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Association between global DNA methylation pattern and some haematological parameters in sickle cell subjects

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ABSTRACT

Background: DNA methylation mechanisms have been implicated in the conversion of HbF to adult haemoglobin and the presence of HbF in sickle cell disease has been reported as good prognostic marker. The objective of this study was to determine the activities of DNA methyltransferase 1 in sickle cell subjects.

Methods: A case-control study was conducted among the sickle cell patients attending Haematology Clinic in Obafemi Awolowo University Teaching Hospital, Ile-Ife and Ladoke Akintola Teaching Hospital, Osogbo. One hundred subjects were recruited for this study. Sixty (60) were sickle cell subjects while 40 were homozygous AA control.

Results: The results showed that DNA methyltransferase 1 activity in SCD subjects (2.347±0.2472) was significantly lower ($p<0.0001$) when compared to control (10.39±2.229). Also total white cell count was significantly higher ($p=0.0305$) in sickle cell subjects (11.53±1.126) when compared to controls (5.39±0.326). Packed cell volume (25.4±0.5601), haemoglobin concentration (8.468±0.1863), red blood cell count (3.462±0.1035) and MCHC (334.9±5.755) were all significantly lower ($p<0.0001$) in sickle cell subjects when compared to controls (PCV (38.3±0.4955); HB (12.83±0.1814); RBC (5.58±0.07424); MCHC (383.9±3.25) respectively. Furthermore, SCD subjects on hydroxyurea (2.342±0.8018) had a significantly lower ($p<0.0001$) DNA methyltransferase 1 activity when compared to SCD subjects on L-glutamine (7.567±0.9179). Those on L-glutamine also had a significantly higher ($p<0.0001$) DNA methyltransferase 1 activities when compared to Crizanlizumab (1.814±0.883), Voxelotor (2.757±1.054), folic acid (2.151±0.245) and pain medication (2.163±0.240.

Conclusion: In conclusion, DNA methyltransferase 1 activity was lower in SCD subjects, especially in subjects on hydroxyurea, crizanlizumab and voxelotor.

Keywords: DNA, DNA methyltransferase 1, Sickle Cell anaemia, Haemoglobin, Hydroxyurea

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1 INTRODUCTION

Sickle cell disease (SCD), one of the most common inherited diseases worldwide, is now understood to be a disorder of global importance and economic as well as clinical significance. It was first reported by Herrick in 1910 even though reports suggest prior description of the disorder [1]; it is the result of homozygous and compound heterozygote inheritance of a mutation in the β-globin gene. A single base-pair point mutation (GAG to GTG) results in the substitution of the amino acid glutamic acid (hydrophilic) to valine (hydrophobic) in the 6th position of the β-chain of haemoglobin referred to as haemoglobin S (HbS) [1]. Phenotypic variation in clinical presentation is a unique feature of Sickle Cell Disease (SCD) despite a well-defined Mendelian inheritance, the first to be molecularly characterised as described by Pauling and confirmed to be due to a single amino acid substitution by Ingram [2] almost 70 years ago. Global DNA Methylation refers to the total level of 5mC (5-Methylcytosine) content in a sample relative to total cytosine content. This occurs at cytosine residues that precede guanine (CpG dinucleotides) by the action of DNA methyltransferase (DNMT) enzymes. It has been known for decades that changes in global Methylation are a feature of human malignancy. Approximately 65% of all CpG dinucleotides in vertebrate DNA are methylated. DNA Methylation has an important role in the regulation of gene expression. Expression of tissue specific genes is associated with an unmethylated state of the gene promoter. Lack of gene expression is associated with DNA Methylation. Fetal haemoglobin (HbF, α2) decreases polymerization of sickle haemoglobin (HbS) and high levels correlate with decreased morbidity and mortality in sickle cell disease (SCD). Silencing of the globin (HbF) gene is associated with DNA Methylation [3]. In Nigeria several studies have examined the association between haematological parameters and sickle cell diseases while none has examined this association in relation to global DNA Methylation in sickle cell, therefore the findings in this study will examine the association between the global DNA Methylation and some haematological parameters in sickle cell and this will add to the body of knowledge in this area of study. Sickle cell disease (SCD) is the commonest monogenetic disease worldwide and its greatest burden is found in Sub- Saharan Africa especially Nigeria [4, 5]. It predominantly affects people of African, Indiana and Arab ancestry [6, 7]. The global impact of sickle cell disease (SCD) has been estimated to be approximately 275,000 births per year [8] and it is equally approximated that it could reach 400,000 births by 2050 in regard to recent projections [9]. It was estimated that over 80% of over 300,000 annual births occur in sub-Saharan Africa (SSA), the largest burden from Nigeria and Democratic Republic of Congo [10]. Although SCD is a red cell disorder, it is well established that white cells and platelets play a significant role in the pathophysiology of this disease. Consistent with this, leukocyte and platelet counts in the stable state can be used together with clinical parameters to predict the outcome of this disorder [11, 12]. The clinical and laboratory profile of patients with sickle cell anaemia are continuously being studied to understand the biology of the disease [13, 14]. The haematological parameters are the most commonly requested investigation to determine the line of management, adjust therapy and predict outcome.

