Serum Inflammatory Markers in Patients with Knee Osteoarthritis

A Proteomic Approach

Giordano, Rocco; Petersen, Kristian Kjær; Andersen, Hjalte Holm; Simonsen, Ole; Arendt-Nielsen, Lars

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Serum inflammatory markers in patients with knee osteoarthritis: a proteomic approach.

Giordano R, M.Sc., [1] Petersen KK, Ph.D., [1, 2] Andersen HH, Ph.D. ,[2] Simonsen O, Ph.D., MD,[3]

Arendt-Nielsen L, Prof, dr. med. Sci., PhD [1, 2*]

[1] Center for Neuroplasticity and Pain (CNAP), SMI, Department of Health Science and Technology, Faculty of Medicine, Aalborg University, Aalborg, Denmark

[2] Center for Sensory-Motor Interaction (SMI), Department of Health Science and Technology, Faculty of Medicine, Aalborg University, Aalborg, Denmark

[3] Orthopedic Surgery Research Unit, Aalborg University Hospital, Aalborg, Denmark.

* Corresponding author: Center for Sensory-Motor Interaction (SMI), Department of Health Science and Technology, Faculty of Medicine, Aalborg University, Fredrik Bajers Vej 7D3, DK-9220 Aalborg, Denmark. Tel.: +45 99408831; fax: +45 9815 4008. E-mail address: lan@hst.aau.dk (L. Arendt-Nielsen).

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ABSTRACT

Objectives: Osteoarthritis (OA) is known to be a slowly progressive disease that alters all tissue compartments of the joint involved with a characteristic degradation of the cartilage, bone remodeling, and inflammation. One of the prominent symptoms in OA patients is pain, but a few radiological, inflammatory or structurally related biomarkers have shown little if any associations to pain. This study aimed to assess serum levels of 92 markers involved in inflammatory pathways in patients with knee OA (KOA) and evaluate their possible associations with the clinical pain intensity.

Methods: Serum samples were collected from 127 knee KOA patients and 39 healthy participants with no knee pain. Each serum sample was analyzed for 92 inflammatory markers using the Proximity Extension Array (PEA) technology. Clinical pain intensity was assessed using a visual analog scale, and patients completed the Knee Injury and Osteoarthritis Outcome Score (KOOS) questionnaire.

Results: Fifteen markers were significantly different when comparing KOA patient and healthy participants. Two markers, fibroblast growth factor-21 (FGF-21) and Eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1), correlated positively with pain intensity (R=0.235, P=0.008; R=0.233, P=0.008). Moreover, a linear regression model showed interleukin-6 (IL-6), macrophage colony-stimulating factor 1 (CSF-1), FGF-21 and tumor necrosis factor superfamily member 12 (TWEAK) as significant independent parameters for pain intensity.

Discussion: The associations between specific cytokines and KOA pain intensities provide new insights into the understanding of the underlying factors driving the pain in OA.

Keywords: knee osteoarthritis, inflammation biomarker, pain, proteomics.

1. INTRODUCTION

Osteoarthritis (OA) is a painful disease mostly affecting the elderly population, one of the leading causes of disability [1], and becoming more prevalent [2]. One of the prominent symptoms in OA patients is pain, and due to the lack of effective and safe therapies this represents a specific unmet medical need [3,4]. OA is known to be a slowly progressive disease that alters all tissue compartments of the joint involved with a characteristic degradation of the cartilage, bone remodeling, and inflammation response [5]. Previously OA has not been defined as a predominantly inflammatory disease [6] but more recent findings indicate that chronic inflammatory processes may be a driver in OA progression and pain [7].

A study has shown that the progression of cartilage disruption and the presence of a reactive inflammatory synovium present a link [8]. Kapoor et al. reviewed the action of secreted inflammatory factors, such as proinflammatory cytokines as a critical mediator of the altered metabolism, and augmented catabolism of the joint tissue in the pathogenesis of OA [9].

It has also been suggested that cytokines may act as biochemical markers of OA severity and pain intensity [10]. Cytokines which are a broad category of small proteins (~5–20 kDa) involved in cell signaling are also proteins that can act as key mediators in inflammatory processes, with numerous cytokines and related signaling pathways involved in the onset and pathogenesis of OA [11,12].

