SERUM LACTATE DEHYDROGENASE LEVEL

IN

CHILDREN WITH SICKLE CELL DISEASE

AT

KENYATTA NATIONAL HOSPITAL

A DISSERTATION SUBMITTED IN PART-FULFILMENT OF REQUIREMENT FOR THE DEGREE OF MASTERS OF MEDICINE IN PATHOLOGY UNIVERSITY OF NAIROBI

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DECLARATION

Student declaration

I **Dr. Germaine S. Makory**, declare that this dissertation for the Masters of Medicine in Pathology is my original work and has not been presented at any other institution of higher learning to the best of my knowledge.

Signed…………………………..

Date……………………………..

DEDICATION

To my family; my husband Charles, my son Angelo and all family members for their support and encouragement.

ACKNOWLEDGEMENT

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ABBREVIATIONS

AST   :   Aspartate Transferase.

EDTA   :   Ethylenediaminetetra-Acetic Acid.

HbC   :   Hemoglobin C.

HbF   :   Fetal hemoglobin.

HbS   :   Sickle hemoglobin.

HbSS   :   Homozygous Sickle hemoglobin.

HPFH   :   Hereditary Persistent Fetal hemoglobin.

IFCC   :   International Federation of Clinical Chemists.

KNH   :   Kenyatta National Hospital.

ERC   :   Ethics and Research Committee.

LDH   :   Lactate Dehydrogenase.

M. Med   :   Master of Medicine.

NAD   :   Nicotinamide Adenine Dinucleotide.

RBC   :   Red Blood Cells.

SCA   :   Sickle Cell Anemia.

SCD   :   Sickle Cell Disease.

SOPs   :   Standard Operating Procedures.

SPSS   :   Statistical Package for Social Sciences.

USA   :   United States of America.

WBC   :   White Blood Cells.

DEFINITION OF TERMS

Allele: Gene occupying a particular location on a chromosome.

Anemia: A reduction in hemoglobin concentration to below the lower unit of normal for a specified age, sex and geographical area.

Complaint: Symptom or distress about which a patient seeks medical assistance.

Complication: Disease or disorder arising as a consequence of another disease.

Crisis: Sudden paroxysmal intensification of symptoms in the course of a disease.

Event: Sudden change in the course of a disease towards improvement or deterioration.

Genotype: Actual alleles present in an individual.

Haplotype: Set of closely linked alleles (genes or DNA polymorphisms) inherited as a unit.

Hemolysis: Destruction of red blood cells with release of hemoglobin in the circulation.

Kinetic method: Analytical method in which the rate of a reaction or a related quantity is measured and utilized to determine concentrations.

Sickle cell anemia (SCA): Inherited form of anemia that occurs in individuals with homozygous inheritance of HbS.

Sickle cell disease (SCD): Genetic disorder that results from the presence of a mutated hemoglobin (Hb S) can be homo or heterozygous.

ABSTRACT

Background

Sickle Cell Disease (SCD) an inherited condition caused by a point mutation in the β- globin gene of hemoglobin. The laboratory diagnosis of SCD at Kenyatta national hospital (KNH) is through hemoglobin electrophoresis. At KNH, the follow up of patients with Sickle SCD aims at preventing malaria, pneumococcal infections and folate deficiency. Hydroxyurea is also prescribed to reduce the occurrence of certain SCD complications. Low socio-economic status coupled with lack of awareness about the disease in many of SCD patients is a hindrance to early diagnosis and contributes to poor adherence to treatment, shortened life expectancy, frequent sickle cell crises and early occurrence of complications. This has stimulated studies looking into the use of already existing, less expensive, effective laboratory tests for the detection of complications with possibilities of recommending their use in the routine follow up of SCD patients. This study was undertaken to look at serum lactate dehydrogenase (LDH) in SCD to find out if as a marker of hemolysis, its elevation would correlate with the clinical manifestations of the disease and the occurrence of complications.

Objective

To characterize the patterns of serum LDH levels among children with SCD aged 1-13 years attending Kenyatta National Hospital (KNH).

Study design and setting

Descriptive cross-sectional study conducted at the pediatric hematology clinic, pediatric filter clinic and the pediatric wards of KNH. The biochemical laboratory tests were done at the KNH-clinical chemistry laboratory and the hematology tests were done at the University of Nairobi (UON) hematology laboratory.

Methods:

A total of 145 children with SCD aged 1 to 13 years were recruited into the study after an informed consent was signed by their parents or guardians. A questionnaire to collect data on age gender, presenting complaint, current medication used and recent medical history in the last one year was filled. Blood samples were collected for the total blood cell count, peripheral blood film, reticulocyte count and the total serum LDH level.

Data handling

The generated data from the questionnaire, the medical examination and the laboratory tests was entered into a computer data base, cleaned and analyzed through statistical software for analysis: SPSS (version 17.0). The results were expressed in graphs and tables and presented in mean/median and percentages.

Results

A total of 145 children were recruited into the study. Males were 57.2% while females were 42.8% and the median age was 5years (IQR: 3.0 - 9.0). The disease duration had a median of 36 months (IQR: 13 - 36 months). Hydroxyurea therapy was documented in 43.4% of the participants. The complications diagnosed at the time of presentation were thrombotic events accounting for 47.4% and these were represented by painful crises in 31.6%, neurological complications in 10.5% and skin ulcers in 5.3%; hemolytic complications were documented in 5.2% of participants while the presence of infections was documented in 47.4% patients.

Serum LDH levels were measured in all the participants and a mean of 628 IU/L (reference range: 180- 360 IU/L) recorded. Majority of participants (93.8%) had serum LDH levels values above the reference range. Anemia was present in 97% participants and leukocytosis in 59% patients. Platelets counts within reference range were recorded in 80 % of the participants. No significant correlation was demonstrated between the serum LDH levels with the disease severity or the presence of complications. The study findings established a significant relationship between the elevation of serum LDH level and gender whereby male participants were predominant in the group with high serum LDH level. There was a significant correlation between the elevated serum LDH levels and presence of anemia. No statistically significant

correlation was found between the serum LDH level, the white blood cells, platelet or the reticulocyte count.

Conclusion:

This study established a mean serum LDH level of 628.8 IU/L in children with SCD attending KNH. The most commonly diagnosed complications at presentation were thrombotic events and infections each documented in 47.4% patients. These complications showed no correlation with serum LDH level. Although a significant correlation between elevated serum LDH with gender and presence of anemia was seen, no significant correlation was found between the serum LDH levels, the WBC count, platelets count or the reticulocyte count. This study demonstrated that in children with SCD at KNH the serum LDH level is not a marker for severity of the clinical manifestation or predictor of complications of the disease.

Study recommendations:

From this study findings, serum LDH level may be used to predict for the presence of severe anemia in children with SCD at KNH. It however cannot be used as a marker of severity or to predict for disease severity and occurrence of complications.

1. INTRODUCTION

SCD, the most common monogenetic disorder worldwide, affects an estimated 30 million persons and represents a major public health concern because of its association with a significant morbidity and mortality (1). Despite extensive studies done about this disease, the sufferers continue to experience a heavy psychological, physical and financial burden in the control of the disease and the prevention of its complications. SCD was declared a public health priority by WHO with emphasis on neonatal diagnosis of SCD (2)

In the developing world, the medical facilities are inadequately equipped and this translates into a reduced life expectancy amongst SCD patients with the majority of them dying before the age of 15 years. Most of the disease complications have been described to occur in early childhood hence the need for early diagnosis (3).

The disease severity is related to two major events, vaso-occlusion and hemolysis which are clinically interlinked (4). There are several laboratory markers of hemolysis but none of them is specific and these are used in combinations of two or more with correlation with the clinical picture (5). Serum LDH level was used in this study as one of the markers of hemolysis and its choice was related to its availability and low cost (6).

This study was undertaken to look at serum LDH level, and see if its elevation can be used to determine the likelihood of complications among children with SCD attending Kenyatta National Hospital.

2. LITERATURE REVIEW

2.1. SCD definition:

Sickle cell disease is an inherited condition, resulting from a point mutation in the β- globin gene of hemoglobin. This leads to substitution of Thymine for Adenine in the 6th codon of the globin gene. The result of this mutation is the formation of abnormal hemoglobin "Sickle hemoglobin or hemoglobin S (HbS)". The HbS has the capacity to polymerize under certain conditions in particular, hypoxia and dehydration (7). The disease occurs in both homozygous

(commonly referred to as sickle cell anaemia: SCA) and heterozygous forms (Sickle cell disease: SCD) (8).

2.2. History of Sickle Cell Disease:

Symptoms of SCD were known in Africa by various names well before it was scientifically documented and this can be traced back to 1670 in one Ghanaian family (9). In 1910, the first scientific description was made by James Herrick who observed "Peculiar elongated sickle shaped red blood cells" in an anemic black medical student. His report led to the recognition of hundreds of hemoglobin synthesis abnormalities and advances in cell biology, physiology and genetics (9, 10).

