# Serum PGLYRP-1 is a highly discriminatory biomarker for the diagnosis of rheumatoid arthritis

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**Abstract.** Peptidoglycan recognition protein-1 (PGLYRP-1) is a part of the innate immune system. It is well-known that dysregulation of innate immune responses is present in patients with rheumatoid arthritis (RA). However, the role of *Pglyrp1*/PGLYPR-1 in RA is poorly understood. Reverse transcription-quantitative polymerase chain reaction was used to detect the level of *Pglyrp1* in peripheral blood mononuclear cells. An ELISA was used to measure the level of PGLYPR-1 in the serum, and correlation analysis was performed to analyze the association between the level of PGLYPR-1 in the serum and clinical characteristics. A receiver operating characteristic (ROC) curve was constructed to evaluate the diagnostic value of PGLYPR-1 in serum. The expression of PGLYPR-1 in the serum of healthy controls compared with PGLYPR-1 in the serum from patients with RA was significantly increased compared with patients with systemic lupus erythematosus (SLE). The level of PGLYPR-1 in serum was correlated with rheumatoid factor and anti-cyclic citrullinated peptide. ROC curve analysis suggested that PGLYPR-1 in the serum may have significant value for RA diagnosis. In addition, the risk score based on PGLYPR-1 in the serum also significantly discriminated the patients with RA from the disease controls (SLE). The present study suggested that increased expression of PGLYPR-1 in the serum from patients with RA may serve as a potential biomarker for RA diagnosis.

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## Introduction

Rheumatoid arthritis (RA), an autoimmune disease, presents as a chronic and systemic inflammatory disorder that affects a number of tissues and organs; however, it frequently attacks synovial joints and leads to systemic bone loss due to excessive osteoclast activity (1,2). In addition, 0.5-1% of the global population suffers from RA (3,4). Early diagnosis and proper treatment may prevent severe disease manifestations in patients with RA (5). However, current diagnostic methods demonstrate various insufficiencies in the early diagnosis of RA, reflecting the need to investigate novel biomarkers that may contribute to improved diagnosis and prognosis.

Peptidoglycan recognition protein-1 (PGLYRP-1; previously termed PGRP-S) is one of the four types of peptidoglycan recognition proteins in humans that is primarily expressed in polymorphonuclear leukocyte granules (6,7). In addition, PGLYRP-1 is a secreted and circulating protein that binds peptidoglycan and promotes inflammation through the activation of innate immune mechanisms (8). A number of studies have demonstrated that an imbalance between the adaptive and innate immune systems driving excessive immune responses is involved in the pathogenesis of RA (9-11). Furthermore, Fodil *et al* (12) reported that single nucleotide polymorphisms (SNPs) of *Pglyrp1* were associated with RA. However, limited data are available regarding the potential role of *Pglyrp1*/PGLYRP-1 in RA.

In the present study, it was hypothesized that the plasma levels of PGLYRP-1, reflecting inflammation, may be associated with RA. This hypothesis was tested by measuring the expression of PGLYRP-1 in the serum and to determine whether PGLYRP-1 in the serum may be used as a novel biomarker for RA diagnosis.

## Patients and methods

*Patient variables.* A total of 62 patients (age, 56.54±11.98 years; 13 males) who fulfilled the revised American College of Rheumatology criteria for RA (13) were admitted to The First Affiliated Hospital of Nanchang University (Nanchang, China) between November 2015 and September 2017. The *Pglyrp1* mRNA expression levels in peripheral blood mononuclear

Table I. Characteristics of RA cases used for detecting gene *Pglyrp1* and protein PGLYRP-1.

