Title: Associations between proinflammatory cytokines in the synovial fluid and radiographic grading and pain-related scores in 47 consecutive patients with osteoarthritis of the knee

Authors:

Sumihisa Orita (sumihisa@silver.email.ne.jp)
Takeshi Mitsuka (mituka.m@fc-h.jp)
Takana Koshi (mocodo5@yahoo.co.jp)
Masayuki Miyagi (masayuki008@aol.com)
Gen Inoue (ginoue@faculty.chiba-u.jp)
Gen Arai (gen.arai.tateyama@hotmail.co.jp)
Tetsuhiro Ishikawa (ishikawa_tetsuhiro@yahoo.co.jp)
Eiji Hanaoka (eihana@aol.com)
Keishi Yamashita (kctha0311.-.-@ezweb.ne.jp)
Masaomi Yamashita (masaomiym@aol.com)
Yawara Eguchi (yawara_eguchi@yahoo.co.jp)
Tomoaki Toyone (toyone@med.teikyo-u.ac.jp)
Kazuhisa Takahashi (19501114@faculty.chiba-u.jp)
Seijit Ohtori (sohtori@faculty.chiba-u.jp)

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Author's response to reviews: see over
Response to the reviewers’ comments.

Response to Referee 1:
We thank you for your constructive comments. Our responses to your queries are given below.

(Response to the major compulsory revisions)

(Major compulsory revisions)

Q1-1
Brenner SS, et al. (Osteoarthritis Cartilage. 2004 Jun;12(6):469-75) published a similar study, but in addition involving synovial tissue, where no correlation between KL score or WOMAC score and IL-6 or TNFα was detected. This study and its discrepancies from the present study must be discussed.

A1-1
Thank you for your constructive suggestion. In the study, TNFα was undetectable, and thus, we cannot determine its exact correlation with the WOMAC score. Some previous studies reported the low detection of TNFα in the synovial fluid from the knees of OA patients, whereas other studies detected this cytokine in the synovial fluid. The present study detected TNFα in the synovial fluid using ELISA. The absolute value of TNFα was comparatively low, and this may have affected the results of the corresponding studies. We should investigate this correlation in future studies with a higher number of samples.

Regarding IL-6, previous studies detected IL-6 in synovial fluid samples, but it did not exhibit a significant correlation with the WOMAC score, indicating that no direct relationships exist between the local levels of typical mediators of inflammation and the clinical state of the disorder. However, regarding IL-6, we found a correlation only with the subscore of stiffness, which can be derived from the constructive degradation of the cartilage, and we also found no correlation with the subscore of pain. Their papers only examined the pain subscore, and thus, we cannot directly compare their work with our
findings.
We modified the manuscript appropriately to address these matters.

Q1-2
Hay CW et al, Am J Vet Res. 1997 Sep;58(9):1027-32 is a highly relevant study of IL-6 and TNF in OA in dogs and should also be discussed.

A1-2
Thank you for the constructive suggestion. The paper you referenced revealed a negative correlation between IL-6 activity and radiographic OA scores in dog OA models, and this finding coincides with the results of the present study. Increased IL-6 activity was associated with increased proteoglycan synthesis in the articular cartilage in dogs with experimental CCL transaction [Venn1993], and thus, IL-6 production is highest in the earlier stages of joint damage. Regarding TNFα, there are some controversies among studies. Some previous studies reported low levels of TNFα in the synovial fluid of OA patients, whereas other studies on experimental models reported its detection. These discrepancies may be attributable to the extremely low value of TNFα and the method of collecting and processing the synovial fluid. However, we can suggest that TNFα is related to OA pathology and clinical evaluations based on the present findings.

Q1-3
The numbers of patients in Table 1 do not add up correctly (16 men, 24 women, but a total of 47)

A1-3
We have edited the table to get rid of this discrepancy.

Q1-4
The lack of NGF detection may be due to a variety of factors including technical failure, as the authors acknowledge. In the absence of the appropriate control experiments (e.g., spiking synovial fluid with recombinant NGF to test whether there are fluid factors that lead to precipitation or inhibit detection; or use of a different assay, etc.) these negative results are not very meaningful. I would recommend to either provide convincing data demonstrating the reason for this lack of detection or to just remove the NGF aspect from the paper and focus on TNF and IL-6.

