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SERUM SOLUBLE KLOTHO, INTACT FGF-23, AND OXIDATIVE STRESS IN RHEUMATOID ARTHRITIS: ASSOCIATIONS WITH DAS28-CRP IN A CASE-CONTROL STUDY

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ABSTRACT

Background: Rheumatoid arthritis (RA) is a systemic autoimmune disease in which chronic inflammation and oxidative stress interact with metabolic and endocrine pathways. Disturbance of the soluble Klotho (s-Klotho)/FGF-23 axis may provide a link between inflammatory burden, redox imbalance, and disease activity.

Objective: To compare serum s-Klotho, intact fibroblast growth factor-23 (FGF-23), MDA measured by the thiobarbituric acid reactive substances (TBARS) assay, and FRAP-derived total reducing capacity between patients with RA and matched healthy controls, and to evaluate their relationships with DAS28-CRP.

Methods: Ninety patients with RA and 90 age- and sex-matched healthy controls were studied. s-Klotho and intact FGF-23 were measured by ELISA. Lipid peroxidation was estimated using the TBARS assay and total reducing capacity using the FRAP assay. Between-group comparisons, effect sizes, Pearson correlations, Holm-Bonferroni correction for 15 pairwise correlations, and a simultaneous multivariable biomarker model were used. Multicollinearity was assessed by variance inflation factors (VIFs).

Results: RA patients had lower s-Klotho (385.4 ± 88.2 vs. 742.1 ± 135.6 pg/mL) and TAC (FRAP assay) (0.81 ± 0.16 vs. 1.48 ± 0.21 mmol/L), but higher intact FGF-23 (88.6 ± 21.4 vs. 41.8 ± 10.5 pg/mL) and MDA by TBARS (5.24 ± 1.12 vs. 1.92 ± 0.41 nmol/mL); all $p < 0.001$. s-Klotho correlated inversely with MDA ($r = -0.642$) and DAS28-CRP ($r = -0.615$), whereas MDA correlated positively with DAS28-CRP ($r = 0.638$). All 15 pairwise correlations remained significant after Holm-Bonferroni correction. In the biomarker model, MDA and s-Klotho showed the strongest independent statistical associations with DAS28-CRP; all predictor VIFs were <2.1.

Conclusion: RA was associated with lower circulating s-Klotho and FRAP-derived reducing capacity together with higher FGF-23 and TBARS-derived lipid peroxidation. The results support an endocrine-redox association with disease activity, but the case-control design and incomplete adjustment for renal, mineral-metabolism, and treatment variables require cautious interpretation.

Keywords: Rheumatoid Arthritis; Soluble Klotho; FGF-23; Oxidative Stress; TBARS; FRAP; DAS28-CRP

1 INTRODUCTION

Rheumatoid arthritis (RA) is a chronic systemic autoimmune disease characterized by persistent synovitis, autoantibody formation, progressive cartilage and bone damage, and clinically important extra-articular manifestations [1-3]. Although modern disease-modifying antirheumatic drugs have transformed disease control, substantial heterogeneity remains in inflammatory burden, treatment response, and systemic complications.

Oxidative stress is an established component of RA pathophysiology. Activated neutrophils, macrophages, and synovial cells generate reactive oxygen species (ROS), while persistent inflammation can weaken enzymatic and non-enzymatic antioxidant defenses [4-8]. Lipid peroxidation can be estimated operationally by thiobarbituric acid reactive substances (TBARS), frequently expressed as malondialdehyde (MDA) equivalents, whereas ferric reducing ability of plasma (FRAP) reflects the total reducing capacity of circulating antioxidants rather than the activity of a single antioxidant system.

The Klotho/FGF-23 endocrine axis provides a biologically plausible interface between inflammation, renal-mineral physiology, and oxidative stress. Alpha-Klotho exists as a membrane-bound protein and as a circulating soluble form generated predominantly by ectodomain shedding. Membrane Klotho participates in high-affinity FGF-23 signaling through FGF receptors, whereas soluble Klotho has additional endocrine, anti-inflammatory, and antioxidant actions [9-16]. Inflammatory mediators can suppress Klotho expression through NF-kappaB-dependent pathways [17,18], and experimental studies indicate that Klotho can enhance oxidative-stress resistance through FOXO-related antioxidant signaling [19,20].

Human evidence in RA remains heterogeneous. Alvarez-Cienfuegos et al. reported altered FGF23-Klotho biology in RA and observed particularly high circulating Klotho among biologic-treated patients [21]. In contrast, a large NHANES-based analysis found an inverse association between systemic immune-inflammation burden and serum Klotho in individuals with RA [22]. Differences in treatment exposure, renal function, mineral metabolism, disease activity, and assay characteristics may explain part of this variability.

