Profiling of inflammatory mediators in the synovial fluid related to pain in knee osteoarthritis

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Abstract

Background: Inflammatory mediators in the synovial fluid (SF) play critical roles in the initiation and development of pain in knee osteoarthritis (KOA). However, the expression of inflammatory mediators is controversial and the role of SF inflammatory mediators in neuropathic pain is not clear. Therefore, the aim of this study is to identify the SF inflammatory mediators associated with nociceptive and neuropathic pain in KOA.

Methods: The levels of IL-1β, IL-6, TNF-α, macrophage colony-stimulating factor, MMP-3, MMP-13, metalloproteinase with thrombospondin motifs 5, calcitonin gene-related peptide, neuropeptide Y, substance P and bradykinin were measured in 86 patients using enzyme-linked immunosorbent assays. Nociceptive pain was measured using the numeric rating scale (NRS), visual analog scale (VAS) and Western Ontario and McMaster Universities Osteoarthritis Index (WOMAC) pain score. Neuropathic pain was measured using the PainDETECT questionnaire. Moreover, knee function was evaluated by the WOMAC score and range of motion (ROM) assessments. Radiological grade was defined using the Kellgren-Lawrence (K-L) grading scale. Results: Pain scores measured using different methods were highly correlated to each other. The worse the pain, the worse the K-L grade and knee function were. The expression of IL-1β and IL-6 was increased in the early stage compared with the late stage. The NRS was positively correlated to age, K-L grade, and the WOMAC score and negatively correlated to ROM and TNF-α expression. The VAS was positively correlated to age, K-L grade, and the WOMAC score but negatively correlated to ROM and the levels of IL-1β, IL-6 and TNF-α. The WOMAC pain score was not correlated to any of the measured inflammatory mediators; it correlated to only ROM. The PainDETECT score correlated to only the WOMAC score. The expression of other inflammatory mediators was not correlated to any of the pain scores. Conclusions: IL-1β, IL-6 and TNF-α play critical roles in pain in the early stage of KOA and correlated to pain.
The measured catabolic enzymes and neuropeptides are not correlated to nociceptive and neuropathic pain. New biomarkers related to pain in the late stage need to be further investigated.

Background

Pain is the most prominent symptom of knee osteoarthritis (KOA) and the major driver of clinical decision-making. Despite great progress in the understanding of the molecular and cellular mechanisms of KOA, the treatment of pain is still a challenge in the clinic (1-4). Therefore, etiological investigation of pain is not only helpful for understanding KOA but also critical for developing new medications to relieve pain.

KOA was previously considered a “wear and tear” disease. In the past decade, inflammation has been found to play a critical role in the pathogenesis of KOA and to contribute to the initiation and development of pain (1, 5, 6). Therefore, inflammatory mediators that serve as important factors that induce or reduce inflammation have attracted interest worldwide. Compared with inflammatory mediators in the serum, inflammatory mediators in the synovial fluid (SF) are thought directly affect inflammation and cartilage metabolism and could be a good target for the treatment of pain.

Numerous types of SF inflammatory mediators, including inflammatory cytokines, matrix catabolic proteases and neuropeptides, play important roles. However, the expression of these cytokines in osteoarthritis pain is still controversial, and different studies have arrived at conflicting conclusions regarding interleukin 1β (IL-1β), interleukin 6 (IL-6) and tumor necrosis factor (TNF-α) (7-9). In addition, the roles of some important inflammatory mediators, such as macrophage colony-stimulating factor (M-CSF) and metalloproteinase with thrombospondin motifs 5 (ADAMTS5), are still not clear. M-CSF was found to be required in the development of pain in a KOA experimental model; moreover, therapeutic neutralization of M-CSF reduced not only pain but also cartilage damage in patients with
ankylosing spondylitis and rheumatoid arthritis (10, 11). ADAMTS5 is an important enzyme involved in the degradation of the extracellular matrix (ECM) protein aggrecan (12), and ADAMTS5-deficient mice do not develop OA after resection of the medial meniscus (13). Therefore, it is necessary to further explore the roles of these inflammatory mediators in pain and identify candidate inflammatory mediators associated with pain.

Furthermore, pain is classified as nociceptive pain and neuropathic pain (4), but few studies have investigated the correlation between SF inflammatory mediators and neuropathic pain. Therefore, another aim of this study was to illustrate the correlations between neuropathic pain and SF inflammatory mediators.

