Cartilage oligomeric matrix protein as a potential biomarker for knee osteoarthritis

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Aims

This study aimed to determine the expression and clinical significance of a cartilage protein, cartilage oligomeric matrix protein (COMP), in knee osteoarthritis (OA) patients.

Methods

A total of 270 knee OA patients and 93 healthy controls were recruited. COMP messenger RNA (mRNA) and protein levels in serum, synovial fluid, synovial tissue, and fibroblast-like synoviocytes (FLSs) of knee OA patients were determined using enzyme-linked immunosorbent assay, real-time polymerase chain reaction, and immunohistochemistry.

Results

COMP protein levels were significantly elevated in serum and synovial fluid of knee OA patients, especially those in the advanced stages of the disease. Serum COMP was significantly correlated with radiological severity as well as measures of body composition, physical performance, knee pain, and disability. Receiver operating characteristic curve analysis unveiled a diagnostic value of serum COMP as a biomarker of knee OA (41.64 ng/ml, area under the curve (AUC) = 1.00), with a sensitivity of 99.6% and a specificity of 100.0%. Further analysis uncovered that *COMP* mRNA expression was markedly upregulated in the inflamed synovium of knee OA, consistent with immunohistochemical staining revealing localization of COMP protein in the lining and sub-lining layers of knee OA inflamed synovium. Most notably, relative *COMP* mRNA expression in knee OA synovium was positively associated with its protein levels in serum and synovial fluid of knee OA patients. In human knee OA FLSs activated with tumour necrosis factor-alpha, *COMP* mRNA expression was considerably up-regulated in a time-dependent manner.

Conclusion

All results indicate that COMP might serve as a supportive diagnostic marker for knee OA in conjunction with the standard diagnostic methods.

Article focus

- We examined cartilage oligomeric matrix protein (*COMP*) messenger RNA (mRNA) and protein levels in the systemic and local joint environment, as well as its association with clinical parameters of knee osteoarthritis (OA) patients, particularly indicators of physical performance as well as knee pain and disability.
- Whether alterations in *COMP* mRNA expression were regulated by a pro-inflammatory cytokine like tumour necrosis factor-alpha (TNF-α) in human

knee OA fibroblast-like synoviocytes (FLSs) was determined.

Key messages

- Positive associations between serum COMP and indicators of physical performance, knee pain, and disability were observed in knee OA patients.
- This in vitro study discovered that up-regulated *COMP* mRNA expression was regulated by TNF-α in human knee OA FLSs.
- COMP might serve as a supportive diagnostic marker for knee OA in conjunction with the standard diagnostic methods.

Strengths and limitations

- This study adds to the currently limited body of evidence regarding the associations between serum COMP and physical performance markers of knee OA, in addition to the importance of COMP in knee OA synovitis.
- Receiver operating characteristic curve analysis with ten-fold cross-validation was conducted to determine the potential of serum COMP as a knee OA biomarker.
- This study could not adequately explore the cause-and-effect relationship between COMP mRNA and protein expressions and knee OA severity, especially synovial inflammation.

Introduction

Knee osteoarthritis (OA), a degenerative joint disease affecting a large proportion of elderly people, is one of the primary causes of severe pain and disability, which imposes a huge economic burden on healthcare systems worldwide.[1,2] Knee OA manifests itself clinically as cartilage degeneration, subchondral bone sclerosis, osteophyte formation, and synovial inflammation (synovitis).[1] Despite intensive research efforts to identify disease markers and develop effective therapies,[3] the progression of joint degeneration cannot yet be halted. As a consequence, the majority of knee OA patients will experience persistent pain, progress to disability, and finally need for a total knee arthroplasty (TKA).[4] The rising incidence of TKA emphasizes the need for the development of disease-modifying medications, and for the identification of possible biomarkers to help in earlier diagnosis, both of which would prolong the time patients have before they become severely disabled. As a rule, radiological alterations are often not detectable in the early stages of knee OA. In that context, weightbearing radiographs are frequently taken after joint degeneration has already occurred, which limits their usefulness in diagnosis and disease monitoring.[5] Given that disruptions in cartilage homeostasis are known to be related to knee OA pathology,[6] it is conceivable that measuring metabolic products of cartilage repair and degradation to reflect variations in joint remodelling might be a useful approach in early diagnosis.

One of the byproducts of cartilage metabolism, cartilage oligomeric matrix protein (COMP), is currently gaining research interest as a possible biomarker for the early detection of knee OA. COMP, often known as thrombospondin-5, is an extracellular matrix (ECM) glycoprotein responsible for the assembly and structure of the cartilage matrix.[7] In the context of knee OA, when the articular cartilage deteriorates over time, the collagen scaffold breaks down, and COMP is released into the extracellular space and eventually excreted in biological fluids including synovial fluid, serum, and urine.[8,9] On the basis of its biological properties, accumulating evidence has uncovered the significant involvement of serum COMP in musculoskeletal diseases like knee OA, in which serum levels of COMP were detected to be significantly elevated in knee OA patients compared to healthy controls.[10-12] In an explant model of articular cartilage treated with pro-inflammatory cytokines including interleukin (IL)-1β and tumour necrosis factor-alpha (TNF-α), COMP protein expression was detected to be significantly increased.[13] From those viewpoints, it is important to note that establishing serum COMP as a diagnostic marker may serve as a crucial tool for initiating early therapy and eliminate the need for unfavourable radiation exposure during diagnosis, monitoring, and treatment of knee OA.

Although the literature indicates that COMP could be a possible biomarker of knee OA,[14] to the best of our knowledge, COMP association with clinical parameters of knee OA, in particular physical performance, knee pain, and disability, has not been thoroughly investigated. Besides this, its significant involvement with synovial inflammation in human knee OA fibroblast-like synoviocytes (FLSs) remains largely unexplored. Accordingly, the purposes of this study were to determine: 1) COMP messenger RNA (mRNA) and protein levels in the systemic and local joint environment, as well as their association with clinical parameters of knee OA patients; and 2) whether alterations in *COMP* mRNA expression were regulated by a pro-inflammatory cytokine, like TNF-α, in human knee OA FLSs.

Methods

The present study was conducted in accordance with the guidelines outlined in the Declaration of Helsinki,[15] and was approved by the Ethical Committee on Human Research at the Faculty of Dentistry/Faculty of Pharmacy, Mahidol University (institutional review board number: MU-DT/PY-IRB 2019/074.2511). All study participants provided informed written consent before being recruited for the research.

