Afr. J. Med. Med. Sci. (2023) **5**2(4)**,** 255-263

Chromatographic pattern of haemoglobin (Hb) types (HbS, HbA2 and HbF) among individuals with sickle cell anaemia and those with sickle cell trait in a tertiary health institution, South eastern Nigeria

NI Ugwu[1], EO Nna[2], CN Ugwu[3], O Clifford[4], ON Benson[5], GC Ugwu[6], RC Ikeagwulonu[7], UU Nnadozie[8], AJ Madu[9], HC Okoye[9] and C Alo[10].

Department of Haematology and Immunology[1], Collehe of Health Sciences, Abakaliki, Safety Molecular Pathology Laboratory[2], Enugu, Departments of Internal Medicine[3] and Paediatrics[4], College of Health Sciences, Abakaliki, Departments of Paediatrics[5], Haematology and Transfusion Medicine[6] and Chemical Pathology[7], Alex Ekwueme Federal University Teaching Hospital, Abakaliki, Department of Surgery[8], Division of Plastic Surgery, College of Health Sciences, Abakaliki, Department of Haematology[9] , University of Nigeria, Ituku-Ozalla, Enugu and Department of Community Medicine[10], College of Health Sciences, Abakaliki, Nigeria.

Abstract

Background: High performance liquid Chromatography (HPLC) has been shown to be reliable in evaluating the different haemoglobin types. This study aimed to determine the mean level of haemoglobin types (HbS, HbA2 and HbF) among individuals with HbSS and HbAS phenotype using HPLC and also to compare Hb types with gender.

*Methods***:** This was a cross-sectional comparative study during which the levels of HbS, HbA2 and HbF of participants with HbSS and HbAS were assayed using HPLC. Data was analyzed using IBM Statistical Package for social sciences (SPSS) software, Version 20.

Results: Eighty three subjects, made up of 60 HbSS and 23 HbAS individuals participated in the study, with mean age of12.9 years ±9.7 and 11.3 years ±8.9 respectively. Mean HbS level among HbSS participants was 80% ±7.9, which was statistically significant compared to that of HbAS, 37.2% ±13.1 (p= 0.001). Mean HbF and HbA2 level of 8.0% ±6.1and 2.6% ±1.9 respectively among HbSS participants were also higher than the value of 3.8% ±3.7 and 2.5% ±1.3 among HbAS participants, though not statistically significant (p>0.05). Among HbSS and HbAS participants, males have HbS values of 80.4% and 37.8% respectively which was higher than that of the females, 79.7% and 36.9% though not significant. Conversely, male HbSS and HbAS have lower HbA2 and HbF levels compared to females, though not significant (p >0.05).

Conclusion: Individuals with HbSS have significantly higher HbS level compared to those with HbAS phenotype. Males have higher HbS but lower HbA2 and HbF compared to females among the two groups, though not significant. Establishment of levels of HbS, HbA2 and HbF will serve as a guide in differencing individuals with HbSS from those with HbAS phenotype.

Keywords: *HaemoglobinA2, Haemoglobin F, Haemoglobin S, High Perfomance Liquid Chromatography, Sickle Cell Anaemia.*

Résumé

Contexte : La chromatographie liquide à haute performance (CLHP) s'est révélée fiable pour évaluer les différents types d'hémoglobine. Cette étude visait à déterminer le niveau moyen des types d'hémoglobine (HbS, HbA2 et HbF) chez les personnes atteintes de phénotype HbSS et HbAS à l'aide de la CLHP et à comparer les types Hb avec le sexe.

Méthodologie : Il s'agissait d'une étude comparative transversale au cours de laquelle les niveaux de HbS, de HbA2 et de HbF des participants avec HbSS et HbAS ont été analysés à l'aide de la CLHP. Les données ont été analysées à l'aide du logiciel IBM Statistical Package for social sciences (SPSS), version 20.