Methods

Patient selection

Patients diagnosed with primary KOA according to the American College of Rheumatology criteria in our outpatient department were enrolled in the study (14). Exclusion criteria included: 1. previous trauma; 2. symptoms of spinal disease; 3. prior treatment in the last 3 months; 4. joint replacement operation on the other knee; and 5. cognitive disorders.

Patient characteristics and pain evaluation

Patient demographics and knee function parameters including, age, sex, range of motion (ROM) and the Western Ontario and McMaster Universities Osteoarthritis Index (WOMAC) score, were recorded (15). The WOMAC score includes 3 subscales related to pain, stiffness and function. The higher the WOMAC score, the worse the joint function is. The radiological grade of OA was evaluated according to the Kellgren-Lawrence (K-L) grading scale (0=none, 1=doubtful, 2=minimal, 3=moderate, and 4=severe) (16). Nociceptive pain was evaluated using the visual analog scale (VAS) and numeric rating scale (NRS). The WOMAC pain score was used to measure function-related pain, while neuropathic pain was measured according to the PainDETECT questionnaire (0-12=unlikely, 13-
SF and an inflammatory mediator assay

SF was harvested with patient approval using a syringe and needle in the outpatient department during their intra-articular injection. The aspirated SF was centrifuged at 3,000 rpm for 10 min to remove cells and stored in a -80°C freezer until use. The concentrations of inflammatory mediators were measured using commercial enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer’s instructions (LanpaiBio, Shanghai, China). The measured inflammatory mediators included inflammatory cytokines such as IL-1β, IL-6, TNF-α and M-CSF; matrix catabolic proteases such as matrix metalloproteinase 3 (MMP-3), matrix metalloproteinase 13 (MMP-13) and ADAMTS5; and neuropeptides such as calcitonin gene-related peptide (CGRP), neuropeptide Y (NPY), substance P (SP) and bradykinin (BK). Due to the low amounts of some samples, all of the cytokines were measured once.

Statistical analysis

Statistical analysis of data was performed using SPSS 20.0 (IBM Corp., Armonk, NY, USA). Data with a normal distribution are reported as the mean and standard deviation. Data with a nonnormal distribution are reported as the mean and 95% confidence interval (CI). Correlation coefficients were determined by the Spearman rank correlation test using two-tailed P values. The comparation of values in different K-L groups were examined by ANOVA or Kruskal-Wallis test depending on the results of normality test and Levene test. The correlation plot was generated using the R package (Version R 3.5.3, R Core Team, Vienna, Austria). P < 0.05 was set as significant difference.

Results

Patient demographics, knee function and radiological assessment

In total, 83 patients including 21 males and 62 females were enrolled in the study. The
average age was 63.4±10.4 years. The average WOMAC score was 25.5 (21.7-29.3), while the average ROM was 112.0° (95% CI 108.0-116.1). The patient demographics, knee function, pain intensity and expression of inflammatory mediators in different K-L grade groups are illustrated in Table 1. With increasing age, ROM became more limited (Figure 1A), and the WOMAC score increased (Figure 1B), moreover, worsening K-L grade also led to limited ROM ($r=-0.499$, $p<0.001$) and increased WOMAC score ($r=0.493$, $p<0.001$) (Figure 2A and Figure 2B), which indicated that knee function was lost gradually.

To explore the differences in inflammatory mediators among different K-L groups, the expression of inflammatory mediators was compared using one-way ANOVA. We found the expression of IL-1β ($p=0.002$) and IL-6 ($p=0.023$) was significantly different among four K-L groups (Figure 3A and Figure 3B). Increased expression of IL-1β and IL-6 was found in the early stage of KOA, which suggested that more severe inflammation may exist in the early stage of KOA. However, no significant differences were found for other inflammatory mediators among the K-L groups.

**Significant correlations among different pain scoring systems**

To evaluate the intensity of pain in KOA, nociceptive pain was measured using the NRS and VAS, while neuropathic pain was determined using the PainDETECT questionnaire. The WOMAC pain score was recorded to measure function-related pain. The NRS and VAS scores were 3.04 (95% CI 2.63-3.44) and 3.55 (95% CI 3.09-4.03) cm, respectively. The WOMAC pain score was 5.05 (95% CI 4.29-5.81), while the PainDETECT pain score was 2.89 (95% CI 2.12-3.67).