Study participants

A total of 270 patients with primary knee OA at the outpatient clinic of the Department of Orthopedics at King Chulalongkorn Memorial Hospital were included in this case-control study; all of them had been diagnosed with knee OA according to the American College of Rheumatology's criteria.[16] The study control group consisted of 93 individuals without any clinical or radiological signs of knee OA. Participants were excluded if they had a history of diabetes, severe hepatic or renal disease, other forms of arthritis, cancer, or other chronic inflammatory disorders, or were currently taking any medications known to interfere with bone metabolism (e.g. corticosteroids or bisphosphonates). Radiographs of knee OA patients were taken while they stood up, knees completely extended, and the X-ray beam focused on the joint. The radiological severity of knee OA was assessed using the Kellgren-Lawrence (KL) system,[17] and all preoperative radiographs were reviewed blinded to the patients' clinical and laboratory data. Typically, individuals were diagnosed

with knee OA if they exhibited radiological signs of knee OA in at least one knee, with a KL grade of 2 serving as an indicator. A retrospective analysis of medical records was conducted to assess the clinical severity and other pertinent characteristics observed during the initial diagnosis.

Body composition

Height, weight, and waist circumference of study subjects were all measured in accordance with established protocols. BMI was calculated by dividing body weight in kilograms (kg) by height in metres squared (m^2). Body composition, including fat mass, was assessed by bioelectrical impedance analysis (BIA) (BC-418 Segmental Body Composition Analyzer; Tanita Corporation, Japan).

Baseline and clinical characteristics of study participants

Baseline and clinical characteristics of study subjects are summarized in Table I. In a comparison of baseline characteristics between healthy controls and knee OA patients, there were no significant differences in mean age, sex ratio, and body compositions consisting of BMI and fat mass. Regardless of physical performance markers, both WOMAC score and TUGT were both significantly greater in knee OA patients than those in healthy controls ($p = 0.030$ and $p < 0.001$, respectively, independent-samples *t*-test), while there were no differences in grip strength, gait speed, STS, or 6MWT. In stratified analysis by KL grade of knee OA patients, the patients with KL grade 4 exhibited significantly higher WOMAC scores than healthy controls ($p = 0.009$, independent-samples *t*-test). Besides this, knee OA patients with KL grade 4, 3, and 2 had significantly greater TUGT scores than healthy controls ($p < 0.001$ for all three grades, analysis of variance (ANOVA)). Based on the radiological severity classified by KL grade, knee OA patients were separated into three subgroups: those with KL grade 2 (n = 73, 27.0%), those with KL grade 3 (n = 75, 31.5%), and those with KL grade 4 (n = 112, 42.5%). Among knee OA patients with different KL grades, there were significant differences in scores of knee pain and disability (VAS and WOMAC; $p < 0.001$ for both, chi-squared test) and measures of physical performance (TUGT, STS, and 6MWT; $p < 0.001$, $p = 0.001$, and $p = 0.018$, respectively, ANOVA). However, mean age, sex ratio, body composition markers (BMI and fat mass), and muscle strength indicators (grip strength and knee extension force) did not differ significantly among knee OA patients with different KL grades.

Knee pain and physical disability

Knee OA patients were evaluated for their levels of pain, stiffness, and physical disability using visual analogue scale (VAS) and the Western Ontario and MacMaster Universities Osteoarthritis Index (WOMAC) scores.[18] Discomfort felt in the joint during motion was measured using a ten-point VAS, where no pain equals a score of 0, and the worst possible pain equals a score of 10. Correspondingly, the WOMAC was employed to assess knee function and disability. The 24 questions on the WOMAC were divided into three categories: pain (5 questions), stiffness (2 questions), and physical function (17 questions), with the total score representing an individual's overall level of impairment. Scores on the WOMAC measure the degree to which a person experiences pain, stiffness, and the inability to do daily tasks.

Muscle strength

Grip, knee flexor, and extensor strengths of the participants were all measured by a physical therapist. A grip strength dynamometer (Takei Scientific Instruments, Japan) was used to measure grip strength. Moreover, a handheld dynamometer (Hoggan Scientific, USA) was used to measure the flexor and extensor strength of the knees. Briefly, the participants were seated on the treatment table with their knees flexed at a 90° angle. The dynamometer was placed 5 cm above the transmalleolar axis and perpendicular to the tibial crest. The participants were instructed by a physical therapist to lift their lower legs and hold a maximal sustained force position for five seconds. An orthopaedic physician provided training to physical therapists on the correct technique for performing handheld dynamometer measurements, with the aim of standardizing procedures.[19]

Physical performance

Functional performance was evaluated using a variety of tests, including gait speed, the Timed Up and Go (TUTG) test, the sit-to-stand (STS), and the six-minute walk test (6MWT), as previously suggested.[20] People who can walk 5 m at a normal pace will be able to take the exam designed for a 4 m gait speed. The entire 5 m distance was timed using a standard stopwatch, with the exception of the first and final 50 cm segments. The time it takes to rise from a seated position, walk 3 m, turnabout, walk back, and sit down again (known as TUTG) is one measure of fundamental balance and mobility, and has been suggested by a previous study alongside the gait speed test.[20] In addition to this, STS was employed as an additional measure of physical performance; individuals were requested to stand and sit five times as swiftly as possible from a standard-height chair (45 cm) with their arms crossed over their chest. The duration of time it took the patient to complete this sequence of activities was recorded. Distance walked in six minutes (6MWT) was the last physical performance measure employed in this analysis.

Cell isolation and culture

Synovial tissues from eight out of 50 knee OA patients who had TKA were enzymatically digested to isolate FLSs, a valuable biological model for investigating the pathogenic physiology of synovitis. Briefly, the synovium was chopped into small pieces using a sterile Bard Parker blade. A 0.33% collagenase type II solution (Sigma-Aldrich, USA) dissolved in Dulbecco's modified Eagle medium (DMEM; Hyclone Laboratories, USA) was then added to the minced tissues. For six hours, the mixture was stirred at 37°C with 5% CO_2 and 95% humidity. Collagenase digestion was proceeded by centrifugation to collect the cell pellet, which was then resuspended in DMEM with high glucose, containing 100 IU/ml penicillin/streptomycin and 10% fetal bovine serum (Hyclone Laboratories). The cell suspension was subsequently seeded at a density of approximately 1×10^6 cells/cm^2 to a tissue culture flask (Nunc, Denmark) measuring 75 cm^2 and containing 10 ml of DMEM supplement. The flask was kept at 37°C in a humidified atmosphere of 5% CO_2, with sterile media changes made every three days until the cells achieved 95% confluence. Passage 1 (P1) cells at a concentration of 2.0×10^4 cells/ml in 24-well plates were employed in further experiments. FLSs were either left untreated or treated with

Table I. Baseline and clinical characteristics of healthy controls and knee osteoarthritis patients with different Kellgren-Lawrence grades.

