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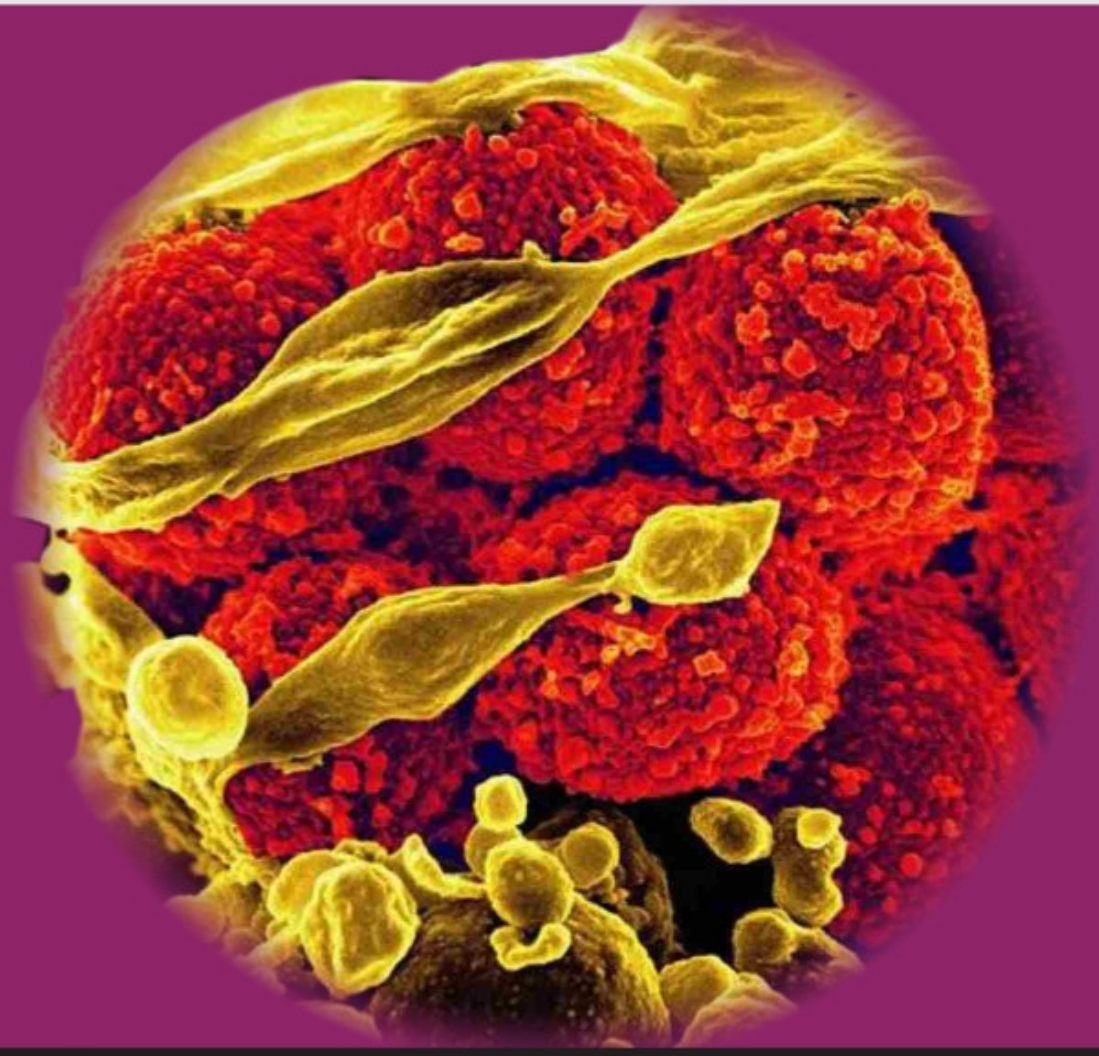

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ORIGINAL RESEARCH ARTICLE

Clinical Phenotypes in a Cohort of Nigerian Patients with Sickle Cell Anaemia: Relationship to Serum Lactate Dehydrogenase

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ABSTRACT

Background:
Distinct phenotypes based on serum lactate dehydrogenase (LDH) levels have been described in sickle cell anaemia (SCA) in studies mainly conducted in high-income countries. We aimed to characterise the phenotypes of Nigerian patients with SCA based on serum LDH distribution and pain episodes.

