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Interplay between foetal haemoglobin, micronutrients and oxidative stress biomarkers in sickle cell anaemia children

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ABSTRACT

Foetal haemoglobin (HbF) has been speculated to have an impact on the quantity of micronutrients and the latter also have a role to play in oxidative stress (OS) in sickle cell anaemia (SCA). No previous study in Ghana has examined the interplay of these factors together among SCA children. This study compared the levels of OS biomarkers (8-hydroxy-deoxyguanosine [8-OHdG] total antioxidant capacity [TAC]) and micronutrients (zinc and copper), and their relationship with HbF in SCA and sickle cell negative, apparently healthy children. This case-control study recruited 58 SCA (out-patients [n = 42] and in-patients [n = 16]) children aged 1–14 years as cases and 62 sickle cell negative children as controls from the Sickle Cell Unit at the Eastern Regional Hospital, Ghana. The micronutrients were measured using the atomic absorption spectrophotometer (AAS) whereas OS biomarkers and HbF were assayed using enzyme-linked immunosorbent assay (ELISA). SCA out-patients had a significantly higher level of HbF compared to HbA patients ($p = 0.035$). SCA in-patients had significantly increased levels of zinc, but a reduced 8-OHdG than SCA out-patients compared to control group ($p < 0.05$). HbF correlated significantly ($r = 0.318$, $p < 0.038$) with zinc in SCA out-patients. Micronutrients are essential in maintaining the redox status in SCA out-patients and HbF can influence some micronutrients.

1. Introduction

Sickle cell disease (SCD) is a genetic disease caused by expression of a mutant haemoglobin called HbS which shows reduced solubility and enhanced polymerization into tactoids under low oxygen tension that deform erythrocytic morphology into sickled-shaped cells [1]. HbS formation is induced by point mutation that replaces adenine with thymine in the β-globin gene sequence resulting in substitution of glutamic acid with valine at position 6 of the β-globin polypeptide chain [2].

Formed HbS polymers, deform morphology of the red blood cell (RBC) into crescent sickle-shape and thus promote RBC targeted haemolysis, vascular endothelial injury and adhesion [3]. The latter, facilitates microvascular occlusion leading to ischemia, tissue damage, impaired organ function and multi-organ failure in SCD patients.

SCD is most prevalent in sub-Saharan Africa with incidence ranging

Abbreviations: 8-OHdG, 8-hydroxy-deoxyguanosine; Cu, copper; HbF, Foetal haemoglobin; OS, Oxidative stress; SCA, Sickle cell anaemia; SCD, Sickle cell diseases; TAC, Total antioxidant capacity; Zn, Zinc.

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from 1 to 5% in most population. In Ghana, 2% of newborns have SCD of which, the severest form (homozygous HbS or sickle cell anaemia) constitute 55% [4].

Biological oxidation in aerobic cells generate energy and free radicals such as reactive oxygen and nitrogen species, critical for defense against microbial infections, cell proliferation, apoptosis, migration induction, inflammatory gene expression and as chemical messengers for signal transduction pathways. However, failure to maintain balance of antioxidant enzymatic and non-enzymatic molecules and reactive oxygen species (ROS); could create oxidative stress (OS) that damage cellular proteins, lipids and nucleic acids affecting both structural and functional integrity [5].

Recent evidence suggests increased oxidative stress in SCA, mediate enhanced severity of pathologies that drive secondary organ dysfunctions in SCD patients. OS, the imbalance of oxidants and antioxidants in the body [6] is attributed to excessive levels of superoxide and peroxide from increased autoxidation of HbS [7], increased free haemoglobin due to chronic haemolysis, injuries associated with recurrent –ischaemic reperfusion and chronic pro-inflammatory state sustained by endothelial injury, hemolysis, pro-inflammatory cytokines and over-expression of adhesion molecules [8].

Repetitive polymerization of the red cells due to reduced oxygen tension triggers a cascade inciting blood cell sickling, adhesion, vaso-occlusion and intravascular haemolysis to sustain debilitating effect of OS [9].