Variable	Healthy controls	Knee OA patients				Model 1*	Model 2†
		Overall	KL grade 2	KL grade 3	KL grade 4	p-value	p-value
Number (%)	93 (100.0)	270 (100.0)	73 (27.0)	75 (31.5)	112 (42.5)	N/A	N/A
Sex (F/M), n (%)	78 (83.9)/15 (16.1)	237 (87.8)/33 (12.2)	63 (86.3)/10 (13.7)	74 (87.1)/11 (12.9)	100 (89.3)/12 (10.7)	0.337	0.808
Mean age, yrs (SD)	67.64 (8.53)	66.17 (7.87)	64.41 (8.15)	67.63 (7.30)	66.13 (7.98)	0.144	0.065
Body composition markers							
Mean BMI, kg/m^2 (SD)	24.33 (10.06)	25.78 (3.96)	25.58 (4.07)	25.83 (4.31)	25.86 (3.63)	0.520	0.249
Mean fat mass, kg (SD)	21.06 (12.26)	22.50 (7.87)	22.08 (7.65)	22.65 (8.09)	22.65 (7.93)	0.461	0.864
Knee pain and physical disability scores							
Mean VAS, 0 to 10 (SD)	N/A	3.89 (2.31)	1.43 (1.12)	3.09 (1.02)	5.92 (1.64)	N/A	< 0.001
Mean WOMAC, 0 to 10 (SD)	1.69 (1.61)	2.68 (1.95)	1.03 (0.77)	2.29 (1.09)	3.93 (2.07)	0.030	< 0.001
Muscle strength indicators							
Mean grip strength, kg (SD)	21.23 (5.71)	22.39 (5.31)	22.59 (4.49)	22.66 (6.60)	22.07 (4.76)	0.375	0.790
Mean knee extension force, N (SD)	N/A	355.99 (79.12)	368.23 (72.25)	350.85 (84.94)	352.40 (78.99)	N/A	0.741
Physical performance indicators							
Mean gait speed, m/s (SD)	1.08 (0.48)	0.96 (0.21)	0.99 (0.20)	1.00 (0.22)	0.92 (0.21)	0.291	0.053
Mean TUGT, s (SD)	7.19 (2.74)	9.88 (2.61)	9.09 (1.74)	9.33 (2.14)	10.75 (3.07)	< 0.001	< 0.001
Mean STS, s (SD)	14.00 (5.75)	14.91 (4.54)	13.44 (3.65)	14.17 (3.99)	16.31 (5.01)	0.424	0.001
Mean 6MWT, m (SD)	351.89 (85.75)	370.18 (78.92)	396.75 (69.27)	370.08 (76.47)	354.45 (82.69)	0.344	0.018

*Model 1: Comparing variables between healthy controls and knee osteoarthritis patients. Statistical differences in continuous variables, including age, body composition markers, Western Ontario and MacMaster Universities Osteoarthritis Index (WOMAC) score, grip strength, and physical performance indicators, were assessed by independent-samples *t*-test, whereas a statistical difference in sex ratio was executed by chi-squared test.
†Model 2: Comparing variables among knee osteoarthritis patients with different groups based on their Kellgren-Lawrence grade. Statistical differences in continuous variables, including age, body composition markers, scores of knee pain and physical disability, muscle strength indicators, and physical performance indicators, were assessed by analysis of variance (ANOVA), whereas a statistical difference in sex ratio was executed by chi-squared test.
COMP, cartilage oligomeric protein matrix; KL, Kellgren-Lawrence; 6MWT, 6 minute walk test; N/A, not available; OA, osteoarthritis; SD, standard deviation; STS, sit-to-stand; TUGT, Timed Up and Go test; VAS, visual analogue scale; WOMAC, Western Ontario and MacMaster Universities Osteoarthritis Index.

10 ng/ml TNF-α for one, three, or seven days in CO_2 incubator with 37°C in 5% CO_2 and 95% humidity. Treatments were administered in serum-free medium with a TNF-α (Biolegend, USA) concentration of 10 ng/ml selected based on previous studies.[21,22]

Enzyme-linked immunosorbent assay

Fasting venous blood samples were obtained from both healthy controls and knee OA patients. The blood samples were collected using clotted blood tubes and subsequently centrifuged to separate the serum. The serum samples were promptly stored at -20°C for subsequent analysis. When TKA was performed on knee OA patients, synovial fluid was aspirated from the knees of knee OA patients using sterile knee puncture. Following centrifugation, cells and debris from the joints were separated from the specimen, and it was then frozen at -20°C for subsequent analysis. Quantitative COMP levels in serum and synovial fluid were measured using a commercial sandwich enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, USA), as per the manufacturer's instructions.

Quantitative real-time polymerase chain reaction

During TKA procedures, synovial tissue samples were harvested from 50 knee OA patients with KL grade 4. A pathologist who was blind to the clinical status and diagnosis of the patients classified 28 of 50 synovial tissues as inflamed synovium and 22 as non-inflamed synovium, based on the presence or absence of three significant morphological alterations: hyperplasia of the synovial lining layer, infiltration of inflammatory cells, and activation of the synovial stroma. Following the manufacturer's instructions, total RNA was extracted from synovial tissues of knee OA patients as well as from cell pellets of knee OA FLSs cultured under the aforementioned experimental conditions using a RNeasy Mini kit (Qiagen, Germany), and complementary DNA (cDNA) was synthesized via reverse transcription using a TagMan Universal PCR Master Mix (Applied Biosystems, USA). Relative *COMP* mRNA expression was determined using a quantitative real-time polymerase chain reaction (PCR) with the QPCR Green Master Mix HRox (Biotechrabbit, Germany) on a StepOnePlus Real-Time PCR System (Applied Biosystems). The $2^{-\Delta\Delta Ct}$ method was used to determine relative *COMP* mRNA

expression, with normalization to glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) as the endogenous control.