Preclinical data shows that proinflammatory cytokines sensitize the peripheral nerve endings, which may eventually contribute to changes in central pain processing [13,14], a manifestation seen in OA patients [15,16]. This indicates that the assessment of inflammatory mediators in circulation could be prognostic, and that pain biomarkers are a way to explore new therapeutic opportunities and pave the way for individualized management. A recent study found that a subgroup of OA patients are characterized by systemic low grade inflammation [17]. More specifically, Interleukin-6 has been

suggested to be up-regulated in patients with high synovitis scores [18], which is also associated with high-pain intensities [19].

To further investigate the influence of inflammatory markers in OA pain, the current study aimed to, 1) evaluate the serum level of inflammatory markers, using a new high throughput proteomic approach to look at the serum levels of 92 polypeptides involved in inflammatory processes in serum of patients with knee OA compared with healthy participants and, 2) evaluate the association between the pain intensity, the Knee Injury and Osteoarthritis Outcome Score (KOOS), and serum biomarker levels.

2. MATERIAL AND METHODS

2.1 Patients

One hundred and twenty-seven patients with KOA scheduled for total knee replacement (TKR) were recruited from the outpatient clinic at Hospital Vendsyssel, Frederikshavn, Denmark. Patients with other diagnosed pain conditions (e.g., hip OA, rheumatoid arthritis, fibromyalgia, and neuropathic pain) or mental impairment were excluded from the study. Radiological KOA was evaluated using the Kellgren and Lawrence (KL) score [20]. The patients were asked not to take any analgesic medication 24 hours before the study visit. The study was approved by The North Denmark Region Committee on Health Research Ethics (N-20120015) and conducted in accordance with the Helsinki Declaration. All patients read and signed an informed consent form prior to enrollment.

Thirty-nine healthy participants were recruited among family members of patients included in the database at CCBR (https://dk.ccbr.com/ccbr-aalborg) and the clinical trial unit C4Pain (www.C4Pain.com), Aalborg, Denmark. The control subjects had X-ray images taken of their knee and KL was evaluated. The exclusion criteria included painful OA, psychiatric conditions preventing the subject from participating in the study, pregnancy, previous/current drugs or alcohol

abuse, previous neurological or musculoskeletal disorders, lack of collaboration ability and subjects unable to abstain from analgesic medication for at least 24 h prior to the knee pain assessment (Demographic showed in Table 1).

2.2 Pain assessment

The peak pain intensities within the last 24 hours were collected (visual analogue scale [VAS]) from both patients and healthy participants. The participants were asked to rate their pain intensity on a scale from "0 -10" in which "0" represents "no pain" and "10" represents "worst pain imaginable".

2.3 Outcome measure

Patients completed the KOOS, a knee-specific questionnaire with five separately reported subscales of pain, other symptoms, function in daily living, function in sports/recreation, and knee-related quality of life [21]. The subscale scores represent the average of all items in the subscale standardized to a score from 0 to 100 with high scores representing less pain and symptoms and high function and quality of life. The KOOS-4 is calculated as the average of each of the four subscales for pain, symptoms, function in daily living, and quality of life and has previously been used in similar KOA studies [22].

2.4 Blood sampling and serum isolation

Venous blood was collected following standard venipuncture procedures between 07:30-09:00 in the morning. Nine ml of whole blood was withdrawn in an untreated tube. After collection the whole blood was left at room temperature for 15 minutes to allow coagulation to occur. The coagulated blood was removed centrifuging at 3000 RPM for 15 minutes and the serum obtained was transferred into a clean tube and stored at -80˚C.

2.5 Cytokines array detection

The relative levels of 92 inflammation-related proteins were analyzed using the inflammation panel (Olink Bioscience, Sweden) based on multiplex extension assay (PEA) [23,24]. Analyses were performed at BioXpedia A/S, Aarhus, Denmark. Proteins that have been assessed and their Uniprot identities are listed in [38]. The multiplex PEA technology allows assessment of protein using paired oligonucleotide-conjugated antibodies as probe for each protein. Each pair of probes recognizes a specific protein and only after the binding the oligonucleotides can hybridize allowing the amplification of the new DNA double strand. The product of the amplification (amplicon) is then detected and quantified using a highly sensitive technology; i.e., the microfluidic real-time PCR platform. This PCR platform cannot be used to report absolute quantification but only relative protein values, allowing comparison between groups of samples.

