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ORIGINAL RESEARCH

Patient-responsive protein biomarkers for cartilage degeneration and repair identified in the infrapatellar fat pad

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ABSTRACT

Objectives: Cartilage defects (CDs) are regarded as early manifestation of osteoarthritis (OA). The infrapatellar fat pad (IPFP) is an important mediator in maintaining joint homeostasis, disease progression and tissue repair, with a crucial role of its secreted proteins. Here, we investigate the proteome of the IPFP in relation to clinical status and response to surgical treatment of CDs.

Methods: In order to characterize the proteome of the IPFP, samples from a cohort of 53 patients who received surgical treatment for knee CDs were analyzed with label-free proteomics. Patients were divided based on validated outcome scores for pain and knee function, preoperatively and at 1-year postoperatively, and on MRI assessment of the defect severity, fibrosis and synovitis.

Results: Specific proteins were differentially abundant in patients with MRI features and better clinical outcome after CD surgery, including a downregulation of cartilage intermediate layer protein 2 (CILP-2) and microsomal glutathione s-transferase 1 (MGST1), and an upregulation of aggrecan (ACAN), and proteoglycan 4 (PRG4). Pathways related to cell interaction, oxidation and matrix remodeling were altered.

Conclusion: Proteins in the IPFP that have a function in extracellular matrix, inflammation and immunomodulation were identified as potentially relevant markers for cartilage repair monitoring.

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KEYWORDS
Cartilage repair; biomarkers; KOOS; VAS; proteomics; mass spectrometry

1. Introduction

Osteoarthritis (OA) has become one of the leading causes of disability worldwide, with an estimated total of 250 million people suffering from the condition [1]. No effective treatment exists in later stages of the disease. Cartilage defects (CDs) are seen as one of the initiating factors of OA, as patients will develop OA later in life if not treated adequately [2]. Surgical treatment to restore and preserve the joint is often advised, with a wide range of available treatment options, such as microfracture, autologous chondrocyte transplantation, osteochondral autograft/allograft transplantation, or focal knee resurfacing implants (FKRI) [3]. Current treatment decision-making for CDs is mainly dependent on subjective assessment and experience of the performing surgeon [3], with no evidence for why some patients respond well to each treatment and others not [4]. However, *in vitro* studies have shown that restoration of cartilage matrix by the chondrocytes in the cartilage is heavily dependent on the homeostasis of the joint [5,6]. Therefore, taking into account the biomolecular status of the joint can be highly relevant for the treatment decision. In particular, proteins play several important roles in the context of OA. Understanding the roles of various proteins in OA pathogenesis is crucial for developing targeted therapeutic interventions aimed at preserving joint function and alleviating symptoms in affected individuals.

Previous research has shown that the different stages of OA have distinctive molecular profiles in synovial fluid [7]. Although this information is valuable for diagnostic purposes, its effect on clinical decision-making is limited if these molecular profiles are not coupled to progression of disease or response to different treatments. Current diagnostic tools like MRI can only assess the present state of the disease, differentiating between healthy and diseased stages at a single point in time, but they cannot predict the future course or prognosis of the disease. Therefore, it is recognized that biomarker research should focus on course of disease, rather than only on status [8]. CD patients are at a crossroad to either progression toward OA or restoration of the cartilage and clinical function. Therefore, in this patient group, biomarkers that are related to enhanced repair can make a clinically

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relevant difference in the treatment decision. However, a recent systematic review only identified 1 study with 17 patients with biomarkers related to outcome of cartilage repair surgery [9].

The most studied source for biomarkers in the OA field is synovial fluid [7], however, in CD patients synovial fluid is difficult to harvest due to frequent absence of synovitis (swollen knee). An underexplored source for biomarker discovery is the infrapatellar fat pad (IPFP), located behind the patellar tendon in the knee joint. It is increasingly recognized that the IPFP plays an important role in OA pathology and development [10,11]. The IPFP interacts with the processes inside the joint, as it mediates inflammation via the synovial layer and fluid [10]. Previously, it was found that the IPFPs of late-stage OA patients have significantly different molecular profiles compared to CD patients, regardless of age [12]. Furthermore, the secretome of the IPFP showed overlap with OA-specific protein profiles of synovial fluid [13]. In addition to this, part of the IPFP is routinely removed to improve visualization during surgery and as such regarded as waste material during knee surgery when accessing the joint. Thus, the IPFP may be a practical source of clinically relevant biomarkers.

To explore the feasibility of this concept, a general overview of the IPFP proteome in the CD population is first needed, and then related to clinical parameters. Therefore, in the present study, we aim to investigate the role of the IPFP in the degeneration and repair of CDs, in relation to patient diagnosis and treatment outcome. To achieve this, we investigated the proteome of IPFP samples from a cohort of CD patients who underwent surgical treatment and correlated the different protein profiles obtained with label-free proteomics with patient outcome.

2. Materials and methods

2.1. Patient samples

Patients were prospectively included if they were scheduled for any type of knee CD surgery in the period November 2018 until July 2021. The local Medical Ethics Committee confirmed that the use of waste material and questionnaires are not subjected to medical-ethical restrictions by national laws (ID: anonymized). Written consent for the use of waste material for research was obtained prior to surgery.

To assess the outcome parameters, we collected patient-reported outcome measures (PROMs). For the PROMs, we recorded the knee injury osteoarthritis outcome scores (KOOS), a measure for knee function, and a visual analog scale (VAS) for knee pain from the patients before surgery and 12 months after surgery. Magnetic Resonance Imaging (MRI) was conducted prior to surgery to evaluate defect and IPFP tissues. The imaging acquisition took place on 1.5 and 3 Tesla whole-body imaging systems utilizing an extremity coil. The MRI protocol included sagittal, coronal, and transversal images through a turbo spin echo sequence with and without fat suppression, as well as a sagittal 3-dimensional proton density sequence. All MRIs were evaluated by a musculoskeletal radiologist (FZ) with 15 years of experience, who was unaware of the clinical findings. The IPFP was graded for severity of fibrosis and synovitis as used in a validated OA MRI grading on a 0–3 scale, 3 being most severe [14]. Furthermore, the MRIs were graded for severity of the cartilage defect using the area measurement and depth & underlying structures (AMADEUS) score [15] on a 0–100 scale, 0 being most severe.