Haematoxylin and eosin and immunohistochemistry

Synovial tissue samples from knee OA patients were immunohistochemically analyzed to determine the localization of COMP protein expression. Tissue samples were fixed in paraffin and sectioned in accordance with standard protocols. Haematoxylin and eosin (H&E) staining was used to assess synovial morphological changes, and COMP protein expression was examined by immunohistochemical staining using specific antibodies. Standard immunohistochemical procedure was carried out using an autostainer (Ventana Medical Systems, USA). Briefly, tissue sections were deparaffinized and rehydrated. Endogenous peroxidase activity was blocked by 0.3% hydrogen peroxide for ten minutes. After performing heat-induced antigen retrieval in 10 mmol/l citrate buffer (pH 6.0) for five minutes, the slides were incubated with pepsin for seven minutes. Following this, the slides were incubated with primary rabbit anti-COMP antiserum (Abcam, USA) diluted at 1:500 for two hours, and control slides were treated with the corresponding nonimmune serum. Afterwards, the sections were stained with goat anti-rabbit IgG (Dako A/S, Denmark) as the secondary antibody conjugated to streptavidin/horseradish peroxidase for 45 minutes at room temperature. Reaction products were visualized using 3,3-diaminobenzidine tetrahydrochloride (Sigma-Aldrich), and the sections were counterstained with haematoxylin.

Statistical analysis

All statistical analyses were performed using SPSS Statistics version 26.0 (IBM, USA) and GraphPad Prism version 9.0 (GraphPad Software, USA). The comparison of normally distributed continuous variables were accomplished using independent-samples *t*-test (for two groups) and ANOVA (for > two groups) with a Tukey post hoc test, while the comparison of abnormally distributed continuous variables was conducted using Mann-Whitney U test (for two groups) and Kruskal-Wallis H test (for > two groups). Based on categorical variables, chi-squared test was used to determine statistically significant differences between groups. To determine the strength of relationship between radiological severity and serum, as well as synovial fluid COMP of knee OA patients, ordinal logistic regression analysis was performed. Pearson correlation coefficient (*r*) was calculated to evaluate possible correlations between serum COMP levels and clinical parameters of knee OA patients, in which *r* values of < 0.3, 0.3 to 0.5, and ≥ 0.5 corresponded to weak, moderate, and strong correlations, respectively.[23] Receiver operating characteristic (ROC) curve was constructed to determine the diagnostic value of serum COMP as a possible biomarker of knee OA, which yielded the area under the ROC curve (AUC), sensitivity, and specificity. Along with ROC curve, K-Fold cross-validation was performed using Python to evaluate the ROC response of the different datasets. Statistical differences in *COMP* mRNA expressions of treated and untreated knee OA synoviocytes at various time points were assessed by Wilcoxon signed-rank test. Data are expressed as mean and standard deviation (SD). A p-value less than 0.05 was considered statistically significant for all analyses.

Results

Serum and synovial fluid COMP levels

Compared to healthy controls, knee OA patients had significantly increased serum and synovial fluid COMP levels ($p < 0.001$ for both, Kruskal-Wallis H test) (Figure 1a). In knee OA patients, median synovial fluid COMP levels were shown to be significantly higher than serum samples ($p < 0.001$, Mann-Whitney U test) (Figure 1a).

Stratified analyses by the disease severity revealed that knee OA patients with either KL grade 3 or 4 had substantially greater serum COMP levels than those with KL grade 2 ($p = 0.002$ and $p < 0.001$, respectively, Kruskal-Wallis H test) (Figure 1b). Compared to healthy controls, knee OA patients with KL grade 4, 3, and 2 exhibited significantly increased serum COMP levels ($p < 0.001$ for all three grades, Kruskal-Wallis H test) (Figure 1b). In accordance with analysis of serum COMP levels, synovial fluid COMP levels were observed to be significantly higher in knee OA patients with KL grades 4 or 3 than those with KL grade 2 ($p < 0.001$ for both grades, Kruskal-Wallis H test) (Figure 1c).

More specifically, ordinal logistic regression analysis demonstrated that serum COMP was significantly associated with a 1.289-fold increase in KL grade in knee OA patients ($p < 0.001$, ordinal logistic regression; odds ratio (OR) 1.289, 95% confidence interval (CI) 1.213 to 1.369). Besides this, synovial fluid COMP was significantly associated with a 1.003-fold increase in KL grade in knee OA patients ($p < 0.001$, ordinal logistic regression; OR = 1.003, 95% CI 1.002 to 1.003). Notably, Pearson correlation analysis displayed a moderate positive correlation between serum COMP and its levels in paired synovial fluid of knee OA patients ($r = 0.248$, $p = 0.014$) (Figure 1d).

Correlations between serum COMP and outcome parameters of knee OA

A heatmap of Pearson correlation matrix between serum COMP and outcome parameters in knee OA patients is depicted in Figure 2a. In knee OA patients, serum COMP levels were strongly correlated with scores of knee pain and disability (VAS: $r = 0.581$, $p < 0.001$; and WOMAC: $r = 0.494$, $p < 0.001$) and weakly correlated with indicators of physical performance (TUGT: $r = 0.211$, $p = 0.006$; and STS: $r = 0.216$, $p = 0.005$) (Figures 2b to 2e).

Serum COMP as a diagnostic biomarker of knee OA

To identify the potential use of serum COMP as a possible biomarker of knee OA, the area under the receiver operating characteristic curve (AUC of ROC) was calculated. ROC curve analysis uncovered that the optimal cutoff value of serum COMP as a possible biomarker for differentiating knee OA patients from healthy controls was defined at 41.64 ng/ml, which yielded a sensitivity of 99.6%, a specificity of 100.0%, and an AUC of 1.00 (95% CI 1.00 to 1.00; $p < 0.001$) (Figure 3a). With ten-fold cross-validation, mean ROC curve uncovered that serum COMP was not influenced by the different datasets (mean AUC = 0.99, sensitivity = 100%, specificity = 99%) (Figure 3b), supporting the potential of serum COMP as

