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Value of tumor necrosis factor-alpha and high-sensitive cardiac troponin-I as early predictors of subclinical atherosclerosis and their relation to disease activity in patients with rheumatoid arthritis: A case-control study

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Abstract

Patients with rheumatoid arthritis (RA) have a higher risk of cardiovascular disease (CVD) compared to the general population, which leads to increased morbidity and mortality. Inflammation is the key in RA and CVD. Our study aimed to refine cardiovascular (CV) risk assessment in RA patients by using carotid intima-media thickness (cIMT) as a marker of subclinical atherosclerosis. We also explored whether proinflammatory cytokines represented by tumor necrosis factor-alpha (TNF-α) and high-sensitivity cardiac troponin I (hs-cTnI), a biomarker of myocardial injury, could be correlated in RA patients. The study included 80 RA patients and 80 control subjects. TNF-α and hs-cTnI levels were measured. Subclinical atherosclerosis was evaluated by cIMT by means of carotid ultrasound. Disease activity score 28 (DAS28) was used to evaluate disease activity. hs-cTnI and TNF-α serum levels were higher in RA patients compared to controls (p=0.001). There was a significant difference in the median of cIMT between cases and controls (median (IQR) 0.9 (0.2) for cases, 0.7 (0.1) for controls, (p=0.001). A significant correlation was found between the level of TNF-α and hs-cTnI (p=0.002). Also, there was a significant correlation between the cIMT level and TNF-α and hs-cTnI (p=0.003 and p=0.002, respectively). Significant correlation was found between cIMT, TNF-α, and hs-cTnI in relation to the DAS28 score (p<0.001, p<0.001, and p=0.001, respectively). In conclusion, patients with RA are more likely to develop subclinical atherosclerosis, as reflected in increased cIMT. Higher levels of hs-cTnI in RA patients may correlate with the presence of occult cardiovascular disease. TNF-α and hs-cTnI correlations can reveal the interplay between disease activity and CVD. Thus, inflammation must be the primary target of various therapeutic approaches.

Keywords: RA, Subclinical atherosclerosis, TNF-α, hs-cTnI, and cIMT.

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Introduction

Risk of mortality is higher in patients with rheumatoid arthritis (RA), which is mainly attributed to cardiovascular (CV) events.[1] CV events are 1.5–2 times more likely to occur in RA patients than in the general population.[2,3,4] Conventional risk factors for cardiovascular diseases (CVD) do not entirely justify this increased CV mortality in RA. Inflammation associated with RA is the main player that leads to atherosclerosis via affecting endothelial function and plaque stabilization. Furthermore, disease activity in RA correlates with mortality and CVD.[5] Therefore, the relationship between markers of inflammation and cardiac biomarkers is of substantial interest.

There is a rising need to explore new and solid biomarkers to monitor the inflammatory activity in RA, especially in early disease, and cytokines can be applicable competitors in this context.[6-9] Tumor necrosis factor-alpha (TNF-α) is among these, which fuels inflammation in the synovium, increases angiogenesis and promotes cartilage and bone resorption. It is also a strong promoter of other proinflammatory cytokines and chemokines.[10] As a result, we can use TNF-α level as a solid possible biomarker for evaluating disease activity even in the early stages, as it reflects clinical activity and structural damage in RA.[11] The relationship between TNF-α and occult plaque load and structure in RA, however, it remains unknown.

Elevations of specific biomarkers for myocardial injury may precisely reflect subclinical myocardial damage in RA. When there is myocardial injury, the levels of cardiac troponins rise, which are part of the cardiomyocyte contractile apparatus. Using high-sensitivity cardiac troponin assays, it is possible to measure troponin levels below the detection threshold. In RA patients, high-sensitivity cardiac troponin I (hs-cTnI) levels are elevated, and this has been connected to the development of long-term CV events even after controlling traditional cardiac risk factors.[12.]

