

Monomeric C-reactive protein level is associated with osteoarthritis

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Abstract. Osteoarthritis (OA) is a chronic joint disease characterized by articular cartilage degeneration and secondary bone hyperplasia. C-reactive protein (CRP) is an acute-phase protein that is widely used as a marker of inflammation. Elevated plasma levels of CRP are commonly observed in patients with OA during the acute phase. Current evidence indicates that CRP dissociating into a monomeric form (mCRP) is the main functional conformation at inflammatory loci. However, it remains unclear whether mCRP is associated with OA and whether mCRP can be used as a biomarker for its pathogenesis. In the present study, the concentration of CRP, mCRP and anti-mCRP autoantibody were detected by performing ELISA. The levels of plasma CRP, mCRP and anti-mCRP autoantibody between healthy subjects and patients with OA were compared. The results revealed that plasma mCRP was strongly associated with OA, while mCRP autoantibodies exhibited little correlation with this condition. Additionally, it was identified that the plasma mCRP levels in Kellgren-Lawrence (KL) grade 4 patients were significantly higher than in those with KL grade 3. Thus, it was revealed in the present study that plasma level of mCRP is associated with OA, which may directly reflect the disease degree of patients.

Therefore, mCRP may be a potential indicator that can be used to monitor the disease activity and evaluate the efficiency of OA therapy.

Introduction

Osteoarthritis (OA) is also known as degenerative arthropathy and proliferative OA. The different names are derived from the pathological manifestations of arthropathy, which include both cartilage degeneration and the formation of new bone (1,2). The clinical manifestations of this disease include joint swelling, pain, joint effusion and hyper-osteogeny (3). The incidence of OA increases with age and it is a common joint disease in the elderly population (4). At present, the pathogenesis of OA has not been determined. Previous studies have demonstrated that inflammatory responses are involved in the pathogenesis of OA and that pathological changes in the synovial structure are similar to that of rheumatoid arthritis at the early stages of onset (5-7). At present, OA is diagnosed mainly through the combination of symptoms and signs with imaging examination. Therefore, the early diagnosis of OA, differentiation of the severity and the search for appropriate markers have become important topics in clinical research.

C-reactive protein (CRP), a member of the pentraxin family, is composed of five identical subunits linked by non-covalent bonds (8). CRP is widely found in vertebrates and invertebrates, and its structure and sequence demonstrate high evolutionary conservation, suggesting that this protein has important biological significance (9). CRP is an acute phase protein whose plasma concentration rises rapidly when the body is affected by tissue damage or bacterial infection (10-13). It has been widely used in clinical practice for the diagnosis and detection of various infectious and autoimmune diseases (11,14). CRP not only serves as an inflammatory marker but also serves numerous important physiological functions in innate immune responses, such as activating complement pathways or regulating macrophage phagocytosis through Fcγ receptors to help clear cell debris and apoptotic cells (15-17). A previous study indicated that monomeric CRP (mCRP) is an allosteric active form of CRP involved in the inflammatory process, while pentameric CRP may serve as a

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protein library capable of transforming into mCRP (18). In the present study, the clinical samples of patients with OA were collected and the differences and associations between CRP, mCRP and anti-mCRP autoantibodies in the serum of healthy subjects and patients were analyzed, with the aim of providing evidence for the pathogenesis of OA and the use of mCRP as a novel clinical marker.

Materials and methods

Materials and reagents. Human CRP (cat. no. BP300.X; lot nos. 361639 and 404353) purified from ascites was purchased from the Binding Site. mCRP was prepared by treating CRP with 8 M urea-EDTA (cat. no. JT8991-1; lot no. 0000221293; Avantor, Inc.) (19) or by recombinant expression and purification as previously described (20,21). Sheep anti-human CRP polyclonal antibody (cat. no. PC044; lot nos. 352325 and 076682) was obtained from the Binding Site. Mouse anti-human CRP monoclonal antibody (mAb) CRP-8 (cat. no. C1688; lot no. 025M4863V) was obtained from Sigma-Aldrich; Merck KGaA. Mouse anti-human CRP mAb 1D6 was generated as previously described (22,23). HRP-labeled goat anti-mouse IgG (cat. no. 115-035-003; lot no. 125229) was purchased from Jackson ImmunoResearch Laboratories, Inc. HRP-labeled donkey anti-sheep IgG (cat. no. ab6900; lot no. GR272029-6) and HRP-labeled mouse anti-human IgG (cat. no. ab99757) were both obtained from Abcam.

