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Functional analysis of synovial fluid from osteoarthritic knee and carpometacarpal joints unravels different molecular profiles

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Abstract

Objective

In this work, we aimed to elucidate the molecular mechanisms driving primary osteoarthritis (OA). By studying the dynamics of protein expression in two different types of OA joints we searched for similarities and disparities to identify key molecular mechanisms driving OA.

Methods

For this purpose, human synovial fluid (SF) samples were obtained from first carpometacarpal (CMC-I) OA and knee joint of OA patients. SF samples were analyzed by label free quantitative mass spectrometry (LC-MS^E). Disease-relevant proteins identified in proteomics studies, such as clusterin (CLU), paraoxonase/arylesterase 1 (PON1) and transthyretin (TTR) were validated by enzyme-linked immunosorbent assays (ELISA), and on the mRNA level by droplet digital polymerase chain reaction (ddPCR). Functional studies were performed *in vitro* using primary chondrocytes.

Results

Differential proteomic changes were observed in the concentration of 40 proteins including CLU, PON1 and transthyretin TTR. Immunoassay analyses of CLU, PON1, TTR, and other inflammatory cytokines confirmed significant differences in protein concentration in SF of CMC-I and knee OA patients, with primarily lower protein expression levels in CMC-I. Functional studies on chondrocytes unequivocally demonstrated that stimulation with SF obtained from knee OA, in contrast to CMC-I OA joint, caused a significant upregulation in pro-inflammatory response, cell death and hypertrophy.

Conclusion

This study demonstrates that differential expression of molecular players in SF from different OA joints evokes diverse effects on primary chondrocytes. The pathomolecular mechanisms of OA may significantly differ in various joints, a finding which brings in a new dimension into the pathogenesis of primary OA.

Keywords

Synovial Fluid; Label free protein quantitation; ELISA; Inflammation; Chondrocytes; Osteoarthritis

Key messages

- Osteoarthritis molecular profile significantly differs amid CMC-I and knee joints,
- Knee osteoarthritis synovial fluid pro-inflammatory properties are reflected by an induced chondrocyte secretion profile and accompanying cell death.

Introduction

Despite decades of research, we still do not have effective, *disease modifying osteoarthritis drugs* (DMOADs). At the core of a problem lies our limited comprehension of regulatory mechanisms of OA development and progression. A limiting factor is the use of radiographic imaging as the main diagnostic tool and a method of following OA progression. Besides the technical limitations of radiography in assessing joints' pathologies, other important OA clinical manifestations such as cartilage structural alteration and inflammation cannot be detected with this technique [1]. On the other hand, targeted research in the OA field involved specific molecules and their physiological concentrations across synovial joints, blood and urine, which led to discovery of OA biomarkers. Molecules, such as cartilage oligomeric protein (COMP) and C-terminal telopeptides of type II collagen (CTX-II) are good examples of their potential applicability for diagnostics, and assessment of OA disease staging and progression [2].

The limitations of radiography and growing evidence of various molecular players in OA lead to an increase in number of proteomic investigations focusing on mechanisms of synovial joint degeneration, primary OA onset and progression [3, 4]. Biomarkers can reflect effector molecules (e.g. MMP13), by-products of cartilage degradation, or both e.g. fibronectin, which is released during cartilage degradation and acts as a stimulus of innate immune responses across synovial joint cells [5].

Several proteomics studies have compared synovial fluid composition (SF) obtained from OA patients and control groups, particularly from knee joints [6, 7]. However, OA pathogenesis might be inherently different between anatomic joints given the evidence from epidemiology, genetic, epigenetic studies and risk factors assessment [8, 9]. This led to the proposition that primary OA pathogenesis may vary depending on joint location. Similarly, in rheumatoid arthritis (RA) there is some preliminary evidence for the existence of joint specific pathogenic processes [10].

Proteomic characterization of molecular differences between the various joint types might bring valuable information about the molecular pathogenesis and disease patterns [11]. Proteomic analyses of SF likely reflect the processes undergoing in synovium and cartilage and thus offer insights into the molecular events of OA pathogenesis. In this work, the molecular composition of synovial fluid derived from first carpometacarpal (CMC-I) or knee OA joints was studied. With the help of quantitative proteomics, fortified by functional bioinformatics/network approaches, and validation by independent assays we aimed at characterizing similarities and disparities and to identify key molecular mechanisms driving primary OA across different anatomical joints.

Methods

Definitions of arthritic groups for synovial fluid collection

Patient´s recruitment, participation and sample collection were obtained after a signed informed consent, approved by the Helsinki and Uusimaa Hospital District ethics committee (Dnr_59/13/03/02/2013) and hospital board of directors (9310/407).

CMC-I osteoarthritis and advanced knee osteoarthritis sampling

SF samples were randomly collected from 12 patients (*N*=12) scheduled for trapeziectomy surgery. All patients had non-erosive OA of the CMC-I, confirmed by radiological features. Two CMC-I patients were excluded due to lack of sufficient synovial fluid amount to be analyzed.

SF samples were gathered from ten patients (*N*=10) scheduled for elective total knee replacement for management of primary idiopathic OA.

Patients with corticosteroid injection within 6 weeks, blood dyscrasias or active malignancy were excluded. The prior use of nonsteroidal anti-inflammatory drugs was not considered as an exclusion criterion. Patients' epidemiological data are presented in supplementary **Table S1**.

Synovial fluid sample collection

SF samples were collected at the time of surgery prior to surgical incision, by needle aspiration, in order to avoid contamination with blood (see supplementary Methods).

Immunodepletion

Hyaluronidase digested SF samples were processed to deplete the highly abundant proteins by ProteoPrep Blue Albumin Depletion Kit (Sigma-Aldrich), according to the manufacturer's instructions (see supplementary Methods).

Mass Spectrometry and bioinformatic data analysis

Depleted SF samples were grouped into two pools (named as *Pool 1* and *Pool 2*) encompassing individual patients' samples collected from CMC-I and knee OA (N=5 SF samples in each group respectively; and amounting to N=20 in total; see supplementary **Table S1**). Following pooling, SF samples were trypsin digested overnight at 37 °C with constant shaking. Three-hundred nanograms of protein digest/run (three technical replicates per pooled sample) were used for randomized LC-MS^E analysis. The peptides were separated with the nanoAcquity UPLC system (Waters) equipped with a 5-µm Symmetry C18 trapping column, 180 µm x 20 mm, reverse-phase (Waters), followed by an analytical 1.7-µm, 75 µm x 250 mm BEH-130 C18 reversed-phase column (Waters), in a single-pump trapping mode. The parameters of the

LC-MS^E runs were described previously [12]. Relative quantification between samples using precursor ion intensities was performed with Progenesis QI for Proteomics software (Nonlinear Dynamics/Waters) and ProteinLynx Global Server (PLGS v3.0) as described [12]. Database searches were carried out against UniProtKB/Swiss-Prot reviewed human (release 2017_6, 48614 entries) with Ion Accounting algorithm and using the following parameters: peptide and fragment tolerance: automatic, maximum protein mass: 500 kDa, minimum fragment ions matches per protein ≥ 7, minimum fragment ions matches per peptide ≥ 3, minimum unique peptide matches per protein ≥ 2, primary digest reagent: trypsin, missed cleavages allowed: 2, fixed modification: carbamidomethylation C, variable modifications: deamidation (N, Q), oxidation of Methionine (M) and false discovery rate (FDR) < 1%. For differential expression analysis, the list was limited to those quantified with fold change, FC > 1.3 and $p \leq 0.05$ by ANOVA for all comparisons. The list of up/down regulated protein changes with their corresponding unique UniProtKB/Swiss-Prot identifiers served as inputs into bioinformatics and other functional analyses.