A surrogate marker for subclinical atherosclerosis is the thickness of the carotid intima-media (cIMT). It is a low-cost, non-invasive, free of radiation, and simple imaging technique. Many studies found elevated cIMT and carotid plaque in RA patients.[13,14] The assessment of RA patients' CVD risk may therefore be enhanced by including cIMT in risk prediction models.

Our study aimed to identify patients with RA at a preclinical stage of cardiovascular involvement. TNF-α, as a pro-inflammatory cytokine was studied to see if it correlated with hs-cTnI, as a marker of myocardial injury, in RA patients with subclinical atherosclerosis as measured by cIMT. This can lead to better CVD risk stratification and optimization of long-term cardiovascular risk prediction, which may allow for the development of preventive strategies.

Subjects and Methods

Study population and ethical statement

From April 2019 through July 2021, a cross-sectional case control study was carried out in Assiut University Hospital. Using the Epi-Info tool, the sample size was estimated under the premise that the study's power was set at 80%, the confidence interval was 95%, the odds ratio was 8%, and the dropout rate was 10%.

In addition to 80 patients with rheumatoid arthritis who met the 2010 ACR/EULAR criteria for classification[15] and attended the Rheumatology Outpatient Clinic of the Internal Medicine Department, 80 apparently healthy control subjects of comparable age and sex were also included in the study. Inclusion criteria included patients over the age of 18 who have active RA (either early with symptoms lasting < 6 months or established disease lasting > 6 months).[16] Exclusion criteria included patients who have previously experienced cardiovascular illness, e.g., heart failure, acute coronary syndrome, revascularization, transient ischemic attacks, and cerebrovascular stroke. Patients who have concomitant hepatic or renal diseases, active infections, malignancy, smoking, hypertension, dyslipidemia, obesity, diabetes mellitus, and hyperuricemia were also excluded from the study. Also, patients who are receiving anti-TNF-α therapy were not included in the study.

The study protocol was reviewed and approved by the Institutional Review Board of the Faculty of Medicine, Assiut University (approval dated March 2019). The confidentiality of all patients admitted to the study was maintained to the greatest extent possible. Before participants were admitted to this study, the goals and nature of the study were made clear to them. A written informed consent was obtained from each participant before included in the study. Blood samples and carotid ultrasound carry minimal risk to the patients.

Demographic, clinical and laboratory characteristics

Each patient was clinically evaluated to determine the onset, course, and duration of their RA. Medical records were also consulted for information on treatment. Demographic data for cases and controls were recorded. Clinical examination included general and joint examinations, and measurement of blood pressure at rest and body mass index. The disease activity score 28 (DAS28) was used for RA cases to evaluate activity status.[17]

Venous blood samples were collected from each study participant. Complete blood count (CBC) was performed on a fully automated high-volume hematology analyzer (ADVIA® 2120i System supplied by Siemens Healthineers, GmbH Henkestr.127 91052 Erlangen, Germany), according to the manufacturer's instructions. The erythrocyte sedimentation rate (ESR) was performed using a fully automated analyzer (Sysmex ALIFAX analyzer, Italy), according to the manufacturer's instructions. The hemoglobin A1C (HbA1c) was performed on a fully automated blood chemistry analyzer (ADVIA® 1800 high volume chemistry analyzer, Siemens Healthineers, GmbH Henkestr.127 91052 Erlangen, Germany), according to the manufacturer's instructions. After blood clotted, serum was separated and used for the following investigations. Rheumatoid factor (RF) and C-reactive protein (CRP), was determined by turbidimetry (ADVIA® 1800 high volume chemistry analyzer, Siemens Healthineers, GmbH Henkestr.127 91052 Erlangen, Germany), according to the manufacturer's instructions. Uric acid, lipid profile (cholesterol, high density lipoprotein (HDL), low density lipoprotein (LDL), and triglycerides), urea, creatinine, alanine transaminase (ALT), aspartate transaminase (AST), serum albumin (ALB), and random blood glucose (RBG) were performed using an automated blood chemistry analyzer (ADVIA® 1800 high volume chemistry analyzer, Siemens Healthineers, GmbH Henkestr.127 91052 Erlangen, Germany), according to the manufacturer's instructions.