Patient selection. A total of 206 patients with OA diagnosed between January 2016 and July 2018 at the Department of Joint Surgery and Joint Reconstruction of Xi'an Hong Hui Hospital (Xi'an, China) were enrolled in the present study. Participants were assessed by rheumatologists and radiologists. Rheumatoid arthritis or any other form of inflammatory arthritis (such as crystal arthropathy or septic arthritis) were considered to be exclusion criteria. A total of 60 age and sex matched healthy individuals were selected from the same hospital between November 2017 and July 2018. The clinicopathological characteristics of patients with OA are listed in Table I. CRP, mCRP and anti-mCRP autoantibodies were detected by ELISA while other clinical features were collected hospital case records. The demographic data of OA patients and healthy individuals are included in Table II. The plasma samples of patients were obtained prior to the initiation of medical treatment. All samples were stored at -80°C in aliquots. Informed consent for blood sample collection was signed by all participants. The present study followed the guidelines of The Declaration of Helsinki and was approved (approval no. 202003055) by the Ethics Committee of the Hong Hui Hospital (Xi'an, China).

Patient classification. The Kellgren-Lawrence (KL) grading system of knee OA is the grading method of knee OA severity. According to the X-ray manifestations of the knee joint, OA severity was divided into grade 0 (normal knee joint), grade 1, 2, 3 and 4 (the most serious knee OA) (24,25) and were described as follows: i) Grade 0: Knee joint X-ray is completely normal. There are no manifestations of OA. No joint space stenosis and no reactive bone changes can be observed. ii) Grade 1: Suspected knee joint space stenosis.

Osteophytes may occur, but only slightly. iii) Grade 2: Small osteophytes and possible joint space narrowing are identifiable on the X-ray of a standing knee joint. iv) Grade 3: KL grade 3 of knee OA is characterized by a large number of moderate osteophytes, clear narrowing of joint space, certain subchondral bone sclerosis (increased white area with joint edges is identifiable via X-ray) and possible knee deformity. v) Grade 4: KL grade 4 of knee OA is characterized by a large number of large osteophytes, severe narrowing of joint space, obvious subchondral bone sclerosis and obvious knee deformity. The patients with OA mentioned in the present study were all patients who received systematic evaluation and treatment at hospital. In the early stages of OA, patients have occasional pain in the knee joint, which affects exercise but rarely daily life. If patients pay attention to rest and exercise properly, they can alleviate their symptoms (26,27). For the aforementioned reasons, there are no hospitalized patients in the early stage of KL grade 1 and KL grade 2. Therefore, patients with KL grade 1 and KL grade 2 were not included in the present study. KL grade 3 and KL grade 4 patients in the middle and late stage exhibit joint degeneration and pain aggravation, which seriously affects their daily life, requiring hospitalization or joint replacement (26,27). These patients were the main research subjects in the present study.

ELISA assay quantifying CRP. The sheep anti-human CRP polyclonal antibody was immobilized onto microtiter wells (1:2,000; cat. no. 42592; lot no. 10917007; Corning, Inc.) in coating buffer (10 mM sodium carbonate/bicarbonate, pH 9.6) overnight at 4°C . All the following steps were conducted at 37°C , and after each incubation step, wells were washed three times with TBS (10 mM Tris, 140 mM NaCl, pH 7.4) containing 0.02% NP-40. Wells were blocked with blocking buffer [TBS containing 1% bovine serum albumin (cat. no. 0322; LABLEAD)] for 1 h. Samples diluted in blocking buffer were added into wells for 1 h. Captured CRP was detected with 1D6 mAb (1:100 in blocking buffer) that specifically recognizes its native conformation and an HRP-labeled goat anti-mouse IgG (1:20,000 in blocking buffer). Wells were incubated with TMB buffer [0.1 mg/ml 3,3',5,5'-tetramethylbenzidine (cat. no. 0759; LABLEAD) and 0.02% H_2O_2 in 0.1 M Na_2HPO_4 , 0.05 M citric acid, pH 4.5-5.5] for 30 min and stopped with 1 M H_2SO_4 . The optical density (OD) of samples were measured at 570 and 450 nm using a microplate reader. The OD value of each sample was calculated as $\text{OD}_{450} - \text{OD}_{570}$ nm. A total of 100 μl volume was used at all incubation steps, while 300 μl volume was used for washing after each incubation step.

