Angiopoietin-2 as A Biomarker For Echocardiographic Abnormalities and Carotid Atherosclerosis In Rheumatoid Arthritis Patients

[1]Hanan Omar, [2]Ahmed Tageldien Abdellah, [3]Housseini Abdalla, [4]Samah Ismail Nasef, [5]Hamdy Omar

Departments of [1]Clinical Pathology, [2]Cardiology, [3]Diagnostic Radiology, [4]Physical Medicine, Rheumatology & Rehabilitation and [5]Internal Medicine, Faculty of Medicine, Suez Canal University, Ismailia, Egypt

Atherosclerosis and cardiovascular diseases (CVD) are increasingly recognised complications of rheumatoid arthritis (RA). Angiopoietin 2 (Ang-2) levels have been associated with clinically overt CVD in general population; we assessed serum Ang-2 levels and its correlation with Echocardiographic abnormalities and carotid intima-media thickness in RA patients. 44 RA patients without clinically overt CVD and 44 healthy controls were assessed by questionnaire and clinical examination. Disease activity score (DAS-28) was calculated. Laboratory investigations included measurement of serum Ang-2, Rheumatoid factor (RF), anti-cyclic citrullinated peptide (Anti-CCP), and C reactive protein (CRP). Doppler Echocardiography and Carotid ultrasonography were done to all patients and controls. Mean age of RA patients was 44.4±9.6 and about (86.4%) 38 were females. Mean levels of Ang-2 was higher in RA patients (17.591±13.9 ng/ml) as compared to controls (7.909 ±4.10 ng/ml) *P*<0.001 and was significantly elevated in RA patients with left ventricular (LV) diastolic dysfunction (23.53±7.75 ng/ml) than those without dysfunction (14.81±15.33ng/ml), *P*<0.05 and was significantly elevated in RA patients with carotid intima-media thickness (cITM) >0.6mm (21.12±14.79 ng/ml), *P*<0.005. Serum Ang-2 correlated positively with disease duration, DAS-28, LV posterior wall thickness, E wave velocity and cIMT. In conclusion, serum Ang-2 level is associated with LV diastolic dysfunction and increased carotid intima-media thickness in RA patients and may be useful biomarker for subclinical CVD and atherosclerosis in RA patients.

Rheumatoid arthritis (RA) is a chronic inflammatory disease associated with a wide range of co-morbidities particularly of cardiovascular origin (Gabriel and Michaud 2009). Several cross-sectional and longitudinal studies have concluded that there is an increased risk of both atherosclerosis and consequent cardiovascular disease (CVD) in RA patients (Tyrrell *et al.,* 2010, Kramer & Giles 2011).

In RA, the risk of death due to CVD was considered to be doubled compared to normal population (Prior *et al.,* 1984). It was found that in RA the traditional risk factors for CVD were increased as elevated blood pressure, hypercholesterolemia and combined with inflammation which is associated with RA this may explain the increased risk of CVD in these patients (Goodson *et al.,* 2004, Panoulas *et al.,* 2008)

In addition to coronary artery diseases, several studies showed that the prevalence of congestive heart failure is increased in RA patients (Pincus & Callahan 1986). A high incidence of pericardial, myocardial and endocardial involvement was also reported in post-mortem studies (Mutru *et al.,* 1989). However, cardiac involvement in RA is not always symptomatic (Wislowska *et al.,* 1998). Doppler echocardiography is a sensitive and non-invasive method of identifying cardiac abnormalities and systolic or diastolic dysfunction (DeMaria *et al.,* 1991).

The inflammation which happens in RA may lead to acceleration of the process of atherosclerosis (Pasceri & Yeh 1999).

Measuring carotid intima media thickness (cIMT) is a non-invasive method of obtaining morphological evidence of atherosclerotic damage to the arterial walls. The values have been verified to be strongly associated with the risk of CVD (Simon *et al.,* 2002, Folsom *et al.,* 2008).

Moreover, carotid plaque prevalence is considered as another important marker of atherosclerosis and a strong predictor of ischaemic cardiovascular events (Xie *et al.,* 2011).