Anti-Cyclic Citrullinated Peptide (Anti-CCP) was measured on an automated immunoassay analyzer (Architect i2000 system autoanalyzer, Abbott Park, IL, USA) using a commercial kit (lot number k083868, Abbot Laboratories, USA), for detection of human IgG autoantibodies to cyclic CCP, according to the manufacturer's instructions. Serum TNF-α was measured using an enzyme-linked immunosorbent assay (ELISA) kit (Cat No. MBS267654, supplied by BioSource, USA), according to the manufacturer's instructions.

Serum hs-cTnI was measured using a sandwich immunoassay, chemiluminometric technique using an automated immunoassay system (Siemens Healthcare's ADVIA Centaur® XPT Immunoassay Systems, GmbH Henkestr.127 91052 Erlangen, Germany ADVIA Centaur High-Sensitivity Troponin I Assay (TNIH), according to the manufacturer's instructions. Sample type (Human serum, plasma (lithium heparin), Sample Volume 100 µL, Assay Range 2.50–25,000.00 ng/L (pg/mL), LoB 0.5 ng/L (pg/mL), LoD 1.6 ng/L (pg/mL), LoQ (20 % CV) 2.5 ng/L (pg/mL), LOQ (10 % CV)6.0 ng/L (pg/mL), Onboard Stability 28 days, Time to First Result 18 min, 99 th percentile (n=2010) Combined: 47.34 ng/L (pg/mL) Male: 57.27 ng/L (pg/mL) Female: 36.99 ng/L (pg/mL).

cIMT measurement

Carotid ultrasound measurement was done using a high-resolution B-mode ultrasound machine (Philips HD15XE, Netherlands). All the measurements were done by a single sonographer. The cIMT was defined as the distance between the media-adventitial interface and the leading edge of the intima-luminal at the carotid artery wall, measured

bilaterally. The measurement obtained was 10 mm (1 cm) proximal to the bifurcation of the common carotid artery (bulb). For each carotid artery, three image readings were obtained. The cIMT thickness was analyzed using the mean average of six measurements. For the present study, a cIMT value of 0.9 mm was considered the cutoff point. Plaque was also sought, defined as focal thickening with at least 50% greater than the surrounding wall or at least 1.5 mm thick.

Statistical methods

The statistical package for the social sciences; SPSS Inc., Chicago, IL, USA, (SPSS Version 21) was used for all statistical calculations. Data were statistically reported using the mean ± standard deviation (SD), median and interquartile range (IQR) for not normally distributed data, and relative frequencies (percentages) and frequencies (number of occurrences), as appropriate. Using the Mann-Whitney U test for non-normally distributed data, quantitative variables were compared. To compare categorical data, the chi-square test was used. When the anticipated frequency was less than 5, an exact test was utilized instead. The Pearson correlation test was used to determine the correlation between different variables. To predict elevated cIMT in RA patients, logistic regression analysis was utilized. The optimal cut-off values for projected subclinical atherosclerosis in RA patients were determined using receiver operating characteristic (ROC) analysis. The p-value was always two-tailed and set to a significant level of 0.05.

Results

Clinical characteristics and laboratory features of study participants

The study included 80 RA patients and 80 controls. RA cases included 68 (85.0%) females and 12 (15.0%) males, their ages ranged from 21 to 60 years, with a mean ±SD of 43.49±12.68. Regarding controls, they were 69 (86.3%) females and 11 (13.7%) males, their ages ranged from 22 to 57 years (mean ±SD of 42.40±12.29). Age and sex did not differ between the two groups (*p*= 0.583 and p=0.822, respectively), indicating that the baseline characteristics were similar in both groups. There were 19 patients (23.8%) who had early RA disease and 61 (76.2%) with established disease. The disease duration ranged from two months to twelve years, as shown in Table 1. Table 2 displays the laboratory features of the study cases and controls.

