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Assessment of thyroid hormone levels among Sickle Cell Disease Patients in steady clinical state at Nnamdi Azikiwe University Teaching Hospital, Nnewi, Nigeria.

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ABSTRACT

Background of study: Sickle Cell Disease (SCD) is an inherited haemoglobinopathy characterized by life-long haemolytic anaemia and vaso-occlussive crisis. Oxidative stress may be linked to its pathophysiology which may lead to several organ damage and organ dysfunctions. **Objective:** To assess the effect of sickle cell disease on thyroid hormone levels using TSH, T4 and T3 as markers. **Materials and methods:** A total of 90 male subjects (consisting of 30 HbSS subjects in steady state, 30 HbAS individuals and 30 normal subjects (HbAA) as the control subjects) aged 18 to 60 years were randomly recruited for this study. The genotypes of the subjects were determined using cellulose electrophoretic procedure and the method adopted for the determination of serum tri-iodothyronine (T3), thyroxine (T4) and thyroid stimulating hormone (TSH) levels was the enzyme-linked immunosorbent assay (ELISA) technique. Full blood count was determined by the Sysmex automated procedure while the disease severity was evaluated using the severity scoring technique. **Results:** There was a significant difference in the mean serum levels of T3, T4 and TSH in the different blood genotype groups (P<0.05). The post-hoc analysis showed a significantly lower mean serum levels of T3 and T4 in HbSS subjects compared with the HbAA individuals (P<0.05) respectively. More so, the mean serum level of TSH in homozygous sickle cell individuals (HbSS) was significantly higher than the control (HbAA) and heterozygous sickle cell subjects (HbAS) (P<0.05). Furthermore, the correlation coefficient between serum levels of T3 (r=0.083) (P=0.692) and TSH (r=0.277) (P=0.191) showed a non-significant positive correlation with disease severity in HbSS subjects. **Conclusion:** There was a significant low serum level of T3 and elevated TSH in the presence of a non-significant linear correlation of T3 and TSH with SCD severity, suggesting a possible primary hypothyroidism in sickle cell disease patients. We thereby recommend regular supplementation with nutrients that could aid the functional activities of thyroid gland in sickle cell disease individuals.

INTRODUCTION

Sickle Cell Disease (SCD) is a hereditary hemoglobinopathy characterized by abnormal hemoglobin production, hemolytic anemia and intermittent occlusion of small vessels leading to acute and chronic tissue ischemia, chronic organ damage and organ dysfunction (1). Haemoglobin A (HbA) is a tetrameric protein that is composed of two α-globin chains and two β-globin chains. In sickle cell anemia, a point mutation on the β-globin gene results in glutamic acid substituting for valine at position 6 of the amino acid sequence. This single amino acid substitution results in the formation of sickle cell hemoglobin (HbS). Hemoglobin S polymerizes upon deoxygenation and this causes erythrocyte rigidity, sickling and early destruction (2). Vaso-occlusion and hemolysis related to these rigid and/or sickled cells lead to disease manifestations, including hemolytic anemia, severe pain episodes from bone marrow ischemia, central nervous system strokes, the acute chest syndrome, pulmonary hypertension, left-sided heart disease, bacteremia, leg ulcers, growth failure, priapism and damage to the spleen, kidneys, liver and bones (2). SCD is among the most common monogenetic diseases worldwide. It is estimated that 312,000 people with haemoglobin SS (HbSS) are born each year throughout the world, with the majority of these births (236,000) in sub-Saharan Africa (3). In Nigeria, carrier prevalence is about 20 to 30% (3,4). The prevalence of SCA in Nigeria ranges from 0.4-3% (2).

The thyroid gland is an endocrine gland located in the neck and secretes thyroid hormones, which primarily influence the metabolic rate (5). Hormonal output from the thyroid is regulated by thyroid-stimulating hormone (TSH) secreted from the anterior pituitary gland (5). Sickle cell anemia patient have been reported to suffer from endocrine dysfunctions which often have an influence on growth,

development and metabolism (6,7). Patients with hemoglobin SS disease and other severe anemia's exhibit a variety of homeostatic responses, including changes in red cell metabolism and an enhancement of oxygen delivery by the red cell (8). The majority of the decrease in hemoglobin oxygen affinity appears to be due to an accumulation of 2, 3-Diphosphoglycerate (DPG) within the red cell (3). Although all of the factors that influence red cell DPG concentration invivo are not known, it is thought that DPG elevations may be caused by increased deoxygenation of the hemoglobin (3) and increased levels of thyroid hormones (triiodothyronine T3 and tetraiodothyronine T4) (4,). The variations in the extent of severity of endocrine abnormality may be related, at least in part, to the severity of the sickle cell disease. In addition to other contributory factors, such as tissue hypoxia secondary to red cell sickling, tissue damage is mainly responsible for dysfunction (9).