In brief, 1 µl of the serum sample was mixed with a 3 µl incubation mix containing 92 pairs of probes, each consisting of an antibody labeled with a unique corresponding DNA oligonucleotide. All samples were spiked with two incubation controls (green fluorescent protein and phycoerythrin), one extension control and one detection control. The mixture was incubated at 4°C overnight. Then, a 96 µL extension mix containing PEA enzymes and PCR reagents was added, and the samples incubated for 5 min at room temperature, before the plate was transferred to the thermal cycler for an initial DNA extension at 50°C for 20 min followed by 17 cycles of DNA amplification. A 96.96 Dynamic Array IFC (Fluidigm, South San Francisco, CA, USA) was prepared and primed according to the manufacturer's instructions. In a new plate, 2.8 µL of sample mixture was mixed with 7.2 µL detection mix from which 5 µL was loaded into the right side of the primed 96.96 Dynamic Array IFC. The unique primer pairs for each protein were loaded into the left side of the 96.96 Dynamic Array IFC, and the protein expression program was run in BioMarkTM HD Fluidigm real-time PCR (Fluidigm, South San Francisco, CA, USA) according to the instructions for Proseek. Results were presented in Normalized Protein Expression (NPX) in

log2. Using NPX Manager, the quantification cycle (Cq) values, generated in the real-time PCR, were normalized against extension and interplate controls and a correction factor. Subtracting the extension control from the Cq-value of every sample corrects for technical variation and subtracting the interplate control compensates for possible variation between runs. Further, the NPX was calculated by normalization against the calculation correction factor. The NPX was calculated in three steps from the Cq-values:

(I) $\Delta Cq_{sample} = Cq_{sample} - Cq_{extensioncontrol}$,

(II) $\Delta\Delta Cq = \Delta Cq_{sample} - \Delta Cq_{interplatecontrol}$,

(III) $NPX = Correction\ factor - \Delta\Delta Cq_{sample}$.

Validation data for all antibodies used in this panel and the performance of each assay are available at the manufacturer's webpage (www.olink.com). With this calculation, NPX values correspond to a relative quantification between samples and with a high NPX value corresponding to a high protein concentration. Limit of detection (LOD) was determined for each biomarker based on the mean value of triplicate negative controls analyzed in each run. Cut-off for LOD was set arbitrarily at 30% in order to avoid false positivity and negativity in the expression of the samples that were used for further statistical analyses.

2.6 Statistical analysis

Distribution of protein values was evaluated using the Shapiro-Wilk normality test. To test for differences in biomarker levels between patients with OA and healthy participants, paired independent-sample t-tests were performed on normally distributed markers, and the Mann-Whitney U signed rank tests were used on non-normally distributed markers. The data is shown in a volcano plot, combining p-values (y-axis) with the fold changes (x-axis), thus highlighting biomarkers with high or low levels of expression.

Correlations between markers' NPX and pain intensity were conducted using Pearson's correlation coefficients for the normally distributed data, while Spearman's correlations were used for the non-normally distributed data. All significant values were corrected for multiple comparisons by the False Discovery Rate (FDR) method with a P_{FDR}-value ≤ 0.008.

Linear regression models were constructed using two models to identify inflammatory markers predicting clinical pain in KOA patients. Model 1 included as predictors all the markers that were significant in the t-tests and Mann-Whitney U tests. In model 2, backwards selection was applied to model 1 to identify independent predictors for clinical pain.

The data analysis was performed in SPSS software V.25 (IBM, Armonk, New York, USA). $P<0.05$ was considered significant. All statistical significance was corrected using the Benjamini and Hochberg false discovery rate (FDR) [25], using GraphPad Prism version 8 for Windows 10 (GraphPad Software, La Jolla, CA, USA).

3. Results

3.1. Differences in serum levels of inflammatory cytokines between healthy participants and patients with osteoarthritis

Seventy-two markers were defined as valuable due to the LOD, representing more than 30% of expression in KOA patients and healthy participants (Supplementary figure 1, Supplemental Digital Content 1, http://links.lww.com/CJP/A626). Moreover, the brain-derived neutrophic factor (BDNF) was not evaluated due to the facility's internal problem. Levels of fifteen cytokines (Table 2) were found to be significantly different in the KOA patients' samples as compared with the healthy participants (Figure. 1).