In 1917, Emmel described the sickling phenomenon in-vitro, among members of one family and suggested the genetic basis for sickling. In later studies, sickling was proven to be due to oxygen deprivation (11). Huck and Sydenstricker in their further detailed analysis concluded the disease to be inherited in a Mendelian autosomal recessive way (12).

2.3. Prevalence and geographical distribution:

In Africa, 230,000 children are born each year with SCA and a high mortality is reported due to malaria, bacterial infections and anemia. There are an estimated 200 million carriers of the sickle cell trait worldwide. Currently, in USA 6-10% of the children born of African American parents have the Sickle cell trait (10).

In Kenya, although studies to evaluate the prevalence of the sickle cell trait have been done in the past, no recent study on the country-wide prevalence of SCD is available. It has been estimated that the sickle cell trait prevalence is variable from one ethnic group to another, from 0% in the northeastern Kenyan population to the highest prevalence of 40% found among the Bantus of the southeastern coast (11).

Majority of SCD patients in Africa die before the age of 15 years while in the developed world SCD patients are living beyond 40 years (10). Despite limited resources in most African countries, life- expectancy is improving with time though mortality remains high (13). This has been attributed to a better understanding of the disease management, early diagnosis and timely application of therapeutic measures.

2.4. Pathophysiology of the sickle cell disease and complications:

Under conditions such as hypoxia or dehydration, HbS polymerizes but this is reversible in the early stages upon reoxygenation or rehydration. In the absence of these corrective measures or in cases of repeated sickling, the RBCs change in shape becomes irreversible and this results in the sickled RBCs acquiring pathogenic properties (3).

The risk factors for the development of sickle cell vaso-occlusion include HbS polymerization, sickle cells deformability, blood viscosity, the fraction of the dense cells, sickle cells endothelial cell adherence, endothelial cells activation, haemostatic activation, vascular tone and contributions from white blood cells and platelets. This is through a multistep process involving the sickle erythrocytes, the leukocytes and the endothelial cells (14).

The other major pathological phenomenon in SCD is hemolysis with both intra and extra vascular hemolysis occurring concurrently, with the latter accounting for two thirds of the total hemolysis (4). The polymerization of HbS leads to RBC's membrane defect, shortened life span and manifests itself as hemolytic anemia. Sickled RBCs through their damaged cellular membrane express abnormal adherence molecules to the vascular endothelium, monocytes, macrophages and are hence earmarked for phagocytosis through complement fixation (3). Okpala et al. in their study on the role of leukocytes in the SCD pathophysiology demonstrated that the recurrent inflammation and vasculopathy present in SCD leads to leukocytes and endothelial cell activation with increase in adhesion molecules expression. Thus, leukocytes specifically neutrophils, contribute to the vaso-occlusion in SCD (15).

2.5. Clinical features:

The disease is present from infancy with a mild hemolytic anemia apparent by 10-12 weeks of age. This lack of clinical manifestations in early life is related to the high level of HbF in post-natal life preventing the sickling of HbS containing RBCs (10). The symptoms are not usually apparent until the second half of the first year of life. Splenomegaly is first noted after the age of 6 months and the first vaso-occlusive episode is experienced before the age of 6 years (16). In early infancy, the hand and foot syndrome is commonly the first sign of SCD. This often occurs

early at the age of 6 to 8 months and resolves spontaneously in 1 to 4 weeks. The hand and foot syndrome is rare in SCD patients above 4years old (17).

At steady state, SCD patients have a persistent hemolytic anemia, interspersed with acute episodes of crises. These crises result from sequestration of blood cells in the lung, liver, spleen and cerebral vessels with subsequent stroke in the later. The severity of symptoms in SCD varies from one patient to another depending on the SCD haplotype. SCD patients are prone to infections affecting different organs and systems associated with the hyposplenism or asplenia accompanying the disease. The responsible micro-organisms are mostly the encapsulated bacteria such as *Haemophilus influenza* and *Staphylococcus Pneumoniae*. These patients also suffer from osteomyellitis caused by *Salmonella* species, *Staphylococcus aureus* or gram negative enterococci (18, 19).

2.6. Complications of SCD:

In SCD, the acute complications occur mainly as crises that are divided into two main groups, painful and hematological crises. There are 4 types of hematological crises, which are the aplastic crises, hemolytic crises, megaloblastic crises and splenic sequestration (3, 8).

SCD patients are prone to infections due to the presence of hyposplenism and asplenia caused by repeated splenic infarctions resulting in loss of mechanical function of the splenic vasculature and defective activation of the alternate pathway of complement. SCD patients need multiple and often emergency blood transfusions putting them at a high risk of HIV and other transfusion-transmissible infections (20, 21).

The long term complications are chronic organ damage, affecting various tissues and systems such as growth and development, bones and joints, central nervous system (delayed language, cognitive development and others), heart (cardiomegaly), the respiratory system (chronic pulmonary disease, pulmonary artery hypertension) and the hepato- biliary and gastro intestinal system (cholelithiasis and others). The other organs affected are the kidneys (nephritic syndrome, renal failure and others), eyes (vaso-occlusive disease of the retina), skin (leg ulcers) and the male genital organs: priapism (22). SCD patients who are pregnant are at a higher risk

for small for age babies and exposed to a high mortality rate of 20-30% for mother and infant without medical supervision (8, 23).

Odero A. found the prevalence of pulmonary artery hypertension among children with SCD at KNH to be 49.4% (CI: 95%) where 86% had mild pulmonary hypertension (24). Another chronic complication of SCD is Iron overload resulting from frequent transfusions used in the long term management of SCD patients and excess circulating iron from severe hemolysis (25).

The determinants of severity in SCD are the level of fetal hemoglobin, the presence of α-thalassemia, the β-globin gene cluster haplotype where the Bantu haplotype has been particularly associated with a severe disease expression and gender (26). Female SCD patients have a higher number of F-cells or RBCs containing hemoglobin F than males making them less prone to sickling events than their male counterparts (27).

Variants of SCD:

I.   Sickle cell trait (HbAS), has no hematological manifestations; it affects 8 - 10% of African - Americans and up to 25-30% of the population in West Africa (3). Its prevalence in some ethnic groups of Kenya is up to 40% (11).

II.   HbSC disease, accounts for 25-50% of patients with SCD of African origin. The HbC is found in West Africa and its prevalence in East Africa is thought to be less than 1%. The vaso - occlusive complications seen in SCD patients with Hb-SC resemble those of Hb SS but are less severe (6, 28).

III.   HbS/β^+-thalassemia has a preponderance of HbS while the HbS/β^0-thalassemia has an elevated HbA_2. These forms are mostly found in eastern Mediterranean region and India (28).

IV.   SCD with coexistent α-Thalassemia has a clinical severity similar to the one seen in Hb-SS. The carrier frequency for α^+ thalassaemia in Kenya is 40- 50% (29).

V.   Sickle Cell-HPFH has elevated Hb-F levels of 20-30% and this makes vaso-occlusive complications rare (3, 30).

In Africa the major forms of SCD observed are defined by the nature of abnormal hemoglobin and these are the hemoglobin SS, SC, HbS β- thalassemia and HbS α- thalassaemia (10, 29). In Kenya, Ojwang et al. (1985) confirmed a high incidence of HbS heterozygocity in the than district of Kisumu (31).

2.7. Diagnosis of SCD:

The diagnosis of SCD begins with a detailed clinical history taking followed by a complete physical examination. Suspicion for the presence of SCD is than confirmed by laboratory investigations.

In patients with SCD, Hb electrophoresis shows presence of HbS with low or absent HbA and in some cases increased HbF. This is the diagnostic method of choice used at KNH. Isolation and quantification of hemoglobin variants can be done by chromatography; which has the advantage of accurately diagnosing both the homozygous and the heterozygous forms of the disease (30).

For antenatal screening, the method of choice for the diagnosis of SCD or sickle cell carrier status is the DNA analysis through PCR using fetal blood or cord blood (32).

The sickling test is positive for HbS solubility. The principle of this test is based on the polymerization of HbS resulting in change of RBCs shape (sickled RBCs) under certain conditions. This test is easily available, but is not used for diagnosis as it does not discriminate between the SCA (HbSS) and the sickle cell carrier status and it may not be reliable in early infancy due to the high level of HbF that can give a false negative result (33, 34).

2.8. Laboratory features:

The total blood count (TBC/ FBC/ CBC) reveals anemia which is apparent from the age of 3 months and persists throughout life with hemoglobin of less or equal 80 g/l. The RBCs indices may show a   variable MCV and MCHC that may be low, normal or high as it depends on several interacting factors including reticulocytosis, presence of other hemoglobin variants(α or β thalassemia), folate deficiency, hydroxyurea therapy and others that tend to increase the MCV. Leukocytosis is also frequently present.