Materials and Methods:
This was a descriptive cross-sectional study of 200 male and female patients with SCA, aged 2-43 years, who were in the steady-state. Medical history was obtained through structured questionnaire and review of clinical notes. Haematological and biochemical investigations included full blood count, reticulocyte count, Hb F quantification, serum LDH and total and direct bilirubin. Data were analysed using *Stata* v15. Patients were categorised into phenotypes based on quartile distribution of LDH and annual pain rates. Subgroup analyses were conducted within the upper and lower quartile groups to elucidate their subtle characteristics.

Results:
There was moderate, but significant correlation between serum LDH and other haemolytic markers. The mean haematocrit, Hb F level, total white blood cell, platelet and reticulocyte counts were statistically different between the upper and lower LDH quartiles (p<0.05), while the annual pain rates were not different (p=0.1117). Within the upper quartile of LDH distribution (i.e. *hyperhaemolysis* group, n=52), 11(5.5%) had concomitant *high* pain rate (painful events ≥ 3/annum) suggestive of a mixed haemolytic/ vasoocclusive phenotype. Similarly, within the lower quartile (*viscosity/vasooclussive* group, n=50), 47(23.5%) had associated low pain rate (painful events < 3/annum) suggestive of a clinically silent phenotype. Patients with LDH levels in the interquartile range had pain rate ranging from *high* to *low*, suggesting a variable phenotype.

Conclusions:
Clinical phenotypes of patients with SCA in Northern Nigeria are potentially more complex than those described outside the African setting. A 'Venn diagram' model is proposed to depict these phenotypes.

Keywords: Phenotypes, sickle cell anaemia, Nigeria, chronic hyperhaemolysis, serum LDH, painful events, Venn diagram model

INTRODUCTION

Sickle cell anaemia (SCA) is a chronic haemolytic anaemia associated with recurrent painful episodes and propensity to develop end organ damage.[1,2] The highest burden of the disease is in sub-Saharan Africa and wide variation exists in clinical presentation and severity of the disease between individuals and in different populations.[3-6] This variation is largely due to genetic and environmental factors.[7-10]

Recent studies from the United States of America (USA) have suggested a unique clustering of clinical features and associated end-organ damage into two broad clinical phenotypes determined principally by serum LDH distribution, namely chronic hyperhaemolysis and viscosity/vaso-occlusive phenotypes.[11-13] Chronic hyperhaemolysis phenotypes coincide with the top quartile of LDH distribution and have lower haematocrit, lower % HbF, higher markers of haemolysis and propensity to develop leg ulcers, pulmonary hypertension, chronic kidney disease and stroke. The top quartile of LDH distribution has also been linked with fewer annual incidents of acute painful episodes and earlier mortality in two separate cohorts of North American SCA patients. [12] The viscosity/vaso-occlusive phenotype, on the other hand, coincides with the lower LDH quartile, has higher haematocrit and propensity to develop recurrent painful crises, acute chest syndrome (ACS) and osteonecrosis.[11,14–16]

However, most studies that established or supported the *chronic hyperhaemolysis* paradigm were conducted outside the sub-Saharan Africa (SSA). Furthermore, some studies conducted in Africa have reported a different pattern of clustering of clinical features, suggesting the possible influence of genetic and environmental factors in the development of these clinical features.[17,18] Other studies have questioned the robustness of serum total LDH and indeed other biochemical surrogate markers in predicting haemolysis, as LDH did not correlate well with directly measured red cell survival in SCA patients.[19,20] While predicting the clinical course and complications of SCD remains essential in planning the appropriate preventive and therapeutic measures. It is also important to validate if the clinical phenotype determined based on LDH categorisation is relevant in patients living in SSA where the disease is most prevalent and the resources for managing its complications are scarce. This study, therefore, aimed at characterising the clinical phenotypes of Nigerian patients with SCA based on some selected markers of haemolysis (LDH, total bilirubin, haematocrit and reticulocyte count) and frequency of painful crises.

MATERIALS AND METHODS

Study Design

This was a descriptive cross-sectional study. Two hundred (200) individuals with SCA, aged 2 years and above were consecutively recruited in the steady state. Steady state was defined as the absence of hyperhaemolysis, vaso-occlusive crisis or active infection in the last 4 weeks and no history of blood transfusion in the preceding 120 days. Vaso-occlusive crisis was defined as pain that lasts for at least 4 hours, for which there is no explanation other than SCA and requires therapy with parenteral analgesics in a hospital setting. Hospitalisation was defined as inpatient hospital care for any reason for at least 24 hours.