The present case-control study evaluated serum s-Klotho, intact FGF-23, MDA by TBARS assay as an operational index of lipid peroxidation, and TAC by FRAP assay as an index of total reducing capacity in RA patients and matched healthy controls. The study further examined biomarker patterns across DAS28-CRP activity strata, multiplicity-adjusted correlations, and simultaneous biomarker associations with disease activity.

2 MATERIALS AND METHODS

2.1 Study Design and Participants

A case-control study was conducted at the Ibn Al-Baladi Specialist Center for Hereditary Blood Diseases in Baghdad, where participants were recruited. The study included 90 patients with RA and 90 age- and sex-matched healthy controls. RA was classified according to the 2010 American College of Rheumatology/European League Against Rheumatism (ACR/EULAR) classification criteria [23]. Controls had no known inflammatory rheumatic disease and were selected to provide a comparable age and sex distribution.

Participants with chronic kidney disease defined as estimated glomerular filtration rate (eGFR) <60 mL/min/1.73 m^2, diabetes mellitus, overt cardiovascular disease, active infection, malignancy, current smoking, or current antioxidant-supplement/calcitriol use during the preceding 3 months were excluded. These prespecified exclusions were intended to reduce major confounding of circulating Klotho, FGF-23, and redox markers. The restrictive criteria also mean that the findings primarily apply to RA patients without these major comorbidities.

2.2 Clinical Assessment

Age, sex, body mass index (BMI), disease duration, rheumatoid factor (RF), anti-citrullinated protein antibody (ACPA) status, erythrocyte sedimentation rate (ESR), and high-sensitivity C-reactive protein (hs-CRP) were recorded. Disease activity was evaluated using DAS28-CRP.

$$DAS28\text{-}CRP = 0.56\sqrt{TJC28} + 0.28\sqrt{SJC28} + 0.36\ \ln(CRP + 1) + 0.014(GH) + 0.96$$

TJC28 denotes the 28-joint tender joint count, SJC28 the 28-joint swollen joint count, and GH the patient global-health visual-analogue score. For severity-stratified analyses, patients were categorized as having moderate activity ($3.2 \leq$ DAS28-CRP ≤ 5.1) or high activity (DAS28-CRP >5.1).

2.3 Blood Collection and Biochemical Assays

After a 12-hour overnight fast, 8 mL of venous blood was collected. Serum was separated by centrifugation at 3000 rpm for 10 minutes and stored at -80 °C until biochemical analysis.

- Serum s-Klotho: quantified using a human soluble Klotho sandwich ELISA kit (Immuno-Biological Laboratories, Japan; analytical sensitivity 6.15 pg/mL).
- Intact FGF-23: measured using an intact FGF-23 ELISA kit (Kainos Laboratories, Tokyo, Japan; analytical sensitivity 3.0 pg/mL).
- MDA by TBARS assay: measured spectrophotometrically at 532 nm and interpreted as an operational index of lipid peroxidation. The TBARS reaction is not chemically specific for MDA alone; therefore, results are described throughout as MDA by TBARS/TBARS-derived lipid peroxidation [24].
- TAC by FRAP assay: measured at 593 nm using the ferric reducing ability of plasma principle and interpreted as FRAP-derived total reducing capacity [25].
- Inflammatory markers: ESR was determined by the Westergren method and hs-CRP by turbidimetric immunoassay.

2.4 Statistical Analysis

Continuous variables are presented as mean ± standard deviation (SD), and categorical variables as number and percentage. Independent-samples t tests were used for two-group continuous comparisons and chi-square tests for categorical comparisons. Mean differences with 95% confidence intervals (CIs) and Cohen standardized mean differences (d) were reported for the principal biomarker comparisons.

Pearson correlation coefficients were calculated among s-Klotho, FGF-23, MDA, TAC, DAS28-CRP, and hs-CRP in the RA group. Because the 6-variable correlation matrix contains 15 unique pairwise tests, family-wise multiplicity was controlled using the Holm-Bonferroni procedure. For reference, the conventional Bonferroni threshold was 0.05/15 = 0.0033. All 15 observed correlations remained statistically significant after multiplicity correction.

DAS28-CRP was modeled as the dependent variable with s-Klotho, FGF-23, MDA, and TAC entered simultaneously. hs-CRP was not entered because CRP is mathematically incorporated into DAS28-CRP. Multicollinearity was evaluated using variance inflation factors (VIFs); VIFs ranged from 1.45 to 2.05, indicating no concerning collinearity among the four biomarker predictors. Two-sided $p < 0.05$ was used for the regression model.