On the peripheral blood film (PBF), the RBCs show poikylocytosis with sickle cells and target cells as well as polychromasia and nucleated RBCs. Howell-Jolly bodies (RBCs inclusions) are

present as a sign of hyposplenism or asplenia. Reticulocytosis is present accompanying the hemolysis (3).

Though bone marrow examination is not necessary for diagnosis of SCD, it shows erythroid hyperplasia with presence of sickle cells and increased macrophages that may contain sickle cells. Foamy macrophages and sea- blue histiocytes may be increased. On Bone Marrow biopsy, the sickle cells are seen inside macrophages within blood vessels (30). In hypoplastic or aplastic crises, the examination of the bone marrow aspirate shows extensive necrosis with erythroblastemia that is usually related to parvovirus B19 infection (3).

Red cell hemolysis is clinically diagnosed by a combination of elevated serum bilirubin, (total and unconjugated, the latter being predominant, LDH, aspartate transferase (AST), reticulocytes count and a marked reduction or absence of haptoglobin in serum. Previous studies done have established that the occurrence of the complications is directly proportionate to the level of hemolysis and suggested the use of hemolysis markers as a way to predict the occurrence of complications and disease severity (33, 34,35). Studies done by O'Driscoll (2007), Makani (2009) and Kato (2006) have demonstrated a correlation between the elevation of serum lactate dehydrogenase levels, reticulocyte count, reduction or absence of haptoglobin in blood , blood flow velocity (trans-cranial Doppler ultrasound) and nitric oxide resistance with the occurrence of SCD complications such as pulmonary hypertension, priapism, leg ulcers and stroke (6, 12, 36).

LACTATE DEHYDROGENASE

Lactate dehydrogenase (LDH) is an enzyme that catalyzes the conversion of pyruvate to lactic acid as a final product of glycolysis when oxygen is absent or in short supply and catalyzes the reverse reaction in the liver when oxygen is present during the Krebs cycle (36). LDH is present in all the body cells and is found in high concentration in muscle cells, kidneys, erythrocytes, leucocytes, lungs, lymph nodes, spleen and brain (37). This makes serum total lactate dehydrogenase a nonspecific marker which has to be interpreted in a context of other markers of disease.

LDH has 5 iso-enzymes found in different organs: LDH-1 in the heart, LDH-2 in the red blood cells, LDH-3 in the lungs, LDH-4 in the kidneys and LDH-5 in the liver and striated muscles (38). The electrophoretic separation of LDH iso-enzymes has been overtaken by other diagnostic tests that are more organs specific and more efficient.

The reference range for serum LDH is 180-360 U/L in children as recommended by the International Federation of Clinical Chemists (IFCC) and 125- 220 IU/L in adults with no gender differences (38). Among the factors affecting the serum LDH levels are physical exercise that leads to temporary increase and blood transfusion which causes a transient increase in the first 24 hours after transfusion. There is a day to day variation of serum LDH of between 5-10% (38).

Serum LDH level is elevated above the reference range in hemolytic states and this has been demonstrated in studies done on SCD patients. These studies have demonstrated a correlation between the serum LDH level and other markers of hemolysis such as bilirubin, reticulocytosis, aspartate amino transferase (AST), haptoglobin and others (6, 36). Similarly, studies done by Steinberg (2005), Kato (2006) and Miller (2000) have demonstrated the existence of a correlation between the serum LDH level with markers for SCD severity, HbS level, leukocytosis, hemoglobin concentration and plasma arginase activity level (35, 36, 37). In 2004, Stuart (et al) found the mean serum LDH levels to be significantly higher in children with HbSS compared with those with HbSC and established that genotype is an important determinant of LDH serum level. In the same study, the level of serum LDH level in children with SCD (HbSS) was found to correlate with presence of anemia, reticulocyte count, AST and creatinine level (39).

A low serum LDH level has been reported in SCD patients on chronic transfusions and in those on continuous hydroxyurea therapy. This is attributed to the effect that hydroxyurea has on prevention of RBCs sickling through increase in HbF level (6, 38).

2.9. Management of SCD:

Approach to management of SCD is through comprehensive medical care focusing on prophylaxic measures aimed at preventing SCD related complications, parental education, psychosocial support, periodic medical assessment with monitoring of chronic organ damage and treatment of acute illness (40). Supportive treatment of SCD is directed at preventing the triggers of vaso-occlusive crises such as infections, fever, dehydration, acidosis, hypoxemia, exposure to cold, strenuous physical exercises and others. Some of the measures used are prevention of pneumococcal infections through penicillin prophylaxis, malaria prophylaxis (in endemic areas for malaria), vaccination against encapsulated bacteria such as pneumococcal vaccine as well as vaccination against transfusion transmissible infections such as hepatitis B and hepatitis A. Dietary advice and supplements such as folate, as well as physiotherapy, psychotherapy, orthopedic care, drug dependency counseling and advice on use of family planning methods   in women with SCD are   part of a comprehensive care. In centers with advanced laboratory equipment, genetic counseling is offered to those parents at risk of producing children with SCD (1, 3). SCD patients on follow up in various local health institutions are on standardized treatment of hydroxyurea therapy, for prevention of SCD complications, folate supplementation and malaria prophylaxis in keeping with the epidemiology of malaria.

There are three major therapeutic options currently available for the treatment of SCD namely blood transfusion, hydroxyurea therapy and bone marrow transplantation.

Blood transfusions are used to top up hemoglobin, for exchange transfusion, hyper transfusion or prior to surgery. A review done by Amrolia (et al) in his study on children with SCD in Philadelphia (2003), found that 50% of SCD patient had received at least one red cell transfusion in their lifetime while 5% of them will be on chronic transfusions (41). Sumba in his M. Med dissertation (2004) reported that 53% of children with SCD attending KNH had at least one transfusion in their lifetime (42).

Hydroxyurea was first synthesized in Germany in 1869 as an anticancer drug and has been used to treat myeloproliferative disorders, leukemia, melanoma and ovarian carcinoma (43). In SCD it exerts its benefits via a number of mechanisms such as activation of Hb F synthesis and arrest in

erythroid precursor cell development. The latter leads to the recruitment of earlier erythroid progenitors with a greater capacity for hemoglobin synthesis thus stimulating increase in macrocytic red blood cells less likely to sickle. Hydroxyurea has also an effect on correction of red blood cells density, improvement of blood viscosity with inhibition of HbS polymerization in erythrocytes and myelosuppression especially of neutrophils. In the intravascular space, Hydroxyurea mobilizes the nitric oxide which acts as a natural vaso-dilator resulting in improved blood flow. The most commonly observed toxicity to hydroxyurea is transient myelosuppression. At the recommended dose of 25mg/kg so far, no severe adverse effects or effects on growth in children have been reported. The factors predicting response to hydroxyurea are baseline Hb F level, baseline Hb level, compliance, maximum tolerable dose and absence of Bantu haplotype (41, 44). In SCD patient care at KNH, the prescription of hydroxyurea is limited to those patients who have had vaso-occlusive episodes requiring admission more than twice in a year. These criteria vary from centre to centre with some centers readily putting patients on hydroxyurea therapy (45).

Although bone marrow transplantation or stem cell transplantation remain the only curative therapy for SCD, stringent selection criteria allow fewer than 10% of children with SCD to undergo the procedure. The major barrier is that even those selected under such criteria have to get a matched sibling donor preferably with an identical human leukocyte antigen (HLA) (41).

The other treatments on trial are short chain fatty acids, for the modulation of Hb F level, gene therapy and administration of modifiers of oxygen affinity, membrane active drugs and drugs interfering with adherence of RBCs to the endothelial cells (44).

3. RESEARCH QUESTION

Can the level of serum lactate dehydrogenase be used as a marker for disease severity and complications in children with sickle cell disease?

4. RATIONALE

Every year in Africa 230,000 children are born with sickle cell disease to already financially burdened families. In Kenya the disease is endemic with a high prevalence in some communities. Majority of children with SCD die before the age of 15 years due to its complications. Complications are related to the hemolysis and vaso-occlusions that characterize the disease. Despite this, few local studies have been done on SCD and this is expressed by a paucity of data on SCD in Kenya.

This study will use the measurement of serum LDH levels in children with SCD aged between 1 and 13 years and attending KNH, as a marker of hemolysis to assess whether its elevation correlates with the disease severity and occurrence of complications. The obtained results will serve as a basis for recommendation on the use of serum LDH levels as a routine test in the follow up of children with SCD.

The expected outcome in the appropriate use of LDH for SCD monitoring will be a reduction in the occurrence of complications and improvement in the life expectancy of the children with SCD attending KNH. In addition, the data collected will be used to establish the reference ranges of serum LDH level in children with SCD at KNH.