A1.4
The involvement of NGF in pain research is important; however, handling NGF is often complicated. Its experimental condition or distribution in the tissue may affect its detection. Considering its importance in pain research, we believe its possible existence should be considered even if it was not detected. However, because we could not detect NGF, we modified the manuscript by deleting Figure1(C).

Q1.5
The authors are correct that the lack of data from normal SF is a weakness. There are certainly ethical ways to obtain normal SF in an orthopedic environment (e.g., during otherwise indicated arthroscopy or surgery from non-OA knees). I would be more enthusiastic about this manuscript if the authors could provide data on normal SF for TNF and IL-6 for purposes of comparison.

A1.5
As you mentioned and as we have already described, it is important to have data on control patients. Clinical surgery or arthroscopy in healthy knees without disease is not feasible, and few previous studies presented this type of data. One study reported relatively low TNF\(\alpha\) levels in the synovial fluid in normal knees (Marks HP, Arthroscopy 2005; 21: 1342-1347.). Measurement of the other cytokines, IL-6 and NGF, was not possible during our investigation. Because IL-6 and TNF\(\alpha\) levels are elevated in the early phase of knee
injuries, it will be important to measure their levels in normal knees. Regarding NGF, its involvement in pain remains to be elucidated, and other studies reporting NGF levels could not be located. We have discussed this matter in the revised manuscript.

**Q1-6**

*There are no data on disease duration. It is inferred that a higher KL score means longer standing disease. Can the authors provide temporal disease duration?*

**A1-6**

In Table 1, we added an additional item, “Disease duration.” As indicated, a higher KL score can indicate longer disease duration. We have included this point in the revised manuscript.

**Q1-7**

*The Discussion needs to be more concise*

**A1-7**

The discussion section of the revised manuscript has been modified in accordance with the constructive comments of all 4 reviewers.

*(Response to Minor comments)*

**Q1-8**

*There are still some rough edges in terms of English language use and grammar.*

**A1-8**

As I am not a native English speaker, I used an English editing service; however, some aspects of English usage and grammar may have been uncorrected. Therefore, I have resubmitted the revised manuscript and this letter to another editorial service.
Q1:9
Caption to presumed Fig. 3 is labeled incorrectly as Fig. 2.

A1:9
We apologize for this error, which occurred due to the removal of the original Figure 2. This has been corrected in the revised manuscript.

Response to Referee 2:
We thank you for the constructive suggestions. Please find below our responses to your queries.

Q2:1
The strengths of this paper are the fact that synovial fluid and not serum cytokines are being measured, as these are most proximal to the disease.

A2:1
We appreciate your comment. As you rightly mentioned, measuring the contents of the synovial fluid is worth investigating because it could be related to OA pathogenesis.

Q2:2
The weaknesses of this paper are: its lack of description of the kits/methods used for measurement of the cytokines, the CVs of the assays and level of detection (critical in case of NGF where nothing was measurable), the number of freeze thaws of the samples or how quickly the samples were stored and at what temperature after they were obtained. One presumes the synovial fluids were direct aspirates and not obtained by lavage (this should be clarified in the text).

Q2:2
We would like to address the suggested items individually.
The CVs for the each measured (%) were as follows:
TNFα, 5.8 ± 1.2% and IL-6, 4.2 ± 0.18%

The CV of NGF was not calculated because NGF was not detectable.

Levels of detection (already described in the manuscript):

TNFα, 0.5 pg/ml; IL-6, <0.70 pg/ml; and NGF, <1 pg/ml

Regarding the samples:

We avoided freeze-thaw cycles. As described in the manuscript, samples of synovial joint fluid were collected with a syringe and needle in our outpatient clinics by experienced orthopedic physicians, and these were immediately stored at -70°C until use. The samples were directly aspirated without lavage.

These points have been addressed in the revised manuscript.

Q2-3

The manuscript requires a native English speaker to edit it.

A2-3

The manuscript has been submitted to an English editing service.

