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

Sumihisa Orita¹,⁴§, Takana Koshi², Takeshi Mitsuka³, Takana Koshi², Masayuki Miyagi⁴, Gen Inoue⁴, Gen Arai⁴, Tetsuhiro Ishikawa⁴, Eiji Hanaoka⁵, Keishi Yamashita³², Masaomi Yamashita³², Yawara Eguchi⁴, Tomoaki Toyone⁶, Kazuhisa Takahashi⁴, Seiji Ohtori⁴

¹Department of Orthopaedic Surgery, Chiba Rosai Hospital, Chiba, Japan,
²Department of Orthopaedic Surgery, Seirei Sakura Citizen Hospital, Chiba, Japan,
³Department of Orthopaedic Surgery, Social Insurance Funabashi Central Hospital, Chiba, Japan,
⁴Department of Orthopaedic Surgery, Graduate School of Medicine, Chiba University, Chiba, Japan
⁵Department of Orthopaedic Surgery, Chiba Social Insurance Hospital, Chiba, Japan,
⁶Department of Orthopaedic Surgery, Teikyo University Chiba Medical Center, Chiba, Japan
$\text{Corresponding author}$

E-mail addresses:

SO: sumihisa@silver.email.ne.jp  TK: mocodo5@yahoo.co.jp  TM: mituka.m@fc-h.jp

TI: ishikawa_tetsuhiro@yahoo.co.jp

GI: ginoue@faculty.chiba-u.jp

KA: gen.arai.tateyama@hotmail.co.jp

MM: masayuki008@aol.com

MM: masayuki008@aol.com

YE: yawara_eguchi@yahoo.co.jp

KE: toyone@med.teikyo-u.ac.jp

SO: sohtori@faculty.chiba-u.jp

TK: 19501114@faculty.chiba-u.jp

TK: 19501114@faculty.chiba-u.jp
Abstract

Background
The exact mechanism for knee pain in OA remains to be unclear. One of the sources of knee pain in osteoarthritis (OA) is considered to be related to local chronic inflammation of the knee joints, involving the production of inflammatory cytokines such as tumor necrosis factor alpha (TNFα), interleukin (IL)-6, and nerve growth factor (NGF) in the synovial membrane, and these cytokines are believed to promote pathological OA. In the present study, correlations between proinflammatory cytokines in the knee synovial fluid and radiographic changes and functional scores and pain scores among OA patients were examined.

Methods
Synovial fluid was harvested from the knees of 47 consecutive OA patients, and the levels of TNFα, IL-6, and NGF were measured using enzyme-linked immunosorbent assays. Knees with OA were classified using the Kellgren-Lawrence (KL) grading (1–4). The Western Ontario and McMaster University Osteoarthritis Index (WOMAC) was used to assess self-reported physical function, pain, and stiffness.

Results
TNFα and IL-6 were detectable in knee synovial fluid from most OA patients, whereas NGF was undetectable. TNFα was not correlated with the KL grade, whereas IL-6 had a significantly negative correlation. Correlation between TNFα and IL-6 showed a moderate significant. We observed differences in the correlations between TNFα and IL-6 with WOMAC scores and their subscales (pain,
stiffness, and physical function). TNFα exhibited a significant correlation with the total score and its 3 subscales, whereas IL-6 exhibited a moderately significant negative correlation only with the subscale of stiffness.

**Conclusions**

The present study demonstrated that the concentrations of proinflammatory cytokines are correlated with KL grades and WOMAC scores in knee OA patients. Although TNFα did not have a significant correlation with the radiographic grading, although it did it was significantly associated with the WOMAC score. IL-6 had a significant negative correlation with the KL grading, whereas it had only a weakly significant correlation with the subscore of stiffness. These 2 cytokines were moderately correlated, and the results suggest that these cytokines play a role in the pathogenesis of synovitis in osteoarthritic knees in different ways: TNFα is correlated with pain, whereas IL-6 is correlated with joint function, and NGF was undetectable in synovial fluid, which induces the importance to develop experiments in a different way in a future study.

