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# Elevated plasma cartilage oligomeric matrix protein (COMP) level are associated with the progression of non-traumatic osteonecrosis of femoral head

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## 1. Introduction

Non-traumatic osteonecrosis of the femoral head (ONFH) is a considerably refractory disease with high morbidity among young patients, causing enormous economic losses worldwide [1]. According to the statistics, there are 20,000 newly diagnosed ONFH patients in the United States every year [2]. For ONFH, researchers have only explored its general pathological processes in which insufficient blood supply leads to femoral head collapse, eventually causing cartilage destruction in the hip joint [3]; its mechanisms, however, have not yet been clarified. The most frequent risk factors for ONFH include alcohol, corticosteroid therapy and trauma [4]. ONFH is characterized by progressive femoral head necrosis and hip degeneration; nevertheless, 36% of the hips with cartilage degeneration cannot be detected by plain radiographs or magnetic resonance imaging (MRI) [5].

Although most studies have focused on the destruction of bone or bone marrow, cartilage destruction that in turn exacerbates the collapse of the femoral head also has a significant effect on ONFH [6]. The fifth member of the thrombospondin (TSP5), cartilage oligomeric matrix protein (COMP) is an extracellular matrix (ECM) glycoprotein derived from cartilage, which has been used to assess the degree of cartilage degeneration in arthritis or knee injury [7]. Abnormal COMP is associated with two rare genetic diseases, pseudoachondroplasia (PSACH) and multiple epiphyseal dysplasia (MED), both of which are autosomal dominant skeletal dysplasias [8]. High levels of COMP in plasma have been used as markers of early-stage osteoarthritis (OA) and rheumatoid arthritis (RA), owing to COMP fragments that are released into the joint fluid after arthritis and cartilage degeneration [9]. Furthermore, many matrix metalloproteinases (MMPs), including COMP, that can degrade the components of the extracellular matrix are strongly expressed in arthritis [10]. To the best of the authors' knowledge, cartilage degeneration also occurs in the process of necrosis of the femoral head. Therefore, increased COMP levels and chondrocyte apoptosis may have a considerable influence on the pathological processes of ONFH. Even so, studies have rarely investigated whether the COMP levels are associated with the collapse of ONFH.

To explore the role of COMP levels in the collapse of non-traumatic ONFH, clinical bone and cartilage histomorphology, COMP expression position and levels, as well as plasma COMP levels were evaluated. The present study aimed to explore the effect of plasma COMP on non-traumatic ONFH and the possibility of using it as a predictive biomarker of disease progression.

## 2. Methods

### 2.1. Study subjects

We adopted a cross-sectional method of large samples. It was approved by the First Affiliated Hospital of Guangzhou University of Chinese Medicine, and a total of 67 non-traumatic ONFH patients were enrolled from May 2016 to November 2016, excluding patients with possible confounding factors, including congenital diseases, smoking, renal dysfunction, HIV infection, diabetes mellitus, cancer and other factors. Osteonecrosis of the femoral head was diagnosed via history, X-ray examinations and MRI, and the stage was determined according to the ARCO staging system [11]. The duration of pain in patients was also recorded. Bone, cartilage and plasma samples of ONFH were collected before arthroplasty, while 61 control plasmas were collected from gender- and age-matched healthy volunteers who underwent a physical examination during the same period. Cartilage sections were dissected

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carefully from the weight-bearing region of the femoral head, and bone sections were excised from the normal tissue or subchondral osteonecrotic areas with 1–4-mm-deep cartilage.

### 2.2. Bone morphology observed by H&E staining

Necrotic bone regions were dissected and were fixed in 4% formaldehyde for over 24 h at room temperature. After being decalcified by subsequently using 10% EDTA over 2 weeks, the samples were embedded in paraffin wax. The embedded specimens were cut longitudinally into 5-μm sections and were stained with hematoxylin-eosin and then were observed through a microscope (BX53, Olympus Corp., Japan).

### 2.3. Cartilage morphology observed by H&E, Safranin O, and Alcian Blue staining

Cartilage samples were excised along the axial plane into pieces of 10 mm*5 mm*7 mm and were fixed independently in 37% formaldehyde. Subsequently, all specimens were decalcified in 10% EDTA, dehydrated and ultimately embedded in paraffin. Those embedded specimens were cut into 40-μm sections and were stained using hematoxylin-eosin (H&E), Safranin-O and Alcian blue and then were observed under the microscope (BX53, Olympus Corp., Japan). The Mankin score was used to evaluate cartilage degeneration according to the three cartilage stains.

### 2.4. Immunoshistochemistry for COMP

Immunofluorescence was performed using standard procedures. As a representative marker of endoplasmic reticulum, rabbit anti-Grp78 antibody (Zen BioScience, Chengdu, China) was used in combination with the anti-Myc mouse antibody (Santa Cruz Biotechnology Inc., CA, USA) in a 1:100 dilution. The anti-rabbit-DyLight 488-labeled antibody and anti-mouse-DyLight 594-labeled antibody (ZSGB-Bio, Beijing, China) were used in a 1:100 dilution as secondary antibodies. Finally, the cells were mounted with DAPI (Sigma, USA) for nuclear staining and then visualized by laser scanning confocal microscopy.