Résultats : Quatre-vingt-trois sujets, composés de 60 personnes HbSS et de 23 personnes HbAS, ont participé à l'étude, l'âge moyen étant de 12,9 ans 9,7 et 11,3 ans

Correspondence: Dr. Ngozi I Ugwu, Department of Haematology and Immunology, Faculty of Basic Clinical Sciences, College of Health Sciences, Ebonyi State University, Abakaliki, Nigeria. E-mail: ngpziugwu5@gmail.com.

8,9 respectivement. Le niveau moyen de SHb chez les participants au SHb était de 80 % 79, ce qui était statistiquement significatif par rapport à celui du HbAS, 37,2 % 13,1 (p = 0,001). Les niveaux moyens d'HbF et d'HbA2 de 8,0 % 6,1 et de 2,6 % 1,9 respectivement chez les participants au programme HbSS étaient également supérieurs à la valeur de 3,8 % 3,7 et 2,5 % 1,3 chez les participants au programme HbAS, bien qu'ils ne soient pas statistiquement significatifs (p>0,05). Chez les participants au programme HbSS et au programme HbAS, les hommes ont des valeurs HbS plus élevées de 80,4 % et de 37,8 % respectivement, ce qui était plus élevé que chez les femmes, 79,7 % et 36,9 % bien que non significatif. À l'inverse, les taux de HbSS et de HbAS des hommes sont plus faibles que ceux des femmes, bien qu'ils ne soient pas significatifs (p >0,05).

Conclusion : Les personnes atteintes de HbSS ont un niveau de HbS beaucoup plus élevé que celles qui ont un phénotype HbAS. Les mâles ont un HbS plus élevé, mais un HbA2 et un HbF plus faibles que les femelles dans les deux groupes, bien que mais pas significatif. L'établissement des niveaux de HbS, de HbA2 et de HbF servira de guide pour différentes personnes atteintes de HbSS par rapport à celles atteintes de phénotype HbAS.

Mots-clés : HaemoglobinA2, hémoglobine F, hémoglobine S, chromatographie liquide à haute perfomance, anémie falciforme.

Introduction

Sickle cell disease (SCD) is a genetic disorder of haemoglobin characterized by the tendency of hemoglobin molecules within the red cell to polymerize and deform the red cell into a sickle (or crescent) shape resulting in characteristic vaso-occlusive events and accelerated haemolysis [1].

It is inherited in an autosomal recessive fashion and only manifests in the homozygous or doubly heterozygous state. The commonest type of sickle cell disease in Nigeria is the homozygous type, also known as sickle cell anaemia (HbSS) [2]. Other types such HbSC, HbSβ-thal are less common [3]. Sickle cell trait, also known as sickle cell carrier (HbAS) is not classified as sickle cell disease because it does not usually manifest with clinical disease but HbS gene can be transmitted to the offspring [4].

Sickle cell disease affects mainly Africa, India and Middle East but has extended to other parts of the world, such as Europe and America due to migration.[5] About 5%–7% of the world population carry an abnormal haemoglobin (Hb) gene, with sickle cell anaemia (SCA) being the most common form of haemoglobinopathy globally [5]. Three quarters of sickle-cell cases occur in Africa and Nigeria has the highest burden globally with 2% of newborns affected, giving a total of 150,000 children born every year with sickle cell anaemia [6]. The carrier frequency ranges between 10% and 40% across equatorial Africa, decreasing to 1–2% on the North African coast and <1% in South Africa [5].