Bioinformatics analysis

Differentially expressed proteins were functionally linked to canonical pathways, associated diseases and functions, and upstream network regulators using Ingenuity Pathways Analysis software (IPA, Ingenuity systems, Redwood City, CA; www.ingenuity.com). The Venn diagram was drawn using Venn calculating software available at: http://bioinformatics.psb.ugent.be/cgi-bin/liste/Venn/calculate_venn.htpl.

Tissue acquisition and primary chondrocyte culture

Patient recruitment, participation and sample collection were approved by the Helsinki and Uusimaa Hospital District ethics committee (Dnr_59/13/03/02/2013) and hospital board of directors (9310/407; see supplementary Methods).

Cartilage tissue extracted exclusively from TKA was used for chondrocyte isolation and primary chondrocyte stimulation studies. Cartilage was sequentially digested in a mixture containing 2.5 mg/ml of pronase and 250 mg/ml collagenase P (Roche, Basel, Switzerland); with PBS wash in-between, for 60 minutes and overnight, respectively under slow agitation at +37 °C. The resulting cell suspension was filtered through a 70-μm nylon cell strainer, centrifuged, washed twice with PBS and seeded at $1.5 \times 105/\text{cm}^2$ in Gibco DMEM/F-12 supplemented with 10 % FCS, 100 U/ml penicillin, 100 U/ml streptomycin and 0.25 μg/ml amphotericin B (Life Technologies). TKA primary chondrocytes cultures from passage 1 were stimulated for 24 hrs with 10% SF from CMC-I and knee OA joints. Cells were balanced for 3–4 days before stimulation using FBS-free cell culture medium.

Droplet digital PCR analysis

Total RNA was isolated from cartilage tissue derived from knee OA and CMC-I OA, and from primary chondrocytes using the RNeasy® Mini Kit (Qiagen, Valencia, CA, USA). One hundred nanograms of the total RNA was used for the cDNA synthesis using iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA). Droplet digital PCR was performed using a Bio-Rad QX200 Droplet Digital PCR system (Bio-Rad), according to manufacturer instructions (see supplementary Methods).

Immunoassays

Protein measurement using Enzyme-linked immunosorbent assay (ELISA)

The concentrations of C-C motif chemokine receptor 6 (CCR6/short name CCR-6), clusterin (CLU), serum paraoxonase/arylesterase 1 (PON1), and transthyretin (TTR) were quantitatively assessed by sandwich enzyme-linked immunosorbent assay (ELISA; USCN Life Science Inc., Hubei, China; see supplementary Methods).

Protein measurements using Luminex xMAP® technology

Determination of protein levels of matrix metalloproteinases, cytokines and chemokines in primary chondrocyte culture supernatants was performed with xMAP® technology (Luminex, Austin, TX, USA) on Bio-Plex 200® (Bio-Rad Laboratories, Hercules, CA, USA; see supplementary Methods).

Protein measurements using Q-plex™ Array Multiplex ELISA technology

To compare the expression levels of cytokines associated to SF OA, several cytokines were measured using the Q-Plex™ Human Cytokine High-Sensitivity Array (Quansys Biosciences, San Diego, CA, USA; see supplementary Methods).

Cell viability

To qualitatively confirm the immediate effect of SF OA treatment on chondrocyte viability and morphology, positive and negative controls (IL1β/IL-1β and 20% EtOH) were used, respectively (see supplementary Methods).

Statistical analysis

The results obtained in ELISA, ddPCR and LUMINEX experiments were evaluated using the non-parametric Mann-Whitney U and Kruskal-Wallis tests. Statistical analyses were performed utilizing GraphPad Prism version 6 software (GraphPad Software). *P* values ≤ 0.05 were considered as significant.

Results

Identification of proteins from synovial fluid of CMC-I and knee OA joints

A workflow illustrating steps employed in the study design and the study outline is shown in supplementary **Fig. S1**.

Mass spectrometry analysis from group pooled SF samples resulted in the identification and quantitation of 2719 peptides corresponding to 122 proteins. The complete list of proteins and peptides with their relative abundance identified from SF of CMC-I and knee OA (both by LC-MS^E and quantitative ELISA/LUMINEX experiments) is summarized in the supplementary **Table S2A-B**. Out of the 40 proteins characterized by differential expression by LC-MS^E, 28 proteins were upregulated and 12 downregulated in knee OA SF (with fold change, FC > 1.3, ≥2 unique peptides used for quantitation; named as differentially expressed proteins, DEP) as compared to CMC-I OA SF (supplementary **Table S2C**). The comparative analysis of proteins identified in this study with those identified in other large-scale OA synovial fluid proteomic profiling surveys is given in the supplementary **Fig. S2**.

Following the identification of differentially expressed proteins we functionally annotated them by utilizing IPA software. It has been revealed that *LXR/FXR/RXR* and *Acute phase response signaling* pathways were highly upregulated in end-stage knee OA in comparison to CMC-I OA synovial fluid, as indicated by positive z-scores and logP values ~13 assigned to these processes, while *Complement system* was downregulated (**Fig. 1 A**). Moreover, *Clathrin mediated endocytosis*, *Glucocorticoid receptor* and *Atherosclerosis signaling* were predicted to be significantly statistically enriched in the end-stage knee OA. Importantly, several categories of cell activation and molecular transport (10 and 16 DEP assigned respectively; **Fig. 1 B** and supplementary **Table S3A**), including *Transport of steroids* and *Fatty acid* *metabolism* were identified. On the other hand, *Quantity and concentration of steroids* and *Cellular infiltration* categories (z score < -2) were downregulated at the end-stage knee OA in comparison to CMC-I OA (**Fig. 1 B** and supplementary **Table S3B**).

Following identification of significantly enriched categories and signaling pathways we performed network analysis, focusing on linking DEP with most significantly up or down regulated functions and canonical pathways. This way we connected 19 DEP with various functional categories encompassing *Inflammation*, *Transport* and *Lipid metabolism* and canonical pathways including *Acute phase response and Atherosclerosis signaling* interconnected to *IL12 signaling* (**Fig. 2**).