Blood transfusion and red blood cell (RBC) destruction leads to iron overload with disruption of tissue vitalization during vaso-occlusive crisis and inflammatory mediators that mostly cause the metabolic and endocrine dysfunction (10). Erythrocyte transfusion therapy is associated with iron overload and possible iron-induced organ damage (11). The human body has no effective physiological mechanism for excreting excess iron. Therefore, in conditions such as sickle cell disease (SCD), where transfusions are frequently indicated, exogenous iron can accumulate, circulate as non-transferrin bound iron (NTBI), enter tissues, form reactive oxygen species (ROS), and result in end organ damage. However, patients with SCD, compared with thalassemic patients, despite a similar transfusion load, may be relatively protected from iron mediated cardiac and endocrine gland toxicity (12,13). The etiology of thyroid dysfunction in SCD is not clear; however,

most affected patients have received multiple transfusions consistent with severe iron overload. Autopsy reports in some patients have shown significant iron deposition in the thyroid gland, suggesting that the etiology of the primary thyroid failure might well be transfusion hemosiderosis and subsequent cellular damage to the thyroid gland (13,15). Therefore, this present study focuses on the assessment of thyroid hormone levels in sickle cell subjects using TSH, T4 and T3 as markers.

MATERIALS AND METHODS
Study site
This research was carried out in Haematology unit in Nnamdi Azikiwe University Teaching Hospital Nnewi, Anambra state, Nigeria.

Study design
This is a case-control study designed to assess thyroid function in sickle cell disease subjects in Nnamdi Azikiwe University Teaching Hospital, Nnewi, Anambra state, Nigeria. A total of 90 subjects within the age range of 17 to 60 years were randomly recruited for this study. This included thirty (30) homozygous sickle cell (HbSS) subjects in steady state, thirty (30) heterozygous sickle cell (HbAS) individuals and thirty (30) normal subjects (HbAA) (control). The selection of the steady state group was dependent on subjects not experiencing crisis for at least 2 weeks and not receiving blood transfusion for at least 3 months prior to the study.

Inclusion and exclusion criteria
Homozygous (HbSS) sickle cell disease subjects in steady state, heterozygous sickle cell (HbAS) subjects and normal healthy subjects (HbAA) were included in this study whereas individuals with recent history of blood transfusion and chronic conditions such as hepato or renal insufficiency or any known endocrine

disease, individuals on medications such as rexinoids, dopamine agonists and iodine supplements which have variable effects on thyroid function and subjects outside the age range of 18 to 60 were excluded.

Ethical consideration
The ethical approval for this research was obtained from the Ethics Committee of Nnamdi Azikiwe University Teaching Hospital, Nnewi, Anambra State (NAUTH/CS/66/VOL.10/24/2017/004) and in accordance with the Helsinki Declaration by the World Medical Association (WMA) on the ethical principles for medical research involving human subjects; informed consent was obtained from the participants prior to study.

Collection of samples
Five (5) ml of venous blood was collected asceptically collected from each subject through venipuncture. 2 ml was dispensed into an EDTA container for the determination of genotype and full blood count whereas the remaining 3 ml was dispensed into a plain container and centrifuged at 5000rpm for 5 minutes. The serum obtained after centrifugation was used for the estimation of T3, T4 and TSH.
Determination of haemoglobin genotype
Haemoglobin electrophoresis was done using cellulose acetate paper as described by Manafa *et al.,* (16).
Electrophoresis
The cellulose acetate plates were pre-soaked in the TRIS-Borate buffer for 20-30 minutes and 100ml of the buffer poured into each side of the zip zone electrophoresis chamber. Making sure there was no air bubble under it, the wick was positioned over the support bridge in the chamber. The pre-soaked cellulose acetate plate was placed between absorbent papers to remove excess buffer before use. Using an applicator, 5µl of the patient haemolysates and 5µl of the haemo-controls were placed on the

cellulose acetate plate. The cellulose acetate plate with the haemolysate was placed in the electrophorectic tank across the bridge and a glass slide was placed on the paper to ensure contact with the wick. After covering the chamber, electrophoresis was run at 350 volts for 20 minutes.