**Background**

Knee osteoarthritis (OA) is a common chronic degenerative disease characterized by the loss of articular cartilage components due to an imbalance between extracellular matrix destruction and repair [1]. The entire joint structure is affected, including the synovial membrane and subchondral bone, and OA can be recognized as an irregularity and deformity of joint spaces in radiographic images. Its main clinical sign is joint pain, which not only contributes to functional limitations and reduced quality of life but is also the leading cause of impairment of mobility in the elderly population [2].
Although the exact mechanism of knee pain in OA is unclear, one of the sources is considered to be related to local chronic inflammation of the knee joints, involving the production of inflammatory cytokines in the synovial membrane, such as tumor necrosis factor alpha (TNFα), interleukin (IL)-6, and nerve growth factor (NGF), which are generally considered to promote pathological OA [3–5]. 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 [6, 7]. 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 [8]. NGF is reportedly upregulated in human osteoarthritic chondrocytes and synovial fibroblasts, suggesting its important role in the pathology of OA [6, 9]. Another report indicated that NGF antagonism is an important mediator of OA pain because its antagonistic effect resulted in analgesia in a murine OA model [10].

Thus, investigations of the dynamic states of these cytokines should be conducted. Additionally, a previous study has indicated a strong association between the radiographic images of knees with OA and with knee pain [11]; however, the association between the cytokines and radiographic features or pain is unclear. Under the hypothesis that relationships between these cytokines and clinical evaluations in OA patients are possible, the present study evaluated the association between
proinflammatory cytokines in the synovial fluid from the knees of OA patients and radiographic severity and pain scale scores.

Methods

Our Institutional Review Board approved the present study. We obtained informed consent from each participating patient.

Patient selection

The present study included adult patients with knee pain who visited each our facility for clinical consultation from between August 2009 toand March 2010. The present study consisted of patients with knee OA diagnosed using the American College of Rheumatology criteria for OA without who had not received any preceding-prior [A2]treatment. Patients with clear clinical evidence of any involvement of trauma or with prior treatment, or other orthopedic diseases including spinal disorders with causing radicular pain in the legs were excluded. Patients diagnosed with rheumatoid arthritis based on physical examination and laboratory data were also excluded.

Synovial fluid sampling and cytokine assay

With the approval of patients, samples of synovial joint fluid were collected using a syringe and needle in our outpatient clinics by experienced orthopedic physicians. The samples of synovial fluid were aspirated directly without lavage and immediately stored at -70°C until use. We avoided freeze-thaw cycles were avoided. Cytokine quantification was performed using a double-antibody sandwich enzyme-linked immunosorbent assay for TNFα, IL-6 (R&D systems, Minneapolis, MN), and NGF
(Boster Biological Tec., Wuhan, China) without dilution according to the manufacturers’ protocols (centrifugation before use: for 15 min at 1000 × g (TNFα and IL-6) and for 20 min at 2000 × g (NGF)). The detection limits of the assays were 0.5 pg/ml for TNFα, <0.70 pg/ml for IL-6, and <1 pg/ml for NGF. All samples were assessed in duplicate.

5 Grading of OA and pain evaluation

Anteroposterior radiographs of the symptomatic knees were obtained. The X-ray beam was aimed at the lower pole of the patella and kept parallel to the joint surface. The grading of radiographs was scored by experienced orthopedic surgeons using the Kellgren-Lawrence (KL) grading scale as follows: grade 1, doubtful narrowing of joint space and possible osteophytic lipping; grade 2, definite osteophytes and possible narrowing of joint space; grade 3, moderate multiple osteophytes, definite narrowing of joints space, some sclerosis, and possible deformity of bone contours; and grade 4, large osteophytes, marked narrowing of joint space, severe sclerosis, and definite deformity of bone contours [12]. The functional status and pain level of each patient were evaluated using the Western Ontario McMaster University Osteoarthritis Index (WOMAC) score [13]. The index consists of 3 subscales: pain, stiffness, and physical function. A higher score on the WOMAC scale represents poorer function or greater pain. The data were arranged according to the KL grade for each cytokine, and the correlations between the cytokines were analyzed. Correlations between the cytokine concentrations and WOMAC score were also analyzed.

