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Plasma Homocysteine, Methyl-Malonic Acid, Vitamin B_{12} and Folate Levels in Adult Nigerian Sickle Cell Anaemia Patients

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Authors' contributions

This work was carried out in collaboration between all authors. Author JAO source and wrote the proposal for the grant. Authors JAO, KSA, ADA and OGA designed the study, Authors JAO and OGA performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Authors KSA and ADA managed the analyses of the study. Author ADA coordinated sample collection while authors JAO and OGA managed the literature searches. All authors read and approved the final manuscript.

Original Research Article

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ABSTRACT

Aims: To compare the mean levels of plasma total Homocysteine (tHct), Methylmalonic acid (MMA), vitamin B_{12}, folate and haematological parameters(PCV, WBC, Platelet counts, MCV, MCH, MCHC) among adult SCA patients in steady state (SS), SCA in (VOC) and age and sex matched controls in order to determine significant differences.
Study Design: Case-control study.
Place and Duration of Study: Department of Haematology and Department of Chemical Pathology, University College Hospital, Ibadan, Nigeria between March 2012 and July 2012.
Methodology: We included 60 SCA patients (30 in VOC, 30 in Steady State; and 30 age and sex matched controls. Plasma tHct, MMA, folate and vitamin B_{12} were assessed using HPLC and haematological parameters were determined using haematological autoanalyzer (Syxmex Kx21).

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Results: The mean plasma tHct, MMA, vitamin B_{12} of SCA patients (VOC and SS) were significantly lower (p=0.000) compared to control population but the mean folate levels were comparable (p=0.085). The SCA (in VOC) had significantly lower (p=0.000) MMA and folate levels compared to SS group but the SS group had significantly lower (p=0.001) tHct level compared to VOC group. While the PCV, Hgb, MCV, and MCH were significantly lower; the WBC, platelet count and the MCH were significantly elevated in SCA patients compared to controls.
Conclusion: A larger, better controlled, multicenter study is required to confirm lower tHct and MMA found in SCA groups compared to control group and higher tHct in SCA (VOC) but higher MMA level in SCA (SS) when VOC and SS groups were compared. The haematological parameters in SCA groups were not in keeping with macrocytic anaemia but were indicative of chronic haemolytic and inflammatory process.

Keywords: Sickle cell anaemia; adult; homocysteine; folate and B_{12} level; methlmalonic acid.

1. INTRODUCTION

Sickle cell disease (SCD) is a sickling syndrome that results from homozygote or double heterozygote inheritance of mutant βs globin gene. This results in abnormal haemoglobin (Hgb) molecules with hydrophobic motif that is exposed in deoxygenated state [1]. The abnormal Hgb cascades processes of abnormal cellular adhesion, inflammation, coagulation and vasoconstriction in SCD. These perpetuate vaso-occlusive condition and promote cumulative damage to organs and tissues [2]. It occurs in high frequency, with a range of 25-45%, in many tropical countries of the world. In Nigeria, the prevalence of SCD is 2-3% and about 25% for the trait (HbAS) [3,4].

In SCD, vaso-occlusion and resultant ischaemic events constitute a major cause of morbidity and mortality but the pathogenesis is yet to be fully understood [3]. Although, Hgb polymerization in deoxygenated state causes abnormal erythrocyte deformability to irreversible sickled state, secondary micro-vascular occlusion has also been postulated but other researchers found poor correlation between extent of HbS polymerization and the frequency of episodes of painful crisis [5,6]. SCD is also characterized by chronic haemolytic process interspersed by hyper-haemolytic crisis leading to rapid exhaustion of folate and vitamin B_{12} required for active DNA synthesis required in rapid compensatory erythropoiesis. Hence, in the face of high demand or inadequate supply of these vitamins, deficiencies of folate and vitamin B_{12} may lead to an elevated plasma homocysteine concentration (hyperhomocysteinaemia) [7-11] which is a recognized risk factor for venous thrombosis and arterosclerosis [12]. In adults, total homocysteine (thcy) and methylmalonic acid (MMA) in serum or plasma are sensitive markers of cobalamin status. Both thct and MMA are used for the diagnosis and follow-up of cobalamin deficiency [13-15]. tHcy is also elevated in folate deficiency and is used as an indicator of this deficiency state [6,16,17] whereas MMA is a sensitive and specific marker of cobalamin deficiency.