Staining

The cellulose acetate plates were removed from the chamber and stained in Ponceau S for 5 minutes. Destaining was done by 3 successive washes in 5% acetic acid. The plates were allowed to stay in each wash until the background is white, the plates were dried and the bands were read against that of haemo-control with a densitometer.

Estimation of T3

The method for the estimation of triiodothyronin (T3) was as described by Tietz (17). The procedure is essentially an enzyme linked immunosorbent assay.

Estimation of T4

The estimation of thyroxin (T4) was based on the ELISA method as described by Tietz (17).

Estimation of TSH

The procedure adopted for the estimation of thyroid stimulating hormone (TSH) was an enzyme linked immunosorbent assay method as described by Tietz (17).

Estimation of full blood count (FBC)

Full blood count was estimated by the method as described by Buttarello and Plebani (18).

Severity scoring system in sickle cell disease

Disease severity score was defined by Manafa *et al.* (16).

Anaemia score

Hb $\geq$ 10g/dl $\qquad \rightarrow$ 0
Hb $\geq$ 8g/d < 10g/dl $\quad \rightarrow$ 1
Hb $\geq$ 6 < 8g/dl $\qquad \rightarrow$ 2
Hb $\geq$ 4 < 6g/dl $\qquad \rightarrow$ 3
Hb< 4g/dl $\qquad\qquad \rightarrow$ 4

Complications score

The complications included stroke, retinopathy, acute chest syndrome, nephropathy, priapism, leg ulcer, pulmonary hypertension, liver failure, and anaemic heart failure.
Each complication was scored 1 except
- Nephropathy – 2
- Stroke – 2

WBC score

- Count $< 9 \times 10^9$ cells/µl $\rightarrow$ 0
- Count $\geq 9 < 11 \times 10^9$ cells/µl $\rightarrow$ 1
- Count $\geq 11 < 15 \times 10^9$ cells/µl $\rightarrow$ 2
- Count $\geq 15 \times 10^9$ cells/µl $\rightarrow$ 3

Transfusion score

Life Transfusion Rate = Total Number of Blood Pint

$\qquad\qquad\qquad\qquad$ Age

Approximate to the nearest whole number

Hospital admission

No of hospital admission per year = No of hospital admission in the last 3 years

3

Approximate to the nearest whole number

Disease severity scores

- ≤ 3 – mild
- $> 3 \ - \leq 7$ moderate
- $> 7 \ $ – severe

Statistical analysis

Data collected was subjected to statistical analysis using the Students` t-test and the Analysis of Variance (ANOVA). Values were deemed significant at p<0.05. Correlation of the parameters with disease severity was determined using the Pearson's correlation coefficient. Statistical analysis was carried out using SPSS version 22.0.

RESULTS

Table 1: Mean ± SD of T3, T4 and TSH levels in different blood genotype groups:
A statistically significant difference was observed in the mean serum levels of T3, T4 and TSH in the different blood genotype groups (P<0.05).

Table 1: Mean ± SD of T3, T4 and TSH levels in different blood genotype groups:

Groups	Number of subjects (n)	T3 (ng/ml)	T4 (µg/dl)	TSH (µIU/ml)
HbSS	30	0.79±0.14	4.73±0.92	2.50±0.96
HbAA	30	0.98±0.20	6.07±1.56	1.72±1.02
HbAS	30	0.88±0.15	6.16±1.65	1.06±0.75
f-value		7.082	7.717	13.502
P-value		0.002	0.001	0.001

Table 2: Variations of the serum levels T3, T4 and TSH in HbSS, HbAS and HbAA Subjects:

The post-hoc analysis showed a significantly lower mean serum level of T3 in homozygous sickle cell disease (HbSS) compared with that in normal control group (HbAA) (P<0.05). However, there was no significant difference in the mean serum level of T3 in homozygous sickle cell disease (HbSS) compared with that in heterozygous sickle cell disease (HbAS) (P>0.05). The same pattern was observed when the mean serum level of T3 in heterozygous sickle cell (HbAS) disease was compared with that in the control group (HbAA) (P>0.05). A significantly lower mean serum level of T4 was observed in homozygous sickle cell disease (HbSS) compared with that in the control group (HbAA) and in heterozygous sickle cell disease (HbAS) (P<0.05). Conversely, there was a non-significant difference between the mean serum level of T4 in homozygous sickle cell disease (HbAA) compared with that in heterozygous sickle cell disease (HbAS) (P>0.05). There was a significantly higher mean serum level of TSH in homozygous sickle cell disease (HbSS) compared with that in the control group (HbAA) and in heterozygous sickle cell disease (HbAS) (P<0.05). However, no significant difference was observed in the mean serum level of TSH in the control group (HbAA) compared with that in heterozygous sickle cell disease (HbAS) (P>0.05).

Table 2: Variations of the serum levels T3, T4 and TSH in HbSS, HbAS and HbAA Subjects:

Groups	Number of subjects (n)	T3 (ng/ml)	T4 (µg/dl)	TSH (µIU/ml)
HbSS vs HbAA	30	P=0.001	P=0.006	P=0.021
HbSS vs HbAS	30	P=0.255	P=0.003	P<0.001
HbAA vs HbAS	30	P=0.183	P=1.000	P=0.077