To further investigate the consistency among different pain scoring systems, correlation coefficients were determined using the Spearman test. As expected, the four measurements of pain were highly correlated with each other and exhibited homogeneity (Figure 4A and Figure 4B).
Pain affected knee function and correlated to age and K-L grade

To determine the correlations between pain and patient demographic parameters, knee function or radiological grade, pain was analyzed for correlations with age, ROM, WOMAC score and K-L grade using the Spearman test. As expected, with an increase in age or K-L grade, the NRS, VAS and WOMAC pain scores increased (Figure 5A and Figure 5B). Correspondingly, knee function was gradually lost, as shown by an increase in the WOMAC score (Figure 5C) and limited ROM (Figure 5D). However, neuropathic pain scores correlated to only the WOMAC score ($r=0.384$, $p<0.000$) and not to age ($r=0.108$, $p=0.331$), K-L grade ($r=0.176$, $p=0.112$) or ROM ($r=-0.179$, $p=0.105$). In conclusion, age and K-L grade were important factors correlated to KOA pain, and KOA pain significantly affected knee function.

Correlations among pain, patient characteristics and inflammatory cytokines

Inflammatory cytokines play critical roles in pain initiation and development (5). Among inflammatory cytokines, IL-1β, IL-6 and TNF-α are the most important inflammatory cytokines involved in KOA. Even though IL-1β, IL-6 and TNF-α have been widely investigated in both experiment models and clinical samples, the expression of these cytokines in KOA pain is still controversial, and different research studies have reached conflicting conclusions (7, 18, 19). Moreover, as illustrated in the introduction, M-CSF has been found to be important in KOA (10, 11). However, whether SF M-CSF is involved in KOA pain is still unknown.

To investigate the correlations between pain and these inflammatory cytokines, the expression of IL-1β, IL-6, TNF-α and M-CSF was measured by ELISA and analyzed by the Spearman test. As is shown in Table 2, IL-1β and IL-6 were very weakly negatively correlated to VAS score, and TNF-α was negatively correlated to VAS and NRS scores. However, M-CSF did not show any correlation to any of the pain scores. Moreover, WOMAC-
assessed pain and neuropathic pain were not correlated to any of the inflammatory cytokines. In short, the above evidence demonstrated that inflammatory cytokines significantly correlated to KOA pain. The expression of IL-1β, IL-6 and TNF-α may be higher in patients with less pain.

To further determine the correlations between the expression of inflammatory cytokines and patient characteristics, the expression of IL-1β, IL-6, TNF-α and M-CSF was analyzed for correlations with age, K-L grade, ROM and WOMAC score using the Spearman test (Table 3). We found IL-1β and IL-6 were negatively correlated to age, K-L grade and WOMAC score, while IL-6 was positively correlated to ROM. In addition, TNF-α was negatively correlated to age. In line with the results shown in Figure 3, the expression of IL-1 and IL-6 was relatively high in the early stage compared with the late stage, and the expression of these two inflammatory cytokines was associated with knee function.

No correlations were found between pain and measured catabolic proteases

OA progression is usually accompanied by high matrix catabolism, therefore, we hypothesized that the high expression of catabolic proteases in the SF enhances the breakdown of cartilage and causes severe synovitis and inflammation, which may lead to more pain. Among these catabolic proteases, MMPs and ADAMTS5 play important roles in KOA through involvement in the degradation of ECM type II collagen and aggrecan, respectively (20, 21). However, whether these proteases are involved in KOA pain is still unknown.

To evaluate the correlations between these catabolic proteases and pain, MMP-3, MMP-13 and ADAMTS5 levels were measured by ELISA and correlated with pain scores using the Spearman test (Table 4). The results showed that only MMP-13 was weakly correlated to NRS score ($r=0.267$, $p=0.015$). However, no correlations were found between pain scores and other measured catabolic enzymes. This result demonstrated that the measured
catabolic cytokines may not be involved in KOA pain.

No correlations were found between pain and measured neuropeptides

It is believed that neuropeptides sensitize knee nociceptors and are related to knee pain (19). SP, NPY, CGRP and BK are considered important neuropeptides involved in OA pain. First, the SF expression of SP was previously found in KOA and rheumatoid arthritis (22). The concentration of SP is significantly higher in painful temporomandibular joints than in painless temporomandibular joints (23). Second, the expression of NPY in dorsal root ganglion was found to be significantly correlated to OA pain in animal models (24), and SF NPY expression was found to be correlated to OA pain (25). Third, CGRP was reported to be involved in the progression and prognosis of KOA and widely distributed in the nociceptive pathways in the peripheral and central nervous systems, which correlated to numerous types of pain (26-28). Last but not least, BK is an inflammatory mediator that can lead to vasodilation and inflammation induction. Although BK has been reported to correlate with biomarkers for cartilage degradation and inflammation in OA (29), the role of BK in pain still needs to be further investigated. Therefore, it is necessary to explore whether these neuropeptides play roles in OA pain.

