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Original Article

Value of combination of 25-(OH)-D3, IL-6, and cyclic peptide containing citrulline antibodies in different stages of rheumatoid arthritis

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Abstract: Objective: To determine the value of a combination of 25-hydroxyvitamin D3 (25-(OH)-D3), interleukin-6 (IL-6), and cyclic peptide containing citrulline (CCP) antibodies in the acute stage and remission stage of patients with rheumatoid arthritis (RA). Methods: A retrospective analysis was made on 80 RA patients who received treatment in Wenjiang District People's Hospital of Chengdu from February 2017 to February 2020. According to their condition, they were identified as acute-stage patients (n=48) or remission-stage patients (n=32). In addition, 40 healthy individuals who received physical examination in our hospital during the same period were enrolled in a control group. Serum 25-(OH)-D3, IL-6, and CCP antibodies in all enrolled participants were quantified, and their levels were compared between RA patients at the acute stage and those at the remission stage before therapy, and also between patients with different efficacy after 3 months of therapy. The correlations of serum 25-(OH)-D3, IL-6, and CCP antibodies with disease activity score in 28 joints (DAS-28) were analyzed. A corresponding joint receiver operating characteristic (ROC) curve was drawn to analyze the diagnostic value of the combination of 25-(OH)-D3, IL-6, and CCP antibodies in the staging of RA patients, and logistic regression was used to establish an efficacy risk model. Results: The highest serum 25-(OH)-D3 level was found in the control group, followed by the remission-stage patients and then acute-stage patients from high to low (all $P<0.05$), and the lowest levels of serum IL-6 and CCP antibodies were also found in the control group, followed by the remission-stage patients and then the acute-stage patients from low to high (all $P<0.05$). The Pearson's test revealed a negative correlation of 25-(OH)-D3 with DAS-28 and a positive correlation of IL-6 and CCP antibodies with DAS-28. According to ROC curve-based analysis, the area under the joint curve of 25-(OH)-D3, IL-6, and CCP antibodies was >0.9. After therapy, patients showed an increase in 25-(OH)-D3 and decreases in IL-6 and CCP antibodies (all $P<0.05$). The logistic model confirmed that the area under the ROC curve of RA affecting the efficacy on patients was >0.8. Conclusion: A combination of 25-(OH)-D3, IL-6, and CCP antibodies can be adopted as a diagnostic indicator in acute and remission stages of RA. A risk factor model of clinical efficacy in RA patients can help us effectively identify high-risk patients before therapy and take intervention measures early.

Keywords: 25-(OH)-D3, IL-6, CCP antibodies, rheumatoid arthritis, efficacy

Introduction

Rheumatoid arthritis (RA) is a systemic disease mainly manifested as a chronic inflammatory joint disease [1]. Its pathologic changes mainly include chronic non-suppurative synovitis, synovial congestion, edema, exudation, inflammatory cell infiltration, and granulation tissue formation, which erode articular cartilage through attenuation and injury [2]. Since structural joint injuries are irreversible, emphasis is placed on early identification and treatment to prevent the progression of related diseases [3]. However, one survey shows that 36-54% of RA patients suffer from radiologically visible joint injuries shortly after the onset of symptoms [4, 5]. Therefore, early and effective diagnosis and timely appropriate treatment can reduce the disability rate of RA patients and improve their prognosis [6].

