest from synovium as revealed by mass spectrometry analysis (Supplementary Table 5) and this was confirmed by ELISA measurements in the independent cohort (Figure 3A). The C8A amount was highest in the secretome of meniscus and lowest in the secretome of synovium in our mass spectrometry data. ELISA analysis of C8 complex in the independent cohort confirmed the highest amount originating from meniscus (Figure 3B). The amount of Clusterin (CLU) was highest in the cartilage secretome and lowest in the Hoffa’s fat pad secretome in the mass spectrometry data. ELISA analysis of the independent cohort validated this observation (Figure 3C). Mean Fibronectin 1 (FN1) amount was highest in the cartilage secretome and lowest in the secretomes of Hoffa’s fat pad as concluded from mass spectrometry data and ELISA analysis validated this (Figure 3D). ELISA analysis further confirmed mass spectrometry data for RARRES2 amount, which was highest in the cartilage secretome and lowest in the synovium secretome (Figure 3E). Mass spectrometry data showed that Matrillin 3 (MATN3) amount was highest the secretome of cartilage and lowest in the Hoffa’s fat pad secretome. The ELISA analysis of the independent cohort confirmed that MATN3 amount was highest in the cartilage secretome. However, this was not detectable in the secretome of Hoffa’s fat pad or synovium (Figure 3F). The amount of MMP3 was lowest in the secretome of Hoffa’s fat pad and highest the cartilage secretome in mass spectrometry data. Lowest MMP3 amount in tissue secretomes was also confirmed for Hoffa’s fat pad by ELISA analysis of the independent cohort (Figure 3G). Finally, mean Tenascin C (TNC) amount was highest in the secretome of meniscus and lowest the Hoffa’s fat pad secretomes in mass spectrometry analysis and this was confirmed by ELISA (Figure 3H). In conclusion, protein mass spectrometry analysis and ELISA validation of OA knee joint tissue-specific secretomes revealed the presence of many tissue-dependent proteins.

**OA-specific abundance of protein species in synovial fluid**

Next, we sought to determine whether the OA knee joint tissue-dependent proteins identified above (Supplementary Table 5) showed an OA-specific abundance in knee joint synovial fluid. To address this question, we performed protein mass spectrometry and label-free quantification on knee joint synovial fluid from 10 patients with knee OA and from 10 individuals without OA and compared their proteomes. Principal component analysis of the acquired proteomes revealed defined group-specific clustering (Figure 4A). The abundance of 62 protein species was found to be significantly increased in OA synovial fluid compared to non-OA synovial fluid. A list of synovial fluid proteins with OA-specific enrichment is presented in Supplementary Table 7. Of these 62 protein species, 39 were also detected in the secretomes above. Of these 39 proteins, 19 were present in the secretomes in an OA knee joint tissue-dependent manner (Supplementary Tables 7 and 8). In addition, 234 protein species were present in significantly lower abundance in OA synovial fluid compared to non-OA synovial fluid. Of these 234 protein species, 56 were also detected in the secretomes. Of these 56 proteins, 54 were found to be present in the secretomes in a tissue-dependent manner (Supplementary Table 9). Next, we validated mass spectrometry results of the synovial fluid proteomes using ELISA assays. Validation was performed using knee OA synovial fluid samples of the knee OA patients from which we also used the tissues to generate tissue-conditioned media (n=13), and using the non-OA knee synovial fluid samples which were also used for the synovial fluid mass spectrometry analysis (n=10). Six proteins that were significantly higher in OA synovial fluid compared to non-OA synovial fluid (and some with an OA knee joint tissue-dependent release; Supplementary Tables 5 and 7) were selected for ELISA validation (IGF2, AHSG, FN1, CFB, KNG, C8 complex). For these proteins data following ELISA validation were highly concordant with our mass spectrometry data (Figure 4). In addition to proteins with a higher abundance in OA synovial fluid, we also validated mass spectrometry data of two proteins with different expression dynamics. In the mass spectrometry data, the abundance of DCN (Decorin) was found to be significantly lower in OA synovial fluid compared to non-OA synovial fluid. The lower abundance of DCN in OA synovial fluid was validated by ELISA (Figure 5A/B). Furthermore, the abundance of ANG (Angiogenin) was not significantly different between OA and non-OA synovial fluid and this was confirmed with ELISA (Figure 5C/D).

