# Serum level of 14-3-3η (eta) protein as a diagnostic marker for rheumatoid arthritis and potential correlation with disease activity

## Abstract

**Background:** New markers are needed for early diagnosis of Rheumatoid Arthritis as Seronegativity in both early and settled RA remains a major limitation of both anticitrullinated protein antibodies and rheumatoid factor. The 14-3-3η the protein may represent a novel biomarker for the detection of RA.

**Aim of the work:** to study serum14-3-3η as an RA-specific marker that complements both RF and ACPA and increasing their diagnostic value.

**Methods:** RA patients (92 patients; 57 with early RA & 35 with established RA) were included and control group (74 subjects; 18 patients with PsA, 14 patients with SLE &42 healthy subject). All demographic, clinical data &serological data (disease activity score in 28 joints (DAS28) and ESR, CRP, RF, and ACPA were recorded. Serum14-3-3η levels were estimated for all.

**Results:** serum 14-3-3η levels in patients with RA (mean &SD 2.72±1.75 ng/ml) were significantly higher (P< 0.0001) as compared to healthy individuals [0.09±0.09 ng/ml] and all controls [0.14±0.21ng/ml)]. ROC curve analysis showed that 14-3-3η in early RA compared to healthy controls had a significant (P<0.0001) AUC of 0.999 (95% CI, 0.997–1.00). At a cutoff of ≥0.39 ng/ml, the ROC curve yielded a sensitivity of 87.7%, a specificity of 97.6%, a PPV of 0.98, and an NPV of 0.85. There was significant positive correlation between serum 14-3-3η levels and DAS28 in early RA patients (P<0.0001).

**Conclusion:** 14-3-3η is an RA specific marker and of great value as a serological marker for early RA.

**Keywords:** serum14-3-3η, RA diagnosis, disease activity

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## Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune disorder. It is induced by multiple factors and shows high variability among patients accompanying the course of the disease. If untreated, RA results in severe joint destruction leading to reduced physical activity and workplace disability.[1,2] It is now widely declared that diagnosis of RA at an early stage, evaluation of disease severity, and implementation of an effective treatment strategy can considerably improve a patient's prognosis.[3] With the appreciation of this, RA classification criteria were established in 2010 focusing on outlining the disease by its earliest features.[4] Seronegativity in both early and settled RA remains the main limitation of both anticitrullinated protein antibodies (ACPA) and rheumatoid factor (RF) stressing the need for new harmonizing markers that will increase diagnostic sensitivity.[5,6] The current markers estimate only for around thirty percent of the total variety in predicting disease outcome.[7] The 14-3-3η protein is a unique biomarker for RA detection.[1] There are seven isoforms of the 14-3-3 intracellular proteins family. They share about 50% amino acid matching among each other and cooperate with a lot of intracellular proteins, thereby governing an array of biological activity including protein synthesis, cellular metabolism, protein trafficking, and cytoskeletal transport.[8] Overall isomers, only 14-3-3η was found in synovial fluid with high levels (at least 5-fold greater than its level in matched sera) incriminating the joint as the likely source of 14-3-3η.[9,10] Soluble 14-3-3η works through signaling cascades as the extracellular kinase and P38 pathway, this leads to stimulation of some proinflammatory cytokines, such as tumor necrosis factor-α (TNF-α), receptor activator of nuclear factor-κB ligand (RANKL), interleukin6 (IL-6), interleukin 1β (IL-1β), and Matrix metallopeptidase -9 (MMP-9).[11] Serum levels of 14-3-3η favor to be high in RA patients, but not in another disorder as gout, psoriasis, Crohn's disease, ulcerative colitis, type 1 diabetes, osteoarthritis, systemic lupus erythematosus, primary Sjögren's syndrome, scleroderma, and multiple sclerosis.[12,13] Early diagnosis of RA can diminish permanent joint damage.[14]

## Aim of the work

Assessment the diagnostic value of serum 14-3-3η as a diagnostic marker for RA. Also, we try to discover likely correlation with disease activity.

