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ORIGINAL ARTICLE

Association of Paraoxonase 1 Polymorphism and Serum 25-Hydroxyvitamin D with the Risk of Cardiovascular Disease in Patients with Rheumatoid Arthritis

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SUMMARY

Background: **Patients with rheumatoid arthritis (RA) have significantly increased cardiovascular (CV) morbidity and mortality that are not accounted for by traditional risk factors alone. Paraoxonase 1 (PON1) and 25-hydroxyvitamin D have been shown to be involved in the pathogenesis of CV diseases.**
Objective: **This study aimed to investigate PON1 gene polymorphism and serum 25-hydroxyvitamin D concentrations in RA patients, and to determine their association with CV risk in RA.**
Methods: **Serum samples from 46 RA patients and 45 healthy controls were tested for PON1 R192Q genotypes and serum vitamin D concentrations. The cardiovascular risks were assessed by Q-risk. Lipoprotein cholesterol levels, traditional CV risk factors, medication use, and RA disease activity status were also assessed.**
Results: **PON1 polymorphism and low serum 25-hydroxyvitamin D were significantly associated with increased CV risk ($p < 0.05$). Compared to patients with either the PON1 QQ genotype or the QR genotype, patients with the RR genotype demonstrated decreased CV risk on multivariate analysis, controlling for traditional CV risk factors, C-reactive protein levels, prednisone use, and cholesterol-lowering medication use ($p < 0.05$).**
Conclusions: **There was a relationship of the genetic determinants of paraoxonase 1 (PON1 192) and serum 25-hydroxyvitamin D to CV risk in RA patients. Paired measurement of paraoxonase 1 genotype and serum 25-hydroxyvitamin D can be used as biomarkers of CV risk in RA patients.**
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INTRODUCTION

Patients with rheumatoid arthritis (RA) have significantly increased cardiovascular (CV) morbidity and mortality that are not accounted for by traditional risk factors alone [1-3]. Recently, both RA and atherosclerosis have been considered as two strictly linked inflammatory diseases [4]. Earlier studies revealed that the in-

creased CV risk occurs even early in the disease course and, therefore, a possible preclinical manifestation of the disease [4]. On this basis, the understanding of commonly shared pathological mechanisms is mandatory for the right treatment of RA in order to reduce atherosclerosis and the subsequent impact of CV disease on these patients.

Rheumatoid arthritis associated synovitis often results in the loss of structural integrity of bone and cartilage [5]. This degradation was reported to be mediated by several proteolytic enzymes, and current evidence suggests that the induction of these catabolic processes is attributed to the pro-inflammatory cytokines [6]. Recently, attention has been focused on the role of reactive oxygen species produced by activated neutrophils during the inflammatory response [7]. It was reported that an increased amount of such substances in the synovial fluid as well as plasma may contribute the destructive proliferative synovitis in RA [8]. Under normal conditions, reactive oxygen species are formed at relatively low concentrations in all cells and tissues, and a variety of anti-oxidative mechanisms serve to control its production. Under pathological conditions, the levels of these reactive oxygen species are altered by increased production and/or inadequate removal, which results in oxidative stress, inducing cell damage and lipid peroxidation [9]. Enzymatic protection against the breakdown products of peroxidized lipids and oxidized protein and DNA are provided by many enzyme systems.

Paraoxonases (PONs) are a group of enzymes involved in the hydrolysis of organophosphates. Paraoxonase 1 (PON1) is a high-density lipoprotein (HDL) associated lipolactonase that is synthesized in the liver and transported along with HDL in the plasma. It functions as an antioxidant; it prevents the oxidation of low-density lipoprotein (LDL) and cell membranes` oxidation and is, therefore, considered to be atheroprotective [10,11]. In addition, PON1 enzyme showed phospholipase A2-like activities including hydrolyzing platelet activation factor and homocysteine thiolactone, factors that play an important role in atherothrombosis [12-14]. Moreover, previous reports showed that mice lacking serum paraoxonase 1 are susceptible to organophosphate toxicity and atherosclerosis [15]. In human, lower PON1 activity was observed in high oxidative stress diseases such as cardiovascular diseases [16], dyslipidemia [17], and inflammatory diseases [18]. Human PON1 has two genetic polymorphisms giving rise to amino acid substitutions; one at codon 55 which is a methionine/leucine substitution (A→T), and the other at codon 192 which is a glutamine/arginine substitution (A→G) [19]. Paraoxonase 1 activity varies substantially in populations, and most of this variation depends on genetic polymorphisms, particularly the Q192R polymorphism in the coding region. This polymorphism has been associated with CV risk in the general population [20] and in rheumatic patients [21].