Despite the fact that patient with SCA has the same molecular defect, there is a great variation in the clinical manifestations among patients with sickle cell anaemia ranging from death in early childhood to a normal life span with few complications. This has been attributed to several factors including genetic, environmental, nutritional and psycho-social factors [7]. Mild disease has been reported to be partly due to the influence of genetic modifiers of SCA such as co-existence of α –thalassaemia [8]. In addition, patients with increased levels of HbF often tend to have a relatively mild clinical course because HbF reduces the tendency of HbS to polymerize within the red cell [9]. The presence of HbA2 in erythrocytes is also known to have a protective effect against damage in SCA by reducing the minimum gelling concentration of haemoglobin S [10]. Normal adult haemoglobin, known as haemoglobin A, has a structure of α2 β2. It accounts for about 97% of total hemoglobin. Other hemoglobin types present in adult include hemoglobin A2 (HbA2) which is α2 δ2 and accounts for 2% of the total haemoglobin while hemoglobin F (α2 γ2) accounts for less than 1% of the total adult hemoglobin [11].

In the past, the procedures available for the diagnosis of sickle cell disorders in Nigeria were limited to a few screening tests such as the sickling test, solubility tests as well as cellulose acetate electrophoresis. However, few centers in Nigeria have introduced the use of high performance liquid chromatography (HPLC) technology which is capable of identifying and quantifying haemoglobin variants. HPLC has been proven to be rapid, sensitive and accurate method for quantifying various types of normal and abnormal haemoglobins despite its limitations, one of which includes co-elution of HbS byproducts with HbA2 resulting in falsely elevated level of the latter [12]. It is however very suitable for HbF estimation with little or no limitations.

As a result of increasing availability of HPLC in Nigeria and scarcity of information on the pattern of different haemoglobin types, there is need to establish the pattern of different haemoglobin types (HbS, HbF and HbA2) among individual with sickle cell anaemia (HbSS) in steady state and those with sickle cell trait (HbAS).This study was aimed to determine the levels of different haemoglobin types such as HbS, HbA2 and HbF among individuals with HbSS and HbAS phenotype.

Materials and methods

Study design

This was a cross-sectional comparative study.

Study setting

The study was carried out at the sickle cell center of Alex Ekwueme Federal University Teaching Hospital, Abakaliki between September 2018 and February 2019.

Study population

This was made up of HbSS (sickle cell anaemia) patients attending sickle cell disease clinics of Alex Ekwueme Federal University Teaching Hospital, Abakaliki as well as individuals with sickle cell trait (HbAS) who were recruited from among staff, medical students and children attending immunization clinics and children out-patient clinics.

Inclusion criteria

Haemoglobin SS (sickle cell anaemia) in steady state and HbAS (sickle cell carrier) individuals who gave their consent. Steady state is defined as the absence of any acute illness or crisis for at least four consecutive weeks and absence of blood transfusion in the previous three months.[13]

Exclusion criteria:

Haemoglobin SC and other double heterozygous phenotype, patients who received blood transfusion in the previous three months, pregnant women and patients on hydroxyurea therapy were excluded from the study.

Sampling technique and sample size

Participants who met the inclusion criteria and had given their consent were recruited consecutively.

The minimum number of participants needed for the study to provide 80% power was calculated using G*Power software version 3.1.9.2.[14]Using a medium effect size of 0.4, alpha level of significance 0.05, a power of 80% and allocation ratio of 0.5, a sample size of 44 for cases (HbSS) and 20 for HbAS were obtained. A medium effect size was based on previous study that reported mean level of 82.8% for HbS with standard deviation of 5.0% [15]. To account for any possible reduction in participants (attrition), 10%of the calculated sample size for each group was added, making a total of 48 for HbSS and 22 for HbAS, with an overall total of 70 participants. However, 83 participants were recruited for this study.

The participants with HbSS were in steady state at the time of recruitment i.e. there was no crisis, infection or fever for at least 4 weeks and no blood transfusion in the preceding 3 months.

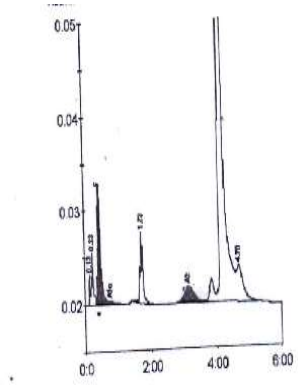
Sample analysis procedure

Two millilitres of venous blood was collected by clean venepuncture from each patient via the antecubital vein using a plastic syringe with minimum stasis, into commercially prepared ethylene di-amine tetracetic acid (EDTA) bottles. Each sample was then mixed gently and thoroughly to ensure anticoagulation and to prevent cell lysis. Collected blood samples were subsequently transported to the haematology laboratory where analysis was done.