Previously identified OA proteins, namely CLU, PON1, and TTR were chosen to be further scrutinized in independent ELISA assays given their high differential change in LC-MS^E measurements on pooled SF patient samples (supplementary **Table S2**), known participation in OA molecular inflammatory events, and no available data on their SF protein expression in two studied joints. CLU (FC= 1.72, $p < 0.01$), PON1 (FC= 1.73, $p < 0.05$) and TTR (FC= 2.35, $p < 0.05$) were present in higher concentrations in SF from knee OA in comparison to that derived from CMC-I OA patients corroborating the LC-MS^E results (**Fig. 3 A**; N≥6 per studied group). To further validate our findings, the extensively studied molecules IL6, IL10 and IFNG with previously established roles in OA, together with a potential novel marker, CCR6 were measured in the SF from both joints [13]. Concentration of IL6 was higher ($p < 0.0001$; fold change, FC= 20.3) in SF from knee OA as compared to CMC-I OA patients, while IFNG showed a similar trend in expression, albeit not statistically significant (**Fig. 3 A**). The concentration of CCR6 was higher in SF obtained from CMC-I OA patients ($p < 0.01$, FC= 1.29; **Fig. 3 A**). Interestingly, the anti-inflammatory cytokine IL10 levels were similar in the SF from both CMC-I and knee OA joints (**Fig. 3 B**). In contrast to IL6 and IFNG, IL10 concentration correlated well with validated levels of PON1 ($r^2 = 0.79$, p= 0.02; **Fig. 3 B**). Since the significant differences at the protein level were observed, we further queried their respective absolute concentration numbers on the mRNA level in native cartilage from CMC-I and knee OA joints. Interestingly, absolute mRNA concentration levels of *PON1* and *CCR6*, with the exception of *TTR*, were fairly similar across cartilage from CMC-I and knee OA patients (low copy concentration levels, **Fig. 4**).

Given the observed pro-inflammatory nature of SF of both CMC-I and knee OA joints, we further studied the effect of SF obtained from these joints on chondrocyte metabolism. In primary chondrocytes, stimulation with SF obtained from OA knee joint significantly increased the absolute mRNA concentration levels of *MMP1*, *MMP3, MMP9, MMP12*, *MMP13*, *CTSK*, *IL8* and *CXCL5* relative to SF obtained from CMC-I OA or controls (**Fig. 5 A-B**). Moreover, the expression of *MMP3* inhibitor, *TIMP3*, was clearly more downregulated by stimulation with SF obtained from OA knee versus OA CMC-I (**Fig. 5 A-B**). Noteworthy, stimulation with SF obtained OA knee led to downregulation of two genes encoding major structural constituents of cartilage, i.e. collagen type II alpha 1 (*COL2A1*) and aggrecan (*ACAN*, **Fig. 5 B**). In cell culture supernatants, the levels of cytokines and chemokines were significantly more upregulated by stimulation with SF from OA knee as compared to stimulation by SF from OA CMC-I. The protein levels of MMP3, -7, -8, -9, and IL8 were also markedly different in cell culture supernatants of chondrocytes stimulated with OA SF obtained from knee joints, CMC-I or unstimulated controls (**Fig. 5 C**).

Beside the OA SF induction of inflammation, the OA SF is known to mediate cell death in chondrocytes [14]. Therefore, we studied if the induced inflammatory responses by OA SF knee and CMC-I disrupted chondrocyte homeostasis by measuring the rate of cell death. In line with literature and our functional annotation network analysis, OA SF from CMC-I and knee

OA caused an increase in cell death, which was more pronounced for a knee OA SF, to a degree similar as the positive control, IL1β (**Fig. 6 A-B**). Interestingly, although IL6 levels were increased in knee OA SF, we observed that IL6 neutralization did not affect the OA SF- induced cell death and hypertrophy rates (**Fig. 6 A-B**).

Discussion

Several studies have demonstrated the importance of synovial fluid for metabolism of knee OA joint tissues and on the function of resident cells [15]. In this study, we compared the molecular composition of SF obtained from two distinct primary OA-affected joints and the stimulatory effect of synovial fluid obtained from these joints on human chondrocytes. Although known constituents of SF OA proteome were identified (supplementary **Fig. S2**) and [6, 16], our analyses also pinpointed several differences in the protein profiles of CMC-I and knee OA SF.

Comparative analysis of proteins in SF derived from CMC-I and knee joints of OA patients distinguished 43 differentially expressed proteins. Among these, 8 were upregulated in CMC-I OA joints whereas 35 were upregulated in OA knee (supplementary **Table S2**). Notably, the identified differences in SF proteomic profile support the findings from previous studies demonstrating dependency of the fluid composition on joint cartilage origin [17]. Functional categorization of differentially expressed proteins revealed that most enriched canonical pathways encompassed several categories of inflammatory response such as *acute phase response signaling, complement system* activation and *IL12 signaling and production in macrophages* among others (**Fig. 1 A**). Notably, inflammation and defense response are at the core of knee OA pathogenesis [18]. The upregulated glucocorticoid receptor category is also of significance, since intra-articular injections of glucocorticoids are effective in knee OA [19], but seem unresponsive in CMC-I OA [20]. The biological response is mediated through glucocorticoids receptors [21]. An upregulation of *cellular infiltration* category (**Fig. 1 B**) in case of hand OA, a classical feature of synovitis, is in striking agreement with synovitis incidence studies, in which a higher rate of hand synovitis (94%) was observed then in knee OA synovitis (46%) [22].

Functional network analysis revealed that differentially upregulated proteins relevant to OA, such as CLU, PON1 and TTR were associated with functional categories of *inflammation* and *lipid transport* (**Fig. 2**). TTR has previously been implicated in OA pathogenesis, inflammatory responses and in inflammatory diseases such as RA and gout [23, 24]. CLU and PON1 are known to possess anti-inflammatory properties. CLU can either act as an anti- or pro-inflammatory molecule, depending on its form [25].

The differential protein expression of TTR, CLU and PON1 detected in LC-MS^E experiments was further validated in quantitative ELISA assays. The additional criterion for validation was a reported dysregulation in OA joint tissues and SF, a putative role in OA, and lack of quantitative data [26-28]. Increased, age-related deposition of TTR on cartilage surface marks it as a potential pathogenic mediator in OA, and was recently shown to promote progression of OA, in an OA mouse model [23]. CCR6 and its ligand CCL20 are known to be upregulated in OA cartilage. CCL20 ligand activation of CCR6 leads to destruction of cartilage and an increased enzymatic activity in osteoblasts [29]. A decreased activity of PON1 has been observed in knee OA [30]. Furthermore, PON1 exerts anti-inflammatory properties, by inhibiting LPS-induced inflammation in macrophages and monocyte-to-macrophage differentiation, and is closely linked to several adipokines, all of which are implicated in OA pathogenesis [31, 32].

Our study demonstrated that the concentration of TTR, CLU, and PON1 was higher in SF derived from knee OA joint as compared to that obtained from the CMC-I joint. The upregulated levels of TTR in OA knee samples are in line with the previous study [28]. Upregulated CLU levels in SF of OA knee vs. SF of OA CMC-I joint were observed in the current work. Similar CLU levels in the SF and plasma samples, and in hip and knee OA patients have been reported, which suggests a difference in CLU levels among weight-bearing and non-weight bearing joints [33]. CLU has been detected by mass spectrometry and by immunohistochemistry in cartilage, with reported upregulation in OA cartilage [27, 33]. Similarly to CLU, the PON1 SF concentration levels in OA knee were significantly upregulated vs. OA CMC-I joints (range from 1.5-3 µg/ml; **Fig. 3 A**). CCR6, instead, was present in higher concentration in SF obtained from CMC-I OA patients. CCR6 and its ligand CCL20 are known to be upregulated in OA cartilage and expressed in numerous joint cells, but have not been reported so far as SF components [29].