As RA is recurrent, patients with it need to have regular reexamination and evaluation [7, 8]. The disease activity score in 28 joints (DAS-28) method is frequently adopted for clinical evaluation of RA patients at the current stage [9]. However, recent research has indicated a high value of the combination of laboratory indicators in assessing the condition of RA patients [10]. Vitamin (Vit) D is a fat-soluble vitamin. 25-hydroxyvitamin D3 (25-(OH)-D3), the main existing form of Vit D *in vivo*, directly reflects the level of Vit D and mainly serves to maintain the balance of calcium and phosphorus *in vivo,* so it is used in the diagnosis of osteoporosis and rickets [11, 12]. Interleukin-6 (IL-6) is an early-found inflammatory factor, mainly secreted by antigen-presenting cells, B cells, T cells and non-hematopoietic cells, which plays a crucial role in inflammation and the immune response [13, 14]. Citrulline is the main antigenic determinant for recognizing anti-filaggrin-associated antibodies in the serum of RA patients [15]. As early as 2010, the American College of Rheumatology (ACR) and European League against Rheumatism (EULAR) have deemed synthetic anti-citrullinated protein antibody (ACPA) as an index for RA diagnosis [16]. There are various CPA antibodies, among which cyclic peptide containing citrulline (CCP) antibodies are the most extensively adopted antibody with favorable sensitivity and specificity [17]. 25-(OH)-D3 and IL-6 are common indexes with a high value in the diagnosis of RA, but their value in different stages of RA is still unclear. In addition, CCP antibodies are a new index introduced by our hospital in recent years, that is effective in the diagnosis of RA, but whether it can be used as an index to distinguish RA stages needs further exploration.

Accordingly, this study was designed to determine the value of the combination of 25-(OH)-D3, IL-6, and CCP antibodies in different stages of RA patients and the evaluation value of the three in clinical efficacy on patients, to provide a reference for clinical diagnosis and efficacy evaluation.

Materials and methods

Clinical data

A total of 80 patients with RA treated in The People's Hospital of Wenjiang Chengdu from Feb. 2017 to Feb. 2020 were enrolled and analyzed retrospectively. They were assigned to the patient group, and identified as acute-stage patients (n=48) or remission-stage patients (n=32) based on their condition. In addition, 40 healthy individuals who received physical examination in our hospital during the same time were identified as the control group. This study was conducted with permission from the Ethics Committee of Wenjiang Branch of Sichuan Provincial People's Hospital, with an ethical approval number of 2020 ethical review (1408).

Inclusion criteria of the patients

Patients who met the diagnosis and classification criteria of RA revised by the ARA in 1987 [18], patients who were diagnosed with RA at the first diagnosis in our hospital, patients who cooperated with treatment and follow-up, and those >18 years old were included.

Patients who did not have detailed case data, who had received anti-rheumatic drugs before this study, who were intolerant of this treatment plan, pregnant women, lactating women, patients who had comorbid fracture or tumor, patients who had expression disorder, patients with other autoimmune diseases, patients with infectious diseases, and patients with diseases that might cause inflammation were excluded.

Individuals who had normal clinical laboratory examination and imaging examination results were enrolled into the control group.

Therapeutic regimen

All patients were treated with methotrexate and leflunomide. Specifically, the patient was injected with methotrexate (Sichuan Huiyu Pharmaceutical Co., Ltd., State Food and Drug Administration (SFDA) approval no.: H20043-647) at an initial dose of 7.5 mg per week, and the dosage was gradually increased to 12.5 mg per week, once a week. The patient was also ordered to orally take 25 mg leflunomide (Changzhou Watson Pharmaceutical Co., Ltd., SFDA no.: H20090330), twice a day, for 3 months.

Determination methods

Fasting venous blood (4 mL) was extracted from each participant, followed by the determination of 25-(OH)-D3, IL-6, and CCP antibodies.

Table 1. Comparison of clinical data among three groups

Factor	acute-stage group (n=48)	remission-stage group (n=32)	control group (n=40)	*P*-value
Age (year)				0.524
≥50 years old	28	20	28	
<50 years old	20	12	12	
Gender				0.685
Male	16	10	10	
Female	32	22	30	
BMI				0.327
≥22 kg/m^2	17	15	12	
<22 kg/m^2	31	17	28	
Past medical history				
Hypertension	27	14	22	0.508
Diabetes mellitus	14	8	10	0.880
Smoking history				0.342
Yes	15	11	8	
No	33	21	32	
Alcohol abuse history				0.582
Yes	10	4	6	
No	38	28	34	

Note: BMI: body mass index.