### 2.5. Western blotting for COMP

Samples were subjected to agarose-SDS-PAGE, transferred to PVDF membranes, blocked (3% bovine serum albumin (BSA) in PBS), and detected with specific rat anti-human COMP (mAb HC484D1, AbD Serotec) as the primary antibody and rabbit anti-mouse immunoglobulins (DakoCytomation) and rabbit anti-rat IgG and IgM (Jackson ImmunoResearch Europe Ltd.) as horseradish peroxidase-conjugated secondary antibodies. Semi-quantitative analysis of the enhanced chemiluminescence (ECL) western blot detection system was used to visualize particular proteins.

### 2.6. COMP measurements by ELISA

According to the manufacturer's instructions, each test was assayed in duplicate and quantitative determination of the COMP concentration in plasma was performed using commercially available enzyme-linked immunosorbent assay (ELISA) (Cusabio, Wuhan, Hubei, China). ROC curves were used to draw data from the results obtained in this study, and the cutoff value was set to provide optimal diagnostic accuracy and likelihood ratios for the level of COMP.

### 2.7. Ethical approval

Informed consent was obtained from all patients and healthy volunteers. This study is in accordance with the ethical standards of the Review Board on Human Research of the Faculty of Medicine and with the Declaration of Helsinki, meanwhile, it was carried out with the approval of the responsibility by the Ethics Committee of the First Affiliated Hospital of Guangzhou University of Chinese Medicine.

### 2.8. Post hoc statistical power calculation

Statistical power (1-β) was calculated by Power and Sample Size.com Calculators(http://powerandsamplesize.com) using the formula given in the following to obtain the data of different mean COMP levels, standard error, and enrolled numbers of patients in each group [12]. Statistic power was regarded strong when > 0.8. This calculator uses the following formulas to compute sample size and power, respectively:

$$1 - \beta = \Phi(z - z_{1-\alpha/2}) + \Phi(-z - z_{1-\alpha/2})$$

$$Z = (\mu_A - \mu_B)/\sigma$$

### 2.9. Statistical analysis

Statistical analysis of data was performed with SPSS version 24.0(IBM Corporation, Armonk, NY, USA). Nonpaired *t*-tests, one-way ANOVA, Pearson correlational analysis were used for statistical analysis. All tests were two-tailed at the 5% level of significance.

## 3. Results

### 3.1. Radiography and pathology evaluation of ONFH patients and control subjects

Fig. 1A shows the regular shape and uniform spherical density of the femoral head together with a normal joint space. Fig. 1B shows the uneven density, local cystic degeneration and mildly narrowed joint space. Fig. 1C shows the collapse of the articular surface, osteosclerosis and preservation of the joint space. Fig. 1D shows the irregular femoral head, subchondral collapse and degenerative arthritis. Fig. 1E shows the normal trabecular bone and smooth surface cartilage in a control subject without evident destruction. Fig. 1F shows the manifest disorganization of the bone trabeculae in the necrotic region along with rough surface cartilage. Fig. 1(G, H) show the substantial collapse of the femoral head and even cartilage stripping in Fig. 1H. The bone specimen became lighter and formed a boundary with normal tissue; concurrently, continuity of the articular cartilage surface was severely destroyed. Fig. 1I shows that the control bone samples displayed complete trabecular architecture and full osteocytes. However, Fig. 1(J-L) show that with the increase in the stages, trabeculae of the bone became sparse and osteocytes were lost in the necrotic region, leading to a larger number of empty lacunae. As shown in Fig. 1 M, the numbers of empty lacunae in the control group were significantly lower than those in the ONFH group ($p < .001$). Although there was no significant difference between stages III and IV in the ONFH group ($P > .05$), the numbers of empty lacuna in both stages III and IV were higher than that in stage II ($p < .001$).

### 3.2. H&E, Safranin O, and Alcian Blue staining for cartilage

Fig. 2 (A, E, I) shows three types of staining of the control group in which articular cartilage could be broadly divided into four layers. Chondrocytes were of normal morphology, neatly regular structure of collagen fibers and were arranged along the direction of the cartilage surface. With the increase in ARCO stages, the cartilage surface showed tiny fissures, while chondrocytes began to appear undesirable, irregular and even necrotic. With further development, cartilage became thinner, partial full-thickness cartilage was lost, the chondrocyte number decreased, and disintegration and death appeared (Fig. 2 (B-D, F–H, J-L)). Mankin scores in the femoral articular cartilage from the control and ONFH groups were measured. All scores in the three stages were