For each of the PROMs and MRI parameters, a threshold was established based on the minimal clinically important difference (MCID) or conventionally used cutoff values (Table 1). Moreover, based on the radiologist's grading of MRI scans, patients were allocated into a low degree of fibrosis group (graded as no fibrosis) or a high degree of fibrosis group (graded as either mild or moderate fibrosis).

During surgery, IPFP samples were immediately washed in phosphate-buffered saline (PBS) to remove any remaining blood from the tissue. Explants were then snap-frozen by immersing them in liquid nitrogen and stored at −80°C until further analysis.

2.2. Protein extraction

From the IPFP samples of 53 CD patients, 15 µm cryosections were obtained using a cryostat (Leica Microsystems Cryotome, Wetzlar, Germany) covered in dry ice to maintain the temperature between −30 and −35°C [16]. A minimum of 20 sections of IPFP from each patient were collected in an Eppendorf tube. All sections were stored at −80°C until protein digestion. Proteins were extracted through an in-house developed protocol, taking into account the considerations of Feist et al. [17], which emphasized selecting a lysis buffer to enhance protein solubility and denaturation without interfering with MS analysis. For this purpose, a lysis buffer containing 5 M urea (GE Healthcare, Chicago, US) and 50 mm ammonium bicarbonate (ABC, Sigma-Aldrich, Saint Louis, US) was used. After briefly spinning down the sample, three freeze-thaw cycles were performed using dry ice and water bath sonication, followed by a brief vortexing at 2000 rpm for 10 seconds, with no incubation period between cycles. The samples were then centrifuged for 30 min at 15,000 × *g* at 10°C to remove insoluble particles. Total protein content was determined through Bradford Protein Assay (Bio-Rad Laboratories, Hercules, US) according to the manufacturer's protocol. Absorption was measured at 595 nm (optical density) using a Spark 10 M microplate reader (Tecan, Männedorf, Switzerland).

2.3. Protein digestion

Protein digestion was performed using an in-solution protocol with 50 µg protein in 50 µl lysis buffer per sample, as previously described [18]. In brief, denaturation and reduction were achieved by adding 5 µl dithiothreitol (DTT, 200 mm in 50 mm ammonium bicarbonate (ABC) buffer), followed by incubation for 45 min at room temperature (RT). Next, 6 µl iodoacetamide (IAM, 400 mm in 50 mm ABC buffer) were added for alkylation, followed by incubation for 45 min at RT in the dark. To quench the excess of IAM, 10 µl of 200 mm DTT was added, followed by a 45 min incubation at RT. Protein digestion was initiated by adding 2 µl trypsin/Lys-C solution in resuspension buffer (Promega, Leiden, the

Netherlands), followed by incubation in a Thermoshaker (Eppendorf, Hamburg, Germany) at 250 rpm and 37°C. After 2 h, 200 µl of 50 mm ABC buffer was added to each sample and incubated overnight at 250 rpm and 37°C. Digestion was stopped by adding 30 µl of 20% acetonitrile (ACN, Biosolve, Valkenswaard, the Netherlands)/10% formic acid (FA, Biosolve, Valkenswaard, the Netherlands). After 30 min of centrifugation at 15,000 × *g*, supernatants were stored at −20°C until LC-MS analysis.

2.4. Liquid chromatography tandem mass spectrometry (LC-MS/MS)

Proteomic analysis was performed on a Thermo Scientific Ultimate 3000 Rapid Separation UHPLC system (Dionex, Amsterdam, the Netherlands), coupled to a Q-Exactive HF mass spectrometer (Thermo Fisher Scientific, Waltham, MA, U.S.A.), equipped with a PepSep C18 analytical column (15 cm, ID 75 µm, 1.9 µm Reprosil, 120 Å). Samples were desalted on a C18 trapping column and separated on an analytical column with a 90-minute linear gradient (5%–35% ACN with 0.1% FA, flow rate 300 nL/min). Mass spectra were acquired in positive ion mode and in data-dependent acquisition mode (DDA) using a mass-to-charge ratio (*m/z*) of 250–1250 at a 12,000 resolution. MS/MS scans were acquired from the 15 most intense ions at a 15,000 resolution [18,19].

2.5. Data analysis

MS-based proteomics data was analyzed using Proteome Discoverer (PD) Software version 2.5 (Thermo Fisher Scientific, Waltham, MA, US). Proteins were identified and quantified using the built-in Sequest HT search engine with SwissProt (Human) database (Homo sapiens, Tax ID 9606) with the following settings: enzyme trypsin, a maximum of 2 missed cleavage sites, a minimum peptide length of 6 and maximum of 144, a precursor mass tolerance of 10 ppm, and fragment mass tolerance of 0.02 Da. Dynamic modifications of methionine oxidation (+15.995 Da) and protein N-terminus acetylation (+42.011 Da) and static modification of carbamidomethylation (+57.021 Da) were used. A false discovery rate (FDR) of ≤ 1% was applied.

2.6. Statistical analysis

The description of each clinical parameter used for evaluation, based on the minimal clinically important difference (MCID), as well as the cutoff values used are depicted in Table 1 [15,20–27]. Normality of data was assessed using Shapiro-Wilk test. If normal distributed, difference in clinical outcome was

Table 1. Summary of the different clinical parameters used in the study, their cutoff values and previous literature using said parameters and thresholds.

