Original Research Article

HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC PATTERN OF CHILDREN WITH SICKLE CELL ANAEMIA IN ENUGU NIGERIA

ABSTRACT

Aim: To identify the quantity of haemoglobin (Hb) 'S'(HbS), 'A2'(HbA2), 'F'(HbF) and other Hb phenotypes of sickle cell anaemia (SCA) children in Enugu Nigeria using High Performance Liquid Chromatography(HPLC).

Introduction: The inheritance of two abnormal Hb genes one of which must be the Hb 'S' gene result in Sickle cell disease (SCD). Co-inheritance of two Hb S genes results in homozygous SCD/SCA) , coinheritance of Hb 'S' with Hb 'C' gene gives HbSC disease and with β-thalassemia allele gives HbSβ-thalassemia(Hb S/β Th). SCA is the most common and most severe of the SCD. The phenotypic expressions of Hb vary unpredictably in the same individual and contribute to the varied clinical severity of SCA among other variables. It is therefore imperative to characterize the Hb variants in children with SCA for proper risk stratification necessary for best outcome management. HPLC is the most validated method for screening, detection and quantification of various Hb subtypes.

Methodology: A cross-sectional, descriptive study involving 75 SCA children aged 6 months to 17years, on follow-up at the clinic. Patients on hydroxyurea or who received blood transfusion within the previous four months were excluded. Following due ethical protocols, the D-10 HPLC machine (BIO-RAD D-10) was used to identfy the Hb phenotype in venous blood

samples based on their ionic gradients and quantify them by the principle of variable absorbance. The participants' sociodemographic data were recorded. Participants were grouped into 3 socioeconomic classes (SECs) as proposed by Oyedeji.

Results: There were 48 females (62.7%) and 27 males (37.3%) in age range 6 months – 17 years in lower (16.0%), middle (57.3%) and upper (26.7%) SECs. Majority had HbF below 10% (46.7%), HbS above 80% (43%) and HbA2 of 4% and below (84%). No other Hb variant was identified. The proportion with HbS/HbA2/HbF levels suggestive of beta thalassemia was 16%, 25% males compared to 10.6% females. Females had higher HbF levels while males had higher HbS and HbA2 levels. However the gender differences in HbF, HbS, HbA2 and SECs did not attain statistical significance. A significant negative relationship was found between age and HbF ($r = -.424$, $p < .001$) while a significant positive relationship between age and HbS ($r = .287$, $p = .013$) and between age and HbA2 ($r = .265$, $p = .022$).

Conclusion: Irrespective of gender, high HbS and low HbF levels at direct variance (HbS) and indirect variance (HbF) with age may be found in children with SCA. Observed Hb phenotypes suggest co-existent β- thalassaemia in this subset of southeast Nigerian SCA children.

Keywords: HPLC, HbS, HbA2, HbF, β- thalassemia, SCA

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Introduction

Sickle cell disease is a heterogenous group of autosomal recessive disorders characterized by the inheritance of at least one Hb S allele[1], a result of mutations in the β-globin gene of the Hb molecule. SCD may result from inheritance of 2 Hb S genes (Homozygous SCD or SCA), co-inheritance of Hb S with a second abnormal β-globin chain variant, such as a Hb C (HbSC disease) or β-thalassemia allele (HbS/β-Th). SCA is the most common of the SCD and has diverse clinical presentation and severity in childhood. It has been suggested that variable and unpredictable phenotypic expressions of Hb variants contribute to the clinical diversity and management challenges in SCA[2]. It is therefore imperative to characterize the Hb variants in children with SCA for proper risk classification and enhanced management. Nigeria has an SCA prevalence rate of 2-3%, the highest in Sub-Saharan Africa. SCA accounts for 20% neonatal mortality rate and 5% of under-5 mortality rate (U5MR) in the African continent. In Nigeria SCD accounts for 4.2% of the national U5MR[3,4,5]. Regardless of the huge burden, the management of SCA in Nigeria is still widely limited to supportive, symptomatic, preventive measures[6]. Furthermore, variation in HbF level, the β-globin gene haplotype locale and the co-inheritance of β or α-thalassaemia and other Hb variants are among the environmental and genetic factors contibuting to the heterogenous clinical severity of SCA[7]. For instance, individuals with Hb S/β Th have one abnormal beta chain, β^S, and a defective beta-globin gene, either in decreased synthesis, β^+, or complete absence of synthesis, β^0. There is production of abnormal Hb, as well as the decreased synthesis of beta globin chains. A patient with Hb S/β Th is indicated by higher than normal HbF, HbA2 and HbS and little to no presence of adult Hb (Hb A). Hence in co-existent beta thalassemia if a small amount of normal Hb is produced, (β^+) an individual may have milder symptoms of SCD. However, if no normal Hb is produced, (β^0), an