The study patients were selected based on previous diagnosis of SCA using Haemoglobin (Hb) electrophoresis on cellulose acetate paper at alkaline pH. This diagnosis was further validated by Hb quantification using high performance liquid chromatography (HPLC). Patients with other forms of haemoglobino-pathies such as Hb SC, as well as those on hydroxyurea therapy, were excluded from the study.

Sample Size

Sample size was calculated using the formula $N = Z^2pq/d^2$ with the highest reported prevalence of sickle cell anaemia in Nigeria of 3%.[4] A minimum sample size of 44 was obtained, but a total of 200 patients were recruited to increase the power of the study.

Setting

Individuals with SCA were recruited from the pediatric and adult sickle cell disease (SCD) clinics of Aminu Kano Teaching Hospital (AKTH), Kano, a public tertiary health institution in Northwestern Nigeria. The clinics operate once a week with an average turnover of 60-100 patients seen for routine follow-up.

Standard of care involves routine full blood count (FBC) every 6-8weeks, Penicillin V prophylaxis from birth to 12 years of age, malaria prophylaxis with daily proguanil, daily folic acid supplementation and annual transcranial Doppler (TCD) ultrasound screening from 2 to 16 years of age. In addition to the routine Nigerian National Program on Immunization (NPI)-recommended childhood immunisations, parents are also encouraged to immunise their children against pneumococcal disease with Pneumovax® and *H. influenza* with Hibrex®, which are routinely offered in the pediatric clinics. Referral services are also offered to those patients who may require care from other specialists including social welfare and nutrition support for children. Routine health education is usually given by the nursing staff at the beginning of each clinic day.

Ethics Considerations

The Research Ethics Committee (REC) of AKTH approved the study protocol. All patients recruited into the study gave written informed consent for participation. Assent was obtained for all participants below the age of 18 years. All aspects of this study were conducted in compliance with the local research ethics guidelines and the Helsinki Declaration for research on human subjects.

Data Collection

Basic Bio-demographic Data

Basic bio-demographic data were obtained from patients or their caregivers using a structured questionnaire and review of patients' clinical notes. Details obtained included age, sex, age at diagnosis, past medical and blood transfusion history.

Haematological Studies

Five milliliter (5ml) of venous blood was drawn by venipuncture aseptically into tubes containing ethylene diamine tetraacetic acid (EDTA) anticoagulant. Haematological studies (measurement of haematocrit and total white blood cell [WBC] and platelet counts) were performed using *Swelab*® Automated haematology cell analyser. Reticulocyte count was estimated manually from a peripheral blood smear stained with new methylene blue and expressed as percentage of the total red cell count using standard operating procedure. Haemoglobin F (Hb F) level was quantified by high performance liquid chromatography (HPLC) using HbGold® analyser (Drew Scientific Ltd. UK).

Biochemical Analyses

Blood for biochemical analyses was collected into clot/gel separator bottles, centrifuged and separated immediately and serum was used to measure LDH, total and direct bilirubin, and serum aspartate aminotransferase (AST) levels.

Data Analysis

The data generated were analysed through calculations of means and standard deviations for variables with normal distribution (reported as mean ± standard deviation), using the STATA version 15 (College Station, Texas). Categorical variables were reported as numbers and percentages. Serum LDH values were divided into quartile groups, and *chronic hyperhaemolysis* was defined as the top quartile, corresponding to 75th percentile, of the distribution. Student's *t*-test was used to compare parameters between the different subgroups while correlation between LDH and other markers of haemolysis was tested using *Pearson's* correlation coefficient.

RESULTS

There were more females (66%) than males (34%) in the study. The median age of the patients was 17 years, range 2-43 years. Approximately half (49%) of the patients were between the ages of 15-19 years. The median age at diagnosis for all patients was 19 months (range 3-288 months). The average annual hospitalisations was 2.49 per patient (median = 2 per patient). When frequency of hospitalisation was considered, 56 (28%) of

patients had never been hospitalized. However, more than half of the patients 118 (59%) had between 1 and 5 hospitalisations per annum while 22 (11%) had between 6 and 10 hospitalisations per annum. Four patients (2%) had more than 10 hospital admissions per annum. Table 1 summarizes pain frequency and transfusion history of all participants. Seven patients (3.5%), all females, were found to have pain frequency of >10/annum, which may be menstruation-induced, but yet to be validated.

