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# Th17 and IL-17 exhibit higher levels in osteonecrosis of the femoral head and have a positive correlation with severity of pain

## Th17 i IL-17 osiągają wyższe stężenia w przebiegu martwicy głowy kości udowej i są dodatnio skorelowane z nasileniem bólu

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### Abstract

**Introduction:** Synovitis associated with osteonecrosis of the femoral head (ONFH) is responsible for several clinical symptoms. However, the mechanisms underlying synovitis and the inflammatory environment remain unclear. This study analysed the proinflammatory mediation expression of IL-17 and Th17, which perform key functions in regulating inflammatory processes in the inflamed synovium and peripheral blood in ONFH.

**Material and methods**: Synovial fluid from the hips of 23 patients and five controls was collected during surgery, and peripheral blood samples were obtained from 34 patients and nine controls. The expression of IL-17 in the synovium was detected by immunohistochemistry, and the levels of Th17 and IL-17 in the blood were measured by flow cytometry and ELISA. Pain assessment was performed for all the patients and controls.

**Results:** An inflamed synovium was characterised by increased leukocyte infiltration and IL-17 expression in comparison with the control. Preoperative levels of Th17 and IL-17 were significantly higher in the peripheral blood of the ONFH group than those in the controls. The symptoms were also positively correlated with the Th17 levels of the ONFH patients.

**Conclusion:** Th17 cells were recruited to an inflamed synovium, and inflammatory cytokine IL-17 was expressed at an increased level in the hip synovium of ONFH patients, which possibly contributed to clinical syndrome development. Overall, this study will help to identify new therapeutic strategies for ONFH, especially the targeting of IL-17 to decrease inflammation and pain. **(Endokrynol Pol 2018; 69 (3): 283–290)**

**Key words:** *T helper 17 lymphocyte; IL-17; osteonecrosis of femoral head; inflammation; pain; visual analogue scale*

### Streszczenie

**Wstęp:** Zapalenie błony maziowej związane z martwicą głowy kości udowej (*osteonecrosis of the femoral head*; ONFH) odpowiada za kilka objawów klinicznych, jednak mechanizmy leżące u podstaw zapalenia błony maziowej oraz środowisko zapalne pozostają niejasne. W niniejszym badaniu poddano analizie ekspresję mediatora zapalenia IL-17 na limfocytach Th17, które pełnią kluczową rolę w regulowaniu procesów zapalnych w objętej stanem zapalnym błonie maziowej i krwi obwodowej w przebiegu ONFH.

**Materiał i metody:** Podczas zabiegów operacyjnych pobrano maź stawową ze stawów biodrowych 23 pacjentów i 5 osób z grupy kontrolnej, natomiast próbki krwi obwodowej uzyskano od 34 pacjentów i 9 osób z grupy kontrolnej. Ekspresję IL-17 w błonie maziowej wykrywano za pomocą metody immunohistochemicznej, a stężenie Th17 i IL-17 we krwi mierzono metodą cytometrii przepływowej i metodą ELISA. U wszystkich pacjentów i osób z grupy kontrolnej oceniono parametr bólu.

**Wyniki:** Cechą charakterystyczną objętej stanem zapalnym błony maziowej był wzrost nacieczenia limfocytarnego i ekspresji IL-17 w porównaniu z grupą kontrolną. Stężenie Th17 i IL-17 przed wykonaniem zabiegów operacyjnych było znacząco wyższe we krwi obwodowej pacjentów z martwicą głowy kości udowej niż grupy kontrolnej. Również objawy były u tych pacjentów dodatnio skorelowane z poziomem Th17.

**Wnioski:** Limfocyty Th17 były rekrutowane do objętej stanem zapalnym błony maziowej, a cytokina zapalna IL-17 ulegała ekspresji na zwiększonym poziomie w błonie maziowej stawu biodrowego pacjentów z martwicą głowy kości udowej, co prawdopodobnie przyczyniło się do rozwoju zespołu objawów klinicznych. Ogólniając, niniejsze badanie może pomóc zidentyfikować nowe strategie terapeutyczne w martwicy głowy kości udowej, w szczególności ukierunkowane na IL-17 w celu zmniejszenia stanu zapalnego i bólu. **(Endokrynol Pol 2018; 69 (3): 283–290)**

