Usefulness of heat shock protein 70 for diagnosis and disease activity monitoring in patients with rheumatoid arthritis

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Introduction
Rheumatoid arthritis (RA) is a systemic autoimmune disease presented by inflammatory polyarthritis, affecting predominantly smaller joints such as hands and feet but can also affect large joints. Heat shock protein 70 (HSP70) is released from damaged cells after stress and has been found in the bloodstream of patients with RA.

Aim
To study the applicability of HSP70 serum levels as a diagnostic factor and a severity indicator in patients with RA

Patients and methods
The study included 59 patients with RA and 14 controls. Patients were classified according to disease activity score 28 into four groups: 10 patients in remission, 12 patients with low disease activity, 18 patients with moderate disease activity, and 19 patients with high disease activity. HSP70 and anti-cyclic citrullinated peptide assessments were done by ELISA technique.

Results
Patients with RA had a higher HSP70 level than the control group ($P < 0.001$), irrespective of presence or absence of rheumatoid factor or anti-citrullinated cyclic peptide. Next, diagnostic accuracy of the HSP70 in diagnosis of RA was evaluated (area under curve = 0.91). HSP70 had 89.8% sensitivity and 92.8% specificity in diagnosis of RA. In addition to diagnostic value, HSP70 can distinguish between high disease activity (76.67 ± 16.06 ng/ml) and mild (25.57 ± 7.39 ng/ml), moderate (35.17 ± 8.34 ng/ml), or remission phase (16.90 ± 4.06 ng/ml).

Conclusion
The study demonstrates an increase in the serum level of HSP70 in patients with RA in comparison with controls.

Keywords:
heat shock protein 70, rheumatoid arthritis, anticyclic citrullinated peptide (antiCCP)

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Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune inflammatory joint disease that affects 0.5–1.0% of the adult population. It is characterized by systemic inflammation, resulting in damaged cartilage, bone, and soft tissue, ultimately leading to restricted movement and disability [1]. As RA is an autoimmune disease, several autoantibodies can be detected in serum of patients with RA, of which rheumatoid factor (RF) and anti-citrullinated protein antibodies are the most prominent. More recently, antibodies against additional posttranslationally modified proteins were discovered, such as anti-carbamylated protein antibodies and anti-acetylated protein antibodies [2].

Heat shock proteins (HSP) are cell stress-inducible molecules that are highly conserved among prokaryotes and eukaryotes [3]. They are classified by gene families according to their molecular mass as HSP100, HSP90, HSP70, HSP60, HSP40, and small HSPs [4].

HSP70 is a high-molecular-weight HSP (70 kDa). It functions as molecular chaperones (proteins that facilitate folding of proteins and provide quality control); they execute essential and protective cellular functions under normal physiologic conditions and in conditions of environmental stress [5]. HSP70 is released from damaged cells after stress and has been found in the bloodstream of patients with autoimmune diseases [6] including RA [7]. HSP70 could alter immune cells' activity through changes in secretion of inflammatory cytokines such as tumor necrosis alpha and interleukin-10, hence protecting joints from progressive destruction [8]. Self-Ags present in damaged cells may be chaperoned by HSP into immature dendritic cells (DCs) for representation by major histocompatibility complex molecules on mature DCs. In RA, physical interactions between HSP70 and major histocompatibility complex class II-shared epitopes suggest that HSP70 participates in the

autoimmune response. Although abnormally elevated HSP70 mRNAs have been described in RA synovial tissue, the distribution of HSP70 in the RA synovial fluid space (which contains the bulk of immature DCs present in the joint and which may be a source of immunogen) is unknown. Also unknown are the potential interactions of HSP70 with immature DCs in RA [9].

Aim

The aims are to evaluate whether serum levels of HSP70 could be used in diagnosis of RA, to detect if there is correlation between HSP70 level and severity of RA, and to correlate the other well-established RA markers [RF and anti-cyclic citrullinated peptide (anti-CCP)] and HSP70 level to see if HSP70 acts as a dependent or independent serum marker in prediction of RA outcome.