Interestingly, pro-inflammatory markers IL6 and IFNG were upregulated in knee OA in contrast to anti-inflammatory cytokine IL10 similar levels in both joints. The measured IL10, IL6 and IFNG knee OA SF concentration ranges corroborated the results of previous studies [34]. To our knowledge, concentrations of IL10, IL6 and IFNG have not been reported in SF from OA CMC-I joints. Furthermore, IL6, IL10 and IFNG molecules are involved in similar molecular pathways as CCR6, TTR, CLU and PON1 [23, 29, 35, 36]. In this work, an inverse relationship encompassing anti-inflammatory molecule, IL10 with CLU and PON1 was observed.

The composition of arthritic SF drives chondrocytes to actively participate in the inflammatory processes [14]. An important conclusion from our study is that SF derived from two different end-stage OA joints evoked different pro-inflammatory responses in chondrocytes. Key catabolic mediators such as MMP3/MMP-3, MMP9/MMP-9 and IL8/IL-8 were upregulated while anabolic mediators COL2A1 and ACAN were downregulated at the gene and corresponding protein levels. These responses were in line with proteomics data, ELISA results and bioinformatics analyses prediction, confirming a higher pro-inflammatory profile of SF derived from knee joint with OA. Furthermore, an increased cell death and chondrocyte hypertrophy rates were observed when stimulated with OA SF, with a higher rate in chondrocytes stimulated with OA SF from knee and comparable to positive control, IL1β. Furthermore, IL6, IL10 and IFNG molecules are involved in similar molecular pathways as CCR6, TTR, CLU and PON1 [23, 29, 35, 36]. In this work, an inverse relationship encompassing anti-inflammatory molecule, IL10 with CLU and PON1 was observed.

Strength of the study

Few studies including the GOGO cohort (Genetics of Generalized Osteoarthritis cohort) have pinpointed specific differences in the systemic concentration of numerous OA- validated biomarkers in relation to anatomical location of the joint [37, 38]. In line with these studies, our proteomics and ELISA findings revealed relevant concentration differences for 44 proteins (40 proteins measured by quantitative proteomics including three validated by ELISA, and 4 proteins assessed in ELISA measurements) in SF from OA knee and CMC-I joints. Taken together, our work postulates to carefully evaluate the concentration threshold of measured biomarkers, taking into consideration the type of affected OA joint [11].

The composition of arthritic SF drives chondrocytes to actively participate in the inflammatory processes [14]. An important conclusion from our study is that SF derived from two different end-stage OA joints evoked different pro-inflammatory responses in chondrocytes. Key catabolic mediators such as MMP3/MMP-3, MMP9/MMP-9 and IL8/IL-8 were upregulated while anabolic mediators COL2A1 and ACAN were downregulated at the gene and corresponding protein levels. These responses were in line with proteomics data, ELISA results and bioinformatics analyses prediction, confirming a higher pro-inflammatory profile of SF derived from knee joint with OA. Furthermore, an increased cell death and chondrocyte hypertrophy rates were observed when stimulated with OA SF, with a higher rate in chondrocytes stimulated with OA SF from knee and comparable to positive control, IL1β. Moreover, IL6 neutralization (despite being a prominent player in OA pathogenesis) did not influence OA SF induced cell death and hypertrophy. Chondrocytes death and hypertrophy markedly undermine the cartilage homeostasis, ECM-degradation and contribute for OA progression [39].

Weakness of the study

Our study demonstrates the complexity of molecular regulatory mechanisms in various primary OA joints. Culprits for such differences could presumably be anatomical features, biomechanics, joint activity, synovial clearance kinetics, as well as genetic and epigenetic variabilities of articular joints [40-43]. Unfortunately, these were not addressed at this stage. Moreover, OA heterogeneity may be joint-specific, particularly the inflammatory subtypes.

Conclusion

In conclusion, this study contributes to further understanding of the heterogeneous nature of OA in the hand/CMC-I and knee. This can eventually lead to new treatment modalities for the complex disease.

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Disclosure statement

The authors have declared no conflicts of interest.

Supplementary data

Supplementary data are available at Rheumatology online.

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Legends

Fig. 1. Top Ingenuity Canonical pathways and disease and related functions annotation of differentially expressed proteins in synovial fluid from knee OA and CMC-I. **(A)** Top canonical pathways with the lowest predicted *P* values corrected using the multiple-testing Benjamin-Hochberg (B-H) procedure) are presented (knee OA/CMC-I ratio). A threshold of 1.3 (green line) represents a p value ≤ 0.05. **(B)** Disease and functional attributes analysis. Differentially expressed proteins were analyzed for their involvement in the disease and participation in cellular processes. The chart presents the statistically enriched associations sorted according to significant *z* values ($z > |2|$; knee OA/CMC-I ratio). Transport of molecules, transport of lipid and fatty acid metabolism represented the most enriched functional categories. For complete list of annotations see supplementary **Table S3**.

Fig. 2. Functional network analysis encompassing differentially expressed proteins in synovial fluid from CMC-I and knee osteoarthritic joints. Sixteen up-regulated ones, including clusterin (CLU) and paraoxonase/arylesterase 1 (PON1) and three differentially down-regulated proteins were functionally linked to *inflammatory* events of the joint, *fatty acid metabolism, transport of steroids* and *IL12 signaling* among others. CP- canonical pathways. Differentially expressed proteins with expression validated in independent assays, namely CLU, PON1 and TTR are indicated (thick red borders). The corresponding fold change ratios, *p* values (by ANOVA) and the number of unique peptides are indicated by each node.

Fig. 3. Quantitative ELISA measurements of protein concentration levels of differentially expressed proteins and pro- and anti-inflammatory molecules. **(A)** CLU, PON1 and TTR levels were statistically significantly higher in the knee OA, corroborating the LC-MS^E results. CCR6 level was lower in the knee OA vs. CMC-I OA, while that of inflammatory marker, IL6 was significantly higher. **(B)** IL10 cytokine levels were similar in CMC-I OA and knee OA, and inversely correlated with CLU and PON1 levels in CMC-I OA SF. *, $p < 0.05$, **, $p < 0.01$),