To validate if serum LDH reliably predicts haemolysis among patients in this centre, a simple linear correlation was conducted between LDH and other markers of haemolysis (namely, Hb level, reticulocyte count, serum total and direct bilirubin). A weak, but significant negative correlation with Hb (r = -0.2406, p = 0.0004) was observed and weak positive correlations with reticulocyte count (r = 0.286602; p = 0.0012), total bilirubin (r = 0.495237; p = 0.0253) and direct bilirubin; (r = 0.256484; p = 0.0001) were obtained (Figure 1).

Table 1. Pain frequency and transfusion history of all participants

Characteristics	n (%)
Average annual frequency of painful crisis	
<3 (Low)	149 (74.5)
3-5 (Moderate)	35 (17.5)
6-10 (Severe)	9 (4.5)
>10 (Very severe)	7 (3.5)
Transfusion history	
Yes	87 (43.5)
No	113 (56.5)

Table 2. Medical, haematological and biochemical parametres of participants by LDH distribution

Variable	Low LDH mean ± SD n = 52	High LDH mean ± SD n = 50	All patients mean ± SD n = 200	*p*-value
Clinico-demographic				
Age (years)	21.1 ± 5.8	12.2 ± 6.3	16.0 ± 8.2	<0.00001
Female: Male ratio	2.18	1.60	1.94	-
Age at diagnosis (months)	49.8 ± 68.3	25.7 ± 36.1	34.6 ± 48.2	0.0285
Annual VOC (per patient)	1.69 ± 2.01	1.14 ± 1.41	1.26 ± 2.23	0.1117[#]
Total lifetime transfusions	0.86 ± 2.42	2.13 ± 2.66	1.34 ± 2.61	0.0132
Haematological				
Haematocrit (%)	23.7 ± 4.8	21.7 ± 4.3	23.5 ± 4.8	0.0287
TWBC ($x10^9$/L)	11.9 ± 4.0	14.6 ±4.2	12.9 ± 4.3	0.0011
Plt ($x10^9$/L)	367.0 ± 140.9	438.0 ± 152.8	370.8 ± 142.5	0.0164
Retic (%)	5.56 ± 3.05	7.26 ± 4.1	6.30 ± 3.7	0.0196
Hb F (%)	7.89 ± 5.25	5.31 ± 3.82	7.21 ± 4.60	0.0057
Biochemical				
LDH (U/L)	381.5 ± 78.7	1034.5 ± 565.4	905.8 ± 370.7	<0.00001
Total Bilirubin (mmol/L)	23.2 ± 21.1	80.1 ± 49.8	54.2 ± 45.2	<0.00001
Direct bilirubin (mmol/L)	7.7 ± 5.6	15.9 ± 13.4	12.5 ± 10.7	0.0001

[#] Statistically not significant

†Abbreviations: TWBC: Total white blood cell count; Plt: Platelet counts; Retic: Reticulocyte count; Hb F: Foetal haemoglobin; LDH: Serum lactate dehydrogenase.