In RA, angiogenesis is a significant pathogenic inflammatory process and occurred in the initial stage of the disease in which the inflammatory cells were recruited and supplied with oxygen and nutrients with an increase in the division of vascular endothelial cells due to increased cytokines levels such as adhesion molecules and growth factor which were enhanced by fibroblasts (Cooles & Isaacs 2011). This process was initiated by stimulation of growth factors such as vascular endothelial growth factor and fibroblast growth factors binding on endothelial cells to their related receptors and generate proteolytic enzymes (Koch & Distler 2007).

Angiopoietins (Ang) are one of the growth factors that enhance angiogenesis. The Ang–Tie ligand receptor system is composed of two tyrosine kinases receptors which are, Tie-1 and Tie-2, and four ligands which called Ang-1, Ang-2, Ang-3, and Ang-4 (Thurston 2003).

Ang-2 is endogenous Tie-2 receptor antagonist as it inhibits Ang-1 and Tie2 binding that leads to inhibition of the process of adhesion between vascular endothelium and cellular wall which results in the initiation of the angiogenesis. Ang-2 enhances endothelial activation, vascular leakage with migration of the neutrophils and as a result it is known as a 'pro-inflammatory factor'. Ang-2 activates TNF-α which lead to monocyte adhesion and increased expression of Ang-2 in endothelial cells to maintain the presence of these vessels (Roviezzo *et al.,* 2005, Sturn *et al.,* 2005).

In RA, chronic inflammation and angiogenic cytokines stimulate endothelial cells to produce Ang-2 and store it in endothelial cell Weibel-Palade Bodies (WPBs). As Ang-2 is released it leads to fast destabilization of the endothelium and initiate endothelium activation and induce vascular permeability in response to inflammatory stimuli (Lemieux *et al.,* 2005, Fiedler & Augustin 2006).

Atherosclerosis and cardiovascular diseases are common significant complications in patients with RA. Angpt-2, as marker of endothelial cell activation, has been proposed as a mediator of angiogenesis, which might play an important role in the regulation of endothelial integrity and inflammation. Increase level of Ang-2 may indicate the beginning of atherosclerotic changes in RA patients and carotid atherosclerosis is the good and early indicator of atherosclerotic changes in the blood vessels in whole the body and also considered as the risk of cardiovascular disorder and subclinical echocardiographic.

The aim of our study is to assess whether Ang-2 level correlats with subclinical echocardiographic and Doppler cardiac abnormalities and examine its relationship with carotid atherosclerosis in RA patients.

Patients and Methods

A case-control, cross sectional study was done at Suez Canal University Hospital in Ismailia. A total of 44 patients with RA were included in the study. The patients were fulfilling the 2010 classification criteria for RA (Aletaha *et* al., 2010). They were recruited from Rheumatology and Rehabilitation and Internal Medicine departments. We also included 44 age and gender matched normal healthy controls. We excluded patients who had other connective tissue diseases, hypertension, suffered cardiovascular or cerebrovascular events, had evidence of cardiovascular

disease, peripheral vascular disease, diabetes mellitus, liver diseases, renal insufficiency, or used drugs affecting cardiovascular system. We only included non-smokers or people who had stopped smoking at least 5 years previously. The study was approved by the ethics committee of the faculty of medicine at Suez Canal University. Written consent was obtained from each patient after detailed discussion with him to explain the aim and the steps of study. Interview Questionnaire was taken for personal history, joints symptoms, cardiovascular diseases and chronic illness. General examination included body mass index (BMI) and blood pressure measurement to all patients and controls. Peripheral joints were examined particularly for number of swollen joints; number of tender joints and patient's assessment of pain was done using visual analogue scale (VAS). Moreover, disease activity scores (DAS 28) was calculated (Prevoo *et al.,* 1995). Cardiac examination was done to all the participants.