| Characteristic | Detecting gene *Pglyrp1* (n=44) | Detecting protein PGLYRP-1 (n=40) |
|---|---|---|
| Age, years (mean ± SD) | 54.82±11.83 | 58.43±12.00 |
| Sex (female/male) | 36/8 | 35/5 |
| DAS28 (mean ± SD) | 4.91±1.64 | 5.50±1.16 |
| ESR, mm/h (mean ± SD) | 60.43±33.77 | 68.45±37.13 |
| CRP, mg/l (mean ± SD) | 49.14±56.51 | 54.25±60.79 |
| RF, IU/ml (mean ± SD) | 364.47±572.54 | 341.89±538.09 |
| ACPA, RU/ml (mean ± SD) | 349.75±586.00 | 449.30±656.55 |
| WBC, $10^9$/l (mean ± SD) | 7.51±2.52 | 6.96±2.08 |
| RBC, $10^{12}$/l (mean ± SD) | 3.80±0.58 | 3.74±0.66 |
| HGB, g/l (mean ± SD) | 106.64±17.44 | 104.08±19.42 |
| HCT, l/l (mean ± SD) | 0.33±0.07 | 0.32±0.07 |
| PLT, $10^9$/l (mean ± SD) | 267.37±127.77 | 285.63±122.81 |
| Lymphocytes, $10^9$/l (mean ± SD) | 1.60±0.73 | 1.35±0.66 |
| Lymphocyte, % (mean ± SD) | 22.57±9.22 | 20.62±9.89 |
| Monocytes, $10^9$/l (mean ± SD) | 0.46±0.21 | 0.44±0.21 |
| Monocytes, % (mean ± SD) | 6.37±2.67 | 6.39±3.34 |
| Neutrophils, $10^9$/l (mean ± SD) | 5.29±2.30 | 6.22±7.42 |
| Neutrophils, % (mean ± SD) | 69.34±12.24 | 70.62±12.88 |

ACPA, anti-cyclic citrullinated peptide; DAS28, disease activity score 28; CRP, c-reactive protein; ESR, erythrocyte sedimentation rate; HCT, hematocrit; HGB, hemoglobin; *Pglyrp1*/PGLYRP-1, peptidoglycan recognition protein-1; PLT, platelets; RBC, red blood cell; RF, rheumatoid factor; WBC, white blood cells; SD, standard deviation.

cells (PBMCs) and the PGLYRP-1 levels in the serum were simultaneously determined in 22 patients. A further 22 patients were employed to detect the *Pglyrp1* mRNA level in PBMCs solely because of the limited serum samples and 18 patients were engaged to identify the PGLYRP-1 level in the serum also solely because of the limited PBMCs samples. In total, 44 specimens [6 from patients with novel-onset RA (14)] were used to detect the *Pglyrp1* mRNA expression level in PBMCs and 40 specimens (6 cases of novel-onset RA) were used to identify the PGLYRP-1 level in the serum. The characteristics of the RA patients (44 specimens for detecting the *Pglyrp1* gene and 40 specimens for detecting the PGLYRP-1 protein ) are presented in Table I. The RA disease activity was measured using the disease activity score 28 (DAS28) (15). In addition, the present study included 91 healthy controls (HCs; age, 53.76±14.39 years; 14 males, 77 females) who were unrelated to the patients and did not have inflammatory or autoimmune diseases. Among them, 51 HCs were employed to detect *Pglyrp1* in PBMCs and 40 HCs were engaged to detect PGLYRP-1 in the serum. RA and HC cases were age- and sex-matched. As an autoimmune disease control, 41 patients with systemic lupus erythematosus (SLE; age, 38.61±12.60 years; 3 males, 38 females) who fulfilled the revised American College of Rheumatology criteria for SLE (16) were also enrolled at The First Affiliated Hospital of Nanchang University between January 2017 and September 2017. The study was approved by the Ethics Committee of The First Affiliated Hospital of Nanchang University (ethical approval no. 019) and was performed in compliance with the Declaration of Helsinki. All participants also signed informed consent forms.

*PBMC preparation and total RNA extraction*. PBMCs were isolated from 2 ml EDTA-anticoagulated blood from each donor using Ficoll-Hypaque density gradients (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) at 25˚C. The cells were frozen in TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at a concentration of $10^6$/ml and stored at -80˚C. Total RNA was extracted from PBMCs using TRIzol reagent, according to the manufacturer's protocol. The concentration and quality of the RNA were assessed by absorbance spectrometry ratios of A260/A280 and A260/A230 using a NanoDrop ND-1000 spectrophotometer (Agilent Technologies, Inc., Santa Clara, CA, USA).