Q2-4

The manuscript would also greatly benefit from major revision of the Discussion section. Currently it is rambling and the ideas should be consolidated and refined and focussed. The authors are encouraged to use the first two paragraphs to discuss the two main important findings, and an additional 1-3 paragraphs to discuss other issues such as limitations.

A2-4

Thank you for the constructive comment. We edited and modified the discussion section according to your comment.

Q2-5

The work of Dr. Wim van den Berg should be cited – this was mouse work showing that
TNFalpha inhibition in a mouse model of OA led to inhibition of synovitis, whereas, IL-1 inhibition led to inhibition of structural change. The authors work is compatible with these data.

Q2-5
Thank you for the suggestion. The work of Dr. Wim van den Berg reported that TNF\(\alpha\) inhibition in a mouse model of OA led to the inhibition of synovitis, and furthermore, IL-6 has a role in reducing cartilage destruction, and these findings add importance to the present findings. We have cited their study in the discussion section.

Q2-6
The WOMAC version and the validity of the Japanese version should be discussed since correlation of TNFalpha with symptoms is a major finding in this paper.

A2-6
The WOMAC score used in the present study is translated from the English version, and thus, we believe that its validity is similar as that reported in a previous study on Asian OA patients [Thumboo2001]. We discussed this matter in the manuscript.

Q2-7
The authors should consider semi-quantitative scoring the radiographs for osteophyte and joint space narrowing radiographic features using the OARSI atlas then evaluating for correlation with TNFalpha and IL-6. KL tends to obscure findings because it inappropriately combines the two separate pathophysiological processes of osteophyte and joint space narrowing. The results can sometimes be that a cytokine correlates with one feature and not another, or in one direction. Using a cohort of consecutive OA patients seen in several practices, the authors investigated whether synovial fluid levels of TNF\(\alpha\), IL-6 or NGF correlated with parameters of radiographic disease progression and pain/disability. While NGF could not be detected in any of the samples, a negative correlation between IL-6
and the KL score, and a positive correlation between TNFa and the WOMAC score, and a negative correlation between IL-6 and the stiffness subscore are reported. These results are of some interest, although not entirely new. with one feature and another direction with the other radiographic feature, leading to little or confusing results with KL.

A2-7
We appreciate your constructive suggestion. As you indicated, KL grading is an inaccurate grading method consisting of 2 parameters, osteophytes and joint space narrowing, and its arbitrary scoring is dependent on the observer. Evaluating the correlation with the more quantitative method OARSI atlas is extremely important; however, we believe this is beyond the scope of this manuscript, and we may complicate our argument by addressing this matter. Thus, we addressed this matter in the limitations section.

Q2-8
Figure 1C should be deleted as NGF was undetectable. More discussion regarding this is required. Has anyone measured NGF is synovial fluid? If so, what is the difference between their method and the one used here?

A2-8
We deleted Figure 1(C) and described its absence in the legend.
Regarding the existence of NGF, we discussed the difficulties in detecting NGF experimentally in the Discussion.

Response to Referee 3:
We thank you for your valuable comments. We have responded to your queries below.

(Response to the Minor Essential Revisions)
Q3-1
Table 1 shows inconsistencies in the number of patients in each group. How could you
obtain 16 total men with 21 KL scores even if a patient did not provide synovial fluid?

A3-1
We apologize for this error, which occurred due to the recalculation of additional data and because the number of the KL 4 patients did not include the patients with no synovial fluid. We have recalculated the number and have also made appropriate revisions in the table and manuscript accordingly.

Q3-2
Figure 1, legend. Again, did you measure cytokines concentration in synovial fluid from 37 or from 47 or 44 patients? Please clarify this in all text, table and legend.

A3-2
This was also mistyped. The correct number is 47. We apologize for this typographical error.

Q3-3
Figure 1. The undetectable levels of NGF need not to be shown on a graph. In the same way, the non-significant differences between groups need not to be written on the graph (A) but mentioned in text and legend.

We deleted the description regarding the non-significance of TNFα and deleted Figure 1(C).

Q3-4
Figure 3. The presentation of results should be simplified. The correlation coefficients and p-values could be presented as a table where significant correlations would be highlighted.