Laboratory investigations: Blood samples were drawn to measure complete blood count (CBC), C-reactive protein (CRP), Erythrocyte sedimentation rate (ESR), Anti Citrullinated Peptides (Anti CCP) by ELISA Kit and Rheumatoid Factor (RF) by Cobas 6000 analyser, *Roche* Diagnostics

Assessment of serum Angiopoietin-2 levels

It was assessed by enzyme-linked immunosorbent assay (ELISA) (CUSABIO BIOTECH CO., Ltd.) according to the manufacturer's instructions. This was an in vitro ELISA kits for the quantitative measurement of human Ang-2 in the serum of patients and controls. A standard curve was used for interpretation of results The kit has a detection range from 1.56 ng/ml to100 ng/ml and no significant cross-reactivity or interference was reported.

Echocardiographic study

We used the General Electric system Vivid-7 machine ultrasound with 2.5 MHZ transducer. Echocardiography was performed to 44 RA patients without clinically overt CVD and 44 control subjects in left lateral position, using parasternal short and long axes views, apical 4 and 5 chamber views. M-Mode and 2-D views were done to assess cardiac valves and to measure left ventricle (LV), left atrium (LA), aortic root, right ventricle (RV), and right atrium (RA) dimensions. LV systolic function was evaluated by assessing ejection fraction (EF) and fractional shortening (FS). LV diastolic function was assessed by peak early wave (E; m/sec), peak atrial filling wave velocity (A; m/sec), and ratio of E to A (E/A). Diastolic dysfunction was defined by the presence of one of the following patterns:

a) Impaired relaxation pattern if E/A ratio was <0.75, DT >240 ms, IVRT >100 ms.

b) Pseudo-normalized pattern if E/A ratio was between 0.75 and 1.5, DT between 140 to 240 ms, IVRT between 60 and 100 ms.

c) Restrictive pattern was considered to be present if E/A ratio was >1.5, DT <140 ms, IVRT <60 ms. (Lang *et al.,* 2015).

Carotid ultrasonography

Carotid Intima-Media Thickness (cIMT) was assessed in 44 RA patients without clinically overt CVD and 44 control subjects by high-resolution B-mode ultrasound using (Philips ClearVUE 360 (Philips, Andover, MA, USA) and bilateral assessment for IMT was done in three areas: common, bulb and internal carotid artery. cIMT more than 0.6 mm is considered as a marker of subclinical atherosclerosis (Veller *et al.,* 1993) and carotid plaques is considered as a marker of advanced atherosclerosis (Gonzalez-Juanatey *et al.,* 2009). Atherosclerotic plaques were defined as any focal thickening of the intima-media complex protruding into the vessel lumen and/or as a focal increase of echogenicity with a homogeneously hyperechoic echotexture within an otherwise hypoechoic intima-media complex (Troitzsch *et al.,* 2012).

Statistical Analysis

We performed descriptive statistics including number, percentage (%), mean and standard deviation (SD). Analytical statistics included Student's t-test that was used to indicate the presence of any significant difference between two groups of quantitative variable, Fisher's exact test that was used to compare between two groups or more regarding one qualitative variable ANOVA test to compare means between the groups and Pearson's correlation analysis that was used to show strength and direction of association between two quantitative variables. P-value, was considered significant difference if $p < 0.05$. We used Receiver operating characteristic (ROC) curves to evaluate the cut-off value, sensitivity and specificity of Ang-2 to predict carotid atherosclerosis and diastolic dysfunction in RA patients. All statistics were calculated by using SPSS 16 software.

Results

Generally, the demographic variables were well comparable between RA patients and control subjects, hence no statistically significant differences observed between them. Clinical and laboratory characteristics of RA and control group are given in (table 1). All RA patients were on medical treatment

which included: steroids, DMARDS as mono/combination therapy. About (39 patients) 88.6% were on methotrexate, (26 patients) 59% were on lefluonamide and (8 patients) 18.1% were on oral steroids with a dose of 10mg or less and duration more than 6 months. No one was on biological therapy.

Table1. Demographic, clinical and laboratory data in RA patients and controls (N=44).