2 MATERIALS AND METHODS

2.1 Materials

2.1.1 Biological materials

The study population comprises sickle cell subjects in Obafemi Awolowo Teaching Hospital Complex (OAUTHC), Ile-Ife and Ladoke AKintola University of Technology (LAUTECH), Osogbo.

2.1.2 Equipment and Apparatus

The instrument for data collection for this study included a standard questionnaire to collect information on the demographic characteristics, past medical history, drugs and blood transfusion history of sickle cell subject. Samples for Global Methylation and Haematological indices were collected using standard procedure

2.2 Methods

2.2.1 Study Area

This study will be carried out in Osun State Nigeria. Osun State is a state in south western Nigeria; bounded to the east by Ekiti and Ondo states, to the north by Kwara State, to the south by Ogun State and to the west by Oyo State. The state was named after the River Osun, a very important river which flows through the state. The state was formed from the southeast of Oyo State on 27 August 1991 and has its capital as Osogbo.

2.2.2 Study Design and Sample Size

A case control study design was used in this study to assess Global DNA Methylation and Haematological pattern in sickle cell subjects attending Haematology Clinic in Obafemi Awolowo University Teaching Hospital, Ile-Ife and Ladoke Akintola Teaching Hospital, Osogbo. One hundred subjects were recruited for this study. Sixty (60) were sickle cell subjects while 40 were homozygous AA control. The sample size was calculated based on prevalence of sickle cell trait in the study area using the formula for calculating the sample size for a case control research study. For this study, the following assumptions are made: The Prevalence of SCD is 10% to 40% (WHO, 2013). Level of confidence will be set at 95% confidence level (standard value for Z=1.96), and maximum margin of error of 5%.

2.2.3 Ethical Approval

The ethical approval required to conduct this study was obtained from ministry of health Ethical Review Board, Osun State Ministry of Health with reference Number OSHREC/PRS/569T/266.

2.2.4 Sample Collection

Six milliliters (6ml) of blood was collected from each subjects and 3 ml placed in a plain containers and EDTA tubes respectively. Samples in plain containers were stored in a refrigerator overnight for proper clot retraction. The samples were then spun to obtain a clear serum. The serum was then stored at -20^{0}C prior to analysis. EDTA samples were analyzed immediately.

2.2.5 Laboratory Analysis

Serum was used to analyse for DNA methyltransferase 1 using ELISA kits from Elabscience Biotechnology, Wuhan, China. Complete blood count was analysed using 3 part haematology autoanalyzer.

2.2.5.1 DNA Methyltransferase 1 (Global DNA Methylation) Analysis

Enzyme-linked Immunosorbent Assay (ELISA) was used in the determination of global DNA Methylation in sickle cell subjects.