Parameter	Description	Cut-off value (Number of patients)	Reference
KOOS	Patient-reported outcome measure related to pain and function of the knee. The questionnaire is divided into 5 subscales with a total of 52 items, from which a score is obtained out of 100, where lower scores represent higher degrees of disability	Preoperative (KOOS0): • ≤45 • >45 t = 1 year (KOOS1Y): • ≤75 • >75 Difference (ΔKOOS= KOOS1Y- KOOS0): • ≤10 • >10	[20–22]
VAS	10 cm visual analog scale for self-reported pain intensity. Higher scores correspond with higher pain intensities	Preoperative (VAS0): • ≤5.8 • >5.8 t = 1 year (VAS1Y): • ≤2.4 • >2.4 Difference (ΔVAS = VAS1Y − VAS0): • > −2.7 • ≤ −2.7	[23,24]
MRI: IPFP fibrosis	Extent of thickening or scarring of the fat pad seen in pre- and postoperative MRI scans as assessed by a radiologist	No fibrosis Mild/moderate	[25–27]
MRI: IPFP synovitis	Extent of fluid accumulation around the fat pad seen in pre- and postoperative MRI scans as assessed by a radiologist	No/low synovitis Moderate/high synovitis	[25–27]
MRI: AMADEUS	Severity of cartilage defect, assessed by a radiologist	Mild/moderate (>25) Severe (≤25)	[15]

assessed between baseline and follow-up PROMs using paired t-tests in IBM SPSS Statistics for Windows (Armonk, NY), and differences between surgical procedures were assessed using one-way ANOVA. Welch's t-test was performed to assess the effect of sex on each clinical parameter. Protein abundance was normalized based on the total peptide amount, using scaling without any imputation. Differences of protein abundance associated with clinical status (IPFP fibrosis, IPFP synovitis, AMADEUS) and clinical outcome measures were compared using pairwise peptide ratios and background-based ANOVA [28] with a Benjamini-Hochberg correction used for hypothesis testing in PD. Proteins were considered to be differentially abundant between patient groups when their abundance ratios were ≥ 1.5 in either group. Only proteins observed in at least 60% of patients in either group with an abundance ratio adjusted p-value of ≤ 0.05 between groups, were considered for further analysis. Data integration for pathway and gene ontology (GO) enrichment was performed with STRING-db [29] and ShinyGO 0.77 (http://bioinformatics.sdstate.edu/go/)

3. Results

IPFPs were collected during cartilage surgery at the anonymized (MUMC+) hospital. Fifty-three cartilage repair patients were included (male/female: 35/18). Patients received a range of surgical procedures, which reflects the clinical practice: regenerative therapies, osteochondral autografting, or FKRI [30]. 50 patients had isolated cartilage defects, with 2 concomitant ACL rupture, and 1 meniscal tear. As the hospital is a tertiary referral center, most cases had complaints for a long time, with only two cases with a known acute trauma in the previous year. No statistically significant differences between surgical procedures were observed through the use of a one-way ANOVA for any of the PROMs or MRI parameters.

Due to hampered patient contact due to COVID-19 restrictions, only from 51 patients MRIs were retrieved, from 43 patients questionnaires could be retrieved at $t = 0$, and from 41 patients at $t = 1$ year. However, of all patients at least one comparison using MRI or PROMs was available. The total number of patients per group can be found in Table 2. The average KOOS score at $t = 0$ (preoperative) was 44.7 ± 18.1 ($n = 43$) and the average VAS was 5.5 ± 2.3 ($n = 43$). At the 1 year follow-up, the average KOOS increased to 67.1 ± 20.3 ($p < 0.001$), and the average VAS was reduced to 3.0 ± 2.9 cm ($p < 0.001$) (Table 2). These data reflect the good average clinical improvement after the surgical treatment, but also that there is a great variance due to non-responders.

3.1. Characterization of the IPFP proteome

A total of 1209 proteins were identified at high confidence level (1% FDR) from the 53 IPFP samples. The biological molecular function of these proteins was enriched using the online bioinformatics tool ShinyGO 0.77. In the IPFP tissues of CD patients, the identified protein functions in the IPFP tissues of CD patients primarily involve cell structure and motility, cellular interaction and signaling, gene expression regulation, redox balance and detoxification of reactive oxygen species,

Table 2. Patient characteristics and clinical parameters.