This damage result in a compromised antioxidants system that causes a reduced total antioxidant capacity (TAC) (vitamin C, vitamin E and glutathione) in SCA [10,11]. Additionally, it has been reported that some micronutrients such as copper (Cu) and zinc (Zn) play very important roles in ensuring optimal antioxidant status [12,13]. In SCA, efficient anti-oxidants system is particularly critical to mitigate chronic red cell destruction due to OS generated by oxidation of iron from haemoglobin [12,14]. For instance, Zn and Cu are required for full activity of superoxide dismutase, an enzyme involved in neutralizing the toxic oxygen metabolite of OS [15].

Foetal haemoglobin (HbF) acts as a major modulator of physiologic and clinical features in SCD. Whilst HbF levels decline to 1–2% of total adult Hb within the first year of post-foetal life in non- SCD individuals, levels could remain persistently very high in SCA patients. Despite high variability of HbF quantities in RBCs of SCD patients, a high level of persistent HbF in post-foetal life reduces erythrocytic sickling tendencies due to its exclusion from the sickle haemoglobin polymer [16,17]. HbF in SCA improve its clinical outcome due to its higher affinity for oxygen. Additionally, HbF dissociates into a dimer to bind HbS to form tetramer, which is, very stable and does not polymerize. Thus, high HbF is reported to associate with milder but not asymptomatic SCD. It has been reported that HbF levels positively correlate serum zinc and magnesium levels but negatively correlate with copper and calcium levels [17]. This suggests that HbF levels in SCA may influence the interplay between oxidative stress burden and micronutrients in SCA.

This study compared the levels HbF, micronutrients (Zn and Cu), and OS biomarkers (8-hydroxy-deoxyguanosine [8-OHdG] and TAC) in SCA patients and sickle cell negative (HbA), apparently healthy children and establish a relationship, if any, between the level of HbF, micronutrients and OS biomarkers.

2. Materials and methods

2.1 Patients. The study site was at the Sickle Cell Unit of the Eastern Regional Hospital, Ghana. This case-control study recruited 120 children from the ages of 1–14 years comprising 58 SCA patients with homozygous HbS (42 out-patients and 16 in-patients) and 62 healthy controls. The controls were apparently healthy sickle cell negative with HbA phenotype.

The SCA patients were those that had only HbS using the haemoglobin electrophoresis, by physical presentation and some haematological parameters. The SCA in-patients were those who had been admitted at the ward. They were given folic acid and were on constant hydration. The SCA out-patients were those who were coming for their regular check up at the hospital and were declared healthy by the paediatrician. They were also on folic acid. SCA patients who had started Hydroxyurea medications were excluded from the study.

2.2 Ethical Consideration. Approval for all protocols in this study was obtained from the Committee on Human Research, Publications and Ethics, Kwame Nkrumah University of Science and Technology, School of Medical Sciences and the Eastern Regional Hospital, Koforidua according to the 2013 version of the ethical guidelines of the Declaration of Helsinki. An informed consent in a form of signature or fingerprint was sought from parents of participants and confidentiality of the participants in this study was maintained.

2.3 Specimen Collection. Five (5) ml of venous blood collected into serum gel separator tube was allowed to clot at room temperature then centrifuged at 3000 rpm for 10 min. The serum was separated into an Eppendorf tube and stored at −80 °C for analysis of HbF, Zn, Cu, 8-OHdG and TAC concentrations.