The Hb genotype of all participants were determined using cellulose acetate paper electrophoresis at pH 8.6. Following the manufacturers' instructions, the percentages of HbS, HbA2 and HbF of all participants were assayed using BIO-RAD® D10 high performance liquid chromatography (HPLC) (Bio-Rad D-10; Bio-Rad Laboratories, Hercules, California, USA)at the Haematology unit of Safety Molecular Pathology Laboratory, The Molecular Pathology Institute, Enugu, Nigeria.

After collection, the blood samples were stored at 2°C –8°C and analysed within 3 days of collection. The sample preparation involves dilution of 5 µl of EDTA anticoagulated blood in 1 ml of hemolysis fluid provided in the kit. Thereafter, the samples were applied in the HPLC instrument. The lysed samples were diluted with the specific hemolyzing/ wash buffer and injected into an assay specific analytic cartridge. The variant dual pumps delivered a programmed buffer gradient of increasing ionic strength to the cartridge, where the Hb fractions were separated based on their ionic interaction with the cartridge material. The separated Hb fractions were passed through a flow cell, where optical absorbance was measured at 415 nm with simultaneous use of secondary wavelength of 690 nm to reduce

background noise. The software delivers a printed report showing the chromatogram, with all the Hb fractions eluted. The integrated peaks were assigned to manufacturer defined "windows" derived from specific retention time (RT). Each haemoglobin variant has its characteristic retention time, which is the time that elapses from the sample injection to the apex of the haemoglobin peak. The"windows" are established ranges, in which common variants have been observed to elute using the Variant beta globin short program. The printed chromatogram shows all the haemoglobin fractions eluted, the RTs, the areas of the peaks and the values (%) of different haemoglobin components (Figure 1). If a peak eluted at a retention time that is not pre-defined, it is labeled as an unknown. Each analytical cycle, from sampling to printing of results, took about 5 minutes.



Peak table - ID:D011770

Peak	R.time	Height	Area	Area %
Unknown	0.13	3214	6869	0.6
A1a	0.23	5828	19065	1.7
F	0.44	12756	76602	7.2
A1c	0.76	334	3901	7.4
A0	1.73	7456	37831	3.3
A2	3.14	1680	33923	3.3
S-Window	4.15	166937	897662	79.4
C-Window	4.70	3760	56248	4.9

Total Area: 1130992

Fig. 1: *Chromatogram showing all the haemoglobin variants eluted, the retention times, the areas of the peaks and the values (%) of different haemoglobin components.*

Data analysis

Data generated from the study were analyzed using IBM Statistical Package for social sciences (SPSS) software, Version 20(IBM SPSS Inc. Chicago, IL). Proportion, percentages, mean ± standard deviation (SD) were generated using descriptive statistics. Tests of statistical significance between variables were analyzed using student's t-test. Level of significance was set at $P \leq 0.05$.

Ethical consideration

Ethical approval was obtained from Research and Ethics Committee of Alex Ekwueme Federal University Teaching Hospital Abakaliki. Written informed consent was obtained from the adult participants as well as parents/caregivers of all HbSS and HbAS participants as the case may be. In addition, assent was obtained from older children (7 years and above) before being included in the study.

Results

Eighty three subjects, made up of 60 patients with sickle cell anaemia (HbSS) and 23 subjects with sickle cell trait (HbAS) participated in the study, with age range of 1 to 52years.Haemoglobin SS participants were made up of 33 (55%) females and 27 (45%) males with mean age of 12.9years ±9.7. Haemoglobin AS participants were made up of 10 (43.5%) males and 13(56.5%) females (Table 1).

