

Article

Assessment of Paraoxonase-1 Activity, Glutathione, and Oxidative Stress Status in Patients with Rheumatoid Arthritis in Al-Shirqat City

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Abstract: Rheumatoid arthritis (RA) is a chronic systemic autoimmune disease in which persistent inflammation and oxidative stress interact to promote synovial injury, structural joint damage, and systemic complications. A combined assessment of enzymatic antioxidant defense, intracellular redox reserve, total oxidant burden, and oxidative DNA damage may provide a more integrated picture of redox disturbance than any single marker alone. Objective to assess serum paraoxonase-1 (PON1) activity, reduced glutathione (GSH), total oxidant status (TOS), and 8-hydroxy-2'-deoxyguanosine (8-OHdG) in patients with RA in Al-Shirqat City, Iraq, and to investigate their relationships with disease activity and inflammatory indices. An analytical case-control study included 70 patients with established RA and 70 age- and sex-matched healthy controls. PON1 activity was assessed spectrophotometrically, GSH was quantified in blood by the DTNB-based method, TOS was determined using the Erel colorimetric principle, and serum 8-OHdG was measured by competitive ELISA. Disease activity was evaluated using DAS28-CRP. Compared with controls, RA patients had significantly lower PON1 activity (131.0 ± 53.0 vs. 164.0 ± 59.0 U/L, $p = 0.001$) and GSH (6.01 ± 0.30 vs. 6.72 ± 0.32 $\mu\text{mol/g}$ protein, $p < 0.001$), together with higher TOS (7.54 ± 3.21 vs. 5.73 ± 2.81 , $p = 0.001$) and 8-OHdG (5.94 ± 2.80 vs. 3.59 ± 2.00 ng/mL, $p < 0.001$). 8-OHdG showed the strongest positive association with DAS28-CRP ($r = 0.461$, $p < 0.001$). In multivariable analysis, DAS28-CRP and CRP remained independent predictors of 8-OHdG. RA patients in Al-Shirqat demonstrate a coordinated redox disturbance involving impaired antioxidant defenses, increased systemic oxidant load, and enhanced oxidative DNA damage. 8-OHdG showed the closest relationship with disease activity and may be a useful adjunctive biomarker for characterizing the molecular burden of active disease.

Keywords: Rheumatoid Arthritis, Oxidative Stress, Paraoxonase-1, Glutathione, Total Oxidant Status, 8-Hydroxy-2'-Deoxyguanosine, DAS28, Iraq

Introduction

Rheumatoid Arthritis (RA) is a chronic systemic autoimmune inflammatory disorder affecting primarily synovial joints, but also having a widespread impact on the cardiovascular, pulmonary, hematologic, skeletal and metabolic systems. Current understanding of RA centers on the non-antibodies and non-synovial nature of this disease, and involves a series of interactions among genetic

susceptibility, environmental exposures, post-translational protein modification, adaptive immune activation, innate immune activation, altered cellular metabolism and persistent inflammatory signaling. The 2010 American College of Rheumatology/European Alliance of Associations for Rheumatology (ACR/EULAR) classification system defined an algorithm for the use of joint involvement, serology, acute-phase reactants, and symptom duration, and helped to identify those who are more likely to have progressive and erosive disease and thus benefit from earlier diagnoses [1]. After onset, RA involves proliferation of macrophages, T cells, B cells, plasma cells and activated fibroblast-LIKE synovial cells in the synovium. These cells release cytokines and chemokines involved in the recruitment of leukocytes, the activation of osteoclasts, cartilage destruction and the development of invasive pannus tissue.

One of the molecular mechanisms linking chronic inflammation to tissue damage is oxidative stress, which is one of the most uniform biochemical abnormalities found in RA. Mitochondrial pathways, NADPH oxidases, myeloperoxidase-dependent reactions and inducible nitric oxide synthase are mechanisms that activated neutrophils and macrophages use to generate reactive oxygen species (ROS) and reactive nitrogen species (RNS). Reactive species are physiologic and are involved in intracellular signaling and host defence at physiologic concentrations. But, when generation occurs in excess or over a longer period of time, it can exceed antioxidant systems and cause modification of lipids, proteins, carbohydrates and nucleic acids [2-4]. The redox imbalance that occurs is of particular interest in RA as ROS may act as a downstream consequence of inflammation as well as an active mediator of inflammatory signaling. Oxidative conditions can activate redox-sensitive transcription factors, such as NF- κ B and AP-1, which then increase the production of cytokines, adhesion molecules, cyclooxygenase products and matrix-degrading enzymes [3,5]. This way, inflammation and oxidative stress can become an autoloop instead of being two separate processes.

There are multiple potential clinical implications of oxidative damage in RA. In the joint, too much ROS can contribute to dysfunction of chondrocytes, degradation of hyaluronic acid, alteration of other proteins in the extracellular matrix, and activation of osteoclastogenic signaling. Oxidative modification of proteins can also generate or reveal new "neo"epitopes which can further activate autoimmune responses. From a systemic perspective, chronic redox imbalance can lead to endothelial dysfunction, changes in the behaviour of the lipoproteins, increased risk of atherosclerosis and the higher cardiovascular risk seen in RA patients [6,7]. This is significant as cardiovascular disease continues to be a significant long-term morbidity in RA. Hence, markers of both oxidative stress and antioxidant protection could give additional data to the traditional inflammatory markers like erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP).

Oxidative stress is not one molecule that can be measured. It is an imbalance of several pro-oxidant processes and several antioxidant processes. For this reason, attempt was made to use a multi-axis biochemical approach instead of a single biochemical marker in the present study. Total oxidant status (TOS) was chosen as an overall indicator of the cumulative oxidizing capacity in serum. The Erel method is a practical colorimetric method that converts a sample of oxidants to ferric ions by reaction with ferrous ion, thus enabling an estimation of the total oxidant content instead of determining the amount of each oxidant separately [8,9]. TOS has been linked to RA disease activity and is a good general indicator of systemic oxidant pressure but does not provide the information to identify individual chemical species of the signal.

A representative of the non-enzymatic intracellular antioxidant defence system used was reduced glutathione (GSH). GSH is a tripeptide containing glutamate, cysteine and glycine which is present at high concentrations in most cells. It contains a sulfhydryl group which allows direct scavenging of reactive species, as well as reducing equivalents for glutathione peroxidase (GPx) detoxification of hydrogen peroxide and lipid hydroperoxides. In addition, GSH is involved in the maintenance of protein thiol status, detoxification reactions, redox signaling, and the regeneration of other antioxidants. Inflammation may lead to the accumulation of chronic inflammatory activity, which can lead to increased usage of GSH and a change in GSH/GSSG ratio, lowering intracellular redox buffering capacity. Previous research in RA has revealed significant changes in the glutathione defense

system, and further recent studies that assessed erythrocyte GSH/GSSG and thiol-disulfide homeostasis have confirmed a continued dis-regulation of redox regulation [10,11].