2.4 Estimation of HbF, Zn, Cu, 8-OHdG and TAC concentrations. Enzyme-linked immunosorbent assay (ELISA) reagents for 8-OHdG, TAC and HbF were purchased from the Shanghai chemical Ltd Building 2, China and then used to measure 8-OHdG, TAC and HbF. All reagents were prepared before starting the assay procedure. Standards were duplicated but the samples were not since the wells were not enough. Briefly, fifty (50) µl of the standards were added first to the standard wells. Ten (10) µl of each sample was dispensed into sample wells. Forty (40) µl of the sample diluent was also dispensed into the sample wells. The sample diluent was not added to the blank wells. Hundred (100) µl of horseradish peroxidase (HRP) conjugate reagent was added to each well and an adhesive strip provided in the kit was used to cover the plate. The covered plate was incubated for 60 min at 37 °C. After incubation, the wells were aspirated and washed thoroughly with the wash solution. This was done for about five times and the liquid was completely removed at each step by decanting and blotting against clean paper towels. Fifty (50) µl of both chromogen A and B solutions were added to each well and was gently mixed and incubated for 15 min at 37 °C. The plate was protected from light as a precautionary measure. Fifty (50) µl of the stop solution were added to each well. There was a colour change from blue to yellow. The reading of the absorbances was done using an ELISA plate reader (Synergy H1 reader, BioTek) at 450 nm within 15 min. The concentrations were calculated using the Beer Lambert's law.

2.5 Estimation of micronutrients (Zn and Cu). Serum Cu and Zn were estimated using the atomic absorption spectrophotometer (AAS) (Analytikjena nova 400P, Germany). Briefly, 1 ml of double distilled water was added to at least 500 µl of the blood serum samples and they were digested with nitric acid and perchloric acid (HNO_3–$HClO_4$) mixture followed by concentrated sulphuric acid (H_2SO_4). The mixtures were heated to 200 °C and kept at that temperature for 30 min till the solution became clearer. After the solutions cooled down, double distilled water was added to them till they reached 50 ml. The solutions were then transferred into glasswares then analysed. The AAS was used to determine the amounts of Zn and Cu.

2.1. Statistical analysis

The IBM SPSS Statistics v. 25 was used to analyse the data. Data was presented as a frequency (percentage) for categorical variables and mean and standard deviation for normally distributed data. Independent sample *t*-test and one way-analysis of variance (ANOVA) test were used to estimate the variations in means between two (in-patients SCA and out-patients SCA) and more than two (controls, in-patients SCA and out-patients SCA) respectively. Pearson correlation analysis followed by Benjamini-Hochberg correction for multiple testing was used to estimate the relationship between HbF concentration, micronutrients, and OS

biomarkers among the SCA participants.

3. Results

3.1. Demographic data of participants and their guardians

Table 1 shows the demographic data of the participants. The SCA participants who were in crisis in-patients [5.7 ± 2.9 years] were significantly ($p < 0.009$) younger rather than the SCA out-patients [6.8 ± 3.4 years] compared to those who were apparently healthy sickle negative control [8.5 ± 3.9 years]. Majority of the participants had a normal body mass index (BMI) with the controls having a greater percentage (63.0%).

3.2. Comparison of HbF in SCA in-patients and SCA out-patients

Fig. 1 shows the HbF levels in both SCA out-patients and in-patients compared to HbA. The SCA out-patient had significantly higher HbF levels compared to the HbA patients (1.501 ± 0.12 μg/ml vs 1.37 ± 0.12 μg/ml, $p = 0.035$). Meanwhile, there were no statistically significant difference in the HbF levels between SCA out-patients and SCA in-patients ($p = 0.293$) as well as between HbA patients and SCA in-patient ($p = 0.288$).

3.3. Comparison of the levels of 8-OHdG, TAC, Zn and Cu amongst the study participants

Fig. 2 below shows the comparisons of 8-OHdG, TAC, Zn and Cu amongst HbA and SCA children. 8-OHdG was statistically different amongst the three groups. 8-OHdG concentrations were statistically significantly ($p < 0.0001$) reduced in SCA in-patients (20.10 ± 10.46 pg/ml) than SCA out-patients (27.85 ± 6.13 pg/ml) compared to sickle cell negative (HbA) control children (34.07 ± 3.04 pg/ml). Conversely, there was no significant difference amongst the three groups in TAC concentration ($p = 0.154$). Zn was statistically significantly ($p < 0.0001$) increased among the SCA in-patients ($1.4 \times 10^{-3} \pm 0.46 \times 10^{-3}$ mg/L) followed by the sickle cell negative (HbA) control ($1.0 \times 10^{-3} \pm 0.057 \times 10^{-3}$ mg/L) and the SCA out-patients ($5.5 \times 10^{-4} \pm 0.59 \times 10^{-4}$ mg/L). However, there were no statistically significant differences between the mean levels of Cu amongst the three groups ($p = 0.300$).