25-(OH)-D3 was quantified using a Roche E601 automatic chemiluminescent analyzer and matching reagent (the chemiluminescence method), and IL-6 was determined using the ELISA and a corresponding kit (Wuhan Elite Biotechnology Co., Ltd. E-EL-H6156). CCP antibodies were quantified using the latex-enhanced immunoturbidimetry with Beckman AU680. All instruments and detection reagents were purchased from the manufacturers, and kits used for quantification were all original kits.

Outcome measures

Primary outcome measures: Serum 25-(OH)-D3, IL-6, and CCP antibodies in patients and the control group were quantified. The patients' stages were evaluated using the DAS-28 as follows: DAS-28 ≥2.6 points indicated the acute stage and DAS-28 <2.6 points indicated the remission stage. The levels of 25-(OH)-D3, IL-6, and CCP antibodies were compared between RA patients at the acute stage and those at the remission stage before therapy. The serum 25-(OH)-D3, IL-6, and CCP antibodies in patients were compared before and after therapy.

Secondary outcome measures: Clinical data of the three groups were compared, and the correlations of serum 25-(OH)-D3, IL-6, and CCP antibodies with DAS-28 were analyzed. A corresponding joint receiver operating characteristic (ROC) curve was drawn to analyze the diagnostic value of the combination of 25-(OH)-D3, IL-6, and CCP antibodies in the staging of RA patients, and logistic regression was used to establish an efficacy risk model.

Statistical analyses

This study adopted SPSS24.0 for statistical analyses of data, and Graphpad8 for visualization of the data into corresponding figures. Counted data were analyzed by the Chi-square test and measured data (mean ± SD) were compared between groups by the independent-samples T test, and compared within groups by the paired t test. ROC curves were adopted to analyze the evaluation value of 25-(OH)-D3, IL-6, and CCP antibodies in RA patients at the acute stage and those at the remission stage. The Pearson's test was adopted to analyze the associations of 25-(OH)-D3, IL-6, and CCP antibodies with DAS-28. Logistic regression analysis was performed to analyze the risk factors affecting the clinical treatment efficacy on patients, and the Hosmer-Lemeshow test was carried out to correct the discrimination and goodness of fit of the ROC curve detection model. P<0.05 denotes a significant difference.

Results

Comparison of clinical data

According to the comparison of clinical data among the three groups, the three groups were similar in age, sex, body mass index (BMI), past medical history, smoking history, and alcohol abuse history (**Table 1**, all P>0.05).

25-(OH)-D3, IL-6, and CCP antibodies in patients

According to the comparison of serum 25-(OH)-D3, IL-6, and CCP antibodies between the con-

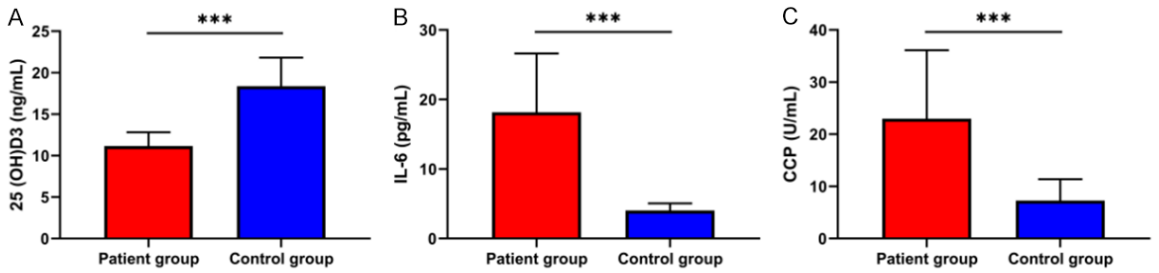

Figure 1. 25-(OH)-D3, IL-6, and CCP antibodies in patients. A. Comparison of serum 25-(OH)-D3 between the patient group and control group. B. Comparison of serum IL-6 between the patient group and control group. C. Comparison of serum CCP antibodies between the patient group and control group. Note: ***indicates P<0.001 in intergroup comparison. The independent-sample t-test was used for the comparison of measured data between groups. 25-(OH)-D3: 25-hydroxyvitamin D3 (25-(OH)-D3); IL-6: interleukin-6; CCP antibodies: cyclic peptide containing citrulline (CCP) antibodies.