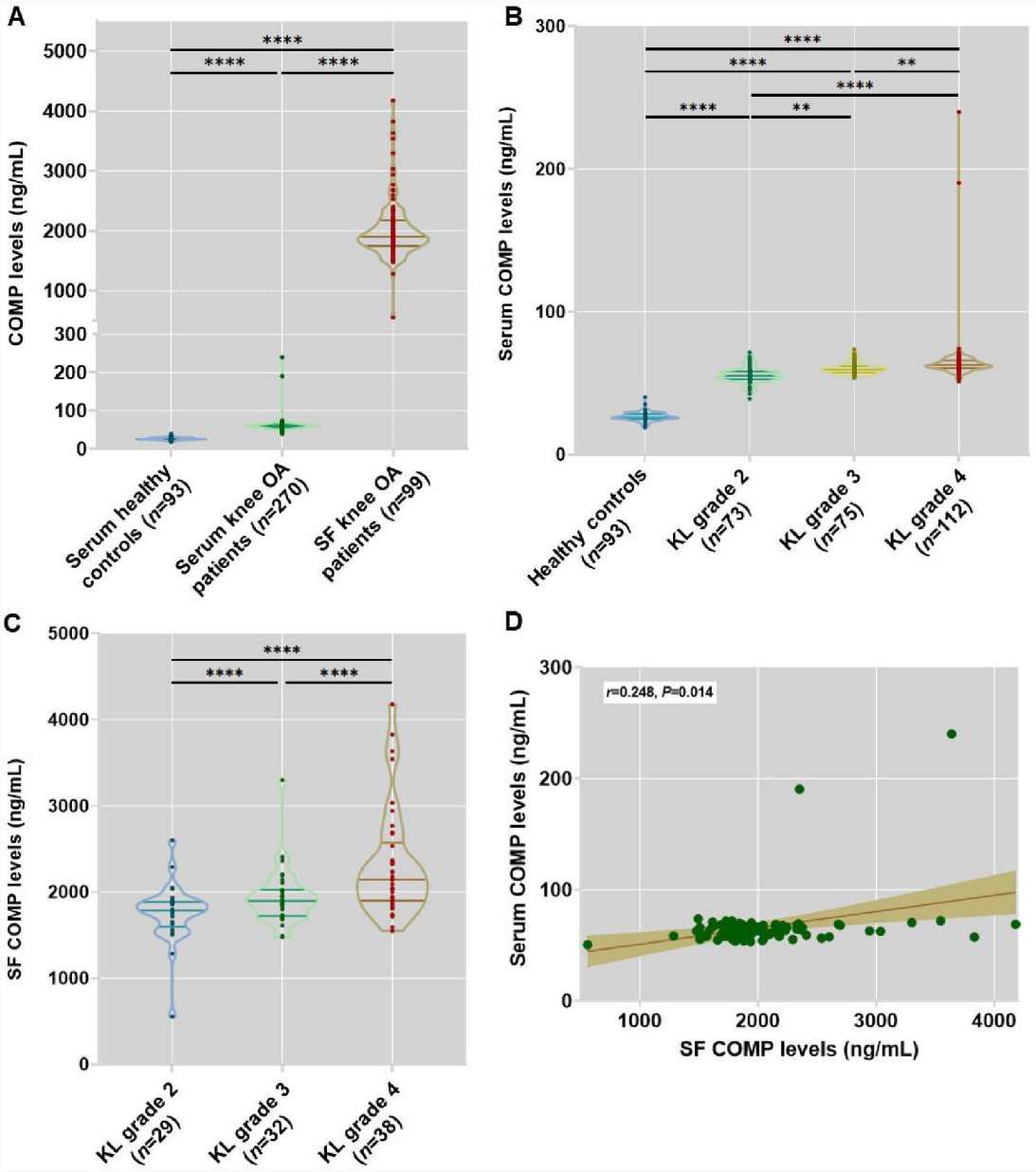


Fig. 1

Cartilage oligomeric matrix protein (COMP) levels in healthy controls and knee osteoarthritis (OA) patients. a) Comparing serum and synovial fluid COMP levels between healthy controls and knee OA patients. b) Comparing serum COMP levels between healthy controls and knee OA patients with different Kellgren-Lawrence (KL) grades. c) Comparing synovial fluid COMP levels among knee OA patients with different KL grades. d) A positive association between serum COMP and its levels in paired synovial fluid of knee OA patients. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 for non-parametric multiple comparisons in independent groups using Kruskal-Wallis H test.

a biomarker for differentiating knee OA patients from healthy controls.

COMP mRNA and protein expressions in non-inflamed and inflamed synovial tissues of knee OA

Given considerable increases in serum and synovial fluid COMP levels in knee OA patients – particularly those with late-stage OA (KL grade 3 to 4) – relative *COMP* mRNA expression in synovial tissues classified into non-inflamed (n = 22) and inflamed synovial tissue (n = 28) samples of knee OA patients was further investigated using real-time polymerase chain reaction. As depicted in Figure 4a, relative *COMP* mRNA expression was significantly up-regulated in the inflamed synovial tissues, compared with the non-inflamed synovial tissues (p < 0.001). Remarkably, relative *COMP* mRNA expression was shown to be strongly correlated with its protein levels in serum and synovial fluid of knee OA patients (r = 0.620, p < 0.001; and r = 0.450, p < 0.001, respectively) (Figures 4b and 4c).

Since *COMP* mRNA expression was shown to be significantly increased in the inflamed synovium of knee OA patients, the localization of its protein expression was subsequently determined using immunohistochemical staining. Knee OA synovial tissues with and without synovitis are depicted histologically in Figures 4d to 4m. In the inflamed synovium of knee OA (Figure 4e), H&E staining displayed synovial lining enlargement (Figure 4f), inflammatory cell infiltration (Figure 4g), and synovial stoma presence