		Fibrosis		Synovitis		AMADEUS		KOOS0		ΔKOOS		VAS0		ΔVAS	
Characteristics	All patients	H	L	H	L	H	L	H	L	H	L	H	L	H	L
Age	29.4 ± 11.7	30.3 ± 11.1	27.3 ± 13.8	30.2 ± 12.6	29.4 ± 11.5	31.9 ± 12.1	26.8 ± 10.7	27.1 ± 10.7	34.3 ± 11.4	29.2 ± 12.3	34.6 ± 9.7	31.2 ± 13.2	30.4 ± 9.9	32.9 ± 12.2	30.3 ± 11.1
BMI	24.3 ± 3.6	23.7 ± 3.5	24.9 ± 3.7	23.8 ± 3.0	24.1 ± 3.8	24.6 ± 3.3	23.3 ± 3.8	23.5 ± 3.1	24.0 ± 3.8	23.0 ± 3.7	25.6 ± 2.8	24.1 ± 4.0	23.4 ± 2.7	24.4 ± 3.5	23.7 ± 3.5
KOOS at $t = 0$	44.7 ± 18.1	43.9 ± 18.3	41.9 ± 16.0	46.7 ± 22.1	42.6 ± 16.5	45.0 ± 15.9	41.7 ± 20.3	59.4 ± 10.2	29.6 ± 9.9	38.8 ± 18.3	52.7 ± 17.8	35.0 ± 13.0	53.9 ± 17.7	46.8 ± 21.6	43.9 ± 18.3
KOOS at $t = 1$Y	67.1 ± 20.3	66.6 ± 20.1	64.5 ± 21.3	69.8 ± 17.0	64.7 ± 21.3	62.4 ± 21.5	69.7 ± 18.6	74.3 ± 19.5	62.1 ± 21.6	74.2 ± 18.5	51.4 ± 19.5	63.2 ± 23.6	72.3 ± 17.9	55.1 ± 24.1	66.6 ± 20.1
ΔKOOS	24.5 ± 21.9	23.9 ± 24.4	26.2 ± 18.1	19.1 ± 19.4	26.0 ± 24.3	19.5 ± 21.9	29.5 ± 23.9	13.4 ± 18.4	32.5 ± 22.8	34.8 ± 17.6	−1.5 ± 9.2	29.9 ± 24.1	17.5 ± 20.0	7.9 ± 16.4	23.9 ± 24.4
VAS at $t = 0$	5.5 ± 2.3	5.3 ± 2.1	5.1 ± 2.0	4.8 ± 2.5	5.5 ± 1.9	5.4 ± 2.1	5.2 ± 2.0	4.0 ± 2.3	6.0 ± 1.9	5.5 ± 2.0	4.7 ± 2.6	6.8 ± 0.9	3.2 ± 1.9	4.4 ± 2.8	5.3 ± 2.1
VAS at $t = 1$Y	3.0 ± 2.9	2.4 ± 2.8	3.4 ± 2.8	1.6 ± 2.3	3.0 ± 2.9	3.1 ± 2.9	2.2 ± 2.8	1.9 ± 2.5	2.6 ± 3.0	1.5 ± 2.5	4.3 ± 2.6	2.9 ± 3.2	1.6 ± 2.3	4.1 ± 3.2	2.4 ± 2.8
ΔVAS	−2.9 ± 2.6	−2.7 ± 2.7	−2.7 ± 2.8	−2.6 ± 2.7	−2.7 ± 2.7	−2.3 ± 2.6	−3.1 ± 2.8	−1.9 ± 2.7	−3.2 ± 2.6	−3.7 ± 2.4	−0.1 ± 1.4	−3.5 ± 2.9	−1.7 ± 2.1	−0.2 ± 1.3	−2.7 ± 2.7
Sex, Female/Male	18/35	12/27	6/6	6/8	12/25	9/19	9/14	5/16	7/15	7/17	3/7	7/15	5/16	3/15	7/9
Previous surgery y/n	25/28	21/18	4/8	7/7	18/19	14/14	11/12	11/10	13/9	11/13	10/0	12/10	12/9	8/10	12/4
Type of surgery, N															
Regenerative	11	8	3	3	8	9	2	5	3	3	3	4	4	3	3
Autograft	27	19	6	8	17	10	15	12	8	13	2	9	11	8	6
FKRI	15	12	3	3	12	9	6	4	11	8	5	9	6	7	7
Total	53	39	12	14	37	28	23	21	22	24	10	22	21	18	16

H = high, L = low. KOOS = knee injury and osteoarthritis outcome score (high score means better function). VAS = visual analog score (high score means more pain). AMADEUS = area measurement and depth & underlying structures score (high score means less severe defect).

molecular degradation and turnover, extracellular and structural support, and protein synthesis (see Figure 1 for the top-20). A comprehensive list of all high-confidence proteins is provided in the supplementary material (Table S1). Many proteins were identified that are directly related to cartilage homeostasis, such as aggrecan (ACAN), collagen type II, IX, XI, Fibronectin (FN1), cartilage intermediate layer protein (CILP), cartilage oligomeric matrix protein (COMP), matrix metalloproteinase 3&9 (MMP3&9) and chondroadherin (CHAD). The identification of many integrins (ITGA2&5, ITGB1&3) indicate activity of mesenchymal stem cells. The presence of oxidative-stress related proteins (e.g. superoxide dismutase (SOD1, SOD2), glutathione peroxidase (GPX1), peroxiredoxins (PRDX1, PRDX2) show the inflammatory state which can be expected in the affected joints.

3.2. Protein profiles associated to MRI assessment

First we assessed the relation between the physiological state of the joint at the time of surgery ($t = 0$) and the protein profiles of the IPFP. Therefore, we analyzed the proteins that were differently abundant between groups based on the three MRI assessments ($n = 51$), scored binary as high/low (Table S2). In relation to the severity of cartilage defect (AMADEUS score), 22 proteins were differentially abundant (Figure 2(a)). Upregulated proteins include matrix-remodeling protein matrix metalloproteinase-3 (MMP3) and actin-related protein 2/3 complex subunit 2 (ARPC2), which plays a role in osmolarity of cartilage [31]. Downregulated proteins included hemoglobin and proteins regulated by hematopoietic progenitor genes. In patients with higher synovitis scores, 23 proteins in the IPFP were found to have differential abundance. Higher synovitis score was related to downregulation of hemoglobin subunits, ACAN, apoptosis (MAP1S) and osteonectin (SPARC) (Figure 2(b)). Both in patients with synovitis and more severe cartilage defect, carbonic anhydrase II (CA2), a protein active in bicarbonate and pH regulation, was downregulated. For patients with high fibrosis, a total of 23 proteins were found to be differentially abundant (Figure 2(c)). Upregulated proteins included CILP2, proteins related to lipid metabolism and prostaglandin synthesis (MGST1), as well as oxidative stress regulator NAD(P)H:quinone oxidoreductase 1 (NQO1), which may play a role in OA development [32]. Proteins related to immune response (immunoglobulins, S100A9, CD5L) were found to be downregulated. Pathway analysis of the proteins differentially associated with any of the three MRI variables revealed that oxidative stress, immune, and extracellular matrix reorganization pathways were the most impacted (Figure 2(d)).

3.3. Protein profiles associated to patient reported outcome measures

Next, we analyzed the differences in protein abundance related to the PROMs (Table S3). Two (COL5A2, COL1A1) and 7 proteins (Figure 3(a)) were found to be differently abundant in the groups with worse KOOS and VAS, respectively, at the preoperative assessment ($n = 43$). Proteins related to biochemical breakdown and remodeling (MMP3, ACTA1, COL1A1, CILP2 and COL5A2) were the most affected. Eleven and 24 proteins were differently abundant in the groups that improved more than the MCID at follow-up ($n = 34$) in the KOOS (Figure 3(b)) and VAS (Figure 3(c)), and thus responded well to treatment. Those proteins included proteins related to extracellular matrix and connective tissue (PRG4, ACAN, CILP2, COL15A1), and markers related to lipid metabolism, oxidation and transport (CD36, CES1, PLA2G2A, MGST1, FABP3). PLA2G2A specifically is linked to OA-related metabolic pathways [19], and there are drug treatments targeting inhibition of this protein [33]. These observations were also reflected in the pathway analysis based on all proteins differentially expressed in any of the comparisons in relation to PROMs (Figure 3(d)), which highlighted connective tissue remodeling and lipid homeostasis, extracellular matrix and exosome signaling.