Table 1. Characteristics of the 80 study RA cases.

Clinical characteristics	N	(%)
Deformities		
No	58	(72.5)
Yes	22	(27.5)
Disease duration (years)		
Early disease < 6mn	19	(23.8)
Established disease > 6mn	61	(76.2)
Extra articular manifestations		
No	64	(80.0)
Yes	16	(20.0)
Treatment		
One line	4	(5.0)
Two lines	24	(30.0)
Triple therapy	28	(35.0)
More than 3 lines	24	(30.0)
DAS 28		
Low (DAS 28 ≥ 2.6 and ≤3.2)	28	(35.0)
Moderate (DAS 28 > 3.2 and ≤5.1)	28	(35.0)
High > 5.1	24	(30.0)

Qualitative data are presented as number (percentage).

Table 2. Laboratory features of the study cases and controls.

Laboratory characteristics	Groups		*p*-value
	Cases (n=80) Median (IQR)	Controls (n=80) Median (IQR)	
Hemoglobin g/dl	12.5 (2.2)	13.9 (3.1)	<0.001
Platelets 103/ul	287.0 (157.5)	317.0 (93.0)	NS
WBCS 103/ul	6.4 (3.2)	6.9 (3.3)	NS
ESR mm/hr	58 (44)	6 (5)	<0.001
CRP mg/l	18 (9)	7 (3)	<0.001
RF IU/ml	59 (63.1)	2 (4.0)	<0.001
Anti-CCP IU/ml	109.5 (117.0)	11.3 (3.0)	<0.001
HBA1c %	4.9 (0.8)	5.0 (0.9)	NS
Random blood glucose mg/dl	106 (29)	109 (12)	NS
HDL mg/dl	69.5 (16.0)	70 (7.4)	NS
LDL mg/dl	112 (32)	112 (19)	NS
Cholesterol mg/dl	119 (94.0)	110 (33.0)	NS
Triglyceride mg/dl	103.5 (43.5)	96.1 (37.0)	NS
Uric acid mg/dl	4.5 (1.68)	4.6 (1.1)	NS

The median (IQR) is used to present quantitative data. p <0.05 is significant.

Comparison of hs-cTnI, cIMT and TNF-α between cases and controls

The levels of hs-cTnI, TNF-α, and cIMT between patients and controls differed significantly (*p*=0.001, for all), as indicated in Table 3.

Correlation of cIMT, TNF-α and hs-cTnI levels with various laboratory and clinical characteristics of RA cases

The correlations between the levels of cIMT, TNF-α, and hs-cTnI with various laboratory and clinical features of RA cases are illustrated in Table 4.

Table 3. Comparison of hs-cTnI, cIMT and TNF-α between cases and controls.

	Cases (n=80) Median (IQR)	Controls (n=80) Median (IQR)	p-value
hs-cTnI ng/ml	0.02 (0.10)	0.0 (0.01)	0.001
TNF-α pg/ml	9.6 (3.9)	8.0 (1.8)	0.001
cIMT mm	0.9 (0.2)	0.7 (0.1)	0.001

The median (IQR) is used to present quantitative data. *p*<0.05 is significant.

Table 4. Correlation of cIMT, TNF-α and hs-cTnI levels with various laboratory and clinical characteristics of the 80 RA cases.

Variable		cIMTmm	TNF-α pg/ml	hs-cTnI ng/ml
Age (years)	r	0.484	0.000	0.017
	p-value	<0.011	0.997	NS
Disease duration (years)	r	0.609	0.229	0.227
	p-value	<0.001	0.041	0.043
DAS2	r	0.417	0.646	0.371
	p-value	<0.001	<0.001	0.001
ESR mm/hr	r	0.337	0.439	0.244
	p-value	0.002	<0.001	0.029

Table 4. Continued.