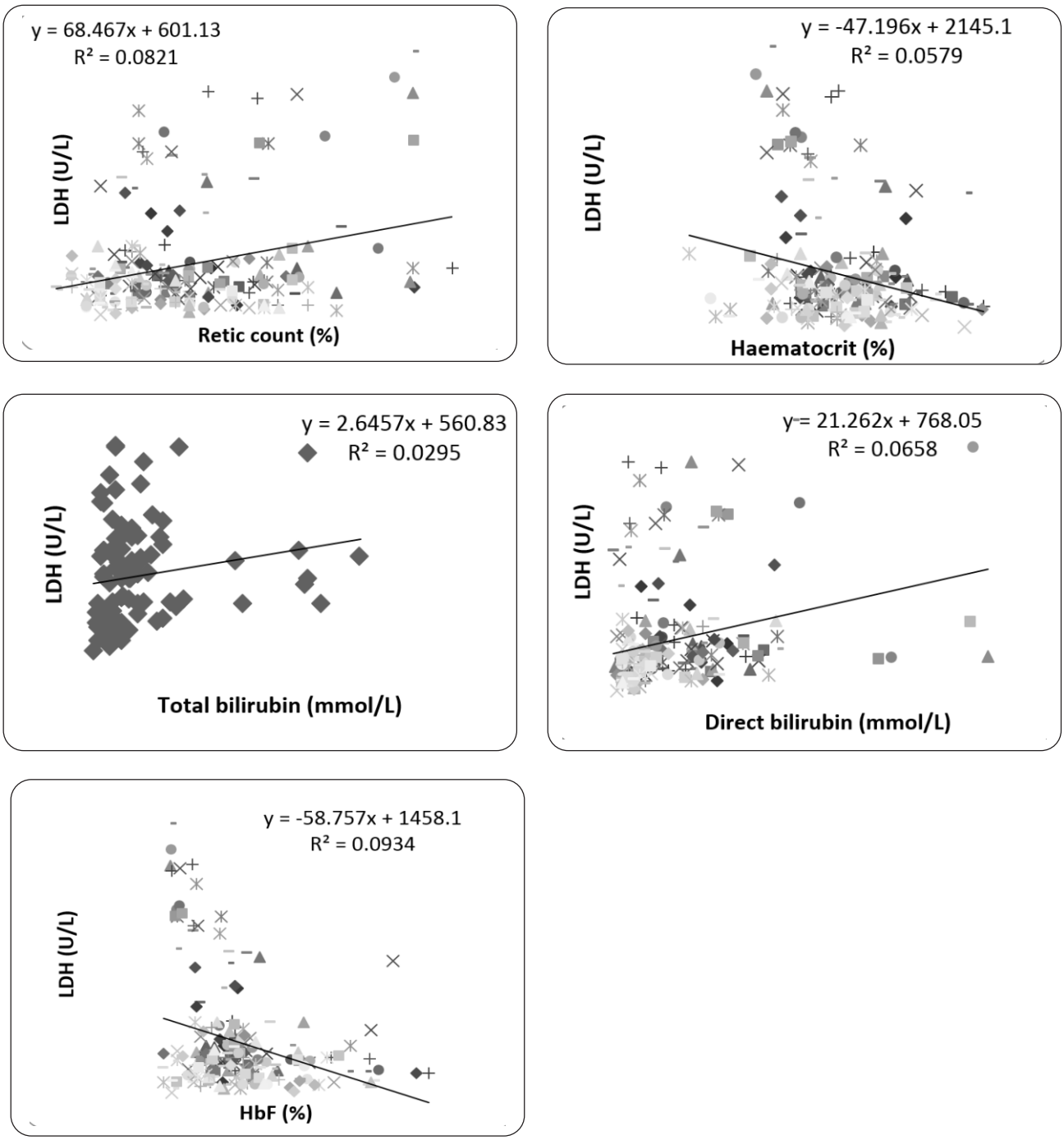

Figure 1: Scatterplots showing weak, but significant correlation between serum LDH and other indirect markers of haemolysis (n = 200)

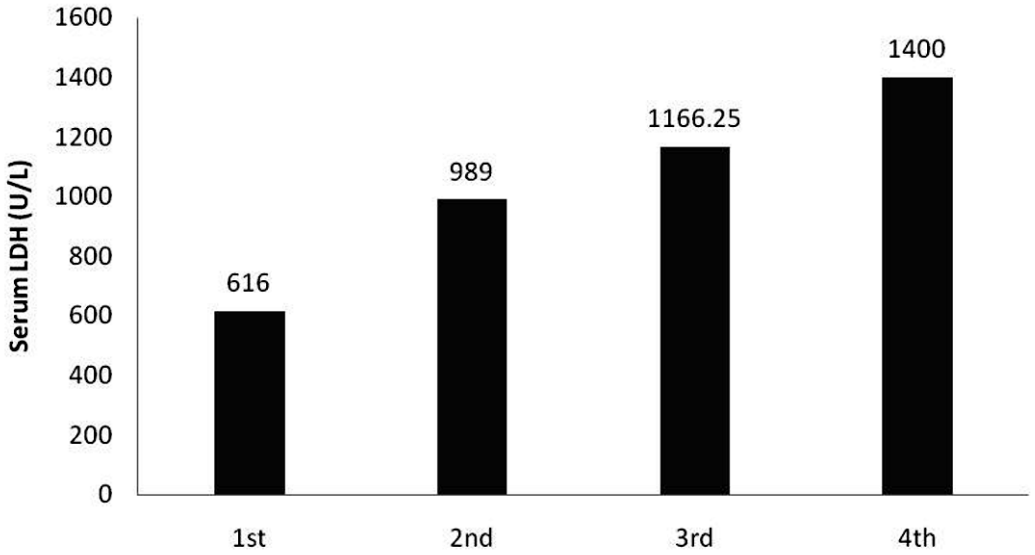

Figure 2: Bar diagram shows the 1st, 2nd, 3rd and 4th quartiles of serum LDH (U/L) of the study subjects. The 3rd quartile, corresponding to the 75th percentile, represents the chronic hyperhaemolysis group.