ELISA assay quantifying mCRP. Mouse anti-human CRP mAb CRP-8 was immobilized onto microtiter wells at 0.3 $\mu\text{l}/\text{ml}$ in coating buffer overnight at 4°C . All the following steps were conducted at 37°C , and after each incubation step, wells were washed three times with TBS containing 0.02% NP-40. Wells were blocked with blocking buffer for 1 h. Samples diluted in blocking buffer were added into wells for 1 h. Captured mCRP was detected with a sheep anti-human CRP polyclonal antibody (1:2,000 in blocking buffer) which can both recognize the CRP, mCRP and an HRP-labeled donkey anti-sheep IgG (1:20,000 in blocking buffer). Wells were incubated with TMB buffer for 30 min and stopped with 1 M H_2SO_4 . Absorbance

Table I. Clinical characteristics of patients with osteoarthritis.

Clinical characteristics	Value (total number, mean \pm SD or median)	
	All OA patients	OA patients that were KL graded
Sex		
Female	159	37
Male	47	12
Age, years	66.55 \pm 8.16	68 \pm 8.28
Grade		
KL3	14	14
KL4	35	35
CRP (μ g/ml)	0.77 (0.37-1.66)	2.17 (0.61-4.97)
mCRP (ng/ml)	12.51 (7.78-24.81)	15.95 (8.45-24.1)
Anti-mCRP autoantibody (OD)	0.52 \pm 0.37	0.46 \pm 0.27
Neutrophil ratio (%)	73.51 \pm 8.49	74.16 \pm 6.86
Lymphocyte ratio (%)	17.1 \pm 6.16	16.71 \pm 6.15
Monocyte ratio (%)	7.1 (5.8-8.3)	7.05 (6.18-8.23)
Uric acid (μ mol/l)	293.06 \pm 70.1	301.63 \pm 75.63
Urea (mmol/l)	5.5 (4.5-6.6)	5.7 (4.57-6.65)
Creatinine (μ mol/l)	58 (50-66.25)	57 (49.75-66.5)
Total cholesterol (mmol/l)	4.46 \pm 0.94	4.47 \pm 0.97
Triglyceride (mmol/l)	1.38 (1.03-1.84)	1.4 (1.1-1.76)
High density lipoprotein (mmol/l)	1.23 (1.09-1.39)	1.26 (1.13-1.4)
Low density lipoprotein (mmol/l)	2.85 (2.33-3.3)	2.77 (2.35-3.29)
Apolipoprotein A1 (g/l)	1.35 \pm 0.24	1.37 \pm 0.22
Apolipoprotein B (g/l)	0.88 (0.75-1.04)	0.84 (0.75-1)
Glucose (mmol/l)	4.88 (4.44-5.42)	4.87 (4.41-5.81)
Antistreptococcolysin O (IU/ml)	34.5 (18-59)	32 (16.5-60.5)
Rheumatoid factor (IU/ml)	7.2 (3.25-8.9)	7.3 (3.17-9.05)

CRP, C-reactive protein; KL, Kellgren-Lawrence; mCRP, monomeric C-reactive protein.

Table II. Comparisons of patients with OA and healthy individuals.

Clinical characteristic	Value (total number or mean \pm SD or median)		P-value
	OA	Healthy	
Sex			-
Female	159	46	
Male	47	14	
Age, years	66.55 \pm 8.16	65.35 \pm 5.35	0.241447
CRP (μ g/ml)	0.77 (0.37-1.66)	0.3 (0.04-0.72)	3.83 \times 10 ⁻⁵
mCRP (ng/ml)	12.51 (7.78-24.81)	5.04 (3.45-9.77)	3.72 \times 10 ⁻⁵
Anti-mCRP Autoantibody (OD)	0.52 \pm 0.37	0.46 \pm 0.24	0.399173

OA, osteoarthritis; CRP, C-reactive protein; mCRP, monomeric CRP.

at OD570 and OD450 nm was measured with a microplate reader. The OD value of each sample was calculated as OD450-OD570 nm. A total of 100 μ l volume was used at all incubation steps, while 300 μ l volume was used for washing after each incubation step.