**Słowa kluczowe:** *limfocyty pomocnicze Th17, IL-17, martwica głowy kości udowej, zapalenie, ból, wizualna skala analogowa*

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## Introduction

Osteonecrosis of the femoral head (ONFH) is a devastating disease for affected patients, with complete collapse of the femoral head occurring in 80% of untreated patients [1, 2]. Trauma, alcoholism, excessive steroid use, sickle cell anaemia, damage from radiation, Gaucher's disease, and exposure to high pressure are reported to be the potential reasons for ONFH [3]. Chronic pain is one of the features of this disease, which is considered to be associated with synovitis caused by a series of inflammatory molecules [4]. To date, the pathophysiology of ONHF remains poorly understood, but secreted inflammatory molecules (such as proinflammatory cytokines) are believed to be among the critical mediators of the pathophysiological processes [5, 6]. However, the release of proinflammatory cytokines in an inflamed synovium has rarely been investigated.

Chronic pain is the most remarkable symptom that seriously disturbs ONFH patients. Osteonecrosis can be categorised into four stages according to a previously published grading system [7]. The level of pain varies among patients. Severe sufferers may complain of a considerable impact on their normal lives, whereas others may only declare discomfort in the hip joint. These findings may be related to synovitis during ONFH pathogenesis; previous studies found that inflammatory synovium is infiltrated with CD4+ T cells, macrophages, and some CD8+ T cells [8]. Inflammatory factors, such as tumour necrosis factor alpha (TNF-$\alpha$) [9], nitric oxide (NO) [10], interleukin (IL)-33 [11], and several adhesion molecules [8], also participated in the pathogenesis of synovitis. Interestingly, these inflammatory cells and cytokines were reported to be involved in pain severity [12–14].

IL-17 has been reported to be expressed at increased levels in inflamed osteoarthritis synovium [15, 16], and closely related to pain. In healthy individuals, approximately 1% of CD4+ T cells in their peripheral blood are Th17 cells [17]. During pathological processes, Th17 cells can be recruited to lesions and produce IL-17 and TNF-$\alpha$ to mediate inflammatory reactions [18–21]. Moreover, IL-17 is proven to be able to stimulate fibroblasts, chondrocytes, and synovial cells to produce other proinflammatory cytokines, including IL-6, as well as prostaglandin E2 (PGE2) and NO [22–24]. All these cytokines are thought to act on local lesions and induce clinical symptoms, such as inflammation and pain. In the present study, we focused on the alteration of Th17 and the expression of IL-17 in ONFH, and we explored the potential relationship between Th17 level and pain severity.

## Material and methods

### *Ethics statement*

Patients were enrolled between October 2012 and May 2014 at Qianfoshan Hospital, Shandong University, China. Our research was approved by the Medical Ethical Committee of Shandong University. Written, informed consent was obtained from each participant.

### *Stages of ONFH*

We divided the ONFH into four clinical stages mainly by iconography and clinical manifestation according to a previously published grading system [7].

### *Immunohistochemistry*

For immunohistochemistry (IHC) analysis, which implied regional expression of targeted molecules, femoral head tissues were collected during surgery from 23 patients (all patients were in Stage 4) with ONFH and five controls with transcervical fracture. Freshly isolated synovium from each patient was immediately fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS). Transverse and coronal sections of 5 $\mu$m thickness were deparaffinised in xylene, rehydrated though graded ethanol, and stained with Alcian blue, as well as eosin and propidium iodide. Prior to staining, the sections were treated with 3% $H_2O_2$ for 10 min to inactivate catalase and with bovine serum albumin to block nonspecific antigens. To identify the inflammatory environment, the sections were incubated with rabbit anti-human TNF$\alpha$ antibody (Abcam Inc., USA) in PBS at a dilution of 1:100. To localise Th17, the sections were incubated with rabbit anti-human IL-17 antibody (5 $\mu$g/ /mL, R&D Systems Inc., USA) in PBS at dilutions of 1:300, 1:500, and 1:300. All the sections were incubated at 4°C overnight. After the sections were thoroughly washed, the bound primary antibody was incubated with secondary antibody (working solution of LiankeBio) for 20 min in Reagent A and 20 min in Reagent B. Colour was developed using diaminobenzidine (Abcam Inc., USA). The negative control for the primary antibody used PBS instead of the antibody.