Table 1
Demographic data of participants.

	Normal = 62 (HbA)	SCA = 42 (Out-patients)	SCA = 16 (In-patients)	*p*-value
Age years (mean ± SD)	8.5 ± 3.9	6.8 ± 3.4	5.7 ± 2.9	0.009
Age group (years)				
≤5	15(24.2%)	15(36.6%)	9(56.3%)	0.226
6–10	26(41.9%)	17(41.5%)	6(37.5%)	0.978
>10	21(33.9%)	9(22.0%)	1(6.3%)	0.168
Gender				
Male	30(48.4%)	17(40.5%)	15(93.8%)	0.162
Female	32(51.6%)	25(60.0%)	1(6.3%)	0.043
BMI z scores (Gender standardized BMI)				
<5th percentile (Underweight)	3(75.0%)	0(0.0%)	1(25.0%)	0.140
5th -<85th percentile (Normal)	46(63.0%)	21(28.8)	6(8.2%)	
85th-<95th percentile (Overweight)	9(100.0)	0(0.0%)	0(0.0%)	
≥95th percentile (Obese)	4(100.0%)	0(0.0%)	0(0.0%)	

(SD – Standard deviation, BMI – Body mass index, BMI percentiles classification is according to World Health Organization BMI classification for children).

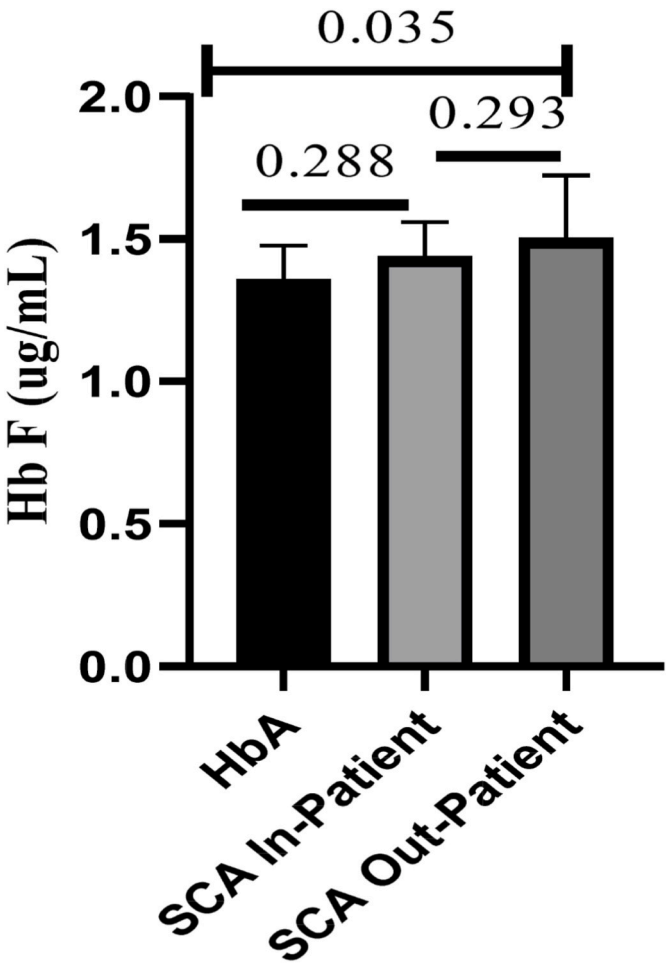

Fig. 1. HbF between the SCA out-patients and the in-patients compared to HbA.