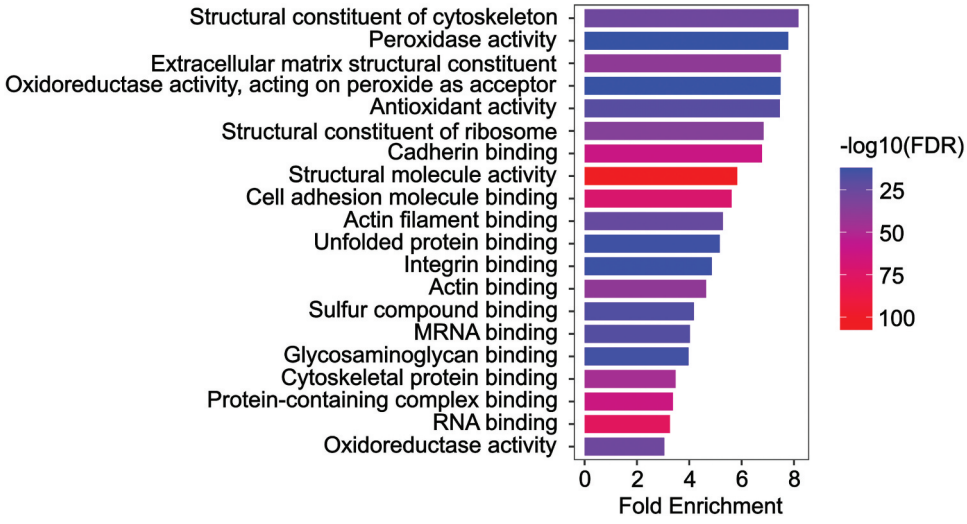

Figure 1. Visualization of functional enrichment analysis results based on 1209 identified proteins found in IPFP samples from 53 CD patients. The top 20 gene ontology (GO) molecular function terms with a fdr-value <0.001 and an enrichment factor ≥ 3 are represented as bars, with colors indicating the -log10(FDR) value.