Paraoxonase-1 (PON1) was selected as a representative enzyme antioxidant system associated with the protection of cardiovascular health and in a direct way with lipid oxidation. PON1 is a calcium dependent enzyme which is synthesized primarily in the liver and carried in the blood bound to high-density lipoprotein (HDL). It contains paraoxonase, arylesterase and lactonase activities and plays a role in the antioxidant and anti-inflammatory activity of HDL. In RA, the composition and function of HDL can change during the acute-phase response, when it can become a less effective or even a non-functional carrier. A recent systematic review and meta-analysis showed that PON1 and arylesterase activity was significantly lower in patients with RA compared to controls, with a high level of between-study heterogeneity [12]. Previous study by Tanimoto and others also revealed a decrease in serum PON1 activity in RA and correlated this decrease with the changes of HDL structure and acute-phase responses [13]. Other factors, such as genetic variation, anti-HDL antibodies, medication use, and metabolic state can also influence PON1 activity [14,15]. Thus, low PON1 could be a clinically relevant overlap area of inflammation, oxidative lipid damage and cardiovascular risk.

The downstream effects of chronic oxidative stress go beyond antioxidants and lipoproteins to affect other pathways as well. ROS, particularly hydroxyl radicals, can react with the nucleobases, and deoxyribose backbone, damaging the DNA. Guanine can be oxidized to 8-hydroxy-2'-deoxyguanosine (8-OHdG) which is one of the most studied markers of oxidative DNA damage [16]. High levels of 8-OHdG have been measured in serum, urine and synovial fluid in RA and oxidative DNA damage has been associated with disease activity and inflammatory burden [17-23]. This is of particular importance for Iraq's context as studies from Duhok and Baghdad have recently reported elevated levels of 8-OHdG or similar markers of DNA damage in Iraqi RA patients [18-20]. These results confirm the usefulness of oxidative DNA damage in the local populations and justify the use of the evaluation of 8-OHdG and other antioxidant/oxidant indexes.

While many studies have focused on oxidative stress in patients with RA, very few have assessed a lipid-associated antioxidant enzyme (PON1), a major intracellular antioxidant (GSH), an integrated oxidant measure (TOS) and a DNA-damage endpoint (8-OHdG) in the same RA cohort. This hybrid design is biologically interesting because the four markers denote different levels of the redox pathway, instead of repeating the same information. PON1 represents the extracellular pool of lipoprotein-associated antioxidant defence, GSH, the cellular pool of reducing reserve, TOS, the cumulative systemic oxidant load, and 8-OHdG the downstream consequence of oxidative attack on macromolecules. The evaluation of these together may thus yield a more coherent biochemical picture of the redox phenotype that accompanies RA [22,23].

The aim of the present study was to evaluate the activity of PON1, GSH, TOS and 8-OHdG in patients with RA from Al-Shirqat City as compared to healthy controls. A secondary objective was to look at the relationships of these biomarkers with DAS28-CRP, ESR, CRP and disease duration. The working hypothesis was that PON1 would be decreased, GSH would be decreased, and both TOS and 8-OHdG would be increased in RA patients and that the extent of the redox disturbance (8-OHdG in particular) would be correlated with clinical disease activity.

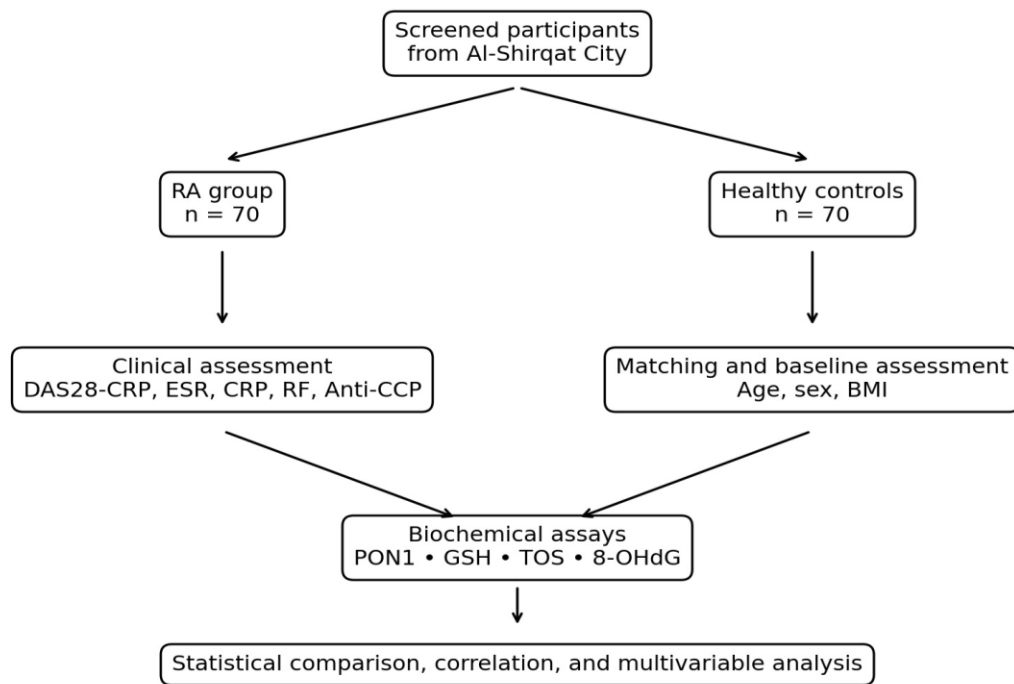


Figure 1. Conceptual redox-inflammatory pathway evaluated in the present study.

Materials and Methods

Study design and setting

This analytical, case-control study was conducted from January to May 2024, in Al-Shirqat General Hospital and affiliated Rheumatology clinics in Salah Al-Din Province, Iraq. Study design was designed to allow direct comparison of biochemical oxidative-stress indices between clinically diagnosed RA patients and a reference population from the same geographic area. It was organized in a reporting structure typical of the principles of observation in biomedical sciences, with the source population and the eligibility criteria well specified, the methods of the laboratory were clearly explained, the outputs were described, and the statistical procedures were explained.

Study population and grouping

A total of 140 participants were involved in the study, and they were split into two groups of half that size. There were 70 adults who had been diagnosed with RA and 70 apparently healthy individuals who comprised the patient and control groups, respectively. The diagnosis and classification of RA was based on clinical evaluation and fulfillment of the 2010 ACR/EULAR classification criteria, a score of $\geq 6/10$ was used to support the classification in the appropriate clinical context [1]. The controls were also recruited from the same geographical area and frequency matched with the patient group for age and sex. To minimise the possibility that the differences observed in the biomarkers could be due to large demographic differences, matching was used.