A3-4
We added Table 2, which shows the statistical data of Figure 2 (the original Figure 3 was renamed Figure 2 because the original Figure 2 was deleted).
Q3-5
Results, second paragraph. Please provide the exact p-values when you found statistical significance.

A3-5
We provided the exact p values as requested.

(Response to the Discretionary Revisions)
Q3-6
Results, third paragraph. Title is in italic.

A3-7
We have unitalicized the subheading as requested.

Q3-7
Figures legend. The legend of figure 3 is listed as figure 2.

A3-7
We apologize for this error, which occurred due to the removal of the original Figure 2. This has been corrected in the revised manuscript.

Response to Referee 4:
We thank you for the instructive comments. Below, please find our responses to your queries.

(Response to the Major Compulsory Revisions)
Q4-1
It is not clearly stated what is the hypothesis of this study
Our hypothesis was that correlations between these cytokines and clinical evaluations in OA patients are possible. We have added this point towards the end of the Background.

In the methods section is not described if the authors centrifuge the synovial fluid prior use. Centrifugation details have been provided in the Methods as follows: Centrifugation before use: 15 min at $1000 \times g$ (TNF$\alpha$ and IL-6) or 20 min at $2000 \times g$ (NGF).

In table 1, why do the authors think is necessary to give information about the total mean age of the patients? The KL grading score should also be shown in frequencies to compare between the classes. How about the information about disease duration and WOMAC score?

Table 1 presents general information regarding patient demographics such as gender and age. In particular, knee osteoarthritis patients tend to be women, and thus, we considered it important to present the gender distribution and total number of the patients. We have included the information regarding disease duration in Table 1.

In the paper the authors always compare the cytokine levels between KL and WOMAC grades but in the table 1 they separate the patients according to gender. It does not make any sense to me not to have information about these subpopulations.

As described previously, Table 1 gives general information. What is important in this study
is the total number of the patients because we did not take gender into account.

Q4-5
I could not understand the rationelle for the correlation between IL-6 and TNFa. What do the authors hope to accomplish?

A4-5
As indicated, the correlation between these 2 cytokines is not essential to the present study, and thus, we deleted Figure 2 and related descriptions in the revised manuscript.

(Minor Essential Revisions)

Q4-6
What statistical program was used for calculation of the statistical data?

A4-6
We used PASW statistics ver. 18 (SPSS Inc (IBM), NY) for statistical evaluation in the present study.

Q4-7
In panel A, when the authors say that TNFa showed no significant correlation they probably want to say that there are no statistical difference between groups. They also say that there is a tendency, however I do not believe that this is the case. In the figure legend they should also mention what happens in group C.

A4-7
As shown in Figure 1(A), the concentration of TNFa was significantly lower in KL grades 2 to 4 than in KL grade 1 after the recalculation. We have modified the caption and legend of the figure accordingly. Regarding NGF, we deleted Figure 1(C) in response to another reviewer’s suggestion.

Q4-8
In the Figure 3 legend, the caption is Figure 2 by mistake

A4·8
Thank you for pointing out this error; we apologize for it. This error has been corrected in the revised manuscript.

(Response to the Discretionary Revisions)
Q4·9
I believe that it should be interesting to access the serum levels of IL-6, TNF and NGF to compare both with the synovial fluid findings and the scores.

A4·9
We agree that these data are essential for clarifying the pathogenesis of OA.
In the discussion section, we discussed this matter as a future priority.

Q4·10
In the introduction section, the role of IL-6 and TNFα in the pathogenesis of OA should be clarified.

A4·10
We moved the description of the role of cytokines in OA pathogenesis from the Discussion to the Background.
List of revisions made:

General Information:
The revised manuscript was checked by native English speaker, and changes added by them are displayed in violet using “recording changes” of Microsoft Word. Minor changes such as plural, article, simple rewording (ex. “three” to “3”, “found” to “detected”, “while” to “whereas”) and spelling are not described in the following list.