The primary regression model is therefore a biomarker-adjusted model rather than a fully clinical-covariate-adjusted model. Continuous eGFR values and individual treatment-exposure variables were not available in the analytical dataset used for this manuscript and were not imputed. Consequently, the regression estimates should be interpreted as independent statistical associations among the measured biomarkers rather than as fully confounder-adjusted causal effects.

With 90 participants per group, the available sample provides approximately 80% power to detect an independent-group standardized mean difference of about 0.42 at a two-sided alpha level of 0.05. This is a design-sensitivity estimate for the available sample.

2.5 Ethical Considerations

The study was conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from all adult participants and from parents or legal guardians of minors; assent was obtained from minors when applicable after a full explanation of the study objectives and procedures.

3 RESULTS

3.1 Demographic and Clinical Characteristics

The RA and control groups were comparable in age, sex distribution, and BMI. Inflammatory activity markers were substantially higher in RA. The RA cohort had a mean disease duration of 6.8 ± 4.2 years and a mean DAS28-CRP of 4.92 ± 1.18.

Table 1: Demographic, clinical, and inflammatory characteristics of RA patients and healthy controls

Parameter	RA (n=90)	Controls (n=90)	Test statistic	p value
Age (years)	48.6 ± 9.4	47.2 ± 8.8	t = 1.031	0.304
Sex, female/male	72/18 (80%/20%)	72/18 (80%/20%)	χ^2 = 0.000	1.000
BMI (kg/m^2)	26.4 ± 3.1	25.8 ± 2.9	t = 1.341	0.182
Disease duration (years)	6.8 ± 4.2	—	—	—
DAS28-CRP	4.92 ± 1.18	—	—	—
RF positive	68 (75.6%)	0 (0%)	—	<0.001
ACPA positive	74 (82.2%)	0 (0%)	—	<0.001
ESR (mm/1st h)	44.2 ± 16.8	11.4 ± 4.2	t = 17.920	<0.001
hs-CRP (mg/L)	18.6 ± 7.4	2.1 ± 0.8	t = 20.915	<0.001

Values are mean ± SD or n (%), as appropriate. BMI, body mass index; RF, rheumatoid factor; ACPA, anti-citrullinated protein antibody; ESR, erythrocyte sedimentation rate; hs-CRP, high-sensitivity C-reactive protein. eGFR <60 mL/min/1.73 m^2 was an exclusion criterion.

3.2 Primary Biomarker Comparisons

All four target biomarkers differed markedly between groups. s-Klotho and FRAP-derived total reducing capacity were lower in RA, whereas intact FGF-23 and MDA by TBARS were higher. The effect sizes were large across all four biomarkers.

Table 2: Primary biomarker differences between RA patients and healthy controls

Biomarker	RA (n=90)	Controls (n=90)	Mean difference (95% CI)	Cohen d	p value
s-Klotho (pg/mL)	385.4 ± 88.2	742.1 ± 135.6	-356.7 (-390.1 to -323.3)	-3.12	<0.001
Intact FGF-23 (pg/mL)	88.6 ± 21.4	41.8 ± 10.5	46.8 (41.9 to 51.7)	2.78	<0.001
MDA (TBARS) (nmol/mL)	5.24 ± 1.12	1.92 ± 0.41	3.32 (3.07 to 3.57)	3.94	<0.001
TAC (FRAP) (mmol/L)	0.81 ± 0.16	1.48 ± 0.21	-0.67 (-0.72 to -0.62)	-3.59	<0.001

Mean difference is RA minus control. MDA was measured by TBARS and should be interpreted as an operational lipid-peroxidation index. TAC was measured by FRAP and represents total reducing capacity.