## Subjects and methods

### Patients with RA and controls

Demographic data of all participants were prescribed (Table 1), also the clinical assessment of the early and established cohorts with RA (Table 2). Serum 14-3-3η levels were measured for 92 patients

with RA diagnosed according to the (ACR) American College of Rheumatology 2010 criteria[4] with mean age 44.32±8.44 years & female /male ratio 8.2/1. Serum 14-3-3η expression in early RA, were measured in 57 patients who had a mean disease duration 1.17±0.47 years. Furthermore, serum 14-3-3η levels were analyzed from a total of 74 controls: 42 apparently healthy subjects and 32 patients with arthritis other than RA; 14 patients with systemic lupus erythematosus and 18 patients with psoriatic arthropathy. All demographic & clinical data, Disease Activity Score 28 (DAS28) and Serological assessment including ESR, CRP, RF and ACPA were recorded. Patients and controls were collected from the outpatient clinic of Rheumatology in Saudi German Hospital, Jeddah; KSA. All participants signed informed consent forms to participate in the study. Also, Ethics Board approval was obtained from IRP, Mansoura School of medicine, Mansoura University, Egypt.

**Table1** Demographic & serological data of studied groups

| Groups(No.) Parameters | Rheumatoid arthritis(92 | Controls(74) | PsA(18) | SLE(14) | Healthy control(42) | P value |
|---|---|---|---|---|---|---|
| Gender (female %) | 89.13(82) | 86.49%(64) | 83.33%(15) | 92.86%(13) | 85.71%(36) | NS |
| Age (years) Mean ±SD | 44.32±8.44 | 41.55±6.75 | 45.16±8.49 | 40.52±5.28 | 40.52±5.89 | NS |
| RF(IU/ml) mean± SD | 78.73±80.61 | 12.52±15.07 | 14.58±19.46 | 20.09±22.45 | 9.12±7.36 | <0.0001 |
| ACPA (U/ml) mean± SD | 186.84±258.05 | 3.59±6.31 | 4.06±5.15 | 4.98±10.70 | 2.95±4.72 | <0.0001 |
| 14-3-3η (ng/ml) mean± SD | 2.72±1.75 | 0.14±0.210.19±0.38 | 0.19±0.38 | 0.17±0.16 | 0.09±0.09 | <0.0001 |

ACPA, anticitrullinated protein antibodies; RF, rheumatoid factor; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus; PsA, psoriatic arthritis; ng, nanogram; U/ml, unit/mille; IU/ml, international unit; η, eta

**Table 2** Demographic & serological data of RA patient group

| Parameters | Early RA, n = 57, mean± SD | Established RA, n = 35 mean± SD | P value |
|---|---|---|---|
| Age | 44.94±8.64 | 43.31±8.13 | NS |
| Gender(female/male) | 50/7 | 32/3 | NS |
| Disease duration | 1.17±0.47 | 7.06±2.58 | <0.0001 |
| RF(IU/ml) | 76.74±80.37 | 81.98±82.08 | NS |
| ACPA (U/ml) | 209.67±256.11 | 149.12±260.64 | NS |
| 14-3-3η (ng/ml) | 2.73±1.79 | 2.70±1.69 | NS |
| DAS 28 | 3.63±1.23 | 3.10±0.78 | 0.027 |
| CRP(mg/L) | 26.71±32.54 | 7.06±8.71 | 0.001 |
| ESR (mm/hour.) | 47.56±25.70 | 31.54±10.33 | 0.001 |

RA, rheumatoid arthritis; DAS28, Disease Activity Score 28-joint count; ESR, erythrocyte sedimentation rate; CRP, C-reactive protein; RF, rheumatoid factor; ACPA, anticitrullinated protein antibodies

## Assay of serum 14-3-3η

Detection of serum 14-3-3η levels was done using the quantitative 14-3-3-η ELISA kits. The 14-3-3η ELISA assay has a high sensitivity, assay accuracy and lack of significant cross-reactivity with other isoforms. The assay sample and buffer were incubated together in a pre-coated plate for one hour. After the incubation period, the wells were decanted and washed five times. The wells were then incubated with a substrate for horseradish peroxidase (HRP) enzyme. The product of the enzyme-substrate reaction formed a blue colored complex. Finally, a stop solution was added to stop the reaction, which turned the solution yellow, the strength of the color was measured spectrophotometrically. A standard curve was plotted relating the intensity of color to the concentration of standards.[15]