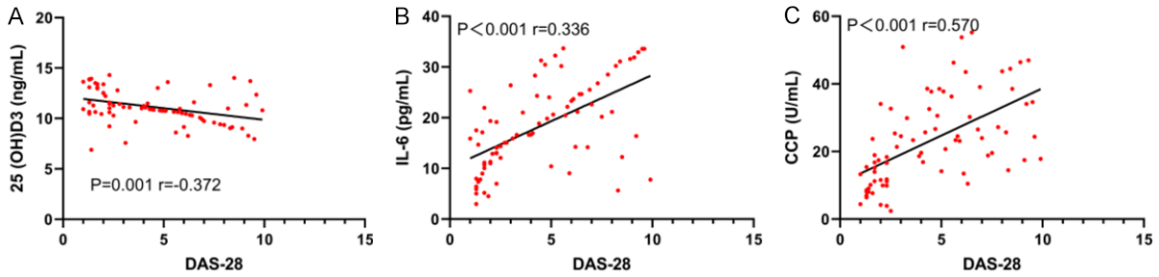

Figure 2. Associations of 25-(OH)-D3, IL-6, and CCP antibodies with DAS-28. A. Analysis of the association of 25-(OH)-D3 with DAS-28. B. Analysis of the association of IL-6 with DAS-28. C. Analysis of the association of CCP antibodies with DAS-28. Note: Pearson's test was adopted for correlation analysis; 25-(OH)-D3: 25-hydroxyvitamin D3 (25-(OH)-D3); IL-6: interleukin-6; CCP antibodies: cyclic peptide containing citrulline (CCP) antibodies.

trol group and the patient group, the patient group showed a notably lower serum 25-(OH)-D3 level (**Figure 1A**, P<0.05) and notably higher levels of IL-6 and CCP antibodies (**Figure 1B**, **1C**, both P<0.001) than the control group.

Correlations of 25-(OH)-D3, IL-6, and CCP antibodies with DAS-28

This study also analyzed the correlations of 25-(OH)-D3, IL-6, and CCP antibodies with DAS-28. There was a negative association of 25-(OH)-D3 with DAS-28 and there were positive associations of IL-6 and CCP antibodies with DAS-28 (**Figure 2A-C**, all P<0.001).

25-(OH)-D3, IL-6, and CCP antibodies in patients at different stages

This study further quantified serum 25-(OH)-D3, IL-6, and CCP antibodies in patients at different stages. According to the results, the patients in acute stage revealed a lower serum 25-(OH)-D3 level (**Figure 3A**, P<0.05) and higher levels of IL-6 and CCP antibodies than remission-stage patients (**Figure 3B**, **3C**, both P<0.01).

Diagnostic value of 25-(OH)-D3, IL-6, and CCP antibodies in patients at different stages

In order to determine the diagnostic value of 25-(OH)-D3, IL-6, and CCP antibodies in patients in acute stage and those in remission stage, we drew corresponding ROC curves for each one. In the ROCs, the area under the curve (AUC)s of 25-(OH)-D3, IL-6, and CCP antibodies in diagnosing patients at different stages were 0.658, 0.694, and 0.789 respectively. The logistic regression was used to fit each index to draw a joint curve, and the AUC of it was 0.969, which suggested that the sensitivity and specificity of detection were notably improved (**Table 2**). The Hosmer-Lemeshow test revealed no statistical difference (P= 0.840), which indicated that the joint detection had high diagnostic value in evaluating patients at different stages (**Figure 4A-D**).