Table 1: showing the age and sex distribution of the participants.

Gender	HbSS Proportion (%)	HbAS Proportion (%)	
Male	27 (45.0%)	10 (43.5%)	
Female	33 (55.0%)	13 (56.5%)	
Total	60 (100%)	23 (100%)	
Mean age	12.9 ±9.7	11.3 ±8.9	P-value 0.259

Result of the analysis of the different haemoglobin types among the participants showed that participants with HbSS have mean HbS value of 80.0 ±7.9 which was significantly higher than that of HbAS participants, 37.2% ±13.1 (p = 0.001) (Table 2). Mean level of HbF among HbSS participant was 8.0 ±6.1 which was higher than the value of 3.8% ±3.7 among HbAS participants though not statistically significant (p = 0.094). Similarly, mean HbA2 value of 2.6% ±1.9 among HbSS participants was higher than that of HbAS participants, though not significant (p = 0.260) (Table 2).

Among HbSS participants, mean HbS, HbA2 and HbF in males were 80.4% ±8.6,2.1% ±1.4, 6.3% ±5.1 respectively which were not statistically significant with

Table 2: Mean values of the HbS, HbA2 and HbF among HbSS and HbAS participants

Haempglobin types	HbSS	HbAS	P-value
HbS (%)	80.0±7.9	37.2±13.1	0.001
HbA2 (%)	2.6±1.9	2.5±1.3	0.260
HbF (%)	8.0±6.1	3.8±3.7	0.094

Table 3: Mean values of the HbS, HbA2 and HbF among HbSS participants according to gender.

Haempglobin types	Male HbSS	Female HbSS	P-value
HbS (%)	80.4±8.6	79.7±7.2	0.979
HbA2 (%)	2.1±1.4	2.8±2.2	0.607
HbF (%)	6.3±5.1	9.7±6.5	0.149

Table 4: Mean values of the HbS, HbA2 and HbF among HbAS participants according to gender.

Haempglobin types	Male HbAS	Female HbAS	P-value
HbS (%)	37.8±12.9	36.9±13.7	0.755
HbA2 (%)	2.3±1.6	2.6±1.2	0.460
HbF (%)	2.6±3.6	4.4±3.7	0.849

those of the females, 79.7% ±7.2, 2.8% ±2.2, 9.7% ±6.5 respectively (p> 0.05) (Table 3). Similarly, among HbAS participants, mean HbS, HbA2 and HbF were 37.8% ±12.9, 2.3% ±1.6 and 2.6% ±3.6 respectively, which were not statistically significant with those of the females, 36.9% ±13.7, 2.6% ±1.2 and 4.4% ±3.6 respectively (p> 0.05) (Table 4).