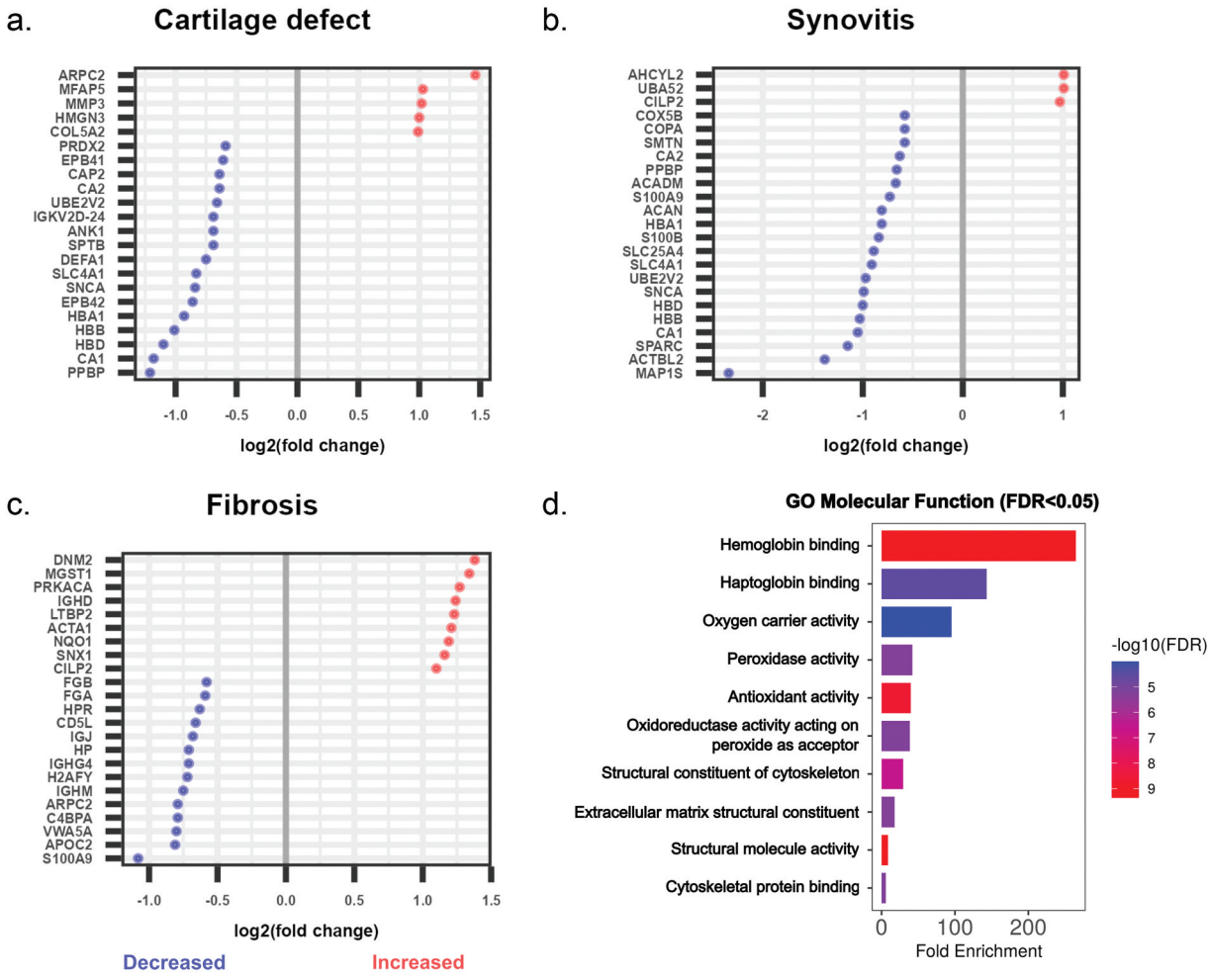

Figure 2. Protein regulation in CD patients associated with three MRI characteristics, including AMADEUS scores, synovitis and fibrosis. (a–c) plots of Log2(fold change) for each of the significantly (abundance ratio adjusted p-value <0.05) dysregulated proteins, based on a quantified level of high versus low cartilage defect severity (a), high versus low synovitis (c), high versus low fibrosis (E). d: functional enrichment analysis on GO molecular function with ShinyGO, shows the significantly altered molecular functions based on the comparisons in a-c. Bars show fold enrichment, with colors indicating the -log10(FDR) value.

3.4. Overlapping proteins

Finally, to assess which markers are related to multiple clinical variables, we summarized the proteins that were identified in multiple comparisons in Figure 4. Several extracellular matrix markers were identified in several comparisons, including MMP3 (pain and defect severity), ACAN (function response and synovitis), COL5A2 (function and defect severity), and CILP2 (pain, function response, fibrosis, and synovitis). Other markers are related to lipid metabolism, oxidation and transport (PLA2G2A (pain and function response), MGST1 (pain response and fibrosis), FABP3 (pain and function response), immune system (IFI30 (pain and function response), DEFA1 (pain response and defect severity), MGST1 (pain response and fibrosis) and cytoskeleton structure (ACTA1, pain and fibrosis). ACAN, CILP2, and MGST1 were the three markers that were related to both a preoperative (MRI) variable and to response (ACAN as positive predictor, CILP2 and MGST1 as positive predictor).

3.5. Effect of confounding factors

Due to an imbalance in sex distribution (18 females, 35 males), a comparative analysis was conducted to assess the effect of sex as potential confounding factor. Therefore, a Welch's t-test was performed to evaluate if outcomes were affected by sex differences. Table S4 shows that no statistical differences were observed between females and males. However, to exclude that sex differences at the molecular level were associated to protein modulation, a comparative analysis on the IPFP proteome was also performed (Table S5, Figure S1). Only Haptoglobin (HP), increased in females, was also correlated with the low-fibrosis group, which had a higher proportion of females (see Table 2). Other proteins such as Ubiquitin-60S ribosomal protein L40 (UBA52), Epoxide hydrolase 1 (EPHX1) and Cytochrome c (CYCS) were detected with higher abundance in females. These proteins were correlated with the low-synovitis (UBA52) and the poor pain recovery group (EPHX1,CYCS). Given that the number of females was decreased in those groups (see Table 2), the observed changes in these proteins are likely less influenced by sex.

4. Discussion

This is the first study to characterize the proteome of the IPFP and its relation to clinical parameters during cartilage repair.