3.2. Associations between Inflammatory makers and pain intensity in patients with KOA

Patient data showed a positive significant Pearson's correlation between clinical pain and FGF-21 (R=0.235, P=0.008, adjusted by FDR) and a positive Spearman's correlation was found between

pain and 4E-PB1 expression (R=0.233, P=0.008, adjusted by FDR). None of the other cytokines evaluated were associated with pain intensity or KOOS-4. Furthermore, linear regression models aiming to predict clinical pain were established to investigate the predictive value of inflammatory markers. Model 1 consists of all the significant variables in the t-tests and Mann-Whitney U tests, with predictive values (R^2) of 15.0% and identified IL6 (P=0.003; 95% CI: -0.7 to -0.15), DNER (P=0.026; 95% CI: 0.02 to 0.42), CSF-1 (P=0.016; 95% CI: 0.06 to 0.56), FGF-21 (P=0.008; 95% CI: 0.07 to 0.43), and TWEAK (P=0.033; 95% CI: -0.51 to -0.02) as significant factors (Table 3). Model 2 used backwards selection of the significant variables included in model 1 and identified IL6 (P<0.001; 95% CI: -0.62 to -0.19), DNER (P=0.05; 95% CI: -0.007 to 0.38), CSF-1(P=0.015; 95% CI: 0.051 to 0.48), FGF-21 (P=0.002; 95% CI: 0.095 to 0.43), and TWEAK (P=0.001; 95% CI: -0.56 – 0.15) as significantly independent parameters for clinical pain with a predictive value (R^2) of 16.0% (Table 3, model 2).

4. Discussion

This study is the first to evaluate a large panel of different inflammatory biomarkers in patients with KOA compared with healthy participants. When compared to healthy participants, patients with KOA were found to have ten cytokines with significantly lowered expression in the serum, and five cytokines with higher expression levels. Further, FGF-21 and 4E-PB1 were found to be significantly correlated to pain intensity in patients with KOA, whereas no correlations were found between marker expression and KOOS-4 in patients. The linear regression model showed IL6, CSF-1, FGF-21, and TWEAK as significant independent parameters for pain intensity.

Cytokines in osteoarthritis with low expression levels

CASP-8

Caspases are usually proteins related with cellular apoptosis, but studies have shown that protein of this family may also be involved in induction of inflammation [26]. Moreover, previous studies have

shown that in particular CASP-8 may drive T cell activation, cell motility, and tumor metastasis, and it has been demonstrated that the early action of CASP-8 is related to the activation of osteoblasts [27,28]. As found in the present study, the significant reduction of this protein in patients with KOA compared with healthy participants could indicate that the possible downregulated osteoblast activity may hamper bone formation.

EN-RAGE and DNER

Newly identified extracellular receptors for advanced glycation end-products (EN-RAGE or Protein S100-A12) and Delta and Notch-like epidermal growth factor related (DNER) are proteins abundantly expressed by immune cells including granulocytes, monocytes, macrophages as well as fibroblasts and specifically DNER is expressed in dendrites and cell bodies of CNS neurons [4]. They generally recruit or stimulate inflammatory mediators, or are part of a cellular network, which influences immunological significance. Furthermore, they are known to be expressed locally in synovial tissues [29]. Strong expression of human EN-RAGE in synovial inflammation is found in synovial fluid and serum in rheumatoid arthritis (RA) and other inflammatory disease [30]. This protein is expressed and secreted at local sites of inflammation in synovitis expressed by granulocytes starting inflammatory processes in the synovium, thus resulting in chronic arthritis [30]. Proteins of the same family have been found in the synovial fluid of OA patients, and the elevated levels of this protein may predict joint destruction [31]. In this study, we showed a significant reduction of these proteins in patients with KOA compared with healthy participants, which may further support the studies showing no association between the extent of the synovitis and the pain [32].

AXIN1

Another marker found to be dysregulated is axis inhibition protein (AXIN1). AXIN1 is a cytoplasmic protein expressed in the cartilaginous area of the axial and appendicular skeleton

during embryonic axis development, and is a negative regulator of the Wnt signaling pathway by downregulation of β-catenin [33,34]. A preclinical study has shown that a deletion of this protein in the chondrocyte of mice leads to OA-like degeneration [35]. In this study, a lower level of serum expression was found in serum of patients with KOA compared with healthy participants confirming that reduction of this marker can be related with OA's pathogenesis.

STAMPB

STAMBP is a deubiquitinase enzyme and a member of the Jab1/MPN metalloenzyme (JAMM) family of deubiquitinating enzymes (DUBs) [36]. STAMBP is involved in the signal transduction of JAK-STAT cascade binding to the SRC Homology 3 domain of the signal-transducing adaptor molecule STAM [37]. STAMBP plays a critical role in cytokine-mediated response and induces the signaling that can de-ubiquitinate early and late endosome cargo proteins from progressing to lysosome compartmentalization [38]. An *in vitro* study shows that the involvement of STAMBP in the regulation of inflammasomes which regulate innate immune responses by facilitating maturation of inflammatory cytokines [37,39]. In the cited study, the authors show how blocking STAMBP deubiquitinase activity decreases levels of specific inflammation cytokines like IL-1b [37]. In this study, the systemic levels of STAMBP were found to be decreased in patients with KOA compared with healthy participants.