Inclusion and exclusion criteria

Eligible for inclusion were adults (18 years or older) with a confirmed diagnosis of RA and adequate clinical information to evaluate the disease activity. Patients had to be clinically stable, be able to have routine venipuncture and give their informed consent. Other systemic autoimmune conditions, uncontrolled diabetes mellitus, advanced chronic kidney disease, chronic liver disease, history of active malignancy, acute infections, pregnancy, current smoking and high dose supplementation of antioxidants were considered as exclusion criteria as they represent major alternative sources of oxidative stress. Those who had incomplete laboratory data were omitted from the final analytical data set. In the healthy-control group, subjects with a history of inflammatory, autoimmune, malignant, or major metabolic diseases were excluded, following the same rationale.

Clinical and demographic assessment

Age, sex, body mass index (BMI), duration of disease, major medication exposure and pertinent medical history were recorded on a standardized data form. Disease activity was measured in RA patients by the Disease Activity Score in 28 joints based on C-reactive protein (DAS28-CRP). The tender and swollen joint counts were collected on the clinical visit and the laboratory CRP value was added as per the calculation of DAS28-CRP. Patients were stratified into low, moderate and high disease-activity groups for descriptive interpretation. Of the 52.9% of patients in the study cohort classified as low activity, 34.3% were classified as moderate activity and 12.8% were classified as high activity.

Routine inflammatory and immunologic measurements

Routine markers of systemic inflammatory activity included ESR and CRP. To characterize the serologic profile of participants, rheumatoid factor (RF) and anti-cyclic citrullinated peptide antibodies (Anti-CCP) were added. Laboratory analyses were conducted in compliance with the manufacturer's instructions on the platforms available, automated or semi-automated. To minimize preanalytical variation, blood sampling and processing conditions were standardized as far as possible between patient and control groups.

Blood sampling and specimen processing

Venous blood samples were drawn from the subjects following an overnight fast of ~10-12 hours. The blood used for serum assays was left to coagulate and then spun at a fixed laboratory setting. Serum was immediately separated to minimize the impact of extended contact with cells and aliquots were distributed to minimize repeated freeze-thaw cycles. A separate anticoagulated blood specimen was used for GSH determination. Samples that were not analyzed immediately were kept frozen at the laboratory conditions suitable for the assay to be performed later in the batch analysis. The preanalytical protocol was the same for patient's and control samples.

Measurement of paraoxonase-1 activity

Specific activity of PON1 was determined spectrophotometrically by measuring p-nitrophenol production from paraoxon. Enzymes activity was expressed in U/L and was monitored spectrophotometrically where an increase in absorbance in response to the formation of the product was recorded, this has been a well-established method to assess functional PON1 activity [13-26]. This study analyzed PON1 because its activity was considered mainly as a functional biomarker of antioxidants, but it was also acknowledged that it may be influenced by genetic polymorphism, HDL composition, metabolism and drugs.

Measurement of reduced glutathione

The classical sulfhydryl-based method of Beutler [27] was used to determine the level of reduced glutathione in the blood. In short, GSH will interact with 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB, Ellman reagent) to produce a yellow product whose absorbance is linear with [SH]² [28]. Analytical calculation was performed in the laboratory and values are expressed in unit of $\mu\text{mol/g}$ protein. Handling of GSH is sensitive to oxidation and the sample preparation was standardized and prolonged exposure to room temperature was avoided.

Measurement of total oxidant status

Serum Total Oxidative Stress (TOS) was determined by colorimetric method based on the principle of Erel [9]. Oxidants in the sample oxidize the ferrous ions to ferric ions in acidic condition which is then reacted with xylenol orange to give colour. The colour intensity is proportional to the overall amount of oxidant molecules in the sample. The original method uses hydrogen peroxide as calibration standard and is intended to give an integrated assessment of serum oxidant burden. In this study, the TOS values were compared across groups and analyzed from the standardized output of the laboratory's assays.

Measurement of 8-hydroxy-2'-deoxyguanosine

The level of 8-OHdG was measured by sensitive competitive enzyme-linked immunosorbent assay (ELISA, Cell Biolabs, USA) as directed by the manufacturer. All the samples and standards were measured using the same assay methodology, and concentrations were determined from the standard

curve and expressed in ng/mL. To act downstream as an indicator of oxidative DNA damage, 8-OHdG was chosen because it has been extensively utilized in human redox studies [16-23] and was known to be a product of oxidative modification of guanine.

Quality assurance

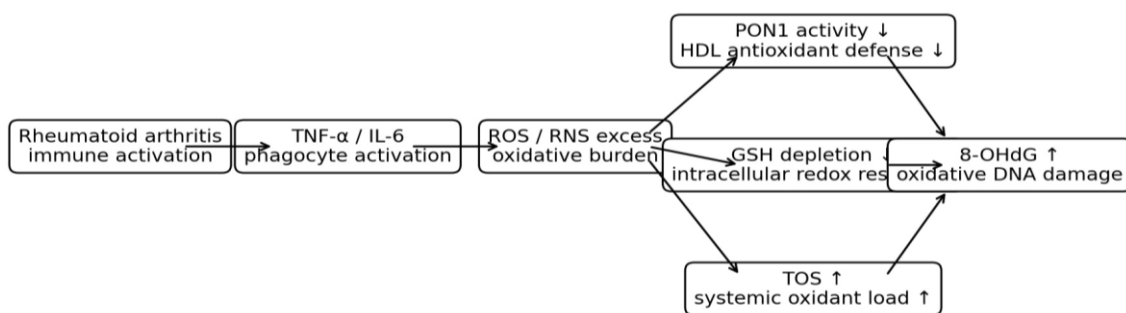
Laboratory quality control emphasized standardization in sample collection, specimen handling, preparation of reagents, instrument calibration and batch analysis. Internal controls, supplied with the reagents for assays, were used when available. Samples were reanalyzed based on laboratory practice when the analytical results were implausible before they were included in the final set of data. To minimize systematic measurement bias, the same measurement procedures and equipment were used for patient and control specimens.

Ethical considerations

Ethical considerations and approval were provided by the Institutional Review Board of University of Tikrit (Ref: Sci.Eth.2024.01). The study goal and methods were explained to all participants and informed consent was obtained in writing before they entered the study. Confidentiality of participants was ensured and samples were coded (with coded study numbers) and not identified by personally identifying information.

Statistical analysis

IBM SPSS Statistics version 26.0 was used for the statistical analysis. Continuous variables were tested for distribution with the Shapiro-Wilk test and by examination of distributional characteristics. The mean $\pm$ standard deviation (SD) was used for normally distributed variables and the median and interquartile range (IQR) for skewed variables. Frequencies and percents were used to report categorical variables. The independent-samples Student t-test was used for approximately normally distributed continuous data, the Mann-Whitney U test was used for non-normally distributed continuous data, and the chi-square test was used for categorical data for between-group comparisons. In the RA group, associations of oxidative-stress biomarkers with clinical variables were analysed with the use of Spearman's rank correlation coefficient. Independent predictors for 8-OHdG and TOS were elucidated by multiple linear regression after adjusting for the demographic and clinical covariates. A p value less than 0.05 was considered statistically significant and a two tailed test was used. To avoid reading statistical significance alone, the direction of the effect, confidence intervals and model R² values were also taken into account.