Patients and methods

This case–control study included 59 patients attending the Rheumatology Clinic, Assiut University Hospital. Patients were diagnosed as having RA according to the 1987 American College of Rheumatology (ACR; inclusion before 2010) [10] or ACR/EULAR 2010 criteria (inclusion after 2010) [11]. Moreover, 14 apparently healthy subjects were taken as control. Patients were classified into four groups, according to disease activity score 28 (DAS28) [12]: group I: 10 patients with RA in remission, group II: 12 patients with RA with low disease activity, group III: 18 patients with RA with moderate disease activity, and group IV: 19 patients with RA with high disease activity. DAS28 is a severity score comprising various variables scaling from 0.49 to 9.07. DAS28 more than 5.1 shows high disease activity, DAS28 more than 3.2 and less than or equal to 5.1 shows moderate disease activity, DAS28 less than or equal to 3.2 and more than 2.6 indicates low disease activity, and DAS28 below 2.6 is considered remission. The study was approved by the Ethical Committee of Faculty of Medicine, Assiut University. Written consents were taken from the patients before enrollment in this study.

Patients with the following conditions were excluded: acute or chronic renal failure, glomerulonephritis, congestive heart failure, acute infections, pregnancy, and hospital admission in last 3 months. Women who are on hormone replacement therapy or patients who have asthma, diabetes, and other autoimmune diseases were also excluded.

Sample collection, storage, and handling

Overall, 8 ml of venous blood was collected under complete aseptic conditions and divided into the following: 2 ml into EDTA-containing tube for complete blood count, about 1.6 ml was added to a tube containing 0.4-ml sodium citrate for erythrocyte sedimentation rate (ESR) measurement, and 4 ml was collected into plain tube. Blood was allowed to clot for 2 h at room temperature, and serum was separated by centrifugation at 3000 rpm for 15 min.

All patients and controls were subjected to the following:

(1) Complete history taking, including general and demographic data such as age, sex, weight, height, family history, and disease duration, was done. BMI (kg/m^2) was calculated.
(2) General and local examinations, including total number of swollen joints, joints with tenderness and extraarticular involvement, were recorded for patients.
(3) Complete blood count was done using cell counters (Ruby CELL DYN, Abbott, Germany).
(4) ESR was performed by Westergren method.
(5) Serum glucose, urea, creatinine, uric acid, and liver function test were done using Dimension RXL max.
(6) RF and C-reactive protein (CRP) were done by Rapid latex slide agglutination test.
(7) Antinuclear antibody (ANA) was done on Alegria [(ORGENTEC Diagnostic GmbH) Mainz, Germany (Elabscience) Hubei, China] using ANA detect kit (ORG200-ORGENTEC), Lot.no. 1712712.
(8) Anti-CCP were done on Alegria (ORGENTEC Diagnostic GmbH), using anti-CCP detect kit (ORG301-ORGENTEC) Lot.no. 1621171.
(9) HSP70 was measured by sandwich ELISA technique using human HSP70 kit (Elabscience, China), Lot.no. AK0017AUG30015.

Statistical analysis

Data were collected and analyzed those using SPSS (Statistical Package for the Social Science, version 20; IBM, Armonk, New York, USA). Continuous data were expressed in the form of mean ± SD or median and range, whereas nominal data were expressed in the form of frequency (percentage). χ^2 test was used to compare the nominal data of different groups in the study, whereas Student *t* test was used to compare mean of different two groups and analysis of variance test for more than two groups in case of normally distributed data. Spearman correlation was used to determine the correlation between different continuous variables in the current study. Diagnostic

accuracy of the HSP70 for diagnosing RA was determined by receiver operating characteristic curve. *P* value was significant if less than 0.05.

Results

Table 1 shows the demographic data of both studied groups. The age of the study group ranged from 18 to 57 years (mean ± SD, 41.36 ± 10.92 years) and age of control group ranged from 25 to 56 years (mean ± SD, 37.57 ± 10.39 years). Most (94.9% of study group and 85.7% of control group) of the enrolled subjects were females.Regarding the demographic data, there were no significant differences between both groups.

Disease characteristics of the study group are shown in Table 2. Duration of RA was between 7 and 300 months, with mean ± SD of 84.48 ± 56.88 months. Family history presented in 20 (33.9%) patients. It was noticed that 19 (32.2%) and 26 (44.1%) patients had extraarticular manifestations and morning stiffness, respectively.

Ten (16.9%) patients were in remission, whereas mild, moderate, and severe activity presented in 12 (20.3%), 18 (30.5%), and 19 (32.2%) patients, respectively.