Title: We changed the title to “Associations between proinflammatory cytokines in the synovial fluid and radiographic grading and pain-related scores in 47 consecutive patients with osteoarthritis of the knee” considering the comments of the reviewers

Abstract
Background (Abstract):
Page 3, line 3: We deleted “The exact mechanism for knee pain in OA remains to be unclear.” and added “of knee pain in osteoarthritis (OA)”.
Page 3, line 4: We added “of knee pain in osteoarthritis (OA)”.
Page 3, line 5: We replaced “in” with “of the”.
Page 3, line 6: We inserted “alpha”.
Page 3, line 7: We added “and these cytokines” and deleted “which” and replaced “considered” with “believed”
Page 3 line 10: We deleted “osteoarthritis”

Methods (Abstract):
Page 3, line 13: We inserted “levels”.
Page 3 line 14: We deleted “ELISA”, and “OA”, and then inserted “with OA”

Results (Abstract):
Page 3, line 18: We deleted “from most OA patients”
Page 3, line 20: We inserted “a” and deleted “Correlation between TNFα and IL-6 showed a
moderate significant.”

Page3, line 21: We replaced “found” with “observed”, and “between” with “in”.
Page3, line 22: We replaced “of” with “between”, and “its” with “their”.
Page3, line 23: We replaced “showed” with “exhibited a”

**Conclusions (Abstract)**:

Page4, line 6: We replaced “can be” with “are”, and “grading” with “grade”, and deleted “the”.
Page 4 line 9: We deleted “the”, and inserted “were”, and “correlated”
Page4 line 10: We deleted “may”.
Page4 line 11: We replaced “osteoarthritis” with “osteoarthritic”, and deleted “respectively, and inserted “is”.
Page4 line 12: We inserted “whereas” and deleted “formal and inserted “is correlated with joint”, and deleted “change and”, “of joint”, and “NGF was undetectable in synovial fluid”.
Page4, line 13: We deleted “, which induces the importance to develop experiments in a different way in a future study”

**Background**

Page 4, line 1: We replaced “with” with “characterized by the”

Page5, line 5: We added “Proinflammatory cytokine mediators have been reported to contribute to OA pathogenesis by increasing cartilage degradation and inducing hyperalgesia by a number of direct and indirect actions. TNFα activates sensory neurons directly via its receptors and initiates a cascade of inflammatory reactions through the production of ILs. IL-6 is reported to have a complex role in OA pathogenesis by initiating inflammatory responses such as the production of tissue inhibitors of metalloproteinase, which may act to limit cartilage damage through negative feedback. NGF is reportedly upregulated in human osteoarthritic chondrocytes and synovial fibroblasts, suggesting their important role in the pathology of OA. Another report indicated that NGF is an important mediator of OA pain because its antagonistic effect resulted in analgesia in a murine OA model” in response to Q4-10.
We replaced “the” with “Additionally, a”.

We replaced “studies” with “study”, “the” with “a”, and deleted “the”.

We inserted “images of knee” and replaced “osteoarthritis” with “OA”.

We deleted “however, the association between the cytokines and radiographic features or pain is unclear”

We added “Under the hypothesis that relationship between these cytokines and clinical evaluations in OA patients are possible,” in response to Q4-1.

**Methods**

*Patient selection*
Page 6, line 8: We replaced “suffering from” with “with”
Page 6, line 9: We deleted “Subjects for”
Page 6, line 10: We replaced “comprised” with “consisted of”

*Synovial fluid sampling and cytokine assay*
Page 6, line 19: We added “The samples of synovial fluid were aspirated directly without lavage, “ in response to Q2-2.
Page 6, line 19: We deleted “were”.
Page 6, line 19: We added “We avoided freeze-thaw cycles.” in response to Q2-2.
Page 6, line 21: We deleted “Quantification of ” and inserted “quantification”
Page 6, line 22: We inserted “enzyme-linked immunosorbent assay” and deleted “(ELISA)”.
Page 7, line 5: We deleted “respectively”, and “the”.

*Grading of OA and pain evaluation*
Page 7, line 10: We inserted “as follows:”.