### *Grading of immunostained sections*

Semi-quantitative grading of IL-17 immunoreactivity in immunostained sections was separately performed by two investigators, who evaluated nine separate 20× magnification fields for each tissue sample. The method used here was described by Shamji et al. [25], and the strategy provided the most complete and comprehensive evaluation of surgical tissue samples. The degree of cytokine immunoreactivity was scored as follows:

0 = no positive cells and 1 = at least one positively labelled cell.

### *Enzyme-linked immunosorbent assay (ELISA)*

The circulating levels of Th17 and IL-17 were evaluated in the blood samples. Peripheral blood was obtained from 34 patients (six for stage 1, 13 for stage 2, six for stage 3, and nine for stage 4) with ONFH and nine controls with transcervical femoral fracture. The samples were analysed using a Human Th17 ELISA kit (eBioscience, BMS2017HS) following the manufacturer's instructions. All procedures were performed at room temperature, and the mean absorbance of the standards and samples was detected in duplicate. Colorimetric reactions were analysed at 450 nm on a Varioskan flash multifunction plate reader (Thermo Scientific, Germany). All the experiments were repeated at least three times.

### *Flow cytometry*

We analysed the percentage of Th17 in the peripheral blood of the patients via flow cytometry. Flow cytometry was used to evaluate the intracellular IL-17 production and identify the Th17 cytokine-producing cells in both the ONFH and transcervical fracture groups. Briefly, heparinised peripheral whole blood (300 $\mu$L) added with an equal volume of Roswell Park Memorial Institute (RPMI) 1640 medium was incubated for 5 h at 37°C, with 5% $CO_2$ in the presence of 25 ng/mL phorbol myristate acetate (PMA) (ENZO, BML-PE160-0001), 1 $\mu$g/mL ionomycin (ENZO, ALX-450-007-M001), and 1.7 $\mu$g/mL monensin (ENZO, 380-026-M100). After incubation, the cells were stained with phytoerythrin (PE)–Cy5-conjugated anti-human CD3 (eBioscience, 15-0038-42) and fluorescein isothiocyanate-conjugated anti-human CD8 monoclonal antibodies (eBioscience, 11-0088-42) at room temperature in the dark for 15 min to delimitate CD4+ T cells. The cells were then fixed, permeabilised (LiankeBio, LK-GAS003, FIX&PERM Kit), and stained with a PE-conjugated anti-human IL-17A monoclonal antibody (eBioscience, 12-7178-41). Isotype controls were used to enable correct compensation and confirm the antibody specificity. Stained cells were analysed by flow cytometry on a fluorescence-activated cell sorter (FACS) cytometer equipped with CellQuest software (BD Bioscience Pharmingen, San Jose, CA). Finally, the CD3+ and CD4+ T cell subsets were gated by flow cytometry, and the proportion of IL-17-producing cells was determined.

### *Pain rating scale*

We used the visual analogue scale (VAS) [26] to evaluate the pain level of the patients. All the patients were given a VAS noted list for self-evaluation of their pain at rest. The scale of pain was matched with the IHC results.

### *Statistical analysis*

Data were expressed as the mean ± standard deviation (SD). Flow cytometry and ELISA data were statistically analysed by one-way analysis of variance (ANOVA) with post-hoc analysis. Differences between study groups in tissue immunoreactivity scores were examined using Chi-squared test. A bivariate correlation was used to determine the relationship between the VAS scores and the Th17 and IL-17 levels. All the tests were performed using SPSS 16.0. A value of $P < 0.05$ was considered statistically significant.

## Results

### *Th17 was expressed in inflamed synovium*

To investigate the expression pattern of Th17 and IL-17, IHC was conducted in synovium samples collected from patients with ONFH and transcervical fracture. Blood vessel hyperplasia, infiltration of inflammatory cells, and upregulation of TNF$\alpha$ were observed in the synovium samples of the ONFH group (Figure 1A, 1B, and 1C). Conversely, substantially milder inflammation was observed in transcervical fracture samples. Furthermore, we confirmed the infiltration of Th17 in ONFH by immunostaining for IL-17. The expression of IL-17 was markedly increased in the ONFH samples (Figure 1D) in comparison with the fracture samples (Figure 1E). Statistical analysis of IL-17 based on IHC indicated that the expression of IL-17 between the two groups presented a significant difference (Figure 1F). Overall, our results reveal that the inflammatory reaction in the synovium of the ONFH patients and the IL-17 level may participate in this process.