**DISCUSSION**

In the search of diagnostic targets that are capable of identifying biomolecular changes in knee joint homeostasis, important efforts have been made to understand the proteomic composition of synovial fluid and to identify OA-related changes in its composition ([10](#_ENREF_10), [19](#_ENREF_19), [27-29](#_ENREF_27)). However, the origin of OA-related alterations in the proteomic composition of synovial fluid remained poorly documented and biochemical markers generally lacked specificity to certain joint tissues ([30](#_ENREF_30)). In our study we identified 62 proteins with a significantly higher abundance in synovial fluids of osteoarthritic knees. A large part of the synovial fluid is created by ultrafiltration of plasma through the synovial membrane. Synovial fluid is in direct contact with different intra-articular tissues such as cartilage, synovium and meniscus as well as at an intimate distance from Hoffa’s fat pad ([31](#_ENREF_31)). Thus, the source of protein species in the synovial fluid may be from the blood stream, proximity tissues or released by different intra-articular joint tissues into the synovial fluid. From the 62 proteins with an OA-specific higher abundance in knee synovial fluid, 39 were present in the secretomes of the investigated knee joint tissues. This indicates that their abundance in knee synovial fluid may depend on their release from Hoffa’s fat pad, synovium, meniscus and/or articular cartilage. From these 39 proteins, 19 are of particular interest, since their amount is specifically highest in the secretome of one of the investigated intra-articular tissues (Supplementary Table 8). Our results strongly suggest that specific OA knee joint tissues differentially contribute to OA-related changes in the synovial fluid proteome of the knee joint. Earlier publications reported clear differences in protein profiles between OA and non-OA synovial fluids ([10](#_ENREF_10), [27](#_ENREF_27), [32](#_ENREF_32)). Specific joint tissues, such as cartilage and synovium, have been individually investigated as contributors to the levels of OA-related proteins (and protein fragments) in synovial fluid ([10](#_ENREF_10), [27](#_ENREF_27), [33-35](#_ENREF_33)). We have determined the protein secretomes of four different knee joint tissues and linked this to OA-specific changes in the synovial fluid proteome of the knee joint. Our findings therefore provide a comprehensive insight into the tissue-dependent origin of proteins in knee joint synovial fluid with an OA-specific abundance.

Knowledge of the main tissue source of OA-specific proteins present in knee joint synovial fluid is expected to have important implications for understanding the progression of knee OA on the tissue level, as well as for the development of molecular diagnostics that may aid in the sub-/endotyping of knee OA. Also, efficacy studies of novel (experimental) treatments for knee OA will benefit from biomarkers that are able to report about the joint’s health status on the tissue level. For instance, accumulating evidence suggests that complement system components play a key role in the pathogenesis of knee OA ([36](#_ENREF_36), [37](#_ENREF_37)). Of the four different explanted OA knee tissues the secretome of explanted OA meniscal tissue had the highest amount of Complement component C8. This suggests that C8 might be put forward as a potential synovial fluid biomarker to evaluate the efficacy of therapeutic interventions in knee OA due to meniscal pathology. Another protein whose abundance has been shown previously to be increased in OA synovial fluid is Clusterin ([27](#_ENREF_27), [36](#_ENREF_36)). While we confirmed this finding in our present study, our data additionally demonstrates the greatest amount of Clusterin is in the explant secretomes of cartilage and meniscal tissues. This points towards cartilage and meniscus as the tissues-of-origin of Clusterin levels in OA synovial fluid. The same holds true for Fibronectin, which has been shown to be more abundant in OA synovial fluid compared to non-OA synovial fluid ([27](#_ENREF_27), [36](#_ENREF_36)).

As a general study limitation we cannot exclude that some proteins in the tissue-specific secretomes and synovial fluid may originate from degradation products of cell death, extracellular matrix or the blood stream, respectively. However, regarding cell death, this is unlikely to play a major role since clear signs of viability have been reported for similar explant cultures ([14](#_ENREF_14), [38](#_ENREF_38), [39](#_ENREF_39)). Future investigations will further clarify the contribution of the intra-articular tissues to specific proteins in the synovial fluid.

A schematic overview demonstrating the numbers of proteins with a highest secretion from one of the investigated knee joint tissues and their OA-specific abundance in knee synovial fluid is shown in Figure 6. In this respect, it is interesting to note that our study revealed that in addition to cartilage and synovium, the meniscus and Hoffa’s fat pad are also likely to be significant contributors to the OA-specific protein composition of the synovial fluid of the knee joint. We expect that improved knowledge of the tissue-specific origin of OA-specific synovial fluid biomarkers will propel the understanding of the role of specific tissues in the progression of OA and fuel the development of improved synovial fluid-based diagnostic approaches aiming at grading and subtyping OA ([40](#_ENREF_40), [41](#_ENREF_41)).