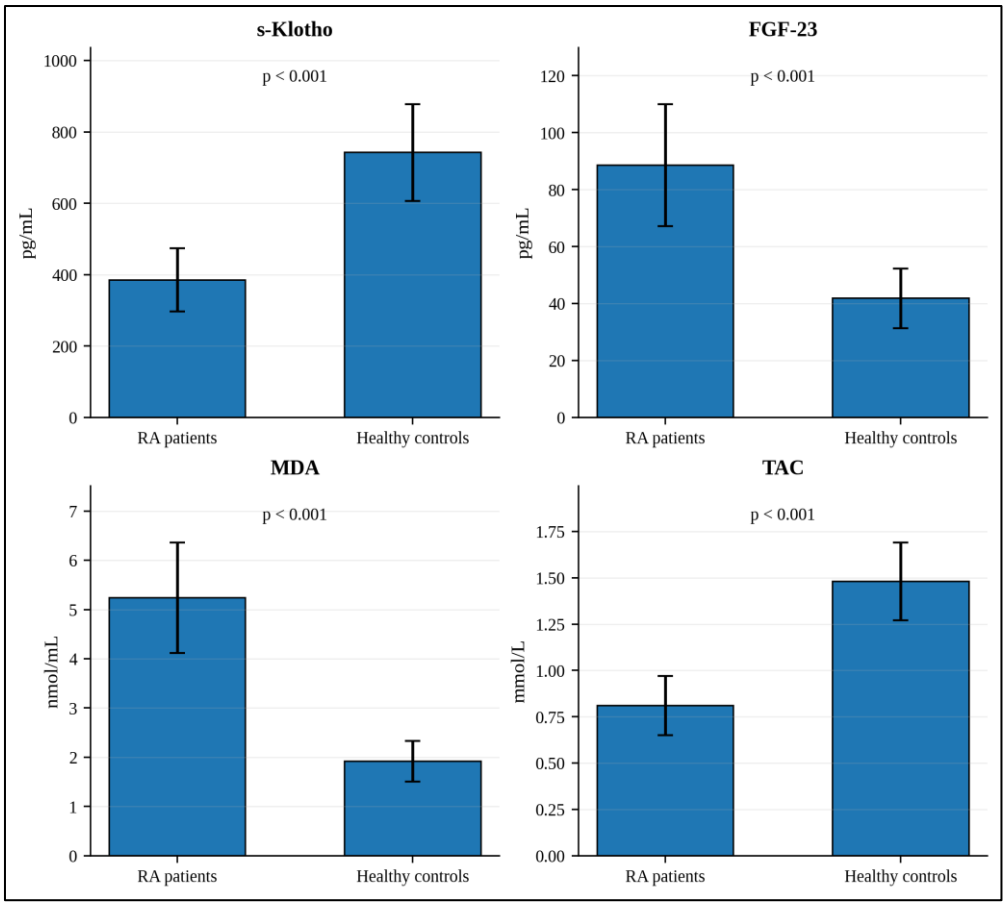

Figure 1: Serum biomarker profiles in rheumatoid arthritis and healthy controls. Bars show mean ± SD. MDA represents TBARS-derived lipid peroxidation and TAC represents FRAP-derived reducing capacity. All four group comparisons were significant at p <0.001.

3.3 Biomarker Patterns by Disease Activity

Patients with high DAS28-CRP activity had lower s-Klotho and TAC (FRAP) and higher intact FGF-23 and MDA (TBARS) than those with moderate disease activity. All subgroup differences were large in standardized magnitude.

Table 3: Biomarker levels according to DAS28-CRP activity strata

Biomarker	Moderate (n=48)	High (n=42)	Mean difference (95% CI)	Cohen d	p value
s-Klotho (pg/mL)	432.1 ± 72.4	332.0 ± 71.8	100.1 (70.2 to 130.0)	1.39	<0.001
Intact FGF-23 (pg/mL)	78.2 ± 16.5	100.5 ± 20.8	-22.3 (-30.1 to -14.5)	-1.20	<0.001
MDA (TBARS) (nmol/mL)	4.68 ± 0.91	5.88 ± 1.04	-1.20 (-1.61 to -0.79)	-1.23	<0.001
TAC (FRAP) (mmol/L)	0.89 ± 0.14	0.72 ± 0.13	0.17 (0.11 to 0.23)	1.26	<0.001

Mean difference is moderate minus high disease activity. MDA, TBARS-derived lipid-peroxidation index; TAC, FRAP-derived reducing capacity.