5. OBJECTIVES

5.1. Broad Objective:

To characterize the patterns of serum LDH levels among children with SCD aged 1-13 years and attending KNH.

5.2. Specific Objectives:

1. Measure the total Serum LDH levels in children with SCD aged 1 to 13 years.
2. Document SCD complications in SCD patients.
3. Correlate the serum LDH levels with occurrence of SCD complications.
4. Relate serum LDH levels with Hydroxyurea therapy.

5. Correlate the total serum LDH levels with: hemoglobin level, WBC count, platelets counts and the reticulocyte count.

6. METHODOLOGY

6.1. Study design:

This was a Descriptive Cross- sectional study.

6.2. Study area:

KNH departments where children with SCD are attended to, hematology clinic, pediatric filter clinic and pediatric wards.

6.3. Study population:

Children with SCD aged 1 to 13 years and attending KNH as out-patients or admitted in the pediatric wards.

6.4. Sample size:

Calculated using the Fisher's formula;

$$n = \frac{Z^2 a/2 \times P(1-P)}{d}$$

n = 139

Where:

n = minimum number of children with SCD recruited.

$Z_{a/2}$ = standard of normal deviate at 5% level of significance (95% CI) is 1.96.

P = known prevalence of high LDH level in patients with SCD at 90%.

D = margin of error precision (5%).

6.5. Inclusion criteria:

1. Children between the age of 1 and 13 years diagnosed to have SCD on Hb electrophoresis.
2. Children with SCD attending the hematology clinic, the pediatric filter clinic or admitted in the pediatric wards at KNH.
3. Children whose parents/guardians have consented to the enrollment into the study.

6.6. Exclusion criteria:

All Children with SCD were enrolled into the study except those whose parents/ guardians declined consent for their enrollment.

6.7. Sampling methods:

SCD patients attending KNH were sequentially enrolled into the study until a study size of 145 was achieved. The higher number was to cater for those who may have insufficient samples or may not meet the criteria.

6.8. Participants recruitment:

1. Children with SCD were consecutively enrolled as they attended KNH hematology clinic, the pediatric filter clinic or were admitted in the pediatric wards.
2. A written consent was obtained from parents/ guardians by the principal investigator (Appendix I).
3. SCD diagnosis was confirmed from the Hb electrophoresis results present in the children's medical records.
4. Data on presenting complaint, expressed by the patient or the guardian; recent medical history (in the last 1year) and current medication used (hydroxyurea) was collected and entered into the study data collection form as well as the findings on physical examination and newly diagnosed complications (Appendix I).

6.9. Blood specimen collection:

1. From each participant a sample of four milliliters of venous blood was obtained and divided into 2 aliquots of 2ml in an EDTA container for full blood count, reticulocyte count and peripheral blood film while the other 2ml collected in a plain blood container was used for measurement of serum LDH level.

2. Transport to the Hematology and the clinical chemistry laboratories was done within 4 hours of sample collection to reduce the changes that occur in anticoagulated blood at ambient temperature. These changes are especially marked in leukocytes, platelets and reticulocytes.

6.10. Laboratory procedures:

1. In the Clinical Chemistry laboratory, the clotted blood in a plain container was promptly separated and the aliquots of serum obtained were stored at room temperature and serum LDH measurement performed within 24 hours.

2. Serum LDH measurement was done by a kinetic method using a two part liquid reagent supplied by the manufacturer. The LDH activity was measured utilizing Pyruvate as a substrate. The activity was determined by the rate of increase in absorbance at 340 nm using the "Olympus-640" (Chema Diagnostica) and the results obtained expressed in international Units per Liter (IU/L). The reference range in pediatric populations is 180-360 IU/L as recommended by the IFCC (38).

3. In the Hematology laboratory, preparations for blood films and reticulocytes counts were done, than the blood cell counts were done within 4 hours. The remaining samples were stored under refrigeration at 4^0 -6^0C for up to 7 days. The hemogram was done by automation using" CELL DYN 1300" (Abbot). This is a multiparameter automated hematology analyzer (Appendix IV).

6.11. Quality assurance:

1. The personnel involved in this study were phlebotomists experienced in dealing with pediatric patients, laboratory technologists and pathologists already working in the Hematology and Blood Transfusion unit.

2. Proper archiving was done.

3. Quality control material was used in all the tests performed.

4. Prompt separation of the plain blood sample was done with consideration that LDH measurement results can be affected by presence of hemolysis, use of plasma instead of serum, delayed separation of red blood cells beyond 2 days at room temperature or at 4^0C due to the leakage of LDH from the platelets.

5. Storage of the samples and reagents was at the recommended temperatures and charts were utilized to record the daily temperatures of the refrigerator.

6. All the tests were done in clinical chemistry and hematology laboratories that have an internal quality control programs and participate in external quality assessment schemes.

7. Peripheral blood films were examined by the principal investigator and checked by a supervisor before the recording of the results.

8. Each 10th reticulocyte preparation was counterchecked by a "blinded" qualified third party (senior technologist).

9. The assay procedures were done as per standard operating procedures (SOPs) established in both the hematology and the clinical chemistry laboratories respectively and observing quality assurance measures.

6.12. Ethical considerations:

1. Approval from the Kenyatta National Hospital- University of Nairobi Ethics and Research Committee (KNH-UON ERC) was first obtained to perform the study (Appendix V).

2. Prior to the recruitment of participants, an informed consent was obtained from the parent/guardian. (Appendix III).

3. Confidentiality was observed.

4. Participants whose parents/guardians refused to give the consent were not denied their usual care.

5. Results were made available to the attending physicians.

6. All care was taken to ensure patients safety and comfort.

6.13. Data management:

The data collected in the data collection form was entered into the data sheet using EXCEL, cleaned and transferred to an SPSS sheet where it was analyzed using the statistical package for social sciences (SPSS) version 17.0.

The study population was described using proportions for categorical variables and means/medians for continuous data. Chi square test was used to find out the associations between serum LDH level categories and the presence of severe disease, complications and hydroxyurea therapy. Odds ratios as estimates of risk were reported alongside the chi square tests. Total blood cell counts were compared between the two levels of total serum LDH using tests to compare the means and tests to compare medians (Mann Whitney U-test). Spearman correlation was performed to correlate the total blood cell counts and the LDH levels.

All statistical tests were performed at 5% level of significance. (Confidence interval=95% with P-value of ≤0.05 considered statistically significant). The findings were presented in percentages, tables and graphs.

7. RESULTS

A total of 145 children aged between 1 and 13years were recruited into the study from July to April 2010. Of these participants, 128 were recruited from the hematology clinic (Clinic no: 23), 11 were admitted in various pediatric wards and 6 from the pediatric filter clinic. The median age was 5 years with an inter-quartile ratio (IQR) of 3.0-9.0. The disease duration had a median of 36 months with IQR of 13-36 months while the mean age at diagnosis was 12 months. The genotype of 25 participants was HbSF while 120 had HbSS.

Gender distribution of the study participants.

Of the 145 recruited participants 83 (57.2%) were male while 62 (42.8%) were female. The female to male ratio was 0.7:1. In all the age groups, the males were more than the females.

Distribution of the participants by age-groups.

The study participants were divided in 4 age groups made up of both males and females. There were a total of 44 (30.4%) participants in the age group of 1- 3 years which was the highest. The lowest number of participants was in the age group of 7-9 years: 28 (19.3%) as demonstrated in the figure 1.

Figure 1: Distribution of participants by age and gender.
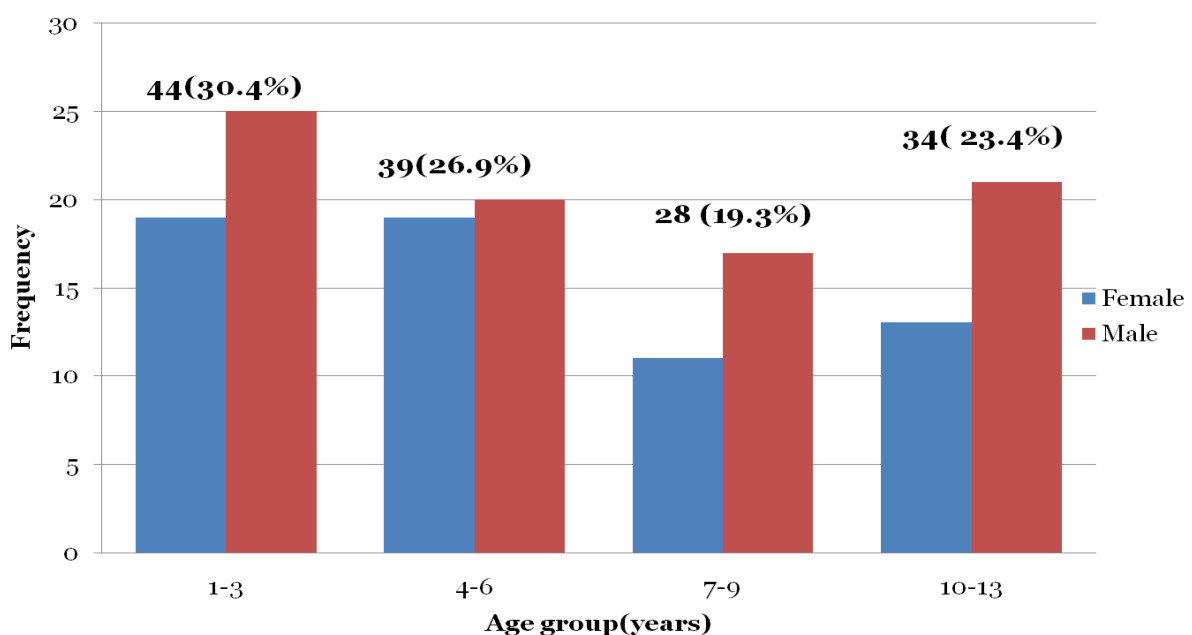
Total serum LDH values