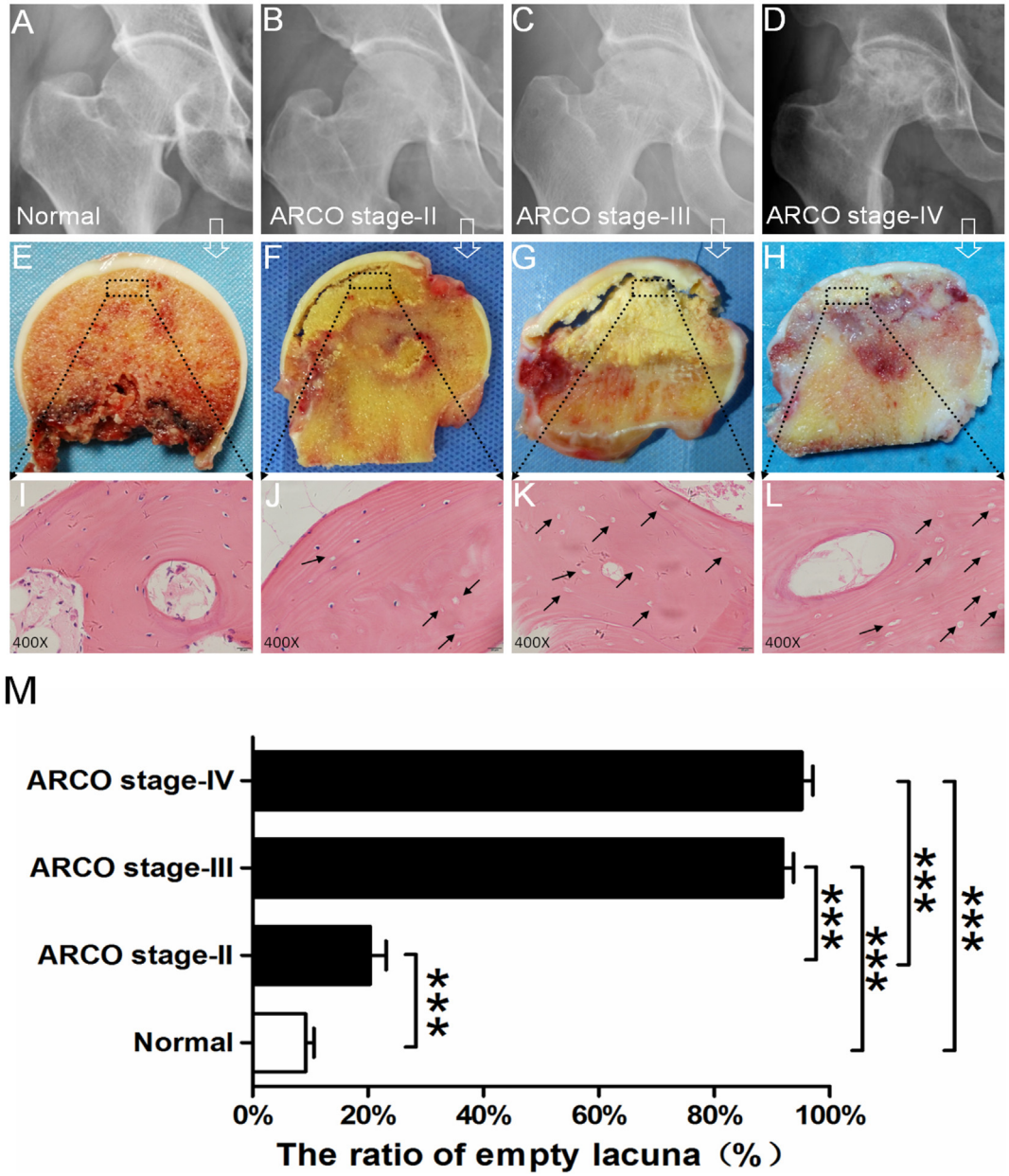


**Fig. 1.** (A-D) Typical X-ray images of control subject and ONFH patients with different ARCO stage. Cartilage destruction and articular degeneration were noteworthily related to the progression of ARCO stages. (E-H) Representative bone and cartilage samples of control subject and ONFH patients with three ARCO stages. The black boxes indicated the regions were removed for detection. (I-L) Histopathological features of control and ONFH bone. The number of empty lacuna in ONFH patients, which had increased with the ARCO stage, was significantly more than the control subject. *** represents $P < 0.001$.

significantly higher than those in the control group, of which group IV was the highest (Fig. 2M). The results indicated that cartilage progressively degraded during the pathogenesis of ONFH.

### 3.3. Immunohistochemistry of ONFH patients and control subjects

The necrotic cartilage region of the femoral head in ONFH positive for COMP was determined by immunohistochemical staining (Fig. 3). Fig. 3(1) clearly shows that the normal articular cartilage could be divided into four layers. Nevertheless, Fig. 3(2–4) shows that the superficial layer of articular cartilage had become rough, disordered and even structurally disappeared with the progression of disease. Moreover, chondrocyte apoptosis considerably increased with a tendency towards higher levels in the secretion of COMP with increased ARCO stage progression.

### 3.4. Western blot quantitative analyses of ONFH patients and control subjects

The expression of COMP was observed by western blotting (Fig. 4). The expression of COMP in cartilage samples of ONFH patients was significantly higher than those of control subjects ($P < .001$). Among the three ARCO stages, the levels of COMP in ONFH patients increased as the severity of X-ray findings increased ($P < .001$).