*Reverse transcription quantitative-polymerase chain reaction (RT-qPCR) analysis*. Total RNA was reverse-transcribed into cDNA using a PrimeScript™ RT reagent kit (Takara Bio Inc., Otsu, Japan). RT assay was set at an initial denaturation step of 37˚C for 15 min, followed by 85˚C for 5 sec. The qPCR was performed on an ABI 7500 Real-time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.), using SYBR® Premix Ex Taq™ II (Takara Bio, Inc.) in 10-$\mu$l reactions containing 1X SYBR-Green PCR Master Mix, 0.4 $\mu$M of each specific forward and reverse primer, and 0.5 $\mu$l cDNA template. The PCR assay had an initial denaturation step at 95˚C for 5 min, followed by 40 cycles at 95˚C for 15 sec and at 60˚C for 1 min, and melt curves were detected to confirm the specificity of amplification and the lack of primer dimers. The primers used in RT-qPCR are presented in Table II. Furthermore, β-actin was used as an internal control.

Table II. Specific gene primers used for reverse transcription-quantitative polymerase chain reaction analysis.

| Name | Sequence (5'→3') |
|---|---|
| *Pglyrp1* | F: CACATGAAGACACTGGGCTGGT |
| | R: CATGAAGCTGATGCCAATGGAC |
| β-actin | F: TACTGCCCTGGCTCCTAGCA |
| | R: TGGACAGTGAGGCCAGGATAG |

*Pglyrp1*, peptidoglycan recognition protein-1; F, forward; R, reverse.

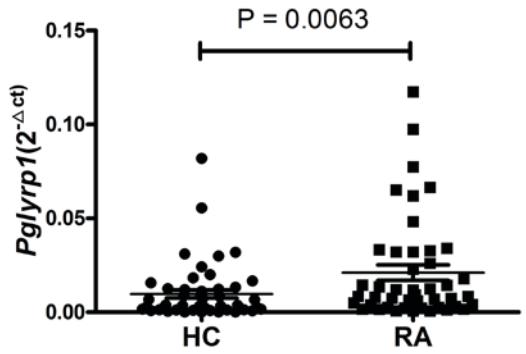


Figure 1. Reverse transcription-quantitative polymerase chain reaction to determine the relative expression levels of *Pglyrp1* in PBMCs from patients with RA and HCs. The expression level of *Pglyrp1* in patients with RA was significantly upregulated compared with the HC group. HCs, healthy controls; PBMCs, peripheral blood mononuclear cells; *Pglyrp1*, peptidoglycan recognition protein-1; RA, rheumatoid arthritis.

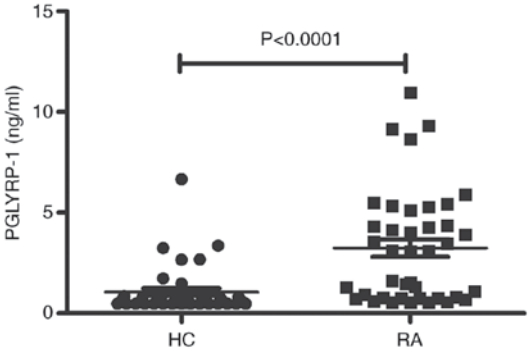


Figure 2. ELISA to determine the relative expression levels of PGLYRP-1 in the serum from patients with RA and HCs. The expression level of PGLYRP-1 in patients with RA was significantly upregulated compared with the HC group. HCs, healthy controls; PGLYRP-1, peptidoglycan recognition protein-1; RA, rheumatoid arthritis.

Following the reactions, the Cq values were determined using the fixed threshold settings.

The relative expression levels of *Pglyrp1* were calculated using the $2^{-\Delta Cq}$ method normalized to the endogenous control, with $\Delta Ct=Ct_{target}-Ct_{reference}$ (17,18).