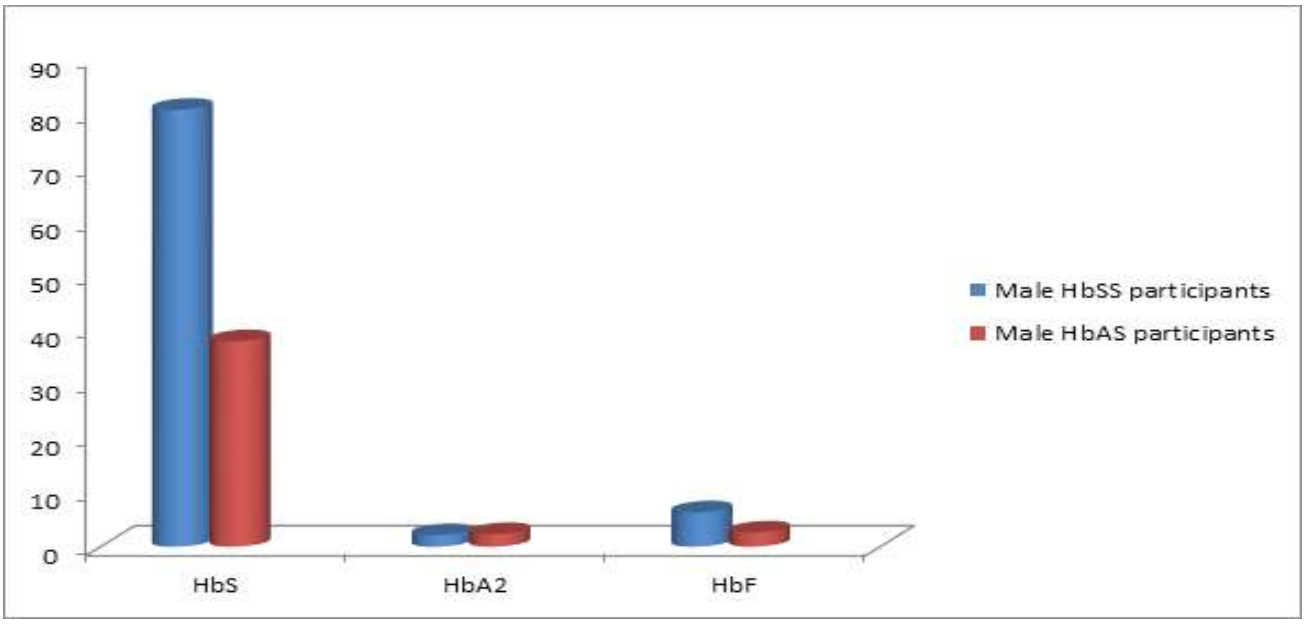


Fig. 2: Haemoglobin (Hb)S, HbA2 and HbF levels among male HbSS and male HbAS participants.

Among male participants, mean HbS level in HbSS individuals was 80.4% ±8.6, which was significantly higher than the value of 37.8% ±12.9 among HbAS individuals (0.026). Male HbSS participants had mean HbA2 value of 2.1% ±1.4 which was lower than the value of 2.3% ±1.6 among male HbAS, though not statistically significant (p = 0.993). Mean HbF level among male HbSS was 6.3% ±5.1 while that of male HbAS participants was 2.6% ±3.8. However, the difference was also not significant (p = 0.388) (Figure 2).

Among female participants, mean HbS level in HbSS individuals was 79.7% ±7.2, which was significantly higher than the value of 36.9% ±13.7 among HbAS individuals (0.004). Female HbSS participants had mean HbA2 value of 2.8% ±2.2 which was higher than the value of 2.6% ±1.2 among female HbAS individuals, though not statistically significant (p = 0.323). Mean HbF level among female HbSS was 9.7% ±6.5 while that of female HbAS participants was 4.4% ±3.7 also not significant (p = 0.388) (Figure 3).

from this study showed that patients with HbSS (sickle cell anaemia) in steady state have significantly higher mean HbS level (80%) than HbAS participants (37.2%). This is in agreement with findings of previous studies [15,16], which also reported high percentage of HbS among patients with sickle cell anaemia and moderate level of HbS among individuals with sickle cell trait. High HbS level is associated with sickling of red cells and other complications seen in sickle cell anaemia. However, reduction of HbS level to less than 30% has been recommended in other to prevent complications such as stroke, acute chest syndrome, sickle nephropathy, osteonecrosis and other complications [17]. The lower HbS level among HbAS participants may explain why individuals with HbAS are usually clinically stable and hardly experience vaso-occlusive crisis and other complications seen in HbSS. Usually, individuals with HbSS phenotype have high concentrations of HbS to about 80 to 90% in red blood cells which is associated with severe disease [15], but when normal adult haemoglobin (HbA) is also present,