### 3.5. Plasma COMP level quantity of non-traumatic ONFH patients and control subjects

Table 1 displays the demographic data of 67 non-traumatic ONFH and 61 control subjects. The age of the patients was 44.2 ± 11.4 years (range 19–65 years), with 52 males and 15 females. Among those

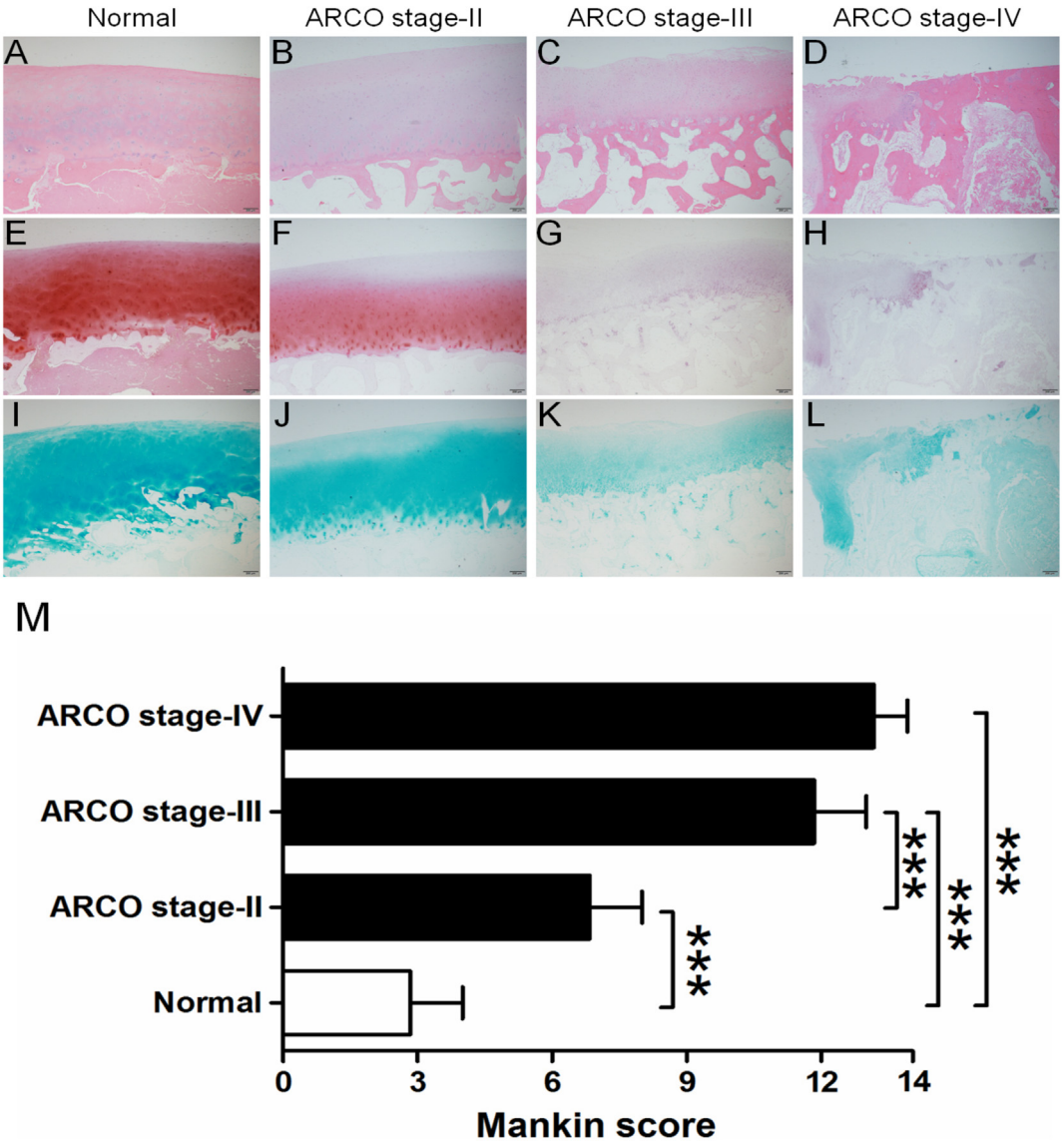


**Fig. 2.** Three kinds of staining of control group and ONFH group. (A-D) Hematein and Eosin (HE) of the femur articular cartilage. (E-H) Safranin O staining of femur articular cartilage. (I-L) Alcian blue staining of femur articular cartilage. With the increase of ARCO stages, the cartilage staining decreased. (M) Mankin score of the femur articular cartilage from control and ONFH group. Three stages all got statistically momentous higher scores than the control group. Group stage III got statistically significant higher scores than group stage II. *** represents $P < 0.001$.