Total serum LDH level was measured in all the participants (n=145). The mean serum LDH level was 628.8 IU/L. The lowest serum LDH value measured was 192 IU/L while the highest was 1583 IU/L. The total serum LDH reference range used in this study was the IFCC recommended for pediatric age: 180- 360 IU/L (37). Out of 145 participants 93.8 % had elevated serum LDH. The highest number of participants, 51.7% (n=75) had serum LDH levels between 361-729 IU/L while the lowest number of participants, 2.8% (n=4) had serum LDH levels between 1441-1800 IU/L. This is illustrated in the table below.

Figure 2: Total serum LDH levels (n=145).
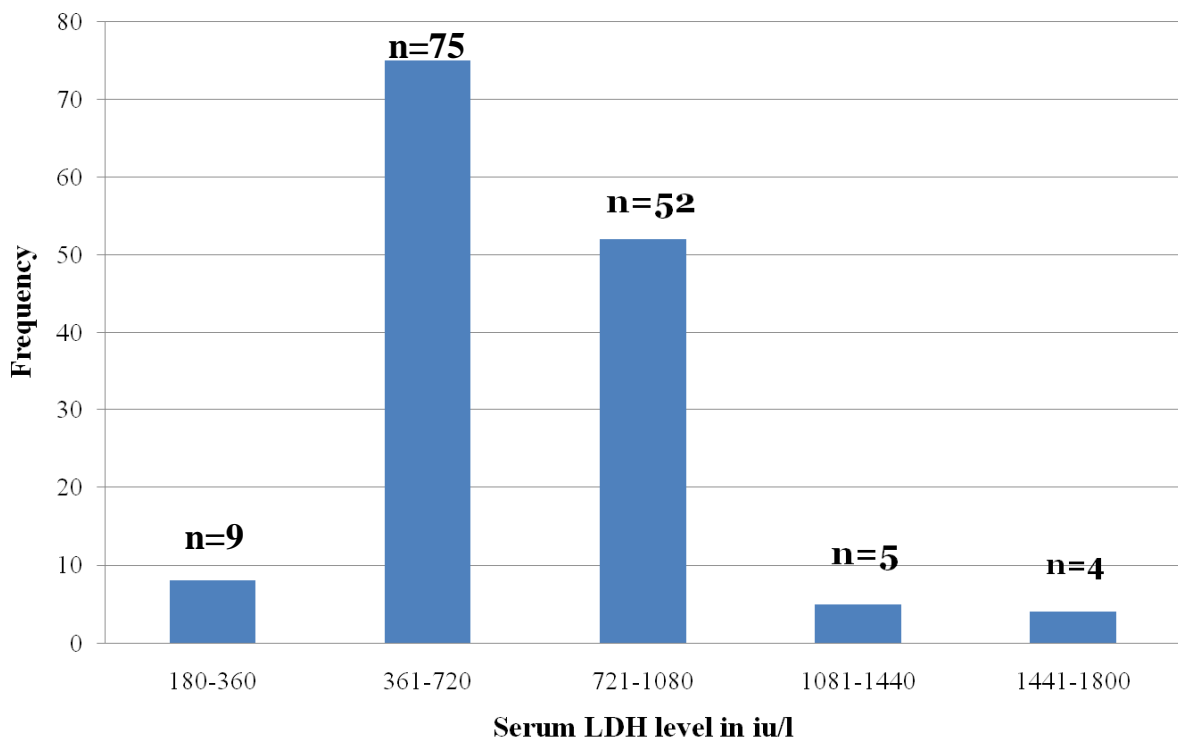
Complications diagnosed at time of presentation

At the time of presentation, various complications were diagnosed among 57 participants (39%). The diagnoses were made using data from the clinical presentation, physical examination, relevant laboratory tests and excluding any pre-existing SCD complication. The predominant complications were thrombotic events in 47.4% that were represented by painful crises, neurological complication and leg ulcers. In equal prevalence, the infections were diagnosed in 47.3% of participants while the hemolytic complications were documented in 5.3% (n=3) who had severe anemia (hemoglobin ≤5g/dl with or without signs of cardiac function decompensation) which was found only in one age group, 4-6 years. The infections were documented in all age groups with a peak in age- group 1-3years. The figure 3 shows the distribution of complications by age groups and frequency of occurrence as they are commonly described in the area of study (KNH).

Figure 3: Complications, frequency by age groups.
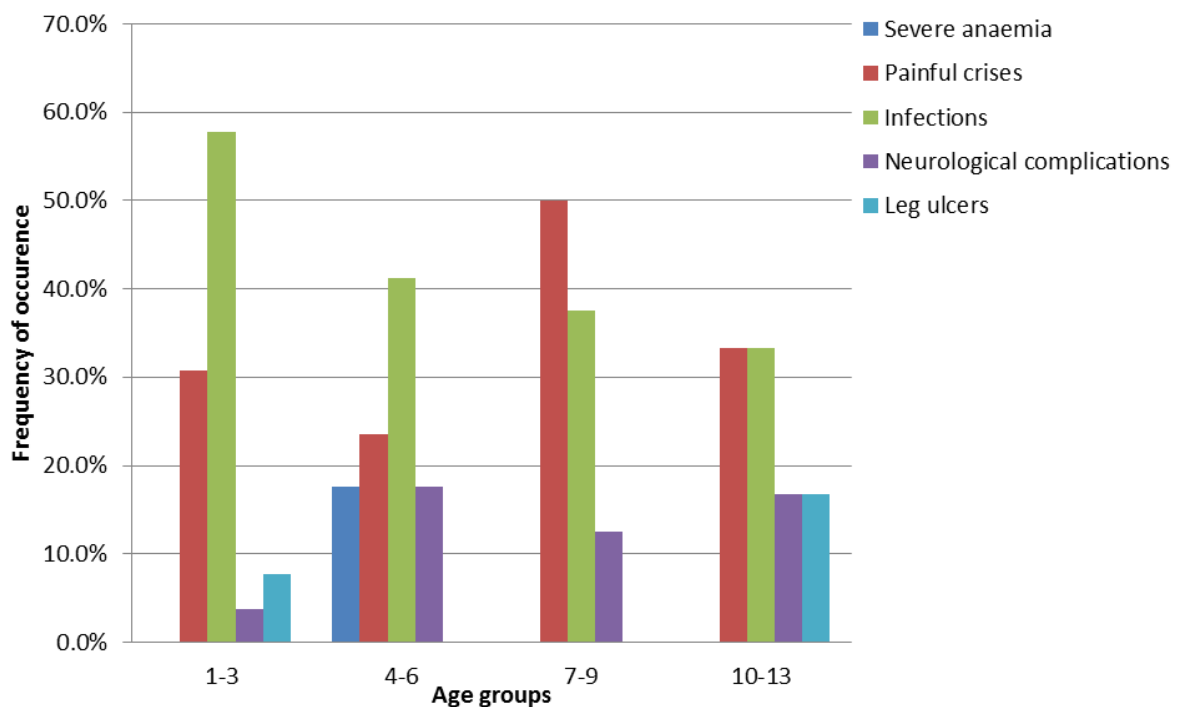
Distribution of total serum LDH level by age groups.

Out of the 145 recruited participants, the lowest median serum LDH was: 561 IU/L. The highest serum LDH value was recorded in age group 7-9 years with a median of 698 IU/L while the lowest was in the age group 1-3 years: 561 IU/L. This is illustrated in the figure 4 below.