To address this issue, the expression of these neuropeptides was analyzed and correlated with different pain scoring systems using the Spearman test (Table 5). However, no correlations were found between any of the measured pain scoring systems and these neuropeptides, which suggests that these neuropeptides may not contribute to KOA pain.

Interactions and correlations among inflammatory mediators

To investigate the associations among the measured inflammatory mediators, correlation coefficients were defined among all of the inflammatory mediators using the Spearman test. The outcome is shown in Figure 6. On the one hand, all the types of inflammatory mediators had internal associations. Among the inflammatory cytokines, IL-1β was highly
positively correlated to IL-6 ($r=0.93$, $p<0.000$), while TNF-α was negatively correlated to M-CSF ($r=-0.362$, $p=0.004$). Interestingly, of the measured matrix-catabolizing proteases, only MMP-3 and ADAMTS5 showed a very weak correlation ($r=0.239$, $p=0.030$), and no other correlations were found among the matrix proteases. To our surprise, almost all of the measured neuropeptides were correlated to each other, which demonstrated the obvious interactions among these neuropeptides. On the other hand, correlations were found among different types of inflammatory mediators. For example, TNF-α and M-CSF were correlated to all of the measured neuropeptides, while MMP-3 and MMP-13 were correlated to only SP. These results demonstrated that inflammatory cytokines, catabolic proteases and neuropeptides interacted with each other.

Discussion

Pain is the major symptom of KOA and still needs to be further investigated. Though complicated mechanisms are involved in OA pain, our study focused on only SF inflammatory mediators because we think that increasing or reducing the levels of certain inflammatory mediators may shed new light on pain treatment.

Unlike the structural changes in OA, the biggest obstacle in pain research is the subjective nature of this field (3, 30). Therefore, to minimize subjective effects, in this study, pain intensity was measured using four different pain scoring systems including the VAS, NRS, WOMAC pain score and PainDETECT score. The VAS and NRS are commonly used pain measurement tools. Although the VAS is commonly used in pain research, it is more complicated than the NRS, especially in use with elderly patients due to cognitive impairments (31). For this reason, we decided to use both pain scoring systems to evaluate pain intensity. A previous study reported correlations between the VAS and NRS ranging from 0.62-0.91 (32). In line with this result, our correlation coefficient was 0.775 ($p<0.000$). Moreover, the WOMAC pain score is widely used in KOA pain research because
it is more precise than the VAS and NRS and can reflect pain at rest and during movement (33). In addition, it has been demonstrated that pain can be classified as nociceptive and neuropathic pain (4). For this purpose, we also evaluated neuropathic pain using the PainDETECT questionnaire. To our knowledge, few studies have focused on the correlation between neuropathic pain and nociceptive pain. In our study, we found that neuropathic pain scores had a weak correlation to the NRS ($r=0.428$, $p<0.000$), VAS ($r=0.422$, $p<0.000$) and WOMAC pain score ($r=0.551$, $p<0.000$). Therefore, these results indicated that all of these pain measurement methods have high internal consistency and are reliable for further research.

Patient characteristics are correlated to pain in OA (30). First, old age is thought to be a systemic risk factor for OA (34, 35) and is correlated to pain (36). In our study, we found that age was significantly correlated to pain scores ($r=0.430-0.474$, $p<0.000$) and played an important role in the genesis of OA. Second, radiological images are important tools for the evaluation of OA severity and predicts knee pain. The positive correlation between knee pain and K-L grade was found in several studies (37, 38). McAlindon reported the correlation coefficient was 0.43. In line with this result, our study proved that K-L grade was significantly correlated to pain scores ($r=0.409-0.479$, $p<0.000$) but not correlated to neuropathic pain scores ($r=0.176$, $p=0.112$). Third, pain significantly affects ROM. Numerous studies have demonstrated that pain affects ROM (36, 37). In agreement with previous reports, our study demonstrated that patient ROM was highly correlated with pain scores ($r=-0.403~-0.451$, $p<0.000$). In summary, as age increases and radiological assessments show worsening results, patient pain increases, and joint function is lost.

