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– ORIGINAL PAPER –

Nitric Oxide, Tumor Necrosis Factor-α, and Lipid Peroxidation as Biomarkers of Endothelial Injury in Sickle Cell Disease

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Abstract

Sickle cell disease (SCD) is a highly prevalent inherited hemoglobinopathy in high-burden regions such as Nigeria, characterized by chronic hemolysis, inflammation, oxidative stress, and endothelial dysfunction, which contribute to vaso-occlusive crises, pulmonary hypertension, and organ damage. This study aimed to assess NO, TNF-α, and MDA as integrated biomarkers of oxidative endothelial injury in SCD. A comparative cross-sectional analytical study was conducted in Benin City among SCD patients and apparently healthy controls. The study included 135 participants (90 SCD patients and 45 healthy controls). Following ethical approval and informed consent, serum NO, TNF-α, and MDA were measured using ELISA, while dietary intake was assessed using a reliable food frequency questionnaire (Cronbach's α = 0.702). Statistical analyses included independent t-tests, Pearson correlation, and multivariable regression ($p < 0.05$). Compared with controls, SCD patients had significantly higher MDA (9.06 ± 1.89 vs. 2.25 ± 0.57 nmol/mL) and TNF-α (45.11 ± 10.26 vs. 5.82 ± 1.92 pg/mL), but markedly lower NO levels (11.20 ± 3.44 vs. 46.22 ± 8.19 µmol/L) (all $p = 0.001$). Regression models ($R^2 = 0.887–0.895$) identified antioxidant supplementation as the strongest predictor of lower MDA and TNF-α and higher NO, while hydration may be associated with lower MDA. These findings support integrated biomarker assessment and emphasize antioxidant support and adequate hydration as practical strategies for improving SCD management in resource-limited settings.

Keywords: *Lipid peroxidation, Oxidative Endothelial Injury, Sickle Cell Disease, Nitric Oxide Depletion, Tumor Necrosis Factor-α Activation*

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Introduction

Sickle cell disease (SCD) is an inherited hemoglobinopathy with the highest burden in sub-Saharan Africa, the Middle East, and India. Some countries report carrier rates up to 25%(Jonani et al., 2025). SCD imposes major economic and healthcare burdens through chronic care, hospitalizations, lost productivity, and acute complications in resource-limited settings(Udeze et al., 2022). Oxidative stress and inflammation are central to SCD. Key biomarkers include nitric oxide (NO), tumor necrosis factor-alpha (TNF-α), and malondialdehyde (MDA). NO depletion from chronic hemolysis impairs vascular tone and promotes vaso-occlusive events, endothelial dysfunction, vasoconstriction, and tissue ischemia, driving disease severity.

TNF-α is a pro-inflammatory cytokine driving systemic and endothelial inflammation in SCD. Elevated levels correlate with leukocyte adhesion, endothelial activation, vaso-occlusion, and organ damage. Studies in sub-Saharan African SCD patients show elevated TNF-α and related IL-1 family cytokines during crisis versus steady state, supporting roles in acute exacerbations and prognostic value for severity (Lekpor et al., 2024; Siransy et al., 2023). TNF-α serves as a biomarker and therapeutic target. MDA indicates lipid peroxidation and oxidative membrane damage. Elevated MDA in SCD signifies oxidative stress, contributing to endothelial injury and organ dysfunction. Its interplay with NO depletion and TNF-α–mediated inflammation drives progression [Liu et al., 2023; Aslan, 2004]. In sub-Saharan Africa, integrating these biomarkers into diagnostics could improve risk stratification and management. Yet testing remains scarce because of limited infrastructure, cost, and assay availability(Lekpor et al., 2024).

Therapies targeting oxidative stress and inflammation in SCD are promising but face hurdles. Hydroxyurea reduces vaso-occlusive crises, has anti-inflammatory effects, and is affordable, yet access in Africa is limited by availability and hesitancy(Odame & Bazuaye, 2024; Riley et al., 2024). Nrf2 activators like CDDO-methyl show beneficial and adverse vascular effects in preclinical models, requiring clinical evaluation (Ihunnah et al., 2022). Point-of-care assays for NO, TNF-α, and MDA could support decentralized monitoring and intervention(Smart et al., 2026), but infrastructure, training, and guidelines remain essential.

Biomarkers of oxidative and inflammatory endothelial injury—NO depletion, elevated TNF-α, and increased MDA—are central to SCD pathophysiology and course. Their diagnostic and therapeutic use could improve outcomes in sub-Saharan Africa, where economic and infrastructural barriers limit use. Affordable platforms, biomarker panels, and access to therapies are urgent [Vona et al., 2021; Kalpatthi & Novelli, 2018]. This study aimed to assess NO, TNF-α, and MDA as integrated biomarkers of oxidative endothelial injury in SCD

Methods

Study Design

We performed a comparative cross sectional analytical study among sickle cell patients and controls in Benin-City. A total of one hundred and thirty-five 135) subjects, comprising ninety (90) sickle cell patients and forty-five (45) healthy controls, were sampled to assess biomarker levels. This design evaluates associations between biomarker levels and clinical endothelial injury in SCD, addressing diagnostic and predictive gaps. Nigeria has the highest prevalence of SCD, with approximately 150,000 sickle cell births in sub-Saharan Africa concentrated within Nigeria (Onavbavba et al., 2025). This strains healthcare resources and underscores health concerns. A study at the University of Nigeria Teaching Hospital, Enugu, found a median monthly burden of nearly N76,711 (approximately US$385) per patient. Out-of-pocket costs contributed heavily, and catastrophic health expenditure affected all groups, especially the poorest quartile. This highlights the need for policy interventions and support(Amarachukwu et al., 2022).