Figure 1: Correlation between triiodothyronine (T3) and disease severity in homozygous sickle cell (HbSS) anemia subjects in steady state**.**
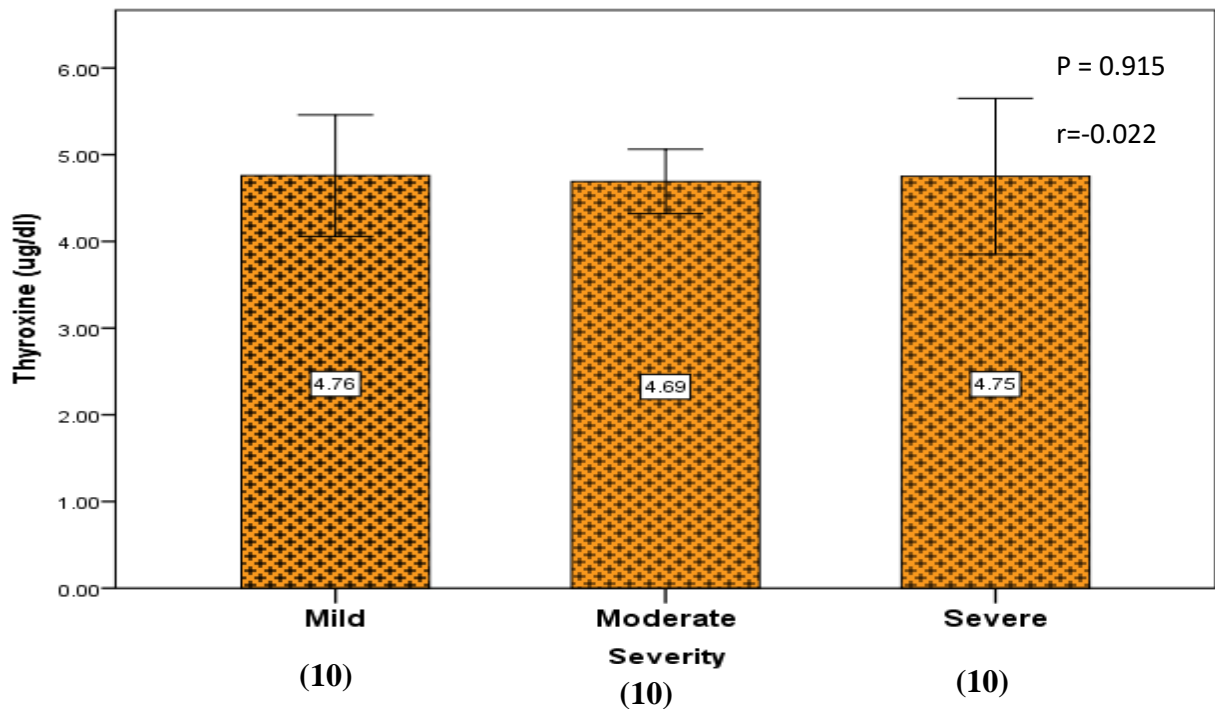There was a non-significant positive association between the serum levels of T3 and disease severity (10 mild, 10 moderate and 10 severe) in subjects with homozygous sickle cell (HbSS) disease in steady state (r=0.083) (P=0.692).
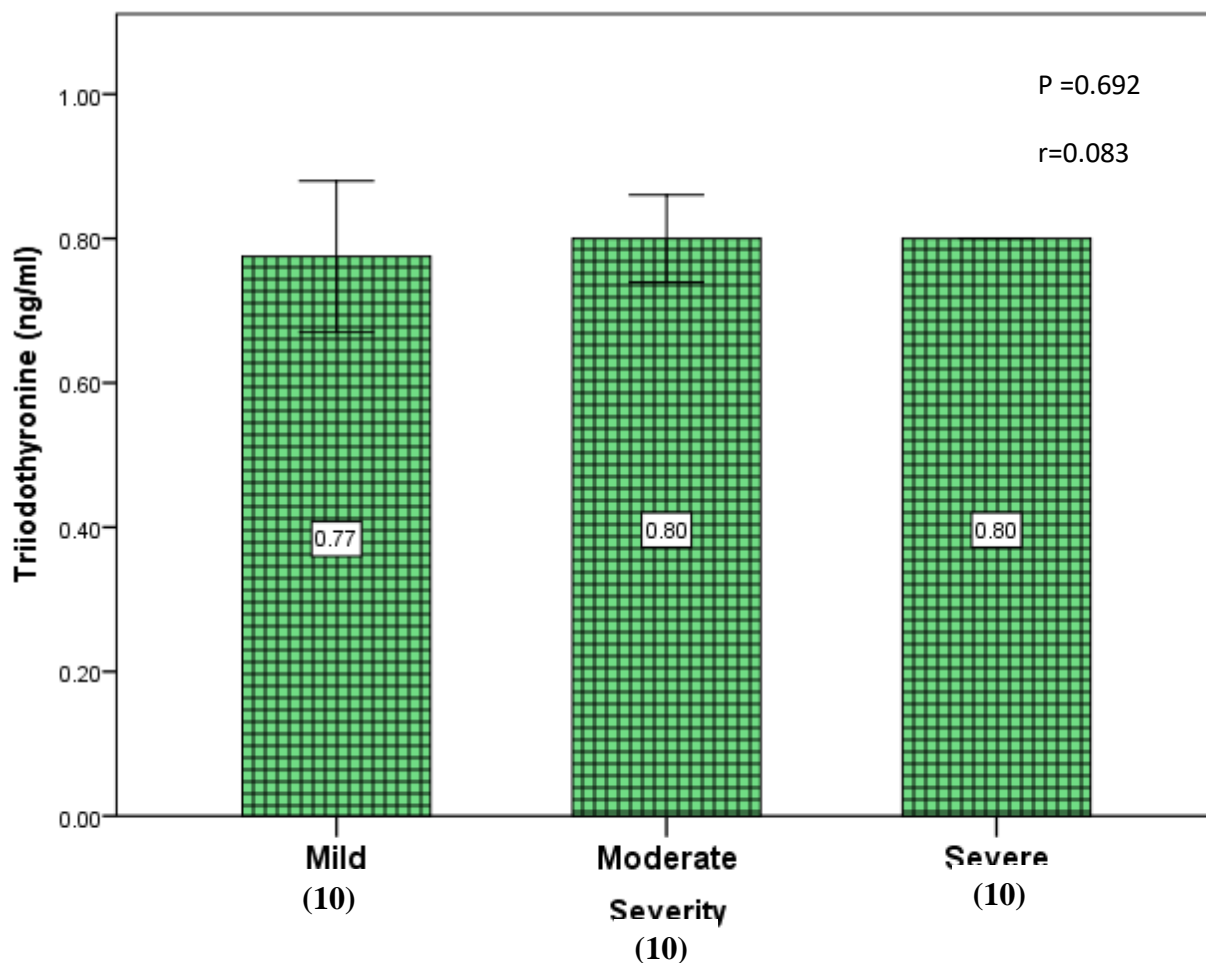
Figure 1: Correlation between triiodothyronine (T3) and disease severity in homozygous sickle cell (HbSS) anemia subjects in steady state.