*Statistical analysis*
Page 6, line 4: We added “(PASW statistics ver18 (SPSS Inc (IBM), Somers, NY))” in response to Q4-6.
Results

Patient demographics
Page 8, line 10 and 13: We changed the number to correct ones: “50” for “47”, and “47” for “44”
Page 8, line 13: We added “Disease duration increased as the KL scores increased.” in response to Q1·6.

Concentrations of the proinflammatory cytokines
Page 8, line 18: We added “(CV value(%):TNF-α 5.8 ± 1.2, IL-6 4.2 ± 0.18%, NGF: unable to be calculate)” in response to Q2·2.
Page 8, line 21: We deleted “no significant distribution, while the lower the KL grade the more the concentrations tended to increase” and replaced with “was significantly lower in KL grade 2 to 4 than in KL grade 1” in response to Q4·7.
Page 9, line 1: We added “pg/ml”.
Page 9, line 2: We deleted “of IL-6 showed a”, and “decreased” and inserted “IL-6” and “was”, “lower”.
Page 9, line 3: We deleted “in comparison with that” and inserted “than”, “grades” and “and 2”.
Page 9, line 4-5: We inserted “pg/ml”.
Page 9, line 5: We deleted “p < 0.05), and it also showed a significant decrease in comparison with that in the KL2” and inserted “vs. KL1” and “vs. KL2”.
Page 9, line 5: We replaced “p < 0.05” with the exact p value, “p = 0.032” in response to Q3·5.
Page 9, line 6: We replaced “p < 0.05” with the exact p value, “p = 0.036” in response to Q3·5.
Page 9, line 6: We changed the sentences as “NGF was not detected in any sample”
Page 9, line 7: We deleted “in any of the samples of the present study (Figure 1(C)) Figure 2 shows the correlation between the concentrations of two detectable cytokines: TNFα and IL-6. There was a moderately significant correlation between the concentrations of these cytokines (r = –0.4, p = 0.009).”.

Results

Patient demographics
Page8, line 10 and 13: We changed the number to correct ones: “50” for “47”, and “47” for “44”.
Page8, line 13: We added “Disease duration increased as the KL scores increased.” in response to Q1·6.

Concentrations of the proinflammatory cytokines
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Page9, line 2: We deleted “of IL-6 showed a”, and “decreased” and inserted “IL-6” and “was”, “lower”.
Page9, line 3: We deleted “in comparison with that” and inserted “than”, “grades” and “and 2”.
Page9, line 4-5: We inserted “pg/ml”.
Page9, line 5: We deleted “p < 0.05), and it also showed a significant decrease in comparison with that in the KL2” and inserted “vs. KL1” and “vs. KL2”.
Page9, line 5: We replaced “p < 0.05” with the exact p value, “p = 0.032” in response to Q3·5.
Page9, line 6: We replaced “p < 0.05” with the exact p value, “p = 0.036” in response to Q3·5.
Page9, line 6: We changed the sentences as “NGF was not detected in any sample”.
Page9, line 7: We deleted “in any of the samples of the present study (Figure 1(C)) Figure 2 shows the correlation between the concentrations of two detectable cytokines: TNFα and IL-6. There was a moderately significant correlation between the concentrations of these cytokines (r = –0.4, p = 0.009).”.
**Correlation between WOMAC score and cytokine concentration**

Page 9, line 12: We changed “Figure 3” to “Figure 2”.

Page 9, line 13: We inserted “shows”

Page 9, line 18: We added “Table 2 shows the exact statistical values.”.

**Discussion:**

Page 9 line 21: We added “The present study examined whether inflammation plays a substantial role in the development of pain in OA.”

Page 9 line 7: We added “The WOMAC score used in the present study is translated from the English version, and thus, we believe that its validity is similar as that reported in a previous study in Asian OA patients”