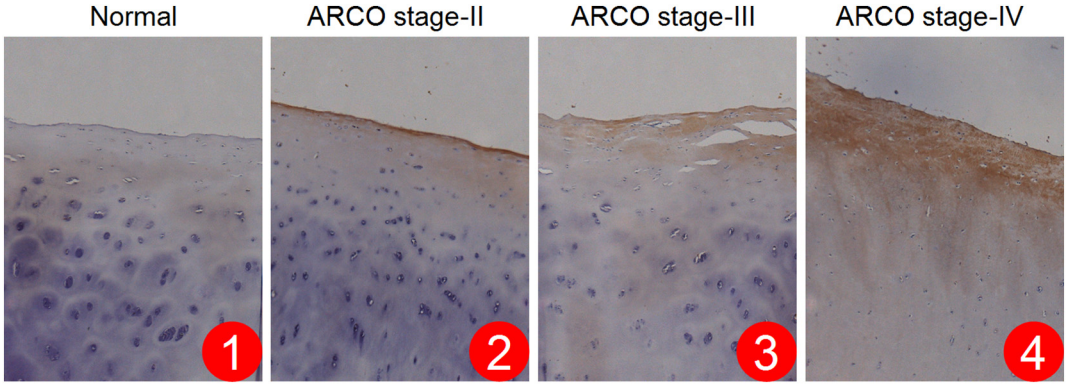


**Fig. 3.** Immunohistochemistry assay for COMP of cartilage samples of control subject and ONFH patients in different stages. The COMP widely scattered in the chondrocytes, but as the disease progresses, chondrocyte apoptosis and COMP secretion increased.

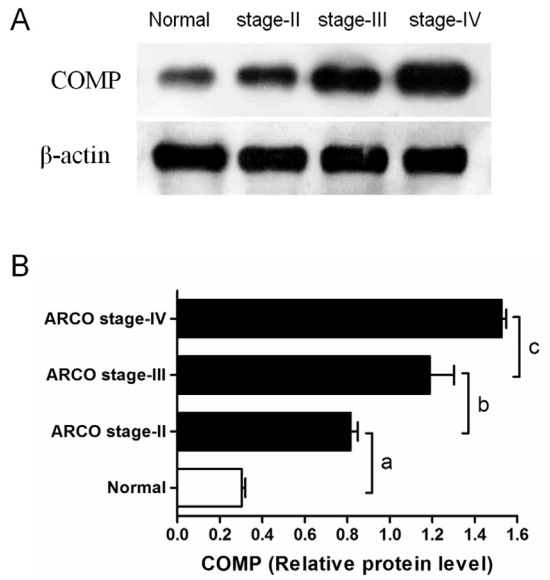


**Fig. 4.** Western blot analysis of cartilage samples for the COMP. Level of COMP is signally increased with the ARCO stage. Values are the means ± SEM. [a]p < 0.001 vs the control group; [b]p < 0.001 vs the stage-II group; [c]p < 0.001 vs the stage-III group.

**Table 1**
Levels of plasma COMP in non-traumatic ONFH Patients and Control Subject and probable relation between other clinical data. Data presented as mean ± SD. Variable are reflected in the absolute and relative frequencies. AI = alcohol-induced; SI = steroid-induced; ID = idiopathic.

| Group | Cases | COMP Level, ng/ml | Comparison | *P* value |
|---|---|---|---|---|
| Control subjects | 61 | 5.863 ± 3.409 | Control vs ONFH | 0.035 |
| ONFH | 67 | 6.959 ± 2.407 | | |
| ARCO stages | | | | |
| Stage II | 17(25.3%) | 5.887 ± 1.696 | II vs III | 0.129 |
| Stage III | 32(47.8%) | 6.958 ± 2.255 | III vs IV | 0.142 |
| Stage IV | 18(26.9%) | 7.974 ± 2.876 | IV vs II | 0.010 |
| Pre-collapse | 17(25.3%) | 5.887 ± 1.696 | Pre- vs Post- | 0.032 |
| Post-collapse | 50 (74.4%) | 7.324 ± 2.516 | | |
| Etiology | | | | 0.835 |
| Alcohol-induced | 31(46.3%) | 7.467 ± 2.559 | AI vs SI | 0.225 |
| Steroid-induced | 21(31.3%) | 6.588 ± 2.503 | SI vs ID | 0.836 |
| Idiopathic | 15(22.4%) | 6.430 ± 1.803 | ID vs AI | 0.166 |

patients, 21 were steroid-induced, 31 were alcohol-induced, and 15 had idiopathic osteonecrosis. There were 17 patients with stage II, 32 with stage III and 18 with stage IV. The age of the control subjects was 43.7 ± 13.2 (range 22–64 years), with 46 males and 15 females. There were no significant differences in age and gender between the groups.

The statistical power was 0.84, suggesting that the sampling ratio of 1 and the sample size of 128 were sufficient to obtain the conclusion (Fig. 5).