Amplified oxidative stress may reinforce synovial inflammation, tissue injury, and cardiovascular risk, creating a self-sustaining redox-inflammatory loop.

Figure 2. Study workflow and analytical sequence.

Results

Finally, 140 participants were analysed (70 patients with RA, 70 healthy subjects). The key findings were that the antioxidant defense was reduced in a coordinated manner and that various markers of oxidants and DNA damage were increased in RA patients. Results are reported in the following four domains: baseline characteristics, between-group comparisons of biomarkers, correlations with disease activity and multivariable models.

Table 1. Demographic and clinical characteristics of the study population.

Variable	RA Patients (n = 70)	Healthy Controls (n = 70)	p-value
Age (years), Mean $\pm$ SD	48.90 $\pm$ 8.20	47.50 $\pm$ 8.50	0.303
Sex (Male/Female), n (%)	29 (41.4%) / 41 (58.6%)	27 (38.6%) / 43 (61.4%)	0.863 ^a
BMI (kg/m ²), Median (IQR)	27.30 (23.35-30.85)	29.50 (25.10-33.90)	0.031
Disease Duration (years), Median (IQR)	5.40 (2.10-9.80)	N/A	-
DAS28-CRP, Median (IQR)	3.45 (2.60-4.80)	N/A	-
ESR (mm/h), Median (IQR)	32.60 (16.70-48.15)	14.45 (5.90-22.38)	<0.001
CRP (mg/L), Median (IQR)	25.97 (13.41-51.12)	2.94 (2.23-4.62)	<0.001
RF (IU/mL), Median (IQR)	147.70 (84.65-216.73)	10.00 (4.30-14.95)	<0.001
Anti-CCP (RU/mL), Mean $\pm$ SD	341.21 $\pm$ 127.41	14.05 $\pm$ 6.80	<0.001

Data are presented as Mean $\pm$ SD, Median (IQR), or n (%). ^a Chi-square test. RA, rheumatoid arthritis, BMI, body mass index, DAS28-CRP, Disease Activity Score in 28 joints, ESR, erythrocyte sedimentation rate, CRP, C-reactive protein, RF, rheumatoid factor, Anti-CCP, anti-cyclic citrullinated peptide.

Table 2. Comparison of oxidative-stress biomarkers between RA patients and healthy controls.

Biomarker	RA Patients (n = 70)	Healthy Controls (n = 70)	p-value
PON1 Activity (U/L), Mean $\pm$ SD	131.0 $\pm$ 53.0	164.0 $\pm$ 59.0	0.001
GSH (μ mol/g protein), Mean $\pm$ SD	6.01 $\pm$ 0.30	6.72 $\pm$ 0.32	<0.001
TOS, Mean $\pm$ SD	7.54 $\pm$ 3.21	5.73 $\pm$ 2.81	0.001
8-OHdG (ng/mL), Mean $\pm$ SD	5.94 $\pm$ 2.80	3.59 $\pm$ 2.00	<0.001

PON1, paraoxonase-1, GSH, reduced glutathione, TOS, total oxidant status, 8-OHdG, 8-hydroxy-2'-deoxyguanosine.

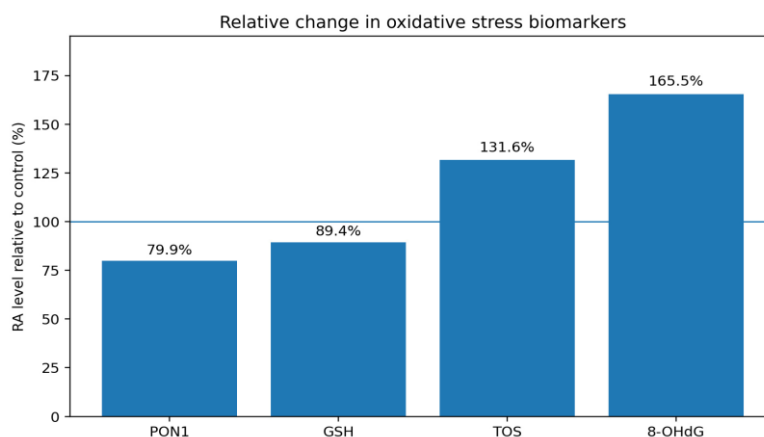
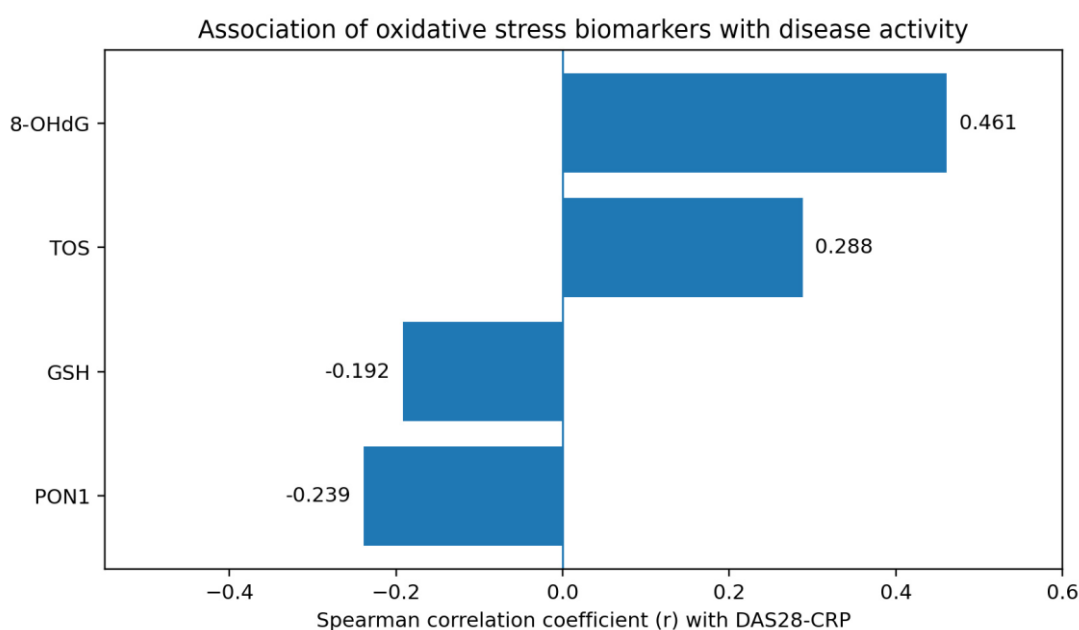
**Figure 3.** Relative biomarker levels in RA patients normalized to the healthy-control mean (control = 100%).