Procedure: The different wells were pre-labelled with standard and samples. Then 100 µL of the standard and samples were added to the pre-determined wells and incubated for 90 minutes at 37^{0}C. After incubation, the liquid was removed and 100 µL of biotinylated detection antibody added and incubated for 1 hour at 37^{0}C. The microplate was then decanted and washed 3 times using a microplate washer. Then 100 µL of HRP conjugate was added to each well and incubated for 30 minutes at 37^{0}C and further washed 5 times using a microplate washer. Thereafter, 90 µL of substrate reagent was added and incubated at 37^{0}C for 15 minutes. Fifty (50) µL of stop solution was then added and the OD measured immediately at 450nm wavelength.

2.2.5.2 Complete Blood Count Analysis

The sample for full blood count was analyzed immediately using sysmex autoanalyzer Kobe, Japan.

2.3 Statistical Analysis and Data Management

Data collected were analysed using Graphpad Prism 10.0 (California, USA). Data were analysed using both descriptive statistics of mean and standard deviation and Student t test for analytical statistics. A value of $p<0.05$ was accepted as significant

3 RESULTS

3.1 Some social demographic data in subjects with sickle cell disease (SCD).

Table 1 shows that 26.67% of SCD subjects in this study were employed, 61.67% were unemployed and 11.7% were self-employed. Most of the SCD subjects were single (71.7%) while 28.3% were married. The data also showed that 25% of SCD subjects in this study had relatives who also had SCD while 75% of the SCD subjects had no relative with SCD. Most of the SCD subject had just one other sibling (16.67%) with SCD, 6.67% of SCD subjects had more than one sibling with SCD while 1.67% of the SCD subject's fathers had SCD. Leg ulcer (81.67%) accounted for the common complication in sickle cell subjects recruited for this study, followed by acute chest syndrome (80%), vaso-occlusive crises (76.67%), osteomyelitis (23.33%), retinopathy (11.67%), priapism (10%) and nephropathy (6.67%). The frequency of complications also shows that most complication happened monthly (50%) while others occurred more that than monthly (23.33%). 21.67% of the SCD were current in one form of crisis. Table 2 shows that most SCD subjects were folic acid (98.33%) and pain relieve drugs (96.67%) were the

drug or medication of choice. 11.67% were on hydroxyurea, 10% were on L-glutamine, 6.67% were on Crizanlizumab and 18.33% were on Voxelotor. Duration of medication showed that 90% of SCD subject have been on one form of medication or the other for more than 6 months while 6.67% have been on medication for lower than 6 months. 40% of the SCD had blood transfusion history while 60% had no transfusion history. Those with transfusion history showed that 20% of them received blood transfusion weekly, 18.33% receives blood transfusion 3 monthly and only 1.67% of them received blood transfusion twice in a year. 63.33% of SCD subjects reported that they engage in one form of exercise daily while 35% of them reported no exercise in their daily routine.

3.2 DNA methyltransferase 1 activity, haematological parameters in subjects with Sickle cells disease

Table 3 showed that total white cell count was significantly higher ($p=0.0305$) in sickle cell subjects (11.53±1.126) when compared to controls (5.39±0.326). Packed cell volume (25.4±0.5601), haemoglobin concentration (8.468±0.1863), red blood cell count (3.462±0.1035) and MCHC (334.9±5.755) were all significantly lower ($p<0.0001$) in sickle cell subjects when compared to controls (PCV (38.3±0.4955); HB (12.83±0.1814); RBC (5.58±0.07424); MCHC (383.9±3.25)) respectively. Also, the results showed that DNA methyltransferase 1 activity in sickle cell subjects (2.347±0.2472) was significantly lower ($p<0.0001$) when compared to control (10.39±2.229). In this study, we also observed the effect of medication on DNA methyltransferase 1 activity. Table 4 showed that SCD subjects on hydroxyurea (2.342±0.8018) had a significantly lower ($p<0.0001$) DNA methyltransferase 1 activity when compared to subjects on L-glutamine (7.567±0.9179). Those on L-glutamine also had a significantly higher ($p<0.0001$) DNA methyltransferase 1 activities when compared to Crizanlizumab (1.814±0.883), Voxelotor (2.757±1.054), folic acid (2.151±0.245) and pain medication (2.163±0.240. Table 5 showed the correction matrix between DNA methyltransferase 1 activity with some haematological parameters. DNA methyltransferase 1 had a positive correlation with total WBC ($r=0.1773$) (figure 1) as shown in scatter plot. There was a negative correction between DNA methyltransferase 1 and neutrophil ($r=-0.03666$) (figure 2), haemoglobin concentration ($r=-0.1805$) (figure 3), PCV ($r=-0.1798$) (figure 4), RBC count ($r=-0.0944$), MCV ($r=-0.06738$), MCH ($r=-0.0965$) and platelet count ($r=-0.0401$). However, no significant correlations were observed.