Variables	RA Patients	Controls	*P value
Gender			
Male (No.)	6 (13.6%)	7 (15.9%)	NS
Female (No.)	38 (86.4%)	37(84.09%)	
Age (year) Mean (±SD)	44.4±9.6	45.5± 8.7	NS
BMI(Kg/cm^2) Mean (±SD)	28.13±2.9	29.24 ± 9.3	NS
Systolic BP (mmHg) -mean (± SD)	133 ± 6	130 ± 9	NS
Diastolic BP (mmHg) -mean (± SD)	76 ± 4	73 ± 7	NS
Disease duration (years) Mean (±SD)	7.68±5.4	---	
Morning stiffness (minutes)	75.4 ± 28.1	---	
DAS28 Mean (±SD)	4.80±0.82	---	
CRP (mg/dl) Mean (±SD)	6.41±8.7	---	
RF positive (number)%	(31)70%	---	
Anti-CCP positive (number)%	(19) 43.1%	---	
Ang-2 (ng/ml)	17.59±13.9	7.909 ± 4.10	0.001
cIMT(mm^2) Mean (±SD)	0.71± 0.15	0.647±0.081	0.007

P value> 0.05 is not significant (NS)

SD= standard deviation, NS= not significant, DAS 28= Disease Activity Score, BMI= Body Mass Index, CRP=C - reactive protein cIMT= carotid intima-media thickness, BP= blood pressure, RF= rheumatoid factor, Anti-CCP= anti-cyclic citrullinated peptide Angio-2= Angiopoietin-2.

Serum Ang-2 levels were significantly higher among RA patients (17.591±13.9 ng/ml) than control group (7.909 ± 4.10 ng/ml) (P<0.001).

The main echocardiographic and Doppler findings in RA patients without clinical evidence of CVD and control subjects are summarised in (Table 2).

Correlation of Ang-2 level with clinical, laboratory, echocardiographic and carotid Doppler data in RA patients is presented in (Table 3).

Table 2. Echocardiographic and Doppler variables in RA patients and controls

Variable	RA Patients (n= 44)	Controls (n= 44)	*P value
	Mean ± SD	Mean ± SD	
Aortic diameter (mm)	33 ± 6.9	31 ± 2.1	NS
LA diameter (mm)	35.7± 3.5	32 ± 2.6	< 0.04
LVESD (mm)	33 ± 2.7	31 ± 2.5	NS
LVEDD (mm)	52 ± 1.9	51 ± 2.5	NS
LVEF (%)	54 ± 4.3	56 ± 3.6	NS
LV PW (mm)	9.8 ± 1.5	8.3 ± 1.27	< 0.05
RVD (mm)	31 ± 2	26 ± 2.3	NS
IVS (mm)	9.6 ± 1.7	8.9 ± 1.2	NS
E wave velocity (cm/s)	67 ± 13	81 ± 14	< 0.03
A wave velocity (cm/s)	77 ± 21	71 ± 18	NS
E velocity/A velocity ratio	0.92 ± 0.3	1.4 ± 0.3	< 0.04
Isovolumetric relaxation time(ms)	82 ± 24	61 ± 25	< 0.05
Deceleration time (ms)	177 ± 45	163 ± 33	< 0.05
Isovolumetric contraction time (ms)	36 ± 14	41 ± 16	< 0.03
Ejection time (ms)	280 ± 24	290 ± 32	NS
Myocardial performance	0.44 ± 0.13	0.35 ± 0.12	< 0.05

P value > 0.05 is not significant (NS).

LA= left atrium, LVESD= left ventricular end systolic diameter, LVEDD= left ventricular end diastolic diameter, LVEF= left ventricular ejection fraction, LV PW= left ventricular posterior wall, RVD= right ventricular diameter, IVS= interventricular septum mm=millimetre, cm/s= centimetre per second, ms= milliseconds.