Table 3. Spearman correlations of oxidative-stress biomarkers with clinical and inflammatory variables in RA patients.

Biomarker	DAS28-CRP	ESR (mm/h)	CRP (mg/L)	Disease Duration (years)
PON1 (U/L)	r = -0.239, p = 0.046	r = -0.300, p = 0.012	r = -0.185, p = 0.124	r = -0.246, p = 0.040
GSH ($\mu\text{mol/g}$)	r = -0.192, p = 0.112	r = -0.262, p = 0.029	r = -0.210, p = 0.081	r = -0.150, p = 0.216
TOS	r = 0.288, p = 0.016	r = 0.251, p = 0.036	r = 0.255, p = 0.033	r = -0.257, p = 0.032
8-OHdG (ng/mL)	r = 0.461, p < 0.001	r = 0.307, p = 0.010	r = 0.385, p = 0.001	r = 0.180, p = 0.135

**Figure 4.** Spearman correlation coefficients between the four redox biomarkers and DAS28-CRP in RA patients.**Table 4.** Multiple linear regression analysis for predictors of 8-OHdG and TOS in RA patients.

Dependent variable	Independent variable	β (standardized)	95% CI	p-value
8-OHdG (ng/mL)	DAS28-CRP	0.410	0.155 to 0.665	0.002
	CRP (mg/L)	0.330	0.065 to 0.595	0.018
	PON1 (U/L)	-0.180	-0.410 to 0.050	0.120
	GSH ($\mu\text{mol/g}$)	-0.145	-0.385 to 0.095	0.230
TOS	CRP (mg/L)	0.385	0.120 to 0.650	0.005
	DAS28-CRP	0.290	0.035 to 0.545	0.026
	PON1 (U/L)	-0.220	-0.450 to 0.010	0.061

8-OHdG model: $R^2 = 0.38$, adjusted $R^2 = 0.34$, overall $p < 0.001$. TOS model: $R^2 = 0.31$, adjusted $R^2 = 0.27$, overall $p < 0.001$. Models adjusted for age, sex, BMI, and disease duration.

Demographic and clinical characteristics

Matching proved to be effective, the patient and control groups were similar with respect to age and sex. Mean age was 48.90 ± 8.20 years among RA patients and 47.50 ± 8.50 years among controls ($p = 0.303$). There was no significant difference in sex ratio between the RA group and the controls ($p = 0.863$) and women made up 58.6% of the RA group and 61.4% of the controls. BMI was slightly different between groups (median: 27.30 kg/m² for the RA patients and 29.50 kg/m² for the control group, $p =$

0.031). This difference, however, was not viewed as an indication of greater adiposity in the patient group and was therefore not interpreted as such in the multivariable analysis.

Not surprisingly, inflammatory and autoimmune indices were significantly elevated in RA patients. Median ESR was also significantly higher in RA (32.60 mm/h) than controls (14.45 mm/h) and median CRP was significantly higher in RA (25.97 mg/L) than controls (2.94 mg/L), RF and Anti-CCP values also showed marked distinction between the groups. The median duration of disease of the RA cohort was 5.40 years, and the median DAS28-CRP was 3.45. The activity scores from this study showed that 37 patients were low activity, 24 patients were moderate activity, and 9 patients were high activity. This distribution allowed for evaluation of the correlation of markers in a clinically relevant spectrum of disease activity, not only at extreme disease activity.

Oxidative-stress biomarkers

The four a priori redox biomarkers were found to be significantly different in patients with RA as compared to controls. The mean level of PON1 activity in the RA group was 131.0 ± 53.0 U/L, which was significantly lower than the 164.0 ± 59.0 U/L in the controls ($p = 0.001$), indicating about 20% lower mean activity in RA patients. GSH was likewise significantly lower in patients (6.01 ± 0.30 $\mu\text{mol/g}$ protein) than controls (6.72 ± 0.32 $\mu\text{mol/g}$ protein, $p < 0.001$). In contrast, systemic oxidant and DNA-damage indicators were increased: TOS was 7.54 ± 3.21 in patients versus 5.73 ± 2.81 in controls ($p = 0.001$), while 8-OHdG was 5.94 ± 2.80 ng/mL versus 3.59 ± 2.00 ng/mL ($p < 0.001$).

After normalization to the mean of the controls, the values for PON1 and GSH in RA were about 79.9% and 89.4% of the controls, respectively, while the values for TOS and 8-OHdG were about 131.6% and 165.5% of the controls, respectively. This pattern suggests that the RA redox phenotype was not restricted to a single biochemical space. Rather, it had decreased antioxidant protection and increased oxidative consequences, with the highest proportional between-group difference found for 8-OHdG.

3.3 Correlations with disease activity and inflammation

In the RA group, the strongest association was found between 8-OHdG and clinical activity of disease. A moderate correlation was observed between 8-OHdG and DAS28-CRP ($r = 0.461$, $p < 0.001$). ESR levels and CRP were also strongly correlated with 8-OHdG ($r = 0.307$, $p = 0.010$ and $r = 0.385$, $p = 0.001$, respectively), confirming the correlation between oxidative DNA damage and systemic inflammatory activity. The correlation with disease duration was positive but not significant ($r = 0.180$, $p = 0.135$), suggesting that inflammatory activity at the moment of measurement might be more closely related with 8-OHdG than just the duration of disease.

TOS correlated positively with DAS28-CRP ($r = 0.288$, $p = 0.016$), ESR ($r = 0.251$, $p = 0.036$), and CRP ($r = 0.255$, $p = 0.033$). The coefficients were significantly less than those found for 8-OHdG, but were always of the same sign, with more oxidant burden being associated with more disease activity. There was a weak inverse correlation between TOS and the disease duration ($r = -0.257$, $p = 0.032$). The result of this study must be considered with prudence since the duration of the disease is also associated with treatment exposure and adaptation and patients' survival under long-term clinical care.

PON1 activity was inversely correlated with DAS28-CRP ($r = -0.239$, $p = 0.046$), ESR ($r = -0.300$, $p = 0.012$), and disease duration ($r = -0.246$, $p = 0.040$), while its association with CRP did not reach statistical significance ($r = -0.185$, $p = 0.124$). There was a strong negative correlation between GSH and ESR ($r = -0.262$, $p = 0.029$) but not with DAS28-CRP, CRP or disease duration at the conventional level of significance. Therefore, the group-level reduction in both antioxidant markers was not as closely correlated with clinical activity as was 8-OHdG, despite the fact that both markers were reduced at the group level.