Vitamin D is an important prohormone for optimal intestinal calcium absorption essential for mineralization of bone. Vitamin D comes in two forms: vitamin D2 (ergocalciferol) and vitamin D3 (cholecalciferol). Vitamin D3 (1,25-dihydroxyvitamin D) is the active form of vitamin D and acts as a steroid hormone by binding to the vitamin D receptor (VDR), which is present in many cells throughout the body, including cardiomyocytes [22], vascular smooth muscle [23], and endothelium [24]. As the vitamin D receptor is present in multiple tissues, there has been growing interest in evaluating other potential functions of vitamin D, particularly in CV diseases. Cross-sectional studies have reported that vitamin D deficiency is associated with increased risk of CV disorders, including hypertension, heart failure, and ischemic heart disease [25,26]. Initial prospective studies have also demonstrated that vitamin D deficiency increases the risk of developing incident hypertension or sudden cardiac death in individuals with pre-existing CV diseases [27]. The mechanism for how vitamin D may improve CV disease outcomes remains obscure; however, many lines of evidence indicate its beneficial effects as membrane antioxidant [28]. Although vitamin D3 (1,25-dihydroxyvitamin) is the more bioactive form for humans, its serum level does not correlate with overall vitamin D status. Meanwhile, serum concentration of 25-hydroxyvitamin (25(OH)D) was reported to be the most accurate indicator of vitamin D status [29].

This study was carried out to investigate the value of measuring paraoxonase 1 and serum 25-hydroxyvitamin D as surrogate markers of CV risk in RA patients.

MATERIALS AND METHODS

Study design

This was a randomized cross-sectional study which included 91 Saudi individuals. All the patients met the 2010 ACR/EULAR criteria for RA classification [30]. Local ethical and methodological protocols (King Abdulaziz University, Jeddah) for approval of the study were followed. All patients who participated in the study signed an informed consent according to the Declaration of Helsinki (October 2008).

Study participants

The study included Saudi adults aged over 18 years attending rheumatology outpatient clinics at King Abdulaziz University Hospital in Jeddah. Sociodemographic characteristics, relevant history, and educational level were taken and recorded. Exclusion criteria were: the presence of chronic illnesses that potentially alter vitamin D metabolism, or precipitate cardiovascular incompetence, pregnant or breastfeeding women, and the use of vitamin D supplements. After the selection and removal of individuals who did not accept participation, 46 RA patients were included in this work for assessment. Forty-five healthy subjects of matched gender and age were included in this work as a control group. Prior to assessment, similar to the study group, they were interviewed and completed the standard pre-assessment

questionnaire. They were then assessed clinically to ascertain their normality. All subjects had laboratory assessments similar to the study group.

Clinical assessment

All participants were interviewed by trained persons with a standard closed-ended questionnaire. The contents of the questionnaire included general demographic characteristics, educational level, including current and past smoking, sun exposure, dairy consumption, clothing status, current and past use of glucocorticoids, biologic and non-biologic disease modifying anti-rheumatic drugs (DMARDs), current use of drugs affecting bone metabolism including bisphosphonates, calcium, and vitamin D supplementation. The patients then completed a copy of the patient reported outcome measures questionnaire [31]. The questionnaire includes 11 domains assessing for functional disability, quality of life, VAS for joint pain, global status, fatigue, duration of morning stiffness, review of the systems, falls and cardiovascular risks, the patient motivation questionnaire, and self-reported joint pain. The patients then were examined in the clinic where parameters of disease activity were recorded. The RA disease activity was calculated using the Disease Activity Score for 28 joints with ESR (DAS28-ESR) equation [32]. All patients were treated according to EULAR guidelines for RA management [33].

Cardiovascular risk assessment

The patient's 10-year risk of developing cardiovascular disease (CVD) was assessed using the QRISK®2 calculator which includes the following parameters (if known - missing values are calculated by a complex averaging procedure called multiple imputation and implemented [24,25]. Patient age (30 - 84), patient gender, smoking status (non, ex, light, moderate, heavy), diabetic, angina or heart attack in a first-degree relative < 60 (yes/no), existing treatment with blood pressure agent (yes/no), a geographical measure of deprivation, BMI (height and weight), systolic blood pressure (use current not pre-treatment value), total and HDL cholesterol, self-assigned ethnicity (should not be confused with nationality), rheumatoid arthritis, chronic kidney disease, atrial fibrillation. The calculator is available at http://www.qrisk.org.