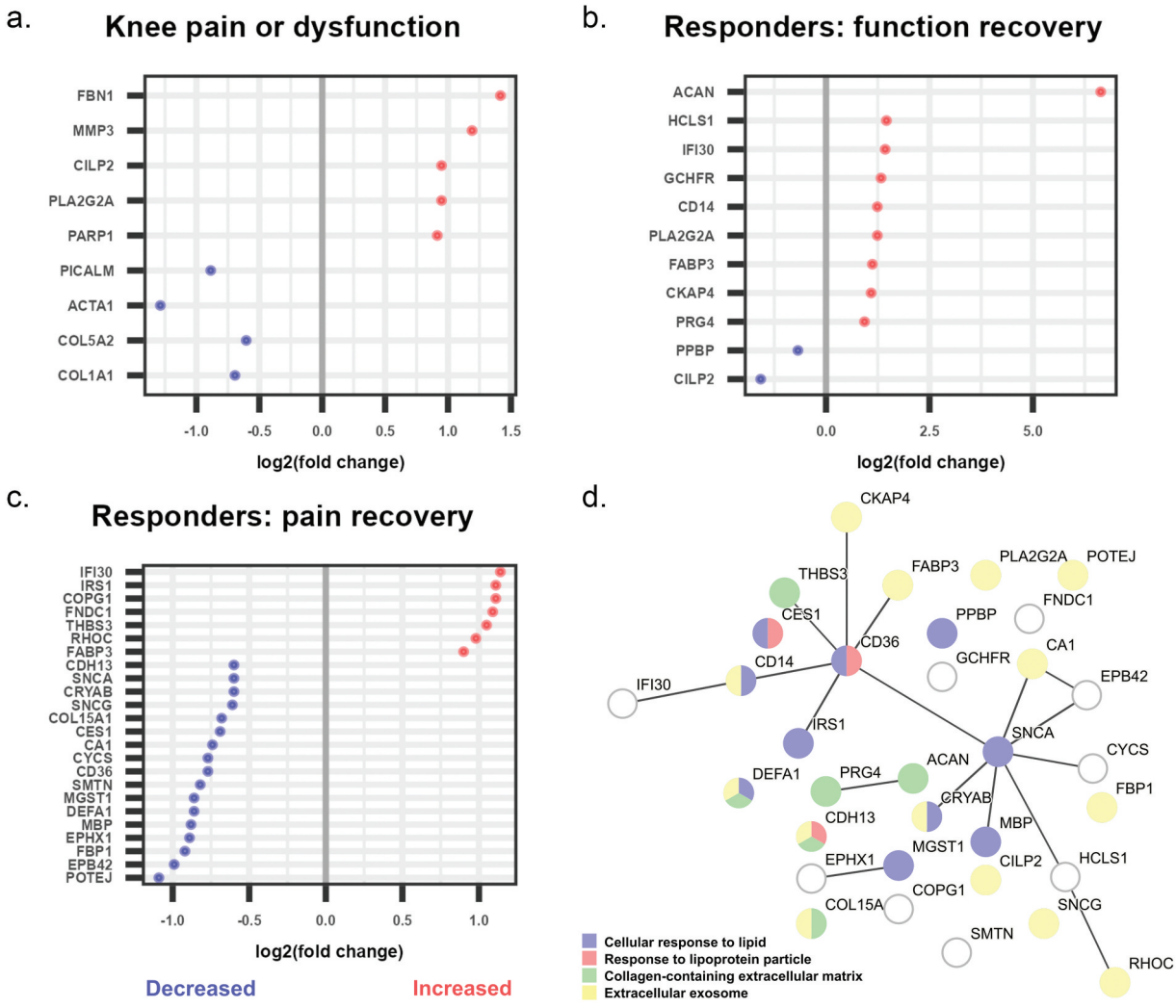

Figure 3. Protein regulation in CD patients associated with knee pain (KOOS) and function (VAS) scores. (a–c) Plots of Log2(fold change) for each of the significantly (abundance ratio adjusted p-value <0.05) dysregulated proteins, based on a quantified level of high versus low pre-operative VAS, or low versus high pre-operative KOOS score (a, COL5A2, COL1A1 found as markers for KOOS, the rest as markers for VAS), surpassing the MCID of the KOOS (b) and the VAS (c). (d) Functional enrichment analysis with STRING database, showing significant protein-protein interactions in IPFP based on the comparisons in (a–c).

The differentially abundant proteins suggest an active interaction between cartilage repair and the IPFP.

Proteins that were found to be differentially abundant in the IPFP were related to the function of cartilage and its formation, such as ACAN, COL5A2, CILP2 and MMP3, including proteins that are classically associated to articular cartilage (PRG4, ACAN, COL12A1) under physiological conditions and those that regulate cartilage production by upregulating production of matrix metalloproteinases (MMP3). ACAN is one of the essential building blocks of healthy cartilage [34], as the protein attracts and binds the water that gives the cartilage its unique mechanical properties, and was found in both functional response to treatment and the synovitis score. Stimulation of ACAN production is therefore a key factor in the cartilage repair process [35]. While ACAN was positively related to treatment outcome, and downregulated with synovitis, the opposite was observed for cartilage inter-layer protein-2 (CILP-2), which has been frequently identified as serum marker for OA progression [36,37]. Metalloproteinases are involved in breakdown of matrix, especially the collagen backbone that is essential for the mechanical stability of the matrix [38]. They are seen as a key driver of cartilage breakdown, leading to irreversible damage [39]. Furthermore, they play key roles in the inflammation response [39]. In another smaller study ($N = 17$), the single non-responder in a cartilage repair study showed also high expression of MMP3 [40].

A second group of proteins that was found to have a different abundance in clinical subgroups are related to bicarbonate metabolism and transport. The most important proteins in this subgroup are the carbonic anhydrase isoforms (CA1, CA2), the bicarbonate transporter AHCYL2, NQO1 and the hemoglobin subunits. Two possible explanations exist for the changed abundance of these proteins: firstly, they can contribute to the acidosis observed in diseased joints; or secondly, increased angiogenesis in the IPFP combined with a poor clearance of blood components increases the abundance of these proteins in the tissue. Previous studies have highlighted the role of acidic environments in the pathophysiology of OA, suggesting that this factor is responsible for worsening symptoms and increased pain scores [41]. Also,

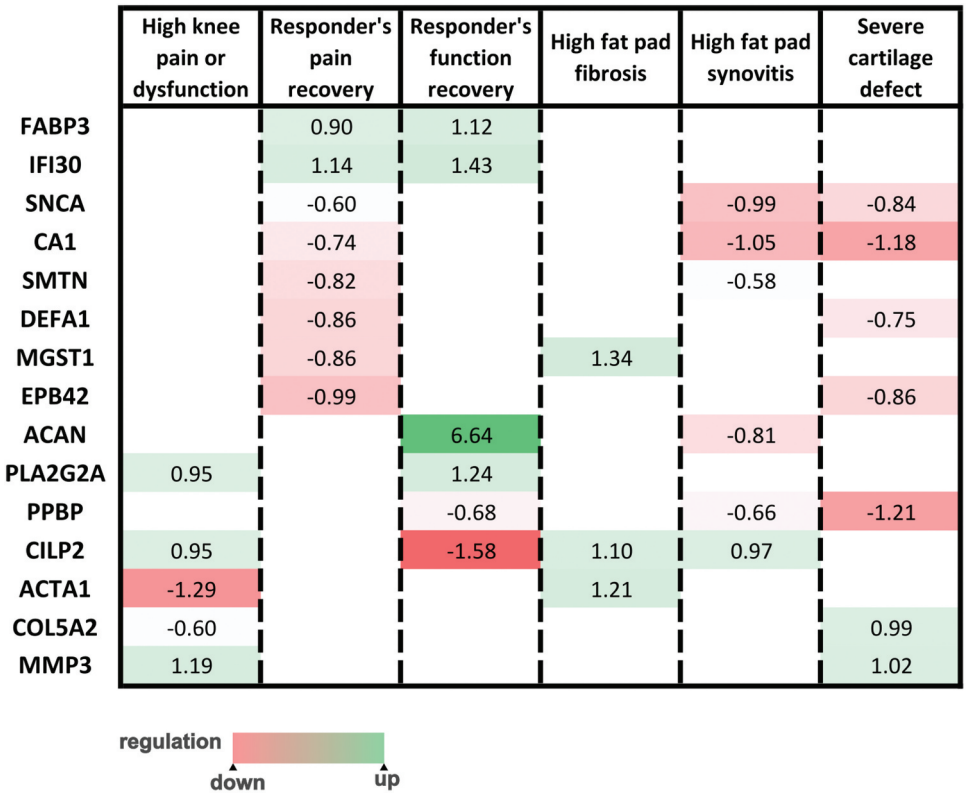

Figure 4. Overlapping protein dysregulation across different clinical status and treatment response groups. The value represents log2 fold change. Green indicates upregulated differences, red indicates downregulation.

oxidative stress of chondrocytes is related to OA formation [32]. In support of the second possibility, it is well recognized that the IPFP of OA patients is more highly vascularized than that of healthy controls, and that clearance of hemoglobin is severely reduced in these patients [42,43]. One last group of proteins includes proteins with inflammatory and immunomodulatory effects. This diverse group of proteins includes S100A9, HP, MBP and EPHX1.