individual is almost clinically identical to SCA. Thus proper classification of patients is pivotal to effective management. HPLC is the most validated method for screening and detection of various hemoglobinopathies. It provides rapid, reproducible, and precise results[8-10] . HPLC also provides precise quantification of HbA2, HbF and other variants [11]. Quantification of HbA2 is suitable for the diagnosis of β-thalassaemia trait. Moreover, HPLC is particularly useful in low income settings in place of the expensive and unavailable but gold standard genetic studies[11]. HPLC however, is unable to detect alpha thalassemia, normal A2 beta thalassemia or other hemoglobinopathies that elute with a similar retention values on HPLC[12]. Establishment of levels of HbS, HbA2, HbF and other Hb phenotypes will serve as a screening guide to possible clinical variability in children with SCA.We assessed the percentages (Hb quantification) of different Hb types (HbS, HbA2 and HbF) in relation to age, sex and socio-economic class (SEC) among children with SCA using HPLC with an aim to establish the co-existent of Hb variants.

Methods:

Study area/design/duration: This was a hospital-based, cross-sectional, descriptive study conducted at the Paediatric Haematology clinic of Enugu State University Teaching Hospital (ESUT-TH), Parklane, ESUT-TH is a tertiary health institution in South-East Nigeria. This study described the quantity of HbS, HbA2, HbF and other Hb phenotypes of children with SCA obtained using HPLC assay. The study population comprised of children with SCA aged 6 months to 17 years, on follow-up at the clinic over 3 years (2019 -2022). Patients on hydroxyurea or who received blood transfusion within the previous four months were excluded. Bio-data (name, age, sex) and socio-demographic history (parents' occupation and education) of all participants were documented at their initial presentation to the clinic. Using standard venipuncture technique whole venous blood samples were collected into vacutainer vials

(K_2EDTA BD Diagnostics, USA)giving K_2EDTA 1.7±0.2mg/ml of blood. Blood specimens were stored up to 4 days at 8 °C or 1 day at room temperature(28°C) and used within 4–5 hours. The samples were automatically diluted (with wash diluent) on the D-10 HPLC machine (BIO-RAD D-10) and injected into the analytical cartridge. The D-10 delivers a programmed buffer gradient of increasing ionic strength (Elution Buffer 1 and 2) to the cartridge, where the haemoglobins (Hbs) are separated based on their ionic interactions with the cartridge material. The separated Hbs then passed through the flow cell of the filter photometer where changes in the absorbance at 415 nm were measured. Two-level calibration (HbA2/F/A1c Calibrator) was used for quantification of the obtained electrophoretic values. A sample report was generated for each sample while quality control values (Lyphochek HbA2 controls levels 1 and 2) were used to compare results. Hb phenotypes and their quantity obtained were documented. The SECs of participants were determined on the basis of their parents' occupation and highest level of education, as recommended by Oyedeji[13]. The 5 SECs initially generated (I to V) were later reclassified into 3 major social classes, namely upper (I and II), middle (III), and lower (IV and V) class[13]. Data was analyzed using IBM Statistical Package for Social Sciences (SPSS) software, Version 20.

Results:

Table 1 present the demographic characteristics of the study participants. 75 SCA children, made up 48 females (62.7%) and 27 males (37.3%) participated in the study. Their age ranged from 6 months – 17 years with mean and standard deviation of 8.13±5.18. Their SECs were lower class (16.0%), middle class (57.3%) and upper class (26.7%).