Table 1: Frequency distribution of employment history, marital status, relatives with sickle cell and complications from sickle cell in Sickle Cell disease subjects.

Employment History	**Frequency Distribution**	**Percentage**
Employed	16	26.67
Unemployed	37	61.67
Self Employed	7	11.7
Retired	0	0
Marital Status		
Single	43	71.7
Married	17	28.3
Divorced	0	0
Widowed	0	0
Any relative with Sickle cell		
Yes	15	25
No	45	75
Relative relationship		
Father	1	1.67
Mother	0	0
1 Sibling	10	16.67
more Than 1 Sibling	4	6.67

Complications from Sickle Cell		
Leg Ulcer	49	81.67
Osteomyelitis	14	23.33
Vaso Occlusive Crises	46	76.67
Acute Chest Syndrome	48	80
Retinopathy	7	11.67
Nephropathy	4	6.67
Priapism	6	10
Frequency of Complication		
> Monthly	14	23.33
Monthly	30	50
Weekly	12	20
currently in crisis	13	21.67

Table 2: Frequency of type of medication used and history of blood transfusion in subjects with Sickle Cell Disease

Medication	Frequency Distribution	Percentage
Hydroxyurea	7	11.67
L-glutamine Oral Powder	6	10
Crizanlizumab	4	6.67
Voxelotor	11	18.33
Folic acid supplement	59	98.33
Pain relieve	58	96.67
Duration of Medication		
< 6 months	4	6.67
> 6 months	54	90
Others	2	3.33
Blood Transfusion		
Yes	24	40
No	36	60
Frequency of Blood Transfusion		
Weekly	0	0
Monthly	12	20
3 monthly	11	18.33
6 monthly	1	1.67
Never	36	60
Regular Exercise		
Yes	38	63.33

No	21	35

Table 3: Some haematological parameters and DNA methyltransferase 1 activity in sickle cell subjects.

Parameters	Control	Sickle Cell Subjects	t value	p value
Total WBC ($X10^9$/L)	5.39±0.326	11.53±1.126	2.209	0.0305
PCV (%)	38.3±0.4955	25.4±0.5601	9.254	<0.0001
Haemoglobin (g/dL)	12.83±0.1814	8.468±0.1863	9.388	<0.0001
RBC ($X10^9$/L)	5.58±0.07424	3.462±0.1035	8.253	<0.0001
MCH (pg)	33.1±0.9713	30.9±0.495	1.721	0.0898
MCHC (g/dL)	383.9±3.25	334.9±5.755	3.445	0.001
MCV (fL)	83.1±2.258	81.52±1.375	0.4521	0.6526
Lymphocyte Count (%)	39.3±2.556	33.05±2.047	1.215	0.2285
Neutrophils (%)	61±2.324	65.53±2.007	0.9013	0.3706
MID (%)	0.7±0.2134	1.767±0.3431	1.255	0.2137
Platelets Count ($X10^9$/L)	254.2±21.46	390.9±18.45	2.955	0.0043
DNMT1 (ng/mL)	10.39±2.229	2.347±0.2472	7.537	<0.0001

Results represent mean±SEM

Table 4: Effect of medication on DNA methyltransferase 1 activity (DNMT 1) in sickle cell subjects

Types of Medication	DNMT 1 (ng/mL)	F value	P value
Hydroxyurea	2.342±0.8018	9.544	0.0001
L-glutamine	7.567±0.9179[a]		
Crizanlizumab	1.814±0.883[b]		
Voxelotor	2.757±1.054[b]		
Folic acid	2.151±0.245[b]		
Pain relieve	2.163±0.240[b]		

Results represent mean±SEM. superscript represents significance with hydroxyurea, b significance with L-glutamine