ELISA assay quantifying anti-mCRP autoantibody. The mCRP was immobilized onto microtiter wells at 2 μ g/ml in coating buffer overnight at 4°C. All the following steps were conducted at 37°C, and after each incubation step wells were washed 3 times with TBS containing 0.02%

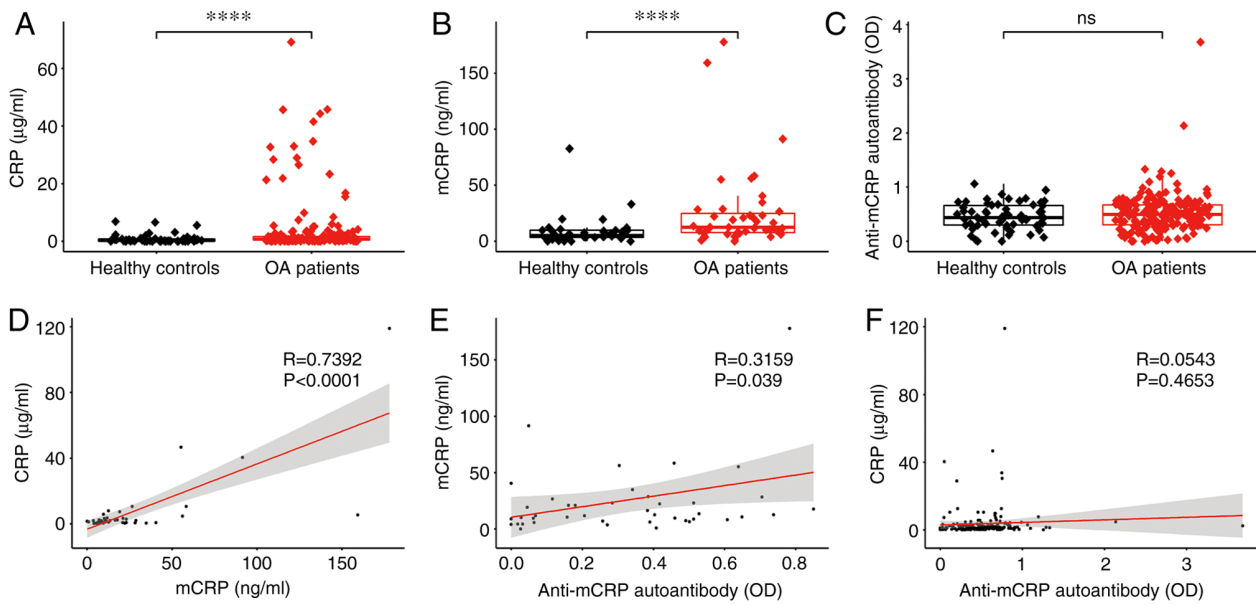


Figure 1. Difference and correlation analysis of serum CRP, mCRP and anti-mCRP autoantibody. The plasma concentrations of (A) CRP, (B) mCRP and (C) anti-mCRP autoantibody in patients with OA were compared with healthy controls. The correlation of (D) CRP with mCRP, (E) mCRP with anti-mCRP autoantibody and (F) CRP with anti-mCRP autoantibody were analyzed using Pearson's correlation test. **** $P < 0.0001$ as indicated. CRP, C-reactive protein; mCRP, monomeric CRP; OA, osteoarthritis; OD, optical density; R, Pearson's correlation coefficient; ns, not significant.

NP-40. Wells were blocked with blocking buffer for 1 h. Samples diluted in blocking buffer were added into wells for 1 h. Captured anti-mCRP autoantibody was detected with an HRP-labeled mouse anti-human IgG (1:20,000 in blocking buffer). Wells were incubated with TMB buffer for 30 min and stopped with 1 M H_2SO_4 . Absorbance at OD570 and OD450 nm was measured with a microplate reader. The OD value of each sample was calculated as OD450-OD570 nm. A total of 100 μl volume was used at all incubation steps, while 300 μl volume was used for washing after each incubation step.

Statistical analysis. Each sample was analyzed three times. The mean value of each sample was used for subsequent analysis. Differences between groups were all analyzed using the Mann-Whitney U-test. Correlations were all analyzed using the Pearson's correlation test. The Univariable logistic regression was used to determine contributors to KL grades in patients with OA. All analyses were performed with R 4.0.3 (R Core Team) (28). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Difference and correlation analysis of serum CRP, mCRP and anti-mCRP autoantibody. The demographic and clinical data of patients are summarized in Table I and continuous characteristics are presented as the mean \pm SD or median (interquartile range). Overall comparisons of patients with OA and healthy individuals are summarized in Table II. The differences in plasma levels of CRP, mCRP and anti-mCRP autoantibody between healthy subjects and patients with OA are revealed in Fig. 1. The level of plasma CRP in patients with OA was significantly higher than that in healthy individuals (Fig. 1A), as well as the