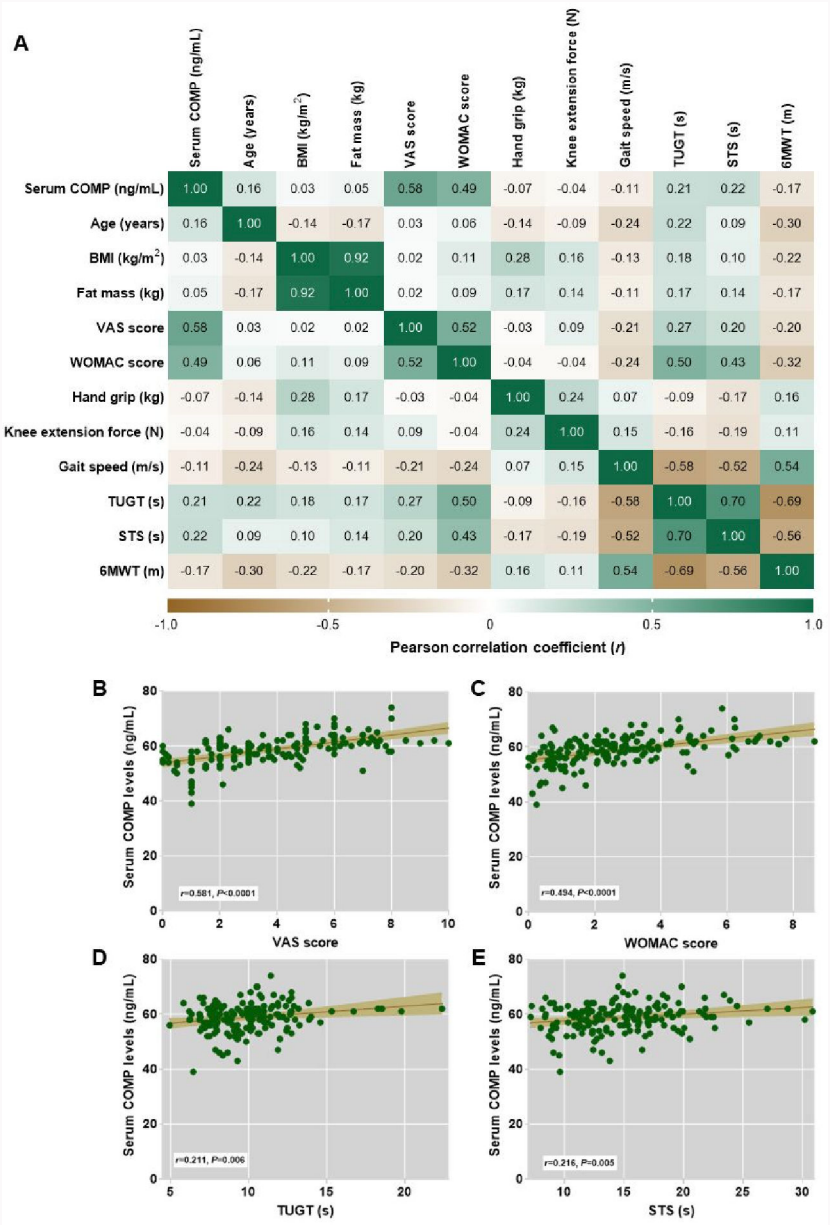


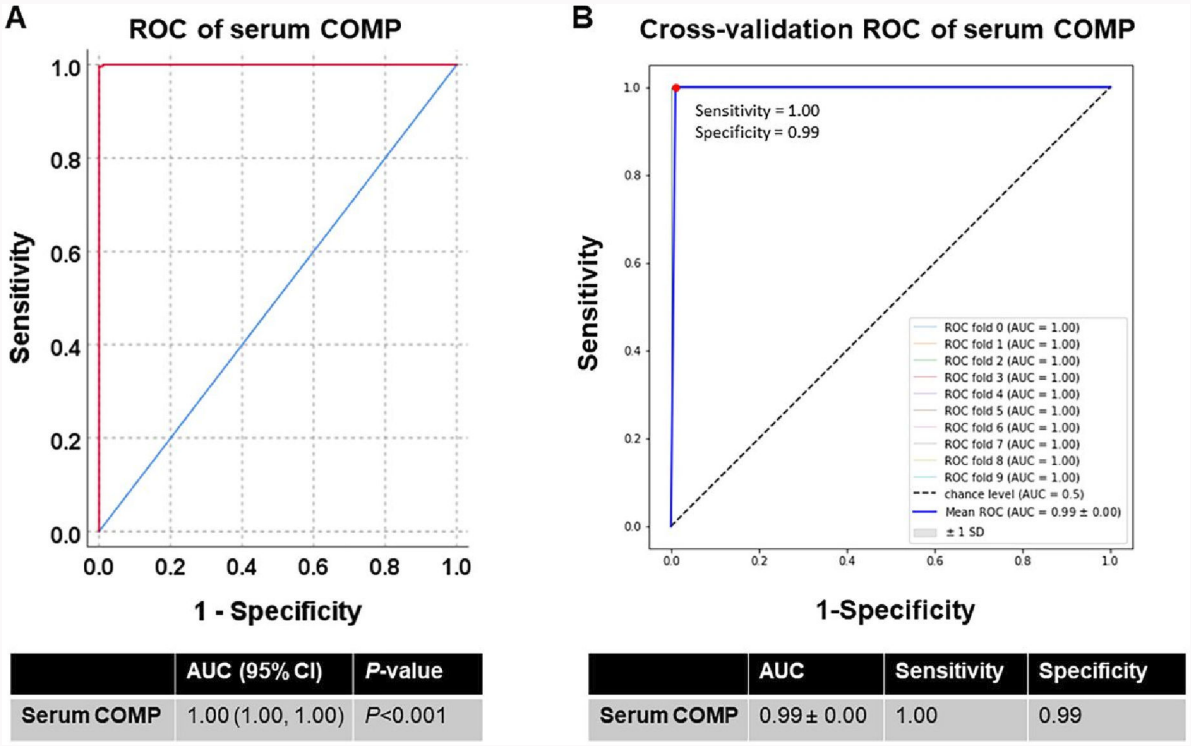
Fig. 2

Cartilage oligomeric matrix protein (COMP) association with outcome parameters of knee osteoarthritis (OA) patients. a) A heatmap of Pearson correlation matrix between serum COMP and clinical parameters of knee OA patients. b) Scatter plot displaying a strong positive correlation between serum COMP and knee pain (visual analogue scale (VAS) score) in knee OA patients. c) Scatter plot displaying a strong positive correlation between serum COMP and physical disability (Western Ontario and MacMaster Universities Osteoarthritis Index (WOMAC) score) in knee OA patients. d) Scatter plot displaying a weak positive correlation between serum COMP and Timed Up and Go test (TUGT) in knee OA patients. e) Scatter plot displaying a weak positive correlation between serum COMP and sit-to-stand (STS) in knee OA patients. 6MWT, distance walked in six minutes.

(Figure 4h). On the contrary, as demonstrated in Figure 4c, the non-inflamed synovium of knee OA lacked synovial lining layer hypertrophy, synovial stoma, and inflammatory cell infiltration. As revealed in Figures 4i to 4m, immunohistochemical analysis revealed that COMP protein expression was abundant in both the synovial lining layer of the inflamed synovial tissues of knee OA patients (Figures 4j and 4k), particularly in FLSs (head arrows) (Figure 4k) and inflammatory cells (dotted arrows) (Figure 4l), and the sub-lining layer of the inflamed synovial tissues of knee OA patients, especially in epithelial cells (arrows) (Figure 4m). In contrast to the aforementioned findings, a faint cytoplasmic staining for COMP protein was observed in the non-inflamed synovium of knee OA patients (Figure 4i).