Table 5: Correction with DNA methyltransferase 1 activity with some haematological parameters

Correlation	r value	p value
DNMT 1 (ng/mL) vs TWBC ($X10^9$/L)	0.1773	0.1754
DNMT 1 (ng/mL) vs. Lymphocyte Count (%)	0.05632	0.6691
DNMT 1 (ng/mL) vs. Neutrophil (%)	-0.03666	0.7809
DNMT 1 (ng/mL) vs. Haemoglobin (g/dL)	-0.1805	0.1676
DNMT 1 (ng/mL) vs. PCV (%)	-0.1798	0.1692

DNMT 1 (ng/mL) vs. RBC Count ($X10^9$/L)	-0.0944	0.4731
DNMT 1 (ng/mL) vs. MCV (fL)	-0.06738	0.609
DNMT 1 (ng/mL) vs. MCH (pg)	-0.0965	0.4633
DNMT 1 (ng/mL) vs. MCHC (g/dL)	0.1517	0.2471
DNMT 1 (ng/mL) vs. Platelet ($X10^9$/L)	-0.0401	0.761

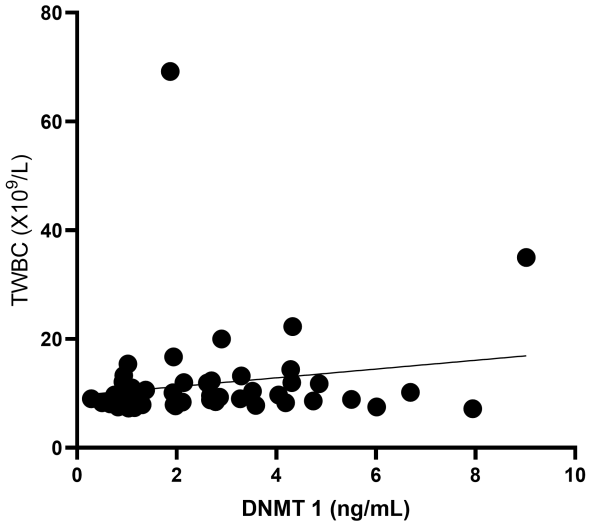

Figure 1: Scatter plot showing linear regression between DNA methyltransferase 1 activity and total WBC.

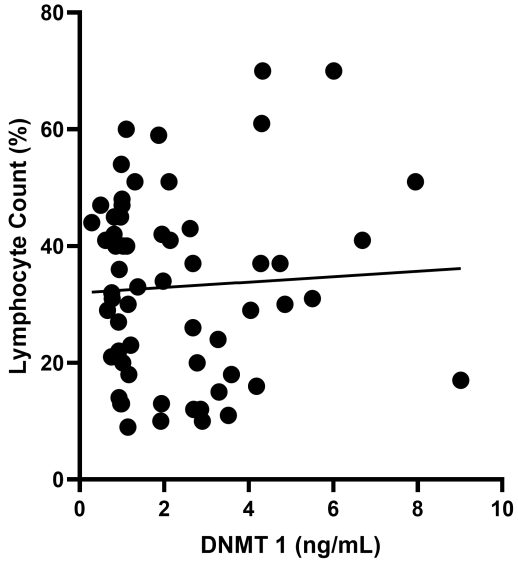

Figure 2: Scatter plot showing linear regression between DNA methyltransferase 1 activity and lymphocyte count.

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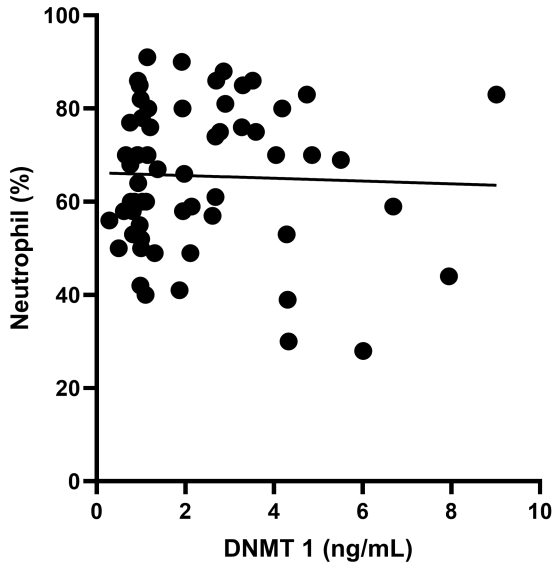