Sample Size Determination

The sample size for this cross-sectional study was determined using Cochran's formula. In the absence of documented prevalence of the outcome among sickle cell patients, a proportion of 0.5 was assumed to ensure maximum variability. At a 95% confidence level and 5% margin of error, the minimum sample size was 384. However, because of limited availability of eligible sickle cell patients during the study period and logistical constraints, 135 participants comprising of 90 SCD and 45 apparent health participants were recruited through consecutive sampling of eligible sickle cell patients and controls, considered adequate for findings. A post hoc power analysis using G Power version 3.1 for a two-tailed independent samples t-test showed that with sample sizes of 90 and 45 participants, an observed effect size (Cohen's d) of 0.85, and $\alpha = 0.05$, statistical power was approximately 99.6% ($1 - \beta = 0.996$). Furthermore, a post

hoc power analysis using G*Power version 3.1 for linear regression models (fixed model, R^2 deviation from zero) showed that with total sample size of 135, eight predictors, and observed $R^2 = 0.887$ to 0.895 (effect size $f^2 = 7.85–8.52$) at $\alpha = 0.05$, statistical power for all models was >0.999 (approximately 100%).

$$\frac{n = Z^2\, p\,(1-p)}{d^2}$$

where:
Z = Z-score corresponding to the confidence level (1.96 for 95%)
p = proportion of 0.5 was assumed
d = margin of error (5%)

$$\frac{n = Z^2\, p\,(1-p)}{d^2} = \frac{1.96^2 \times 0.5(1-0.5)}{0.05^2} = 384$$

Population and sampling

The population for this study consists of individuals diagnosed with sickle cell disease (SCD) in Benin City, Nigeria, a region with a high prevalence and significant public health burden of SCD. This choice is justified by the study's focus on oxidative endothelial injury in SCD, making it essential to target a population where the disease is endemic and its complications are clinically relevant. Nigeria, having the largest global burden of SCD, provides an appropriate setting to investigate the integrated biomarkers (NO, TNF-α, MDA) in a representative and high-risk cohort **(Kalpatthi & Novelli, 2018)**

The sampling involves 135 subjects: 90 SCD patients and 45 age- and sex-matched healthy controls without hemoglobinopathies or chronic inflammatory conditions. This comparative cross-sectional analytical design efficiently evaluates associations between biomarker levels and endothelial injury by comparing affected individuals with healthy counterparts. The sample size was statistically determined using a formula based on the assumed proportion (50%), a 95% confidence interval, and a 5% significance level, yielding a minimum sample size of 384, However, limited availability of eligible sickle cell patients during the study period and logistical constraints resulted in 135 participants, comprising 90 SCD and 45 health participants. Cases are SCD patients with clinical evidence of endothelial dysfunction or vascular complications, while controls are apparently healthy subjects without such evidence and other confounders. Measuring plasma and serum biomarker levels in both groups assesses correlations with endothelial injury.

Data/sample collection

Qualitative data were collected using a semi-structured, self-administered questionnaire, while quantitative data were collected using immunoassay. The questionnaires consist of three (3) sections with open-ended questions and probes which aimed at exploring the socio-demographic profile. Section A (Socio-Demographic characteristics), Section B (Clinical information) and Section C (Lifestyle and Environmental). The data collection was carried out from December 2025 - June, 2026.

Sample Collection and Preparation

After informed consent was obtained, sample collection for this study involved 5mls of blood samples collected from subjects with sickle cell disease, as well as apparently healthy control subjects. Under aseptic conditions, the blood samples were collected using standard veni-puncture techniques from the antecubital vein of the subjects with sickle cell disease and apparently healthy control subjects with sterile disposal needle and syringe, of which the 5mls was dispensed into plain container (for nitric oxide, tumor necrosis factor- **α** and malondialdehyde test). The blood sample in the plain containers were allowed to stand at room temperature until it was clotted and then centrifuged at 3000rpm for five (5) minutes and the serum was separated into another plain container, labeled properly and stored frozen (-20^0C) until time for analysis.

Variables

Serum nitric oxide (NO), tumor necrosis factor-alpha (TNF-alpha), and malondialdehyde (MDA) were measured in this study because they serve as important biomarkers related to oxidative stress, inflammation, and

cellular damage thereby supporting the investigation of disease pathophysiology or treatment effects.