Statistical analysis
Statistical differences between the 2 groups were determined using the Mann-Whitney \( U \) test followed by Bonferroni’s correction for multiple testing, and the statistical significance among the groups was determined using the Kruskal-Wallis test.

The significance of correlations was determined by the Spearman’s rank correlation test (PASW statistics ver. 18 (SPSS Inc (IBM), Somers, NY)). A \( p \) value \(< 0.05\) was considered significant.

**Results**

**Patient demographics**

Table 1 shows the patient demographics. Among the 47 patients enrolled in the present study, we could not obtain any fluid from the knees of 3 patients with osteoarthritic knees classified as KL grade 4, and thus, we analyzed the other 44 samples. Disease duration increased as the KL scores increased.

**Concentrations of the proinflammatory cytokines**

Figure 1 shows the concentrations of proinflammatory cytokines in the synovial fluid of the knee joints in relation to the radiographic findings of these joints. Measurable levels of TNF\( \alpha \) and IL-6 were detected in all samples, whereas NGF was not detectable in any of the samples (CV value (%): TNF\( \alpha \), 5.8 ± 1.2; IL-6, 4.2 ± 0.18; NGF: unable to be calculated).

The concentration of TNF\( \alpha \) was significantly lower in KL grades 2 to 4 than in KL grade 1 with no significant distribution, while the lower the KL grade the more the concentrations tended to increase (KL 1, 6.5 ± 2.0 pg/ml (mean ± S.E.); KL 2, 3.6 ± 0.72 pg/ml; KL 3,
4.2 ± 0.48 pg/ml; and KL 4, 3.2 ± 0.86 pg/ml) (Figure 1A). The IL-6 concentration was significantly lower in KL grades 3 and 4 than in KL grades 1 and 2 (KL 1, 401.6 ± 33.2 pg/ml; KL 2, 292.6 ± 42.2 pg/ml; KL 3, 162.9 ± 46.5 pg/ml; and KL 4, 78.6 ± 62.8 pg/ml) ($p = 0.032$ vs. KL 1; $p = 0.036$ vs. KL 2, $p < 0.05$) (Figure 1B). NGF was not detected in any sample in any of the samples of the present study (Figure 1(C)). Figure 2 shows the correlation between the concentrations of two detectable cytokines: TNF-$\alpha$ 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**

Figure 2 shows the correlations between the detectable cytokines and the WOMAC score. Group A shows TNF-$\alpha$, and group B shows IL-6. TNF-$\alpha$ exhibited a moderately significant positive correlation with the total WOMAC score (A-1) and with each subscale (pain (A-2), stiffness (A-3), and physical function (A-4) ($p < 0.01$)). IL-6 exhibited a moderately significant negative correlation only with stiffness (B-3) ($p < 0.05$), whereas it did not exhibit any significant correlation with the other factors. Table 2 shows the exact statistical values are shown in Table 2.

**Discussion**

The present study examined whether inflammation plays a substantial role in the development of pain in OA. We demonstrated that TNF-$\alpha$ and IL-6 were measurable in the synovial fluid sampled from the knees of OA patients, whereas NGF was undetectable. TNF-$\alpha$ was not correlated with the KL grade, and IL-6
had a relatively significant negative correlation with KL grading. A moderate correlation between TNFα and IL-6 was found. Some differences were found between TNFα and IL-6 regarding their correlations with the WOMAC score and its subscales. TNFα exhibited a moderately significant correlation with the total score and its 3 subscales, whereas IL-6 exhibited a weakly significant negative correlation with the subscale of stiffness. The WOMAC scoring method used in the present study was 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 [14].