**Evidence of proinflammatory cytokines in the synovial fluid samples**

Page 10 line 11: We deleted “The proinflammatory cytokine mediators have been reported to contribute to OA pathogenesis by increasing cartilage degradation and inducing hyperalgesia by a number of direct and indirect actions. TNFα activates sensory neurons directly via its receptors and initiates a cascade of inflammatory reactions through the production of ILs [9, 10].” to move to the background, and added “The result of the present study are comparable with those of previous studies reporting that TNFα is related to synovitis and that IL-6 has a role in reducing cartilage destruction using a mouse zymosan-induced OA model. These studies add importance to the present findings that TNFα inhibition may improve the WOMAC score and that increased IL-6 activity in earlier phases of OA prevents cartilage destruction. Brenner et al performed a similar experiment using the synovial membrane and fluid from OA patients and reported that TNFα was undetectable in their synovial fluid and that there were no correlations between the IL-6 levels and WOMAC pain subscores. Regarding TNFα, there are some controversies among studies. Some previous studies reported low levels of TNFα in the synovial fluid of OA patients, whereas other studies including experiment models reported its detection. The present study detected TNFα in the synovial fluid. These discrepancies may be attributable to the extremely low value of TNFα and the method of collecting and processing synovial fluid. However, we can suggest that TNFα is related to OA pathology and clinical evaluations based on the present findings, although we need additional investigation with a greater number of samples.”.

Page 11, line 7: We deleted “IL-6 is reported to have a complex role in OA pathogenesis by initiating inflammatory responses such as the production of tissue inhibitors of metalloproteinase(MMP)-1, which may act to limit cartilage damage through negative feedback [12]. NGF is reported to be upregulated in human osteoarthritic chondrocytes and synovial fibroblasts, suggesting their important role in the pathology of OA [9, 13]. Other
reports indicate that NGF is an important mediator of OA pain because its antagonism showed an analgesic effect in a murine osteoarthritis model [14].”, and added “As described in the Background, NGF is considered an important factor in OA pathogenesis, and”.

Page 12 line 2: We added “obtained”

Page 12, line 6: We replaced “wound be disadvantageous to exist in” with “, and this is disadvantageous for its existence in”.

Page 12, line 8: We deleted “regarding”.

Page 12, line 9: We replaced “besides” with “in addition to examining the”

Page 12, line 12: We replaced “And then” with “More over”

Page 12, line 14: We added “Evaluating the levels of these cytokines in the synovial fluid from completely normal knees is important but also difficult for ethical reasons. Alternatively, we can evaluate the cytokine levels in the synovial fluid from knees with injuries such as anterior cruciate ligament (ACL) injuries; however, the data may not be useful because proinflammatory cytokine levels are elevated in response to any degradation or injury in the joint. However, we can indicate those partly from a previous study. One study evaluating the cytokine levels in the knees of patients with chronic ACL deficiency reported that TNFα concentrations were lower in the injured knees than in the normal knees, and the TNFα levels reported in that study were also low compared with those in the present study. Because IL-6 and TNFα levels are elevated in the early phase of knee injuries, it will be important to measure their levels in normal knees.” in response to Q1-5.

Correlation between cytokine concentration and Kellgren–Lawrence grading

Page 13 line6: We deleted “TNFα and IL-6 cytokine levels in the synovial fluid were higher in patients with RA than in those with OA, and”.

Page 13 line 7-8: We deleted “synovial fluid”, and added “in the synovial fluid”

Page 13, line 9: We replaced “RA” with “rheumatoid arthritis”.

Page 13 line16: We deleted “IL-7 secreted from human articular cartilage may contribute to cartilage destruction in joint diseases including OA [19], and IL-1-receptor polymorphisms are reported to contribute to disease severity in knee OA related to the KL grading [20]”

Page 13 line22: We added “Increased IL-6 activity has been reported to be associated with increased proteoglycan synthesis in articular cartilage in dogs with experimental CCL transaction, and thus, IL-6 production is highest in the earlier stages of joint injury.”

Page14, line 5: We replaced “Taking these reports into account” with “Considering these reports”

Page14, line 6: We deleted “that the earlier the score of the KL grade the more”, and added “occurs at the greatest rate in earlier KL grade”

Page 14 line9: We deleted “Thus IL-6 may be a marker molecule in early OA patients, in
addition to others such as MMP-3 and homocysteine [15, 24, 25].”