Plasma levels of COMP were higher in patients with ONFH than that in healthy volunteers ($p = .0363$), and the contrast between the ONFH and control groups is demonstrated in Fig. 6A. The levels of COMP were 5.88 ± 1.69 ng/ml, 6.95 ± 2.25 ng/ml and 7.97 ± 2.87 ng/ml in ONFH patients with ARCO stage II, stage III and stage IV, respectively. The levels of COMP were significantly different among patients with ONFH at various ARCO stages ($p = .035$). For example, the level of COMP in stage II was considerably lower than that of stage IV ($p = .010$) (Fig. 6.B). COMP levels and ARCO stages were analyzed using Pearson's correlation test, subsequently proving that COMP levels correlated with ARCO stages ($r = 0.316$, $p = .009$) (Fig. 6.C). The

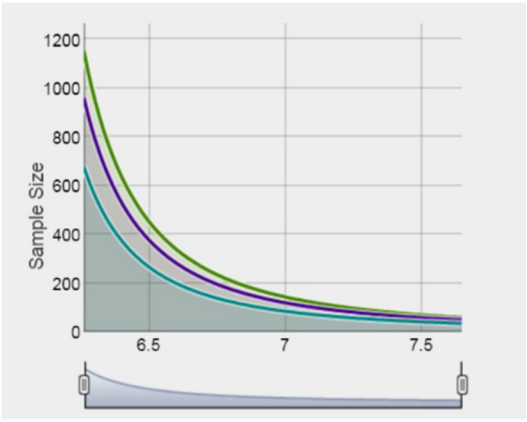


**Fig. 5.** Statistic power determined by mean and sample size. Statistic power: blue line for 0.7, purple line for 0.8 and green line for 0.9.

COMP level of the group after collapse was substantially higher than that of the group before collapse, and differences between the two groups were statistically significant ($p = .032$) (Fig. 6.D). Despite different ONFH etiologies, there were no significant differences between the cases with respect to the COMP levels ($p = .359$). In addition, pain duration was also confirmed to have a significant relationship with COMP levels using Pearson's test ($r = 0.2883$, $p = .0189$) (Fig. 7). The areas under the curve (AUC) were calculated by ROC curve analysis to determine the values for COMP levels in ONFH compared with those in the control group (0.629, 95% CI 0.540–0.713, $p = .0119$) (Fig. 8.A), where the sensitivity was 39.34% and specificity 98.51% (cutoff, 4.192 ng/ml). By analyzing these results, the effects of the collapse on the AUC for COMP levels were validated (0.689, 95% CI 0.564–0.786, $p = .0086$) (Fig. 8.B) with a sensitivity of 48% and specificity of 88.24% (cutoff, 7.158 ng/ml).

## 4. Discussion

The overall objective of the current study was to characterize the relationship between COMP levels and disease process in patients with non-traumatic osteonecrosis. In this cross-sectional study, the increase in chondrocyte apoptosis and the COMP level were strikingly higher in the ONFH group, especially the post-collapse group, and it is worth noting that the level of COMP was positively associated with ACRO stages. To the best of our knowledge, this is the first study to successfully demonstrate the correlation between COMP concentration and the severity of non-traumatic ONFH. The findings of this study suggest that COMP could possibly be used as a biomarker to estimate the process of the femoral head.

Cartilage oligomeric matrix protein (COMP), an extracellular matrix protein secreted by chondrocytes, is found in various tissues, including cartilage fibroblasts, tendons and ligaments [13]. Chondrocytes and extracellular matrix constitute an important part of cartilage; normal cartilage homeostasis and structure, accounting for only 5% of the total volume of cartilage, are also dependent on chondrocytes [14]. COMP is primarily produced by chondrocytes, and it decomposes into joint fluid and blood by way of metalloproteinase (MMP) catalysis during cartilage injury [15]. The metabolic imbalance between chondrocytes and extracellular matrix resulting from various causes of articular cartilage loss [16] leads to increased levels of COMP; therefore, COMP plays a determining role in chondrocyte signaling. To date, the exact pathogenesis of ONFH has not been clarified, and some studies suggest that the hip cartilage destruction is a critical factor in the deterioration of ONFH [17]. In the present study, we evaluated cartilage degradation levels in ONFH and control patients by the Mankin score, and examined the expression of COMP. The results suggested that the levels of COMP expression and the Mankin score showed comparable trends, prompting

