Original Research Article

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

ABSTRACT

High Performance Lipid Chromatographic pattern of children with Sickle cell anaemia in Enugu Nigeria.

Introduction:The inheritance of two abnormal hemoglobin genes one of which must be the hemoglobin S (Hb S) result in Sickle cell disease (SCD). SCD may result from inheritance of 2 Hb S genes (Homozygous SCD or SCA) or co-inheritance of Hb S with a second abnormal β-globin chain variant, such as a hemoglobin C (HbSC disease) or β-thalassemia allele (HbSβ-thalassemia). SCA is the most common and most severe of the SCD. The clinical severity of SCA is varable and dependent on environmental, geographical as well as genetic factors. Such factors include the variable and unpredictable phenotypic expressions of Hb variants in the same individual. It is therefore imperative to characterize the Hb variants in children with SCA as this will enable proper risk classification necessary to enhanced management. HPLC is the most validated method for screening and detection of various hemoglobinopathies. HPLC also providesprecisequantificationofhaemoglobinA2, haemoglobin Fand variant haemoglobin.

Methodology: This was a cross-sectional study involving 75 children with sickle cell anaemiaaged 1 to 17years, on follow-up at the clinic. Patients on hydroxyurea or who received blood transfusion within the previous four months were excluded. This study described the

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quantity of HbS, HbA2, HbF and other Hb phenotypes (if any) of children with HbSS obtained using HPLC assay. Following due ethical protocols, the D-10 HPLC machine (BIO-RAD D-10) was used to separate the haemoglobin in venous blood samples based on their ionic gradients and quantify them by the principle of variable absorbance.The participants' sociodemographic data were recorded.

Results: There were 48 females (62.7%) and 27 males (37.3%) in age range 6 months – 17 years and socioeconomic classes lower (16.0%), middle (57.3%) and upper (26.7%). Majority had HbF below 10% (46.7%), HbS above 80% (43%) and HbA2 of 4% and below in 84%. 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 but no statistical significance existed in these relationships as well as between the socioeconomic classes. 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 ($r = .265$, $p = .022$). No other Hb variant was identified.

Conclusion: High HbS and low HbF levels imply adverse clinical course for this group of patients,moreso in the older age groups. This is irrespective of gender.Co-existent β-thalassaemia traits may be the rare Hb variant in SCA children in South-east Nigeria.

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

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Introduction

Sickle cell disease (SCD) is a heterogenous group of autosomal recessive disorders characterized by the inheritance of at least one hemoglobin S (Hb S) allele[1], a result of mutations in the β-globin gene of the haemoglobin (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 hemoglobin C (HbSC disease) or β-thalassemia allele (HbSβ-thalassemia). 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.

Comment [SC6]: Define the full meaning of SCA first before abbreviating.

Introduction: 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]. In addition, 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 sickle cell beta thalassemia (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 hemoglobin, as well as the decreased synthesis

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of beta globin chains. A patient positive for sickle cell beta thalassemia is indicated by higher than normal fetal hemoglobin (HbF), HbA2 and HbS and little to no presence of adult hemoglobin (Hb A). Hence in co-existent beta thalassemia if a small amount of normal hemoglobin is produced, β^{+} an individual may have milder symptoms of sickle cell disease. However, if no normal hemoglobin, β^{0}, is produced, an individual is almost clinically identical to sickle cell anemia. 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 providesprecisequantificationofhaemoglobinA2, haemoglobin Fand variant haemoglobin[11]. QuantificationofhaemoglobinA2 is suitablefor thediagnosisof β-thalassaemiatrait.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 haemoglobin types (HbS, HbA2 and HbF) in relation to age, sex and socio-economic class (SEC) among individuals with sickle cell anaemia (HbSS) using HPLC in order to determine the distribution of co-existent Hb variants among the groups.