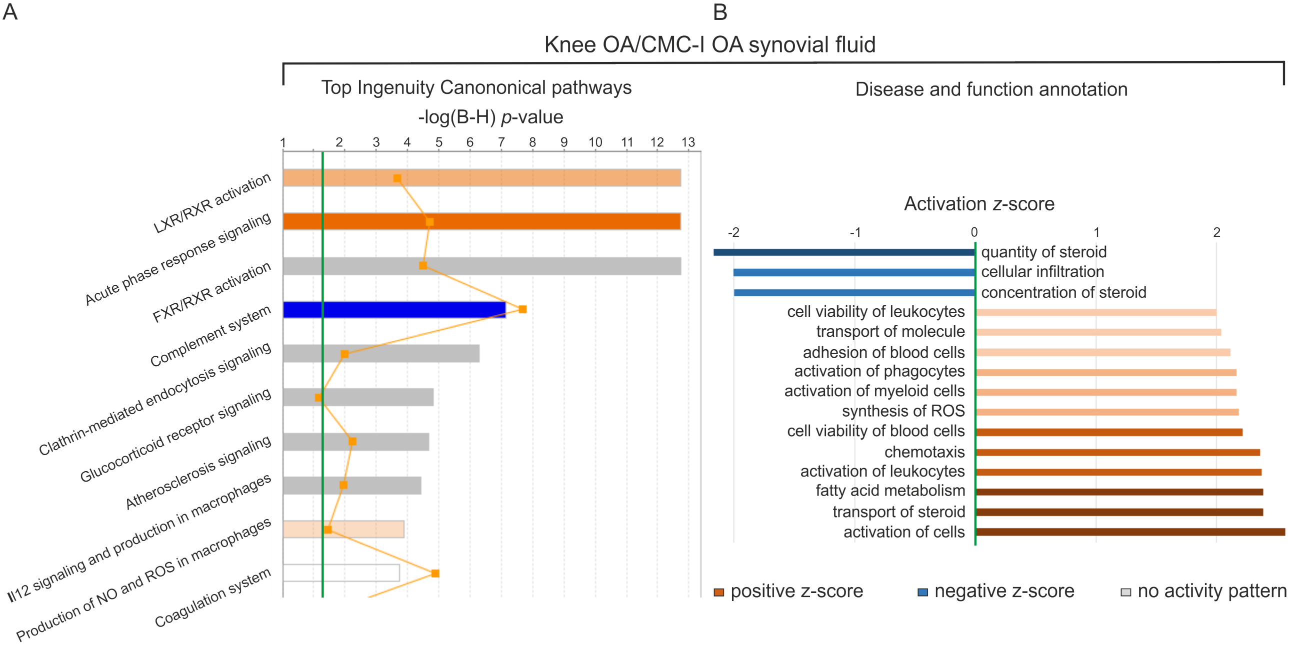
***, $p < 0.001$, **** $p < 0.0001$. Mean values (± the standard deviation [SD]) from at least six patient SF samples per studied group are presented.

Fig. 4. Absolute mRNA copy numbers of differentially expressed proteins in osteoarthritic cartilage from CMC-I and knee joints. **(A)** Dot plot showing fluorescent amplitude on y-axis, x-axis- number of events. Blue droplets were classified as positive by QuantaSoft manual threshold and black droplets as negative. **(B)** The quantitation of absolute mRNA copies concentration of *PON1*, *CCR6* and *TTR*. The error bars associated with each point represent the 95 % confidence interval, following Poisson distribution. Data was obtained from at least 4 patients per each OA joint group.

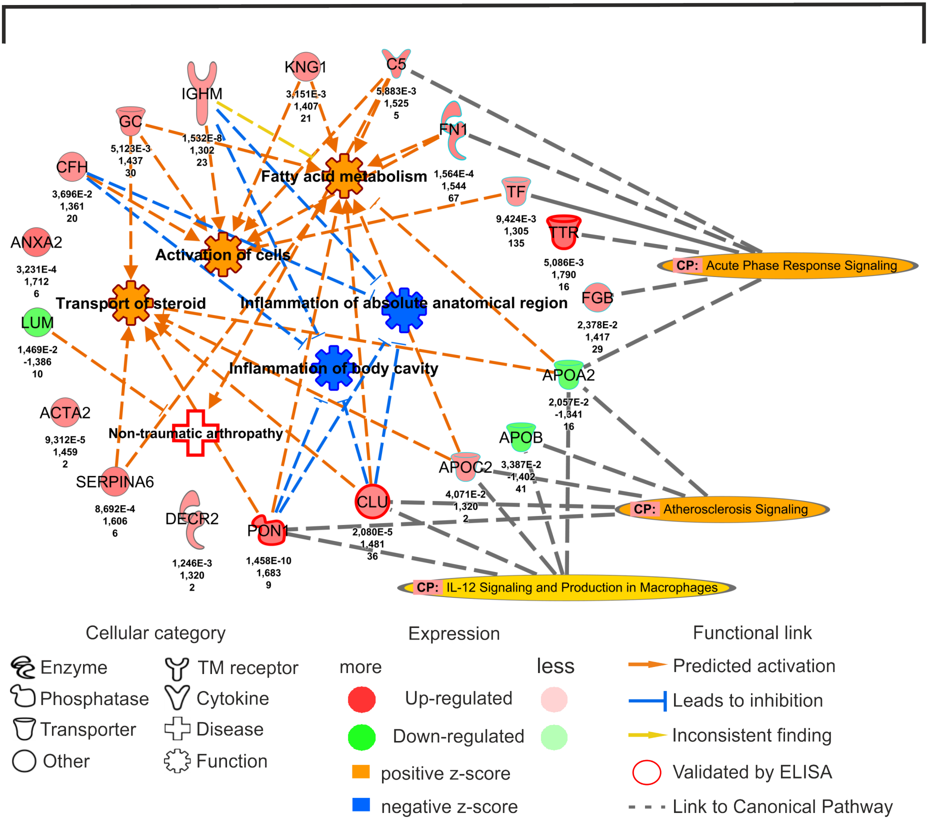
Fig. 5. Osteoarthritis synovial fluid affects catabolic and inflammatory factors in primary chondrocytes. **(A)** Dot plot of a fluorescent amplitude. Blue droplets- positive events, black droplets- negative events, as assessed by ddPCR. **(B)** Absolute mRNA copy numbers of *MMPs*, *TIMP3*, *CTSK*, *IL8*, *ACAN*, *COL2A1* and *CXCL5* genes. Error bars represent 95 % confidence interval. **(C)** Chondrocyte-mediated production of catabolic and inflammatory protein markers upon synovial fluid stimulation. The protein levels were quantified by Luminex xMAP® technology. Mean values (± the standard deviation) from 3 independent experiments (using SF samples from three different biological donors) are presented. Samples were measured in technical duplicates and averaged; *- $p < 0.05$, **- $p < 0.01$ and ***- $p < 0.001$ vs. non-stimulated controls using Kruskal-Wallis test.