There is an increasing body of evidence implicating a high tHcy concentration as a predisposing factor to occlusive vascular disease and other conditions like cognitive dysfunction, adverse pregnancy outcomes and malformations [18-20]. Since SCD patients are fundamentally prone to vaso-occlusion, chronic haemolysis and consequent folate and vitamin B_{12} deficiencies, hyperhomocysteinaemia may occur in them. Therefore, strategies

to reduce modifiable factors predisposing to hyperhomocysteinemia may have a health-promoting effect on SCD.

This study, therefore, investigated whether adult SCA patients in steady state (SS), in vaso-occlusive crisis (VOC) have significantly different mean levels of tHct, MMA, Vit B_{12}, and folate status when compared with control groups. If elevated homocysteine is found, it may explain the observed varying clinical pattern in the manifestations of painful ishaemic crisis, cerebrovascular events, ishaemic necrosis of femoral heads and many other complications in individual patients with SCA. It also examined the pattern of haematological parameters in SCA groups compared to the control group.

2. MATERIALS AND METHODS

This was a prospective study whereby 60 SCA (HbSS) patients; 30 in crisis (VOC), 30 in steady state (SS) and 30 (HbAA), age and sex matched controls were consecutively recruited [Table 1].The SCA patients were those being followed up regularly at the Haematology clinic and at our Day Care Unit (HDCU) of the University College Hospital, Ibadan, Nigeria. The routine folic acid, 5mg daily, regularly prescribed to these SCA patients was not stopped prior to sampling.The control (CC) group were the same ethnic background with the study group [Table 1].

Table 1. Characteristics of study groups

Characteristics	Age(years)	Male	Female	BMI
Control(N=30)	26.1±4.8	14	16	22.5±2.6
HbS(SS) (N=30)	27.1±6.3	18	12	20.8±3.1
HbS(VOC) (N=30)	24.9±4.9	13	17	22.3±8.9

Ethical approval was obtained from UI/UCH Ethical Board and informed consent was obtained from individual patients prior to recruitment into the study.

2.1 Sample Collection

Ten milliliters of venous blood was collected in the morning from each of the subjects. Two milliliters was put into EDTA anti-coagulated bottles for the determination of full blood count (FBC). The remaining 8mls was put in heparinized bottle and centrifuged immediately at 3000 RPM at 4ºC. The supernatant decanted into plain bottle was stored at -20ºC until analyzed.

2.2 Diagnostic Method

Total plasma homocysteine was determined using high performance liquid chromatography (HPLC) according to the method of Cornwell *et al*. [21]. In brief, plasma disulfides and mixed disulfides and protein bound homocysteine were reduced with tri-n- butylphophine. The total reduced homocysteine was gerivatized with 7-fluoro-benzo-tr2-oxa-1-3-diazole-4-sulfonamide (ABDF). The resulting homocysteine ABDF conjugate was quantified fluorometrically, with an excitation wave-length of 38nm and an emission wave-length 515nm. Separation of the thiols was accomplished by High Performance Liquid Chromatography (HPLC) on a reverse phase column. Quantitation was accomplished by comparison of sample peak heights with those of authentic samples. Vitamin B_{12} and folate

levels were determined using HPLC method for the determination of B vitamins by Rada Amidizic *et al*. [22].

2.3 Statistical Analysis

Data were presented in Mean ± SD. Student t-test was used to determine the differences between the means. $P < 0.05$ was considered significant.

3. RESULTS AND DISCUSSION

According to Table 2, While the mean plasma homocysteine levels were significantly reduced in both HbS (VOC) ($p = 0.000$) and HbS (SS) ($p = 0.000$) compared to control (CC) group, the HbS (SS) had significantly lower ($p = 0.000$) mean plasma homocysteine compared to HbS (VOC). The mean vitamin B_{12} levels were significantly reduced in both HbS (VOC) ($p = 0.000$) and HbS (SS) ($p = 0.000$) compared to control (CC) but there was no significant difference ($p = 0.165$) in the mean of HbS (VOC) group (206.44±13.50)ng/dl compared with HbS (SS) group (201.10±15.79)ng/dl. The mean folate level was significantly lower ($p = 0.000$) in HbS (VOC) group compared to HbS (SS) group. Although HbS (VOC) group and HbS (SS) group have reduced mean folate levels compared to control, these were not statistically significant [$p = 0.067$ and $p = 0.095$ respectively].