Table 3. Correlation of serum Angiopoietin-2 level with clinical, laboratory, echocardiographic and carotid Doppler data in RA patients.

	r	B	*P* value
Age	0.213	0.147	NS
Disease duration	0.469	0.183	0.001
DAS 28	0.422	0.025	0.004
BMI	0.205	0.043	NS
LV PWT	0.379	0.008	0.01
E wave velocity	-0.498	- 0.007	0.01
cIMT	0.425	0.005	0.004
Carotid plaques	0.322	0.009	0.033

**P*-values >0.5 is not significant (NS).

DAS-28= Disease Activity Score, BMI= Body Mass Index, LV PW= left ventricular posterior wall thickness, cIMT= carotid intima-media thickness.

About 18 RA patients (40.9%) exhibited left ventricular diastolic dysfunction. Diagnosis of diastolic dysfunction was made by the criteria that were mentioned in the methods. Furthermore, we compared the clinical data and laboratory parameters of RA patients with and without diastolic dysfunction and summarized the results in (table 4).

Table 4. Clinical and laboratory data in RA patients with and without diastolic dysfunction.

Variables	RA with diastolic dysfunction (N=18)	RA without diastolic dysfunction (N=26)	*P value
Age (year) Mean (±SD)	45.14±9	44.1±9.9	NS
Disease duration (years) Mean (±SD)	9.43.±6.67	6.87±4.66	NS
BMI(Kg/cm^2) Mean (±SD)	27.53±2.92	28.41±2.96	NS
DAS28 Mean (±SD)	5.04±0.42	4.68±0.94	NS
cIMT(mm^2) Mean (±SD)	0.80± 0.13	0.67± 0.14	0.007
CRP (mg/dl) Mean (±SD)	8.20±9.5	2.57±5.1	0.045
Angio-2 (ng/ml)	23.53±7.75	14.81±15.33	0.05

*- *P*-values>0.05 is not significant (NS)

DAS 28= Disease Activity Score, BMI= Body Mass Index, CRP=C - reactive protein

cIMT= carotid intima-media thickness, Angio-2= Angiopoietin-2.

In RA patients group there were 32 patients (72.7 %) with cIMT more than 6 mm which denotes subclinical atherosclerosis. And 8 patients (18.1%) exhibited carotid plaques. We have made subgroup analysis between patients with cIMT of more than 6 mm and less than 6mm and the results are presented in (table 5). (Figure 1 and 2)

Table 5. Clinical data and laboratory parameters in RA patients in relation to carotid intima-media thickness

Variables	cIMT<0.6mm (n=12)	cIMT>0.6mm (N=32)	*P value
Age (year) Mean (±SD)	43.42±9.71	44.88±9.64	NS
Disease duration (years) Mean (±SD)	5.83.±2.69	8.38±6.05	NS
BMI(Kg/cm2) Mean (±SD)	27.59±2.66	28.33±3.06	NS
DAS- 28 Mean (±SD)	4.32±0.82	4.98±0.76	0.017
CRP (mg/dl) Mean (±SD)	3±7.45	7.69±8.91	NS
Ang-2 (ng/ml)	8.16±2.62	21.12±14.79	0.005

*- *P* values >0.05 is not significant (NS)

DAS 28= Disease Activity Score, BMI= Body Mass Index, CRP=C - reactive protein

cIMT= carotid intima-media thickness, Angio-2= Angiopoietin-2.