Methods:

Study area/design/duration: This was a hospital-based, cross-sectional study conducted at the PaediatricHaematology clinic of Enugu State University Teaching Hospital (ESUT-TH), Parklane, ESUT-TH is a tertiary institution in the South-East Nigeria. This study described the quantity of HbS, HbA2, HbF and other Hb phenotypes of children with HbSS obtained using

HPLC assay. The study population comprised of children with sickle cell anaemia aged 1 to 17 years, on follow-up at the clinic over 3 years (2019 -2022).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/patient. 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 at which time whole venous blood samples were collected into vacutainer vials (K_2EDTA, BD Diagnostics, USA) using standard venipuncture technique. Blood specimens were stored up to 4 days at 2–8 °C or 1 day at room temperature (15–30 °C/?20–25°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 hemoglobins are separated based on their ionic interactions with the cartridge material. The separated hemoglobins 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 socioeconomic class for the parents was determined on the basis of their occupation and the highest level of education, as recommended by Oyedeji[13]. The 5 socioeconomic classes initially generated (I to V) were later reclassified into 3 major social classes, namely upper (I and II), middle (III), and

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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 children under study. 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 socioeconomic class was lower class (16.0%), middle class (57.3%) and upper class (26.7%).

Table II shows that HbF of the patients ranged from 1.9-32.8 with mean and standard deviation, 11.77±7.31 and majority with HbF below 10 (46.7%). 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 III 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 = .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 = .341);(p = .125)] for HbS and HbA2 respectively.

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

There were no statistically significant differences in the levels of HbS, HbA2 and HbF between the socio-economic classes. The proportion of participants with higher than normal HbS and HbF with HbA2 (>4%) and low levels of Hb A [suggestive of Beta thalassemia] was 16%, 25% males compared to 10.6% females (Table V), this gender difference was not significant (p >0.05).No other haemoglobin 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 HbSS participants. This runs contrary to an earlier study in the South-western part of Nigeria where α and β-thalassaemia traits as well as other haemoglobin 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 HbSS which suggest a possibility of co-existing thalassaemia[16,17]. Elevated HbA2 level is said to reduce the minimum gelling concentration of HbS[18] thereby ameliorating its effect. Thus, HbSS individual with co-existent β^{+}-thalassemia where small amount of normal hemoglobin is produced have been reported to have milder symptoms of sickle cell disease[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, osteonecrosis and other complications[19]. 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 run a more severe disease course. Poor outcomes at the adult transition age and in adolescents with SCA have been previously documented, being attributed to poor health-seeking and poor selfcare in them[22,23]. High concentrations of HbS of 80 to 90% in red

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blood cells is usual in individuals with HbSS phenotype and is associated with severe disease[24]. One may only presume that the (older males) in this study will have more clinically significant adverse outlook than the females since their higher HbS levels was not significantly different from the female levels statistically .

The mean (±SD) HbF level of 11.77±7.31%, range 1.9 - 32.8% in our Hb SS 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% have been noted in other previous 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 Hb SS females than males, these been with statistical significance (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 among males and lower HbA2 and HbF when compared to females. Common to these investigators, these gender differences were not statistically significant. The autosomal recessive mode of inheritance of HbSS with 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 Hb SS patients ($r = -.424$, $p < .001$) 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. This lends credence to the fact that symptoms associated with SCA do not fully manifest until hemoglobin switch from fetal to adult takes place around six months of age[39,40]. In conclusion the chromatographic pattern of haemoglobin types in SCA patients should be established as a prelude to evaluation of disease severity.

CONCLUSION ?????????

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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 II: 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 III: 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

Comment [SC15]: State the units of quantification. Applicable to all Tables.

Table IVa: 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

Comment [SC16]: Write the zero before the points. Ie 0.001, 0.424, 0.287 etc.... applicable to all Tables.

TableIVb: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

Comment [SC17]: STATE n in each group for better comprehension and statistical inference… Applicable to all.

Table V: 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	