All control subjects were negative for CRP and RF, whereas 44 (74.5%) and 33 (55.9%) patients with RA were positive for CRP and RF, respectively ($P < 0.001$). It was noticed that ESR, anti-CCP, and HSP70 were significantly higher in patients with RA in comparison with the control group ($P < 0.001$). A total of nine (15.2%) patients with RA were positive for ANA, and all control subjects were negative for ANA ($P = 0.19$) (Table 3).

Table 4 shows some studied parameters in relation to RA disease activity. It was noticed that ESR is significantly higher in patients with severe activity in comparison with patients with other severity grades ($P = 0.00$). Moreover, ESR was significantly higher in those with remission in comparison with the control group ($P = 0.04$). Positive CRP and RF were more frequently in patients with severe activity in comparison with other groups. There is no significant difference in the number of ANA-positive patients in different severity grades of RA. Anti-CCP had no significant differences between different groups with exception of those with moderate activity, who had significantly higher anti-CCP in comparison with control group ($P = 0.02$), and those with severe activity had significantly higher anti-CCP in comparison with the control group ($P = 0.01$).

It was noticed that HSP70 significantly increases as the disease activity increase, where it was significantly higher in patients with severe activity ($P = 0.00$) in comparison with other groups. Moreover, even patients on remission had still significantly higher level in comparison with the control group ($P = 0.00$).

It was noticed that 33 (56%) patients with RA had positive RF, whereas 39 (66.1%) patients had positive anti-CCP. There were no significant differences in the level of HSP70 between RF-positive and RF-negative patients and also between anti-CCP-positive and anti-CCP-negative patients (Table 5).

At a cutoff point more than 15.48 ng/ml, HSP70 had 89.8% sensitivity, 92.8% specificity and 90.4% diagnostic accuracy for diagnosing RA with area under curve of 0.91, whereas anti-CCP at a cutoff point more than 15 U/ml had 70% sensitivity, 79% specificity, and 71.2% diagnostic accuracy for diagnosing RA, with area under curve was 0.81 (Table 6 and Fig. 1).

Table 1 Demographic data of studied groups

Variables	Study group (*n*=59)	Control group (*n*=14)	*P*
Age (years)			
Mean±SD	41.36±10.92	37.57±10.39	0.08
Range	18-57	25-56	
Sex [*n* (%)]			
Male	3 (5.1)	2 (14.3)	0.05
Female	56 (94.9)	12 (85.7)	
BMI (kg/m^2)			
Mean±SD	24.98±6.09	20.84±4.89	0.43
Range	16.30-29.8	18-25.3	

Table 2 Disease characteristics in the study group

Variables	Study group (*n*=59)
Duration (months)	
Mean±SD	84.48±56.88
Range	7-300
Family history [*n* (%)]	20 (33.9)
Extraarticular manifestation [*n* (%)]	19 (32.2)
Morning stiffness [*n* (%)]	26 (44.1)
Disease activity [*n* (%)]	
Mild	12 (20.3)
Moderate	18 (30.5)
Severe	19 (32.2)
Remission	10 (16.9)

Table 3 Serum levels of some studied parameters

Variables	Study group (*n*=59)	Control group (*n*=14)	*P*
Positive RF	33 (55.9)	0	<0.001
ESR (mm/h)	40.84±10.34	6.21±2.51	<0.001
Positive CRP	44 (74.5)	0	<0.001
Positive ANA	9 (15.2)	0	0.19
Anti-CCP (U/ml)	297.08±59.08	11.89±3.59	<0.001
HSP70 (ng/ml)	43.49±12.32	7.92±2.98	<0.001

Data was expressed in form of mean±SD and n (%). ANA, antinuclear antibody; anti-CCP, anti-cyclic citrullinated peptide; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; HSP70, heat shock protein 70; RF, rheumatoid factor.