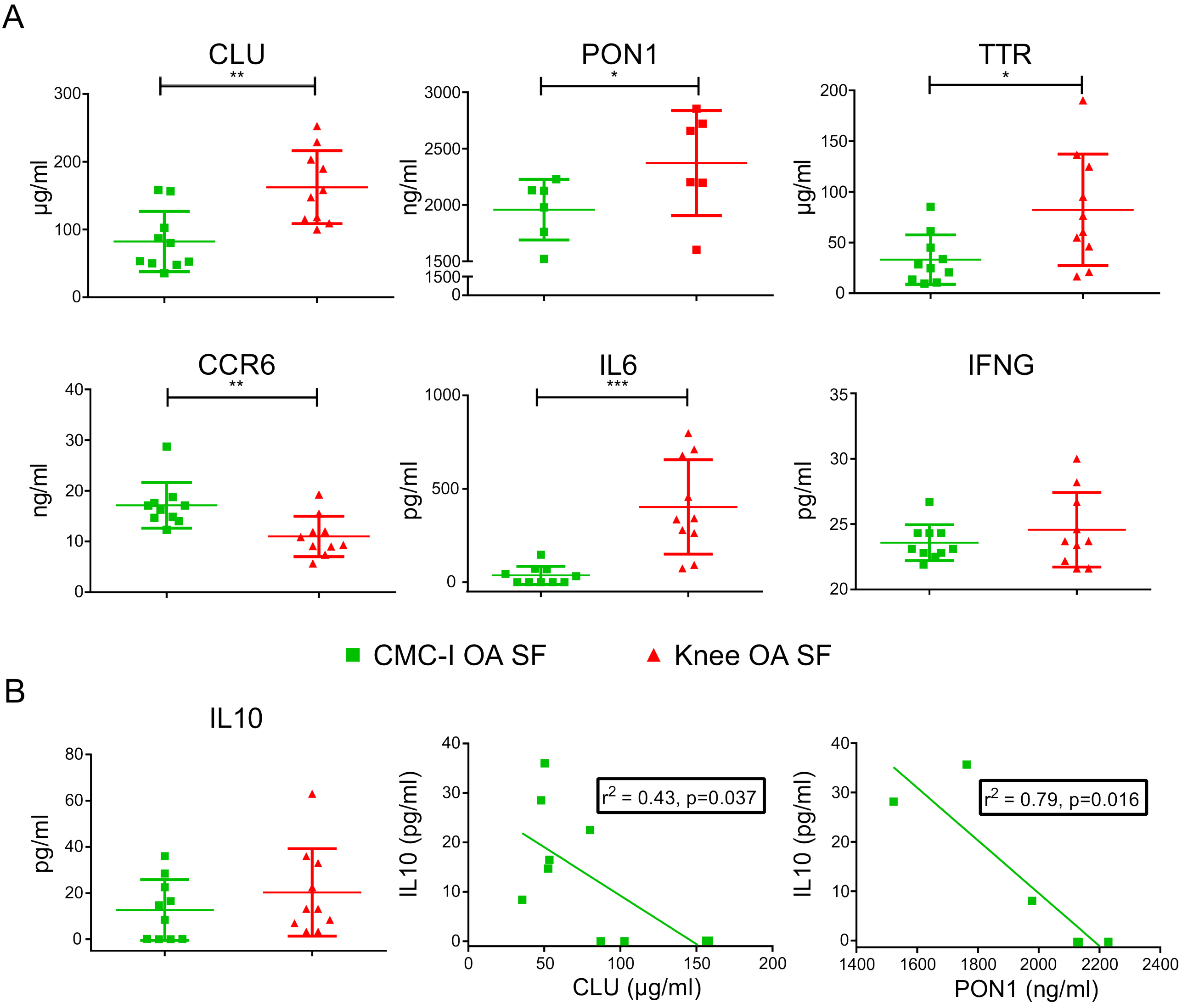
Fig. 6. Osteoarthritis synovial fluid effects on chondrocyte cell death and hypertrophy are IL6 independent. **(A)** Chondrocytes stimulated with SF from knee OA patients exhibited an increased cell death and hypertrophy, similar to IL1β stimulation alone. Addition of neutralizing IL6 antibody (+Nab IL6) did not influence cell viability. Arrows point to

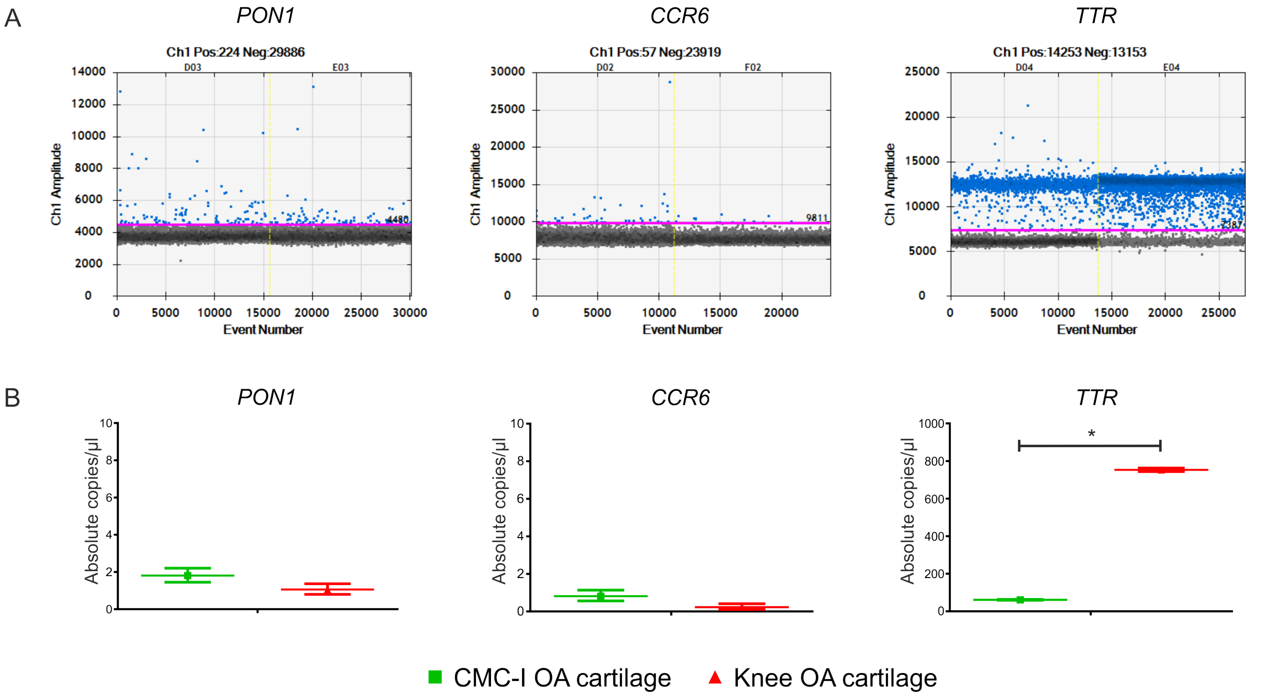
hypertrophic chondrocytes. Images are representative of 3 independent experiments. **(B)** A pronounced reduction of cell viability upon SF OA from knee patients was observed, independent of IL6 (+Nab IL6). Mean values (± the standard deviation) from 3 independent experiments (using SF samples from three different biological donors per joint control group) are presented.