The mean levels of Methylmalonic acid were significantly reduced in both HbS (VOC) Group ($p=0.000$) and HbS (SS) group ($p = 0.006$) compared to control group. Methylmalonic acid in HbS (VOC) group was significantly reduced ($p = 0.005$) compared to HbS (SS) group.

Table 2. Changes in the levels of plasma homocysteine, vitamin B_{12}, Folate and methyl malonic acid in HbS (VOC&SS) compared with controls (CC)

	HbS (VOC)	**HbS (SS)**	**CC**	**p-value**	**p-value**	**p-value**
	×±SD	**×±SD**	**×±SD**	**VOCvs** SS	**VOCvs** CC	**SSvs** CC
n	30	30	30			
t-Hcy[um/l]	6.34±0.72	5.24±0.59	9.13±0.75	0.000*	0.000*	0.000*
Vit- B_{12}[um/l]	206.44±13.50	201.10±15.79	318.44±25.47	0.165	0.000*	0.000*
Folate[ng/dl]	170.76±44.44	205.63±20.02	546.18±1099.16	0.000*	0.067	0.095
MMA[um/l]	2.24±0.33	2.51±0.38	2.77±0.32	0.005*	0.000*	0.006*

According to Table 3 Expectedly, the mean PCV of HbS (VOC and SS) were significantly lower ($p = 0.000$) than control. Although the mean PCV of HbS (SS) was lower than that of HbS (VOC); it was not statistically significant ($p = 0.085$). The mean WBC was significantly ($p=0.000$) higher in HbS (VOC and SS) compared to control. The mean WBC was also significantly ($p = 0.000$) higher in VOC compared to SS. The mean platelet count in HbS (VOC and SS) was significantly higher ($p = 0.000$) than CC. Also, mean platelet count was significantly higher ($p = 0.001$) in HbS (SS) compared to HbS (VOC).The mean MCV in HbS (VOC & SS) was significantly lower ($p = 0.000$)($p = 0.05$) compared with CC but it was significantly higher ($p = 0.001$) in VOC compared with SS. The mean MCH is only statistically higher in VOC compared to control. The mean MCHC level was significantly higher in HbS (VOC and SS) compared to control but no significant

difference was found between HbS (VOC) and HbS (SS) mean levels.

Table 3. Variation of haematological parameters in study population

	HbS (VOC) ×±SD	HbS (SS) ×±SD	CC ×±SD	p-value VOCvs SS	p-value VOCvs CC	p-value SSvs CC
PCV[%]	24.2±5.8	21.7±4.5	40.1±3.5	0.085	0.000*	0.000*
WBC[x10^9 /mm^3]	14.0±4.7	10.7±3.9	5.3±1.5	0.000*	0.000*	0.000*
PLT	266.8±137.3	359.7±141.6	204.5±48.1	0.001*	0.000*	0.001*
Hgb[g/dl]	8.3±1.5	7.7±1.7	12.9±1.2	0.165	0.000*	0.000*
MCV[FL]	80.8±10.8	74±10-3	86.7±12.4	0.001*	0.000*	0.05*
MCH[pg/cell]	29.8±7.9	26.8±4.2	28.9±3.3	0.04	0.031*	0.88
MCHC[g/dl]	35.5±3.2	35.8±2.1	31.9±1.6	0.82	0.000*	0.000*

The haemopoietic cells, like other labile cells, highly require cobalamin and folate for DNA synthesis, growth and development. Most data on the diagnostic utility of tHcy and MMA, and the health effects of hyperhomocysteinemia are based on clinical and epidemiologic studies in normal adults, middle-aged, and elderly populations [9]. Similar studies on SCA patients, in this setting, are sparse and the available related study was on children with SCD [23]. The study documented significant reduction in serum cobalamin level in children.