Table 4 Some studied parameters in relation to rheumatoid arthritis disease activity

Variables	Mild (*n*=12)		Moderate (*n*=18)		Severe (*n*=19)		Remission (*n*=10)		Control (*n*=14)	
Positive RF	5 (41.7)		12 (66.7)		14 (73.6)		2 (20)		0	
ESR (mm/h)	23.83±3.32		37.22±6.39		68.26±13.79		15.70±2.83		6.21±2.51	
Positive CRP	6 (50)		16 (88.9)		19 (100)		3 (30)		0	
Positive ANA	0		1 (8.3)		4 (22.2)		4 (21.1)		0	
Anti-CCP (U/ml)	247.26±55.41		381.65±45.11		397.54± 57.09		110.3±10.1		11.89±3.9	
HSP70 (ng/ml)	25.57±7.39		35.17±8.34		76.67±16.06		16.90±4.06		7.92±2.98	
Significance	*P*1	*P*2	*P*3	*P*4	*P*5	*P*6	*P*7	*P*8	*P*9	*P*10
Positive RF	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
ESR	0.00	0.00	0.00	0.04	0.00	0.00	0.00	0.00	0.00	0.04
Positive CRP	0.01	0.00	0.01	0.00	0.01	0.01	0.00	0.00	0.00	0.03
Positive ANA	0.48	0.35	0.21	0.61	0.06	0.19	0.11	0.17	0.22	0.27
Anti-CCP	0.98	0.43	0.88	0.41	0.99	0.28	0.02	0.40	0.01	0.95
HSP70	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Data was expressed in form of mean±SD and n (%). ANA, antinuclear antibody; Anti-CCP, anti-cyclic citrullinated peptide; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; HSP70, heat shock protein 70; RF, rheumatoid factor. *P*1: compared between mild and moderate disease. *P*2: compared between mild and severe disease. *P*3: compared between mild disease and remission. *P*4: compared between mild disease and control. *P*5: compared between moderate and severe disease. *P*6: compared between moderate disease and remission. *P*7: compared between moderate disease and control. *P*8: compared between severe disease and remission. *P*9: compared between severe disease and control. *P*10: compared between remission and control.

Table 5 Level of heat shock protein 70 based on rheumatoid factor and anti-cyclic citrullinated peptide

	Level of HSP70	*P*
RF		
Positive (*n*=33)	47.21±10.56	0.32
Negative (*n*=29)	38.78±9.45	
Anti-CCP		
Positive (*n*=39)	48.43±8.45	0.12
Negative (*n*=20)	33.25±12.56	

Data were expressed in the form of mean±SD. Anti-CCP, anti-cyclic citrullinated peptide; HSP70, heat shock protein 70; RF, rheumatoid factor.

Table 6 Diagnostic value of heat shock protein 70 and anti-cyclic citrullinated peptide in prediction of rheumatoid arthritis

Indices	HSP70 (%)	Anti-CCP (%)
Sensitivity	89.8	70
Specificity	92.8	79
Positive predictive value	98.1	93
Negative predictive value	68.4	38
Diagnostic accuracy	90.4	71.2
Cutoff point	>15.48	>15
Area the curve	0.91	0.81

Anti-CCP, anti-cyclic citrullinated peptide; HSP70, heat shock protein 70.

Discussion

The present study included 59 patients who were diagnosed as having RA: 56 (94.9%) of them were females and three (5.1%) were males. Similar to the results of our study, El Tanawy *et al.* [13] reported that females (95%) had higher percentage than males (5%). The predominance of RA in women may originate from hormonal factors, and hormonal factors influence premenopausal and postmenopausal women differently in RA development [14].

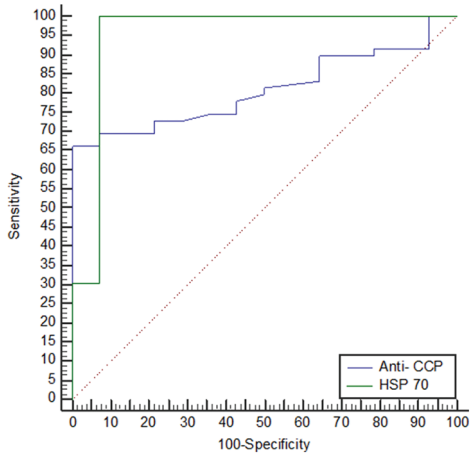
RA is a systemic inflammatory disease that can involve other tissues and organs as well as synovial joints [15]. In the current study, extraarticular manifestations were detected in 19 (32.2%) of 59 patients with RA. These results are consistent with the study done by Nass *et al.* [16] in which 34.8% of patients with RA had extraarticular manifestations.