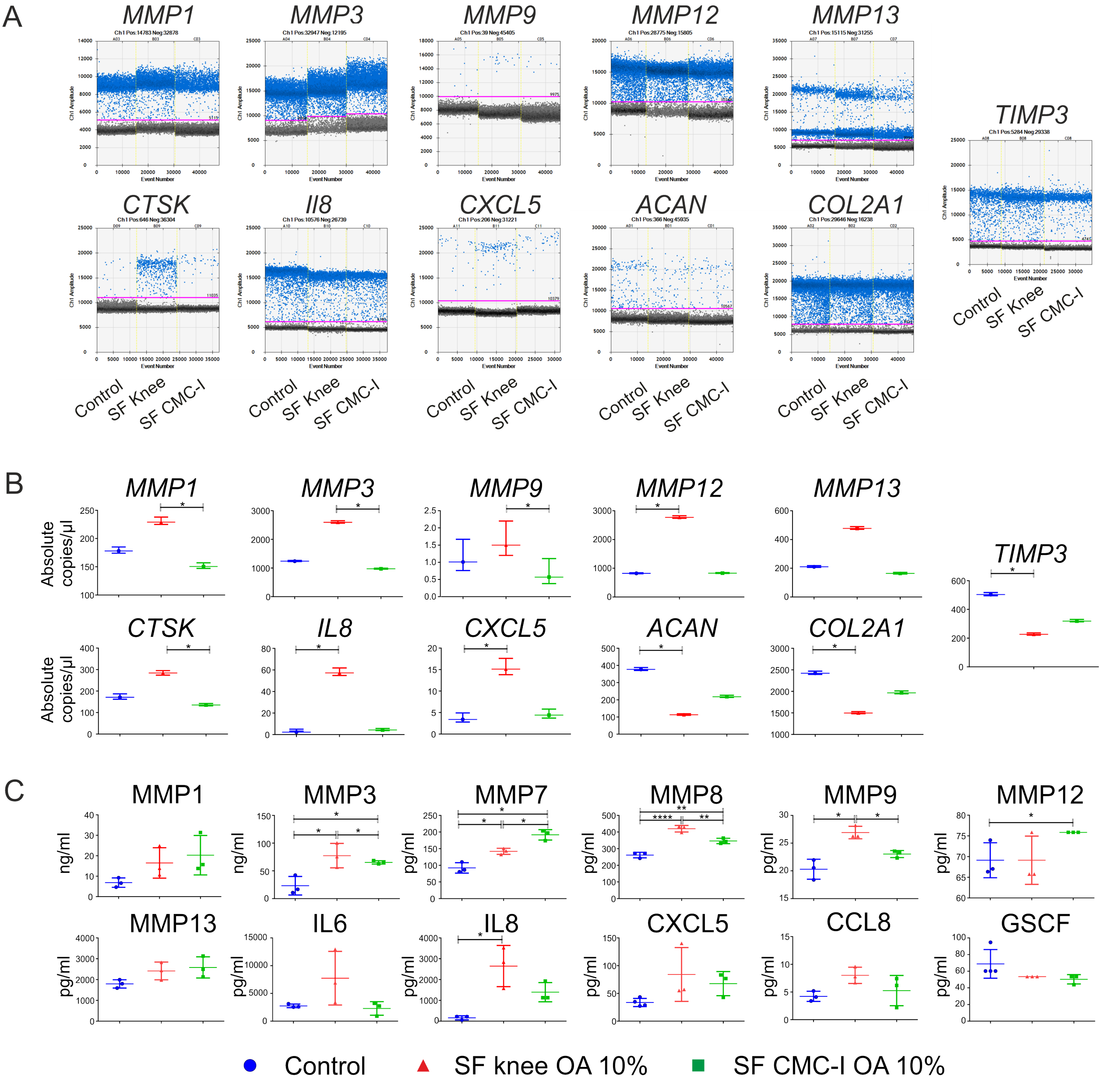

A
B
Knee OA/CMC-I OA synovial fluid
Top Ingenuity Canononical pathways
-log(B-H) p-value
1
2
3
4
5
6
7
8
9
10
11
12
13
LXR/RXR activation
Acute phase response signaling
FXR/RXR activation
Complement system
Clathrin-mediated endocytosis signaling
Glucocorticoid receptor signaling
Atherosclerosis signaling
Il12 signaling and production in macrophages
Production of NO and ROS in macrophages
Coagulation system
Disease and function annotation
Activation z-score
-2
-1
0
1
2
quantity of steroid
cellular infiltration
concentration of steroid
cell viability of leukocytes
transport of molecule
adhesion of blood cells
activation of phagocytes
activation of myeloid cells
synthesis of ROS
cell viability of blood cells
chemotaxis
activation of leukocytes
fatty acid metabolism
transport of steroid
activation of cells
positive z-score
negative z-score
no activity pattern