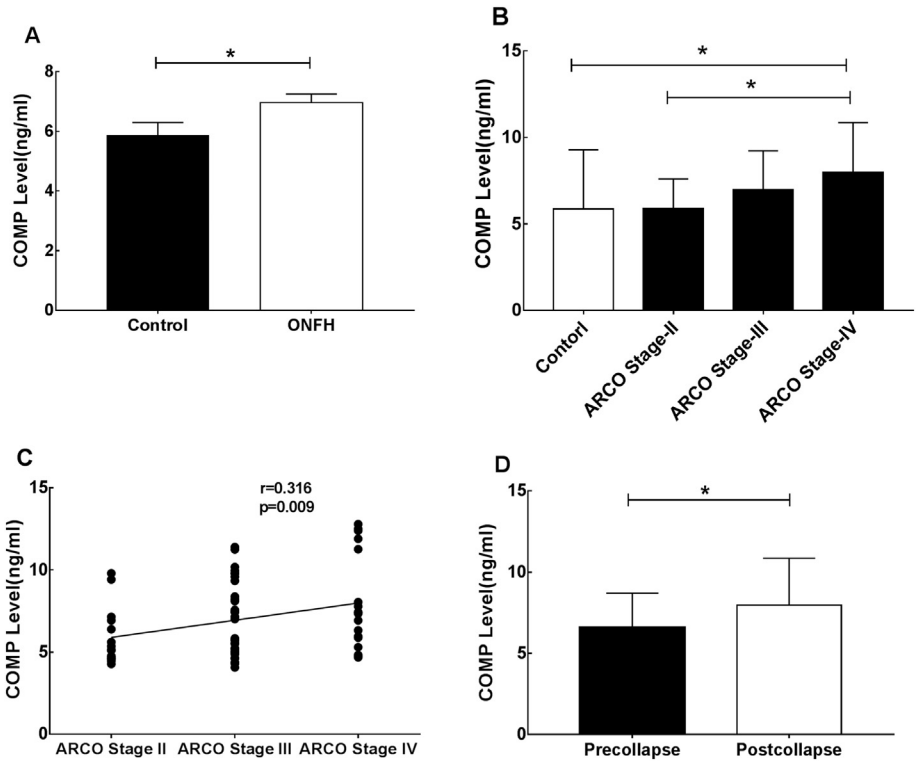


**Fig. 6.** (A-D) Overview shows COMP level in the plasma of non-traumatic ONFH patients and control subjects. (A) COMP levels of non-traumatic OFNH patients were markedly higher than that of control subjects. (B) COMP levels of stage-IV patients were prominently increased compared to controls and stage-II patients. The overall COMP level trend was generally on the rise, but not statistically significant. (C) Scattergram showing the positive correlation between COMP levels and ARCO stages. (D) COMP levels were notably increased in post-collapse group. * represent $P < 0.05$.

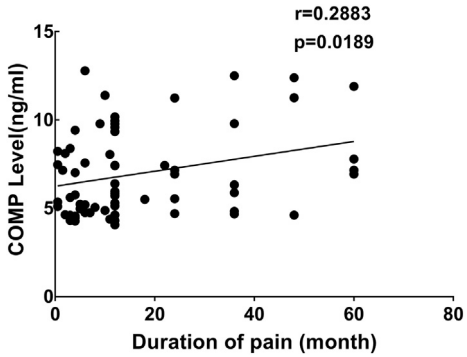


**Fig. 7.** The duration of pain which was recorded in patients with ONFH had the positive correlation with the level of COMP.

us to speculate as to whether COMP expression became notably larger with the degradation of cartilage (Figs. 2 and 4). Theoretically, the secretion of COMP should increase following the chondrocyte apoptosis caused by ONFH. According to the ROC test between the ONFH and control groups, we found that the specificity was extremely high, suggesting the existence of cartilage injury and high secretion of COMP in the process of femoral head necrosis; furthermore, the COMP observed in this study increased with disease progression in non-traumatic ONFH patients.

In addition to these findings, a correlation between COMP and the duration of disease-associated pain was also defined in the present study. As reported in previous studies, there were significant differences in the articular cartilage above the femoral head between the collapse and non-collapse groups in terms of T1 rho values [18], and the T1 rho value in the cartilage necrotic zone positively correlated with the interval from pain onset to MRI examination in the collapsed ONFH group [19]. Moreover, COMP is the first extracellular matrix protein that

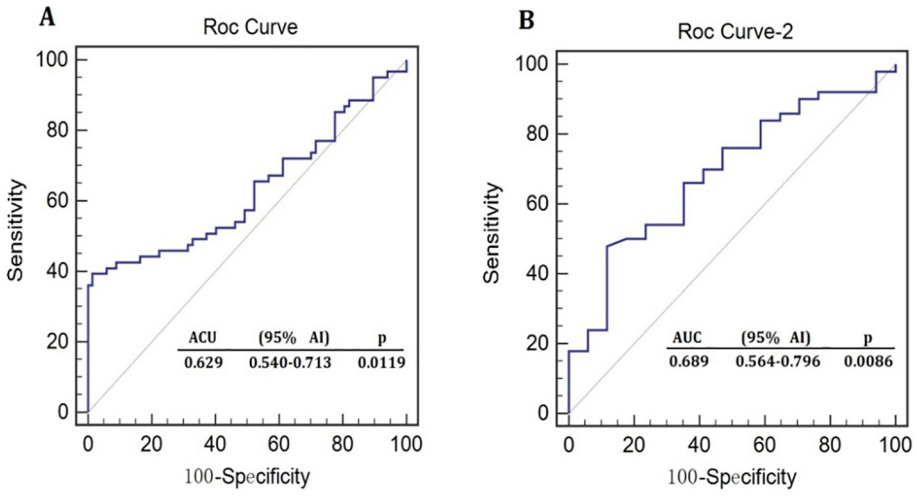


**Fig. 8.** (A) Sensitivity, specificity, receiver-operating characteristic (ROC) curves were determined between the level of COMP in ONFH and that in control subjects.(B) The Receiver operating characteristic (ROC-2) analytical curve was performed to explore the diagnostic value of COMP for radiographic progression of collapse in ONFH.

exhibited an active effect on inflammation in vivo, possibly also causing pain at high levels [20]. This suggests that the degeneration of articular cartilage occurred during the collapse of the femoral head, which was positively associated with the duration of pain. As a consequence, we inferred that the duration of pain was associated with the COMP level, probably reflecting the degree of femoral head collapse.