Variable		cIMTmm	TNF-α pg/ml	hs-cTnI ng/ml
CRP mg/l	r	0.459	0.256	0.092
	p-value	<0.001	0.022	0.417
RF IU/ml	r	0.352	0.114	0.346
	p-value	0.001	0.314	0.002
Anti CCP IU/ml	r	0.524	0.213	0.281
	p-value	<0.001	0.058	0.012
TNF-α pg/ml	r	0.332	-	0.347
	p-value	0.003	-	0.002
cIMT mm	r	-	-	0.337
	p-value	-	-	0.002

P > 0.05 is not significant (NS). , r=correlation coefficient.

The optimal cutoff, sensitivity, and specificity for RA patients using TNF-α and hs-cTnI to diagnose subclinical atherosclerosis

TNF-α had a sensitivity of 74.0% and a specificity of 73.3% for the diagnosis of subclinical atherosclerosis in RA patients at a cut-off of > 9.05 pg/ml (95% CI 0.657-0.866; AUC 0.761). The sensitivity and specificity of hs-cTnI were 62.0% and 60.0%, respectively, for the diagnosis of subclinical atherosclerosis in RA patients at a cut-off of > 0.01 ng/ml (95% CI 0.567-0.802; AUC 0.684). TNF-α was much superior predictor for identifying RA patients with subclinical atherosclerosis, (*p* <0.001) with a higher AUC, as displayed in Figure 1 and Table 5.

Table 5. The optimal cutoff, sensitivity, and specificity for diagnoses of subclinical atherosclerosis in the 80 RA patients using TNF-α and hs-cTnI.

	Cut off	95%CI	Sensitivity	Specificity	AUC	*p*-value
TNF-α pg/ml	9.05	0.657- 0.866	74.0%	73.3%	0.761	<0.001
hs-cTnI ng/ml	0.01	0.567 – 0.802	62.0%	60.0%	0.684	0.006

CI (confidence interval), AUC (area under the curve). *p*<0.05 is significant.

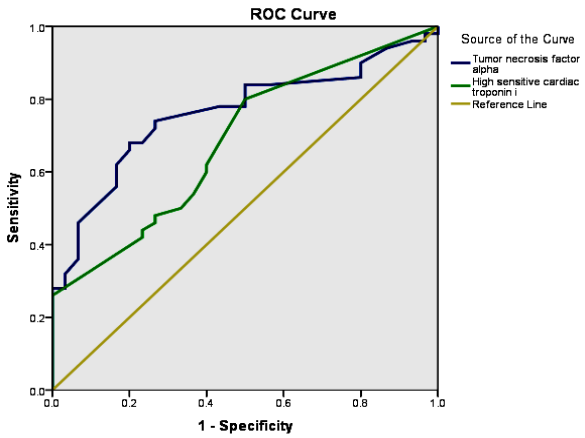

Figure 1. Receiver operating characteristic (ROC) curves for TNF-α and hs-cTnI -based diagnosis of subclinical atherosclerosis in RA patients.

Univariate and multivariate logistic regression analysis for the prediction of increased cIMT in RA patients

Univariate logistic regression analyses for predicting increased cIMT in RA patients revealed that older age, established disease greater than 6 months, and higher TNF-α were significant predictors of increased cIMT in RA patients. Multivariate analysis supported this finding by showing that older patients were more likely to have elevated cIMT (OR = 1.084, 95% CI 1.022-1.151, *p* = 0.008). Established disease was the highest prognostic marker for prediction of increased cIMT among RA patients, where patients with disease duration > 6 months were about 60 times more likely to have increased cIMT compared to patients with

shorter disease duration (OR = 60.442, 95% CI 5.869–622.473, *p*=0.001). Furthermore, patients with TNF-α levels ≥ 9.05 pg/ml were approximately six times more likely to have increased cIMT than patients with TNF-α levels <9.05 pg/ml (OR = 6.461, 95% CI 1.551-26.920, *p*= 0.010), as shown in Table 6.