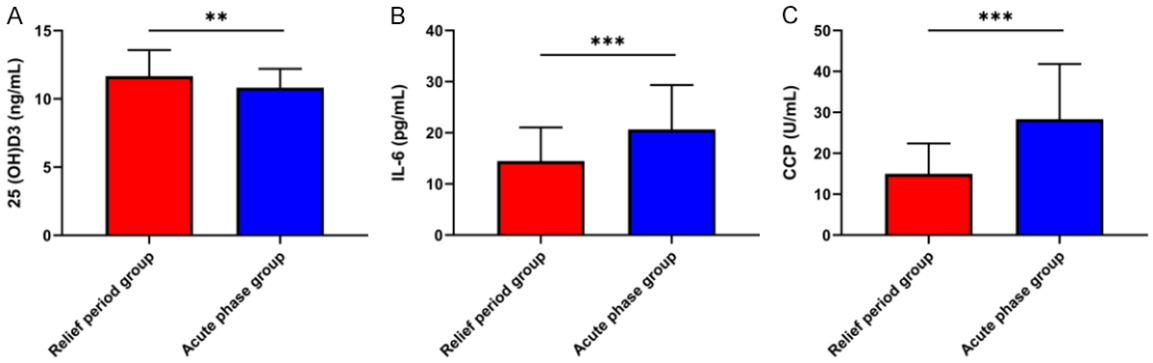

Figure 3. Levels of 25-(OH)-D3, IL-6, and CCP antibody in patients at different stages. A. Comparison of serum 25-(OH)-D3 between the acute-stage group and the remission-stage group. B. Comparison of serum IL-6 between the acute-stage group and the remission-stage group. C. Comparison of serum CCP antibodies between the acute-stage group and theremission-stage group. Notes: **indicates P<0.01 in inter-group comparison; ***indicates P<0.001 in inter-group comparison. The inter-group comparison of measurement data was conducted using the independent-samples t test; 25-(OH)-D3: 25-hydroxyvitamin D3 (25-(OH)-D3); IL-6: interleukin-6; CCP antibodies: cyclic peptide containing citrulline (CCP) antibodies.

Table 2. ROC measurements

Index	AUC	95 CI%	*P*-value	Specificity	Sensitivity	Youden index
25-(OH)-D3	0.658	0.526~0.791	0.016	59.37	77.08	36.45
IL-6	0.694	0.581~0.907	0.003	87.50	45.83	33.33
CCP antibodies	0.789	0.693~0.886	<0.001	90.62	58.33	48.95
Combination of the three	0.969	0.928~1.000	<0.001	97.36	93.02	90.39

Notes: AUC: area under curve; 25-(OH)-D3: 25-hydroxyvitamin D3 (25-(OH)-D3); IL-6: interleukin-6; CCP antibodies: cyclic peptide containing citrulline (CCP) antibodies.