3.4. Correlation between HbF, micronutrients (Zn & Cu) and OS biomarkers

Table 2 displays the correlation between HbF and Cu, Zn, TAC and 8-OHdG. Zn ($p < 0.038$). and TAC ($p < 0.029$) had a weak positive correlation with HbF in the SCA out-patients and this correlation was significant.

4. Discussion

SCA patients are more prone to oxidative stress as their antioxidants are readily used to mop up the ROS. They usually have greater amounts of ROS due to the pathophysiology of the disease [9,18]. Some micronutrients make up these antioxidants and help in their function [12]. There is a speculation that these micronutrients are regulated by HbF [17].

Our findings showed that SCA out-patients had a higher amount of HbF than the SCA in-patients. There was a significant correlation between HbF and zinc and TAC among the SCA out-patients. SCA in-patients had increased levels of Zn, Cu and TAC as compared to the SCA out-patients and controls. In the case of 8-OHdG, SCA in-patients had lower levels as compared to the other groups.

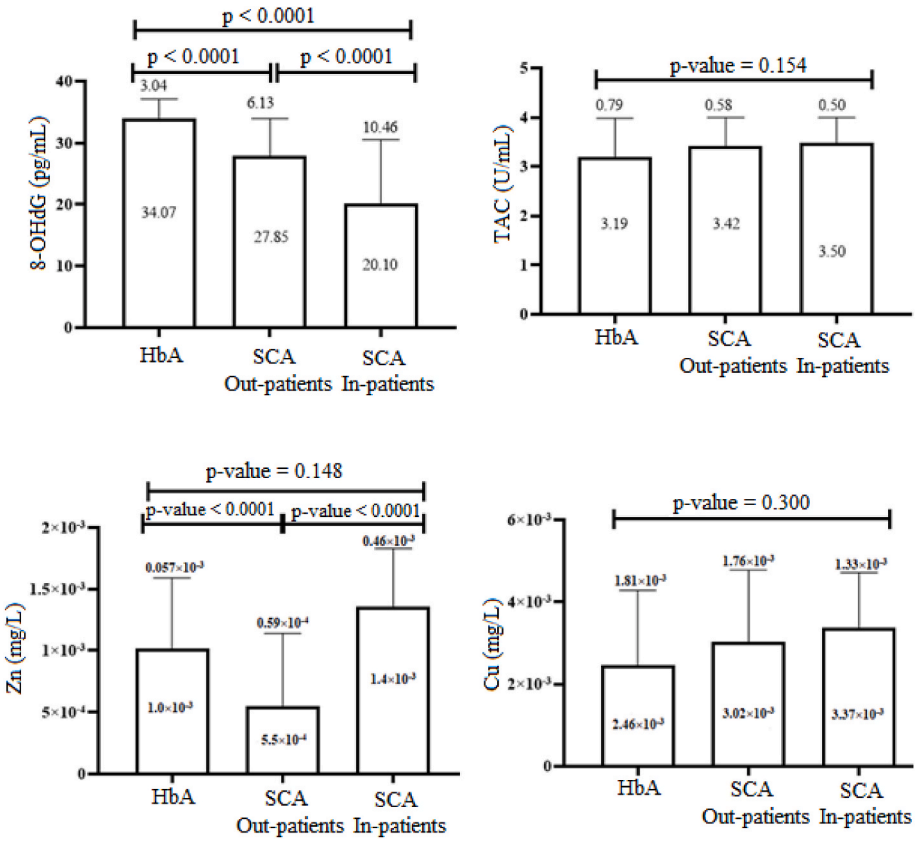

Fig. 2. Comparison of concentrations of 8-OHdG, TAC, Zn and Cu amongst the three groups.

Table 2
Correlation between HbF, and Zn, Cu, TAC and 8-OHdG levels.