Multivariable analysis

To find the independent predictors of oxidative DNA damage and total oxidant burden, multiple linear regression was performed. In the model for 8-OHdG, DAS28-CRP remained independently associated with higher 8-OHdG (standardized $\beta = 0.410$, 95% CI 0.155-0.665, $p = 0.002$), and CRP was also independently associated with higher 8-OHdG ($\beta = 0.330$, 95% CI 0.065-0.595, $p = 0.018$). These

independent associations were no longer present after adjusting for PON1 and GSH. The model of 8-OHdG accounted for 38% of the variation observed (adjusted $R^2 = 0.34$, overall $p < 0.001$).

For TOS, CRP ($\beta = 0.385$, 95% CI 0.120-0.650, $p = 0.005$) and DAS28-CRP ($\beta = 0.290$, 95% CI 0.035-0.545, $p = 0.026$) were independently associated with higher values, while PON1 showed an inverse trend that did not reach statistical significance ($\beta = -0.220$, $p = 0.061$). The TOS model explained 31% of variance (adjusted $R^2 = 0.27$, overall $p < 0.001$). These analyses support the concept that the clinical/immunological activity is closely related to the biochemical level of oxidative stress, especially oxidative damage to DNA.

Discussion

In the present study, an integrated multi-level disturbance of redox homeostasis in Al-Shirqat City patients suffering from RA was demonstrated. The biochemical profile included the decrease in PON1 activity, the decrease in GSH content and the elevation in TOS and serum 8-OHdG. Notably, the study did not consider these markers alone. Instead, it was the association of an HDL-associated antioxidant enzyme, one of the major reducing agents in the cell, a whole-body oxidant burden, and a downstream marker of oxidative DNA damage. The integrated design offers a more biologically informative view than study of a single marker, since it includes a reduction in defense and an accumulation of molecular damage.

The first one was a large decrease in PON1 activity in patients suffering from RA. This is in keeping with a large body of literature that has associated impaired antioxidant function of HDL with inflammatory rheumatic diseases. In 2022, a systematic review and a meta-analysis of 17 studies on PON1 activity revealed statistically significantly reduced levels of PON1 activity in individuals with RA compared to healthy controls, but there was considerable heterogeneity between the studies [12]. In a previous study, Tanimoto et al. demonstrated that serum PON1 activity was decreased in RA patients without significant difference in total lipid levels compared to healthy controls [13] indicating that the qualitative abnormality of HDL rather than the quantitative abnormality of lipid levels may be more informative. Other studies have demonstrated anti-HDL antibodies in RA, which also affect PON1 activity in addition to the rs662 polymorphism, and suggests that there is both a genetic and inflammatory regulation [14].

The decrease in PON1 activity found in the present cohort may be due to several reasons. PON1 is primarily associated with HDL and inflammation can significantly alter the composition of HDL. Serum amyloid A can be accumulated in HDL particles, and the composition of apolipoproteins and the associated enzymes are changed during the acute-phase response. Therefore, HDL can lose some of its antioxidant and cholesterol-efflux abilities and be functionally impaired. One further step is that oxidized lipids can contribute to this dysfunction and thus form a vicious circle where reduced PON1 facilitates further lipid oxidation and lipid oxidation reduces the protective role of HDL [15]. As stated in the previous study, the negative correlation between PON1 and ESR in the present study seems to be a consequence of inflammation causing a decrease in enzyme activity.

The link between PON1 and the joint is more than just a connection. RA is linked to elevated risk of cardiovascular disease, which is present beyond what is accounted for by traditional risk factors. This excess risk is attributed to persistent inflammation, endothelial dysfunction, altered function of lipoproteins, oxidative modification of LDL and prothrombotic signaling [6,7]. A decrease in PON1 activity may thus be of clinical relevance, but not necessarily specific to RA. In the current study, the activity of PON1 was about one-fifth lower in the patients than in controls. However, this difference does not prove cardiovascular risk, but it adds weight to the notion that the antioxidant properties of HDL are impaired in RA and that standard measurements of lipoprotein concentrations may not accurately reflect lipoprotein quality.

The second principal finding was the reduced GSH levels in patients with RA. GSH plays a role in intracellular antioxidant defense and is involved in detoxification of hydrogen peroxide and lipid hydroperoxides, maintenance of protein sulfhydryl groups, xenobiotic conjugation, and regulation of redox sensitive signaling via its interaction with various proteins [8,10]. Chronic inflammatory

activation may lead to a faster consumption of GSH than its regeneration, and inflammatory cells may have a significant oxidative demand. Previous studies by Hassan et al. showed that the glutathione defense system was depleted in RA and reduced glutathione levels along with changes in glutathione-dependent enzymes were decreased [10]. More recently, Alisik et al. have reported that RA not only affects the absolute concentrations of antioxidants, but also that the balance of reduced/oxidized glutathione (GSH/GSSG) of erythrocytes and the serum thiol-disulfide balance is disturbed as well [11].

The present finding of lower GSH is also confirmed by recent evidence in Iraq. A study in Baghdad in 2026 found decreased GSH and increased MDA and inflammatory cytokines levels in Iraqi RA patients [32]. Numerical differences between the results in the two studies are limited by variations in specimen matrix, assay method, patient characteristics, and treatment exposure, but the direction of the effects is quite consistent. This regional convergence is significant since it suggests that this glutathione defence is not only present in populations from Europe or East Asia, but also for clinical populations from Iraq.

Within patient, GSH was significantly correlated with ESR, but not with DAS28-CRP or CRP. There are various possible explanations. First, GSH is controlled by its synthesis, recycling, availability of dietary substrates, and export from the cell, and a single concentration may not be a perfect measure of short-term inflammatory changes. Second, the GSH/GSSG ratio might not just reflect the state of GSH, but rather the dynamic redox changes. Third, the use of drugs and nutrition can affect glutathione metabolism. The strong case-control difference in GSH thus indicates a population level decrease in antioxidant reserve and the weaker correlations indicate that GSH could not be used as a sole activity marker but is more appropriate to be combined with other markers of redox status into a redox panel [28].

The TOS was much higher in RA patients, which shows that the cumulative oxidizing capacity of serum is greater in RA. The benefit of TOS is its conceptual simplicity: rather than measure several unstable oxidants separately it uses a standard colorimetric reaction to estimate the contribution of a number of these oxidants [9]. The increased TOS observed in patients with active RA and significant correlation between oxidant/antioxidant status and disease activity reported by Zhou et al. [24] suggest such a correlation. Recent studies have also demonstrated that the total oxidant or antioxidant status is abnormal in RA and other autoimmune disease groups [25]. The present study has further confirmed these observations for patients in Al-Shirqat City and found that TOS was positively correlated with DAS28-CRP, ESR and CRP.

The magnitude of the correlation between TOS and disease activity was low and not high. This is biologically plausible as it is dependent on several circulating oxidants and because other factors associated with TOS (metabolic, dietary, environmental and medication) are not included in the DAS28. Moreover, oxidative stress does not only occur in inflamed joints. Circulating oxidant burden can result from liver metabolism, vascular inflammation, adipose tissue, mitochondrial function and comorbid conditions. Therefore, TOS should be viewed as a systemic integrative marker and not a disease-specific marker.