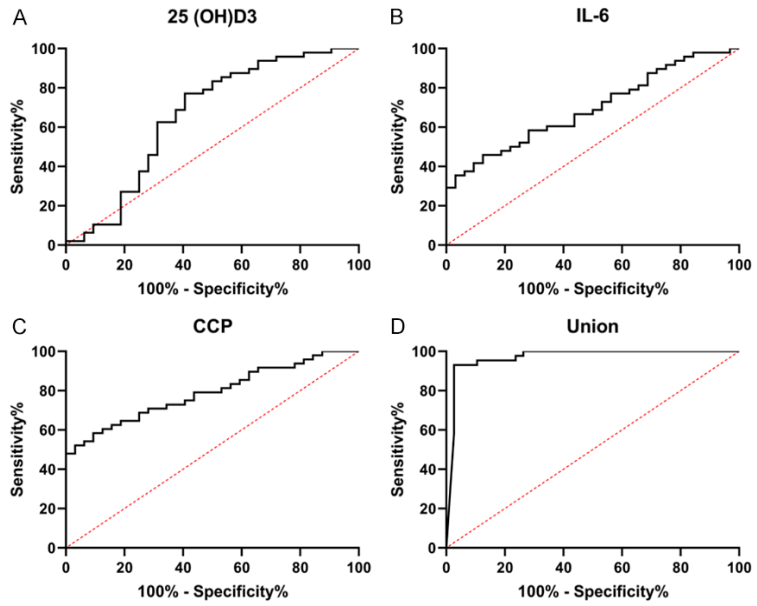

Figure 4. Diagnostic value of 25-(OH)-D3, IL-6, and CCP antibodies in patients at different stages. A. ROC curve of 25-(OH)-D3 in diagnosing RA patients at different stages. B. ROC curve of IL-6 in diagnosing RA patients at different stages. C. ROC curve of CCP antibodies in diagnosing RA patients at different stages. D. Joint ROC curve of the combination of 25-(OH)-D3, IL-6, and CCP antibodies in diagnosing RA patients at different stages; 25-(OH)-D3: 25-hydroxyvitamin D3 (25-(OH)-D3); IL-6: interleukin-6; CCP antibodies: cyclic peptide containing citrulline (CCP) antibodies.

Comparison of serum 25-(OH)-D3, IL-6, and CCP antibodies before and after therapy

The serum 25-(OH)-D3, IL-6, and CCP antibodies in RA patients were quantified before and after therapy. According to the results, 25-(OH)-D in the patient group increased significantly after therapy (**Figure 5A**, P<0.05), and IL-6 and CCP antibodies in the groups decreased significantly after therapy (**Figure 5B**, **5C**, both P<0.05).

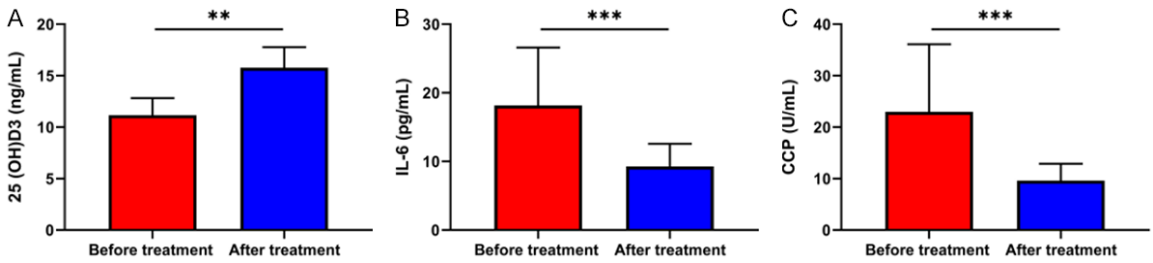

Figure 5. Comparison of serum 25-(OH)-D3, IL-6, and CCP antibodies before and after treatment. A. Comparison of serum 25-(OH)-D3 in the patient group before and after treatment. B. Comparison of serum IL-6 in the patient group before and after treatment. C. Comparison of serum CCP antibodies in the patient groupbefore and after treatment. Notes: **indicates P<0.01 in inter-group comparison; ***indicates P<0.001 in inter-group comparison. The inter-group comparison of measurement data was conducted using paired t test; 25-(OH)-D3: 25-hydroxyvitamin D3 (25-(OH)-D3); IL-6: interleukin-6; CCP antibodies: cyclic peptide containing citrulline (CCP) antibodies.

Table 3. Assignments

Factors	Assignment
Age (Y)	≥50=1, <50=2
Gender	Male =1, female =2
BMI (kg/m^2)	≥22=1, <22=2
Course of disease (Y)	≥2=1, <2=2
DAS-28	≥2.6=1, <2.6=2
25-(OH)-D3	<11.39=1, ≥11.39=2
IL-6	<21.15=1, ≥21.15=2
CCP antibodies	<24.52=1, ≥24.52=2
Efficacy	Markedly effective + effective =1, ineffective =2

Notes: 25-(OH)-D3: 25-hydroxyvitamin D3 (25-(OH)-D3); IL-6: interleukin-6; CCP antibodies: cyclic peptide containing citrulline (CCP) antibodies.