*ELISA*. Stored serum obtained from each categorical subject was thawed and assayed for PGLYRP1 by using a commercial ELISA kit (cat. no. SED937Hu; USCN Life Science Inc., Wuhan, China) (19).

*Serum C-reactive protein (CRP), rheumatoid factor (RF), anti-cyclic citrullinated peptide (ACPA), erythrocyte sedimentation rate (ESR) and routine blood measurement*. The concentrations of serum CRP and RF were determined by nephelometry methods, according to the manufacturer's protocol (IMMUNE800; Beckman Coulter, Inc., Brea, CA, USA). ACPA of immunoglobulin G in serum was measured using a commercial ELISA kit (cat. no. EC180701; Shanghai Kexin Biotech Co., Ltd., Shanghai, China). ESR and routine bloods were determined erythrocyte sediment rate analyzer and hematology analyzer according to the manufacturer's protocols.

*Statistical analysis*. Statistical analysis and graphical presentation were performed using GraphPad Prism version 5.0 (GraphPad Software, Inc., La Jolla, CA, USA) and SPSS software version 16.0 (SPSS Inc., Chicago, IL, USA). A Shapiro-Wilk test was used to assess whether the data were normally distributed. In addition, a Student's t-test was used where the normality test passed; otherwise, the nonparametric Mann-Whitney test was used to analyze the data. Likewise, the Pearson method or the nonparametric Spearman method was used for correlation analysis. Receiver operating characteristic (ROC) curves were constructed to evaluate the diagnostic value of PGLYRP-1 in the serum of RA patients compared with the HCs. $P<0.05$ was considered to indicate a statistically significant difference.

## Results

*Expression of Pglyrp1 is upregulated in PBMCs from patients with RA*. The expression of *Pglyrp1* in PBMCs from 44 patients with RA and 51 HCs was first analyzed using RT-qPCR. As presented in Fig. 1, compared with the HCs, the expression level of *Pglyrp1* in patients with RA was significantly upregulated (P=0.0063).

*Expression of PGLYRP-1 is increased in the serum from RA*. To determine the expression of the *Pglyrp1* gene encoding the PGLYRP-1 protein in serum from RA, additional serum samples of RA patients (n=40) and HC (n=40) were obtained and ELISAs were performed. As illustrated in Fig. 2, a significant increase in the serum levels of PGLYRP-1 in the RA group was demonstrated compared with the HC group ($P<0.0001$).

*Potential diagnostic values of PGLYRP-1 in the serum in RA*. Next, analysis was conducted to evaluate the potential diagnostic value of PGLYRP-1 in serum. As presented in Fig. 3, the PGLYRP-1 level in the serum from patients with RA was significantly correlated with RF (r=0.50, P=0.0011) and ACPA (r=0.45, P=0.0039), which are antibody hallmarks of RA and may reflect the severity of the disease (20,21). However, the PGLYRP-1 level in the serum from patients with RA was not correlated with ESR, CRP or DAS28 (data not shown).

It was hypothesized that the PGLYRP-1 level in the serum may be used as a biomarker of RA. Therefore, a ROC curve was constructed. The area under the ROC curve (AUC) was up to 0.832 [95% confidence interval (CI): 0.745-0.919; $P<0.0001$; Fig. 4] calculated using 0.7306 as the cutoff