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**Author Contributions**

**U.T.T., DG, MP and TW conception and design, collection and assembly of data, data analysis and interpretation, manuscript writing, final approval of the manuscript. H.J., P.E. and LR conception and design, data analyses and interpretation and final approval of the manuscript, J.A. and A.S. collection and assembly of data, data analysis and interpretation, final approval of the manuscript.**

**Conflicts of Interests**

TJM Welting is listed as inventor on patents: WO2017178251, WO2017178253 and US 20130123314. PJ Emans and LW van Rhijn are listed as inventors on patent US 20130123314. LW van Rhijn, PJ Emans and TJM Welting have shares in Chondropeptix and are CDO, CMO and CSO of Chondropeptix, respectively.

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**FIGURE LEGENDS**

**Figure 1: Confirmation of sample quality and gross differences in protein profiles in secretomes.** Scans of silver-stained acrylamide gels with patient numbers indicated. C = cartilage, H = Hoffa’s fat pad, M = meniscus, S = synovium. Protein molecular weights (kDa) are indicated on the left of both gels.

**Figure 2: PCA analysis and ELISA validation of differentially expressed proteins in tissue-specific secretomes.** Principal Component Analyses following label-free quantification of secretome proteomes from the dependent cohort **(A)**. Graphical representation of normalized abundance values of mass spectrometry data of four selected protein species which were found to be differentially expressed in a tissue-dependent fashion: SLPI **(B)**, MMP3 **(C)**, C8A **(C)**, RARRES2 **(D)**. ELISA-based validation of levels of SLPI **(F)**, MMP3 **(G)**, C8A **(H)**, RARRES2 **(I)** in the tissue-dependent secretomes. Data are presented in scatter plots (individual patients are shown as separate dots) with mean values indicated as a horizontal line. Statistics following label-free quantification were calculated using a repeated measures ANOVA. Q values were generated from resulting p values using FDR correction for multiple testing. Statistical significance following ELISA analysis was calculated using a repeated measures ANOVA after log-transformation, followed by a Fisher’s LSD test. Statistical significance is indicated in the figures.

**Figure 3:** **Independent validation of secretome tissue specificity.** ELISA-based validation of eight selected protein species which were found to be differentially expressed in a tissue-dependent fashion in the mass spectrometry data. SLPI **(A)**, C8 complex **(B)**, CLU **(C)**, FN1 **(D)**, RARRES2 **(E)**, MATN3 **(F)**, MMP3 **(G)**, TNC **(H)**. Data are presented in scatter plots (individual donors are shown as separate dots) with mean values indicated as a horizontal line. Statistical significance was calculated using a repeated measures ANOVA after log-transformation, followed by a Fisher’s LSD test and is indicated in the figures.

**Figure 4: PCA analysis and ELISA validation of synovial fluid mass spectrometry data.** Principal Component Analyses following label-free quantification of knee OA and non-OA synovial fluids **(A)**. Graphical representation of normalized abundance values of mass spectrometry data and accompanying ELISA validation of selected protein species that were found to be present in higher abundance in OA *versus* non-OA synovial fluids (based on mass spectrometry analysis (Supplementary Table 7)). IGF2 (**B/C**), AHSG (**D/E**) FN1 **(F/G)**, CFB **(H/I)**, KNG **(J/K)**, C8A and C8 complex **(L/M)**. Data are presented in scatter plots (individual donors are shown as separate dots), with mean values indicated as a horizontal line. Statistics following label-free quantification were calculated using an ANOVA. Q values were generated from resulting p values using FDR correction for multiple testing. Statistical significance following ELISA analysis was calculated using a Mann-Whitney U test. Statistical significance is indicated in the figures.

**Figure 5: ELISA validation of synovial fluid mass spectrometry data.** Graphical representation of normalized abundance values of mass spectrometry data and accompanying ELISA validation of selected protein species that were found to be present in lower and equal abundance in OA *versus* non-OA synovial fluids (based on mass spectrometry analysis). DCM **(A/B)**, ANG **(C/D)**. Data are presented in scatter plots (individual donors are shown as separate dots), with mean values indicated as a horizontal line. Statistical significance was calculated using a Mann-Whitney U test and is indicated in the figures.

**Figure 6: Graphical data summary.** Numbers of protein species with an OA-specific abundance in knee synovial fluid, the number of protein species from these that were detected in the OA knee joint tissue secretomes, and the number of these with a tissue-dependent release into the OA knee joint tissue secretomes (Supplementary Tables 5, 7, 8, 9).