Blood sampling

Six milliliters of blood were withdrawn from each RA patients and controls into ethylenediaminetetraacetic acid (EDTA) tubes. Serum was separated within 12 hours by centrifugation at 1500 rpm and 4°C for 5 minutes. Serum samples were stored at -80°C until analyzed.

Biochemical analyses

At inclusion, the following variables were measured for all subjects included in this work: full blood count, liver and kidney functions, bone profile (calcium, phosphorus and alkaline phosphatase), inflammatory markers (ESR, CRP) as well as rheumatoid factor (RF) using standard lab tests.

Serum vitamin D level assessment

Estimation of vitamin D was performed using an ELISA kit produced by eBioscience company (eBioscience, San Diego, CA, USA) according to manufacturer's protocol. Briefly, the microtiter plate was pre-coated with a monoclonal antibody specific for 25(OH) vitamin D. Standards or samples were then added to the microtiter plate wells and 25(OH) vitamin D, if present, will bind to the antibody pre-coated wells. In order to quantitatively determine the amount of 25(OH) vitamin D present in the sample, a standardized preparation of horseradish peroxidase (HRP)-conjugated polyclonal antibody, specific for 25(OH) vitamin D was added to each well to “sandwich” the 25(OH) vitamin D immobilized on the plate. The microtiter plate was left for an incubation period of 4 hours, and then the wells were thoroughly washed to remove all unbound components. Next, substrate solutions were added to each well. The enzyme (HRP) and substrate were allowed to react over a short incubation period. Only those wells that contain 25(OH) vitamin D and enzyme-conjugated antibody will exhibit a change in color. The enzyme-substrate reaction is terminated by addition of a sulphuric acid solution and the color change is measured spectrophotometrically at a wavelength of 450 nm.

Genotyping

Genomic DNA was extracted from whole blood samples, in a biosafety cabinet, using QIAamp DNA Blood Mini Kit (QIAGEN, USA, Cat. no. 51104). The extracted DNA was stored at -20°C until subjected to PCR amplification. The concentration and purity of the extracted DNA was calculated automatically by a Nanodrop 2000c instrument from Thermo Scientific (USA). PON1 Q192R polymorphisms were detected by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method as previously described [19]. Briefly, extracted DNA was amplified using PCR with different primers (forward and reverse). The forward primer 5' TAT TGT TGC TGT GGG ACC TGA G 3' and reverse primer 5' CAC GCT AAA CCC AAA TAC ATC TC 3' were purchased from (Biolegia, Netherlands). Reaction conditions were carried out in a thermal cycler GeneAmp® PCR System 9700 (Applied Biosystem, Japan) at 95°C for 4 minutes followed by 35 cycles of 95°C for 60 seconds, 59°C for 60 seconds, and 72°C for 60 seconds. For the PON1 192 genotype, PCR product was digested with 8 U Alw1 (BspI) restriction endonuclease (MBI Fermentas) overnight at 55°C. The digested products were separated by electrophoresis on 2% agarose gel and visualized using ethidium bromide. The B-genotype (arginine) contains a unique (Alw1) BspI restriction site which results in 66 and 33 bp products that represent QR and RR genotypes. Whereas the A-genotype (glutamine) will not be cleaved by this re-

Table 1. Baseline laboratory parameters in both the RA and control groups.

Parameter	RA	Control	p-value
Age	49.4 (13.1)	45.1 (12.6)	NS
BMI	34.26 ± 4.16	33.84 ± 5.51	NS
Disease duration (months)	89.62 ± 13.88	-	
Dairy consumption	8 (20%)	14 (35%)	NS
Sun exposure	5 (13%)	7 (18%)	NS
Sun block	2 (5%)	11 (28%)	0.01
Serum vitamin D (ng/mL)	12.03 ± 10.66	21.37 ± 14.98	0.021
Calcium	2.24 (0.09)	2.29 (0.11)	0.024
Phosphorus	1.06 (0.13)	1.05 (0.20)	NS
Alkaline Phosphatase	90.8 (40.2)	77.1 (24.8)	NS
PTH	7.35 (3.8)	7.23 (2.8)	NS

Values represent Mean (SD).