Figure 2 shows the correlation between thyroxine (T4) and disease severity in homozygous sickle cell (HbSS) anemia subjects in steady state.

There was a non-significant negative relationship between the serum levels of T4 and disease severity in subjects with homozygous sickle cell (HbSS) disease in steady state (r=-0.022) (P=0.915).
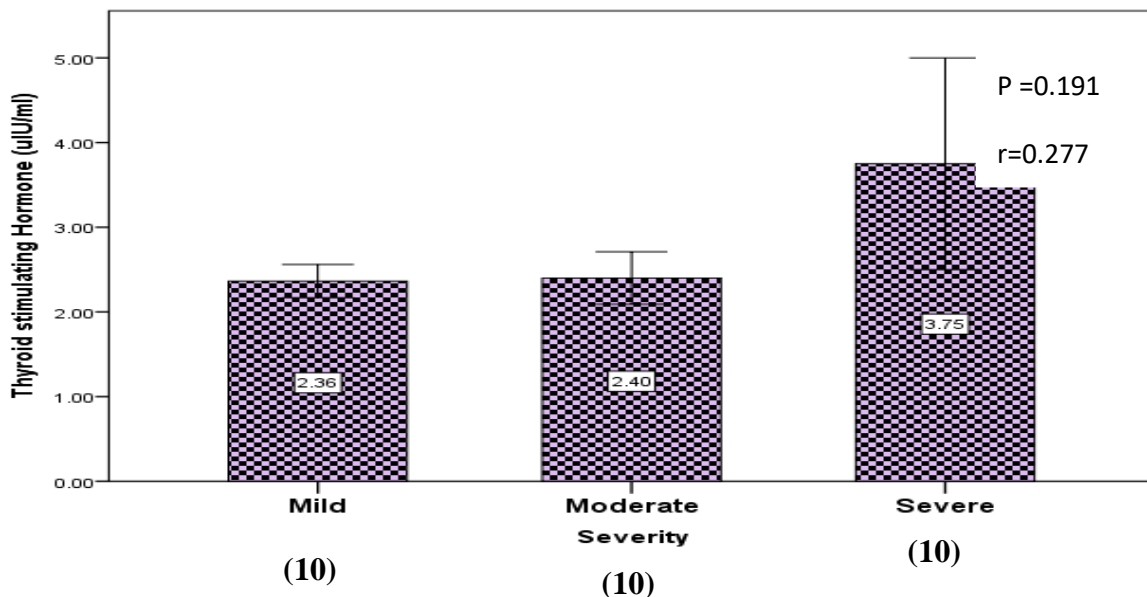
Figure 2: Correlation between thyroxine (T4) and disease severity in homozygous sickle cell (HbSS) anemia subjects in steady state.

Figure 3 shows the correlation between thyroid stimulating hormone (TSH) and disease severity in homozygous sickle cell (HbSS) anemia subjects in steady state. There was a non-significant positive association between the serum levels of TSH and disease severity in subjects with homozygous sickle cell (HbSS) disease in steady state (r=0.277) (P=0.191).

Figure 3: Correlation between thyroid stimulating hormone (TSH) and disease severity in homozygous sickle cell (HbSS) anemia subjects in steady state.

DISCUSSIONS

Sickle cell anemia is an inherited blood disorder affecting approximately 2 to 3% of the Nigerian population (20). Increasing evidence points towards an oxidative stress response responsible for increased pathophysiology of secondary dysfunctions in sickle cell patients (21). The metabolic effects of thyroid hormones (THs) are directly linked to ROS production and oxidative stress and these conditions are also associated with sickle cell disease (22). Both hyperthyroidism and hypothyroidism have been shown to be associated with OS and special cases are the autoimmune thyroiditis or the functional picture of low-T3 syndrome (23,24).