Variable		HbF in Out-patients	HbF in In-patients
Cu	Spearman r	0.085	−0.186
	Sig. (2-tailed)	0.578	0.564
Zn	Spearman r	0.318	−0.193
	Sig. (2-tailed)	0.038	0.549
TAC	Spearman r	0.329	0.218
	Sig. (2-tailed)	0.029	0.497
8-OHdG	Spearman r	0.624	0.348
	Sig. (2-tailed)	0.080	0.223

(Cu – Copper, Zn – Zinc, HbF – Foetal haemoglobin).

4.1. Comparison of HbF in SCA in-patients and SCA out-patients

The SCA out-patients had a higher HbF than SCA in-patients. The presence of HbF in SCA prevents the polymerization of HbS thereby lowering the effects of the disease [19]. An earlier study found that low production of HbF can result in increased polymerization leading to crisis [20] An observation in a prospective study for management of SCA patients done in Nigeria demonstrated that HbF was elevated in SCA patients in a clinical steady state than those with vaso-occlusive crisis (VOC) but the difference was not significant just as it was in our study [21]. This could mean that differences in HbF concentrations in SCA children in crisis and in a steady clinical state are not that major.

4.2. The relationship between HbF and Zn and Cu

Zn correlated positively with HbF in SCA out-patients but negatively with HbF in the SCA in-patients. Cu correlated positively with HbF in SCA out-patients and negatively in the SCA in-patients. Both correlations pertaining to Cu were insignificant. Only the correlation of HbF and Zn in SCA out-patients was significant and this is similar with a previous case control study in Nigeria [17]. HbF is known to inhibit the polymerization of erythrocytes containing HbS and this would prevent the destruction of the red cells thereby preventing the release of Zn. The red blood cells are important storage sites for Zn [17]. In the case of Cu correlating positively with HbF in the SCA out-patients, Emokpae's finding was not in agreement with our study as their study found a negative correlation between Cu and HbF [17].

4.3. Comparison of the levels of 8-OHdG, TAC, Zn and Cu amongst the three groups

The present study indicated that SCA in-patients had the least amount of 8-OHdG as compared to the other groups. SCA in-patients had a smaller amount of 8-OHdG and this may be due to the fact that they were well managed at the ward. Majority of the patients received fluid replacement therapy and this inhibits the polymerization of HbS [22]. Erythrocyte dehydration leads to an increase in cellular haemoglobin increasing the propensity of RBCs containing HbS to sickle [22]. The decrease of plasma volume causes the VOC to be severe resulting in dehydration. The intracellular dehydration further manifests into haemoglobin polymerization and more sickling [23]. The fluid replacement could also be a reason for the low 8-OHdG concentration as the DNA degradation would be lessened as VOC induced OS is prevented [24]. Few of the patients were given haemoglobin or haemotransfusion and these reduce the levels of HbS [23]. The reduction of HbS will ameliorate the effects of the sickle cell disease which includes OS. The SCA in-patients that were given painkillers that were nonsteroidal anti-inflammatory drugs (NSAIDs) would have anti-inflammatory effect thereby curbing OS by reducing ROS. Therefore, DNA damage would also decline. Finally, folic acid drops 8-OHdG levels. OS injury results in endothelial cell damage and this leads to DNA damage. 8-OHdG levels in this situation are curbed by folic acid. Folic acid is an oxidized form of

folate and has a high bioavailability. It is a part of one carbon metabolism comprising of nucleotide metabolism, methylation metabolism and maintenance of cellular redox status. Folic acid deficiency can cause a loss in the function of superoxide dismutase (SOD). The increased production of ROS is a common feature in SCA and this damages the biomolecules especially DNA inducing apoptosis [25]. According to a previous research work [26], folic acid given in low doses can improve vascular function. It therefore acts as an antioxidant and improving the functions of antioxidants. This may explain why SCA patients had a lower 8-OHdG concentration than HbA children.