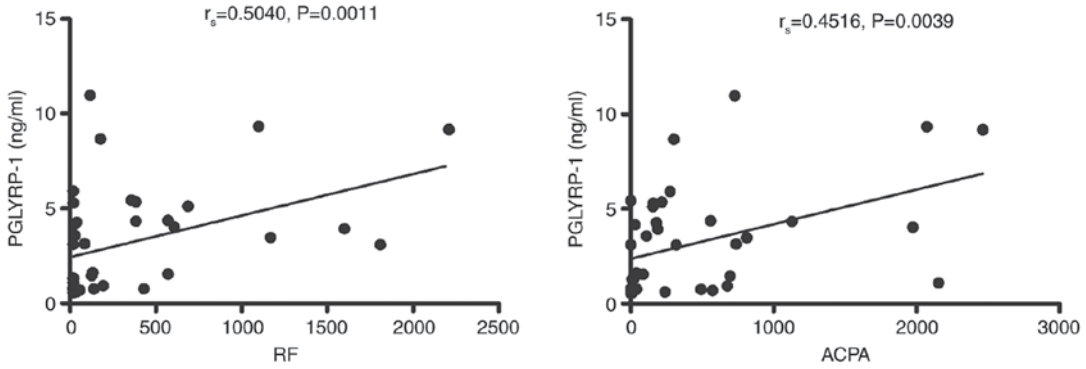


Figure 3. Correlation of PGLYRP-1 in the serum from RA cases with RF and ACPA. ACPA, anti-cyclic citrullinated peptide; PGLYRP-1, peptidoglycan recognition protein-1; RA, rheumatoid arthritis; RF, rheumatoid factor.

point. The sensitivity and specificity were 85.0 and 65.0%, respectively.

*PGLYRP-1 level in the serum in patients with RA and SLE.* The level of PGLYRP-1 was significantly increased in patients with RA compared with patients with SLE (P<0.0001; Fig. 5A). Subsequently, a risk score based on PGLYRP-1 in serum was further assessed in RA patients and all controls (HC + SLE). The AUC for the risk score was 0.780 (95% CI, 0.719-0.880; P<0.0001; Fig. 5B). The sensitivity and specificity were 85.0 and 64.2%, respectively. The risk score also significantly differentiated the patients with RA from disease controls (SLE) and the AUC was 0.768 (95% CI: 0.665-0.871; P<0.0001; Fig. 5C). The sensitivity and specificity were 87.5 and 61.0%, respectively.

Due to the difference in the ages of high incidence between RA and SLE (the incidence of RA is high in people of 50-60 years, and the incidence of SLE is high in women of childbearing age), patients with RA and SLE were not age-matched in the present study. No correlation between PGLYRP-1 serum levels and age or sex was observed in SLE, RA and HC (Fig. 6).

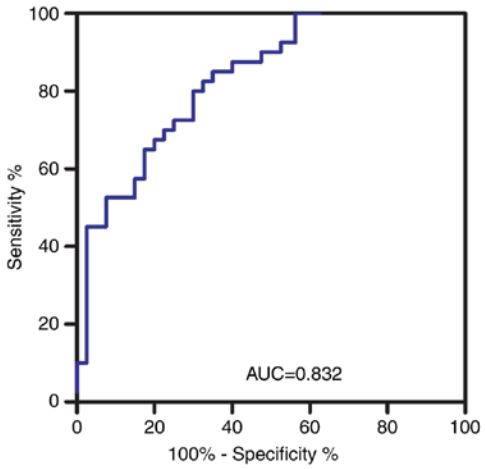


Figure 4. ROC analysis of PGLYRP-1 in the serum from patients with RA. AUC, area under the curve; PGLYRP-1, peptidoglycan recognition protein-1; RA, rheumatoid arthritis; ROC, receiver operating characteristic.

## Discussion

The *Pglyrp1* gene encodes an innate immunity protein (PGLYRP-1) that directly breaks down the structure of microbial cell wall peptidoglycan and serves an important role in antibacterial defenses, and several inflammatory diseases (22). To the best of our knowledge, only one study has demonstrated that SNPs of *Pglyrp1* are associated with RA (12), which suggested that the *Pglyrp1* gene was correlated with RA. However, there are no reports regarding the assessment of the *Pglyrp1*/PGLYRP-1 levels in the peripheral blood from patients with RA, to the best of our knowledge. The present study is among the first studies to report the level of *Pglyrp1* in PBMCs, in which the level of PGLYRP-1 in the serum was increased in patients with RA. These results indicated that *Pglyrp1*/PGLYRP-1 may be associated with the pathogenesis of RA.