Figure 4: Serum LDH levels by age groups.
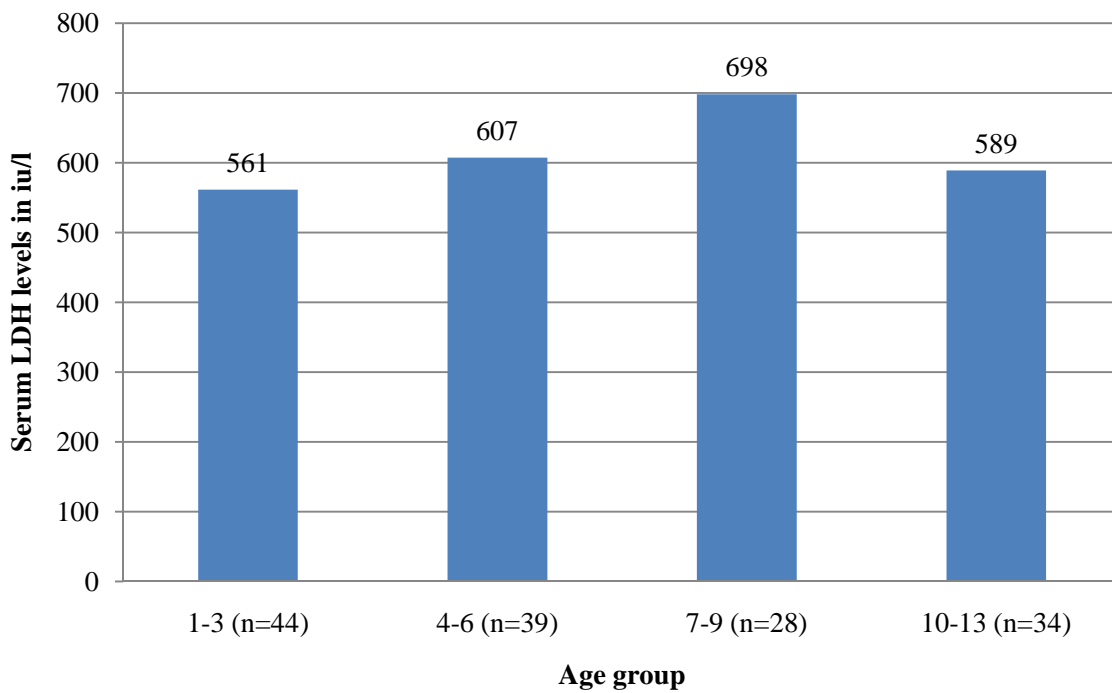
Total serum LDH levels by age.

The next graph shows individual distribution of the serum LDH levels by age. (r = 0.149 and a P-value of 0.075.) There was trend in the distribution of serum LDH by age as shown in the graph below.

Figure 5: Individual serum LDH values by age.
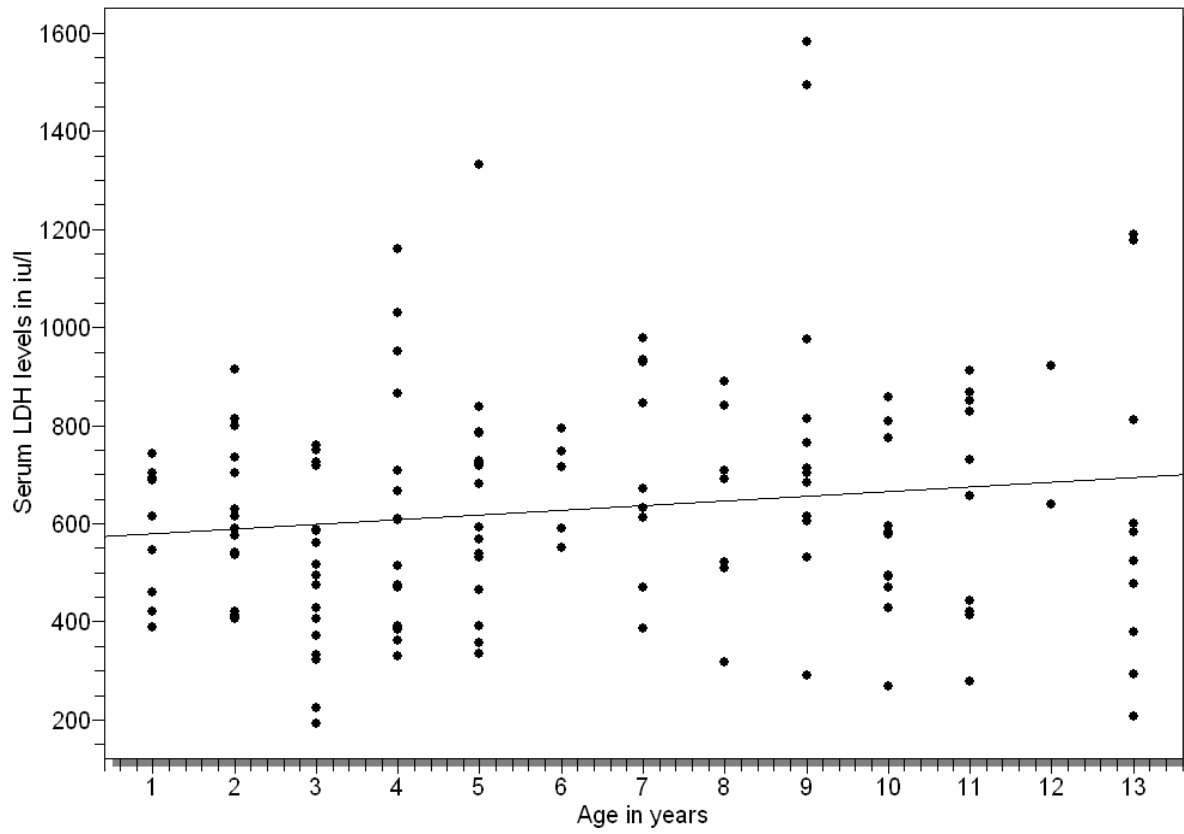
Complaints at the time of presentation:

The presenting complaints were reported by the older participants themselves or their parents or guardians in 39.4% (57) cases. The major complaints reported were cough and fever. These were categorized into 3 groups according to severity. Mild, where treatment may not be needed and the participant, parent or guardian was reassured and included cases such as running nose, dry cough without fever. Moderate, where the condition was more severe but could be managed at home with treatment; and severe, where urgent admission for in-patient care was needed.

The serum LDH median amongst the children who presented with complaints was 600 IU/L (IQR: 256.1) while those who did not report any complaint had a median of 659 IU/L (IQR: 261.4). The P-value was: 0.183 (statistically not significant).

The table 1 shows the various complaints reported.

Correlation between the complaints severity and the serum LDH level: the median of serum LDH was 591 IU/L in children who presented with complaint of mild severity, 610 IU/L in those with moderate severity complaints and 524 in those who reported severe complaints.

Table 1: Complaints at time of presentation

Complaints	Nature of complaints	N:	Serum LDH level (IU/L)
Attributed to SCD.	-Pain: joints and bones.	16	725
	-Body weakness and malaise.	8	563
	-Convulsions.	2	587
Related to infections.	-Cough and fever.	19	549
	-Abdominal discomfort: nausea, diarrhea and vomiting.	7	572
	-Dysuria.	2	460
Others.	-Skin lesions.	2	599
	-Injuries.	1	509
Total.		**57**	

Admission in the last 1 year and transfusion status.

In the last 1 year, 70 (48.2%) out of the 145 participants have been admitted with various diagnoses. These were SCD related conditions and others not related to SCD. The most common diagnosis was severe anemia diagnosed in 20 children (13.7%), followed by painful crises and others as shown in the table 2 below.

Of the admitted children (n=70), 36 (24.6%) were transfused with the majority of them (n=33, 22.7%) receiving 1 unit of packed red blood cells each, while the rest received each 2, 3 and 4 units respectively.

Table 2: Reasons for admission in the last 1 year.

Related to the SCD crises:	N:	Serum LDH level: IU/L
-Severe anemia.	20	570
-Painful crises.	16	624
-Hemolytic crises.	2	761
-Cerebro- vascular accidents.	2	530
-Hemiparesis.	1	357
-Acute chest syndrome.	1	615
Not related to SCD crises:		
-Respiratory tract infections.	6	549
-Trauma.	6	536
-Gastro-enteritis.	4	689
-Febrile illness.	4	725
-Malaria.	4	621
-Urinary tract infections.	4	587
Total:	**70**	

Reticulocyte counts in participants.

The reticulocyte count was done in 142 participants as 3 samples were rejected due to inadequacy for evaluation. The counts were expressed in both absolute counts ($x10^9$/L) and percentage. The median was 285.5 x 10^9 /L. (IQR= 196-419 x 10^9/L). 10 participants (7%) had counts within the reference range by age as published by Dacie and Lewis (50-100x10^9/L) (46). The rest of the participants 130 (93%) had elevated reticulocyte counts.

Blood cell counts.