Nutritional deficiency of folic acid and vitamin B_{12} are well described acquired causes of elevated plasma homocysteine concentrations [14-16] and folic acid deficiency is a well-recognized complication of sickle cell disease [9,17]. Previous studies had established that virtually all patients with clinical abnormalities due to cobalamin deficiency that are correctable by cobalamin therapy have elevated serum methylmalonic acid (MMA) [24-28] or total homocysteine (tHcy) [24-26] concentrations or both, even when serum cobalamin concentrations are in the normal range [26]. Homocysteine concentration is elevated in most folate-deficient patients with megaloblastic anemia [24] despite serum folate concentrations in the low-normal range in <25% of them [29]. Therefore, routine supplementation of folate at a dose of 5mg daily orally has become a standard practice in this setting. However, there exists contrary school of thought that regards supplementation unnecessary since most patients have plasma and red cell folate concentrations within the usual reference range. Although it was proven that 1mg folate orally daily suffices as supplement, a study observed and suggested that the dose of folic acid required to normalize plasma homocysteine level in SCA may be higher than the 1mg oral daily dose that is usually prescribed, and that patients with SCA may have a higher nutritional requirement for folic acid than previously thought.

This study showed that significant changes exist in the mean levels of homocysteine, Methylmalonic acid, vitamin B_{12}, folate and haematological parameters in adult SCA patients in crisis (VOC), in steady state (SS), compared to control group. Although mean folate level was comparable in both SCA groups and the control group, the control group had significantly higher mean levels of tHct, vitamin B_{12}, MMA than the SCA groups (VOC and SS). However, between the SCA in VOC group and SS group, mean vitamin B_{12} level was comparable while mean folate and MMA were higher in SS group and tHct was higher in the VOC group. Since tHcyt and MMA were lower in SCA groups, it is safe to believe that elevated serum tHcy and methylmalonic acid are unlikely to be common in our SCA patients. This lower level could be attributable to routine folate and vitamin B complex

supplementation being administered to these patients. On the contrary however, the comparable (not significant) serum folate is not surprising since serum folate is highly labile, unlike red cell folate and for the fact that intake of vitamins was not restricted prior to sampling. The lower mean levels of tHcy and MMA may be a reflection of regular folate supplementation in these patients. This could also reflect compliance, on the part of the patients, to the prescribed folate and vitamin B_{12} supplements. Therefore, routine use of these nutrients should be strongly advised. It was reported that oral supplementation with folate has been shown to lower the plasma homocysteine concentration even in the absence of folic acid deficiency [27].

However, in SCA in vaso-occlusive crisis compared to SCA in steady state; plasma homocysteine, folate, methylmalonic acid were significantly reduced. The low serum B_{12} level found in vaso-occlusive crisis group was not significant when compared with the steady state group. This is difficult to explain but several therapeutic interventions like intake of routine drugs shortly before presentation, rehydration either per oral or by intravenous infusion; urgent blood transfusion etc. may be accountable for this outcome.

As expected, the haematological parameters of control group clearly showed significant differences when compared with SCA groups. The mean haematocrit of control was twice that of SCA. This is likely attributable to chronic haemolytic process that is expectedly on-going in SCA groups. On the other hand, the mean white cell count ($10.0x10^9$/L) of SCA patient was almost twice that of the control group ($5.3x10^9$/L). This is a direct reflection of chronic inflammatory process on-going in SCA patients [30]. These two parameters explained the higher demand for folate and vitamin B_{12} needed for rapid DNA synthesis in highly rapid cell turn over state [9, 17]. There was also relative thrombocytosis in SCA groups compared to control group. This is also as a result of inherent inflammatory process in SCA group [30, 31]. In steady state SCA, lower MCV value, a reflection of small cell size, was in keeping with iron deficiency anaemia whereas the mean MCV in SCA in VOC group was within normal. The SCA patients in VOC group might have received treatment in the form of rehydration, folate replacement, blood transfusion around sampling period and this could have influenced the outcome of the MCV, MCH and the MCHC.

The limitations of this study include failure to use fasting samples for all the groups, failure to restrict vitamin supplementation, blood film review for each participant was not included, and sample size is not large enough to allow for meaningful conclusion.

4. CONCLUSION

A larger, better controlled, multicenter study is required to confirm lower tHct and MMA found in SCA compared to control group and higher tHct in SCA (VOC) but higher MMA level in SCA (SS) when VOC and SS groups were compared. The haematological parameters in SCA groups were not in keeping with macrocytic anaemia but were indicative of chronic haemolytic and inflammatory process.

CONSENT

All authors declare that 'written informed consent was obtained from the patient (or other approved parties) for publication of this case report and accompanying images.

ETHICAL APPROVAL

All authors hereby declare that all experiments have been examined and approved by the appropriate ethics committee and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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