SIRT2

Srtuin2 (SIRT2) is a member of the sirtuin family, which is a group of nicotinamide adenine dinucleotide-dependent deacetylases [40], mostly involved in the regulation of different processes like proliferation, metabolism, apoptosis, and inflammatory responses [41,42]. Studies have reported that SIRT2 is involved in the regulation of neurological diseases and neuroinflammation [43,44]. Moreover, SIRT2 inhibits inflammation through de-acetylation of the NF-κBp65 protein and inhibits NF-κB-dependent pro-inflammatory cytokine expression [45,46].

A preclinical study in an animal model of arthritis has confirmed SIRT2 action as an arthritis suppressor, showing that a deficiency of SIRT2 led to a severe arthritic phenotype [47]. A recent preclinical study revealed the important role of SIRT2 in regulating neuroinflammation and neuropathic pain development, highlighting that a high expression of SIRT2 alleviated neuropathic pain through the regulation of inflammatory cascade leading to an inhibition of NF-κB-mediated neuroinflammation [48].

In this study, lower expression levels of SIRT2 were found in serum from patients compared with controls confirming that the inhibition of this marker is not only related to the pro-inflammatory response in these patients but also to their painful condition.

SCF

The stem cell factor (SCF) is produced by cells such as fibroblasts and it plays an important role in the differentiation and proliferation of cells by acting in a paracrine manner [49]. Preclinical studies show SCF as a ligand for c-Kit which is a receptor tyrosine kinases expressed on a specific subpopulation of sensory neurons, and may relate to the action of SCF with pain regulation both peripherally and centrally [50,51]. SCF has been found to be downregulated in patients with KOA compared with healthy participants, which could confirm the involvement of this factor in the activation of the pathways involved in the regulation of pain present in these patients.

4E-BP1

The eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1) inhibits the cap-binding translation initiation factor eIF4E from interacting with other elongation factors [52]. This is a key regulatory process in translation involved in the regulation of cell physiology through the modulation of protein synthesis [52]. 4E-BP1 is the target of action of intracellular signaling, the serine–threonine kinase mammalian target of rapamycin (mTOR), which regulates cell proliferation, cell growth, and synaptic plasticity downstream of multiple stimuli such as glutamate,

growth factors, and cytokines [53,54]. 4E-BP1 is also expressed in neurons in the dorsal horn of the spinal cord, and in peripheral nerves [55,56]. Preclinical evidence shows that removal of 4E-BP1 leads to enhanced eIF4F complex formation in the spinal cord and to mechanical hypersensitivity and increased response to noxious chemical and inflammatory stimuli [57]. The present study shows that patients with KOA present significantly lower serum expressions of this protein, and found it positively correlated with pain intensity. This may confirm the connection between pain, inflammation process, and the reduction of this protein showed in the literature.

TWEAK

Tumor necrosis factor-like weak inducer of apoptosis (TWEAK) is a member of the TNF superfamily, which has effects including pro-inflammatory activities on epithelial and endothelial cells [58] as well as proliferation enhancing effects on endothelial cells and astrocytes [59,60]. A previous study demonstrated that TWEAK can induce the production of pro-inflammatory cytokines and chemokines by normal human dermal fibroblasts and synoviocytes obtained from RA and advanced osteoarthritis patient tissues [61]. In contrast to the pro-inflammatory functions of this protein, our results highlight a significant reduction in the sera expression in patients with KOA. Moreover, linear regression showed that TWEAK is an independent factor for pain intensity in KOA patients.

uPA

The urokinase plasminogen activator (uPA) is one of the two types of plasminogen activators (PAs) identified in mammals, which catalyze the conversion of the zymogen plasminogen into plasmin [62]. Plasmin is a protease with the capacity of degrading most of the extracellular proteins, including proteoglycans, directly or indirectly through the activation of pro-collagenase into collagenase [63]. Clinical evidence suggests a contribution of the PA system to joint pathogenesis in osteoarthritis (OA) and RA showing that expression levels of active uPA protein in OA synovium are not detectable in patients with OA [64]. In this study, significantly lower levels of uPA were found in the

serum of patients with KOA compared with healthy participants, which could confirm previous findings in which lower levels of this marker were associated with the OA state.