It is well known that inflammatory cytokines play critical roles in OA pain. On the one hand, although M-CSF is a very important inflammatory cytokine, to our knowledge, no previous research has focused on the correlation between SF M-CSF and pain intensity.
Therefore, we hypothesized that an increased SF M-CSF level would lead to more severe pain because M-CSF induces inflammation. However, no significant correlation was found between pain and the concentration of M-CSF in this study. Therefore, the strategy of eliminating the expression of M-CSF may not be effective for reducing OA pain. On the other hand, the roles of some common SF inflammatory cytokines, such as IL-1β, IL-6 and TNF-α, are still controversial. Radojcic reported that IL-6 in the SF is significantly positively associated with knee pain using the WOMAC pain score (B = 0.022; 95% CI 0.004-0.040) (8). In contrast, Brenner reported that IL-6 levels have no correlation with the WOMAC pain score (7). Orita reported that TNF-α is positively related to pain, but IL-6 is not (9). Therefore, different research studies have obtained conflicting results. This discrepancy may be due to the subjective nature of pain. In this study, we found that IL-1β and IL-6 were negative correlated to K-L grade and pain scores. We hypothesized that in the early stage of KOA, inflammation in the knee joint may be the major cause that promotes patients to seek help in an outpatient department. In addition, in the late stage of KOA, pain may not come from inflammation but rather from other sources that need to be further investigated. This finding is consistent with the results of previous reports. Barker found that in the early stage of knee OA, the expression of TNF-α and IL-6 in the serum is significantly higher than that in the serum during the late stage of knee OA (39). Orita reported that IL-6 in the SF has a relatively significant negative correlation with K-L grade but that TNF-α has no correlation to K-L grade (9). Ene found that compared with those in the late stage of OA, the expression of inflammatory mediators and infiltration of mononuclear cells in the early stage of OA were enhanced (40). Combining these results and our results, we suggest that anti-inflammatory treatment may be more useful in the early stage than in the late stage and that a new treatment strategy for controlling pain should be considered for the late stage of knee OA.
High catabolism and high anabolism coexist in OA patients, but the degree of catabolism is more extensive than that of anabolism (41). In addition, it is important to evaluate catabolism-related proteinases to monitor the progression of OA. Therefore, we hypothesized that high expression of catabolic proteinases may produce fragmentation of the matrix, which could induce inflammation and cause more pain. A previous study showed that MMP-3, MMP-13 and ADAMTS5 in the SF can be detected in KOA (42, 43). Although ADAMTS5 plays a critical role in degrading aggrecan, to our knowledge, this is the first report to explore the correlation between ADAMTS5 and pain. However, after comparing ADAMTS5 with various pain scoring systems in this study, no correlation was found between ADAMTS5 and pain. In addition, we found that only the expression of MMP-13 had a weak correlation with the NRS. These results are similar to previous results. Bay-Jensen reported that serum COL3/ADAMTS was weakly associated with pain scores ($r=-0.13-0.17$, $p<0.05$) (44). Sun compared MMP-3 and NRS but did not found any correlations (45). No other correlations between SF catabolic proteases and pain were found in a previous study except for the above-mentioned results. For this reason, we concluded that the measured catabolic proteases may not be involved in OA pain and that more catabolic proteases should be analyzed in the future.

Neuropeptides have been reported to be involved in OA pain in many experimental models and clinical patients (3, 19), but whether these neuropeptides are correlated to pain is still controversial. Therefore, we measured the expression of these neuropeptides and tried to identify correlations with pain. First, NPY is an important neuropeptide involved in pain occurrence. Wang et al. reported that NPY in the SF is related to OA pain (25). However, we did not find any correlations after comparing four types of pain scoring systems with NPY expression. This disagreement may be due to the study by Wang et al. using Hideo Watanabe's pain score, which only includes 5 levels of pain and is not so
accurate as the VAS, NRS or WOMAC pain score. Correlation coefficients for different pain subgroups were not available in their research. Therefore, the role of NPY still needs to be further investigated. Second, SF SP has been reported in KOA patients (22), and after treatment with medication, the level of SP has been correlated to pain relief (46). The SP receptor (TACR1) gene was also demonstrated to correlate to pain in KOA (47). However, until now, no study has reported a link between SP and pain intensity. Our study showed no correlation between SF SP and OA pain. Third, CGRP is an important neuropeptide produced in both peripheral and central neurons and involved in numerous types of pain, such as headache (26). It has been reported that increasing the SF expression of CGRP contributes to the progress of arthritis in developmental dysplasia of the hip (48). Takano reported that the elevation of CGRP levels in the synovium may contribute to OA pain (49). Dong reported that CGRP is correlated to KOA pain using the WOMAC pain score \( r=0.524, \ p<0.001 \) (27). However, we did not find a correlation between CGRP and pain intensity. Last, BK is an inflammatory mediator that can lead to vasodilation and inflammation induction. SF levels of BK have been reported to correlate with biochemical markers of cartilage degradation and inflammation in KOA (29). However, in our study, BK was not correlated to any pain score. This result is consistent with previous results. After evaluating correlations between BK and pain in OA, Bellucci did not find any correlations (29). In short, the measured neuropeptides were not correlated to OA pain in our study.