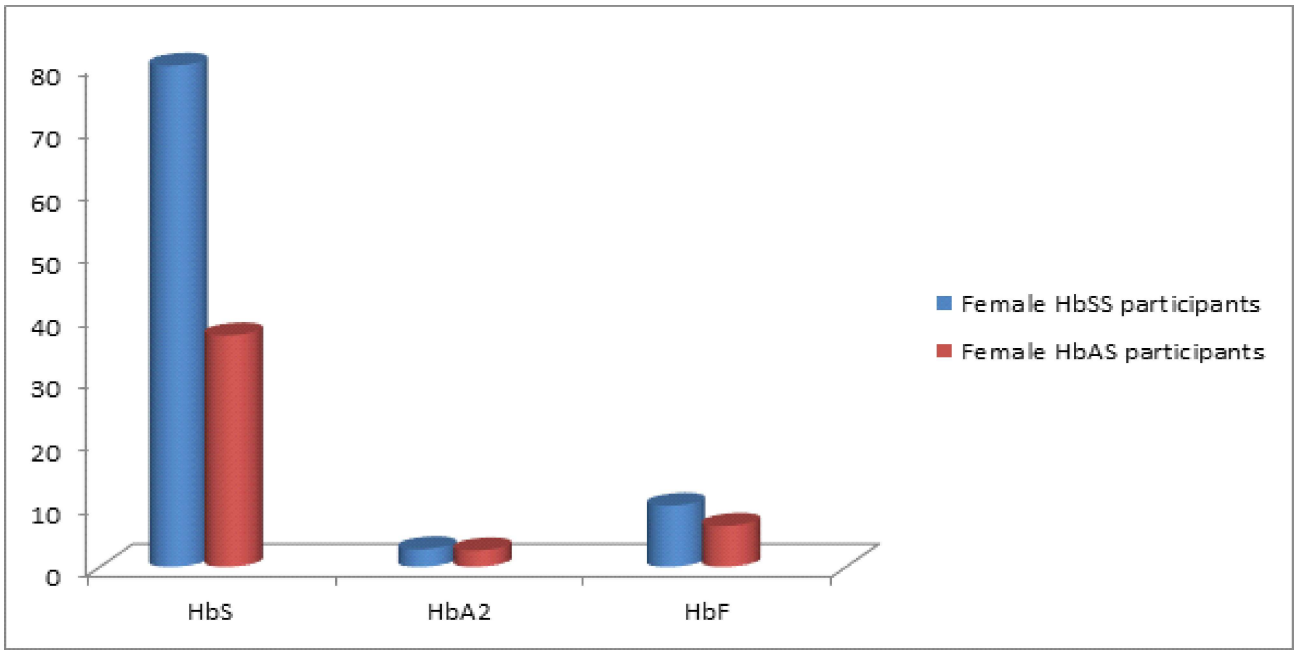


Fig. 3: Haemoglobin (Hb)S, HbA2 and HbF levels among female HbSS and female HbAS participants.

Discussion

We compared the percentages of different haemoglobin types (HbS, HbA2 and HbF) among individuals with sickle cell anaemia (HbSS) and sickle cell trait (HbAS) using HPLC in order to differentiate between a carrier and a person with sickle cell anaemia. The findings

HbS is found at levels of 20–45% [18], defining individuals with sickle cell trait (HbAS).

This study also found that participants with HbSS phenotype have mean HbA2 value higher than that of HbAS participants, though not statistically significant. This collaborates with the report from previous

studies.[19,20] Some studies have also reported HbA2 values of over 4% among some patients with HbSS and this suggest a possibility of co-existing thalassaemia [20,21]. Elevated HbA2 level has been reported to ameliorate the effect of HbS and hence improve the clinical course of SCA patients since it is known to reduce the minimum gelling concentration of HbS [10] Omoti and Omuemu [10] reported significantly higher HbA2 level in sickle cell anemia in steady state and this is in contrary to our report which has shown no significant difference. The difference in HbA2 levels may be associated with the methodology involved in the estimation of HbA2 as Omoti and Omuemu used a formula to calculate HbA2 level while this study used HPLC, a more accurate method. Generally, increased expression of HbA2 in patients with sickle cell anaemia has been associated with compensation for the β-globin production with resultant amelioration of the clinical severity of sickle cell disease [21].