The association of weak inverse nature between TOS and disease duration should be interpreted with caution. The cross-sectional association may not be the best way to establish a true decrease in oxidant burden over time. Higher levels of cumulative exposure to disease-modifying antirheumatic drugs (DMARDs), better treatment optimisation, lower inflammatory activity in survivors, and differences in disease phenotype may all be related to longer disease duration. Certain longitudinal studies have shown decreases in oxidative DNA damage after successful treatment with methotrexate and biologic agents, which act through anti-inflammatory properties [20-25]. Thus, the association with disease duration should be interpreted as a hypothesis-generating rather than definitive evidence of a decrease in oxidative stress over time.

The most remarkable result of the current study was the increase in 8-OHdG. For the 4 major biomarkers, the mean serum concentration was higher than the control group, with the highest proportional difference in RA patients, at 65%. 8-OHdG is produced as a result of oxidative modification of guanine residues in DNA and is widely assumed as an indicator of oxidative DNA

damage [16]. The significance of its biological impact is different from TOS, because 8-OHdG represents a late product, as a consequence of the oxidative attack on an important macromolecule, while TOS is a measure of the oxidizing potential of the circulating environment. This difference could contribute to the correlation of 8-OHdG with DAS28-CRP, which was stronger than that of the other markers measured.

The results of 8-OHdG are in accordance with both the international literature and the Iraqi literature. Altindag et al., showed that RA had higher DNA damage and oxidative stress [17]. A large sample of Iraqi patients was included in Hassan's study in Duhok in 2024, which showed significantly higher levels of 8-OHdG in RA patients than in controls, with higher levels being associated with higher disease activity [18]. Al-Jumaily et al. [19] also found abnormal levels of oxidative DNA damage parameters in Iraqi RA patients. Both Salman and Al-Jumaily found some abnormalities in oxidative DNA damage parameters in Iraqi RA patients [19]. Recently Al-Bassam and his group from the University of Baghdad reported that 8-OHdG is a promising biomarker in RA field, which also led to an increased local interest in oxidative DNA damage [20]. These independent Iraqi investigations make the new Al-Shirqat discoveries so relevant as they allow comparison within the same national context and yet constitute a separate regional group.

The positive correlations of 8-OHdG with DAS28-CRP, ESR and CRP confirm the hypothesis that oxidative damage to DNA is correlated with inflammatory state. One mechanistic explanation is that the activated leukocytes generate ROS in a dose-dependent fashion depending on the amount of inflammation, thus causing more cells in the blood and tissues to be exposed to these oxidants. Mitochondrial dysfunction could also exacerbate ROS production in synovial cells, and impaired antioxidant defence mechanisms could lead to lower ROS neutralizing capacity [4]. DNA oxidation can then accumulate if the rate of damage is higher than the rate of repair and removal of the damage. The moderate correlation with DAS28 is indicative of a correlation between 8-OHdG and clinical disease activity, but not a redundancy. This is desirable because if it merely matched perfectly with CRP or DAS28 then there would be little added value to it as a complementary biomarker.

This is confirmed by multivariable analysis. Multivariate analysis showed DAS28-CRP and CRP, but not PON1 or GSH, to be independent predictors of 8-OHdG after controlling for age, sex, BMI, disease duration. These results indicate that oxidative DNA damage has a tight association with the present inflammatory state. They do not imply that PON1 and GSH are of no biological relevance. Rather, antioxidant depletion could be a part of a complicated network whose impact is mediated by total redox balance, not by a linear dependency on 8-OHdG. The adjusted R^2 of 0.34 also suggests that there is a considerable unexplained variation, to be expected when a biomarker is influenced by many genetic, environmental and metabolic factors.

Inflammation and oxidative stress may be inter-related. Cytokines, such as the tumor necrosis factor alpha (TNF- α) and Interleukin-6 (IL-6), have been shown to stimulate ROS production, and oxidative conditions can trigger redox-sensitive transcription and inflammasome pathways that can continue to stimulate cytokine production. ROS also can affect proliferation, angiogenesis, degradation of ECM and osteoclast differentiation of the fibroblast-like synoviocytes [2-5]. Therefore, high TOS and 8-OHdG levels are not to be considered as just laboratory phenomena. They are molecular markers of pathways that might be involved in the pathogenesis of RA that can lead to systemic effects and chronic symptoms.

In recent years, Iraqi research has also confirmed the presence of oxidative and inflammatory abnormalities. Hamodat and Abdullah analyzed 120 RA patients and 100 healthy controls and confirmed that oxidative stress markers were more elevated with the severity of disease along with elevated IL-6 [33]. Increased MDA and inflammatory cytokines along with decreased GSH and total antioxidant capacity was also reported by another recent Iraqi case-control study [32]. Although the biomarkers and the methods used with the assays are different, the similarity of these patterns to the results found in the current Al-Shirqat study further corroborates external coherence. Simultaneously, regional replication is not redundant, as environmental exposures, diet, treatment availability, socioeconomic factors, and local demographic profiles can all impact redox markers.

The association of PON1 and GSH in the present study is particularly informative as it represents two antioxidant compartments. The extracellular lipoprotein-associated antioxidant activity (LCAA) is mostly represented by PON1, while the intracellular reducing buffer is mostly represented by GSH. These reductions were observed at the same time, indicating that antioxidant dysfunction in RA is not restricted to one biochemical pathway. This pattern may contribute to an understanding of how increased oxidant burden escalates to DNA damage. As a consequence, the following simplified mechanistic series of events is plausible: chronic immune activation leads to elevated production of ROS and RNS, antioxidant defenses, such as PON1 and GSH become relatively low, oxidative load from ROS and RNS build up and oxidative modification of DNA becomes more prominent as shown by 8-OHdG. A summary of this conceptual pathway is shown in figure 2.

The question that is important is whether these markers are in any way diagnostic or monitoring. The current case-control study aimed to evaluate but not validate a diagnostic test and the results should not be used to determine clinical cut-off levels. RA diagnosis and measurement of activity continues to be based on the use of RF, Anti-CCP, CRP, ESR and DAS28. Redox biomarkers could, however, complement the information from conventional markers, since they measure biological information indirectly. For example, 8-OHdG could provide the understanding of the molecular effects of chronic oxidative damage, and PON1 could provide the understanding of altered lipoproteins mediated by their antioxidant function and potential cardiovascular vulnerability.

The most promising in the available clinical monitoring data set is 8-OHdG which had the greatest case-control difference and highest association with DAS28-CRP. However, a number of actions would have to be taken before the routine use of it. Assays should be standardized between laboratories, biologic variability should be well defined, clinically relevant thresholds need to be determined, and longitudinal studies should show that alterations in 8-OHdG reflect alterations in disease activity or predict outcomes beyond currently available markers. When using TOS, care should be taken as well. Although it is easy to measure, standardization of the platform and clear understanding of units are required to make it a part of routine care.