## Statistical methods

Statistical analysis was performed using SPSS version 21.0 (SPSS Inc., Chicago, IL). Data are stated as mean value ±SD. Differences among groups in clinical & serological data were compared by one-way ANOVA test for normally distributed values. The Spearman rank-order correlation was estimated between variables. Receiver-operating characteristic (ROC) curves were used to evaluate the diagnostic

utility of 14-3-3η as estimated by the area under the curve (AUC), and the corresponding positive and negative predictive values (PPV; NPV). Further, sensitivity and specificity of RF, ACPA, and RF and/or ACPA with and without the inclusion of 14-3-3η was calculated for both the groups with early and established RA concerning the healthy controls and all controls (healthy plus disease controls). P<0.05 was considered to be statistically significant.

## Results

### Compared to control group

Serum 14-3-3η levels in RA patients (mean & SD 2.72±1.75 ng/ml) were significantly higher (P<0.0001) as compared to healthy persons (0.09±0.09 ng/ml) and all controls (0.14±0.21ng/ml). RF and ACPA levels in RA patient were significantly higher (P<0.0001) as compared to all controls. There was the insignificant difference between RA patients and all controls as regard to age and gender distribution. Although levels of serum 14-3-3η in PsA and SLE patients were numerically higher than healthy individuals control group but it was insignificant (P>0.05) (Table1). ROC curve analysis comparing patient with RA with all control demonstrated a significant (p<0.0001) AUC of 0.988 (95% CI, 0.977–0.999; Figure 1). At a cutoff of ≥0.39 ng/ml, the ROC curve yielded a sensitivity of 90.2%, a specificity of 94.6%, a PPV of 0.98, and an NPV of 0.73 (Figure1).

### Among patients' groups

There were no significant differences in14-3-3η serum levels between the early and established RA groups (2.73±1.79 vs. 2.70±1.69ng/ml). Early RA had significantly higher levels of CRP, ESR, and DAS28 (P=0.001, P= 0.001& P=0.027 respectively) compared to established RA. There were no significant differences in RF and ACPA levels between the early and established RA groups. ROC curve analysis comparing early RA with healthy controls demonstrated a significant (P<0.0001) AUC of 0.999 (95% CI, 0.997–1.00). At a cutoff of ≥0.39 ng/ml, the ROC curve yielded a sensitivity of 87.7%, a specificity of 97.6%, a PPV of 0.98, and an NPV of 0.85. ROC curve analysis comparing established RA with healthy controls demonstrated a significant (P<0.0001) AUC of 0.990 (95% CI, 0.976–1.00). At a cutoff of ≥0.39 ng/ml, the ROC curve yielded a sensitivity of 91.4%, a specificity of 97.6%, a PPV of 0.96, and an NPV of 0.93.