In order to categorise patients based on intensity of haemolysis and to determine those who possibly have a haemolytic phenotype, the LDH concentrations were divided into quartiles as described by Taylor *et al*.[12] Figure 2 shows the quartile distributions of serum LDH of the patients. *Chronic hyperhaemolysis* was defined as LDH values that fall within the top quartile (i.e. > 75th percentile).[12] Fifty-two (52) patients had serum LDH above the upper quartile of 1166.25U/L signifying *chronic hyper-haemolysis*, with a mean value of 1034.56 ± 565.44U/L within the group. Fifty (50) patients had serum LDH concentration below the lower quartile of 616U/L with a mean of 381.54 ± 78.66 U/L within this group. The number of patients in the interquartile range (IQR) = 98. Table 2 compares the medical, haematological and biochemical characteristics of patients based on quartile distribution of serum LDH and these were compared statistically using Student's *t*-test. All the clinical, biochemical and haematological parameters compared were significantly different between the two quartile groups except for the annual VOC rates, which was not statistically different between these two quartiles (*p*-value = 0.1117; Table 2).

In order to determine if the upper LDH quartile truly represents a *hyperhaemolysis* group, LDH was correlated with the other markers of haemolysis (i.e. Hb level, retics, serum total and direct bilirubin) within the upper and lower LDH quartile subgroups. Among the patients in the upper LDH quartile group, statistically significant correlation coefficients were obtained for Hb, retics and direct bilirubin (*r* = -0.482112, 0.649010 and 0.557113 with *p*-values 0.0462, 0.0117 and 0.0237 respectively), except for serum total bilirubin, which, although numerically higher, did not correlate significantly (*r* = 0.312222, *p* = 0.0553). In the lower LDH quartile, the correlation coefficients were numerically higher than those of the whole group (*r*= -0.578882, 0.499829, 0.4952370 and 0.333176), but only the correlation between LDH and Hb level was statistically significant (*p*-values 0.0172*, 0.0571, 0.1782 and 0.0757 for Hb, retics, total bilirubin and direct bilirubin respectively).

The pain rates of patients within the upper and lower quartile groups of the LDH distribution were compared. Pain rate of less than 3 episodes per annum was considered "*low*", while pain rate of 3 or more per annum was considered "*high*" as proposed by Platt *et al*.[21] The "low" pain group (i.e. VOC < 3/annum) also included patients who did not experience acute sickle cell-related pain within the preceding

twelve calendar months or more as categorised by the study questionnaire. Within the upper quartile (high LDH group, *n* = 52), 11 patients (5.5%) had a VOC rate of≥ 3 per annum suggesting a *high* pain rate. Similarly, within the lower quartile of LDH distribution (low LDH group, *n* = 50), 47 patients (23.5%) had pain rate of less than 3 per annum suggesting a *low* pain rate. Individuals whose LDH level fall in the IQR had variable pain frequencies ranging from *high* to *low*. Based on this result, the sickle cell phenotypes in these patients were classified using a "Venn Diagram" model presented in Figure 3 with details in Table 3.

Table 3: Haematological and biochemical characteristics among the different phenotypic subgroups

Variable	Mainly Haemolytic* Mean ± SD *n* = 23	Mainly Vaso - occlusive# Mean ± SD *n* = 12	Mixed Haemolytic/ vaso-occlusive† Mean ± SD *n* = 11	Silent§ Phenotype Mean ± SD *n* = 47	Variable‡ Phenotype Mean ± SD *n* = 107
Age (years)	11.9±7.4	22.9±10.9	11.0±5.4	19.8±6.2	15.2±7.9
PCV (%)	23.0±5.7	25.5±4.4	20.7±3.7	23.9±5.0	23.8±3.8
Hb (g/L)	7.7±1.9	8.5±1.5	6.9±1.2	8.0±1.7	7.9±1.3
TWBC (x10^9/L)	13.6±3.8	10.3±3.8	15.7±4.0	12.2±4.19	10.1±2.8
ANC (x10^9/L)	8.2±4.7	4.4±1.0	9.5±3.7	7.36±2.81	6.99±2.83
Plt (x10^9/L)	401.2±160.4	378.3±88.1	430.3±123.3	351.8±147.1	339.9±127.5
Retic (%)	6.9±4.6	6.0±3.6	8.4±4.4	6.0±3.1	6.0±3.7
Hb F (%)	6.11±5.05	7.51±4.97	4.94±5.26	9.11±6.13	7.12±3.63
LDH (U/L)	2526.2±826.4	470.1±135.3	2807.5±1110.2	417.3±101.1	823.5±305.1
TBil (mmol/L)	70.3±49.8	46.2±46.1	94.1±40.8	30.5±29.6	57.9±43.6
DBil (mmol/L)	6.0(3.0-52.0)¥	9.9±6.9	7.0(4.0-43.0)¥	4.0(3.0-72.0)¥	13.9±10.9

Key: *High LDH plus Low VOC; #Low LDH plus High VOC; † High LDH plus High VOC; § Low LDH plus Low VOC; ‡LDH in the interquartile range plus any VOC rate; ¥ Median (range) reported here for skewed datasets.