There was no significant difference between TAC levels in the three groups (Fig. 2). SCA in-patients had an elevated TAC concentration followed by SCA out-patients, then lastly HbA patients. This is due to the management of the sickle cell patients at the ward and there would be less ROS for the antioxidants to mop up [23]. Folic acid which is taken regularly by the SCA patients acts as an antioxidant by inhibiting vascular peroxidase 1 through epigenetic mechanism [25]. Cu and Zn are important cofactors in SOD and may clarify why the in-patients had a greater TAC level [15].

Zn was higher in SCA in-patients than the other groups (Fig. 2). This result was different in an earlier study [27]. The Zn levels of the controls were greater than SCA out-patients. The SCA in-patients had elevated Zn concentrations than SCA out-patients and the controls. It was discovered in another research that Zn was lower in SCA patients in a steady state and those with history of VOC as compared to normal controls [28]. This could be due to the fact that the previous work was done in adults who are less prone to Zn deficiency. In this study, Zn level was significantly lower in SCA out-patients than the in-patients. The in-patients were well hydrated due to the fluid replacement therapies and as such VOC would be less severe. The effect is that haemolysis is decreased and Zn would not be released from the RBCs. The erythrocytes are key storage sites for Zn [17]. In another similar work [15], Zn was rather lower in SCA in-patients than the out-patients. Their results were different from this study because their exclusion criteria consisted of SCA patients on Zn medication. This current work included those on Zn medication as it were administered to them as an immune booster and in cases of diarrhoea. The HbA children had a greater mean concentration of Zn than the SCA out-patients ($p < 0.0001$). This observation was similar as to research work by Emokpae and Musa [17], Emokpae et al., in 2019 [15] and also with other studies [29,30]. Zn is likely to be low in SCA children since they normally suffer from chronic haemolysis and there is increased demand of Zn [15].

Similarly, Cu was greater in the SCA in-patients than the other categories of the participants. The differences between the quantities of Cu in the SCA patients and the controls did not vary significantly. Though the differences in the levels of Cu were insignificant, SCA out-patients had greater amounts than the HbA patients. There was a particular research that had an opposite finding to this and their findings were not significant [12]. This current study's result was consistent with earlier research works [15,17,29]. This might be because the SCA candidates had a higher amount of TAC since Cu is essential in the formation of SOD. On the other hand, SCA out-patients had lower levels of Cu as compared to the in-patients and this is similar to a preceding research [15]. Also, in a prior study, the mean concentrations of Cu in SCA patients in crisis and SCA patients in a steady state were lower than the normal controls [28].

5. Conclusion

Sickle cell patients on admission had lower levels of 8-OHdG. Also, the zinc and copper concentrations were generally higher in SCA in-patients than others. Likewise, their resulting TAC too was also greater than the SCA out-patients and controls. This suggests that high amounts of micronutrients are essential in maintaining the redox status in SCA patients. In addition, the management of SCA children in the form of hydration and other medications when on admission is crucial and beneficial. There was a significant correlation between HbF and zinc and TAC among the SCA out-patients. HbF might influence some micronutrients.

Author contributions

Conceptualization, A.G.O.P., D.G., R. K. D. E., J.G.A. and O.A.M.; data curation, A.G.O.P. and J.G.A; formal analysis, A.G.O.P.; B.S. and E. OA., funding acquisition, A.G.O.P.; investigation, A.G.O.P. and R.K.D.E.; methodology, A.G.O.P. and R.K.D.E.; project administration, A.G.O.P., E.T. and O.A.M.; resources, A.G.O.P. and J.G.A.; software, A.G.O.P.; supervision, D.G., E.T., B.S., E.O.A. and O.A.M; validation, A.G.O.P., D. G., B.S. and E.O.A.; visualization, A.G.O.P, D.G., R.K.D.E., writing-original draft, A.G.O.P. and E.O.A.; writing-review and editing, A.G.O. P., D.G., E.T., R.K.D.E., B.S., W.I.O.B., M.E.A.A., E.O.A. and O.A.M.

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Declaration of competing interest

There are no conflicts of interest pertaining to this study.

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