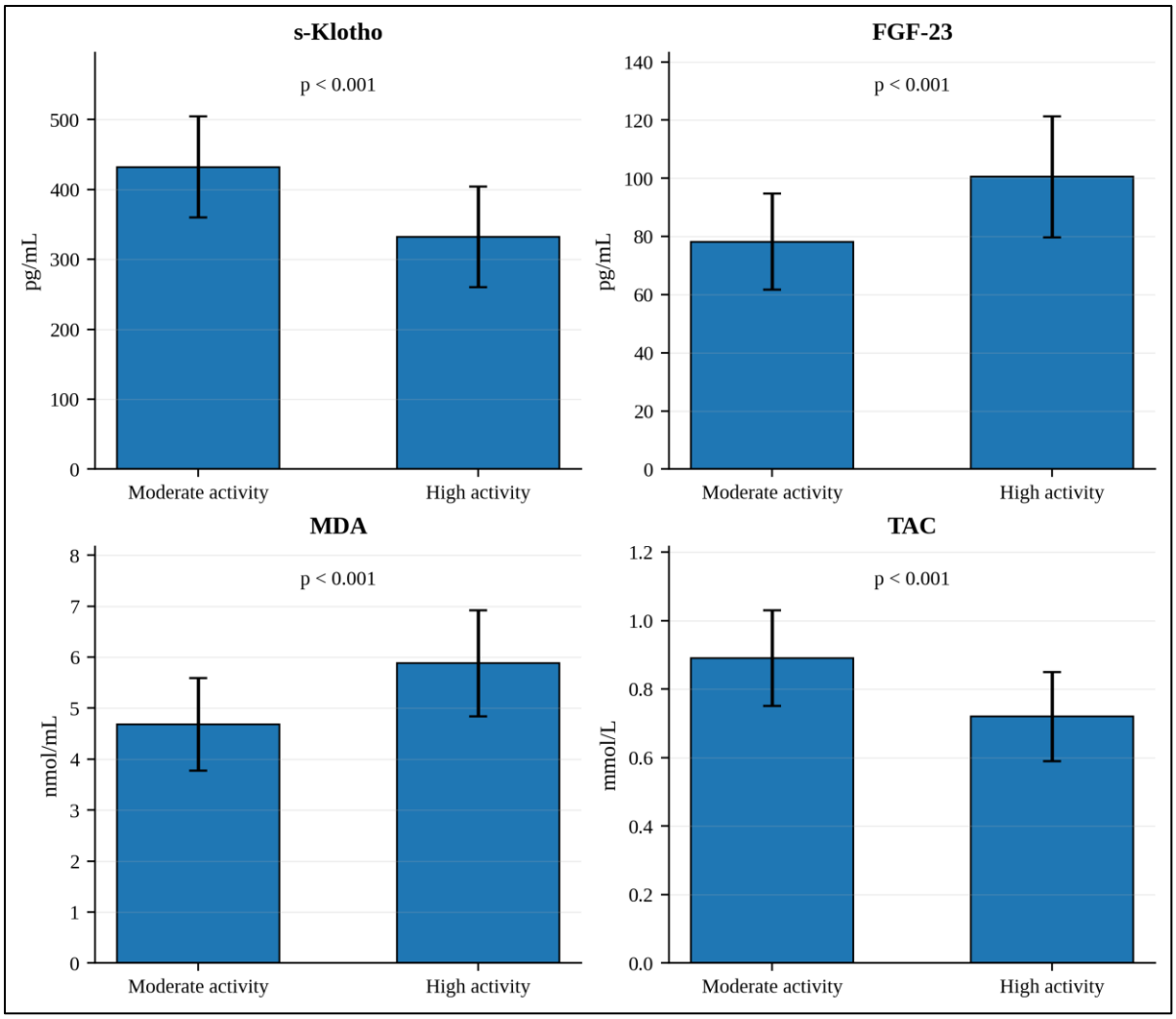

Figure 2: Target biomarker levels in moderate and high RA disease activity. Bars show mean ± SD. Each subgroup comparison yielded p <0.001.

3.4 Correlation Analysis and Multiplicity Control

The correlation pattern showed a coherent endocrine-redox gradient. Lower s-Klotho was associated with higher MDA, lower TAC, higher hs-CRP, and greater DAS28-CRP. MDA showed the strongest positive biomarker correlation with DAS28-CRP, while s-Klotho showed a strong inverse correlation. The smallest absolute correlation was between FGF-23 and TAC (r = -0.421), and the largest was between DAS28-CRP and hs-CRP (r = 0.712).

Table 4: Pearson correlation matrix in the RA group (n=90)

Variable	s-Klotho	FGF-23	MDA	TAC	DAS28-CRP	hs-CRP
s-Klotho	1.000	-0.512	-0.642	0.588	-0.615	-0.544
FGF-23	-0.512	1.000	0.485	-0.421	0.510	0.462
MDA (TBARS)	-0.642	0.485	1.000	-0.610	0.638	0.571
TAC (FRAP)	0.588	-0.421	-0.610	1.000	-0.529	-0.490
DAS28-CRP	-0.615	0.510	0.638	-0.529	1.000	0.712
hs-CRP	-0.544	0.462	0.571	-0.490	0.712	1.000

There were 15 unique pairwise correlations. Two-sided raw p values ranged from 3.6×10^-15 to 3.6×10^-5. All 15 associations remained significant after Holm-Bonferroni correction; all also remained below the conservative Bonferroni threshold of 0.0033.