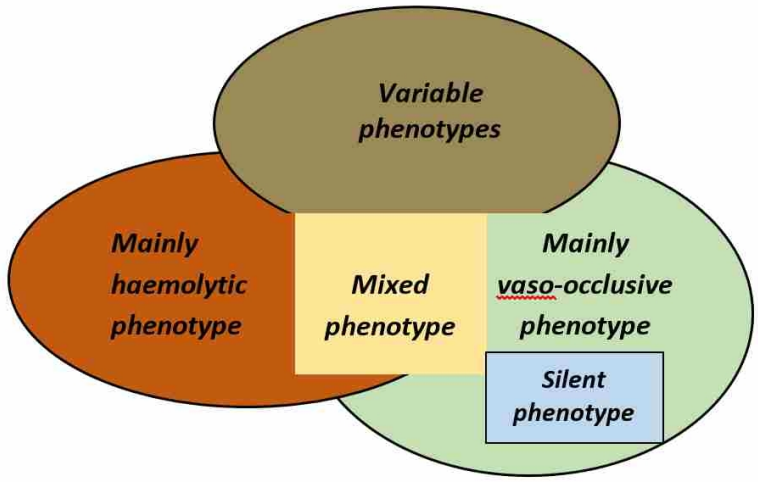

Figure 3: '*Venn Diagram*' model of sickle cell phenotypes in Africa. Based on serum LDH distribution and annual pain rates, patients may be categorised into five distinct phenotypes namely: Mainly haemolytic; Mainly vasooclusive; Mixed haemolytic/vasooclusive; Clinically Silent and Variable phenotypes.

DISCUSSION

Recent insight into disease mechanisms in SCD has increasingly recognized the role of chronic intravascular haemolysis in the pathogenesis of some of its complications. Serum LDH concentration has now emerged as an important marker of haemolysis in SCD and the quartile distribution of LDH is frequently used to categorise patients into clinical sub-phenotypes such as *chronic hyperhaemolysis* (represented by the top quartile of LDH distribution) or *viscosity/vasoocclusion* (represented by the lower quartile) sub-phenotypes. Taylor and colleagues (2008) suggested that these clinical groups have distinct associated spectrum of complications and that the steady state serum LDH measurements might be used to predict those patients at risk of these presentations.[12] This categorisation is important because it helps clinicians to anticipate complications and plan appropriate treatment early.

This study found about a quarter (up to 26%) of individuals having their LDH values above the upper quartile. The mean steady state LDH concentration (Table 2) found in this study is significantly higher than both 356 U/L and 875 U/L found in Saudi Arabia in the UK, respectively. [22,23] Within the top quartile of the LDH distribution in this study, the mean LDH value of 1034.56 ± 565.44 U/L obtained is significantly higher than both 610.6 U/L reported in the American National Institutes of Health (NIH) and 680.8 U/L in the CSSCD cohorts of the same category.[24] This finding is significant and suggests that patients in our environment appear to have significantly higher steady state LDH levels than their counterparts elsewhere. Considering the concomitant finding of higher pain rates, patients with SCA in this environment appear to have a phenotype that is characterised by both high pain rates and high haemolysis. Indeed, our data found that the LDH categorisation into upper and lower quartiles did not show a significantly different VOC rates between the dichotomous quartile groups. This suggests that LDH distribution may not be a reliable predictor of pain rates in our SCA population and therefore prompted further scrutiny of subgroups of patients in this study. Firstly, to validate the utility of LDH in defining haemolysis, the relationship between serum LDH and other markers of haemolysis of the whole patient group showed weak correlation coefficients, which were quite significant. Secondly, higher correlation coefficients were obtained in the upper LDH quartile subgroup thus verifying that it truly represents a *hyperhaemolysis* group. Although the correlation with serum total bilirubin in this analysis was unexpectedly found to be not statistically significant, it was numerically higher notwithstanding. The exact reasons for this is, however, not clear.