In our study, we also investigated correlations among inflammatory cytokines, catabolic proteases and neuropeptides. We found that IL-6 and IL-1\( \beta \) were highly correlated to each other. This result is similar to the results in Brenner’s reports, which showed that IL-6 and IL-1\( \beta \) are highly correlated to each other \( r=0.73, \ p=0.001 \) (7). However, we did not find any correlations between IL-6 and other biomarkers. This result is similar to results in Bellucci’s research, which did not find any correlations after comparing IL-6 with IL-8,
MMP-1, MMP-3 and cartilage oligomeric matrix protein (COMP) (29). This study is the first to explore associations between M-CSF and inflammatory mediators. We found that the expression of M-CSF was positively correlated to CGRP, NPY and BK but negatively correlated to SP, while TNF-α was negatively correlated to CGRP, NPY and BK but positively correlated to SP. These results indicate possible interactions between inflammatory cytokines and neuropeptides. However, the catabolic proteases had minor interactions with the inflammatory cytokines and neuropeptides.

The present study has some limitations. First, the number of patients with an advanced K-L grade was limited. This limitation prevented us from further exploring the factors that influence pain in the late stage of KOA. Second, because patients likely with neuropathic pain was defined as a PainDETECT score higher than 18, we did not identify any patient likely having neuropathic pain in this study. This limitation indicates we need to further explore the risk factors for neuropathic pain.

Conclusions

IL-1β, IL-6 and TNF-α but not M-CSF play critical roles in pain in the early stage of KOA and correlated to pain which indicates anti-inflammation may be more efficient in releasing pain in the early stage. The measured catabolic enzymes including MMP-3, MMP-13 and ADAMTS5 and neuropeptides including CGRP, NPY, SP and BK are not correlated to nociceptive and neuropathic pain. New biomarkers related to pain in the late stage need to be further investigated.

Declarations

*Ethics approval and consent to participate:* The experimental protocol was approved by the Ethics Committees of the Second Affiliated Hospital of Wenzhou Medical University. Informed consent was obtained from all patients.
Consent for publication: Not applicable

Availability of data and material: The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests: The authors declare that they have no competing interests

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Author contributions: Study conception and design: LL and PF. Collection and assembly of data: LL, ZXL, YYL, QW, ZY, PF. SPSS statistical analysis: LL and PF. Analysis and interpretation of data: LL and PF. Manuscript: LL and PF. All authors approved the final version to be published.