Page 14 line 12: We deleted “Generally, synovial fluid concentrations of mediators do not reflect direct cell-to-cell interactions, autocrine or paracrine pathways, and may only partially reflect local concentrations on the surface of adjacent bone, which is often covered by pannus. Furthermore, cytokine levels in synovial fluid vary with time [26]. It must be noted that cytokine determinations in the present study were performed using immunological methods, resulting in data not reflecting the bioactivity of these mediators. We should take the bioactivity of the molecules into account as an explanation for the fluctuation in the present study.”

Correlation between cytokine concentration and the WOMAC score
Page 45 line 23: We added “However, IL-6 was not correlated with the WOMAC score excluding the subscore of stiffness, which indicates that IL-6 primarily affects the progress of the degeneration of the joint cartilage in OA that leads to stiffness of the joint. Furthermore, we found a correlation only with the subscore of stiffness, which can be derived from the constructive degradation of the cartilage, and we found no correlation with the subscore of pain. A previous paper reported a negative correlation between IL-6 activity and radiographic OA score in dog OA models, and this coincides with the results of the present study.” in response to Q1-1, Q1-2, and Q2-5.

Page 15 line 8: We deleted “Considering other aspect, an animal study supports the finding that TNFα increased the proportion of neurons that express the TRPV1, a receptor for acid or noxious heat, in cultured dorsal root ganglia neurons, and may thus contribute to the generation of inflammation-evoked hyperalgesia in a rodent model of antigen-induced arthritis [27]. To further investigate the correlation of TNFα and pain, inhibiting the cytokine may be helpful. A previous study suggested that intraarticular injection of an anti-TNFα agent into osteoarthritic hands may provide an analgesic effect in humans, while the overall result did not indicate a significant improvement for an adequate number of patients [28].” to simplify the manuscript.

Page 15 line 16: We deleted “However, IL-6 does not correlate with the WOMAC score except for the subscore of stiffness, which indicates that IL-6 may mainly affect the progress of the degeneration of joint cartilage in OA that leads to stiffness of joint”.

Page 15 line 18: We deleted “The result also supports the finding that IL-6 showed moderately significant negative correlation with radiographic evaluation of the OA joint. A previous study has already indicated the importance of increased IL-6 in OA patients, concluding that synovial fluid IL-6 levels may help to classify OA patients before any categorization of end-stage OA [29].

Inflammatory cytokines are suggested to be good indicators of the degree of rheumatoid arthritis activity, and are not always investigated in OA patients. The increased IL-6 levels seen in the early KL grades may predict the OA activity of the joint: however, further
investigations including other cytokines should be made in future studies: IL-7 is such a cytokine reported to contribute to cartilage destruction in OA [19]. MMP-1 and -3 are reported to induce cartilage degeneration in normal cartilage in beagle dogs [30]. ”

Page 16 line 15: We added “Precise evaluation using more quantified grading such as OARSI atlas should be performed in future studies”.

Page 16 line 19: We added “We should evaluate knees with other injuries or degradations in future studies.” in response to A1-5.

Page 16 line 20: We added “Lastly, we only examined synovial fluid. It will be important to assess the serum levels of these cytokines and compare them with both the levels of cytokines in the synovial fluid and the grading scores.” in response to A4-9.

References:
We changed the order of the references according to the modifications made and added the following citations:


18. van de Loo FA, Joosten LA, van Lent PL, Arntz OJ, van den Berg WB: Role of interleukin-1, tumor necrosis factor alpha, and interleukin-6 in cartilage proteoglycan metabolism and destruction. Effect of in situ blocking in murine antigen- and


**Figures and figure legends**

**Table 1:** We recalculate the data and added, “..., and thus, the numbers in parenthesis indicate the effective number for the present study.”
Additionally, we added “Disease duration” and “**Described as mean ± SEM” in response to another reviewer’s comments.

**Table 2:** We added Table 2, “Statistical data of Figure 2,” to present the exact statistical values.

**Figure 1:** We deleted the label “Non-significant (NS.) among the groups” and added “*: P < 0.05 vs. KL 1” in Figure1(A) in response to reviewer questions and deleted Figure 1(C).

**Figure 1 legend:** We added, “NGF was undetectable in all patients.”

**Figure 2 and its legend:** We deleted the figure along with its legend.

**Figure 3:** We have now presented this as Figure 2.