COMP mRNA expression in fibroblast-like synoviocytes of knee OA

As TNF-α is a key molecule responsible for synovial inflammation by enhancing the production of inflammatory mediators, we further determined whether upregulation of *COMP* mRNA expression was modulated by TNF-α in human knee OA FLSs, which are important cellular components of the inner layer of the synovium and play a critical role in persistent inflammatory joint diseases like knee OA. On days 1, 3, and 7, relative *COMP* mRNA expression was detected to be significantly



	AUC (95% CI)	*P*-value
Serum COMP	1.00 (1.00, 1.00)	*P*<0.001

	AUC	Sensitivity	Specificity
Serum COMP	0.99 ± 0.00	1.00	0.99

Fig. 3
Diagnostic value of serum cartilage oligomeric matrix protein (COMP) as a biomarker of knee osteoarthritis (OA). a) Receiver operating characteristic (ROC) curve displaying serum COMP as a biomarker for differentiating knee OA patients from healthy controls. b) ROC curve with ten-fold cross-validation displaying the reliability of serum COMP as a knee OA biomarker. AUC, area under the curve; CI, confidence interval; SD, standard deviation.

upregulated in knee OA FLSs treated with TNF-α, compared to untreated FLSs ($p < 0.001$ for all days, Wilcoxon signed-rank test) (Figure 4n).

Discussion

One of the pathological aspects driving the development of cartilage degeneration in knee OA is synovial inflammation, which has also been demonstrated to be linked to clinical symptoms.[24] Among the numerous approaches currently used to diagnose and characterize synovial inflammation, histological and imaging examinations are only two diagnostic approaches. Although recent advancements in MRI have enabled the acquisition of high-quality images that offer enhanced visualization of both early and progressive stages of various diseases, this imaging method is not commonly employed for routine OA diagnosis due to financial constraints and limited availability of instruments in most countries. Hence, it is becoming more important to detect knee OA in its earliest stages, when it is still characterized by synovial inflammation-induced degenerative processes. Given that advances in biological markers may possibly give the tools for proper stratification, the present study focused on examining the expression and production of COMP, one of the byproducts of cartilage metabolism, in the systemic and local joint environment of knee OA patients. Significant increases in serum and synovial fluid COMP levels were seen in knee OA patients, especially those with late-stage OA, and significantly correlated with outcome parameters of knee OA. Apart from this, *COMP* mRNA expression was observed to be significantly upregulated in the inflamed synovial tissues of knee OA, compared with the non-inflamed synovial tissues. Additionally, this study uncovered for the first time that alterations in *COMP* mRNA expression were regulated by TNF-α in human knee OA FLSs. Taken together, the above results lend credence to the idea that COMP might be not only a potential biomarker of knee OA, but possibly also a molecule involved in an inflammatory process in knee OA.

In support of our findings regarding significant increments in serum and synovial fluid COMP levels in knee OA patients, several studies have unveiled a diagnostic potential of COMP for arthritis including knee OA.[12,25–27] In knee OA patients, serum COMP levels were found to be significantly elevated and correlated with radiological severity and degree of synovitis.[28–33] In addition to this, serum COMP levels were found to be significantly increased in knee OA patients with bone scan abnormalities,[34] suggesting that alterations in COMP serum levels might reflect changes in the turnover of knee OA tissues like the synovium. This hypothesis was addressed by our additional finding, showing a significant upregulation of *COMP* mRNA expression in knee OA synovium – predominantly the inflamed synovial tissues. In parallel with this, *COMP* mRNA expression was detected to be positively correlated with its serum and synovial fluid levels. Our result of upregulated *COMP* mRNA expression in the inflamed synovium of knee OA is supported by a previous study showing that COMP was expressed in the synovium and cartilage.[35] In both human FLSs and chondrocytes, COMP expression has been reportedly activated by transforming growth factor-β.[35] Furthermore, it has been shown that COMP was released into the cartilage secretome because of matrix