Laboratory method

Blood samples were collected from sickle cell patients to assess nitric oxide (NO), tumor necrosis factor-alpha (TNF-alpha), and malondialdehyde (MDA) as oxidative endothelial injury biomarkers using Enzyme-Linked Immunosorbent Assay. Serum NO, MDA, and TNF-α concentrations were quantified by commercially available sandwich enzyme-linked immunosorbent assay (ELISA) as described by Abou-Diwan et al., 2024; Tanaka et al., 2025; Yi et al., 2025), according to the manufacturers' instructions. ELISA kits were obtained from Elabscience Biotechnology Inc. (Houston, TX, USA; NO Catalog No. EL-M0456, TNF-alpha Catalog No. E-EL-H0109, and MDA Catalog No. E-EL-0060. All samples and standards were assayed in duplicate, and mean absorbance was read at 450nm using a microplate reader. Calibration curves from serial dilutions of manufacturer-provided standards were used to calculate analyte concentrations by four-parameter logistic (4-PL) regression (Hinsberger et al., 2023). Internal quality control included low- and high-concentration control sera at the beginning and end of each run, and assays were accepted only when control values were within the manufacturer's acceptable ranges: NO (20-70 umol/l), TNF-alpha (0.1-10 pg/ml) and MDA (1.0- 4.0 nmol/l). Analytical precision was assessed using intra-assay and inter-assay coefficients of variation (CV); intra-assay CVs were <10% and inter-assay CVs were <12% for NO, MDA, and TNF-α(Tanaka et al., 2025; Yi et al., 2025). Duplicate measurements differing by more than 15% were repeated, and no assay exceeded the 15% CV threshold for ELISA-based biomarker determination. Pre-analytical error was minimized by standardizing collection time, processing within two hours of venipuncture, protecting samples from prolonged light exposure where applicable, and avoiding hemolyzed specimens to ensure analyte stability and measurement reliability(Abou-Diwan et al., 2024; Yi et al., 2025). ELISA was used because of its sensitivity, specificity, reproducibility, ease of use, and capacity to process multiple samples simultaneously for biomarker analysis(Tanaka et al., 2025; Yi et al., 2025)..

Dietary Assessment

Dietary intake was assessed using a Food Frequency Questionnaire (FFQ), The instrument consisted of two (2) food items categorized fruits, vegetables, dairy products, meat and fish, Participants were asked to report their usual dietary intake over the preceding week.

Each food item was assessed using a response format as

- 2–4 times per week
- 5–6 times per week
- Once per week

Scoring Algorithm

Responses were converted into quantitative intake estimates according to the instrument's scoring protocol on scale of 0-10 comprising 5 for once per week, 8 for 2-4 per week and 10 for 5-6 per week respectively. Frequency responses were transformed into average weekly consumption values. Total dietary scores with higher scores indicating higher intake of specific diets.

Statistics Analysis

Descriptive statistics were used to summarize the baseline characteristics and biomarker levels (NO, TNF-alpha, MDA) in both sickle cell disease subjects and controls. Independent t-tests were used to assess significant differences between groups. Pearson correlation analysis (Pearson) was used to explore relationships among biomarkers and clinical parameters as data were normally distributed. Multivariate regression was used to adjust for confounding factors and identify independent predictors of oxidative stress and inflammation after obeying assumption (collinearity). All statistical tests were two-tailed. A p-value < 0.05 was considered statistically significant. These statistical methods enable precise quantification and comparison of biomarker levels, addressing variability and potential confounders in the data by validating the association between oxidative stress, inflammation, and disease status, thus supporting the study's aim to elucidate pathophysiological mechanisms in sickle cell disease**.**

Ethical Approval

The study was reviewed by the Edo state Ministry of Health ethical committee and ethical clearance was obtained prior to commencement with ethical code (HA/737/25/D/12021026, dated 2nd December 2025). All procedures involving human subjects were conducted in accordance with the ethical standards of the institution and the Declaration of Helsinki. Written informed consent was obtained from all participants or their legal guardians before sample collection. Participants did not receive compensation or reimbursement for involvement in the study. Confidentiality and anonymity of participant data

were strictly maintained throughout the study to ensure privacy and compliance with ethical research guidelines.

Result

Reliability

The dietary assessment instrument reliability was tested using Cronbach's alpha. The Cronbach's alpha is 0.702 which indicates strong reliable instrument for the study.

Table 1 *Reliability Test*

Cronbach's Alpha	*Cronbach's Alpha Based on Standardized Items*	*N of Items*
.702	*.900*	*20*

The socio-demographic and clinical characteristics of the study participants are summarized in Table 2. The study included 90 sickle cell disease (SCD) patients and 45 healthy controls. Most SCD patients were aged 21–31 years (71.1%), while controls had 66.7% aged 21–31 years and 26.7% aged 32–42 years. Females comprised similar proportions in both groups (55.6% of cases and 53.3% of controls). Regarding disease severity, 84.4% of patients reported more than four vaso-occlusive crises in the preceding year, and 68.9% reported severe pain during the month. No participant had received a blood transfusion in the preceding three months. All SCD patients reported diets rich in fruits and vegetables, with a mean dietary score of 8.09 ± 1.70 versus 7.00 ± 2.18 among controls. Most patients reported low physical activity, with 66.7% rarely and 28.9% never exercising. All patients were receiving hydroxyurea for more than five years, 97.8% reported antioxidant supplementation, mainly vitamins C and E, and 91.1% consumed more than 2 L of water daily for SCD management.

Table *2 Socio-Demographic and Clinical Characteristics*

Parameters	Case	control
Age		
10-20	26(28.9%)	3(6.7%)
21-31	64(71.1%)	30(66.7%)
32-42	0(0.0%)	12(26.7%)
Sex		
Female	50(55.6%)	24(53.3%)
Male	40(44.4%)	21(46.7%)
How often have you experienced sickle cell crisis in the past year?		
1-2times	6(6.7%)	
3-4times	8(8.9%)	
>4 times	76(84.4%)	
Average pain level over the past month		
Moderate	28(31.1%)	
Severe	62(68.9%)	
Have you received a blood transfusion in the past 3 months?		
No	90(100.0%)	45(100.0%)
How would you describe your diet?		
High in fruit and Vegetable	90(100.0%)	0(0.0%)
High in meat and diary product	0(0.0%)	45(100.0%)
How often do you take this diet		
Once per week	16(17.8%)	
2-4 times per week	46(51.1)	
5-6 times per week	28(31.1%)	
Diet Score	8.09±1.70	7.00±2.18
How often do you engage in physical activity?		
1-2/week	4(4.4%)	17(37.8%)

