



## RESEARCH ARTICLE

# Correlation of lipid peroxidation and nitric oxide metabolites, trace elements, and antioxidant enzymes in patients with sickle cell disease

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## Abstract

**Background:** Lipid peroxidation plays a very important role in sickle cell pathophysiology. The formation of malondialdehyde (MDA) in patients with sickle cell disease (SCD) may lead to endothelial dysfunction. Nitric oxide (NO) is a known vasodilator which plays a role in endothelial function. The current study determined the association between MDA and NO metabolites (NOx), trace elements, and antioxidant enzymes (SOD and CAT) in patients with SCD. The ratio of MDA/NOx was also determined as an index of oxidative stress in the study groups.

**Methods:** This was a cross-sectional study involving 90 patients with SCD and 50 "healthy" controls. Blood samples (n = 140) were collected from the study groups. The plasma, sera, and red cells were kept at -20°C for biochemical analyses. Hemoglobin (Hb) and NOx levels were determined in the plasma using Labsystem Multiskan MS and Griess reagent system, respectively. Super oxide dismutase (SOD) and catalase (CAT) levels were determined in the red cells using assay kits from Cayman chemicals. Lipid peroxidation biomarker MDA was determined in the sera using the TBARS assay. Levels of iron (Fe), copper (Cu), and zinc (Zn) were also determined in the sera using Variant 240FS. MDA and NOx ratio was computed for the study groups and compared.

**Results:** Levels of Hb, NOx, SOD, CAT, and Zn were significantly lower in the patients with SCD ( $P < .001$ ). MDA, Fe, and MDA/NOx ratio were, however, significantly higher in the patients with SCD ( $P < .001$ ). There was no significant correlation between MDA and NOx, SOD, CAT, Fe, and Zn in the study groups. MDA, however, correlated positively and significantly with Cu in the HbSS patients with vaso-occlusive crises (VOC). Gender did not affect the levels of oxidative stress markers.

**Conclusions:** Findings from this study suggest a link between lipid peroxidation and Cu in HbSS patients with VOC. Increased MDA/NOx ratio may contribute to sickle cell pathophysiology by promoting oxidative stress.

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## KEYWORDS

endothelial dysfunction, lipid peroxidation, nitric oxide metabolite, oxidative stress, sickle cell disease

## 1 | INTRODUCTION

Sickle cell disease (SCD) is a red cell disorder, characterized by intravascular hemolysis, impaired perfusion, and tissue infarction. The genetic mutation (GTG for GAG) in the gene for  $\beta$ -globin on chromosome 11, which leads to the replacement of a glutamic acid residue with valine, is associated with the disease.<sup>1</sup> This leads to the production of abnormal Hb (HbS). The replacement of glutamic acid residue with lysine at the same position on the chromosome, gives rise to HbC. In SCD, patients may have a homozygous form (HbSS) or heterozygous form, such as HbSC. The red cells become rigid and result in episodes of VOC.<sup>2</sup> The sickling process of the erythrocytes is induced by polymerization of the deoxygenated HbS, leading to clinical presentations such as intravascular hemolysis and VOC, which are the hallmarks of the disease,<sup>3</sup> as well as endothelial dysfunction and vasculopathy.<sup>4</sup>

Nitric oxide is a potent vasodilator, which plays a role in endothelial function. In patients with SCD, NO bioavailability is impaired due to the ongoing intravascular hemolysis and the underlying chronic condition.<sup>5</sup> During this process, arginase is liberated from the erythrocytes, which destroys the substrate for NO synthesis (arginine). Thus, arginase activity is increased in patients with SCD. Levels of antioxidants and trace elements such as Fe, Cu, and Zn may be altered in this hemoglobinopathy. It will be interesting to know how the levels of NOx affect lipid peroxidation as well as important trace elements in SCD.