RA is a chronic, debilitating systemic autoimmune disease with unclear etiology. In 2010, the American College of Rheumatology/European League Against Rheumatism classification criteria for rheumatoid arthritis, ACPA, RF, CRP and ESR were adopted to diagnose RA (23). However, all of these biomarkers suffer from a weak correlation with the disease severity of RA. Novel biomarkers with a satisfactory correlation with the disease activity of RA are required for the evaluation of the curative effect of treatment or disease development (5). The present study revealed that the level of PGLYRP-1 was increased and the regression model from PGLYRP-1 demonstrated that the AUC was high. Furthermore, the risk score based on PGLYRP-1 indicated that it may significantly discriminate patients with RA from those with SLE. The results supported the level of PGLYRP-1 in the serum being used as a candidate biomarker of RA.

It is well-known that RA is a systemic autoimmune disease characterized by increased auto-antibodies, including RF and ACPA (24). Additionally, the RF/ACPA titer is frequently positively correlated with disease activity and the severity of joint destruction (25). In the present study, the serum levels of RF and ACPA were first determined and analyzed due to their association with the level of PGLYRP-1 in the serum. The obtained data demonstrated that the level of PGLYRP-1 was positively correlated with RF and ACPA in the serum of patients with RA and suggested that the level of PGLYRP-1 in the serum may be associated with the autoimmune responses of RA and disease activity.

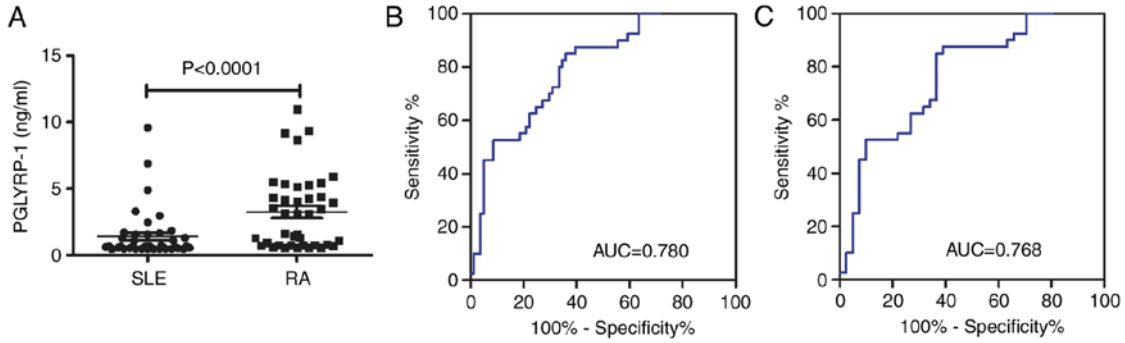


Figure 5. ROC curve analysis of the risk-score of PGLYRP-1 in the serum. (A) The expression level of PGLYRP-1 in the serum in patients with RA was significantly upregulated compared with the SLE group. (B) ROC curve analysis of PGLYRP-1 in the serum for the risk-score in RA patients vs. all controls (HC and SLE). (C) ROC curve analysis of PGLYRP-1 in the serum of the risk score in patients with RA vs. patients with SLE. AUC, area under the curve; HCs, healthy controls; PGLYRP-1, peptidoglycan recognition protein-1; RA, rheumatoid arthritis; ROC, receiver operating characteristic; SLE, systemic lupus erythematosus.