3-4/week	0(0.0%)	2(4.4%)
5-6/week	0(0.0%)	1(2.2%)
Rarely	60(66.7%)	24(53.3)
Never	26(28.9)	1(2.2%)
Are you currently taking any medication for Sickle Cell Disease?		
Yes	90(100.0%)	0(0.0%)
No	0(0.0%)	45(100.0%)
how long have you been on drug?		
>5 years	90(100.0%)	0(0.0%)
None	0(0.0%)	45(100.0%)
Do you take antioxidant supplement?		
Yes	90(100.0%)	0(0.0%)
No	0(0.0%)	45(100.0%)
What antioxidant supplement do you take?		
Vitamin C & E	88(97.8%)	
Zinc	2(2.2%)	
What medication are you taking for Sickle Cell Disease?		
Hydroxyurea	90(100.0%)	0(0.0%)
None	0(0.0%)	45(100.0%)
Average daily water intake:		
1-2L/day	8(8.9%)	
>2L/day	82(91.1%)	

Categorical variables are expressed as percentages; continuous variables as mean ± SD.

Table 3 compares malondialdehyde (MDA), nitric oxide (NO), and TNF-α levels between sickle cell disease subjects and healthy controls. Mean MDA was significantly higher in sickle cell disease subjects (9.06 ± 1.89 nmol/mL) than controls (2.25 ± 0.57 nmol/mL) (t = 23.13, p = 0.001), indicating increased oxidative stress. In contrast, NO levels were significantly lower in sickle cell disease subjects (11.20 ± 3.44 µmol/L) than controls (46.22 ± 8.19 µmol/L) (t = −26.46, p = 0.001). TNF-α levels were significantly higher in sickle cell subjects (45.11 ± 10.26 pg/mL) than controls (5.82 ± 1.92 pg/mL) (t = 25.257, p = 0.001), indicating elevated inflammation. These findings show oxidative stress and reduce nitric oxide bioavailability in sickle cell disease.

Table 3: *Comparison of Oxidative Stress Marker (Malondialdehyde) , Nitric Oxide and Tumor necrosis factors- alpha Levels between Sickle Cell Disease Subjects and Healthy Controls*

Parameters	SCD n =90	Controls n =45	t-value	p-value
Malondialdehyde (nmol/mL)	9.06±1.89	2.25±0.57	23.13	P= 0.001
Nitric Oxide (umol/L)	11.20±3.44	46.22±8.19	-26.46	P= 0.001
TNF-α (pg/mL)	45.11±10.26	5.82±1.92	25.257	P= 0.001

Values are expressed in mean±SD, p <0.05 -Significant, p>0.05- non-significant
SCD- Sickle cell disease, TNF- α - Tumor necrosis factors- alpha

Table 4 presents a multiple linear regression model of malondialdehyde (MDA) predictors, explaining 88.9% of MDA variance (R^2 = 0.889, F = 80.77, p < 0.001), indicating that the predictors accounted for oxidative stress variability. After adjustment, antioxidant supplement type was the strongest predictor of MDA concentration (B = -2.54, β = -1.03, p < 0.001), showing an inverse association with oxidative stress. Daily water intake was also associated with lower MDA concentrations (B = -2.96, β = -0.17, p < 0.001). Frequency of vaso-occlusive crises was positively associated with MDA levels (B = 0.82, β = 0.16, p = 0.028). Age (p = 0.455), sex (p = 0.214), pain score (p = 0.950), physical activity level (p = 0.720), and dietary frequency score (p = 0.713) were not independently associated. Collinearity diagnostics showed tolerance and VIFs < 10, indicating no multicollinearity

Table 4. *Multiple Linear Regression Analysis of Independent Predictors of Malondialdehyde (MDA)*

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.	95.0% Confidence Interval for B		Collinearity Statistics	
		B	Std. Error	Beta			Lower Bound	Upper Bound	Tolerance	VIF
1	(Constant)	15.04	2.65		5.67	0.001	9.77	20.32		
	Age (Years)	0.22	0.30	0.03	0.75	0.455	-0.37	0.81	0.68	1.46
	Sex	-0.36	0.29	-0.05	-1.25	0.214	-0.94	0.21	0.89	1.13
	How often have you experienced sickle cell crisis in the past year?	0.82	0.37	0.16	2.24	0.028	0.09	1.56	0.26	3.83
	pain score over past month	0.01	0.18	0.01	0.06	0.950	-0.35	0.37	0.14	7.17
	How often do you engage in physical activity?	0.05	0.13	0.02	0.36	0.720	-0.20	0.29	0.63	1.58
	What antioxidant supplement do you take?	-2.54	0.29	-1.03	-8.63	0.001	-3.12	-1.95	0.20	1.36
	Average daily water intake:	-2.96	0.79	-0.17	-3.75	0.001	-4.53	-1.39	0.70	1.42
	how often do take your diets	-0.08	0.21	-0.02	-0.37	0.713	-0.49	0.34	0.68	1.47

a. Dependent Variable: Malondialdehyde (nmol/mL) F= 80.77, P=0.001, R=0.889, R2= 0.889

predictors. Low VIF values supported stability.