Another finding of this study was that participants with HbSS phenotype in steady state have elevated mean HbF level compared toHbAS participants. The finding was however, not statistically significant. Previous studies conducted in other parts of Nigeria reported similar findings [22,23]. However, significantly higher HbF reported by Isa *et al* [23], may be because they compared HbF level in patients with HbSS to those with HbAA while this study compared with individuals with HbAS phenotype. Secondly, differences in methodology by which HbF was assayed may have contributed to the slight difference too. Their study used Berke's method while HPLC was used for this study. High level of HbF has been associated with mild disease due to inhibition of deoxy-HbS polymerization with consequent retardation in the sickling process with reduction of acute painful episodes, acute chest syndrome, fewer leg ulcers, less osteonecrosis, and reduced disease severity [24].

Among males, participants with HbSS phenotype were found to have significantly higher mean HbS level than male HbAS participants. Male HbSS participants also have higher mean HbF level than the male HbAS participants, though not statistically significant. Conversely, Male HbSS participants have marginally lower HbA2 level compared to that of male HbAS participants. Likewise among females, participants with HbSS phenotype were found to have significantly higher mean HbS level than female HbAS participants. Female HbSS participants also have higher mean HbF and HbA2 level than the female HbAS participants, though not statistically significant. This may be due to compensatory increase in HbF and HbA2 in patients with HbSS. This further support the finding that individuals with sickle cell anaemia have higher levels of HbS, HbA2 and HbF than those with HbAS phenotypes irrespective of their gender as also reported by previous studies [23,25].

This study has also revealed that among participants with HbSS and HbAS phenotypes, males have higher HbS level but lower HbA2 and HbF levels compared to females, though not statistically significant. Reports from previous studies also showed similar findings [24,25]. This is because sickle cell anaemia (HbSS) is an autosomal recessive disease (caused by abnormalities in the β-globin gene located on chromosome 11). It is not a sex-linked disorder and so both sexes are therefore equally affected. Conversely, inheritance of HbFis dependent on many gene loci outside the β-globin cluster on chromosome 11, including Xp22.2 locus on the X chromosome and that may have contributed to higher HbF level found among females in both groups [26]. It has been noted that symptoms associated with SCA do not fully manifested until hemoglobin switch from fetal to adult takes place [27]. This is in support of the notion that elevated levels of HbF is inversely related to the level of HbS and to the degree of severity in SCA [24].

Conclusion

Chromatographic pattern of haemoglobin types (HbS, HbA2 and HbF) showed significantly higher mean level of HbS among participants with HbSS compared to those with HbAS. Similarly, there was higher mean level of HbA2 and HbF among HbSS than in HbAS participants, though not significant. In addition, HbS was higher among males while HbA2 and HbF were lower compared to females, though not statistically significant. Chromatographic pattern of haemoglobin types should be established to differenciate between sickle cell anaemia and sickle cell trait as it will serve as a guide in diagnosis especially in cases that are confusing clinically.

Authors' contributions

UNI conceived and designed the study, and participated in the laboratory analysis and interpretation of results. NEO participated in the laboratory analysis and interpretation of results.UCN and OHC participated in the study design and writing up the manuscript, IRC participated in laboratory analysis and review of the manuscript, AC and AJM performed the statistical

analysis and interpretation of results. UGC and OC participated in the patients' recruitment and laboratory analysis.NUU and ONB participated in writing up the manuscript and patient recruitment. All authors have read and approved the final manuscript.

Acknowledgements

The authors are grateful to the management of Alex Ekwueme Federal University Teaching Hospital, Abakaliki for creating enabling environment for this study to be carried out. We are also grateful to the staff of the Sickle cell center of the hospital for their support and assistance during the study.

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Received = 10th May 2021
Accepted = 19th July 2021