### *Levels of Th17 and IL-17 in peripheral blood were elevated in patients with ONFH*

Representative flow cytometry was conducted to detect the Th17 level in peripheral blood (Figure 2). The results showed that the percentage of Th17 cells significantly increased in the ONFH group compared with that in the transcervical fracture group. The IL-17 expression level in the peripheral blood was also tested by ELISA, and the expression of IL-17 in the ONFH group was markedly promoted (Figure 3). These results imply that apart from regional infiltration in the synovium, upregulation of Th17 also occurs in the peripheral blood, and the latter may be a prerequisite for the former.

### *Th17 level is positively correlated with VAS pain scores and clinical stages of ONFH*

We evaluated the association between Th17 and pain sensation via a semi-quantitative pattern. We found that higher grading of synovial Th17 distribution is observed with higher VAS score of pain (Figure 4A). Pearson's

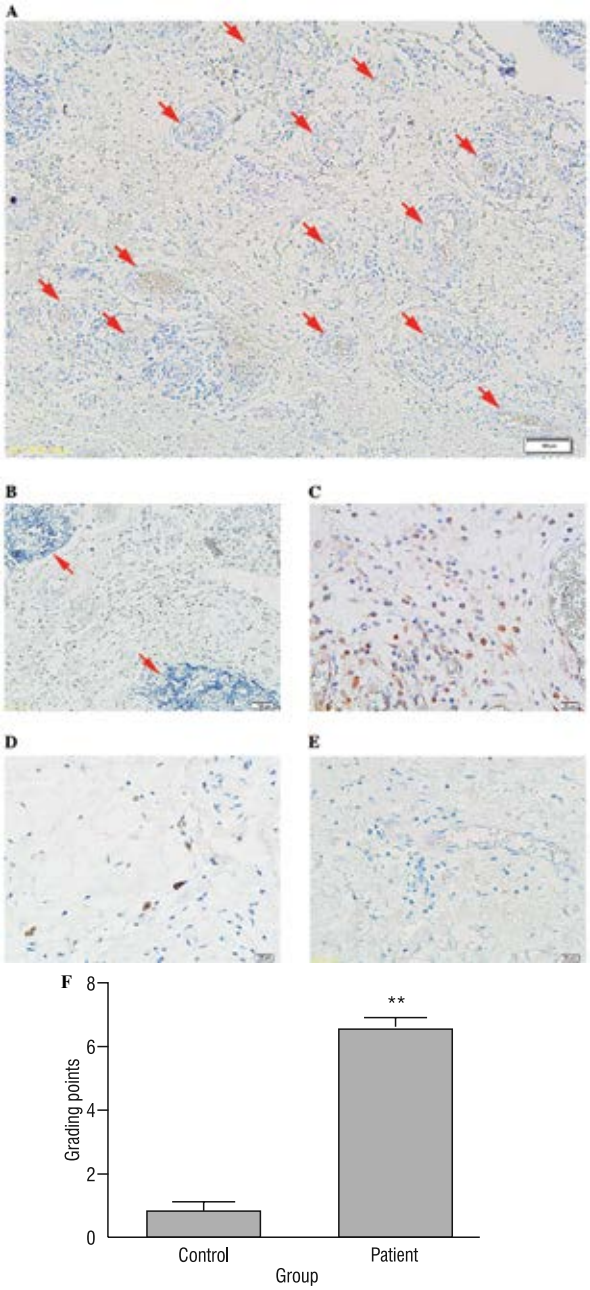


**Figure 1.** *Patients with ONFH displayed inflammation environment and immunohistochemical localisation of IL-17 in synovium surrounding the femoral head.* ***A*** *and* ***B****: severe blood vessel hyperplasia and infiltration of inflammatory cells in samples with ONFH, as detected through immunohistochemistry. Twenty-three synovial tissue samples from patients with ONFH and five from patients with transcervical fracture as controls were analysed. Representative results from each group are shown;* ***C.*** *Expression pattern of TNF-α pathological tissue of ONFH, as assayed by immunohistochemistry;* ***D.*** *Representative IL-17 expression in samples from the ONFH group;* ***E.*** *No distinct IL-17 expression was detected in the control group;* ***F.*** *Semi-quantitative grading methods were used to analyse the immunohistochemical results. Compared with the control group, the expression of IL-17, as shown by the mean grading scores of the positive cells, significantly increased in the ONFH group (F, P < 0.0001, significantly different from the controls)*