Figure 3: Scatter plot showing linear regression between DNA methyltransferase 1 activity and total Neutrophil count

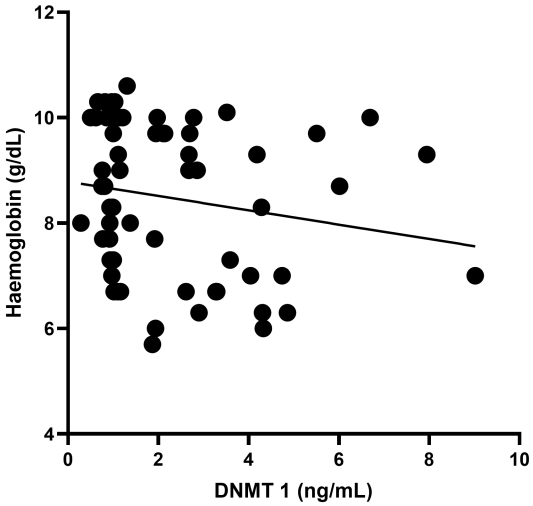

Figure 4: Scatter plot showing linear regression between DNA methyltransferase 1 activity and haemoglobin concentration.

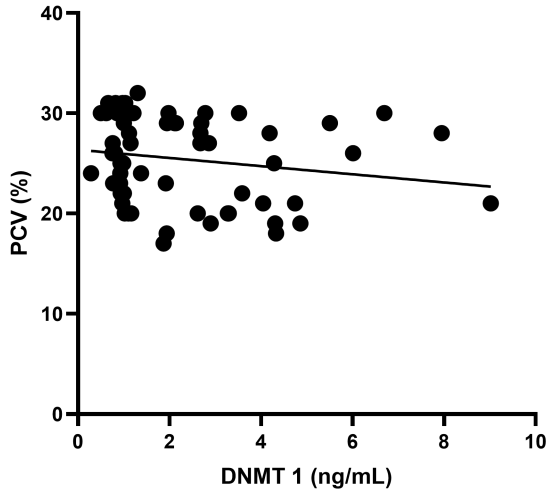

Figure 5: Scatter plot showing linear regression between DNA methyltransferase 1 activity and packed cell volume.