Table 2 shows HbF range of 1.9-32.8 with mean and standard deviation, 11.77±7.31, majority (46.7%) had HbF levels below 10. For HbS, majority of them were above 80%; the range was 48.4-93.7 and the mean and standard deviation was 79.58±9.44. For HbA2, the range was 0.8-9.0, the mean and standard deviation was 3.39±1.07 and majority were 4 and below.

Table 3 compared the proportions of HbF, HbA2 and HbS between male and female participants. Females had higher HbF (12.24±7.26) than males (10.98±7.45) although the difference was not significant (p =0 .472). For HbS the males had higher values (80.93±7.73) than the females (78.77±10.32) similar to HbA2 (3.63±0.76) for males and (3.24±1.21) for females. These differences likewise were not statistically significant[(p = 0.341);(p =0 .125)] for HbS and HbA2 respectively.

There was a significant negative relationship between age and HbF (r = -.424, p <0 .001). There was also a significant positive relationship between age and HbS (r = .287, p =0 .013) and between age and HbA2 (r = .265, p =0 .022). This is represented in Table 4.

There were no statistically significant differences in the levels of HbS, HbA2 and HbF between the SECs. The proportion of participants with a combination of higher than normal HbS, HbF, HbA2 (>4%) and low levels of Hb A [suggestive of Beta thalassemia] was 16% total, 25% males compared to 10.6% females (Table 5), this gender difference was not significant (p >0.05).No other Hb variant was identified in this study.

Discussions:

Our data [combination of high Hb S and Hb F, reduced Hb A in the presence of high Hb A2(>4%)], suggests co-existence of only β- thalassaemia trait[14] in 16% of our SCA participants. This is contrary to an earlier study from south-west Nigeria where α and β- thalassaemia traits as well as other Hb variants such as HbC, Hb D Punjab were found to frequently co-exist with HbS[15]. However, the present study unlike the previous neither evaluated the peripheral blood smear nor the full blood count (FBC) to establish the co-inheritance of β- thalassaemia. Other investigators have also reported HbA2 values of over 4% among some patients with SCA which suggest a possibility of co-existing β^+-thalassaemia[16,17]. Elevated HbA2 level is said to reduce the minimum gelling concentration of HbS[18] thereby ameliorating its effect. Thus, SCA individuals with co-existent β^+-thalassemia where small amount of normal Hb is produced have been reported to have milder symptoms of SCD[16,17]. This is thus the expected outcome for those participants whose HbF, HbA2 and HbS levels seem to mimic $S\beta^+$-thalassemia.

HbS levels less than 30% has been associated with reduced incidence of complications such as stroke, acute chest syndrome, sickle nephropathy and osteonecrosis[9]. Ordinarily such low levels are achieved with the use of therapeutic options like chronic blood transfusion[20] and hydroxyurea[21]. However, majority of our participants had high HbS levels >80% especially the males and those in age groups 5-8 and 13-17 years.This connotes that these class of participants may present a more severe disease course. Poor outcomes at the adult transition age and in adolescents with SCA have been previously documented, having been attributed to poor health-seeking and poor selfcare in them[22,23]. High concentrations of HbS of 80 to 90% in red blood cells is usual in individuals with SCA phenotype and is associated with severe disease[24]. One

may only presume that the (older males) in this study will have more adverse clinical course than the females since statistically their higher HbS levels was not significantly different from the female levels .

The mean (±SD) HbF level of 11.77±7.31%, range 1.9 - 32.8% in our SCA population is comparable to 8.05±5.07%, range 0.4 - 25.5% observed by Adeyemo and co[15] in south-west Nigeria. In contrast, lower ranges of 7.4 - 9.5% were documented in some other Nigeria studies[25,26]. Variation in HbF levels have been attributed to differing haplotypes in people of different races[27,28] as well as methodological differences of the various studies within a race[25,26,29,30]. Such methodological differences include the use of the more precise HPLC as in our study and that of Adeyemo and co[15] and the use of alkali denaturation method of Hb F estimation which predisposes to under estimation[31]. It is noteworthy that all of these studies reported a close association between HbF levels and clinical presentations of their participants.