Cytokines in osteoarthritis with high expression levels

IL-6

Interleukin-6 (IL-6) is a pro-inflammatory cytokine that plays a role in innate immunity and joint inflammation in arthritic diseases [65]. IL-6 is a ligand for IL-6 receptor (IL-6R), which is expressed on the cell surface or can be found in the circulation in its soluble form, IL-6 receptor (sIL-6R); the bind of IL-6 to its receptors can activate different pathways on several different cell types which includes immune cells and osteoclasts [65,66]. Studies in animal models show that IL-6 also acts on nociceptive neurons, highlighting it as a potential pain modulator [67,68]. Further, IL-6 has been found to be highly expressed in animal models and in patients with painful KOA [18,19,69]. This study confirmed the augmented expression of serum levels of IL-6 in patients with KOA compared with controls adding evidence to the inflammation present in this pathology. Moreover, the linear regression highlighted its negative association with the pain intensity, suggesting that higher serum levels of this cytokine can have a different mode-of-action in the joint and in the circulation in relation to pain in the KOA cohort.

CSF-1

CSF-1, a member of the hematopoietic CSF family, is a key regulator for the survival, proliferation, and differentiation of macrophage populations, including osteoclasts [70,71]. CSF-1 acts on its receptor, CSF-1R, and this signaling is evaluable also at central nervous system level because of the expression on microglia and possibly neurons [72–74]. Signaling of CSF-1/CSF-1R has been found to be involved in several pathological conditions [72,75] and in a model of arthritis [76]. CSF-1 may play a critical role in pain and arthritic diseases [72,77], and previous studies proposed its action on microglia to control nerve injury–induced neuropathic pain [78,79]. Moreover CSF-1 can also act peripherally to

regulate pain, probably by its action on macrophages in tissues and possibly in nerves [80]. In this study, highly significant levels of CSF-1 were found in serum of patients with KOA, and the linear regression model explained its independent positive correlation with the pain intensity in these patients confirming the findings present in the current literature [81].

FGF-21

Fibroblast growth factor-21 (FGF-21) is a member of the FGF superfamily that acts as an endocrine hormone with the function of a metabolic regulator [82]. FGF-21 can be released into the circulation and it has been reported that FGF-21 can be detected in serum and synovial fluid (SF) samples of patients with RA and OA, rather than at the site of local joint tissue in OA patients [83]. A recent study showed how the FGF-21 concentration in serum from patients with OA was independently correlated with the severity of knee OA [84]. Our results confirm that high expression levels of this hormone circulate in the serum of patients with KOA, thus indicating that it is a significantly correlated and independent factor for pain intensity in evaluated patients.

MCP-3

Monocyte chemotactic protein 3 (MCP-3, known as CCL7) is one of 28 members of the CC subfamily of chemokines. A previous animal study showed its involvement in neuropathic pain and pain plasticity in the spinal cord of the rat after nerve ligation [85]. After dorsal root ganglia activation, IL-6 signaling causes a high upregulation of the MCP-3 gene which activates spinal microglia and which may promote the spinal sensitization and facilitation of ascending pain transmission [86]. In this study, high levels of MPC-3 were found in serum of patients with KOA in accordance with previous literature pointing towards the cooperative action of IL-6 and MPC-3 in pain sensation.

LAP TGF-beta-1

TGF-β1 is secreted as a homodimer together with its latency-associated pro-peptide LAP. Its association with LAP prevents binding of the TGF-β1 to cell receptors which uncouples the time of growth factor secretion from the time of growth factor action [87]. In this study, the level of the latent form of TGF-β1 was found significantly increased in serum from patients with KOA compared with healthy participants. This may indicate that the active polypeptide could be released and act in the area of the pathology.