In this study, a statistically significant difference was observed in the mean serum levels of T3, T4 and TSH in the different blood genotype groups (P<0.05). The post-hoc analysis showed a significantly lower mean serum level of T3 in homozygous sickle cell disease (HbSS) compared with that in normal control group (HbAA) (P<0.05). There was a significantly higher mean serum level of TSH in homozygous sickle cell disease (HbSS) compared with that in the control group (HbAA) and in heterozygous sickle cell disease (HbAS) (P<0.05). According to Ekuma-Okereke *et al.* (25), T3 represents the metabolically active thyroid agent that possibly has a vasodilatory effect on the vascular muscle cells. Therefore, its significant decrease (P<0.05) in homozygous sicklers (HbSS) (0.79±0.14) than in heterozygous (HbAS) (0.88±0.15) and control (HbAA) (0.98±0.20) subjects could be as a result of the inhibitory effect of SCD on T3 producing enzyme thereby causing the significant elevation of TSH as obtained. This is because SCD as an autoimmune

disorder could cause impaired production of vasodilators which has also been implicated in reduced thyroid function (11). Therefore, the significant decrease in the serum level of fT3 could be due to the relative inhibition of T3 secretion; a resultant effect of endothelia dysfunction associated with increased serum TSH resulting in the development of primary hypothyroidism as demonstrated in this study. This finding is in line with the findings of (26,27) who observed that male patients with the SCD had significantly lower endogenous T3 and higher TSH levels than their comparison groups. However, some researchers did not find significant difference in the levels of T3 (triiodothyronine), T4 (thyroxine), or TSH (thyroid stimulating hormone) among the HbSS and HbAA hemoglobin carriers (27,28).

More so, there was a non-significant positive correlation between the serum levels of T3 and TSH with disease severity in homozygous sickle cell disease (HbSS). This suggests that the more the disease severity, the greater the endothelial dysfunction hence, the higher the organ dysfunction. This is evidently demonstrated by the finding of Ramirez (29) who reported a linear correlation between the mean serum levels of T3 and T4 with the severity of renal failure (30). Thus, there appears to be high preponderance of hypothyroidism in CKD. The liver plays an important role in the metabolism of THs, as it is the most important organ in the peripheral conversion of T4 to T3 by deiodinase (31). Moreover, it is involved in conjugation and circulation of THs by synthesis of thyroid binding proteins (32). Nonetheless, there is evidence of an association between chronic liver diseases and changes in the thyroid gland and it have been postulated that levels of THs and their binding

proteins are altered in patients with hepatic disorders, especially in cirrhosis (33). The most frequent change in plasma levels of THs is a decrease in T3 concentrations, which is reported to be associated with the severity of hepatic dysfunction (34). The low T3 levels may be regarded as an adaptive hypothyroid state that serves to reduce the basal BMR within hepatocytes and preserve liver function and total body protein stores (35). Many mechanisms exist by which THs decrease during illness (36). For instance, decreased peripheral conversion of T4 to T3 by suppression of 5′-deiodinase is thought to be a result of systemic illness and a response to increased serum concentrations of glucocorticoids and inflammatory cytokines (37-41). Therefore, increase concentrations of inflammatory biomarkers may inhibit deiodinase and impair TH synthesis but the mechanisms by which they do so remain unknown. However, iron overload as a result of frequent blood transfusion has been implicated as a cause of endocrine complications such as thyroid dysfunction in SCD (42). Autopsy reports in some patients have shown significant iron deposition in the thyroid gland, suggesting that the etiology of the primary thyroid failure might well be transfusion-hemosiderosis and subsequent cellular damage to the thyroid gland (26). As a result, hypothyroidism may be partly related to the accumulation of iron in thyroid glands due to blood transfusion by iron overload leading to gland dysfunction (11).

In the light of these findings and from several other studies we hypothesize that thyroid hormone levels are relatively altered in sickle cell disease. The suggestive implication of iron overload in patients with multiple transfusions cannot be rule-out as possible contributors. This is because, it is believed that primary hypothyroidism is secondary to iron deposition, based on TSH (thyroid-stimulating hormone) elevation after stimulus with TRH (thyrotropin-releasing hormone) and in necropsy data (13,15). This therefore implies that primary thyroid dysfunction should be considered in adult sickle cell disease patients.

Conclusion
There was a significant low serum level of T3 and elevated TSH in the presence of a non-significant linear correlation of T3 and TSH with SCD severity, suggesting a possible primary hypothyroidism in sickle cell disease patients. We thereby recommend regular supplementation with nutrients that could aid the functional activities of thyroid gland in sickle cell disease individuals.

Conflicts of interest
The authors boldly declare that there is no conflict of interest herein. This original research has neither been published nor being considered for publication elsewhere.

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