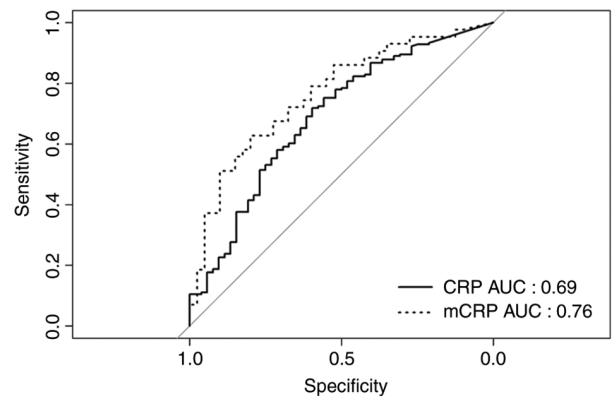


Figure 2. Receiver operating characteristic curves of CRP and mCRP for diagnosing osteoarthritis. mCRP (dotted line) has improved predictive performance than CRP (solid line). CRP, C-reactive protein; mCRP, monomeric CRP; AUC, area under the curve.

level of plasma mCRP (Fig. 1B). No significant difference in anti-mCRP autoantibody between patients with OA and healthy subjects was identified (Fig. 1C). The relationships among CRP, mCRP and anti-mCRP autoantibody are also illustrated in Fig. 1. CRP was strongly correlated with mCRP (Fig. 1D), probably due to mCRP being produced by the depolymerization of pentamer CRP at the inflammatory loci in the lesion area. mCRP was correlated with anti-mCRP autoantibody (Fig. 1E) most likely due to the fact that anti-mCRP autoantibody is generated by mCRP stimulation. No correlation was found between CRP and anti-mCRP autoantibody (Fig. 1F), which can be explained by CRP and anti-mCRP autoantibody not being directly related. In order to further investigate the correlation of CRP and mCRP with the risk of OA, receiver operating characteristic (ROC) curve analyses was performed (Fig. 2). The