The most commonly encountered abnormalities were: leukocytosis recorded among 59% of participants with a mean of 15.3 x 10^9/l (SD: 4.8) and low hemoglobin level among 97% with a mean Hb of 7.7g/l (SD: 1.6). These values are summarized in the table 3.

Table 3: Blood cells counts in children with SCD.

Parameters	Mean(SD)/ Median(IQR)	Low N (%)	Within reference ranges N (%)	High N (%)
WBC (x10^9/l)	15.3 (4.8)	2 (1)	57 (40)	85 (59)
Hemoglobin (g/dl)	7.7 (2.4- 3.3)	140 (97)	4 (3)	0
Platelets	347 (112)	8 (6)	112 (80)	20 (14)
Differential WBC (x10^9)				
Neutrophils	6.2 (2.7)	4 (3)	103 (73)	34 (24)
Lymphocytes	7.5 (5)	0	106 (75)	35 (25)
Monocytes	0.6 (0.3- 1.1)	8 (6)	94 (66)	39 (28)
Eosinophils	0.3 (0.1- 0.5)	19 (14)	114 (80)	8 (6)
Basophils	0	0	134 (95)	7 (5)

Status of hydroxyurea therapy.

Sixty eight (47%) of the recruited participants were on hydroxyurea therapy at different doses. Out of these, 50 (73%) were on a dose of 500mg on alternate days while 18 (27%) were on 500mg once a day. From the findings in this study, hydroxyurea therapy was initiated from the age of 4-6 years at a dose of 500 mg on alternate days though smaller number of participants in this age group was on 500 mg once a day. A similar tendency is noted in the next age-group of 7-9 years. In the older age-group of 10-13 years, there is a similar number of children on hydroxyurea 500mg on alternate days and on 500mg once a day. This is illustrated in the figure 6.

Figure 6: Hyydroxyurea doses by age groups.
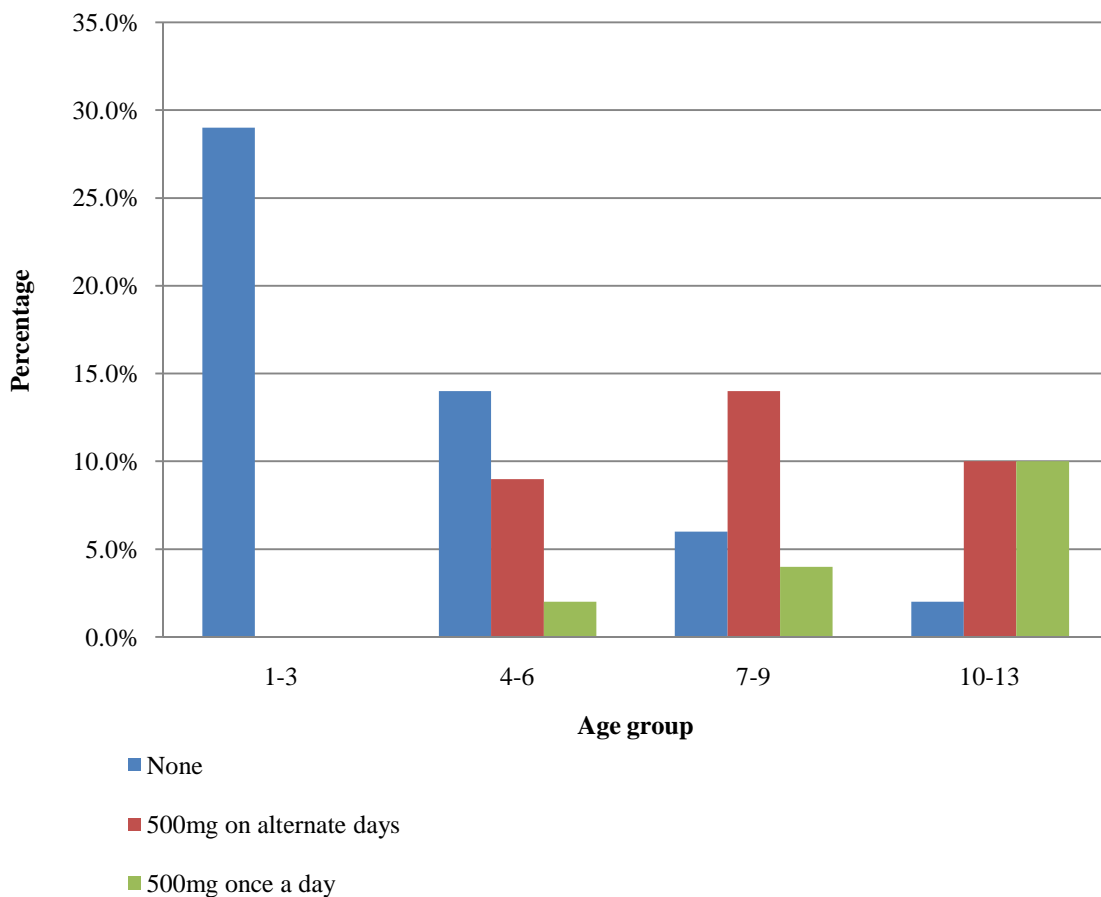
Correlation of LDH level with the presence of complications.

Correlation of serum LDH by gender shows that male participants were more than female likely to have a high serum LDH, on correlation by presence of complications, the serum LDH level was high among the participants with complications as well as those where no complication was diagnosed. Similarly, on comparison of serum LDH levels by genotypes, those participants with HbSS and HbFS had elevated serum LDH values. Gender was an important factor in serum LDH elevation as males participants were more likely to have a high serum LDH level with a statistically significant P-value of 0.025.

Table 4: Correlation between the total LDH levels with gender and presence of complications.

Serum LDH levels with gender	Females	Males	P-value
Serum LDH levels Mean(SD)	577.7 (194.5)	667.5 (258.0)	0.025
Serum LDH levels with complications	**Yes**	**No**	**P-value**
Serum LDH levels Mean (SD)	600.0 (256.1)	647.7 (221.9)	0.238

Correlation between the serum total LDH level and hydroxyurea therapy.

Table 5 shows a summary of the correlations of serum total LDH levels compared in groups on different doses which had a P-value of 0.466. A comparison was done between the groups with a high serum total LDH levels at different doses of hydroxyurea and the obtained P-value was 0.597 (both P-values were not statistically significant).

The participants who were on a dose of 500mg of hydroxyurea on alternate days had a mean serum LDH level of 695 IU/l (± 242: SD), while the ones who were on hydroxyurea 500mg once a day had a mean serum LDH level of 669 IU/L (± 258: SD). On comparing the two groups, the P-value was 0.720.

The table below demonstrates the correlation between the groups on various doses of hydroxyurea among the participants who had elevated serum LDH levels.

Table 5: Correlation between the serum LDH levels with hydroxyurea therapy.

	500 mg on alternate days.	500 mg once a day.	P-value
Mean(SD)	672 (256) IU/L	618 (280) IU/L	0.466
High LDH	46 (93.9%)	15 (88.2%)	0.597

Correlation between the Serum LDH levels and reticulocytes count.

 Absolute reticulocyte counts were recorded in different age groups. In the age group of 1-3 years, all participants had reticulocytosis (>100 x 10^9/l : APPENDIX I) with a group mean of 334.1 x 10^9/l while in the age group of 7-9 there were more participants with reticulocytosis compared to the ones who had counts within reference range. The group mean in this age group was 265.2 x 10^9/l. In the other age groups (4-6 and 10-13 years), more participants had counts within the reference range.

The correlation between the reticulocyte counts and the total serum LDH levels is demonstrated in figure 7 below. The Pearson correlation coefficient was: - 0.048 while the P-value was 0.611. Both were not statistically significant.