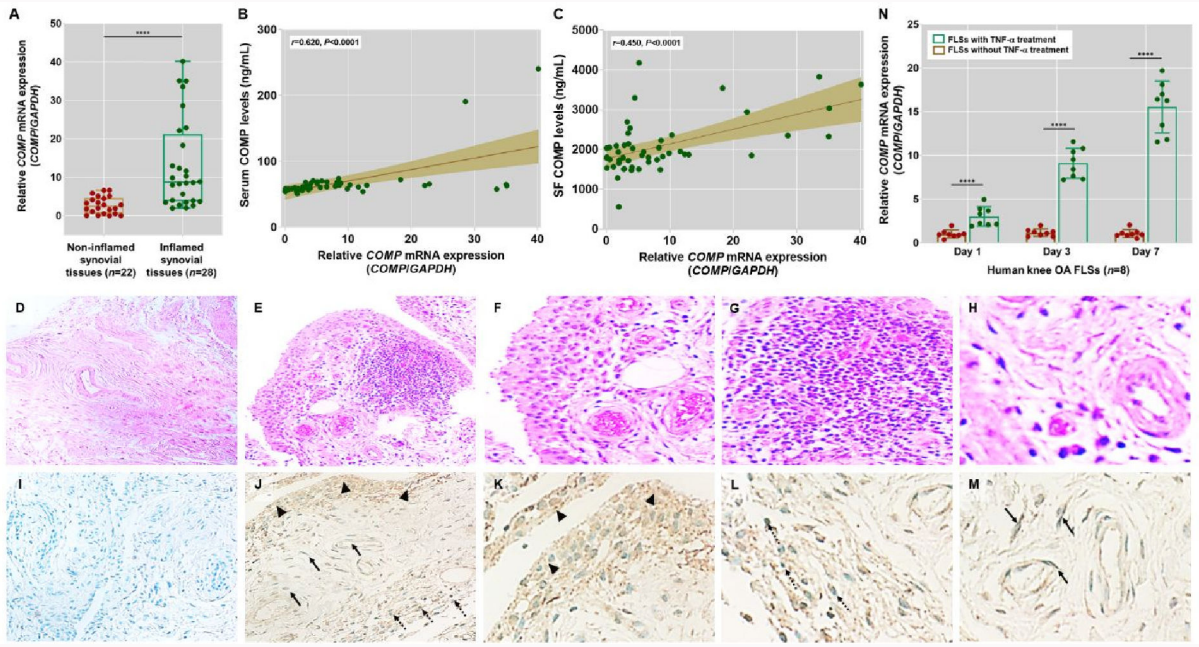


Fig. 4

Cartilage oligomeric matrix protein (COMP) messenger RNA (mRNA) and protein expressions in synovial tissues and fibroblast-like synoviocytes (FLSs) of knee osteoarthritis (OA). a) Relative *COMP* mRNA expression in the non-inflamed and inflamed synovial tissues of knee OA patients. b) Positive association between *COMP* mRNA expression and its protein levels in serum of knee OA patients. c) Positive association between *COMP* mRNA expression and its protein levels in synovial fluid of knee OA patients. d) Histopathological staining of knee OA non-inflamed synovium. e) Histopathological staining of knee OA inflamed synovium. f) Hypertrophy of the lining layer of knee OA inflamed synovium. g) Infiltration of inflammatory cells. h) Stromal activation. i) Immunohistochemical staining for COMP protein expression in knee OA non-inflamed synovium. j) Immunohistochemical staining for COMP protein expression in knee OA inflamed synovium. k) Immunohistochemical staining for COMP protein expression in the lining layer of knee OA inflamed synovium, predominantly in fibroblasts (head arrows). l) Immunohistochemical staining for COMP protein expression in knee OA inflamed synovium, especially in inflammatory cells (dotted arrows). m) Immunohistochemical staining for COMP protein expression in the sub-lining layer of knee OA inflamed synovium, predominantly in epithelial cells (arrows). n) *COMP* mRNA expression in human knee OA FLSs either untreated or treated with 10 ng/ml tumour necrosis factor alpha (TNF-α). ****p < 0.0001 for non-parametric comparisons in independent groups using Mann-Whitney U test. GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

degradative activity induced by pro-inflammatory cytokines including IL-1β and TNF-α.[13] More recently, an in vitro study uncovered a time-dependent upregulation of *COMP* mRNA expression in human knee OA FLSs treated with TNF-α, one of the pro-inflammatory cytokines responsible for synovial inflammation and cartilage degradation.[36] From our aforementioned findings, it is conceivable that alterations in transcriptional and translational production of COMP would reflect the severity of synovitis in knee OA patients. Given that changes in characteristics of articular cartilage and loss of matrix components are central to knee OA pathology, it has been postulated that biological indicators of cartilage metabolism might be exploited for knee OA diagnosis. In an effort to achieve the sensitive, specific detection of knee OA, the present study unveiled the clinical utility of serum COMP as a potential biomarker for distinguishing knee OA patients from healthy controls; ROC curve analysis yielded the optimal serum COMP cutoff value of 99.6% sensitivity and 100.0% specificity. This offers additional evidence for the clinical value of serum COMP as an arthritic biomarker.[12,27,37,38]

Considering our results presented herein, it is tempting to speculate that increased mRNA and protein expressions of COMP in the systemic and local joint environment of knee OA patients, particularly those in the advanced stage of the disease, may be due to compensation mechanisms by the body in response to an imbalance between the anabolic and catabolic processes in the joint, which contributes to synovial inflammation and subsequent cartilage destruction. As a result, joint tissues, especially the cartilage matrix, may release COMP into the synovial fluid and bloodstream. This notion is reinforced by prior research showing that COMP was released into joint fluid following injury,[39] and further corroborated by our findings obtained from in vitro and clinical studies. Although our results demonstrated positive associations between *COMP* mRNA expression in knee OA synovium and its protein levels in the serum and synovial fluid of knee OA patients, further experimental studies are necessary to ascertain whether COMP expression in specific joint tissues can influence serum or synovial fluid COMP levels.

It is important to recognize that there are certain limitations to this study, despite the fact that it has demonstrated several important results. Because of its cross-sectional design and relatively limited sample size, this study could not adequately explore the causal relationships between COMP mRNA and protein expressions and knee OA severity – especially synovial inflammation. An additional limitation pertains to the absence of data regarding knee flexor muscle strength. From this, it is challenging to draw conclusive findings regarding the comparison of knee flexor muscle strength between healthy controls and knee OA patients. Aside from this, the potential significance of alternative pro-inflammatory cytokines like IL-1 in stimulating the inflammatory process in knee OA FLSs needs to be identified. Furthermore, collection of synovium and joint

fluid specimens from healthy subjects operated under ethical constraints; this may be largely nullified in future studies if they include people who have had knee surgery for reasons other than arthritis. Additionally, there was a lack of information regarding the specific number of knee OA patients who had either unilateral or bilateral knee OA. As a result of this limitation, we faced challenges in conducting a comparative analysis of serum COMP levels and pain/functional outcomes between the patients with unilateral and bilateral knee OA. Additionally, it is evident that performing a COMP assay would be beneficial in confirming the existence of knee OA in a patient who has already been diagnosed with OA using established clinical methodologies. Moreover, it is worth noting that the conclusions derived from the ROC curve analysis are favourable. This is due to the fact that the comparison was conducted between individuals with normal (non-diseased) conditions and those with advanced stages of knee OA. It is important to acknowledge that this analysis did not include subclinical or early-phase conditions. Despite the limitations, this study adds to the currently limited body of evidence regarding the associations between serum COMP and physical performance markers of knee OA. This study also provides important insights into the significant involvement of COMP in knee OA inflammation, by which alterations in *COMP* mRNA expression were driven by TNF-α in human knee OA FLSs. Another strength of our study is the fact that ROC curve with ten-fold cross-validation was conducted to determine whether a diagnostic value of COMP is influenced by the different datasets, thereby highlighting the reliability of serum COMP as a knee OA biomarker. In that context, our results would shed light on the clinical pathological association of COMP for diagnosis and treatment of knee OA.

In summary, this study provides supporting evidence for diagnostic value of serum COMP as a novel biomarker of knee OA. More specifically, positive associations between serum COMP and indicators of physical performance, knee pain, and disability were observed in knee OA patients. Subsequent results from an in vitro study discovered that up-regulated *COMP* mRNA expression was regulated by TNF-α in human knee OA FLSs. This accumulated evidence from clinical and in vitro studies raises the possibility that COMP might have potential as a biomarker of knee OA and would be a possible molecule implicated in the inflammatory process in knee OA. Further studies are required to elucidate molecular mechanisms behind the role of COMP in synovial inflammation of knee OA, which would pave the way for potential treatment targets aimed at counteracting synovitis and ultimate cartilage degradation in knee OA patients.

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Data sharing

The data that support the findings for this study are available to other researchers from the corresponding author upon reasonable request.

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Ethical review statement

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