Smooth cartilage surfaces minimize friction and reduce the incidence between joints; however, cartilage degeneration occurs after the collapse of necrotic bone stock [21]. Furthermore, some studies demonstrated that chondrocyte apoptosis was related to the occurrence and severity of articular cartilage degradation [22]. On the basis of bone and cartilage morphology, osteocytes and chondrocytes were reduced, bony trabeculae were changed, and cartilage regions were substantially damaged during the progression of osteonecrosis of the femoral head that was aggravated with the development of disease. Apart from providing nutrients and substrates for chondrocytes, the extracellular matrix of cartilage (ECM), which is elastic and has a reliable resistance to pressure, also protects chondrocytes. The ECM receptor is a transmembrane protein complex. The focal adhesion maintains several links to intracellular organelles through the cytoskeleton, while it simultaneously attaches chondrocytes to enclose extracellular matrix. Furthermore, interaction and focal adhesion pathways are significantly enriched in expression in ONFH, and inhibition of focal adhesion synchronously prevented chondrocyte death [23]. COMP acts as a bridging-molecule between a protein of interest and its activating partner, or between the proteins and the cell surface. Recent studies have shown that COMP binds to various ECM components, and this intriguing activity suggests its possible involvement in influencing vital cell functions such as cell attachment, proliferation and differentiation, by modulating the ECM and cells [24,25]. One relevant study reported an opposite conclusion, suggesting that the inhibition of COMP-degrading enzymes slows down or blocks the initiation and progression of cartilage degeneration [25]; it also arrived at the conclusion that COMP stimulated chondrocyte proliferation and chondrogenesis [26]. Combined with these different studies, it could be suggested that too little or too much COMP may be detrimental and may result in tissue-specific cartilage destruction [13]. Therefore, it can be speculated that high levels of COMP promote apoptosis in chondrocytes and that the degeneration of articular cartilage in turn destabilizes the structure and exacerbates the collapse of the femoral head [6]. In general, cartilage injury occurs during the process of femoral head necrosis, and the apoptosis of chondrocytes would cause high levels of COMP, further aggravating the degeneration of cartilage, raising the risk of collapse and creating a vicious circle.

We confirmed again that COMP plays an important role in the process of chondrocyte apoptosis. The extreme expression of COMP following apoptosis of chondrocytes promotes focal adhesion, which in turn increases chondrocyte apoptosis. Moreover, increased levels of COMP activate the chondrocyte apoptosis pathway, as a recent study reported that most acute chondrocyte mortality occurs in FAK-dependent and SFK-dependent manners [23], thereby increasing chondrocyte apoptosis, resulting in the evolvement of the femoral head.

This study also suggested that non-traumatic ONFH patients with ARCO stage IV had significantly higher plasma COMP levels than did those with stage II. Furthermore, the levels of COMP in the post-collapse group were substantially higher than those in the pre-collapse group. These high levels of COMP may be the result of apoptosis of chondrocytes during the process of ONFH [13,22,23,25]. A positive correlation between COMP levels and ARCO stages was suggested by the results of correlation analysis, and these results indirectly showed that the increase in COMP may be linked to the progression of ONFH and may be a predictive factor of chondrocyte apoptosis and even of the collapse of the femoral head. Nevertheless, the relationship between Arco II and III stages remains uncertain. Therefore, the cutoff value calculated from the ROC curves is useful to define the specific concentration of collapse. What is feasible is that the cutoff value calculated from the ROC curves contributes to evaluation of the specific concentration of collapse. More specifically, reaching this concentration is a dangerous omen of collapse, while exceeding it is an omen of severe apoptosis of chondrocytes or the progressive stage.

In addition to the findings summarized above, this study also found that there were no significant differences in COMP levels among the non-traumatic ONFH patients with various etiologies, probably due to the increase in chondrocyte apoptosis that can be caused by various types of ONFH [27]; this study reached the same conclusion.

## 5. Conclusion

Our findings suggested that the increased expression of COMP may perform an important function in the process of non-traumatic ONFH. The cutoff concentration may be used as one of the sensitive indexes to evaluate the disease progression of non-traumatic ONFH and to guide clinical treatment.

## Acknowledgment

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## Conflict of interest

This article is the sole work of the authors. All authors report no conflict of interest.

## Availability of data and materials

The datasets, we got during this study are not publicly available, but the data may be available from the corresponding author if there are reasonable requirements.

## Author contributions

All authors read and agreed to the final manuscript. Q.W., S.L., S.G. and X.C. concept and design of the study and wrote the manuscript. S.G., X.C., Z.C., X.H., F.P., J.H. implemented the main experiments. Y.Z. and Y.Q. prepared all materials. S.G. and X.C. analysis and interpretation of the data.

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