The results also have therapeutic implications. While this antioxidant supplementation is appealing from a biological point of view, oxidative stress in RA is related to and integrated in the immune activation process and cannot be assumed to be sensitive to this easy-to-give treatment. However, systematic reviews of interventions aimed at antioxidants have yielded inconsistent results, some show a benefit on oxidative markers, and others show no clinical benefit [30,31]. So, the focus continues to be the effective treatment of underlying inflammatory disease with evidence-based RA therapy. If studied, antioxidants should be used in addition to DMARDs, not instead of them. Future trials should target patients with suspected redox imbalance, based on objective data, and determine if biochemical improvements result in clinical and/or cardiovascular benefits.

The PON1 gene could be of interest for cardiovascular-risk studies. It has been reported to be less active in RA and inflammation and genetic factors can alter its function [12-14]. Future studies could therefore utilize PON1 as a marker of HDL and also the HDL functionality, oxidized LDL, apolipoprotein composition, and vascular endpoints. This method could help to determine if low PON1 is simply a marker of inflammation or independently represents patients with a greater vascular risk. The present data does not provide an answer to this question, but the dramatic decrease in the level of PON1 does give ample justification for further investigation.

The study has a number of methodological advantages. The four biomarkers were chosen as they relate to different but biologically related parts of the redox system. Second, there were no two important sources of confounding as the age and sex of both patient and control groups were matched. Third, this study used DAS28-CRP along with other conventional clinical parameters, which allowed for the biochemical findings to be integrated with other measures. Fourth, multivariable analysis was performed to assess for the presence or absence of relationships after adjustment for potential confounders. Finally, the findings of the study are from the Al-Shirqat City which represents some regional evidence to the under-represented biomedical research field in Iraq.

There are also a number of restrictions to be made. Case-control, cross sectional design does not allow for temporal or causal inference. It does not know if oxidative stress leads to aggravating disease activity or if it is primarily as a result of inflammation. This would be better addressed with longitudinal sampling before and after the escalation of treatment. The sample size is sufficient for detailed subgroup analyses of medication class, serologic phenotype, sex, and disease-activity category for the principal case-control comparisons, but is not large enough for detailed subgroup analyses of these other variables. Future studies should be appropriately powered to compare methotrexate, biologic, corticosteroid and combination-treatment groups due to the possibility of treatment exposure impacting on oxidative biomarkers. Another restriction of the study is that the activity of PON1 is influenced by common polymorphisms in the gene, such as Q192R, that were not genotyped in the present study [14]. Genotyping might be useful for differentiating genetic from inflammatory suppression. Similarly, the HDL function and concentration, oxidized LDL, and serum amyloid A, would provide further interpretation of the PON1 pathway. The simultaneous measurement of GSSG and calculation of GSH/GSSG ratio would give a more complete picture of intracellular redox state than GSH alone for glutathione [11]. To allow for a direct comparison with the TOS/TAS approach [24,25] it would be useful to add total antioxidant status and calculate an oxidative stress index.

There are also some limitations with the use of serum 8-OHdG. 8-OHdG can be found in serum, plasma, urine, cellular DNA and other biological matrices, and values may not be comparable. The urinary 8-OHdG has advantages in estimating the whole-body oxidative DNA damage, and may not be influenced by some short-term fluctuations, while the serum measurement may more closely reflect the recent circulating release. Absolute values may also differ depending on the analytical method (ELISA, HPLC, mass-spectrometric). Thus, the presented results should be considered on the basis of the current matrix and the current assay method and should not be compared numerically among all published studies without qualification. Even with these restrictions, the overall pattern is internally coherent, with antioxidant defense markers being low, oxidative burden markers being high and the downstream DNA-damage marker being most related to disease activity. There is a logical and strong supporting evidence for this pattern from systematic reviews, international cohort studies, and several studies conducted in Iraq [12,18-20,30,32,33]. These lines of evidence converge and indicate that redox imbalance is a reproducible property of RA and is worthy of further investigation as part of the systemic biology of RA.

What the present study brings to the publication area is not the finding of oxidative stress in RA, which is well known, but the finding that this oxidative stress leads to the other symptoms. The significance is that it allows four markers that complement each other to be evaluated simultaneously in a defined cohort of Iraqi patients and that these markers do not act in identical ways in relation to clinical activity. In particular, the antioxidant markers do not seem to be as reflective of the downstream molecular effects of active inflammation as does 8-OHdG, and the antioxidant capacity reductions shown by PON1 and GSH are not as clear in this context. The results are presented in this holistic framework, neither exaggerating the novelty nor offering merely a scientific justification, but rather a contribution that is relevant to the region.

Conclusions

The RA patients in Al-Shirqat City had a definite systemic redox imbalance with significantly decreased PON1 activity and GSH and significantly elevated TOS and 8-OHdG compared with healthy controls of similar age and sex. The integrated pattern provides evidence that in this group of patients, RA is linked with a decrease in both extracellular lipid associated and intracellular antioxidant defence, along with higher circulating oxidant burden and oxidative damage to DNA. In all the measured biomarkers, 8-OHdG had the highest correlation with DAS28-CRP, and also remained independently associated with clinical and inflammatory activity in multivariable analysis. The results indicate that a multi-marker redox approach should be used in RA research and suggest that 8-OHdG is the most promising marker studied to date for longitudinal evaluation. The biomarkers should be used in addition to, not in place of, standard clinical and inflammatory markers.

Recommendations

Future studies should confirm the findings in larger Iraqi cohorts and longitudinal measures should be taken before and after therapy changes for RA. A mechanistic profile including simultaneous assessment of PON1 genotype, HDL function, GSH/GSSG balance, total antioxidant status and oxidative stress index would provide a more comprehensive profile. Medication specific analyses are also necessary since methotrexate, corticosteroids and biologic medications can affect oxidative biomarkers in different ways. To enhance analytical validity, urinary 8-OHdG or chromatographic confirmation of oxidative DNA damage may be performed. Last, perhaps, the use of antioxidants comes into the realm of investigation only within well-designed controlled trials, and should be seen as an adjunct to standard disease-modifying therapy, rather than as an alternative approach.

Study Limitations

The main limitations are that the study is a cross-sectional case-control study, the study was conducted in one geographic area, the study sample was relatively small for some of the subgroups and that the study design was prone to residual confounding from diet, medication exposure, and unmeasured environmental factors. Functionality of HDL was not measured and PON1 polymorphisms were not genotyped. GSH was measured without simultaneous determination of GSSG and TOS was not reported together with total antioxidant status to calculate an oxidative stress index. ELISA was used to measure serum 8-OHdG but it was not a reference method. These restrictions are not an indication of invalidity of the observed group differences, but should be taken into account when generalizing the findings and when considering the biomarkers as monitoring tools for clinical use.

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