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

The results of the present study are comparable with those of previous studies reporting, which using a zymosan-induced mouse OA model, reported that TNFα is related to synovitis and that IL-6 has a role in reducing cartilage destruction using a mouse zymosan-induced OA model [15-18]. 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 membranes 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 [19]. Regarding TNFα, there are some controversies among studies. Some previous studies reported low levels of TNFα in the synovial fluid of OA patients [20-22], whereas other studies including experiment models reported its detection [23-25]. 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 investigations with a greater number of samples are needed.

Additionally, TNFα was also reported to not be regulated in the joints in late OA, and this is consistent with the finding of the present study [26].

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]. In addition, TNFα was also reported to not be regulated in the joints in late OA, and this is consistent with the finding of the present study [26].

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]. As described in the Background Introduction, NGF is considered an important factor in OA pathogenesis, and thus, it is important to discuss why NGF was not detected in the present study. We hypothesize that, excluding any technical errors, NGF production is insufficient for detection in the synovial fluid obtained from the knees of OA patients. According to a previous report, the mRNA
expression of neurotrophins including NGF and its receptors was confirmed in the synovial fluid and tissues of patients with OA, whereas NGF mRNA expression was low [27]. Furthermore, NGF is a basic protein, and this is disadvantageous for its existence in the relatively acidic milieu of the synovial fluid [28]. Another study reported the diagnostic usefulness of biopsied tissue rather than as opposed to the use of synovial fluid [26]. Thus, in addition to examining the synovial fluid, it may be important to investigate NGF expression in the subchondral tissue where inflammatory cytokines are reported to be produced [29]. Moreover, to investigate any correlation between proinflammatory cytokines including NGF, multivariable analysis among these cytokines should be performed in the future. 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 injured knees with injuries such as those with 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 infer their levels from a previous study. One study evaluating the cytokine levels in the knees of patients with chronic ACL deficiency deficiencies reported that TNFα–α 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 [Marks2005] [30]. 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.
Correlation between cytokine concentrations and KL grading

A previous study indicated that TNFα and IL-6 cytokine levels in the synovial fluid were higher in patients with RA than in those with OA, and the levels of TNFα in the synovial fluid were positively correlated with Larsen’s radiographic grading of bone destruction in rheumatoid arthritis patients, whereas no correlation between the concentration of TNFα and Dahlgren’s radiographic OA grade was found [31]. The report is consistent with some of the results of the present study regarding TNFα but not IL-6. The present study revealed that TNFα is not clearly correlated with joint degeneration as assessed by KL grading, whereas IL-6 is negatively correlated with KL grading, suggesting that IL-6 has an important role in OA progression. Generally, members of the IL family are reported to be related to the severity of cartilage destruction [32–33]: 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]. The present study indicated that IL-6 production might be increased in the early stage of joint destruction in OA patients. Other studies have reported that TNFα induces IL-6 upregulation, and thus, IL-6 may still be correlated with OA progression [34–36].

Increased IL-6 activity has been reported to be associated with increased proteoglycan synthesis in articular cartilage in dogs with experimental anterior cruciate ligament transaction [24], and thus, IL-6 production is highest in the earliest stages of joint injury.

Considering these reports, IL-6 may be related to the formal pathogenesis of OA, suggesting that active cartilage destruction occurs at the greatest rate in earlier KL grades.
In other words, late-stage KL grading may indicate the “burnt ruins” acquired after active inflammation where IL-6 is more directly involved than TNFα. 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].