**Rycina 1.** *Pacjenci z martwicą głowy kości udowej wykazywali stan zapalny oraz lokalizację immunohistochemiczną IL-17 w błonie maziowej otaczającej głowę kości udowej.* ***A, B.*** *Poważny przerost naczyń krwionośnych oraz nacieczenie komórek zapalnych w próbkach z martwicą głowy kości udowej wykryte za pomocą metody immunohistochemicznej. Analizie poddano 23 próbki tkanki maziowej od pacjentów z martwicą głowy kości udowej oraz 5 próbek od pacjentów ze złamaniem szyjki kości udowej. Przedstawiono reprezentatywne wyniki z każdej grupy;* ***C.*** *Wzorzec ekspresji TNF-α w patologicznej tkance objętej martwicą głowy kości udowej, wykryty za pomocą metody immunohistochemicznej;* ***D.*** *Reprezentatywna ekspresja IL-17 w próbkach z grupy pacjentów z martwicą głowy kości udowej;* ***E.*** *W grupie kontrolnej nie wykryto wyraźnej ekspresji IL-17;* ***F.*** *Do analizy wyników immunohistochemicznych wykorzystano półilościowe metody klasyfikacji. Jak pokazują średnie wyniki ocen komórek dodatnich, ekspresja IL-17 znacząco wzrosła w grupie pacjentów z martwicą głowy kości udowej w porównaniu z grupą kontrolną (F, P < 0,0001, znacząco różne od grupy kontrolnej)*