Inflammatory markers and pain in knee osteoarthritic patients

Several previous clinical and preclinical studies have shown pro-inflammatory cytokines IL-1b, IL-6, IL-8, and TNFα and anti-inflammatory cytokines such as IL-10 in synovial fluid (SF), serum, and at different cellular levels (e.g., sensory neurons and chondrocyte) associated to the degree of osteoarthritic and OA pain [18,88–90]. The current study found that FGF-21 and 4E-BP1 were positively associated with pain intensity and highlighted TWEAK, FGF-21, CSF-1, and IL6 as independent factors predicting pain intensity in KOA patients. High levels of IL-6, in synovial fluid and serum were previously found to be positively associated with pain outcomes [16,90]. On the contrary, the present work highlighted a negative association of IL-6 serum levels with clinical pain, indicating that IL-6 might have different mode-of-action locally (the synovial fluid) and systemically (e.g. in serum). Moreover, growth factor FGF-21 has been shown to be highly expressed in serum and SF in patients with RA and also with detectable levels in patients with OA [83]. This study confirms the high serum levels of FGF-21 in patients with OA, the positive correlation and its significant value as an independent factor for the pain intensity, thereby giving the first indication of a potential involvement of this growth factor in the pain status of OA patients. Moreover, this study showed low expression levels of 4E-BP1, a factor involved in the mTOR pathways, which in a preclinical study has been shown to be expressed in neurons in the dorsal horn and in peripheral nerves, and the depletion of which can lead to mechanical hypersensitivity and increased response to noxious stimuli [55,56]. Future studies are encouraged to assess the associations between inflammatory profiles and pain hypersensitivity in patients with OA.

Limitation in this study can be represented by levels of tumor necrosis factor alpha (TNFα), a marker involved in the inflammation and pain process although previously described as a detectable marker in OA [14], that were not higher than the minimum level of detection (LOD). To avoid false positive or negative results during the statistical data processing, this specific marker was excluded from the analysis.

In conclusion, the present study found patients with KOA to have ten cytokines with significantly lowered expression in the serum and five cytokines with higher expression levels, when compared to healthy participants. Specifically, FGF-21 and 4E-BP1 were associated with pain in KOA, and TWEAK, FGF-21, CSF-1, IL6 were identified as independent predictors for pain intensity. Further studies are needed in order to validate the specific systemic involvement of these inflammatory markers with the pain in KOA patients.

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Figure legend

Figure 1. Volcano plot of differential serum inflammatory marker expressions. Statistical significance versus fold change is shown on the y and x axes, respectively. The volcano plot of data shows the Normalized Protein eXpression (NPX) for the 72 markers in KOA patients compared with healthy controls. a) Black full dots shows significant markers with P_{FDR}-value <0.01 (circles represent results obtained with Mann-Whitney U; triangles represent results obtained with *t*-test). b) Empty circles represent significant markers with P_{FDR}-value >0.01 and non-significant markers with P-value >0.05. Normalized Protein eXpression (NPX) is an Olink's arbitrary unit expressed in Log2 scale for markers expression levels.

Table 1. Demographics

	Controls (n = 40)	KOA patients (n = 127)
Gender (% females)	61,5%	57,8%
Age (years)	68.46 ± 3.13	68.82 ± 9.15
Kellgren Lawrence (1-4)	1.43 ± 0.59	3.74 ± 0.49
BMI (kg/m2)	26.71 ± 4.21	29.37 ± 4.79

Table 1. Demographics of patients and controls enrolled in the study. Data for age, Kellgren Lawrence scale, and BMI are presented as mean ± standard deviation (SD).

Table 2. Expression markers profile

Lowly expressed markers	P_{FDR}*-value*
Caspase-8 (CASP-8)	2.19e-015
Extracellular newly identified receptor for advanced glycation end-products (EN-RAGE)	9.29e-008
Delta and Notch-like epidermal growth factor-related receptor (DNER)	0.00006
Axin-1 (AXIN1)	7.4e-007
STAM-binding protein (STAMBP)	0.00001
SIR2-like protein 2 (SIRT2)	0.00005
Stem cell factor (SCF)	0.0002
Eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1)	0.0003
Tumor necrosis factor (Ligand) superfamily, member 12 (TWEAK)	0.002
Urokinase-type plasminogen activator (uPA)	0.004
Highly expressed markers	
Interleukin-6 (IL-6)	0.00002
Macrophage colony-stimulating factor 1 (CSF-1)	0.001
Fibroblast growth factor 21 (FGF21)	0.001
Monocyte chemotactic protein 3 (MCP-3)	0.006
Latency-associated peptide transforming growth factor beta-1 (LAP TGF-beta-1)	0.009