The pathophysiology of the disease can be affected by a number of factors including inflammation and oxidative stress.<sup>6,7</sup> As a result of intravascular hemolysis, usually encountered by patients with SCD, free heme is released from free Hb after oxidation. Meanwhile, free heme has been associated with VOC and inflammation, in sickle cell mice.<sup>4</sup> Thus, free heme plays a role in oxidative stress in SCD. Other forms of Hb (ferric and ferryl forms) that are produced in SCD have also been linked with increased oxidative stress.<sup>4</sup>

Malondialdehyde has been noted to be one of the final products of lipid peroxidation and plays a role in the modification of the physical properties of the cell membrane. As a result, there is increased permeability of  $H^+$  ions among other polar substances, causing changes in the electric potential of the membrane.<sup>8</sup> The formation of MDA in patients with SCD may inhibit the activity of carrier proteins, as well as membrane enzymes.<sup>9</sup> Alterations in the levels of MDA may cause destruction to the cell membrane and provoke the clinical presentations in patients with SCD.<sup>10</sup>

The aim of the study was to determine the correlation of lipid peroxidation biomarker MDA and NOx, trace elements (Zn, Cu, and Fe), and antioxidant enzymes (CAT and SOD) in patients with SCD.

## 2 | MATERIALS AND METHODS

### 2.1 | Study groups

The study was conducted at the Korle-Bu Teaching Hospital (KBTH) in the Greater Accra Region of Ghana. The KBTH is a leading tertiary facility in the country, with a 2000 bed capacity. There are several departments in the hospital. The study was a cross-sectional one, comprising of patients with SCD, who were recruited from the sickle cell clinic and healthy controls, recruited from the Accra Southern Area Blood Centre. While some of the patients were in the steady state, others were in the VOC state. The patients with SCD were with HbSS and HbSC genotypes. Steady state was defined as a period where patients are not in crisis for at least 1 month. VOC was also defined as pains in the bones, muscles, and joints, which could not be attributed to any other cause. It was assessed by the physician on duty. The study was conducted between January and April 2017. Patients who presented with clinical conditions, such as coronary artery disease, diabetes mellitus, hypertension, renal failure, pregnancy, and recent blood transfusion (3 months prior to the study), were not recruited into the study. Using the cellulose acetate Hb electrophoresis, the genotypes of the study groups were determined. Ethical approval for the study was sought from the Ethical and Protocol Review Committee of the College of Health Sciences, University of Ghana with protocol identification number CHS-Et/M.8-P 3.2/2016-2017 on 30 March, 2017. The consent of study groups was sought before questionnaire was administered and blood samples taken.

### 2.2 | Laboratory investigations

Five milliliters (5 mL) of venous blood sample was collected from each of the study participant into EDTA and plain tubes. The EDTA tubes were spun at 1500 g for 10 minutes, and the plasmas were kept at  $-20^{\circ}\text{C}$  for determination of Hb level (using LabSystems Multiskan MS, Amersham Bioscience Ltd.) and plasma NOx levels (using the Griess reagent system).<sup>5</sup> The concentration of the nitrite was determined for each sample and compared to the Nitrite Standard reference curve. The nitrite was assessed using a spectrophotometer. SOD and CAT levels were determined in the red blood cells using assay kits from Cayman chemicals. The erythrocytes were first lysed with cold water. Superoxide dismutase is a metalloenzyme that catalyzes the dismutation of the superoxide anion to molecular oxygen and hydrogen peroxide. It forms a crucial part of the cellular antioxidant defense mechanism.<sup>11</sup> A tetrazolium salt is used for the detection of superoxide radicals generated by xanthine oxidase and hypoxanthine. Catalase plays a role in the detoxification of hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) to water and oxygen. The method is

based on the reaction of the enzyme with methanol in the presence of an optimal concentration of  $H_2O_2$ . A spectrophotometer is used to measure the formaldehyde produced.<sup>12</sup> The tubes (containing sera) were centrifuged at 1500 *g* for 10 minutes, and the sera were kept at  $-20^\circ C$  for analyses of MDA (using the TBARS assay [R&D systems]) and Fe, Cu, and Zn levels (determined by Variant 240FS manufactured by VARIAN Australia Pty Ltd.).<sup>13</sup> MDA reacts with thiobarbituric acid (TBA) in the presence of heat and acid to produce a colored end product with a light absorption at 530–540 nm. The intensity of the color at 532 nm corresponds to the level of lipid peroxidation in the sample.<sup>14</sup>

## 2.3 | Data analyses

Data were entered into Microsoft Excel 2010, and analysis was carried out using SPSS version 20 (IBM corporation). Mean plus or minus standard error of mean and frequencies were used to represent nominal data (the age of the study groups and the number of participants with regard to sex, respectively). ANOVA was used to compare the means between the study participants, followed by Turkey's post hoc analyses, and *P*-values  $<.05$  were considered significant. Pearson's correlation was also used to determine correlations of MDA and NO<sub>x</sub>, trace elements (Fe, Cu, and Zn), and antioxidant enzymes (CAT and SOD).