In our study, ESR was significantly higher in patients with RA in comparison with control group ($P < 0.001$). In agreement with our results, Shen *et al.* [17] found that ESR is significantly higher in patients with RA in comparison with control group ($P < 0.01$). In this study, nine (15.2%) of 59 of patients with RA had positive ANA test result. This result was close to the result of the study by Codreanu *et al.* [18] who found that low percentage of patients with RA (26.9%) had positive ANA results.

In our study, 33 (55.9%) of 59 patients with RA had positive RF test result. This result was close to the result by Küçüksaraç *et al.* [19] and Porto *et al.* [20] who found that 59.3 and 55.4% of patients with RA, respectively, had positive RF test. However, Shen *et al.* [17] reported that 91.7% of patients with RA had positive RF test result. This discrepancy in the results was owing to the difference in the method used in their studies than ours. Moreover, 44 (74.5%) of 59 patients with RA had positive CRP test result in our study, and this is consistent with the study done by Küçüksaraç *et al.* [19], who found that 72.6% of patients with RA had positive CRP results.

In our study, anti-CCP was positive in 39 (66.1%) of 59 patients with RA. This result is consistent with Porto *et al.* [20] who found that 66% of patients with RA had positive anti-CCP results. In our study, anti-CCP was

Figure 1



ROC curve of diagnostic value of HSP 70 and Anti- CCP in Prediction of RA.

significantly higher in patients with RA in comparison with control group ($P < 0.001$). In agreement with our results, Shen *et al.* [17] found that anti-CCP is significantly higher in patients with RA in comparison with control group ($P < 0.01$).

HSPs are among major factors considered to participate in autoimmune disease. Substantial evidence points to a role of immunity to HSP70 in RA [21]. In this study, HSP70 was significantly higher in patients with RA in comparison with the control group ($P < 0.001$). In agreement with our results, Hayem *et al.* [22] showed increased concentration of HSP70 in patients with RA and Selman [23] found that HSP70 is significantly higher in patients with RA in comparison with the control group ($P \leq 0.001$). This is contrary to a study done by Sedlackova *et al.* [24] who showed insignificant difference in serum HSP70 levels in patients with RA compared with control subjects. However, the participants in their study were only RF positive, whereas in our study, both RF-positive and RF-negative patients were enrolled.

In our study, HSP70 significantly increased as the disease activity increased, where it was significantly higher in patients with severe activity in comparison with other groups of disease activity ($P = 0.00$), and there was a significant difference in its level between patients in remission and patients with mild or moderate disease activity ($P = 0.00$). Interestingly, even patients on remission had still significantly higher level in comparison with the control group ($P = 0.00$). These results are in agreement with Najafizadeh *et al.* [25] and Al-Sayed *et al.* [26].

Given these results supporting the relative specificity of HSP70 in distinguishing high disease activity from other severity grades and patients in remission from healthy subjects, we then compared anti-CCP results between patients with RA with different severity grades (to further address advantages and disadvantages of application of HSP70 in patients with RA). Unlike HSP70, anti-CCP, had no significant differences between patients with RA with different severity grades. In agreement with our results, Shakiba *et al.* [27] found that anti-CCP titer was not different in patients with different disease activities.

In our study, there were no significant differences in the level of HSP70 between RF-positive and RF-negative patients ($P = 0.32$), and also between anti-CCP-positive and anti-CCP-negative patients ($P = 0.12$). These results are consistent with Najafizadeh *et al.* [25] who found that increased serum levels of HSP70 in patients with RA were independent of serum RF and anti-CCP status.

In the current study, HSP70 had 89.8% sensitivity and 92.8.8% specificity in diagnosing RA. These results are close to the study done by Al-Sayed *et al.* [26], who found that the sensitivity and specificity of HSP70 were 76.09 and 87.84%, respectively. However, the sensitivity and specificity of anti-CCP in diagnosing RA in our study were 70 and 79%, respectively, and these results are close to the results of the study done by Sun *et al.* [28], who found that the sensitivity and specificity of anti-CCP were 75.14 and 86.73%, respectively. Therefore, HSP70 is more sensitive and more specific than anti-CCP in diagnosing RA.

Conclusion

We concluded that serum levels of HSP70 can be used as diagnostic test in RA. We found that HSP70 significantly increases as the disease activity increase, supporting the relative specificity of HSP70 in distinguishing between patients with RA with different disease activities. The increased serum levels of HSP70 regardless of the serum RF and anti-CCP status point to the importance of HSP70 as an independent serum marker in predicting the outcome of RA.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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