Table 2. Cardiovascular risk in RA patients versus the control subjects.

Parameter	RA	Controls
DAS-28	4.84 ± 1.28	1.78 ± 0.36
QRISK	42.3 ± 4.2%	9.5 ± 5.6% *
CRP mg/L	14.1 ± 15.25	5.03 ± 3.64 *
ESR mm/hour	37.5 ± 23.37	22.69 ± 10.69 *
RF IU/L	110.5 ± 66.57	5.5 ± 3.79 *
Cholesterol mmol/L	4.67 ± 1.01	2.94 ± 0.71 *
LDL mmol/L	3.6 ± 1.46	2.81 ± 0.93 *
HDL mmol/L	1.02 ± 0.61	1.1 ± 0.49
TGs mmol/L	1.2 ± 0.62	1.63 ± 0.79

Values represents Mean (SD), * $p < 0.05$.

Table 3. Disease characteristics and medication use in the RA patients with high CV risk compared to the RA patients with CV risk.

Variable	Cardiovascular risk		p-values
	Low (20/46)	High (26/46)	
Age	42.8 (6.4)	48.6 (3.7)	0.26
DAS-28	3.7 (0.6)	4.9 (0.4)	0.01
Functional disability	1.6 (0.43)	2.1 (0.51)	0.01
Vitamin D (ng/mL)	19.1 (3.2)	12.7 (2.8)	0.01
PON1 genotype QQ	41%	69%	0.01
Medications			
NSAIDs	10	12	0.34
Prednisolone	8	18	0.01
Biologic therapy	12	16	0.46

Values represents Mean (SD).

Table 4. Logistic regression analyses of variables associated with high cardiovascular risk in the patients with RA.

Variable	OR (95% CI)
Age, years	1.07 (1.03 - 1.24)
BMI, kg/m^2	0.91 (0.85 - 1.03
Female	0.24 (0.07 - 0.56)
Hypertension	4.58 (1.77 - 11.21)
Prednisone use	2.02 (0.73 - 6.23)
Serum Vitamin D	-1.14 (0.78 - 1.21)
DAS-28	4.31 (0.72 - 1.06)
PON1 QQ genotype	0.99 (0.96 - 0.99)

Table 5. Joint association of PON1 QQ genotype and Vitamin D deficiency with CV risk.

Variable	n	Cardiovascular risk	
		Absent	Present
QQ + Vit D3 < 10.6	5	0 (0.0%)	5 (100%)
Otherwise	35	20 (57.1%)	15 (42.9%)
p-value	0.047 *		

n = number of individuals, * Chi-square test.

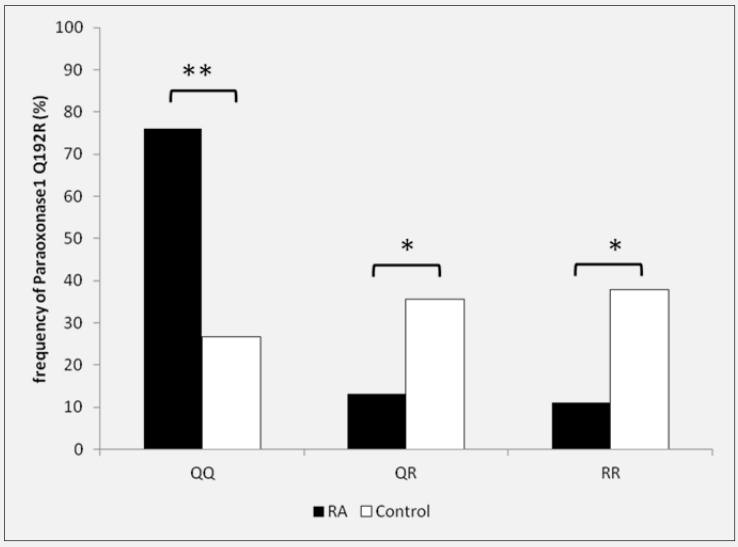

Figure 1. Genotype frequency of paraoxonase 1 Q192R in the RA and control groups. * p < 0.05, ** p < 0.01.

striction enzyme resulting in one band at 99 bp allowing the determination of the PON1 QQ genotype [19].