## 3 | RESULTS

### 3.1 | Demographic characteristics of the study groups

A total of 140 participants were recruited to this study; 90 were patients with SCD, and 50 were "healthy" controls. The male-to-female ratio of the SCD patients was noted to be 1:1. Among the enrolled, SCD patients were those with HbSS and HbSC genotypes. Of these two genotypes, those at the steady state were more than those with VOC at the time of sampling. The controls, with HbAA genotype, were made up of 30 females and 20 males. Table 1 represents demographic characteristics of the study groups.

### 3.2 | Levels of Hb and oxidative stress markers of the study groups

The levels of Hb were significantly reduced in the patients with SCD, compared to the "healthy" controls. It was observed in the patients that, the Hb levels were further reduced in patients with VOC. Turkey's post hoc test showed that, there was no significant difference in the levels of Hb between HbSS VOC and HbSC VOC patients ( $P = .087$ ). It was also noted that, there was no significant difference in the levels of Hb between HbAA and HbSC steady state ( $P = .946$ ). Similar to what was observed from the analyses of Hb levels in the

study groups, SOD levels were also noted to be significantly higher in the controls ( $P < .001$ ). Patients with HbSS VOC were observed to have further reduced levels of SOD. CAT levels were significantly higher in the "healthy" controls, compared to the sickle cell patients. From the post hoc test, it was observed that, there was no significant difference in the CAT levels between HbAA and HbSC steady state ( $P = .517$ ), HbAA and HbSC VOC ( $P = .269$ ), and HbSC steady state and HbSC VOC ( $P = .910$ ). Analyses of NO<sub>x</sub> levels in the study groups revealed a similar trend, as what was observed in the other antioxidants. NO<sub>x</sub> levels between HbAA and HbSC steady state patients were not significantly different ( $P = .241$ ), HbSS steady state, and HbSC steady state ( $P = .241$ ). MDA levels were significantly higher in the sickle cell patients, compared to the controls ( $P < .001$ ). Of the sickle cell patients, MDA levels were more elevated in sickle cell patients with the HbSS genotype with VOC. There were no significant differences in the levels of MDA between HbAA controls and patients with HbSC genotypes both at the steady state ( $P = .533$ ) and the VOC state ( $P = .559$ ). The MDA and NO<sub>x</sub> ratio was significantly higher ( $P < .001$ ) in patients with HbSS VOC compared to those at the steady state with the same genotype. The ratio was also significantly different between HbAA and HbSS VOC ( $P < .001$ ), and HbSS VOC and HbSC VOC ( $P < .001$ ). The levels of Cu and Fe herein assessed were significantly higher in the patients with SCD, particularly those with the HbSS genotype. Of the patients with the HbSS genotype, the levels were further observed to be elevated in patients with VOC. Unlike the levels of Cu and Fe, Zn levels were significantly reduced in the sickle cell patients, especially in the HbSS VOC patients as presented in Table 2.

As presented in Table 3, it was observed from this current study that, levels of Hb were significantly lower in female patients with the HbSC genotype ( $P = .001$ ). The levels of the antioxidants did not differ significantly between males and females. Meanwhile, NO<sub>x</sub> levels approached significance ( $P = .058$ ). The levels of Fe were significantly higher in the female patients with the HbSC genotype at the steady state, compared to their male counterparts ( $P < .001$ ).

### 3.3 | Correlation of MDA with Hb levels, levels of trace elements (Fe, Cu, and Zn), and levels of antioxidant enzymes (SOD and CAT) of the study groups

Correlation analyses between MDA and the antioxidants showed that, MDA correlated positively and significantly with Cu in the HbSS VOC patients ( $r = .609$ ;  $P = .016$ ) (Figure 1). As seen in Table 4, no significant correlations were observed between MDA and Hb level, SOD, CAT, NO<sub>x</sub>, Fe, and Zn ( $P > .05$ ) Table 4.

## 4 | DISCUSSION

The present study reiterates increased oxidative stress in patients with SCD in Ghana. It was shown from this study that, patients with SCD,

**TABLE 1** Demographic characteristics of the study groups

Parameter/Groups	Control group	Study group			P-value	
	HbAA (n = 50)	HbSC steady state (n = 30)	HbSC VOC (n = 11)	HbSS steady state (n = 34)		HbSS VOC (n = 15)
Mean age (y)	32.8 ± 1.5	38.20 ± 2.8	23.3 ± 2.9	25.0 ± 1.5	21.3 ± 1.2	<.001
Male n (%)	20 (40.0)	13 (43.3)	5 (45.5)	20 (58.8)	5 (33.3)	
Female n (%)	30 (60.0)	17 (56.7)	6 (54.5)	14 (41.2)	10 (66.7)	.431

Note: Values are expressed as mean ± standard error of mean (SEM).