Table 5 presents a significant regression model for nitric oxide (NO) levels (F = 88.74, p < 0.001), explaining 88.7% of NO variance (R^2 = 0.887). Antioxidant supplementation was the strongest predictor of NO bioavailability (B = 10.67, β = 0.86, p < 0.001), with a positive association with nitric oxide. Age related positively to NO concentration (B = 3.08, β = 0.09, p = 0.035). However, sex (p = 0.577), sickle cell crisis frequency (p = 0.613), pain score (p = 0.745), physical activity (p = 0.541), water intake (p = 0.648), and dietary frequency score (p = 0.702) were not significant

Table 5. *Multiple Linear Regression Analysis of Independent Predictors of Nitric Oxide (NO)*

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.	95.0% Confidence Interval for B		Collinearity Statistics	
		B	Std. Error	Beta			Lower Bound	Upper Bound	Tolerance	VIF
1	(Constant)	-3.76	12.86		-0.293	0.771	-29.34	21.82		
	Age(Years)	3.08	1.44	0.09	2.14	0.035	0.22	5.94	0.68	1.46
	Sex	0.79	1.41	0.02	0.56	0.577	-2.01	3.59	0.89	1.13
	How often have you experienced sickle cell crisis in the past year?	0.90	1.78	0.04	0.51	0.613	-2.64	4.45	0.26	3.83
	pain score over past month	-0.29	0.88	-0.03	-0.33	0.745	-2.04	1.46	0.14	7.17
	How often do you engage in physical activity?	0.37	0.61	0.03	0.61	0.541	-0.84	1.58	0.63	1.58
	What antioxidant supplement do you take?	10.67	1.42	0.86	7.49	0.001	7.84	13.51	0.10	1.36
	Average daily water intake:	-1.75	3.83	-0.02	-0.46	0.648	-9.37	5.86	0.70	1.42
	how often do take your diets	-0.39	1.01	-0.02	-0.38	0.702	-2.40	1.62	0.68	1.47
Dependent Variable: Nitric Oxide (umol/L) F=88.74, P=0.001, R=0.947, R2= 0.887										

Table 6 presents the multivariable regression model for factors associated with serum TNF-α concentration, which was statistically significant ($F = 85.95$, $p < 0.001$) and accounted for 89.5% of the variation in TNF-α levels ($R^2 = 0.895$). Frequency of vaso-occlusive crises (VOC) was independently associated with TNF-α levels ($B = -8.39$, $\beta = -0.29$, $p < 0.001$). Antioxidant supplementation also showed a strong inverse association with TNF-α concentration ($B = -11.84$, $\beta = -0.84$, $p < 0.001$). In contrast, age, sex, pain score, physical activity level, daily water intake, and dietary frequency score were not significant predictors after adjustment. Collinearity diagnostics showed acceptable tolerance and VIF values, indicating no substantial multicollinearity.

Table 6. *Multiple Linear Regression Analysis of Independent Predictors of Tumor Necrosis Factor-α (TNF-α)*

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.	95.0% Confidence Interval for B		Collinearity Statistics	
		B	Std. Error	Beta			Lower Bound	Upper Bound	Tolerance	VIF
1	(Constant)	89.24	14.71		6.07	0.000	59.972	118.508		
	Age(Years)	-0.08	1.64	0.00	-0.05	0.963	-3.348	3.196	0.683	1.463
	Sex	-1.21	1.61	-0.03	-0.75	0.456	-4.412	1.998	0.888	1.126

How often have you experienced sickle cell crisis in the past year?	-8.39	2.04	-0.29	-4.12	0.001	-12.440	-4.331	0.261	3.827
pain score over past month	-1.87	1.01	-0.18	-1.85	0.067	-3.875	0.134	0.139	7.169
How often do you engage in physical activity?	0.50	0.70	0.03	0.71	0.478	-0.888	1.879	0.631	1.585
What antioxidant supplement do you take?	-11.84	1.63	-0.84	-7.26	0.001	-15.085	-8.598	0.197	1.361
Average daily water intake:	3.20	4.38	0.03	0.73	0.467	-5.513	11.904	0.702	1.424
how often do take your diets	-0.41	1.16	-0.02	-0.35	0.725	-2.708	1.891	0.679	1.473

a. **Dependent Variable: TNF-alpha (pg/mL) F= 85.95, P=0.001, R =0.946, R2=0.895**

Sensitivity analyses were performed to evaluate the robustness of the observed inverse association between how often you have experienced sickle cell crisis in the past year and TNF-α. The primary regression model was repeated after sequential exclusion of potential confounders (age, sex, pain score over past month, physical activity, antioxidant supplement, daily water intake, and how often do take your diets), with minimal changes observed in the regression coefficients and statistical significance. Influential observations were not identified using Cook's distance (CookD<0.03). and leverage statistics (0.07, which indicate average leverage). The persistence of the association across these analyses supports the robustness of the study findings.