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Tables

**Table 1.** Patient demographics, knee function, pain intensity and inflammatory mediator expression in different K-L grade groups.
|                     | 1          | 2          | 3          | 4          | Average      |
|---------------------|------------|------------|------------|------------|--------------|
| Male/Female         | 5/15       | 15/33      | 1/11       | 0/3        | 21/62        |
| Age (years)         | 56.6±9.2   | 63.3±8.8   | 72.1±11.2  | 74.0±6.6   | 63.4±10.4    |
| ROM (°)             | 121.5 (117.7-125.2) | 114.4 (110.1-118.6) | 92.1 (75.1-109.1) | 91.7 (44.6-138.7) | 112.0 (108.0-116.1) |
| WOMAC               | 15.2 (10.6-19.7) | 23.8 (19.7-27.9) | 41.8 (28.2-55.3) | 58.3 (37.2-79.5) | 25.5 (21.7-29.3) |
| NRS                 | 2.0 (1.4-2.5) | 2.9 (2.5-3.3) | 4.6 (3.3-5.9) | 6.3 (-0.8-13.5) | 3.04 (2.63-3.44) |
| VAS (cm)            | 2.5 (1.6-3.5) | 3.5 (2.9-4.0) | 4.7 (3.4-6.0) | 7.0 (2.7-11.3) | 3.55 (3.09-4.03) |
| WOMAC Pain          | 3.0 (2.0-4.0) | 4.8 (4.0-5.6) | 7.8 (4.7-10.8) | 11.7 (5.9-17.4) | 5.05 (4.29-5.81) |
| Neuropathic Pain    | 2.7 (0.9-4.5) | 2.4 (1.5-3.4) | 4.3 (1.5-7.0) | 6.0 (-0.6-12.6) | 2.89 (2.12-3.67) |
| IL-1β (pg/mL)       | 40.7 (37.4-44.0) | 38.8 (36.8-40.9) | 32.4 (28.7-36.2) | 29.0 (5.40-52.7) | 38.0 (36.3-39.6) |
| IL-6 (pg/mL)        | 30.3 (28.3-32.3) | 29.7 (28.6-30.8) | 26.7 (24.6-28.7) | 25.9 (14.8-37.1) | 29.3 (28.4-30.1) |
| TNF-α (pg/mL)       | 666.3 (591.2-741.5) | 669.7 (616.6-722.8) | 575.3 (473.2-677.4) | 524.1 (64.4-1112.6) | 650.0 (611.1-688.8) |
| M-CSF (pg/mL)       | 302.7 (273.7-331.8) | 282.9 (266.2-299.6) | 321.1 (293.8-348.4) | 339.1 (321.6-356.6) | 295.2 (282.7-307.8) |
| MMP-3 (ng/mL)       | 337.3 (351.4-403.2) | 372.2 (355.2-389.2) | 368.3 (328.0-408.6) | 420.7 (321.8-519.6) | 374.6 (362.0-387.2) |
| MMP-13 (ng/mL)      | 208.0 (192.8-233.2) | 213.0 (200.6-225.4) | 228.6 (219.7-246.5) | 250.7 (146.9-354.5) | 215.4 (206.9-224.0) |
| ADAMTS5 (U/mL)      | 45.3 (41.7-49.0) | 42.6 (39.9-45.2) | 44.6 (37.1-50.2) | 41.5 (27.6-55.3) | 43.5 (41.6-45.4) |
| BK (ng/mL)          | 5.67 (5.05-6.28) | 5.68 (5.33-6.03) | 6.15 (5.37-6.93) | 6.28 (5.41-7.16) | 5.77 (5.50-6.03) |
| CGRP (pg/mL)        | 94.7 (84.8-104.5) | 91.4 (84.2-98.6) | 95.1 (78.6-111.6) | 109.9 (25.6-194.2) | 93.4 (88.1-98.7) |
| SP (pg/mL)          | 109.7 (98.4-121.0) | 105.6 (98.9-112.2) | 103.3 (85.2-121.4) | 96.2 (6.03-186.4) | 105.9 (100.6-111.2) |
| NPY (ng/mL)         | 170.5 (155.2-185.8) | 177.7 (168.9-186.4) | 180.6 (161.9-199.2) | 180.1 (111.3-249.0) | 176.5 (169.9-183.1) |

ROM: range of motion, NRS: numeric rating scale, VAS: visual analog scale, WOMAC: Western Ontario and McMaster Universities Osteoarthritis Index, IL-1β: interleukin 1β, IL-6: interleukin 6, TNF-α: tumor necrosis factor α, M-CSF: macrophage colony-stimulating factor.
factor, **MMP-3**: matrix metalloproteinase 3, **MMP-13**: matrix metalloproteinase 13,
**ADAMTS5**: metalloproteinase with thrombospondin motifs 5, **CGRP**: calcitonin gene-
related peptide, **NPY**: neuropeptide Y, **SP**: substance P, **BK**: bradykinin.

**Table 2.** Correlations between inflammatory cytokines and pain scoring systems.

|               | IL-1β | IL-6  | TNF-α | M-CSF  |
|---------------|-------|-------|-------|--------|
| VAS           | -0.280* | -0.272* | -0.249* | 0.018  |
| NRS           | -0.203  | -0.186 | -0.270* | -0.019 |
| WOMAC Pain    | 0.185   | -0.139 | -0.186 | -0.044 |
| Neuropathic Pain | -0.019  | -0.034 | -0.115 | -0.001 |