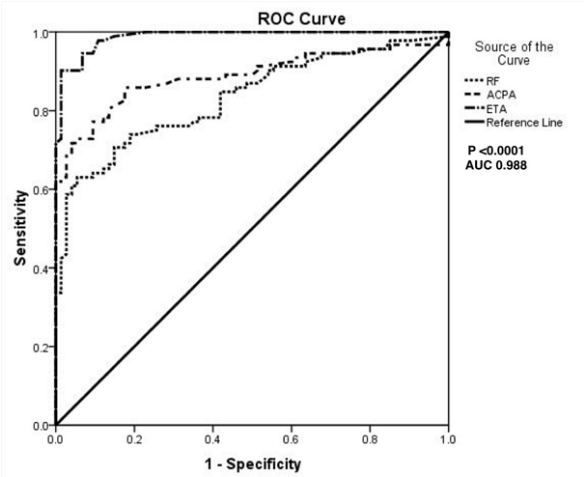


**Figure 1** Receiver operating characteristic curves (ROC) of 14-3-3η, RF and ACPA for RA diagnosis.

### Correlations of serum 14-3-3η with clinical & serological measures in RA patients

The correlation offered in Tables 3A & 3B showed the association between the levels of 14-3-3η and clinical and serological variables in both the early and established RA cohorts, Serum 14-3-3η correlated positively with the titers of ACPA and RF (P=0.001 & P=0.034 respectively). Also, there were significant positive correlations between serum 14-3-3η levels and CRP, ESR and DAS28 (P=0.032, P=0.004 and P<0.0001, respectively).As expected, a significant correlation between ACPA and RF was observed in both the early and established RA. Of the 57 patients assessed with early RA, 37(64.9%), 39(68.4%), and 47(82.5%) were positive for RF, ACPA, and 14-3-3η, respectively. As expected in patients with advanced RA, the proportion of patients with positivity in all three markers was greater in the cohort with early RA (RF 25 (71.4%), ACPA 30(85.7%) and 14-3-3η 30(85.7%). When assessing the patients with early & established RA, The hopeful benefit of adding 14-3-3η to each of the markers was assessed; adding 14-3-3η to RF and/or ACPA increased diagnostic detection for RA ( for more details see Table 4).

**Table 3A** A Correlation coefficients (r) of 14-3-3η with clinical and serological measures in patients with early RA (Spearman's rank correlation)

| Variables | DAS28 | RF | ACPA | 14-3-3 η | CRP | ESR |
|---|---|---|---|---|---|---|
| **DAS28** | ------ | | | | | |
| **RF** | <0.0001 | -------- | | | | |
| **ACPA** | <0.0001 | <0.0001 | ------- | | | |
| **14-3-3 η** | <0.0001 | 0.01 | <0.0001 | ------- | | |
| **CRP** | <0.0001 | <0.0001 | <0.0001 | 0.038 | -------- | |
| **ESR** | <0.0001 | 0.002 | 0.001 | 0.02 | <0.0001 | -------- |

RA, rheumatoid arthritis; DAS28, Disease Activity Score, 28-joint count; ESR, erythrocyte sedimentation rate; CRP, C-reactive protein; RF, rheumatoid factor; ACPA, anticitrullinated protein antibodies

**Table 3B** Correlation coefficients (r) of 14-3-3η with clinical and serological measures in patients with established RA (Spearman's rank correlation)

| Variables | DAS28 | RF | ACPA | 14-3-3 η | CRP | ESR |
|---|---|---|---|---|---|---|
| **DAS28** | | | | | | |
| **RF** | 0.026 | ------- | | | | |
| **ACCP** | 0.027 | 0.012 | ------ | | | |
| **14-3-3 η** | NS | NS | 0.002 | ------ | | |
| **CRP** | <0.0001 | NS | 0.043 | NS | ------ | |
| **ESR** | 0.03 | 0.034 | NS | NS | 0.05 | ----- |

RA, rheumatoid arthritis; DAS28, Disease Activity Score, 28-joint count; ESR, erythrocyte sedimentation rate; CRP, C-reactive protein; RF, rheumatoid factor; ACPA, anticitrullinated protein antibodies

**Table 4** Complementary between diagnostic markers (RF, ACPA, 14-3-3η) in patients with early and established RA

| Diagnostic Markers | Early RA, n=57 | Incremental Benefit | Established RA, n=35 | Incremental Benefit |
|---|---|---|---|---|
| **RF** | 37(64.9%) | ----- | 25(71.4%) | ----- |
| **ACPA** | 39(68.4%) | ----- | 30(85.7%) | ----- |
| **14-3-3 η** | 47(82.5%) | ------ | 30(85.7%) | ----- |
| **RF and /or ACPA** | 39(68.4) | 2 | 30(85.7%) | 5 |
| **RF and/or 14-3-3 η** | 47(82.5%) | 10 | 30(85.7%) | 5 |
| **ACPA and/or 14-3-3 η** | 49(85.9%) | 11 | 32(91.4%) | 2 |
| **RF and/or ACPA and/or 14-3-3η** | 50 (87.7%) | 11 | 32(91.4%) | 2 |

RF, rheumatoid factor; ACPA, anticitrullinated protein antibodies; RA, rheumatoid arthritis.