Compare Standardized Coefficients

Sensitivity analysis	β (TNF-α)	95% CI	P value	Interpretation
Primary model	-8.39	-12.44 to -4.33	<0.001	Reference model
Without Age	-8.80	-12.85 to -4.77	<0.001	Association unchanged
Without sex	-9.01	-12.92 to -5.01	<0.001	Robust
Without pain score over past month	-8.88	-12.95 to -4.80	<0.001	Robust
Without physical activity	-8.79	-12.85 to -4.73	<0.001	Robust
Without antioxidant supplement	-24.80	-27.94 to -21.66	<0.001	Inverse association persists
Without daily water intake	-8.125	-11.88 to -4.37	<0.001	Inverse association persists
Without how often do take your diets	-8.98	-12.88 to -5.09	<0.001	Inverse association persists

Discussion

This study shows that SCD is associated with higher MDA and TNF-α and lower NO than controls. Multivariable regression showed vitamins C and E were associated with lower MDA and TNF-α and higher NO, while hydration was linked to lower oxidative stress and vaso-occlusive crisis frequency to greater oxidative damage. These findings support MDA, NO, and TNF-α as markers of endothelial injury and inflammation and support antioxidant supplementation and hydration. Cronbach's alpha of 0.702 indicates acceptable reliability for the dietary construct and supports data collection (Rocha & Lenz, 2022). However, because it is near the acceptable threshold, further refinement could improve stability (Rocha & Lenz, 2022). Young adults aged 21–31 predominated

in both groups, with balanced gender distribution, consistent with SCD epidemiology (Almarghalani et al., 2024; Mathias et al., 2021). Frequent VOC and severe pain highlight clinical burden(Jang et al., 2021; Seth et al., 2024). No recent transfusions suggest stable management, possibly influenced by hydroxyurea (Almarghalani et al., 2024; Kanter et al., 2021).

Higher fruit and vegetable intake in SCD patients versus controls (8.09 ± 1.70 vs. 7.00 ± 2.18) supports antioxidant intake to mitigate oxidative stress, a key SCD component (Alabed et al., 2023; Bou-Fakhredin et al., 2022; Starlard-Davenport et al., 2025). Antioxidant supplementation, including vitamins C and E, may support redox balance and reduce inflammatory sequelae (Jafri et al., 2022; Sahun et al., 2025); (Bou-Fakhredin et al., 2022). Physical inactivity was common and may impair vascular function and increase vaso-occlusive crises(Connes et al., 2024; Irwin et al., 2024); controlled moderate exercise may be safe with assessment, gradual supervision, and hydration (Connes et al., 2024)(Irwin et al., 2024). Long-term hydroxyurea use reflects standard SCD management, reducing painful crises, VOCs, sickling, and transfusion needs by increasing fetal hemoglobin (López Rubio & Argüello Marina, 2024; Riley et al., 2024); (Riley et al., 2024). Antioxidant supplementation and water intake may complement hydroxyurea by targeting oxidative stress, supporting hydration, and reducing viscosity and vaso-occlusive risk (Starlard-Davenport et al., 2025). Crises and pain result from microvascular obstruction causing ischemia and inflammation(Jang et al., 2021; Palomarez et al., 2022). Vitamins C and E and fruit and vegetable intake support antioxidant capacity against hemolysis-related oxidative damage(Bou-Fakhredin et al., 2022; Chauhan & Zennadi, 2023).

However, low physical activity worsens vascular dysfunction and VOC risk by impeding blood flow and promoting inflammation (Connes et al., 2024; Irwin et al., 2024). Sedentary behavior is a modifiable risk factor in SCD. Young adults with recurrent pain crises align with established SCD epidemiology (Almarghalani et al., 2024; Mathias et al., 2021). Hydroxyurea reduced transfusion needs and VOC frequency, supporting its role as a therapeutic cornerstone (López Rubio & Argüello Marina, 2024; Riley et al., 2024). Higher fruit and vegetable intake supports nutrition's complementary role in oxidative stress management, though intervention studies remain limited(Bell et al., 2024; Bou-Fakhredin et al., 2022). Widespread inactivity conflicts with recommendations for tailored moderate exercise(Connes et al., 2024; Irwin et al., 2024). High antioxidant supplementation aligns with suggested benefits of vitamins C and E, but clinical outcome evidence still remains mixed (Bou-Fakhredin et al., 2022; Starlard-Davenport et al., 2025).

The biochemical markers and clinical associations described in sickle cell disease (SCD) elevated malondialdehyde (MDA), reduced nitric oxide (NO), and increased tumor necrosis factor-alpha (TNF-α) illustrate the interplay of oxidative stress, endothelial dysfunction, and chronic inflammation in SCD. MDA is a marker of lipid peroxidation, reflecting oxidative membrane damage caused by reactive oxygen species (ROS) (Zoroddu et al., 2026). In SCD, recurrent vaso-occlusion and ischemia-reperfusion injury promote excessive ROS generation, damaging lipid membranes and elevating MDA (Jang et al., 2021). ROS also contributes to painful vaso-occlusive crises (VOC), and higher VOC frequency is associated with elevated MDA (Jang et al., 2021). Antioxidant supplementation, particularly vitamins C and E, scavenges ROS, reduces lipid peroxidation, and lowers MDA levels (Adly et al., 2024; Muñoz-Sánchez et al., 2023; Xie et al., 2022). Hydration may also decrease MDA through enhanced clearance of oxidative metabolites and improved microvascular circulation (Zhang et al., 2022). NO is a vasodilator produced by endothelial nitric oxide synthase (eNOS), maintaining vascular tone and health. In SCD, NO bioavailability is reduced by scavenging from radicals and hemolysis-derived hemoglobin, which consumes NO (Hebbel & Vercellotti, 2021; Młynarska et al., 2025). This causes endothelial dysfunction and vaso-occlusion. eNOS dysregulation in SCD involves arginase depletion, oxidative stress, inflammatory mediators, and microparticles (Hebbel & Vercellotti, 2021). Endothelial dysfunction drives vascular complications and is worsened by mitochondrial ROS and inflammation (Gutierrez-Huerta et al., 2024). Preserving eNOS or reducing oxidative stress is important, but SCD endothelial perturbations challenge single-target treatment (Hebbel & Vercellotti, 2021). Trimetazidine may improve endothelial function by modulating eNOS and reducing oxidative stress and inflammation (Kamisah & Che Hassan, 2024). ChAT+ T cells producing acetylcholine can support endothelial NO activity and vasodilation (Tarnawski et al., 2023).TNF-α is a proinflammatory cytokine central to tissue injury in SCD. Elevated TNF-α reflects endothelial injury and inflammation, linking it to VOC and end-organ damage

(Crorkin et al., 2022; Dri et al., 2023). It promotes leukocyte adhesion, inflammatory signaling, and endothelial activation, perpetuating vascular inflammation(Dri et al., 2023). Increased TNF-α correlates with severity and complications.