NRS: numeric rating scale, **VAS**: visual analog scale, **WOMAC**: Western Ontario and
McMaster Universities Osteoarthritis Index, **IL-1β**: interleukin 1β, **IL-6**: interleukin 6, **TNF-α**: tumor necrosis factor α, **M-CSF**: macrophage colony-stimulating factor. * $p<0.05$  ** $p<0.01$

**Table 3.** Correlations between inflammatory cytokines and patient characteristics.

|               | Age  | K-L grade | ROM  | WOMAC |
|---------------|------|-----------|------|-------|
| IL-1β         | -0.272* | -0.363** | 0.214 | -0.317** |
| IL-6          | -0.261* | -0.291** | 0.219* | -0.297** |
| TNF-α         | -0.240* | -0.149   | -0.033 | -0.119  |
| M-CSF         | 0.085  | 0.123     | -0.033 | -1.00   |

IL-1β: interleukin 1β, **IL-6**: interleukin 6, **TNF-α**: tumor necrosis factor α, **M-CSF**: macrophage colony-stimulating factor, **K-L grade**: Kellgren-Lawrence grade, **ROM**: range of motion, **WOMAC**: Western Ontario and McMaster Universities Osteoarthritis Index. * $p<0.05$  ** $p<0.01$

**Table 4.** Correlations between catabolic cytokines and pain.
|                | MMP-3 | MMP-13 | ADAMTS5 |
|----------------|-------|--------|---------|
| VAS            | 0.000 | 0.216  | -0.031  |
| NRS            | 0.079 | 0.267* | 0.041   |
| WOMAC Pain     | 0.205 | 0.102  | -0.056  |
| Neuropathic Pain| 0.175 | 0.209  | 0.180   |

NRS: numeric rating scale, VAS: visual analog scale, WOMAC: Western Ontario and McMaster Universities Osteoarthritis Index, MMP-3: matrix metalloproteinase 3, MMP-13: matrix metalloproteinase 13, ADAMTS5: metalloproteinase with thrombospondin motifs 5.

* p<0.05  ** p<0.01

**Table 5. Correlations between neuropeptides and pain.**

|                | SP    | NPY   | CGRP  | BK    |
|----------------|-------|-------|-------|-------|
| NRS            | -0.163| 0.018 | 0.070 | 0.051 |
| VAS            | -0.103| -0.011| -0.089| -0.064|
| WOMAC Pain     | -0.008| -0.150| -0.149| -0.013|
| Neuropathic Pain| 0.007 | -0.173| -0.131| 0.019 |

NRS: numeric rating scale, VAS: visual analog scale, WOMAC: Western Ontario and McMaster Universities Osteoarthritis Index, CGRP: calcitonin gene-related peptide, NPY: neuropeptide Y, SP: substance P, BK: bradykinin. * p<0.05  ** p<0.01

**Figures**
Figure 1

Correlations between age and knee function. (A). Correlation between age and ROM. (B). Correlation between age and WOMAC score. The correlation coefficient was calculated by Spearman rank correlation test using two-tailed $P$ values and shown above the dot plots.
Figure 2

Knee function in different K-L groups. (A). ROM in different K-L groups. The comparison of values were examined by Kruskal-Wallis test. (B). WOMAC score in different K-L groups. The comparison of values were examined by one-way ANOVA. * p<0.05 and ** p<0.01.
Figure 3

Expression of IL-1β and IL-6 in different K-L groups. (A). The expression of IL-1β in different K-L groups. (B). The expression of IL-6 in different K-L groups. The comparation of values were examined by one-way ANOVA. * p<0.05 and ** p<0.01.
Figure 4

Pain scores measured using different methods and correlations among the methods. (A). Pain scores measured using different methods. (B). Correlation coefficients between different pain scoring systems. The different colors and the circle areas represent the correlation coefficient between pain scoring systems. Correlation coefficients are calculated by Spearman rank correlation test using two-tailed P values.
Figure 5

Correlations between pain and patient characteristics. (A). Correlation between age and different pain scoring systems. (B). Correlation between K-L grade and different pain scoring systems. (C). Correlation between the WOMAC and different pain scoring systems. (D). Correlation between ROM and different pain scoring systems. Correlation coefficients are calculated by Spearman rank correlation test using two-tailed P values. Fitting curves with correlation coefficients are shown in the plots.
Correlations among inflammatory mediators. Correlation coefficients are calculated by Spearman rank correlation test using two-tailed P values and shown in the plot. The different colors and circle areas represent the correlation coefficients between inflammatory mediators. Red represents positive correlations, while blue represents negative correlations.