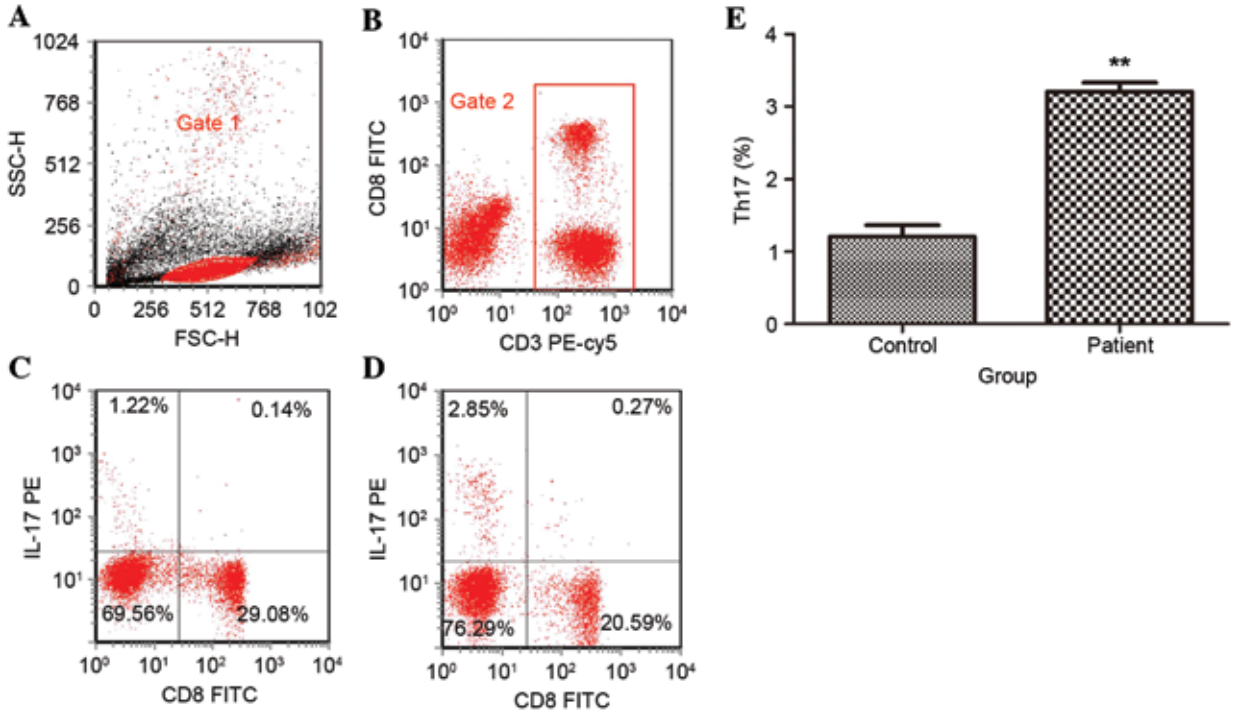


**Figure 2.** *Th17 frequency in ONFH patients and control group. Peripheral blood samples were collected from experimental and control groups, and Th17 cells were isolated.* ***A.*** *Lymphocytes were gated by flow cytometry;* ***B.*** *CD3+ T subsets were gated by flow cytometry. Plots in the internal box represent CD3+ T cells;* ***C*** *and* ***D****. The proportions of Th17 to CD3+CD8−(CD4+) subsets from representative controls and ONFH group are shown, respectively. The percentage of circulating Th17 cells was significantly higher in ONFH patients than in the control group (E, P < 0.0001, significantly different from the control)*

**Rycina 2.** *Częstość występowania limfocytów Th17 u pacjentów z martwicą głowy kości udowej i w grupie kontrolnej. Próbki krwi obwodowej zostały pobrane od osób z grupy eksperymentalnej i grupy kontrolnej, jak również limfocyty Th17 zostały wyizolowane.* ***A.*** *Limfocyty były bramkowane metodą cytometrii przepływowej;* ***B.*** *Subpopulacja limfocytów T CD3+ była bramkowana metodą cytometrii przepływowej. Wykresy w wewnętrznym polu przedstawiają limfocyty T CD3+;* ***C, D.*** *Proporcje limfocytów Th17 do subpopulacji CD3+CD8−(CD4+) z reprezentatywnej grupy kontrolnej i grupy z ONFH, odpowiednio. Odsetek krążących komórek Th17 był znacząco wyższy u pacjentów z martwicą głowy kości udowej niż w grupie kontrolnej (E, P < 0,0001, znacząco różne od grupy kontrolnej)*

R-test was performed, and the positive correlation (R2 = 0.9207) between grading points of synovial Th17 and VAS scores was evaluated. We also found a positive correlation (R2 = 0.6473 and 0.7333, correspondingly) between the levels of Th17 and IL-17 in the peripheral blood and VAS points (Figure 4B and 4C). At different stages of ONFH, the peripheral IL-17 level also varied. In stage 1 to stage 3, the levels of IL-17 showed relatively low levels, whereas in stage 4, IL-17 increased to a much higher level (Figure 4D and 4E).

## Discussion

Although synovitis is an integral component of ONFH, the mechanism responsible for its development and its precise contribution to ONFH initiation and progression is far from clearly understood. On the one hand, ONFH synovium inflammation has been suggested to be associated with macrophage and CD4(+) T-cell infiltration [4, 8]. On the other hand, synovitis may favour ONFH progression via proinflammatory cytokines.

In the present study, we investigated the involvement of Th17 lymphocytes in ONFH through a comparison of pathological specimens and peripheral blood samples from patients with ONFH and controls with transcervical fracture. ONFH synovium samples showed diffuse inflammation. The ratio of blood vessel hyperplasia and infiltration of inflammatory cells was significantly larger in the ONFH synovium than in the transcervical fracture samples. Additionally, a considerable amount of leukocyte clusters existed in the ONFH synovium compared with those in the transcervical fracture samples. The higher levels of Th17 and IL-17 in the ONFH synovium indicated the correspondence between biochemistry and inflammation in synovitis. Previous studies have proven that IL-17 can induce the expression of PGE2, IL-6, and NO [22–24], which are critical mediators of inflammation and pain. We aimed