Interesting to note, three markers were related to both the pre-operative status of the patient (MRI features) and to the clinical recovery: ACAN, CILP2 and MGST1. While ACAN and CILP2 are well-established OA markers [34–37], this is not the case for MGST1. However, MGST1 has a crucial role in prostaglandin E_2 synthesis [44,45], which is one of the key mediators of the joint homeostasis and the inflammatory response [46]. Prostaglandin release in the IPFP has been shown to play an important role in the development of OA [47,48]. This process can be mediated using cyclooxygenase-2 inhibitors [18].

To the best of our knowledge, our investigation is the largest proteomic study performed on a cohort of cartilage defect patients [9]. Recently, clinical associations were found by multiple groups between IPFP features on MRI and status or progression of OA. T2 signal intensity of the IPFP was associated with OA score and cartilage injury in an OA cohort [49]. Furthermore, T2 signal intensity of the IPFP was positively correlated with several urine and serum biochemical markers related to cartilage health [50]. Signal intensity alterations were also significantly different in patients who progressed in OA severity over 48 months, compared to stable patients [51]. Together with the results from this study, the role of the IPFP in diagnosis of degenerative diseases in the knee is rapidly emerging. Recent research suggests that the IPFP plays an active role in the progression of OA and other degenerative joint disorders [52]. The protein profiles found in this study give more evidence to this hypothesis. Proteins that classically are labeled as cartilage- and inflammation-related were found differently abundant in the IPFP, related to clinical state and process, showing the strong involvement of the fat pad. This suggests cross-talk between the cartilage and the fat pad, as has been hypothesized before due to the strong involvement in the OA process [53]. Fortunately, the IPFP is a promising site for the identification of biomarkers as obtaining tissue biopsies from it is less invasive than from cartilage. Of note, the IPFP has garnered interest in the past years as a source of mesenchymal stem cells (MSCs), and early preclinical studies have shown promising results for their use in cartilage restoration [54,55]. That we found MSC-related proteins such as cadherins (CDH13) and integrins.

The majority of evidence for the involvement of proteins in cartilage pathophysiology comes from studies in OA patients. Most biomarker studies in the OA field have studied late-stage OA patients, as the total knee replacement is a convenient moment of sample collection [7]. However, the early-stage CD patients is of high clinical relevance as there are still clinical treatment decisions to be made, and the clinical outcome is unpredictable [56]. Thus, CD patients are a very interesting population to study the disease progression and possible post-traumatic OA development. Our findings suggest that the IPFP could play a role in promoting a pathogenic environment upon CD injury, priming it for development into OA. However, there

is a high degree of heterogeneity among CD patients, for example, in the type of surgery that they received, and previous surgery. This heterogeneity is the limitation of the current study: Our results represent a wide range of patients and are not specific for the outcome of one particular treatment. The markers presented give therefore an overview of the important proteins in the general cartilage damage and repair processes, but cannot be used yet as diagnostic or prognostic markers for individual patients. In addition, most of the clinical variables are linked in one way or the other, which makes the proteomic comparisons not independent. For example, previous surgery is a known negative prognostic factor for clinical outcome [4,57], but may also induce fibrosis in the IPFP. We cannot conclude from our data whether these effects are causal or associated. How previous surgery and potential damage/fibrosis of the IPFP relates to clinical outcome and joint homeostasis is unknown and subject to future research. The presence of these confounders is the major limitation of this study. Confounders that were analyzed were sex, which showed HP as fibrosis marker to be possibly influenced by imbalance in sex ratio in the low and high fibrosis groups. Size of the defect, a known influencing variable of clinical outcome [4], was analyzed as part of the AMADEUS score. Furthermore, previous research using matrix-assisted laser desorption ionization mass spectrometry imaging (MALDI-MSI) showed different lipid profiles between IPFP areas [12]. Single-cell RNA-sequencing also identified 11 cell-types in the IPFP [58] A mixed protein profile of these cells can therefore be expected in the samples. The ultimate goal of our research is to equip surgeons with additional tools to support their daily decision-making. In clinical practice, using heterogeneous biopsies remains the most feasible approach, making the markers identified in this study especially relevant for real-world applications. As more data becomes available, finer subgrouping will be possible, enhancing diagnostic accuracy.

5. Conclusion

Overall, in the present study we showed that the IPFP proteome is substantially altered in different CD patient subgroups, demonstrating its potential for biomarker identification. Several pathways have been described herein, particularly that poor recovery after surgery is related to dysregulation in immunomodulation, inflammation and cartilage formation pathways. Affected pathways were related to cell interaction, oxidation and extracellular matrix maintenance and remodeling. ACAN, CILP-2 and MGST1 specifically were related to both pre-operative clinical status and response to treatment.

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Declaration of interest

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

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Ethical statements

The local Medical Ethics Committee confirmed that the use of waste material and questionnaires are not subjected to medical-ethical restrictions by national laws (ID: METC 2018–0963, 2018). Written consent for the use of waste material for research was obtained prior to surgery.

Author contributions

Conception and design: KE, MH, RH, PE, GK, BC
Collection and assembly of data: KE, LH, MH, FZ, JS
Analysis and interpretation of the data: KE, LH, MH, FZ, RH, BC
Drafting of the article: KE, LH, MH, JS
Critical revision of the article for important intellectual content: KE, LH, MH, JS, FZ, GK, RH, PE, BC
Provision of study materials or patients: PE
Final approval of the article: KE, LH, MH, JS, FZ, RH, GK, PE, BC

Data availability statement

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE [59] partner repository with the dataset identifier P×D057555 and 10.6019/PXD057555.

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