4 DISCUSSION

Sickle cell disease (SCD) is one of the most common inherited hemoglobinopathies, affecting millions of people worldwide. Reactivation of HBG (*HBG1, HBG2*) gene expression and induction of fetal hemoglobin (HbF) is an important therapeutic strategy for ameliorating the clinical symptoms and severity of SCD [7]. During fetal development and until shortly after birth, erythrocytes preferentially express an alternative hemoglobin tetramer termed fetal hemoglobin (a2γ2; HbF) that is composed of two γ-globin chains paired with a-globin chains rather than β-globin chains. The genes encoding for γ-globin, haemoglobin gamma subunit 1 (HBG1) and 2 (HBG2), lack the mutation that causes SCD. Consequently, symptoms of SCD first manifest several months after birth following the "hemoglobin switch", the transition from HbF to HbA, or to HbS in the case of SCD patients [15]. During the transition from HbF to HbA/HbS, the genes encoding for γ-globin, *HBG1* and *HBG2*, are repressed by transcriptional complexes that include *GATA1, TR2/TR4, MYB, KLF1, Sox6, BCL11A, LRF, DNMT1, and HDAC1/2* [16, 17]. The objective of this study was to determine DNA methyltransferase 1 activities and some haematological parameters in sickle cell subjects, in Osun State, Nigeria. The results from this indicate that DNA methyltransferase 1 activity was reduced in sickle cell subjects when compared to controls. Furthermore, sickle cell anaemia subjects on hydroxyurea also had a significantly lower DNA methyltransferase 1 activity when compared to those on L-glutamine therapy. Similarly, Estepp *et al*. [18] supports the use of hydroxyurea (HU) in children with SCD, with the preferred dosing strategy targeting an HbF endpoint >20% to reduce clinical complications and hospitalizations. Individuals with naturally occurring hereditary persistence of HbF variants have significantly higher HbF levels in the majority of their red blood cells (pancellular) producing benign clinical phenotypes. Thus, to achieve maximal clinical benefit, it is also critical that therapeutic agents produce a pancellular distribution of Hb F. Methylation of mammalian genomic DNA is catalyzed by DNMTs. The mammalian DNMT family includes four active members: DNMT1, DNMT3A, DNMT3B, and DNMT3L [19, 20]. The DNMT1 is the major enzyme responsible for maintenance of the DNA methylation pattern. DNMT1 is also often referred to as maintenance methyltransferase, because it is believed to be the primary enzyme responsible for copying methylation patterns after DNA replication [21]. DNMT1 is located at the replication fork and methylates newly biosynthesized DNA [22]. Decitabine and 5-aza cytidine are potent inhibitors of DNA methyltransferases (DNMT) and these two potent inhibitors also induce HbF. DNMT1 is the primary maintenance methyltransferase that propagates the pattern of DNA methylation to daughter cells during cell division [23, 24]. In this study, total WBC was significantly higher in sickle cell subjects Akinbami *et al*. [25] reported a similar findings in homozygous sickle cell subjects in Lagos. The leucocytosis in sickle cell disease patients may due to auto splenectomy resulting from recurrent splenic vessels occlusion, which makes patients more vulnerable to overwhelming infections particularly, encapsulated organisms like *Streptococcus pneumonia* and *Haemophilus influenza* [26]. Also, the increased total white cell count may be due to re-distribution of the white cells between the marginal and circulating pools, pain, nausea and vomiting and anxiety, which common signs and symptoms in sickle cell subjects have been reported to cause leucocytosis in the absence of infection [27]. It was also observed in this study, that platelet count was significantly higher in sickle cells subjects.

This is probably due a negative feedback effect on erythropoietin production in subjects as a result of the anaemia could be responsible for the thrombocytosis. Erythropoietin has a structural homology with thrombopoetin, although the latter is considerably larger than the former but roughly half of thrombopoetin has identity with or similarity to erythropoietin at the N-terminal region [28]. It is therefore, well recognized that thrombocytosis is associated with anaemia of chronic disease and several types of anaemia. Reduced or absent splenic sequestration of platelets as a result of hyposplenism in sickle cell disease also contribute significantly to higher mean platelet counts in sickle cell disease compared with controls [29]. In this study, PCV, haemoglobin concentration, red cell count were all lower in sickle cell subjects when compared to controls this probably due severe haemolysis in sickle cell subjects as a of haemoglobin polymerization. This agrees with work of other researchers, who reported decreased MCHC, RBC count, haemoglobin concentration and haematocrit value in sickle cell subjects both in crises and steady state [25, 30]. Conclusively, the results from this study indicate that sickle cell subjects had a lower DNA methyltransferase 1 activity. Also treatment with hydroxyurea, folic acid, Voxelotor and Crizanlizumab also shows a lower DNA methyltransferase 1 activity when compared to those subjects administered L-glutamine which is suggestive of their DNA Methylation pattern in sickle cell subjects. RBC count, haematocrit and haemoglobin concentration were lower in sickle cell subjects. In addition, total WBC and platelet count were significantly higher in sickle cell subjects.

DECLARATIONS

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Conflict of Interest
The authors declare that they had no conflict of interest in the course of this research

Authors' contributions
This work was carried out and approved in collaboration between all the authors who takes responsibility for its intellectual contents, accuracy and integrity. AIA, OLO and KEA designed the study; AOA and VOO sourced for funding; AIA, OLO AOA and VOO wrote the protocol; AIA and KEA contributed in literature search; AIA, AOA and VOO participated in the Laboratory experiments; AIA did the statistical data analysis. All authors contributed in the discussions. AIA and OLO drafted the manuscript. All authors read and approved the final manuscript.

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