## Discussion

Both ACPA and RF markers are included in the ACR/EULAR classification criteria for RA (4). Despite their sensitivity, patients who developed erosive RA may remain negative for both markers.[16] Thus; other markers for RA have been sought. 14-3-3η is expressed at significantly higher levels in the synovial fluid of patients with arthritis naming the joint as the likely source of serum 14-3-3η[8] it was reported a positive association between 14-3-3η and MMPs suggested that 14-3-3η may have a role in the pathogenesis of RA.[17]

In agreement with these data, our study showed that serum 14-3-3η levels were significantly higher in RA patients than all control which in line with a recent study showed that 14-3-3η is significantly altered in patients with early RA and other autoimmune disorders, and healthy controls (14) & also with another study which reported that 14-3-3η is an RA-specific marker.[17,18] We found that by the addition of serum 14-3-3η to both RF and ACPA markers; the detection sensitivity in subjects with early RA was increased by 19.3% & 12.3% respectively. Seven (50%) of 14 patients who were seronegative for RF and ACPA were 14-3-3η positive and also we reported that using serum 14-3-3η assessment in detection of early RA had the upper hand over either RF or ACPA by 17.6% and 17.5 %, respectively (Table 4) which was matched with a studies demonstrated that serum 14-3-3η assessment enhanced the detection of RA over either RF or ACPA by 32% and 22%, respectively, in patients with early RA[14] and with a previous study stated that in an early RA cohort, 60% of patients were positive for 14-3-3η, 32% for RF, 44% for ACPA and 72% for at least one of those three markers.[17] We stated that the testing of 14-3-3η together with RF and ACPA may assist in detecting those patients who require an early referral to a rheumatologist. Maksymowych and colleagues found that adding 14-3-3η (cutoff ≥0.19 ng/ml) to RF and CCP antibody testing increased diagnostic sensitivity for early RA patients.[14] ROC curve analysis showed that 14-3-3η in early RA compared to healthy controls had a significant (P<0.0001) AUC of 0.999 (95% CI, 0.997–1.00). At a cutoff of ≥0.39 ng/ml, the ROC curve yielded a sensitivity of 87.7%, a specificity of 97.6%, a PPV of 0.98, and an NPV of 0.85 meaning that 14-3-3η is more specific in early RA with high NPV. On the hand, ACPA has a PPV 95% for the development of RA in patients with undifferentiated arthritis, though its NPV is only about 60–70%.[19,20] We also reported that serum 14-3-3η levels had a significant positive correlation with DAS28 in early RA and not in established RA since it was previously reported

that 14-3-3η is modifiable by both anti-TNF and standard DMARD therapies.[21,22] Also, we found a significant positive correlation between serum 14-3-3η levels and other serological markers (RF, ACPA, CRP, and ESR) (Tables 3A & 3B). These data matching with other studies which stated that serum 14-3-3η correlate with CRP and mildly correlated with ACPA and RF in the early RA cohort.[11] On the other hand, previously shown that serum 14-3-3η expression is not strongly correlated with standard clinical and serological measures in both early and established RA.[23] In vitro data stated that 14-3-3η stimulated a lot of proinflammatory signaling flows related to RA.[11] Serum 14-3-3η can induce MMP-9 in vitro, and associates with MMP expression in serum and synovial fluid in RA, signifying that it may play a role in the joint damage cascade.[8,11] Many RA-related transcripts were stimulated by 14-3-3η and involved pro-inflammatory cytokines.[11] Small sample size and the lake of radiographic progression scoring system are the main study limitation. Patients with undistinguishable seronegative arthropathy are in need of testing serum 14-3-3η for early detection of RA which will be of great advantage. Finally, we recommended ongoing evaluation of the diagnostic utility of 14-3-3η biomarker as an early marker for RA diagnosis in a large sample size which can provide earlier therapy with DMRD to prevent joint affection and we can conclude that 14-3-3η is an RA specific early diagnostic marker and correlate with RA disease activity.

## Conflict of interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

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