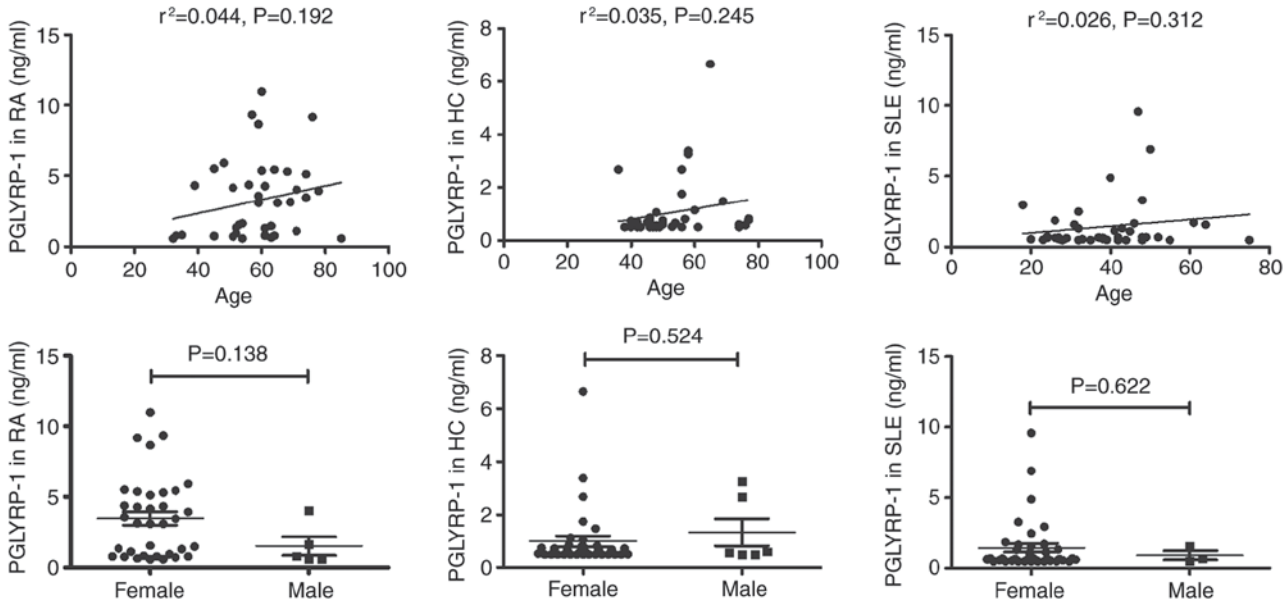


Figure 6. Correlation of PGLYRP-1 in serum with age and sex. No correlation between PGLYRP-1 serum levels and age was observed in RA, HC or SLE. No correlation between PGLYRP-1 serum levels and sex was observed in RA, HC or SLE. HCs, healthy controls; PGLYRP-1, peptidoglycan recognition protein-1; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus.

However, there are a number of limitations to the present study. The first one is the relatively small sample size of patients with novel-onset RA, which results in some uncertainty as to whether the use of corticosteroids or immunosuppressive drugs affect the expression of PGLYRP-1 in the serum. The second issue is the relatively small sample size of patients with RA, and the present study is limited as only patients from one hospital were included, which may restrict the representativeness of the results. Third, due to the lack of data on RF and ACPA in HC, the values of PGLYRP-1 in serum plus RF/ACPA in RA diagnosis were not evaluated. Fourth, the roles of PGLYRP-1 in RA pathogenesis in this study were not investigated. However, the increased expression of PGLYRP-1 and its correlation with RF and ACPA suggest that PGLYRP-1 may serve specific roles in the pathogenesis of RA, which will be investigated in the authors' future study.

In conclusion, to the best of our knowledge, the level of PGLYRP-1 in serum was demonstrated for the first time to be upregulated in RA patients. The results of the present study established an association between the level of PGLYRP-1 in the serum and RA disease activity. Serum PGLYRP-1 is a promising diagnostic biomarker for RA.

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**Availability of data and materials**

All data generated or analyzed during this study are included in this published article.

**Authors' contributions**

QL, XL, ZH and JL conceived and designed the experiments. QL, XL, LZ, FY, ZD, CQ, RS, JX and YG performed the experiments. QL, XL, ZH and JL analyzed the data. QL, XL, ZH and JL wrote the manuscript. FY, ZD, CQ, RS, JX and YG contributed reagents, materials and analytical tools. All authors read and approved the final manuscript.

**Ethics approval and consent to participate**

The study was approved by the Ethics Committee of the First Affiliated Hospital of Nanchang University (approval no. 019) and was performed in compliance with the Helsinki Declaration. All participants signed informed consent forms.

**Patient consent for publication**

All participants signed informed consent forms.

**Competing interests**

The authors declare that they have no competing interests.

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