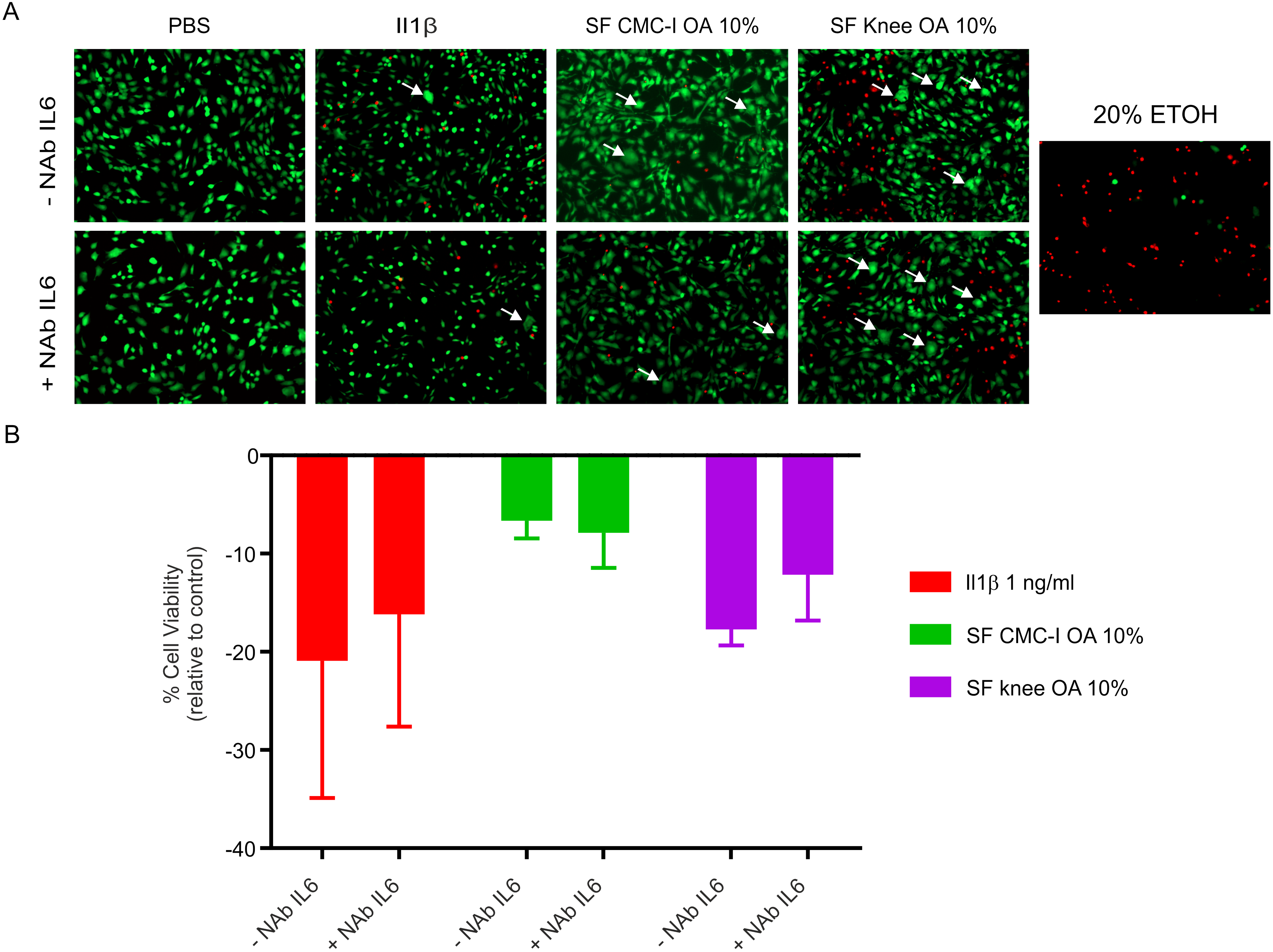
Knee OA/CMC-I OA synovial fluid

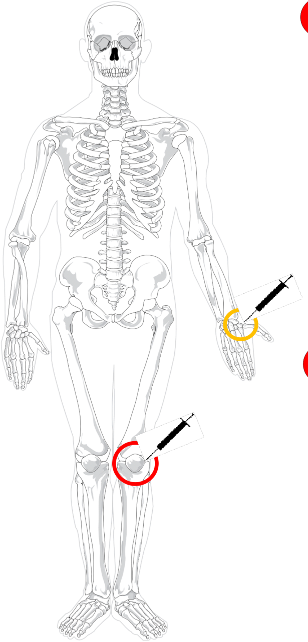



A
CLU
**
300
200
100
0
µg/ml
PON1
*
3000
2500
2000
1500
1500
0
ng/ml
TTR
*
200
150
100
50
0
µg/ml
CCR6
**
40
30
20
10
0
ng/ml
IL6
***
1000
500
0
pg/ml
IFNG
35
30
25
20
pg/ml
■ CMC-I OA SF
▲ Knee OA SF
B
IL10
80
60
40
20
0
pg/ml
40
30
20
10
0
IL10 (pg/ml)
r2 = 0.43, p=0.037
0
50
100
150
200
CLU (µg/ml)
40
30
20
10
0
IL10 (pg/ml)
r2 = 0.79, p=0.016
1400
1600
1800
2000
2200
2400
PON1 (ng/ml)

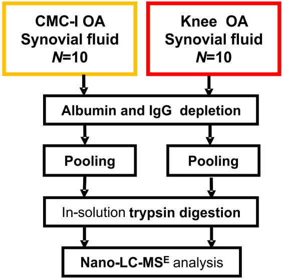

A
PON1
Ch1 Pos:224 Neg:29886
D03
E03
Ch1 Amplitude
Event Number
4480
CCR6
Ch1 Pos:57 Neg:23919
D02
F02
9811
TTR
Ch1 Pos:14253 Neg:13153
D04
E04
7387
B
PON1
CCR6
TTR
Absolute copies/µl
*
CMC-I OA cartilage
Knee OA cartilage


A
MMP1
Ch1 Pos:14783 Neg:32878
MMP3
Ch1 Pos:32947 Neg:12195
MMP9
Ch1 Pos:39 Neg:45405
MMP12
Ch1 Pos:28775 Neg:15805
MMP13
Ch1 Pos:15115 Neg:31255
TIMP3
Ch1 Pos:5284 Neg:29338
CTSK
Ch1 Pos:646 Neg:36304
Il8
Ch1 Pos:10576 Neg:26739
CXCL5
Ch1 Pos:206 Neg:31221
ACAN
Ch1 Pos:366 Neg:45935
COL2A1
Ch1 Pos:29646 Neg:16238
Ch1 Amplitude
Event Number
Control
SF Knee
SF CMC-I
B
MMP1
MMP3
MMP9
MMP12
MMP13
TIMP3
CTSK
IL8
CXCL5
ACAN
COL2A1
Absolute copies/µl
*
C
MMP1
MMP3
MMP7
MMP8
MMP9
MMP12
MMP13
IL6
IL8
CXCL5
CCL8
GSCF
ng/ml
pg/ml
*
**
****
● Control
▲ SF knee OA 10%
■ SF CMC-I OA 10%


A
PBS
Il1β
SF CMC-I OA 10%
SF Knee OA 10%
20% ETOH
- NAb IL6
+ NAb IL6
B
% Cell Viability
(relative to control)
0
-10
-20
-30
-40
- NAb IL6
+ NAb IL6
- NAb IL6
+ NAb IL6
- NAb IL6
+ NAb IL6
🟥 Il1β 1 ng/ml
🟩 SF CMC-I OA 10%
🟪 SF knee OA 10%



1 Proteomic profiling



CMC-I OA Synovial fluid *N*=10

Knee OA Synovial fluid *N*=10

Albumin and IgG depletion

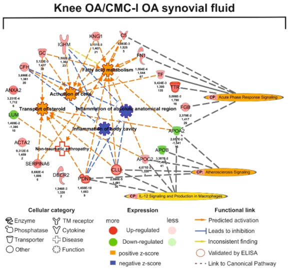
Pooling

Pooling

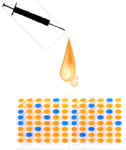
In-solution **trypsin digestion**

Nano-LC-MS^E analysis

2 Bioinformatic analysis



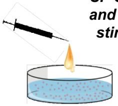
3 Protein expression validation



Sandwich and multiplex ELISA

4 Functional studies

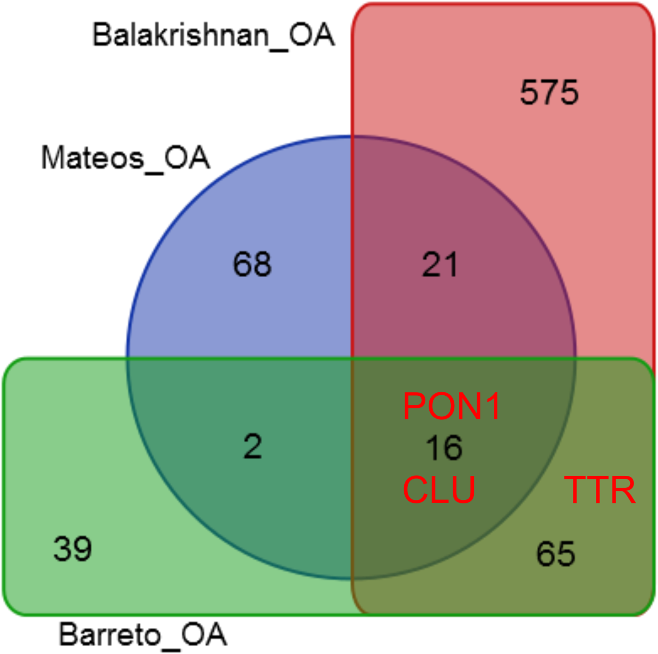
SF CMC-I OA and Knee OA stimulation



Primary chondrocytes

mRNA ddPCR

Protein Luminex xMAP technology


Balakrishnan_OA
575
Mateos_OA
68
21
PON1
2
16
CLU
TTR
39
65
Barreto_OA