In addition, chronic TNF-α elevation may worsen endothelial dysfunction, further reducing NO bioavailability and increasing oxidative damage (Dri et al., 2023; Gutierrez-Huerta et al., 2024). Anti-TNF therapies modulate this pathway in other inflammatory conditions, though their use in SCD requires caution (Liao et al., 2021; Souza et al., 2023). TNF-α's immunomodulatory and pleiotropic roles underscore the balance between protective and pathological inflammation (Crorkin et al., 2022; You et al., 2021). A multiple linear regression model explaining 88.9% of MDA variance supports a strong link between oxidative stress markers and clinical/environmental predictors in SCD. Antioxidant supplement type was the strongest inverse predictor, highlighting dose-dependent efficacy in reducing lipid peroxidation (Adly et al., 2024; Xie et al., 2022). Water intake showed a protective effect, while VOC frequency was positively associated with MDA, confirming disease activity's contribution to oxidative damage (Jang et al., 2021). Age, sex, pain score, physical activity, and dietary frequency did not independently predict MDA, suggesting oxidative stress in SCD is driven more by mechanistic than demographic or lifestyle factors.

Antioxidant supplementation with vitamins C and E protects against oxidative stress in SCD by limiting lipid peroxidation and neutralizing ROS. Vitamin E protects membranes from lipid peroxidation (Berardesca & Cameli, 2021; Munné-Bosch et al., 2022). Vitamin C scavenges ROS in aqueous environments (Zarei et al., 2021). Together, they reduce oxidative stress markers across disease contexts. In SCD, chronic hemolysis causes excessive ROS generation, overwhelming antioxidant defenses (Starlard-Davenport et al., 2025). Supplementation mitigates oxidative damage (Wang & Zennadi, 2021) and improves redox balance, lipid peroxidation products, and antioxidant enzyme activity after vitamin supplementation in SCD patients (Shabalala et al., 2022; Yu et al., 2025). Adequate hydration is also critical for reducing oxidative stress and vaso-occlusive severity in SCD. It relieves sickle-cell occlusion and limits ischemia-reperfusion injury (Zhang et al., 2022). Studies show hydration lowers occlusion events linked to oxidative stress and vascular damage in SCD (Zhang et al., 2022). Low water intake is associated with greater free radical production and impaired antioxidant defense, underscoring hydration's role in maintaining redox balance(Sim et al., 2025).

The association between vaso-occlusive crises (VOC) and oxidative stress is established. Recurrent VOCs promote ROS production and endothelial injury in SCD, worsening inflammation and vascular dysfunction (Beckman et al., 2024; Wang & Zennadi, 2021). Sickle RBCs generate ROS through autoxidation of hemoglobin S and other enzymatic sources, causing membrane damage and pathological sequelae (Orrico et al., 2023). ROS accumulation reduces NO availability, contributing to endothelial dysfunction and vaso-occlusion (Starlard-Davenport et al., 2025).Markers of endothelial activation, including circulating endothelial cells, E-selectin, and VCAM-1, rise during pain crises, indicating oxidative stress-mediated damage (Agouti et al., 2022; Beckman et al., 2024).The lack of effects of age, sex, and physical activity on oxidative stress and NO levels may reflect disease-specific mechanisms, therapeutic interventions, and eNOS dysfunction in SCD, where hemolysis, ROS, and inflammatory mediators predominate (Hebbel & Vercellotti, 2021). Antioxidant supplementation may directly influence NO bioavailability. Exercise requires prescription because of oxidative stress and vaso-occlusion risks, though exercise may offer long-term benefits (Connes et al., 2024). Consequently, activity may have limited influence if management is adequate.

The multiple linear regression model explained 88.7% variance in NO levels, indicating that antioxidant supplementation and age are associated with NO bioavailability in this cohort. Antioxidants preserve NO by scavenging ROS that degrade it, maintaining vascular homeostasis (Huang et al., 2022; Starlard-Davenport et al., 2025). The positive association with age may reflect compensatory increases in NO production or antioxidant responses, although this finding may be cohort-specific (Bulboac\U0103 et al., 2026). The lack of associations with vaso-occlusive crisis frequency or pain intensity suggests that acute severity is less predictive of NO bioavailability when antioxidant status is optimized, possibly through disease management, including supplementation and therapeutic agents (Starlard-Davenport et al., 2025). These results support a central role for oxidative stress in NO-mediated vascular function in SCD. Oxidative stress impairs endothelial nitric oxide synthase function, leading to reduced NO bioavailability and endothelial dysfunction (Li et al., 2025; Młynarska et

al., 2025). Agents that reduce oxidative stress restore NO signaling and vascular function(Splendore et al., 2025).