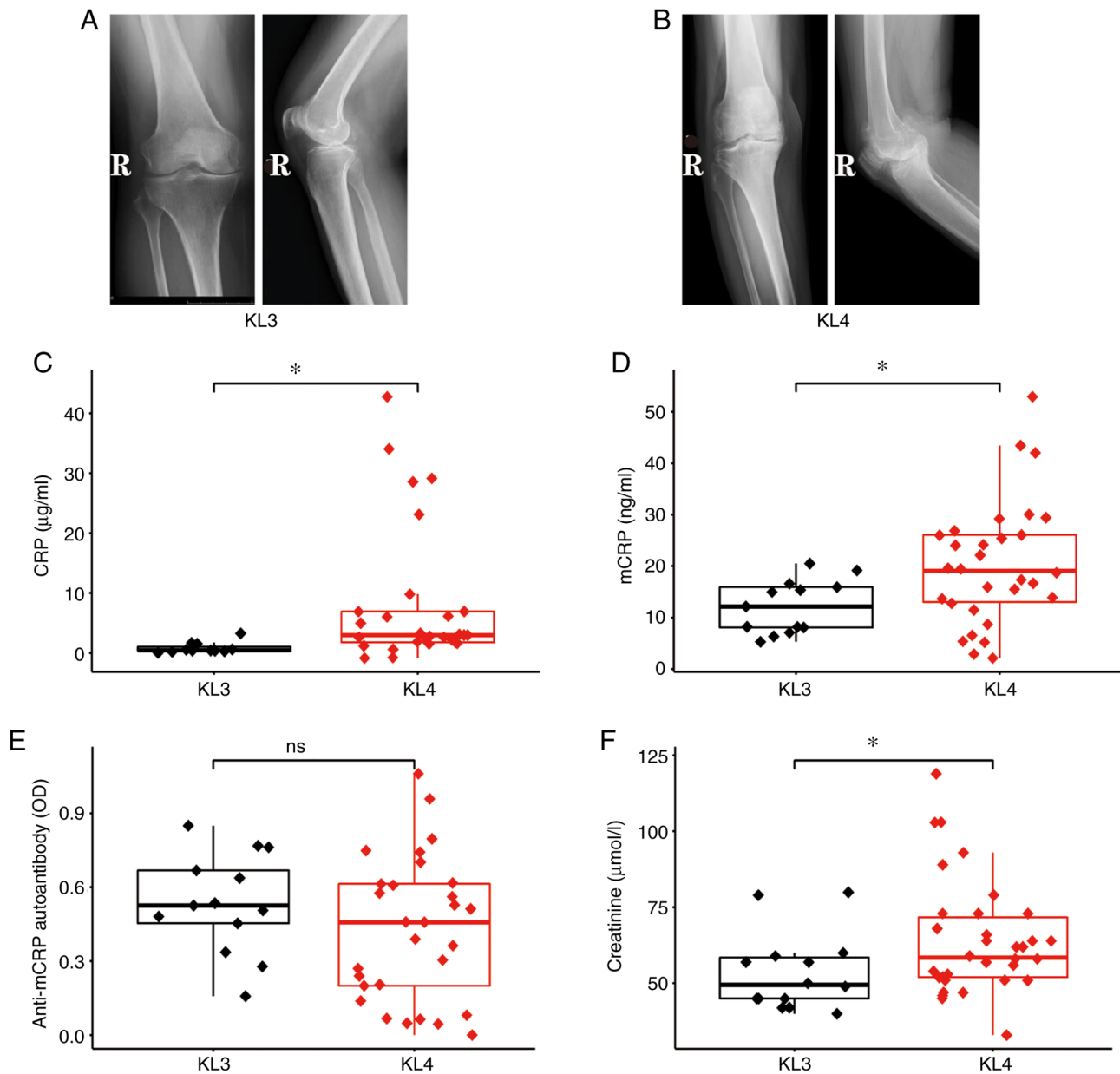


Figure 3. Differences in CRP, mCRP, anti-mCRP autoantibody and the clinical features between KL grades in patients. Typical diagnostic images of (A) KL3 and (B) KL4 grades in patients with OA. Plasma concentrations of (C) CRP, (D) mCRP, (E) anti-mCRP autoantibody and (F) creatinine in patients with OA with KL grades. * $P < 0.05$ as indicated. CRP, C-reactive protein; mCRP, monomeric CRP; KL, Kellgren-Lawrence; OA, osteoarthritis; OD, optical density; R, right-hand side; ns, not significant.

area under the CRP curve was 0.69, and the area under the mCRP curve was 0.76, which demonstrated that mCRP has improved predictive performance.

Differences in CRP, mCRP, anti-mCRP autoantibody and clinical features between KL grades in patients with OA. Images of different KL grades are presented in Fig. 3A and B. Compared with those with KL grade 3, patients with KL grade 4 had a narrower articular cavity and more evident joint deformities. CRP and mCRP were revealed to be significantly higher in KL grade 4 than in KL grade 3 (Fig. 3C and D). These findings suggested that CRP and mCRP can be used as indicators of disease severity. There was no significant difference in anti-mCRP autoantibody between KL grades (Fig. 3E), suggesting

that anti-mCRP autoantibody is not associated with the severity of OA. A significant difference in creatinine level between KL grades was also observed (Fig. 3F), while all other clinical features are not significantly different (data not shown). Univariate logistic regression was performed (Table III) and the results confirmed that CRP and mCRP are significantly associated with KL grades. This finding suggested that CRP and mCRP may be contributors to OA pathogenesis.

Associations of CRP, mCRP and anti-mCRP autoantibody with age and sex in patients. As it can be observed in Fig. 4, the differences in CRP, mCRP and anti-mCRP autoantibody between sex are all not significant (Fig. 4A-C). Thus, the patients were not grouped according to sex for further

Table III. Univariate analysis of Kellgren-Lawrence grades of patients with osteoarthritis.

Clinical feature	P-value	OR	CI95 lower limit	CI95 upper limit
Sex	0.753	1.269	0.307	6.534
Age	0.161	1.057	0.980	1.148
CRP	0.018	2.342	1.312	5.386
mCRP	0.021	1.034	1.009	1.069
Autoantibody	0.227	0.206	0.013	2.488
Neutrophils Ratio	0.295	1.051	0.958	1.156
Lymphocytes Ratio	0.227	0.939	0.843	1.039
Monocytes Ratio	0.845	1.030	0.773	1.421
Uric Acid	0.447	1.003	0.995	1.013
Urea	0.326	1.248	0.825	2.029
Creatinine	0.077	1.049	1.002	1.115
Total Cholesterol	0.318	1.422	0.732	3.007
Triglyceride	0.822	0.923	0.466	1.972
High Density Lipoprotein	0.569	2.321	0.871	49.317
Low Density Lipoprotein	0.431	1.369	0.656	3.250
Apolipoprotein A1	0.602	2.215	0.119	54.015
Apolipoprotein B	0.536	2.480	0.161	58.439
Glucose	0.523	0.844	0.488	1.466
Antistreptococcolysin O	0.720	0.997	0.983	1.013
Rheumatoid Factor	0.438	1.022	0.993	1.127

CRP, C-reactive protein; mCRP, monomeric CRP; OR, odds ratio.