Several studies have observed higher Hb F levels in SCA females than males, these been with statistically significant difference (P=0.02) in some[15,28,32,33] but not in others[34-36]. Similar to this study, Ugwu and co-investigators[34], in an earlier study in the same region as well as other reseachers[35,36] noted higher HbS levels among males and lower HbA2 and HbF levels when compared to females. Common to these investigators, these gender differences were not statistically significant. The autosomal recessive mode of inheritance of SCA with its equal sex affectation[35,36] as well as multiple gene loci (including Xp22.2 locus on the X chromosome)[37] which the inheritance of HbF is dependent on has been postulated as the reasons for these observed gender differences for HbS and HbF respectively.

Similar to our study a significant negative correlation between age and HbF levels in SCA patients($r = -.424$, $p < 0.001$) have been observed by other Nigerian investigators ($r = -0.169$, $P=0.038$)[15,38]. HbF levels inversely proportional to HbS levels has also been reported[35] as did our study. These data lend credence to the literature that symptoms associated with SCA do not fully manifest until Hb switch from fetal to adult takes place around six months of age[39,40]. It is recommended that the Hb chromatographic pattern of SCA patients should be established as a prelude to evaluation of disease severity. Further studies are required to establish the exact relatinship between the two

Conclusions:. Irrespective of gender, high HbS and low HbF levels at direct variance (HbS) and indirect variance (HbF) with age may be found in children with SCA. Observed Hb phenotypes suggest co-existent β- thalassaemia in this subset of southeast Nigerian SCA children.

Ethical Approval and Consent

Ethical approval for the study was obtained from the institutional review board, with Institutional Ethical Clearance (IEC) number ESUTHP/-MAC/RA/034/VOL.3/197. Written informed consent was obtained from each parent of the patient.

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Table 1: Demographic Characteristics of the study participants n = 75

	Male (n = 28)	Female (n = 47)	Total
Age			
- ≤ 4	9(32.1)	13(27.7)	22
- 5-8	7(25.0)	10(21.3)	17
- 9-12	3(10.7)	13(27.7)	16
- 13-17	9(32.1)	11(23.4)	20
Range	10mths-16yrs	6mths -17yrs	6mths-17yrs
M±SD	7.77±5.15	8.35±5.24	8.13±5.18
SEC			
- Lower	4(14.3)	8(17.0)	12
- Middle	15(53.6)	28(59.6)	43
- Upper	9(32.1)	11(23.4)	20

Table 2: Descriptive Summary of HbF, HbS and HbA2

	Frequency	Percent	Range	M±SD
HbF			1.9-32.8	11.77±7.31
- < 10	35	46.7		
- 10-19.9	30	40.0		
- ≥ 20	10	13.3		
HbS			48.4-93.7	79.58±9.44
- < 50%	1	1.3		
- 50-80%	31	41.3		
- > 80%	43	57.3		
HbA2			0.8-9.0	3.39±1.07
- ≤ 4	63	84.0		
- > 4	12	16.0		

Table 3: Comparing HbF, HbS and HbA2 of Males and Females

	Male (n = 28) M±SD	Female (n = 47) M±SD	t	p-value
HbF	10.98±7.45	12.24±7.26	-.724	.472
HbS	80.93±7.73	78.77±10.32	.957	.341
HbA2	3.63±0.76	3.24±1.21	1.553	.125

Table 4a: Correlation between age and HbF, HbS and HbA2

	HbF	HbS	HbA2
Age			
- Pearson Correlation	-.424	.287	.265
- p-value	< .001	.013	.022
- N	75	75	75

Table 4b:Distribution of HbF, HbS and HbA2 according to age

	≤ 4	5-8	9-12	13-17	F	p-value
HbF	16.79±7.68	9.55±5.06	11.16±7.96	8.62±5.19	6.404	.001
HbS	74.54±7.55	82.61±8.02	79.16±11.04	82.87±9.19	3.909	.012
HbA2	2.91±0.61	3.49±0.97	3.40±0.85	3.81±1.50	2.721	.051

Table 5: Participants whose HbA2(>4%), HbS(high), HbF(high), HbA(some) suggestSβ-Thal

	Suggestive of co-existing Beta Thalassemia			Fishers Exact
	Yes	No	Total	p-value
Sex				.116
- Male	7(25.0)	21(75.0)	28	
- Female	5(10.6)	42(89.4)	47	