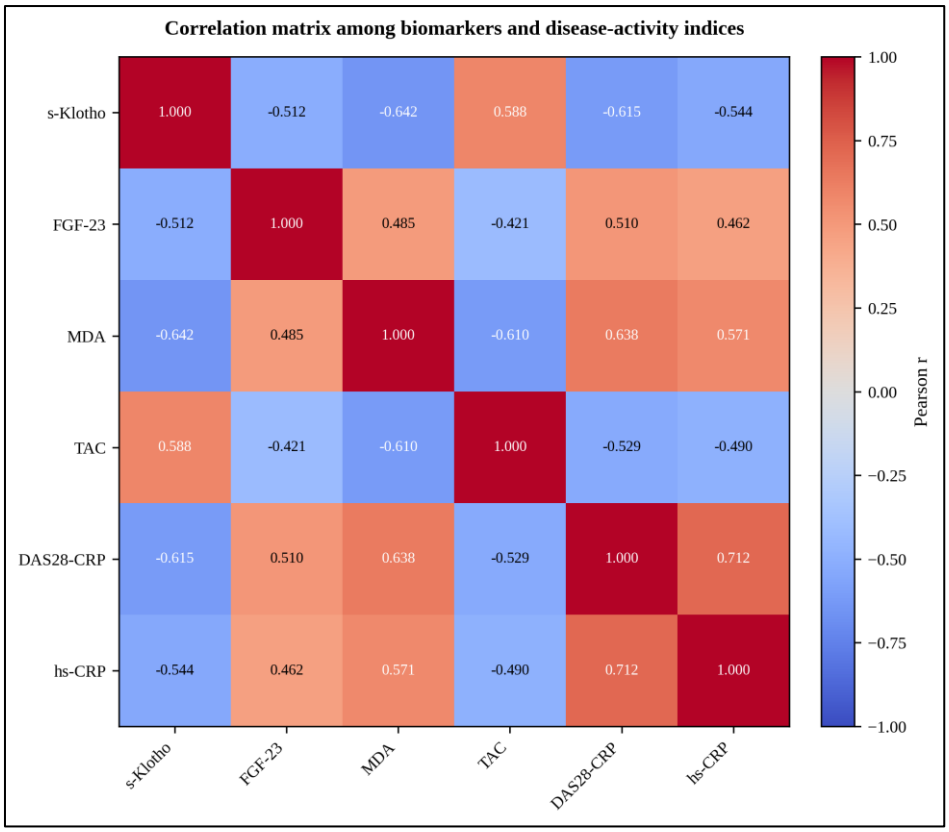

Figure 3: Correlation heatmap for endocrine, oxidative-stress, inflammatory, and disease-activity variables in RA. Cell values are Pearson correlation coefficients. All 15 unique pairwise correlations remained statistically significant after Holm-Bonferroni correction.

3.5 Multivariable Biomarker Model for DAS28-CRP

The simultaneous four-biomarker model was statistically significant and explained 51.0% of the variance in DAS28-CRP (R^2 = 0.510; adjusted R^2 = 0.487; F(4,85) = 22.10; p <0.001). MDA showed the strongest positive independent statistical association with disease activity, while s-Klotho showed the strongest inverse association. FGF-23 was borderline and TAC did not retain an independent association after accounting for the other biomarkers. VIFs were low for all predictors (1.45-2.05), indicating that the observed regression coefficients were not materially distorted by multicollinearity.

Table 5: Simultaneous multivariable biomarker associations with DAS28-CRP (n=90)

Predictor	B (95% CI)	SE	Std. β	t	p value	VIF
s-Klotho	-0.00338 (-0.00626 to -0.00049)	0.00145	-0.252	-2.329	0.022	2.04
FGF-23	0.00983 (-0.00019 to 0.01985)	0.00504	0.178	1.951	0.054	1.45
MDA (TBARS)	0.34079 (0.11287 to 0.56871)	0.11463	0.323	2.973	0.004	2.05
TAC (FRAP)	-0.79790 (-2.28938 to 0.69359)	0.75014	-0.108	-1.064	0.290	1.79

Dependent variable: DAS28-CRP. hs-CRP was excluded because CRP is incorporated into the DAS28-CRP formula. Model statistics: R^2 = 0.510; adjusted R^2 = 0.487; F(4,85) = 22.10; p <0.001. VIF <5 was prespecified as indicating no concerning multicollinearity. This is a biomarker-adjusted model; age, sex, BMI, continuous eGFR, and treatment exposure were not included because the required individual-level covariate data were not available in the analytical dataset.