Risk factors for efficacy in patients

The patients were grouped according to clinical efficacy. Patients with markedly effective outcomes and those with effective outcomes were assigned to the marked effect group (n=65) and those with ineffective outcomes were assigned to the ineffective group (n=15). The clinical data of patients were collected and evaluated (**Table 3**). Univariate analysis revealed that the course of the disease, DAS-28, IL-6, and CCP antibodies were risk factors for the efficacy in patients (all P<0.05), and multivariate analysis revealed that all four were independent risk factors for efficacy (**Table 4**, all P<0.05). According to the results of multivariate logistic regression, a risk prediction equation was established: logit(p)= -7.718+1.872×X1+2.219×X2+1.679×X3+2.292 ×X4. The Hosmer-Lemeshow test was used to test the goodness of fit of the regression equation (P=0.872). The established model was used to determine the lower area of the ROC curve of clinical efficacy on RA patients, and it was 0.889 (**Figure 6**, 95% CI: 0.812-0.967, P<0.001). According to the established risk prediction score model, the probability of a markedly effective outcome in RA patients with a course of disease ≥2 years after therapy was 70.76%, and the probability of an ineffective outcome in RA patients with a course of disease <2 years after therapy was 33.33%.

Discussion

RA is a chronic autoimmune disease triggered by the combined action of genetic background and various risk factors such as environmental factors, and is also a major disabling disease [19]. Prior research has revealed that for patients with RA, there is only a short treatment period in the first year of onset, during which the synovitis changes of patients are reversible, so this period is an effective period to relieve and control the disease [20]. Accordingly, it is of high significance to pursue an early diagnosis of RA, and serological indicators with high sensitivity and specificity are the primary basis for improving the correct rate of early diagnosis.

Over the past few years, many studies have revealed serologic detectionto bes the first choice in the diagnosis of RA [21]. Since cases with RA are abnormal in inflammation and immunity, joint determination of multiple serological indicators can improve diagnostic accuracy [22]. In addition, judging the patient's se-

Table 4. Logistic regression analysis

Factor	Univariate analysis			Multivariate analysis		
	P-value	OR value	95 CI%	*P*-value	OR value	95 CI%
Gender	0.560	1.400	0.452-4.337			
Age (Y)	0.939	0.955	0.290-3.146			
BMI (kg/m^2)	0.560	0.714	0.231-2.213			
Course of disease (Y)	0.010	4.842	1.460-16.064	0.016	6.502	1.423-29.716
DAS-28	0.032	0.179	0.037-0.860	0.033	0.119	0.017-0.846
25-(OH)-D3	0.712	0.800	0.245-2.614			
IL-6	0.003	6.000	1.810-19.891	0.027	5.360	1.213-23.688
CCP antibodies	0.026	5.926	1.238-28.380	0.011	9.896	1.689-57.986

Notes: BMI: Body Mass Index; 25-(OH)-D3: 25-hydroxyvitamin D3 (25-(OH)-D3); IL-6: interleukin-6; CCP antibodies: cyclic peptide containing citrulline (CCP) antibodies. Univariate analysis was carried out by the forward method in logistic regression, and multivariate analysis by the backward LR method in logistic regression.

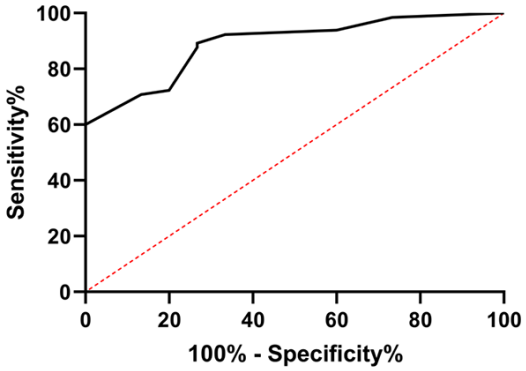

Figure 6. Joint curve of DAS-28, IL-6 and CCP antibodies. Note: DAS28: disease activity score in 28 joints; IL-6: interleukin-6; CCP antibodies: cyclic peptide containing citrulline (CCP) antibodies.