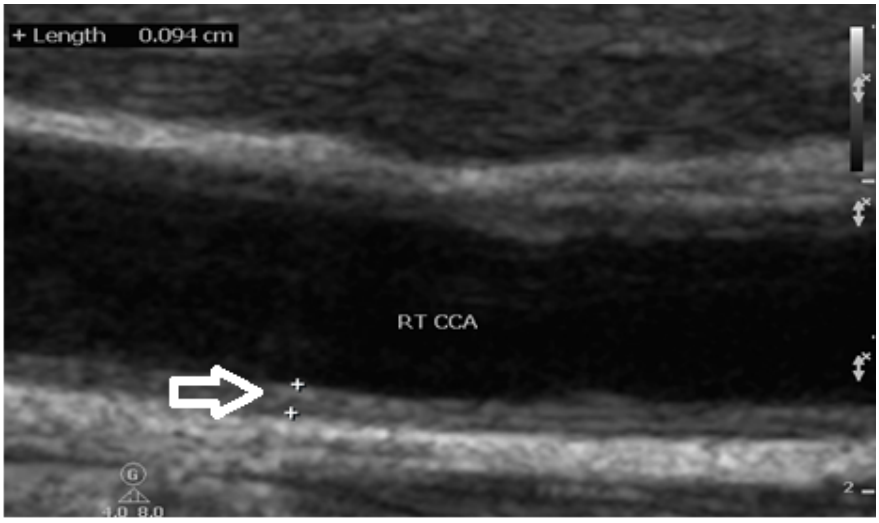

Figure 1. B-mode (gray scale) ultrasound image shows thickened intima media of Rt common carotid artery (RT CCA) = (0.094) in RA patient.

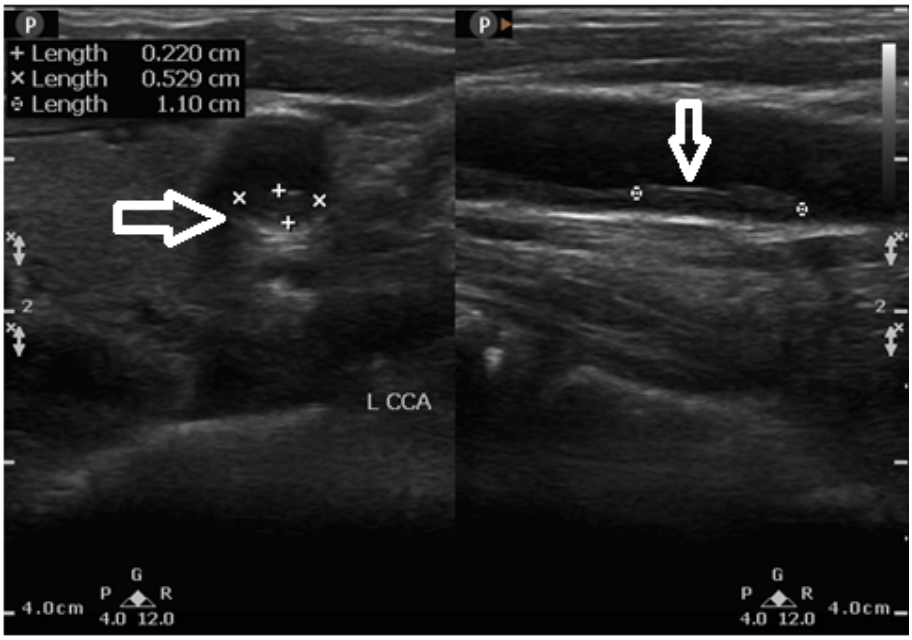

Figure 2. B-mode (gray scale) ultrasound image of longitudinal (Rt arrow) and transverse (LT arrow) scan of Lt common carotid artery (L CCA) shows atheromatous plaques in RA patient.

Receiver operating characteristic (ROC) curve analysis (Fig.3) revealed that a cut-off value of Ang-2 at 10.75 ng/ml (sensitivity:81% and specificity: 86%) can predict Subclinical atherosclerosis (cIMT>0.6mm), a cut-off value of Ang-2 at 16 ng/ml (sensitivity:65% and specificity: 70%) can predict Advanced atherosclerosis (plaques present) and a cut-off value of Ang-2 at 17.5 ng/ml (sensitivity:72% and specificity:84%) can predict diastolic dysfunction in RA patients.