Abbreviations: HbAA, hemoglobin AA; HbSC, hemoglobin SC; HbSS, hemoglobin SS.

**TABLE 2** Levels of Hb, trace elements, and oxidative stress markers of the study groups

Parameter/Groups	Control group	Study group			P-value	
	HbAA (n = 50)	HbSC steady state (n = 30)	HbSC VOC (n = 11)	HbSS steady state (n = 34)		HbSS VOC (n = 15)
Hb (g/dL)	11.525 ± 0.215	11.303 ± 0.266	8.218 ± 0.232	8.935 ± 0.188	6.920 ± 0.162	<.001*
SOD (U/g Hb)	7953.490 ± 85.066	7411.967 ± 70.205	6819.455 ± 114.830	6415.500 ± 90.123	5174.533 ± 213.203	<.001*
CAT (k/g Hb)	5.044 ± 0.282	4.494 ± 0.245	4.035 ± 0.333	2.453 ± 0.207	1.079 ± 0.171	<.001*
MDA (µmol/L)	0.726 ± 0.972	0.993 ± 0.103	1.100 ± 0.145	1.289 ± 0.149	2.307 ± 0.273	<.001*
NOx (µmol/L)	52.424 ± 2.798	45.113 ± 2.687	32.4791 ± 3.575	37.223 ± 1.950	12.196 ± 1.386	<.001*
MDA:NOx	0.016 ± 0.002	0.023 ± 0.004	0.041 ± 0.003	0.036 ± 0.075	0.267 ± 0.010	<.001*
Zn (µg/dL)	101.422 ± 1.343	85.047 ± 1.254	76.882 ± 1.56	66.527 ± 1.001	51.333 ± 1.556	<.001*
Cu (µg/dL)	114.014 ± 2.332	179.653 ± 4.360	194.573 ± 3.803	220.927 ± 4.764	277.907 ± 10.76	<.001*
Fe (µg/dL)	106.000 ± 1.811	123.750 ± 1.120	139.936 ± 2.084	164.250 ± 1.235	175.836 ± 1.380	<.001*

Note: One-way ANOVA was used for the data, followed by Turkey's post hoc test. Values are expressed as mean ± standard error of mean (SEM); significant at  $P \leq .05$ .

Abbreviations: CAT, catalase; Cu, copper; Fe, iron; Hb, hemoglobin; HbAA, hemoglobin AA; HbSC, hemoglobin SC; HbSS, hemoglobin SS; MDA, malondialdehyde; NOx, nitric oxide metabolites; SOD, superoxide dismutase; Zn, zinc.

\*P is significant.

Parameter/Sex	Males (n = 13)	Females (n = 17)	P-value (Male vs Female)
Hb (g/dL)	12.239 ± 0.445	10.588 ± 0.196	.001*
SOD (U/g Hb)	7437.231 ± 102.777	7392.647 ± 98.248	.759
CAT (k/g Hb)	4.489 ± 0.238	4.497 ± 0.399	.987
MDA (µmol/L)	0.894 ± 0.145	1.068 ± 0.145	.410
NOx (µmol/L)	39.522 ± 1.806	49.656 ± 4.386	.058
MDA:NOx	0.023 ± 0.004	0.024 ± 0.004	.848
Zn (µg/dL)	84.662 ± 1.920	85.341 ± 1.704	.738
Cu (µg/dL)	177.092 ± 7.680	181.612 ± 5.139	.517
Fe (µg/dL)	120.115 ± 1.708	126.529 ± 1.105	.001*

Note: Values are expressed as mean ± standard error of mean (SEM); significant at  $P \leq .05$ .

Abbreviations: CAT, catalase; Cu, copper; Fe, iron; Hb, hemoglobin; HbAA, hemoglobin AA; HbSC, hemoglobin SC; HbSS, hemoglobin SS; MDA, malondialdehyde; NOx, nitric oxide metabolites; SOD, superoxide dismutase; Zn, zinc.

\*P is significant.