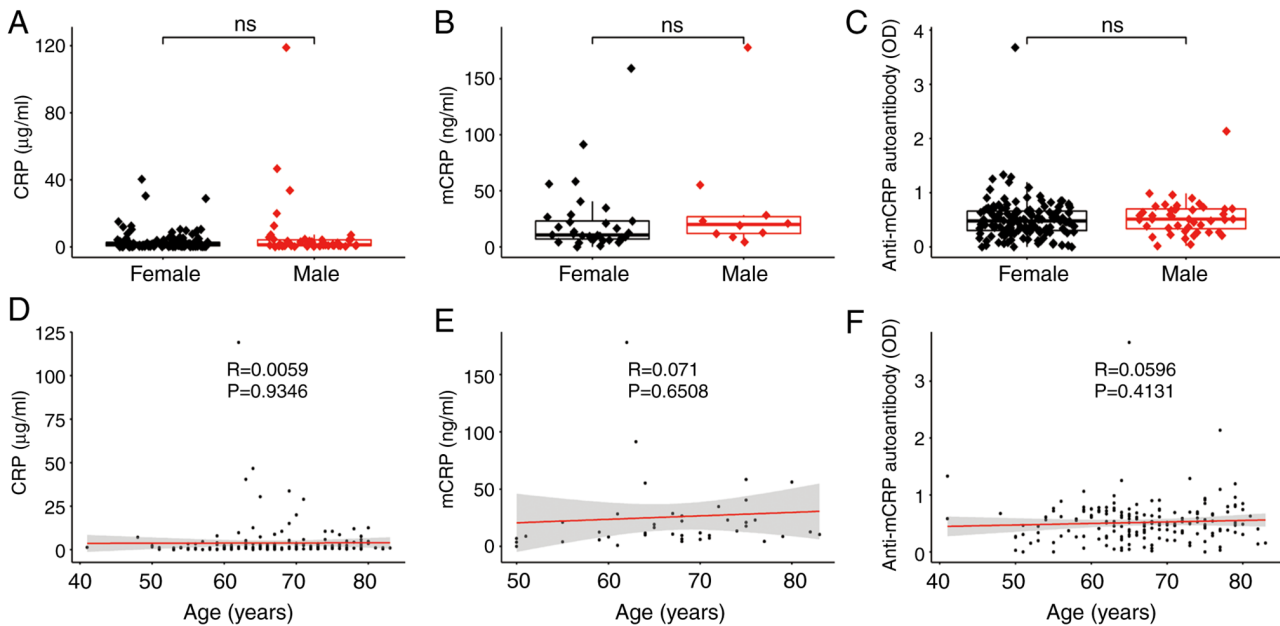


Figure 4. Association of CRP, mCRP and anti-mCRP autoantibody with the age and sex of patients with OA. Plasma concentrations of (A) CRP, (B) mCRP and (C) anti-mCRP autoantibody in male and female patients with OA. The correlations of age with (D) CRP, (E) mCRP (F) and anti-mCRP autoantibody were analyzed using Pearson's correlation test. CRP, C-reactive protein; mCRP, monomeric CRP; OA, osteoarthritis; R, Pearson's correlation coefficient; ns, not significant; OD, optical density.

subgroup analysis. Since the correlation of CRP, mCRP and anti-mCRP autoantibody with age was also not significant (Fig. 4D-F), the patients were again not grouped according to their age.

Discussion

According to a survey performed by the World Health Organization, the incidence of OA has reached ~10% in people

aged >60 years (29). At present, the diagnosis of OA mainly depends on imaging methods. Therefore, the development of convenient early methods of diagnosis for OA is a direction worthy of study.