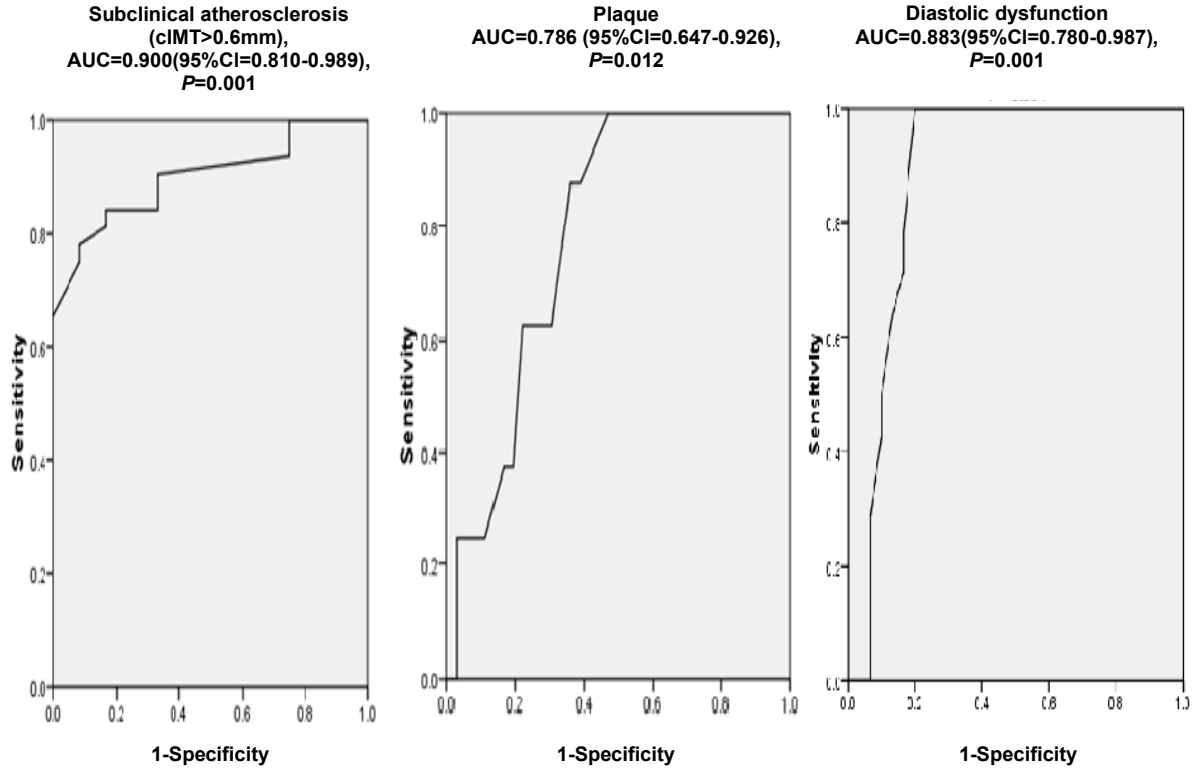

Figure 3. Receiver operating characteristic (ROC) curves showing the ability of serum angiopoietin-2 in predicting subclinical, advanced atherosclerosis and diastolic dysfunction in RA patients.

Discussion

Atherosclerosis and elevated risk of CVD were well documented in RA patients (Gonzalez-Gay *et al.,* 2005). Early detection of subclinical atherosclerosis and prediction of the development of CVD in RA patients is very essential (Abu-Shakra *et al.,* 2005). Though widely examined, the underlying causes of the elevated prevalence of CVD among RA patients remain to be fully explored (Kramer & Giles 2011).

Angiopoietins are vital endothelial growth factors binding to the Tie-2 receptor, which is exceedingly expressed in the endothelium (Eklund & Olsen 2006). Angiopoietin-2 regulates the endothelial integrity and inflammation as it is a good mediator of angiogenesis and significant biomarker of activation of endothelia cells (López-Mejías *et*

al., 2013). Growing experimental and clinical evidences suggest substantial roles for angiopoietins and their receptor Tie-2 in the occurrence of CVD (Ward & Dumont 2002). Moreover, serum Ang-2 predicts cardiovascular mortality in the general population; even after adjustment for major cardiovascular risk factors. The exact mechanisms underlying this elevated cardiovascular risk is not entirely understood (Lorbeer *et al.,* 2013).

Given these information, we aimed to assess the association of serum Ang-2 with the presence of subclinical atherosclerosis and echocardiographic abnormalities in a set of RA patients without clinically evident CVD. Up to our knowledge, this is the first study to explore this association in RA patients.