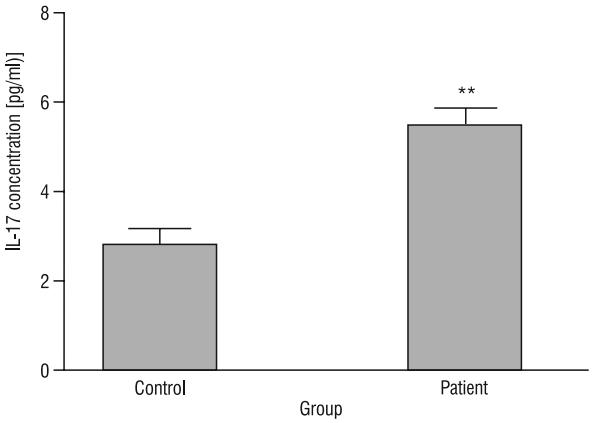


**Figure 3.** *Comparison of IL-17 level in peripheral blood between the two groups. The level of IL-17 was apparently greater in the ONFH group than that in the control group (P < 0.0017, significantly different from the controls)*

**Rycina 3.** *Porównanie stężenia IL-17 we krwi obwodowej obu grup. Stężenie IL-17 było niewątpliwie wyższe u pacjentów z martwicą głowy kości udowej niż w grupie kontrolnej (P < 0,0017, znacząco różne od grupy kontrolnej)*

to determine whether Th17 also performs a function in the ONFH, i.e. in manifestation of pain. The present results showed that IL-17 is expressed in the inflamed synovium of ONFH. As a control, the expression of IL-17 in the patients with transcervical fracture remained at a relatively low level. Another significant observation is the elevated levels of CD3+, CD4+, and IL-17+ cells in the peripheral blood of ONFH patients; consistently, the level of IL-17 in the peripheral blood also increased in association with pain severity. The results showed that patients with higher VAS scores presented enhanced levels of Th17 and IL-17, both in the regional synovium and peripheral blood. Overall, these findings indicated that Th17 cells may participate and perform a key function in the inflammatory process and clinical symptoms of ONFH.

Inflammation in the ONFH group was apparently more severe than that in the controls, with higher levels of accumulation of inflammatory cells, hyperplasia of the blood vessels, and high expression of TNF$\alpha$ [27].