Statistical analysis
Data analysis used the Statistical Package for Social Sciences (SPSS 16), SPSS Inc., Chicago, IL, USA. Descriptive data were expressed as mean and standard deviation (SD). One-way analysis of variance (ANOVA) was used to examine the variation in different PROMs variables with the serum 25(OH) vitamin D level. Differences between the genotypes were assessed by using the *t*-test. Categorical variables were presented as counts and percentages and were compared by chi-square test. Where significant p-value was generated, the odds ratio (OR) was calculated. Association between the disease and genotypes were assessed by calculating odds ratios and 95% confidence intervals (CI). Statistical significance was set up at p-values < 0.05.

RESULTS

Participants' characteristics
There was no statistically significant difference among the RA group and control group with regard to age, gender, disease duration, socioeconomic status, or other comorbidities. Serum calcium and 25(OH) vitamin D concentrations were significantly lower in the rheumatoid arthritis group, whereas there was no significant difference between both groups on assessment of serum phosphorus, alkaline phosphatase, blood sugar and parathyroid hormone (Table 1). Serum cholesterol and LDL cholesterol were significantly higher in the RA group (Table 2).

Cardiovascular risk
RA patients were divided into two subgroups according to QRISK calculations, one with high cardiovascular risk, which were older, more likely to be male, and had a greater prevalence of hypertension than RA patients with low CV risk (Table 3). Trends toward higher total cholesterol and triglyceride levels in patients with plaque compared to those without plaque were noted, although these were not statistically significant. HDL and LDL cholesterol levels were notably similar between the two groups (Table 3). There was no significant difference in the prevalence of diabetes mellitus in both subgroups. Measures of disease activity, DAS-28, was significantly higher in the RA subgroup with cardiovascular risk. Functional disability score was also significantly higher than the RA subgroup with lower cardiovascular risk (Table 3). Use of prednisone and cholesterol-lowering medications was more common in patients with high cardiovascular risk. No differences were observed with the use of DMARDs and biological therapy on comparing the RA subgroups.

Serum vitamin D level
25-Hydroxyvitamin D level was significantly lower in the RA patients in comparison to the control group ($p < 0.01$). On comparing RA patients with high CV risk to those with lower risk, serum 25(OH) vitamin D level was significantly lower in the higher risk group.

PON1 genotype results
There were significant differences in paraoxonase 1 genotypes in the RA subgroup with high CV to the other subgroup. Patients with the RR genotype had the lowest CV risk, followed by patients with the QR genotype whereas the patients with the QQ genotype were at highest risk (Figure 1). Compared to patients with either the QQ or the QR genotype, patients with the RR genotype demonstrated a significantly decreased risk of CV risk after controlling for traditional CV risk factors.

Association of PON1 and serum vitamin D with CV risk
To assess for the potential relationship of PON1 and serum 25(OH) vitamin D level to CV risk in RA patients, multivariate logistic regression analysis showed a significant positive association of PON1 QQ genotype with high CV risk in RA patients. On the contrary, there was a significant negative association between serum 25(OH) vitamin D and high CV risk (Table 4). A new variable has been created combining both PON1 QQ genotype and 25(OH) vitamin D less than cutoff (10.6 nmole/L) as risk group for CV disease. Difference is based on combined PON1 QQ and 25(OH) Vitamin D < 10.6 between RA patients with and without CV risks were found significant ($p = 0.047$) when analyzed by the Chi-square test (Table 5).

DISCUSSION

In the last decades, rheumatoid arthritis has been linked to cardiovascular morbidity and mortality that it is not fully explained by other classic cardiovascular risk factors [3]. One of the main mediators of CVD is oxidative stress [34]. RA is accompanied by accumulation of high levels of reactive oxygen species produced by activated neutrophils during the inflammatory response, which induce vascular endothelial dysfunction and generate a spectrum of proatherogenic changes [35]. In addition, several reports indicate that defences against ROS-induced damage are compromised in RA patients [36,37]. The paraoxonase (PON) family of enzymes, in contrast to the rest of antioxidant enzymes, is characterized its specific activity on the vascular system and protection against coronary artery disease because it hydrolyses lipid peroxidation products and contributes to the prevention of low-density lipoprotein oxidation [38,39]. An inverse association between PON1 activity and oxidative stress in serum and macrophages has been suggested [40]. Case-control studies of PON1 activity and cardiovascular disease have revealed a clear association

of CV risk with polymorphism and functional activity of the PON1 gene [20,41]. In agreement with these data, results of the present work revealed significant differences between RA and control subjects regarding PON1 Q192R polymorphisms. The frequency of the QQ genotype was significantly higher in RA patients than in normal subjects. Mackness, et al. [42] reported that there was a difference in paraoxonase enzyme activities related to Q192R polymorphism. It was found that QQ genotype expressed the lowest enzyme activity, while QR and RR genotypes expressed moderate and highest enzyme activities, respectively [42].