The upper and lower LDH quartiles as well as the interquartile range for pattern of pain rates within these subgroups were examined. Patients with pain rates of less than 3 per annum were considered to have *low* pain rates, while those with 3 or more episodes of pain per annum were considered to have *high* pain rate.[21] Based on this data, at least five distinct phenotypic subgroups were observed within this cohort of patients with SCA, namely:

i) **Mainly haemolytic phenotype**: consisting of patients with high haemolysis (top LDH quartile) plus low Pain rate (< 3/annum);

ii) **Mainly vaso-occlusive phenotype**: consisting of patients with low haemolysis (low LDH quartile) plus high Pain rate≥(3/annum);

iii) **Mixed haemolytic/vasoocclusive phenotype**: consisting of patients with high haemolysis (top LDH quartile) plus high Pain rate (≥ 3/annum);

iv) **Clinically silent phenotype:** consisting of patients with low haemolysis (lower LDH quartile) plus low Pain rate (< 3/annum) and

v) **Variable phenotype**: consisting of those who do not fall in to any of the above specific categories, with LDH in the IQR and any Pain rate.

Based on these findings, we propose a '*Venn diagram model*' (Figure 3) to describe sickle cell phenotypes especially as obtains in Northern Nigeria.

Our findings appear to lend credence to recent observations that the clinical realities of sub-phenotypes of SCD that are based on the paradigm of chronic hyperhaemolysis might be rather different in the African setting.[25,26] This divergence is likely to be the result of environmental influences such as the high prevalence of infections and infestations like malaria, higher ambient environmental temperature and nutritional factors among others. Known genetic modifiers of severity of sickle cell disease prevalent in African populations such as G6PD deficiency and alpha thalassaemia trait are also expected to provide an additional layer of complexity or diversity of the observed phenotypes in the setting of these environmental influences.

Some salient differences in the haematological and biochemical parameters were noticeable among these patients. Patients with the *mixed haemolytic/vaso-occlusive* phenotype were found to have the lowest haematocrit and Hb F levels and the highest reticulocyte count, total WBC count, absolute neutrophil count (ANC), platelet count and serum LDH levels, all of which are known features of clinical severity in SCD.[27-31] The *silent* phenotype, on the other hand, had the highest Hb F levels and the lowest platelet counts, total bilirubin and LDH levels. Expectedly, the mainly *vaso-occlusive* phenotype had the highest haematocrit (which contributes to higher blood viscosity) and the lowest total WBC count and direct bilirubin levels. The *mainly haemolytic* and the *variable* phenotypes had haematologic and biochemical characteristics intermediate between these. Indeed, the largest number of patients were found to have the *variable* phenotype. Based on these findings, it could be hypothesised that the clinically *silent* phenotype group is likely to comprise of those patients with concomitant inheritance of alpha-thalassemia trait, elevated Hb F level or both. The converse is likely to be true in those with the *mixed* phenotype. Indeed, the clinically silent phenotype in this study may be likened to the Arabian/Indian haplotypes with higher Hb F levels or those with compound heterozygous Hb S and hereditary persistence of fetal haemoglobin (HPFH) mutation where patients have a milder form of SCA.[31]

Important limitations of this study, however, include the relatively small sample size of some phenotypic sub-groups, the lack of inclusion of the pattern of end-organ damage as well as the lack of genetic information on the status of alpha-thalassemia trait or the haplotypes among these patients. Neither G6PD status, nor malarial parasite count was included, since the patients were recruited in steady state. These could potentially affect the steady state LDH levels of the patients. Additionally, reliance on the questionnaire to obtain retrospective history of painful events and frequency of hospitalisation might be beset by a recall bias. This information could be better assessed in a prospective study design. It would be interesting to explore these phenotypes in more elaborate studies involving larger sample size and additional genetic information in order to validate these observations.

CONCLUSION

Generally, observations in this study suggest that the phenotypes of SCA in patients living in a typical African setting might be quite different and more complex than the relatively simplistic or dichotomous chronic hyperhaemolysis and viscosity/vaso-occlusive phenotypes described in patients living outside Africa. This provides insight into the potential complexity of phenotypes in these patients.

Conflict of Interest: The authors declare no conflict of interest.

Author's Contributions: AAY, IB, AK and SGA contributed in study conceptualisation, design and discussion; AAY & IB contributed in data collection; AAY & IMI contributed in data analysis; All authors contributed to the writing and critical evaluation of manuscript.

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