verity based on clinical symptoms can improve the patients' prognosis. Vitamin (Vit) D itself has no biological activity, and must undergo two consecutive hydroxylations in the liver and kidney to become 1,25-dihydroxy vitamin D with biological activity, among which 25-(OH)-D3 is the main circulating form of Vit D in the body [23, 24]. IL-6 is a kind of polypeptide consisting of two glycoprotein chains, which are mainly secreted by fibroblasts, MC and T cells [25]. It plays an important part in the inflammatory reaction, hematopoiesis, and immune regulation as a multi-effect cytokine [26]. CCP is a polypeptide fragment of cyclic filament protein that is essentially immunoglobulin, which can bind to immunoglobulin secreted by plasma cells to form an immune complex and induce inflammatory response [27]. Recent studies have revealed that 25-(OH)-D3, IL-6, and CCP antibodies have become crucial auxiliary observation indexes for clinical diagnosis of RA because of lower 25-(OH)-D3 level and higher levels of IL-6 and CCP antibodies in RA patients than healthy individuals [28-30]. Similar to the above studies, RA patients showed a lower 25-(OH)-D3 level and higher levels of IL-6, and CCP antibodies than healthy individuals in our study, which implied that 25-(OH)-D3, IL-6 and CCP antibodies might be all involved in the development of RA. According to our correlation analysis, 25-(OH)-D3, IL-6 and CCP antibodies were bound up with DAS-28, which also suggested the associations of the three with the development of RA.

Prior research has discovered the clinical significance of 25-(OH)-D3, IL-6 and CCP antibodies in diagnosing RA [31, 32]. However, the value of the combination of the three in RA patients at different stages lacks reports. In our study, acute-stage patients presented a lower 25-(OH)-D3 level and higher levels of IL-6 and CCP antibodies than remission-stage patients. Similar to our study, Haga and Gottenberg et al. [33, 34] have also discovered such differences between RA patients and healthy individuals in 25-(OH)-D3, IL-6 and CCP antibodies. In addition, each one of them demonstrated crucial value in distinguishing patients at different stages based on ROC curves, but the sensitivity and specificity of each index are greatly different. In order to improve the diagnostic value, we drew a joint curve of the three indexes. As a result, the combination of the three delivered greatly higher specificity and sensitivity, and the AUC of it was notably higher than that of each index detected alone. The results suggest that the combination of these three can be adopted as an auxiliary index to distinguish different stages of RA.

The patients' prognosis can be improved by timely intervention based on the early prediction of the clinical efficacy on patients after therapy. Therefore, a simple and effective scoring model of clinical efficacy risk factors for RA patients was established to predict the risk of ineffective treatment for RA patients in advance, which was of guiding significance for active intervention for high-risk patients. The Hosmer-Lemeshow test was used to test the goodness of fit and ROC curve, which verified that the model fitted well, indicating the favorable clinical practical value of the prediction equation. According to the further analysis of the prediction equation, the probability of a markedly effective outcome in RA patients with a course of disease ≥2 years after therapy was 70.76%, and the probability of an ineffective outcome in RA patients with a course of disease <2 years after therapy was 33.33%.

This study determined the diagnostic value of the combination of 25-(OH)-D3, IL-6, and CCP antibodies in patients at different stages of RA, and further determined that the course of the disease, DAS-28 score, IL-6, and CCP antibodies were independent risk factors affecting the efficacy on patients. However, this study still has some limitations. As a retrospective study, the results may be biased. RA is a long-term disease, but we have not conducted a long-term follow-up, and it is not enough to determine the efficacy on patients only by inquiring about the patient's medical records and outpatient review records. Therefore, we hope to carry out prospective research in the future to improve the conclusions.

In sum, the combination of 25-(OH)-D3, IL-6, and CCP antibodies can be adopted as a diagnostic index in acute and remission stages of RA patients. A risk factor model of clinical efficacy on RA patients can help us effectively identify high-risk patients before therapy and take intervention measures early.

Disclosure of conflict of interest

None.

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