Table 6. Univariate and Multivariate logistic regression analysis for prediction of increased cIMT in RA patients.

Variables	N	Univariate analysis			Multivariate analysis		
		OR	95% CI	*p* value	OR	95% CI	*p* value
Age	80	1.085	1.039 – 1.133	<0.001	1.084	1.022 – 1.151	0.008
Disease duration							
Early disease	19	ref			ref		
Established disease	61	73.50	8.908 – 606.431	<0.001	60.442	5.869 – 622.473	0.001
DAS 28					Not included in the final model		
Low	28	ref					
Moderate	28	2.400	0.818 – 7.039	0.111			
High	24	NA	0.0 - NA	0.998			
TNF-α							
< 9.05	35						
≥ 9.05	45	7.827	2.804 – 21.852	0.000	6.461	1.551 – 26.920	0.010
hs-cTnI					Not included in the final model		
< 0.01	37						
≥ 0.01	43	2.447	0.968 – 6.185	0.058			

hs-cTnI: high-sensitivity cardiac troponin I; cIMT: Carotid intima media thickness; TNF-α: tumor necrosis factor alpha; CI: Confidence interval; OR: Odds ratio; NA: not achieved. ref: reference in logistic regression analysis, $p \leq 0.05$ is significant.

Discussion

RA patients have a higher chance of developing CVD, according to a vast body of research.[18] Chronic inflammation is closely tied to atherosclerosis, and the burden of inflammation is linked to both clinical and subclinical CVD.[19] In the current study, we investigated the interaction of pro-inflammatory cytokines represented by TNF-α and hs-cTnI, as biomarkers of myocardial injury in RA patients with subclinical atherosclerosis, to reveal the dual impact of chronic inflammation on RA progression and CVD risk. TNF-α is a potent pro-inflammatory cytokine that is required for the production of other cytokines and the induction of chronic inflammation.[20, 21]

In line with previous research, our study found a significant difference in TNF-α levels between RA patients and controls. [8, 22] In this study, TNF-α levels differed significantly between disease activity groups, as patients with high DAS28 scores had significantly higher levels of TNF-α than patients with moderate or low disease scores. This finding was consistent with previous research,[11,23] which suggested that TNF-α is a possible indicator of RA activity and severity. This is because TNF-α can cause the release of prostaglandins, reactive oxygen products, and neutral proteinases that degrade proteoglycans, resulting in cartilage destruction.[8] TNF-α also stimulates the release of tissue-destroying matrix metalloproteinase and inhibits the production of its inhibitors, resulting in joint damage.[24]

TNF-α has been shown to be involved in endothelial activation, angiogenesis, and other endothelial function and repair factors. As a result, TNF-α plays a critical role in endothelial dysfunction and promotes atherogenesis in RA patients.[9,25] We used ultrasonographic measurement of cIMT as a method for quantifying subclinical atherosclerosis and assessing CVD risk. Our findings support the findings of Vázquez-Del Mercado et al., 2015, who found a significant correlation between TNF-α and cIMT.[26] These highlights not only the

importance of TNF-α as a principal mediator of inflammation in RA, but also its critical role in the emergence and progression of subclinical atherosclerosis.

The advent of hs-cTnI assays has allowed for risk classification in people with occult or stable coronary artery disease and has facilitated earlier detection of myocardial injury.[27] Furthermore, as hs-cTnI levels rise, the risk of cardiovascular death is elevated in both patients with stable coronary artery disease and the general population.[28, 29] In the current study, RA patients had considerably higher levels of hs-cTnI than did controls, which was also demonstrated in a previous study.[30] According to a previous study, RA patients had less myocardial mass than controls of the same age as evidenced by cardiac MRI, which proposes a state of myocyte loss or chronic myocardial injury in RA.[31] Our finding of elevated levels of hs-cTnI could be concordant with this hypothesis.