In our work, the QQ genotype was significantly higher in the RA patients who had high CV risk. These results are in agreement with recently published studies showing a relationship of the genetic determinants and activity of paraoxonase 1 to CV risk in RA patients, as assessed by the presence or absence of carotid plaque [43].

Analysis of this study data also revealed a significant association of the PON1 Q192R polymorphism with increased CV risk in RA patients after controlling for traditional CV risk factors. Patients with the RR genotype had less CV risk compared to patients with the QR or QQ genotype. The PON1 Q192R polymorphism and the functional activity of paraoxonase 1 have previously been associated with both prevalent coronary artery disease and incident CV events in the general population. In a prospective study of 1,399 patients undergoing diagnostic coronary angiography, patients with the QQ genotype had an increased risk of major cardiac events, and patients with paraoxonase 1 activity in the highest quartile had the lowest risk of events [20]. In different study, a significant association of the PON1 Q192R polymorphism with carotid plaque was observed in RA patients after controlling for traditional CV risk factors and other significant correlates of plaque noted on bivariate analysis. Patients with the RR genotype had less risk of carotid plaque compared to patients with the QR or QQ genotype [43].

A significant correlation between PON1 and systemic inflammation as measured by the DAS-28 score as well as functional disability in the RA high CV risk group was observed in this study. This agrees with the results of an earlier study which depicted a modest but significant correlation between plasma paraoxonase 1 activity and systemic inflammation as measured by the high sensitivity CRP level. Higher CRP levels were associated with lower paraoxonase 1 activity. A previous proteomics study of HDL demonstrated a trend toward decreased levels of paraoxonase 1 protein in association with abnormal HDL with poor antioxidant capacity in patients with active inflammatory RA [30]. In addition, Popa et al. have reported increased paraoxonase 1 activity following therapy with infliximab in a cohort of 45 RA patients [44].

Previous investigations indicated that vitamin D deficiency increases the risk of developing incident hypertension or sudden cardiac death in individuals with pre-existing CV diseases [27,45]. In addition, 25(OH)D3 plasma levels have been found inversely correlated at least with the RA disease activity showing a circannual rhythm (more severe in winter) [46]. In agreement with these studies, our data revealed significantly lower vitamin D level in RA patients. There also was a negative association between serum 25(OH) vitamin D level and increased CV risk. These results are in agreement with earlier published data which revealed that cardiovascular risks appear to be associated with vitamin D levels less than 20 ng/mL, defined as vitamin D deficiency [47,48]. When combined with PON1 QQ genotype, 25(OH) vitamin D concentrations less than 10.6 ng/mL significantly ($p = 0.047$) differentiate between RA patients with and without CV risk.

The mechanism for how vitamin D may protect against CVD has not been fully elucidated. Proposed mechanisms include effects on the renin-angiotensin system, on glycemic control, inflammatory cytokines, direct effects on the vasculature and regulation of PTH levels, and calcium deposition in vascular smooth muscle [49, 50]. However, many lines of evidence indicate that the improvement of CV disease outcomes by vitamin D is related to its antioxidant effects [28].

CONCLUSION

Our findings suggest a significant association of the PON1 Q192R polymorphism activity with CV risk in RA patients. Concomitant measurement of serum 25(OH) vitamin D level and PON1 may be used as biomarkers to identify the subgroup of RA at high CV risk. Further large-scale CV outcome studies are warranted to validate the utility of paraoxonase 1 in standard clinical practice.

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Authors Contribution :
S. Khoja, A. Iyer, Y. El Miedany, S. Bahlas and, K. Balamash, and Prof. Elshal conceived and designed the experiments; A. Iyer, S. Bahlas, K. Balamash, and MF Elshal performed the experiments; All Authors analyzed the data; MF Elshal, and Y El Miedany wrote the paper. All the authors reviewed the manuscript.

Ethics Approval:
King Abdulaziz University Research Ethics Board, Jed-

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Informed Consent:
Informed consent was obtained from all individual participants included in the study.

Declaration of Interest:
The authors have no relevant financial disclosures.

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