In our study we found that the Ang-2 is significantly elevated in RA patients versus the control this is in accordance with the results of Hares Ghazaly *et al.,* (Hares Ghazaly *et al.*, 2016). The same results were obtained from a study done on Egyptian RA patients in 2014 which revealed that serum angiopoietin-2 was significantly higher in patients compared to the control ($P < 0.001$) (Soliman *et al.,* 2014).

We found that Ang-2 is positively correlated eith RA disease variables including disease duration and disease activity (DAS-28). This was in accordance with the work of Westra et al in 2011 indicating a close relationship between Ang-2 and inflammation in RA patients. However, no correlation was found between Ang-2 and age, BMI or CRP level.

We have observed a wide spectrum of echocardiographic and Doppler abnormalities in RA patients without clinically evident CVD including a significantly increased left atrium (LA) diameter and left ventricular posterior wall (LV PW) thickness in comparison to control subjects ($P< 0.05$). Moreover, Left ventricle diastolic function Doppler indices including reversed E/A ratio and prolonged myocardial performance index (MPI) are significantly common in RA patients in comparison with their control subjects ($P<0.05$). About 40.9% of RA patients had left ventricular diastolic dysfunction. In accordance to our results Some Italian investigators described the presence of diastolic function abnormalities in both men and women with RA (Corrao *et al.,* 1996) As with our patients diastolic dysfunction was observed despite normal left ventricular systolic function. In 2004 Carlos Gonzalez-Juanatey and his co-workers found higher frequency of left ventricular diastolic dysfunction in patients with RA (about 66% of patients)(Gonzalez-Juanatey *et al.,* 2004). This can be explained by the age of patients as the mean age our patients was (44.4±9.6) while the mean age of the patients in the study of Carlos Gonzalez-Juanatey et al. was (59.2 ±12.5 years) and it is well known that the prevalence of diastolic dysfunction increases with the age.

Our work revealed that there was a significant positive correlation between Ang-2 and diastolic dysfunction in RA patients. Westra *et al.* (2011) reported that Ang-2 could be predictive for the development of CVD since Ang-2 levels were significantly higher in CVD patients than in non-CVD patients. They used different methodology in their work as they retrospectively investigated whether markers of endothelial cell activation as Ang-2 at the onset of disease were related to the development of CVD in RA patients (Westra *et al.,* 2011). Similarly, López-Mejías *et al.* (2013) investigated classic cardiovascular risk factors and CVD in 290 RA patients and found that Ang-2 levels were higher in RA patients with CVD than in RA patients without cardiovascular complications (López-Mejías *et al.,* 2013). However, we confirmed for the first time that Ang-2 is higher in RA patients with diastolic

dysfunction and found positive correlation between Ang-2 level and diastolic dysfunction. Moreover, we found that Serum Ang-2 levels more than 17.5 ng/ml can predict the development of diastolic dysfunction in RA patients with sensitivity 72% and specificity 84%.

In our study, we observed a positive association of Ang-2 with cIMT. Additionally, we observed an association of Ang-2 levels with the number of atherosclerotic plaques in the carotid artery. With respect to the results of Gonzalez-Juanatey *et al.* (2009) who found that increased cIMT predicts the development of cardiovascular events in RA patients, this can support the concept that higher Ang-2 levels is associated with an increased risk of CVD in RA patients.

We found that serum Ang-2 levels more than 10.75 ng/ml can predict subclinical atherosclerosis with sensitivity 81% and specificity 86% and serum and Ang-2 levels more than 16 ng/ml can predict advanced atherosclerosis (presence of plaques) with sensitivity 65% and specificity 70%.

In conclusion, serum Ang-2 is significantly elevated in RA patients with diastolic dysfunction and elevated carotid intima-media thickness. Serum Ang-2 can be a biomarker of atherosclerosis and predicts diastolic dysfunction to help us to detect those at risk of cardiovascular disorders in patients with RA. These observations lend further support to the concept that Ang-2 is an important biomarker of subclinical CVD and atherosclerosis in RA patients.

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