Regarding systemic inflammation, the multivariable regression model explaining 89.5% variance in serum TNF-α levels indicates strong capture of inflammatory status. Tumor necrosis factor-alpha (TNF-α) is a pro-inflammatory cytokine mediating immune responses and disease severity in SCD, especially during VOC (Yilmaz et al., 2025; You et al., 2021). TNF-α correlates with organ damage and inflammation in SCD and acts via NF-κB signaling to propagate inflammation (Schubert et al., 2021; You et al., 2021). The inverse association between VOC frequency and TNF-α concentration contrasts expectations and may reflect hydroxyurea and antioxidant supplementation, which suppress TNF-α production (Abramson et al., 2025; Riley et al., 2024; Walden & Creary, 2024). Hydroxyurea reduces inflammatory markers, including TNF-α (Abramson et al., 2025; Riley et al., 2024). Antioxidants inhibit MAPK and NF-κB signaling, limiting TNF-α synthesis (Daghero et al., 2022; Schubert et al., 2021; Simu et al., 2022). The lack of significant effects of age, sex, pain score, physical activity, water intake, and dietary frequency suggests that disease-specific mechanisms and targeted treatments dominate inflammation regulation in this SCD cohort rather than lifestyle factors (Starlard-Davenport et al., 2025; Tanhehco et al., 2022).

Inverse VOC-TNF-α association remained robust after confounder adjustment, with no outliers by Cook's distance or leverage. TNF-α to VOCs and treatment remains complex and may reflect adaptive downregulation in chronic disease states (Młynarska et al., 2025; You et al., 2021). During VOC in SCD, TNF-α mediates inflammation, increases von Willebrand factor (VWF) activity, and promotes leukocyte adhesion and vascular occlusion (Shi et al., 2022); (Gotardo et al., 2024; Torres & Hidalgo, 2022). Acute VOC show elevated TNF-α, and frequent VOC relate to overall systemic inflammation (Seth et al., 2024; Siransy et al., 2023). Hydroxyurea (HU) reduces leukocyte counts, pro-inflammatory cytokines, and VOC frequency and severity (López Rubio & Argüello Marina, 2024; Riley et al., 2024). HU therapy decreases inflammation markers including CXCL10, VEGF-A, and IFN-γ, and mitigates hemolysis-induced kidney injury by downregulating inflammatory mediators (Agbozo et al., 2025). HU use also correlates with fewer emergency visits and hospitalizations for VOC, consistent with lower systemic inflammation (Kang et al., 2022). Antioxidants inhibit NF-κB, which controls TNF-α and other pro-inflammatory cytokines(Ma et al., 2024). Sodium butyrate and betulinic acid suppress NF-κB activation (Alruhaimi, 2023; Yan et al., 2022). Frequent VOC may trigger compensatory anti-inflammatory responses; TGF-β1 reduces TNF-α-induced leukocyte adhesion and vaso-occlusion, potentially explaining reduced TNF-α levels (Torres et al., 2022). This inverse association may reflect adherence to hydroxyurea and antioxidant supplementation. In treated SCD, hydroxyurea and other therapies reduce inflammation and vaso-occlusion, so inflammation may not mirror VOC frequency and TNF-α may not positively correlate with crises under therapy (Federti et al., 2023; Rossato et al., 2022).

Conclusion

This study shows that antioxidant supplementation and adequate hydration are modifiable factors associated with reduced oxidative stress, improved endothelial function, and lower systemic inflammation in individuals with sickle cell disease (SCD). Antioxidant supplementation was associated with lower serum malondialdehyde (MDA) and tumor necrosis factor-alpha (TNF-α) levels and increased nitric oxide (NO) bioavailability, suggesting potential to mitigate endothelial injury and disease complications. These findings support further investigation of MDA, NO, and TNF-α as candidate biomarkers for monitoring SCD progression and treatment response. The inverse relationship between vaso-occlusive crisis frequency and TNF-α warrants mechanistic investigation and biomarker-guided therapeutic approaches.

Strengths and Limitations

The study's strengths include integrated assessment of MDA, NO, and TNF-α as biomarkers of oxidative endothelial injury, validated ELISA methods with quality control, inclusion of steady-state and crisis SCD patients, and dietary assessment to identify modifiable lifestyle factors. Robust multivariable regression explained 88–89% of biomarker variability, while standardized sample collection and ethical approval enhanced validity. However, the sample size, cross-sectional design, recall bias from food frequency questionnaires, The study was conducted in a single urban center where all patients received hydroxyurea and most received antioxidant supplementation, limiting generalizability to untreated or rural populations. Multicenter studies including diverse geographic, socioeconomic, and treatment contexts are

needed to validate these findings and uncontrolled confounders, require confirmation through larger longitudinal and mechanistic studies.

Author Contributions

FOO: Conceptualization, methodology, investigation and data collection.

FOO, GOB, KFO and JEO: Writing—original draft preparation.

FOO, BIGA, KIE, EOA and AOO: Methodology, investigation and data collection,

FOO: Supervision

FOO, TGL, JOE and EOA: Validation, formal analysis, data curation.

FOO, VI, SE, OEI and BIGA: Writing—review and editing.

FOO: Resources, project administration and funding acquisition.

All authors read and approved the final manuscript.

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Conflict of Interest

The authors declare no conflicts of interest.

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Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request. Due to ethical restrictions and the need to protect participant confidentiality, the data are not publicly available.

Ethics Consideration: The authors declare that all the procedures and experiments of this study respect the ethical standards in the Helsinki Declaration of 1975, as revised in 2008(5), as well as the national laws. Written informed consent was provided by the participants in this study.

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