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 concentrations and the WOMAC score**

The present study demonstrated that TNFα is significantly correlated with the WOMAC score including the subscores. 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 joint 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 scoring in dog OA models [20], and this coincides with the results of the present study.
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]. 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. 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].
The present study has some limitations. First, we did not examine the gene expression of each cytokine. Variations in several genes that regulate inflammation have been reported to be associated with the differential expression of inflammatory mediators [37–39], some of which have been associated with OA pathology [40–44]. Thus, further studies including genetic investigations are needed, especially particularly for NGF. Second, the obscurity of KL grading, which is based on an unclear definition of the joint space findings, could have affected the results of the present study. Precise evaluation using more quantified grading systems such as the OARSI atlas should be performed in future studies. Third, we could not examine normal knees because it may be technically difficult and ethically improper to obtain control synovial fluid from intact knee joints. We should evaluate knees with other injuries or degradations in future studies. Lastly, we only examined the 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.

Conclusions
The present study demonstrated that the concentrations of proinflammatory cytokines can be correlated with the KL grading grades and WOMAC scores of knee OA patients. TNFα did not have a significant correlation with the radiographic grading, whereas it did with the WOMAC scoring. IL-6 had a significant negative correlation with KL grading, whereas it had only a weakly significant correlation with the subscore of stiffness. These 2 cytokines were moderately correlated, and the results suggest that these cytokines play a role in the pathogenesis of synovitis in osteoarthritic knees in different
ways, TNFα is correlated with pain, whereas IL-6 is correlated with joint function. NGF was undetectable in the synovial fluid, illustrating the need for differently designed experiments in future studies.

5 Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
SO designed and performed all of the experiments, analyzed data, and drafted the paper. TK, TM, TK, MM, GI, GA, TI, EH, KY, MY, and YE harvested synovial fluid in their outpatient clinic and prepared for the performed experiments. TT, KT, and SO supervised the project and edited the manuscript. All authors contributed to data interpretation and have read and approved the final manuscript.

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Figure legends

**Figure 1**
Concentrations of proinflammatory cytokines in the synovial fluid from the knee joints of 47 patients with OA in relation to the radiographic findings of these joints grouped according to the KL grade. NGF was undetectable in all patient samples. (A) The concentration of TNFα exhibited no significant correlation, whereas with KL grading, although there was a tendency for the concentration to be increased TNFα concentrations at lower KL grades. (B) The concentration of IL-6 exhibited a significant decrease was significantly decreased in KL grades 3 and 4 compared with those in KL grades 1 and 2.
**Figure 2**
Correlation between the concentrations of detectable cytokines (TNFα and IL-6). There was a moderately significant correlation between the concentrations of these 2 cytokines.

**Figure 23**
Correlations between the detectable cytokines and the WOMAC score. Group A shows TNFα, and group B shows IL-6. TNFα exhibited a moderately significant positive correlation with the total WOMAC score (A-1) and with the each subscale (pain (A-2), stiffness (A-3), and physical function (A-4) (p < 0.01)). IL-6 exhibited a weakly significant negative correlation only with stiffness (B-3) (p < 0.05), whereas it exhibited no correlation with the other subscales.
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<th>28 (26)</th>
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<td>4</td>
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<td>7 (5)*</td>
<td>12 (9)*</td>
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*No fluid was obtained from 3 patients (1 man and 2 women), and thus, the numbers in parenthesis indicate the effective numbers for the present study.

**Described as mean ± SEM
Table 2 – Statistical data of Figure 2

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<td>0.0038</td>
<td>-0.16</td>
<td>0.89</td>
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</table>
Figure 1
Figure 2

(A) TNFα

(A-1) WOMAC Score (WS) Total

\[ r = 0.69, P < 0.01 \]

(A-2) WS-Pain

\[ r = 0.57, P < 0.01 \]

(A-3) WS-Stiffness

\[ r = 0.56, P < 0.01 \]

(A-4) WS-Physical function

\[ R = 0.64, P < 0.01 \]

(B) IL-6

(B-1)

\[ r = -0.18, P = 0.09 \]

(B-2)

\[ r = -0.23, P = 0.31 \]

(B-3)

\[ r = -0.48, P < 0.05 \]

(B-4)

\[ r = -0.16, P = 0.89 \]