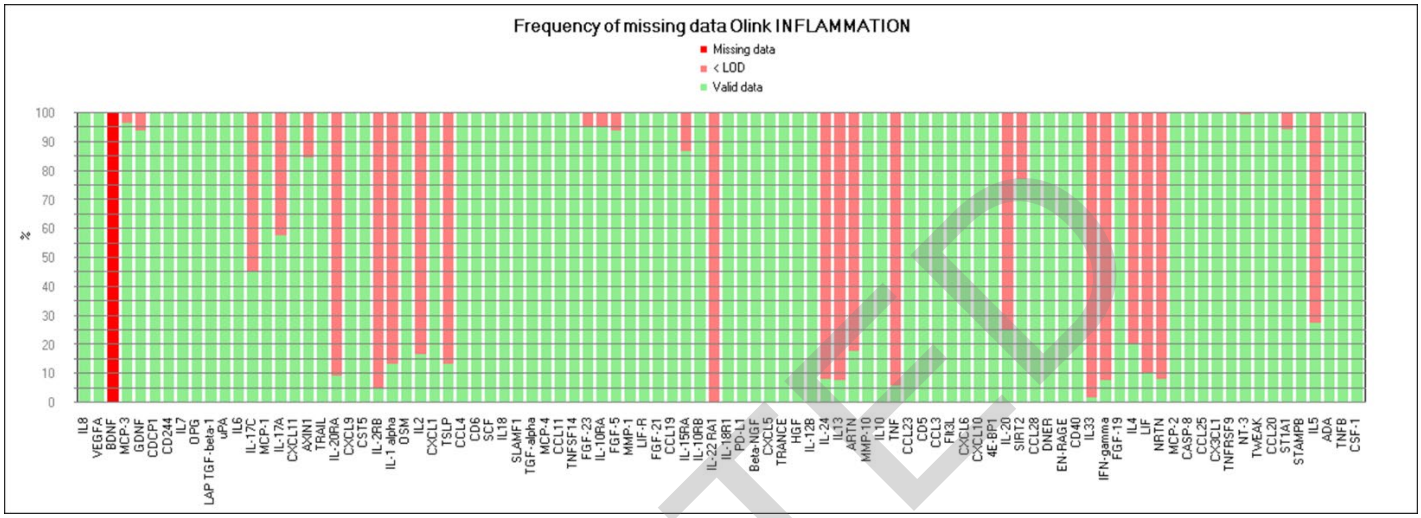
Table 2. Markers significantly dysregulated between KOA patients and healthy controls. The P-values showed are corrected using the False Discovery Rate (FDR) methods [84].

Table 3. Linea regression models

Model	Variable	Standardized coefficient	P-value	R^2
1				**0.154**
	CASP-8	-0.123 [95% CI: -0.40 to 0.15]	0.385	
	EN-RAGE	0.187 [95% CI: -0.07 to 0.44]	0.163	
	AXIN1	-0.092 [95% CI: -0.33 to 0.14]	0.448	
	STAMPB	0.176 [95% CI: -0.19 to 0.55]	0.354	
	IL6	-0.427 [95% CI: -0.7 to -0.15]	**0.003**	
	SIRT2	-0.009 [95% CI: 0.38 to -0.36]	0.963	
	DNER	0.227 [95% CI: 0.02 to 0.42]	**0.026**	
	SCF	-0.142 [95% CI: -0.35 to 0.07]	0.197	
	4E-BP1	0.093 [95% CI: -0.19 to 0.37]	0.509	
	CSF-1	0.306 [95% CI: 0.06 to 0.56]	**0.016**	
	FGF-21	0.246 [95% CI: 0.07 to 0.43]	**0.008**	
	TWEAK	-0.265 [95% CI: -0.51 to -0.02]	**0.033**	
	uPA	-0.127 [95% CI: -0.34 to 0.09]	0.247	
	MCP-3	-0.120 [95% CI: -0.34 to 0.10]	0.275	
	LAP TGF-beta-1	-0.034 [95% CI: -0.23 to 0.16]	0.732	
2				**0.163**
	IL6	-0.406 [95% CI: -0.62 to -0.19]	**<0.001**	
	DNER	0.185 [95% CI: -0.007 to 0.38]	0.058	
	CSF-1	0.264 [95% CI: 0.051 to 0.48]	**0.015**	
	FGF-21	0.264 [95% CI: 0.095 to 0.43]	**0.002**	
	TWEAK	-0.355 [95% CI: -0.56 – 0.15]	**0.001**	

Table 3. Linear regression models of the inflammatory markers showing association between marker serum levels and pain intensity. Model 1 consists of all the parameters and model 2 has been designed using backwards selection. R2 indicates variance of pain intensity. Standardized coefficients refer to how many standard deviations a dependent variable will change per standard deviation increase in the predictor variable. 95% confidence intervals of standardized coefficient.

Supplementary material



Sup.Figure.1 Limit of detection determined for each marker based on the mean value of triplicate negative controls analyzed in each run. Only 72 markers with values above LOD of more than 30% were analyzed.