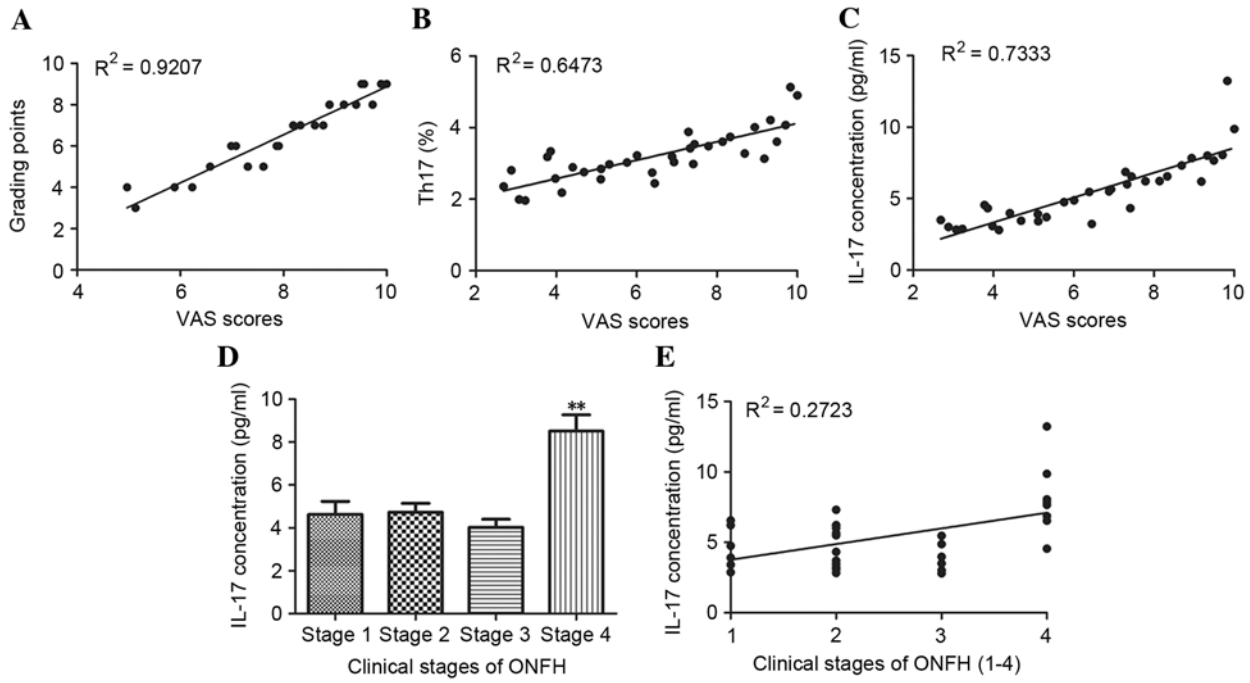


**Figure 4.** *Scatter plots of the correlation of VAS pain score and IL-17 level in regional tissue and peripheral blood. The semi-quantitative grading scores of IL-17 in the pathological tissue of ONFH patients increased along with VAS scores (A, $R^2 = 0.9207$, significantly positive correlation between the two factors). The percentage of Th17 and the concentration of IL-17 in the peripheral blood of ONFH patients also trended in a positive pattern with the VAS scores (B, $R^2 = 0.6473$; C, $R^2 = 0.7333$, evident positive correlation between two factors). IL-17 levels differed markedly in four different clinical stages (D, P < 0.05, significant difference between stage 4 and other stages), but the linear correlation between IL-17 and clinical stage was weak (E, $R^2 = 2723$, weak positive correlation between the two factors)*

**Rycina 4.** *Wykresy punktowe przedstawiające zależność między wizualną skalą analogową nasilenia bólu a stężeniem IL-17 w tkance miejscowej i krwi obwodowej. Półilościowe wyniki klasyfikacji IL-17 w tkance patologicznej pacjentów z martwicą głowy kości udowej wzrastały razem z wynikami wizualnej skali analogowej (A, $R^2 = 0,9207$, istotna dodatnia korelacja między obydwoma czynnikami). Odsetek limfocytów Th17 i stężenie IL-17 we krwi obwodowej pacjentów z martwicą głowy kości udowej również wykazywały tendencje dodatnie z wynikami wizualnej skali analogowej (B, $R^2 = 0,6473$; C, $R^2 = 0,7333$, ewidentna dodatnia korelacja między obydwoma czynnikami). Stężenia IL-17 różniły się znacznie w czterech różnych stadiach klinicznych (D, p < 0,05, istotna różnica między stadium czwartym a pozostałymi stadiami), lecz korelacja liniowa między IL-17 a stadium klinicznym była słaba (E, $R^2 = 2723$, słaba dodatnia korelacja między obydwoma czynnikami)*

These findings support the view that ONFH represents a typical "inflammatory disease" and implies a potential network among inflammatory cells and cytokines. Th17, a typical inflammatory cell type with its mediated IL-17, is defined as a key player in various autoimmune diseases [16, 23, 24]. In the current study, the local level of IL-17 in the synovium of the ONFH group was found to be markedly higher than that in the controls, suggesting a local accumulation of Th17. This result implies a positive relationship between Th17 and inflammatory ONFN. Circulating Th17 and IL-17 are well-established biomarkers for the severity of inflammatory diseases. Interestingly, higher levels of circulating Th17 and IL-17 were detected in the ONFH group, indicating a "circulating source" of the regional Th17 [28]. As a chronic pathological process, ONFH may induce the inflammatory reaction of the organism and produce more Th17 and IL-17, which in turn exacerbates inflammation. Collectively, the expression of Th17 may exist as a consequence of ONFH-induced inflammation while participating in pain severity among patients.

One of the dominant factors of ONFH is pain severity [29]. Moreover, severe pain is a key driver for ONFH treatment, and pain relief is a preliminary goal of therapy [30].

We performed a correlation analysis between IL-17 and VAS scores in our study, and the results revealed a typical positive association between the two parameters. This finding suggests that Th17 may be involved in the pain induction of ONFH. Subsequently, we performed a similar analysis between Th17 and IL-17 in the peripheral blood and VAS scores, and we obtained similar results. In summary, Th17 and its induced IL-17 are closely related to ONFH pain. We assume that the peripheral Th17 cell was recruited to the local synovium to induce inflammation, and the secreted IL-17 may stimulate synovial cells to produce PGE2 and NO, thereby causing pain.

In conclusion, our research indicates that Th17 and IL-17 may participate in synovium inflammation of ONFH, as well as in the pathogenesis of ONFH. Th17 and IL-17 possibly contribute to pain sensation in ONFH. Overall, our study may provide a potential therapeutic target for ONFH and pain relief.

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