**TABLE 3** Levels of Hb, trace elements, and oxidative stress markers of the patients with HbSC genotype at the steady state

especially those with the HbSS genotype, had elevated MDA and reduced NOx, CAT, and SOD, which are markers of oxidative stress. These biomarkers are important in determining ongoing destruction of cell

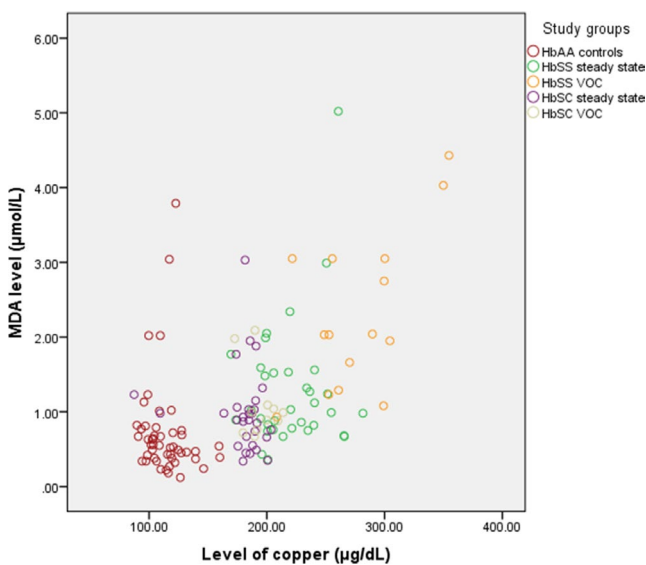
contents, including proteins, lipids, and DNA. There is increased lipid peroxidation biomarker MDA in patients with SCD. In another study, it was noted that, levels of NOx were depleted in patients with SCD.<sup>5</sup>

In line with other similar studies,<sup>15-17</sup> MDA was reported to increase significantly in the patients with SCD compared to their “healthy” counterparts. Levels of antioxidants have also been reported to be significantly reduced in patients with SCD in a previous study,<sup>18</sup> as a result of generation of large reactive oxygen species. These reactive oxygen species levels may be aggravated by several factors including hypoxia and inflammation.<sup>19</sup> The ongoing inflammation in these patients (particularly those with the HbSS genotype in VOC) may have accounted to the enhanced oxidative stress.<sup>15</sup> It is worth mentioning that, the results of this current study suggest in part that, of all the patients with SCD assessed, HbSS patients in VOC may experience intensified inflammation and hemolysis.<sup>15</sup> These patients were also noted to have significantly lower levels of Hb. It would have been interesting to correlate lipid peroxidation status with frequency of crises, to assess any possible link for better

management of crises. However, this was not done in the current study, since the frequency of VOC was not completely gathered from all the patients. It was observed that, there was no significant correlation between MDA and NOx in the study groups. This is in agreement with the work conducted by El-Ghamrawy et al<sup>20</sup> in children with SCD, who also reported lack of association between MDA and nitrite. Nitrite is part of the NO metabolites which was determined in the current study. Findings from the work of El-Ghamrawy et al<sup>20</sup> and our study suggest in part that, there seems to be no significant association between MDA and NOx in patients with SCD (whether in pediatrics or adults).

While gender did not affect the oxidant-antioxidant status in all the study groups, it was, however, observed in the HbSC patients that, Hb level was significantly lower in the female groups. The lower Hb level is in line with other studies.<sup>5,21</sup> Contrary to the results of our study on the relative levels of NOx between the male and female groups, a previous study<sup>22</sup> reported reduction of basal and stimulated NO in male SCD patients. Levels of NOx, as well as MDA, and their ratio were comparable between the male and female groups. The comparable levels of NOx in the male and female groups could partly be as a result of a similar generation of reactive oxygen species that would have depleted the NOx in these patients. One of the important limitations of the current study that needs to be highlighted was the lower sample size realized when gender and the sickle cell genotypes were analyzed separately.