We proved a strong connection between the level of hs-cTnI and disease activity (r = 0.371, *p*= 0.001), keeping with previous research.[32] TNF-α and hs-cTnI serum levels were also found to be significantly correlated (r = 0.347, *p*=0.002). As a result, it is possible that ongoing inflammation is the main reason for the troponin elevation in RA. Curiously, a prior study had shown no evidence of a significant relationship between hs-cTnI levels and disease activity score or inflammatory cytokines.[12] However, they explained that this finding may be due to the relatively well-controlled disease in the study patients and the likelihood that increased inflammatory levels affect uncontrolled disease-related hs-cTnI concentrations. hs-cTnI concentrations are probably influenced by the fluctuating amounts of inflammatory mediators that occur during disease exacerbations.

Our study found a strong association between hs-cTnI and cIMT levels (r = 0.337, *p* = 0.002). This finding may reveal that subclinical atherosclerosis and higher plaque load may be reflected in the rise of specific biomarkers for myocardial injury known as hs-cTnI. It is not quite clear how RA causes higher troponin levels. The release of cardiac troponin in RA is thought to occur through a number of different mechanisms, such as apoptosis, myocyte necrosis, and myocardial hibernation, all brought on by subendocardial ischemia.[33,34] Cardiomyocyte injury caused by inflammatory cytokines is still a reliable and well-supported theory.[33,34] This lends credence to the powerful synergy between elevated hs-cTnI levels and TNF-α in the early detection of cardiac affection in RA, they represent the same pathological process.

Logistic regression analysis indicated that older age, established disease > 6 months, and higher TNF-α were significant predictors of increased cIMT in RA patients. These findings suggest a possible role of TNF-α in the pathogenesis of atherosclerosis in RA patients. More research is required to determine whether there is an association between TNF-α serum level and potential development of cardiovascular events in RA patients and to determine whether these indicators are indicative of a poorer CVD outcome. Further larger studies are needed to ascertain whether TNF-α marker is indicative of a worse clinical prognosis and comorbidities like CVD among RA patients.

The nature of our study is its main limitation, as optimizing cardiovascular risk prediction in RA patients would need long-term follow-up through utilizing CVD risk factors uniquely associated with RA. Follow-up will aid in determining the impact of inflammatory load on the development of future CV events, as well as the value of using TNF-α inhibitors in controlling disease activity and CVD prevention. To validate our results, methods for measuring myocardial structure and inspecting the coronary arteries are recommended.

Finally, we may conclude that TNF-α can be used as a surrogate marker for RA activity, reflecting the extent of cardiovascular disease and correlating with the presence of subclinical atherosclerosis. hs-cTnI is correlated with TNF-α as a biomarker of myocardial injury, indicating that the persistent level of systemic inflammation and cardiac affection in RA are two sides of the same coin. hs-cTnI may act as an adjunct predictive biomarker in cardiovascular risk determination in RA. This can

be used to investigate how different RA treatments, particularly TNF-α inhibitors, affect cardiac inflammation, thereby killing two birds with one biological stone.

Author Contributions

EMI and SRM, contributed to material preparation, writing the original draft, data collection and analysis. SRM and NMT contributed to the study design, concept, and methodology. EMM, contributed to radiological assessment. AMEE, contributed to methodology and laboratory investigations. RFAA, provided clinical supervision. All authors read and approved the final manuscript.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Ethical approval

The study was recorded under the NCT03821090 ID on ClinicalTrials.gov. The study protocol was reviewed and approved by the Institutional Review Board of the Faculty of Medicine, Assiut University (original approval dated March 2019).

Informed consent

A written informed consent was obtained from each study participant before included in the study.

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