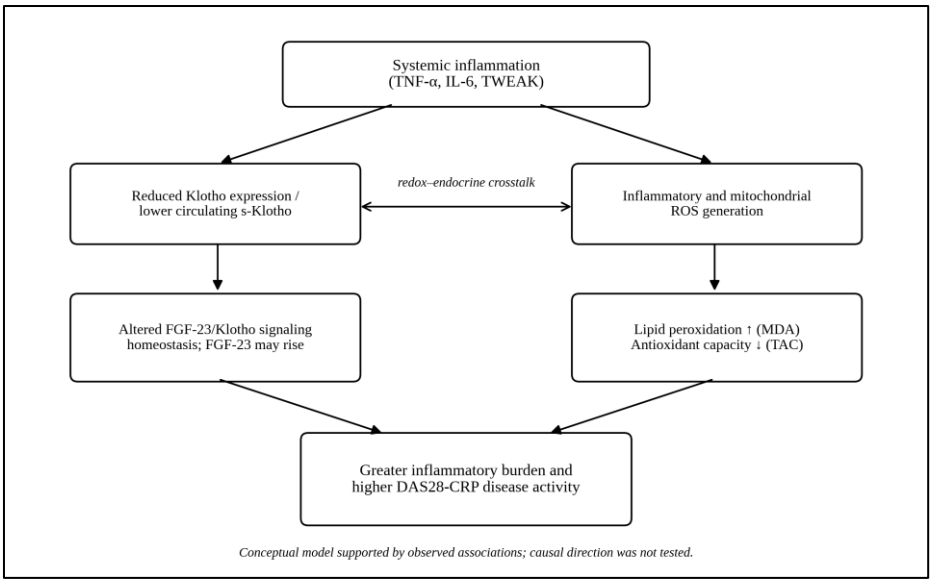

Figure 4: Conceptual framework linking systemic inflammation, Klotho/FGF-23 dysregulation, oxidative stress, and RA disease activity. The model distinguishes observed associations from proposed biological pathways and does not imply that causal direction was established.

4 DISCUSSION

This case-control study identified a pronounced endocrine-redox pattern in RA: circulating s-Klotho and FRAP-derived reducing capacity were lower, whereas intact FGF-23 and TBARS-derived lipid peroxidation were higher than in matched controls. The same directional pattern was seen across moderate and high DAS28-CRP activity groups. All pairwise biomarker and disease-activity correlations survived family-wise multiplicity correction. In the simultaneous biomarker model, MDA and s-Klotho showed the strongest independent statistical associations with DAS28-CRP, with no evidence of problematic multicollinearity.

4.1 Interpreting the Klotho/FGF-23 Axis

An important distinction is required between soluble Klotho, which was measured in this study, and membrane-bound Klotho, which participates directly in classical FGF-23 receptor signaling. The two forms are biologically related but are not interchangeable measurements [9-16]. Accordingly, lower circulating s-Klotho does not by itself establish functional FGF-23 receptor resistance. The present findings are better interpreted as evidence of altered circulating Klotho/FGF-23 homeostasis associated with RA activity rather than direct proof of receptor-level resistance.

Inflammatory signaling can suppress Klotho expression through NF-kappaB-dependent mechanisms [17,18], while Klotho has recognized anti-inflammatory actions and may counter several inflammatory pathways [16]. Experimental work also supports a redox-protective role through FOXO-related antioxidant responses [19,20]. These mechanisms provide biological plausibility for the observed inverse associations of s-Klotho with MDA, hs-CRP, and DAS28-CRP, but mechanistic causality cannot be inferred from circulating measurements in a case-control design.

The human RA literature is not uniform. Alvarez-Cienfuegos et al. described altered FGF23-Klotho biology and observed higher Klotho particularly among biologic-treated RA patients [21], whereas Zhao et al. found lower serum Klotho with increasing systemic immune-inflammatory burden in a large RA sample [22]. Treatment exposure, renal function, age, disease duration, inflammation, mineral metabolism, and assay platform are all plausible sources of heterogeneity. This inconsistency reinforces the importance of treatment- and renal-adjusted analyses in future cohorts.

4.2 Oxidative Stress: Measurement and Interpretation

The elevation of TBARS-derived MDA and reduction of FRAP-derived reducing capacity are consistent with prior evidence of redox imbalance in RA [4-8,26-29]. Activated inflammatory cells can generate ROS that promote lipid, protein, and nucleic-acid oxidation and can amplify redox-sensitive inflammatory pathways. However, the biochemical meaning of the assays must be stated precisely. TBARS is an operational index of lipid peroxidation and is not fully specific for MDA [24], while FRAP estimates aggregate ferric-reducing capacity rather than total antioxidant biology in its entirety [25].