Patients who presented with VOC had lower levels of Zn and higher levels of Cu and Fe. Meanwhile, a previous study had also reported lower level of Zn and higher Cu level in children with SCD in the steady state.<sup>23</sup> Gender only affected levels of Fe in the HbSC patients in the steady state. In such patients, it was observed that, Fe levels were significantly higher in the female patients. To the best of our knowledge, this is the first study to report a positive correlation of MDA and Cu in HbSS patients in VOC in Ghana. Al-Naama<sup>24</sup> also reported a significant positive correlation of MDA and Cu in patients with sickle cell anemia in Southern Iraq. Cu has been reported to compete for the same binding site on



**FIGURE 1** Correlation of lipid peroxidation biomarker MDA and Cu in the study groups. The correlation of lipid peroxidation biomarker and Cu was analyzed with Pearson correlation

**TABLE 4** Correlation of MDA with Hb levels and the levels of antioxidants (and trace elements) of the study groups

Parameter/Groups	Control group	Study groups			
	HbAA (r-value; P-value)	HbSC steady (r-value; P-value)	HbSC VOC (r-value; P-value)	HbSS steady (r-value; P-value)	HbSS VOC (r-value; P-value)
Hb	-.074; .612	-.280; .134	-.347; .296	-.197; .263	-.346; .206
SOD	-.224; .122	-.056; .770	.440; .176	-.226; .199	-.423; .116
CAT	.165; .258	.186; .326	-.281; .403	.020; .912	-.143; .610
NOx	-.085; .561	.181; .348	-.394; .230	.264; .132	-.262; .346
Zn	-.029; .841	.056; .768	.018; .959	-.268; .125	.251; .367
Cu	-.106; .467	-.108; .569	-.354; .286	.175; .324	.609; .016*
Fe	.105; .474	-.151; .427	.134; .694	-.152; .391	-.021; .940

Note: The data were assessed using Pearson correlation test. r-value = Pearson's correlation coefficient; significant at  $P \leq .05$ .

Abbreviations: CAT, catalase; Cu, copper; Fe, iron; Hb, hemoglobin; HbAA, hemoglobin AA; HbSC, hemoglobin SC; HbSS, hemoglobin SS; MDA, malondialdehyde; NOx, nitric oxide metabolites; SOD, superoxide dismutase; Zn, zinc.

\*P is significant.

proteins with Zn,<sup>25</sup> and an increase in plasma concentration of Cu consequently leads to Zn deficiency (often as a result of elevated urinary excretion of Zn).<sup>26</sup> Thus, as more Cu is generated, due to a possible loss of Zn, there could be higher chance of lipid peroxidation, which will affect the cell membrane, and possibly lead to episodes of VOC. Meanwhile, in a previous study, serum Cu was significantly correlated with disease severity score in patients with SCD in Nigeria.<sup>27</sup>

Lipid peroxidation biomarker MDA and NOx ratio has been studied in other clinical complications,<sup>28</sup> but not sickle cell. Since MDA levels are expected to increase in patients with SCD partly due to an ongoing lipid peroxidation, and NOx levels are expected to reduce as a result of scavenging of NO by cell-free plasma Hb and other factors, the computation of their ratio could also give an idea of the extent of oxidative stress. This study reports for the first time, significantly higher MDA/NOx ratio in patients with SCD. The higher MDA/NOx ratio in the patients with SCD suggests increased oxidative stress in these patients. More NOx may have been depleted as a result of at least in part, the chronic inflammatory condition. Indeed, the ratio was even higher in patients with HbSS in VOC, suggesting an aggravated chronic and hemolytic condition in such patients.

In summary, elevated MDA levels and reduced NOx in the patients with SCD suggest in part increased oxidative stress and ongoing endothelial dysfunction. Gender did not affect the levels of oxidative stress markers in the study groups. There was no significant correlation between MDA and NOx in the patients with SCD. However, the significant correlation of MDA and Cu in HbSS patients in VOC and the high MDA/NOx ratio in the patients with SCD suggest their possible involvement in the pathophysiology of oxidative stress.

It is important to state that, the sample size used in the current study was small. Therefore, a similar study involving large sample size is necessary to confirm results obtained from our study. The authors could not also determine levels of other hemolytic markers and their association with oxidative stress markers in the patients with SCD studied. Nonetheless, the findings from this study add to the literature of sickle cell pathophysiology.

#### AUTHOR CONTRIBUTIONS

CA-B conceived the idea, designed the study, and analyzed the data. CA-B, GBD, and RA drafted the first manuscript. CH-B, RA, and GBD did clinical characterization, recruited the patients, and collected data. GBD and RA carried out laboratory analysis. CA-B entered and interpreted the data collected. GA and ADC revised the drafted manuscript. All authors read and approved the final version of the manuscript.

#### DATA AVAILABILITY STATEMENT

The data are stored electronically with all the results from the analysis and available from the corresponding author upon reasonable request.

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