CRP is an important marker of the inflammatory response, which increases rapidly in the acute stage of infection and tissue injury (10-13,30). Studies have also revealed that CRP was directly involved in the development of certain diseases. In myocardial infarction, for example, CRP mediates tissue damage mainly by activating complement pathways (31,32). In addition, mCRP is the monomeric form of CRP and the main active conformation of local tissues (33-35). Furthermore, anti-mCRP autoantibody is generated by mCRP stimulation. Previous studies have determined that anti-mCRP autoantibody is associated with the degree of injury and prognosis of patients (36,37). To study the relationship between CRP, mCRP, anti-mCRP autoantibody and OA, CRP, mCRP and anti-mCRP autoantibody were selected as observation objects to detect the difference and correlation between patients and healthy controls. Significant differences in plasma CRP and mCRP levels between patients and healthy controls and KL grades were identified, suggesting that they may be involved in the development of OA and could be used as severity markers of OA. There was a difference between CRP and mCRP, due to CRP being a non-specific inflammatory marker protein secreted by the liver and has a high concentration in plasma; mCRP is considered to play a role by depolymerizing pentamer CRP into monomer form in the local tissue of the lesion, and in addition, most of mCRP still resident in the local tissue (18,38), so the concentration of mCRP in blood is low. In clinical application, it is easier to detect CRP with higher concentration by ELISA; However, compared with CRP, mCRP may be a more sensitive indicator in a specific disease. Combined with the ROC curve, mCRP has higher accuracy and prediction sensitivity.

The mCRP is the monomer form of pentamer CRP and inflammatory conditions in tissues can promote the dissociation of CRP to mCRP (39). In the process of dissociating to mCRP, CRP can expose certain new antigen epitopes, giving mCRP biological activities different from the pentameric protein. Previous studies have demonstrated that mCRP has a stronger biological effect. For example, mCRP, the main isomer form of CRP, can bind with natural and modified low density lipoprotein (40) and regulate complement activation more effectively (41). Furthermore, mCRP has a stronger activation effect on endothelial cells (7) and neutrophils (42), which means that mCRP formed by the structural rearrangement of CRP is a favorable complement of CRP function. These findings suggested that mCRP is the dominant form of CRP, functioning under pathological and physiological conditions. The present experimental results delineated that there was a strong correlation between serum mCRP and CRP in OA and that there were significant differences in mCRP between patients and healthy controls, as well as in different stages of OA, suggesting that CRP in OA may be involved in the development of the disease through dissociation into mCRP. The mostly commonly investigated pathways associated with OA are the Wnt, Notch, NF- κ B, PI3K/Akt/mTOR and OPG/RANK/RANKL pathways (43-53). It has been revealed that mCRP can activate the NF- κ B signaling pathway

in mouse and human chondrocytes and induce the expression of proinflammatory cytokines (54). Boras *et al* (55) determined that mCRP can co-activate PI3K signaling pathway with Notch-3. A previous study demonstrated that mCRP induced osteoclast differentiation by binding to RANKL (56). The aforementioned studies demonstrated that the NF- κ B, PI3K/Akt/mTOR and OPG/RANK/RANKL pathways may be associated with mCRP function.

In conclusion, the present results revealed that there are significant differences in the levels of CRP and mCRP between patients with OA and healthy controls, and in different disease stages. Thus, CRP and mCRP may be important serological markers for the early diagnosis and disease evaluation of OA. Additionally, it was also identified in the present study that CRP is significantly correlated with mCRP in OA, which is consistent with previous studies that CRP plays a vital role in diseases through dissociation into mCRP (18,39). However, whether mCRP is directly involved in the pathogenesis of OA needs further investigation. The present study may have certain limitations, which are reflected in the relatively small sample size and lack of KL1 and KL2 patients. Further expanding the number of patients with KL grades can provide an improved basis and reliability for follow-up research. Whether the combined analysis with other serum markers of OA, such as cartilage oligomeric matrix protein or C-telopeptide fragments of type II collagen, can improve the accuracy of association and prediction remains to be studied in the future. At present, there is no validation of the mCRP in animal models and related mechanism research. In future, using OA animal models can further investigate whether mCRP regulates the pathogenesis of OA. The present study suggested that mCRP may be used as a novel biomarker to help the early diagnosis and severity evaluation of OA and also lays a foundation for in-depth exploration of the pathogenesis of OA, particularly with respect to the role played by mCRP, and future clinical research for therapeutic targets.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

HL and PX designed the research. YL, KX and WL performed the experiments. XL and PY analyzed the data and wrote the article. HL, PX and YL confirm the authenticity of all the raw

data. All authors reviewed the results and read and approved the final version of the manuscript.

Ethics approval and consent to participate

The present study followed the guidelines of The Declaration of Helsinki and was approved (approval no. 202003055) by the ethics committee of the Hong Hui Hospital (Xi'an, China). Informed consent was obtained from all subjects involved in the present study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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