The inverse s-Klotho-MDA association and positive s-Klotho-TAC association are biologically plausible in light of experimental studies showing that Klotho can increase resistance to oxidative stress through modulation of insulin/IGF-1/PI3K/AKT signaling and FOXO-dependent antioxidant gene expression [19,20]. These results support endocrine-redox crosstalk but should not be interpreted as proof that altered Klotho directly caused the oxidative abnormalities observed in RA.

4.3 Disease Activity and Statistical Independence

The biomarker pattern tracked RA activity in both categorical and continuous analyses. MDA correlated positively with DAS28-CRP ($r = 0.638$), whereas s-Klotho correlated inversely ($r = -0.615$); both relationships remained highly significant after multiplicity correction. After adjusting for the other biomarkers in the simultaneous model, MDA and s-Klotho showed the strongest independent statistical associations with DAS28-CRP. However, because treatment exposure and continuous renal-function measures were not included and the study is cross-sectional with respect to biomarker-disease activity relationships, these findings should be viewed as hypothesis-generating rather than causal or prognostic evidence.

hs-CRP was intentionally not entered as a predictor of DAS28-CRP because CRP is already embedded in the DAS28-CRP equation. Avoiding this mathematical coupling provides a more interpretable estimate of the contribution of the target biomarkers to variation in the composite disease-activity score.

4.4 Clinical Confounding, Generalizability, and Limitations

Several limitations are relevant. First, the study design cannot determine temporal direction; it is not possible to establish whether lower s-Klotho precedes greater disease activity or results from the inflammatory state. Second, although participants with eGFR <60 mL/min/1.73 m^2 were excluded, residual renal confounding remains possible because continuous eGFR/creatinine was not included in the multivariable model. Third, phosphate, calcium, parathyroid hormone (PTH), and 25-hydroxyvitamin D were not available, preventing complete physiological characterization of the FGF-23/Klotho mineral-metabolism axis.

Fourth, detailed treatment exposure was not included in the analytical dataset. Methotrexate, glucocorticoids, conventional DMARD combinations, and biologic or targeted synthetic therapies may alter inflammatory activity and may also influence circulating Klotho or redox status. A treatment-adjusted model and sensitivity analyses excluding biologic-treated or high-dose glucocorticoid patients would therefore be valuable in a patient-level dataset. Treatment-line stratification should be interpreted cautiously because treatment intensity is itself strongly influenced by disease severity and duration.

Fifth, the strict exclusion of smokers and individuals with diabetes, overt cardiovascular disease, significant renal impairment, active infection, or malignancy reduced major biological confounding but limits external generalizability. The results apply most directly to RA patients without these common comorbidities and should not be extrapolated uncritically to the entire RA population. Sixth, TBARS and FRAP are practical global redox assays but do not provide the molecular specificity of chromatographic MDA measurement or individual antioxidant-enzyme assays.

4.5 Future Research

Prospective cohort studies should combine repeated s-Klotho, intact FGF-23, TBARS/MDA, and FRAP measurements with continuous eGFR, creatinine, phosphate, calcium, PTH, and 25-hydroxyvitamin D. Detailed treatment exposure, including biologic therapy and glucocorticoid dose, should be incorporated as prespecified covariates. Such studies would clarify whether Klotho changes precede, accompany, or follow changes in DAS28-CRP. Patient-level datasets would also permit externally validated ROC analyses for distinguishing high from moderate disease activity and would allow assessment of whether combined biomarker panels add clinical information beyond standard inflammatory measures.

5 CONCLUSION

Rheumatoid arthritis was characterized by lower circulating s-Klotho and FRAP-derived total reducing capacity together with higher intact FGF-23 and TBARS-derived lipid peroxidation. The abnormalities were more pronounced in high disease activity, and all pairwise correlations remained significant after multiplicity correction. In a simultaneous biomarker model with low VIF values, MDA and s-Klotho showed the strongest independent statistical associations with DAS28-CRP. These findings support a clinically relevant endocrine-redox relationship in RA while remaining hypothesis-generating because renal, mineral-metabolism, and treatment covariates were incompletely characterized. Prospective, treatment-adjusted studies with repeated biomarker and mineral-metabolism measurements are required to establish temporal direction and clinical utility.

STATEMENTS ON COMPLIANCE WITH ETHICAL STANDARDS

Disclosure of conflict of interest

The authors declare no conflicts of interest regarding this manuscript.

Statement of informed consent

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

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