Figure 7: Reticulocyte counts correlated with serum LDH levels.
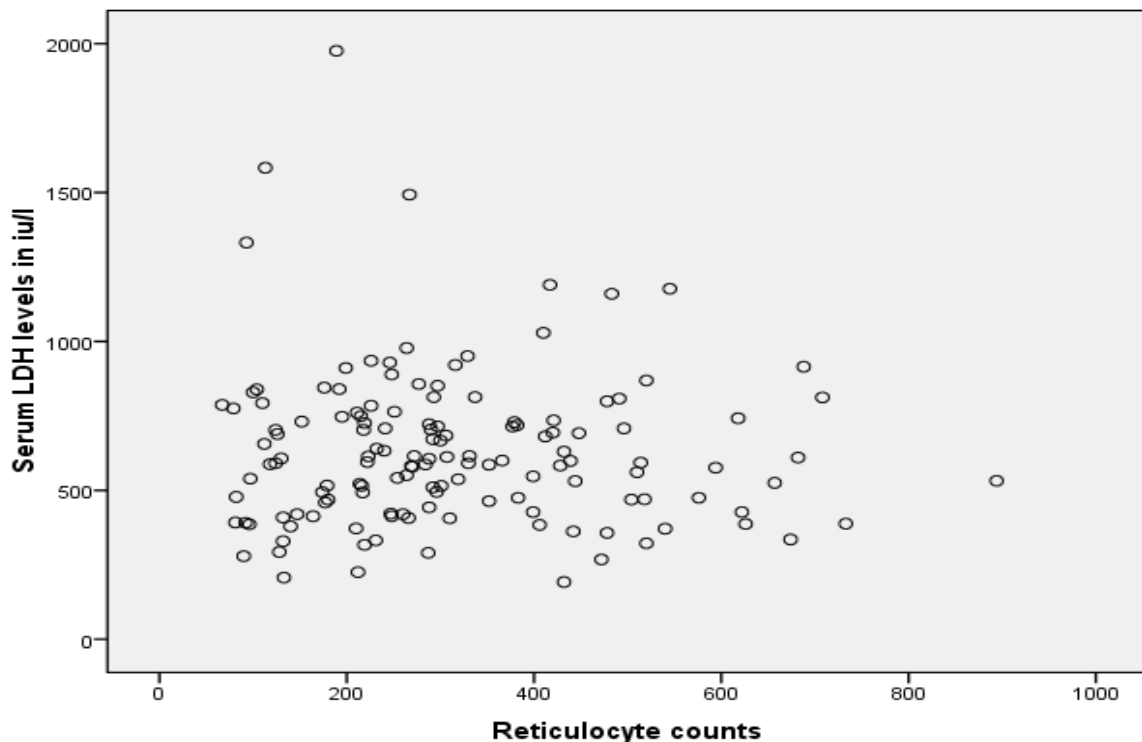
Correlation between the total serum LDH levels with blood cell counts.

The table 6 summarizes the correlation between the blood cell counts and the total serum LDH levels. This was done using the Spearman correlation. A significant correlation between the high total serum LDH levels and the low hemoglobin levels was demonstrated with a P-value of 0.014.

Table 6: Correlation between laboratory results with the serum LDH levels.

Parameters	Spearman correlation	P-value
WBC	0.70	0.402
Hemoglobin	-0.204	**0.014**
Platelets	-0.133	0.118
Neutrophils	0.135	0.110
Lymphocytes	0.017	0.840
Monocytes	0.077	0.366
Eosinophils	0.097	0.254
Basophils	0.070	0.410

8. DISCUSSION

The diagnosis of SCD in Africa is usually made in the first year of life. This corresponds to the occurrence of the first SCD complication (10). In this study, the mean age at diagnosis was 12 months and in most children it coincided with their first admission. The lack of awareness about the SCD in both the general population and the primary medical care providers plays a role in the late disease diagnosis. There are no screening programs in most developing countries and SCD diagnosis at birth remains rare. Children with SCD start experiencing symptoms in their first year of life, but even the presence of these symptoms fails to raise the index of suspicion among the primary medical providers whose attention is more directed towards infectious diseases rather than hemoglobinopathies. This is due to several factors like the non-specificity of SCD symptoms, lack of diagnostic facilities combined with poor evaluation of blood picture in most of the health facilities, long distance between these facilities and the population settlements and the poor socio-economic status of the majority of the population.

In this study, 145 participants were recruited. All were homozygous for SCD and aged 1 to 13 years. The mean age at diagnosis was 12 months. This finding compares to that of Diallo et al. who found that in Africa, SCD is diagnosed in the first year of life and this coincides with the occurrence of the first SCD crisis (10). Participants had a median age of 5years with a ratio of male to female: 0.7:1. On distribution by age groups, there were two peaks: in age group 1-3years (30.4%) and age 10-13 years (23.4%). A notable drop in number of participants in age group of 4-9 years may not be related to SCD. It could be related to other causes of mortality in children in that age group that prevail in this area and population that has long been documented to be associated with diseases such as bacterial, viral and parasitic infections as well as malnutrition (10).

In the last one year, 70 (48.3%) participants had been admitted with various diagnoses. The most common diagnosis directly related to the sickle cell crises was severe anemia which was defined as hemoglobin below 5g/l with or without signs of cardiac function decompensation and

necessitating urgent medical intervention; this was diagnosed in 13.7% of participants (n=20) while those admitted due to various infections were 19.3% (n=28).

On the status of transfusion, in the last 1 year, 36 (24.8%) participants had been transfused at least one unit of blood. This high figure for frequency of transfusions is related to the fact the major indication for transfusion in SCD is severe anemia and in some cases hyper transfusion therapy is used for prevention of sickle cell complications such as stroke. (3, 8, 44).

The mean serum LDH level in this study was 628.8 IU/L (IFCC reference range for children: 180- 360 IU/L) (38). This value correlates with the findings of O'Driscoll who compared the serum LDH levels between children with Hb SS and Hb SC and found the mean serum LDH levels to be 581 IU/L among the children with the homozygous form of SCD (Hb SS) (6). In a Nigerian study, by Aliyu et al, the mean serum LDH level was 441 IU/L (47). In this study, the serum LDH levels was predominantly high in the age group of 7 to 9 years with a median LDH level value of 698 IU/L, no reason was found to explain this high value of serum LDH at this particular age. The serum LDH levels were further classified for ease of comparison, using doubling values with a baseline as the reference range recommended by the IFCC. Only 6.2% of the participants had serum LDH values within range, with the rest having elevated serum LDH levels where there was a peak of 51.7% children who had serum LDH values between 361-720 IU/L.

At the time of presentation, various complaints were reported in 57 (39.4%) participants. The most common complaint reported was cough and fever in 19 (13%) participants. By the intensity of these complaints participants were divided in 3 groups. The 1st group had mild complaints; the 2nd group had moderately severe complaints while the 3rd group was made up of those participants with severe complaints. The mean serum LDH level was high in all these 3 groups and no trend was found between the elevation of serum LDH levels with the presence or absence of complaints. The correlation between the serum LDH levels among the participants without complaints and those with complaints was not statistically significant (P=0.183).

SCD related complications were documented in 39% (n=57) participants at the time of presentation. Infections were the most commonly diagnosed complications in 47.3% while the least encountered complication was skin ulcers in 5.3% of participants. This predominance of infections has already been reported in other studies done in Africa by Diallo et al (10). In SCD the immune system is compromised by a defective alternate pathway of the complement system and the presence of hyposplenism or asplenia (3). In addition, the "Bantu haplotype" is the most prevalent in this region and is characterized by a severe expression of SCD due to low expression of Hb F by the patients and this could explain the high prevalence of thrombotic events (3, 10, 48). The presence of complications did not seem to correlate with the high serum LDH level and the P-value was 0.931.

Hydroxyurea therapy has revolutionalized the management of SCD since its introduction but this is not without barriers as it is associated with side effects such as myelosuppression which can have devastating consequences, hence the reservations about its use. In this study 66 participants (43.4%) were on various doses of hydroxyurea. From this study findings, the hydroxyurea treatment was initiated at age 4-6 years. As the age advanced there was a notable increase of participants on hydroxyurea, though the increase in dose prescribed did not seem to be adjusted as per guidelines. Of note was a tendency of poor adherence mostly due to unavailability of the drug or socioeconomic reasons, thus no conclusive decision can be made on correlation between the total serum LDH levels and hydroxyurea therapy.

Other markers of hemolysis that were measured in this study were the reticulocyte count which had a mean value of 11.2% or 312 x 10^9/l. This correlated with the findings of other studies done in Nigeria (2008), where a mean reticulocyte count among SCD patients was found to be 11.1% (44), in Kilifi (2009), Makani et al. published a mean reticulocyte count of 11.5% (12) while at KNH (2009), Odero in his M.Med dissertation found a mean count of 11.1% (24). Similarly, O'Driscoll et al. found an average reticulocyte count of 305 x 10^9/l (6). The serum LDH level was elevated in 136 (90.3% with n=145) participants while the high reticulocytes was found in 130 (92.8% with n=140). There was no linear correlation, the Pearson coefficient correlation was: -0.048 with a P-value of: 0.611. Both, the serum LDH and the reticulocyte count were used

in this study as markers of hemolysis and were found to be elevated in the majority of participants. This can partly be explained by the fact that both markers are not specific for hemolysis: reticulocytosis acts as a marker of bone marrow response to hemolysis, while the total serum LDH that was used in this study can be elevated in diseases affecting various organs. These are organs like the heart, lungs, kidneys, liver and striated muscles. Already in SCD these organs variably affected by the disease manifestation or complications (8, 24, 36)

The other findings demonstrated on blood cell counts were leukocytosis in 85 participants (59%) and anemia in 140 participants (97%). These findings correlate with the findings of the studies done in Nigeria that found a mean WBC of 13.2 x 10^9/l and a mean hemoglobin level of 8.5 g/l (44). In Kilifi, Makani et al. reported a mean WBC count of 19.2 x 10^9/l and mean hemoglobin level of 7.3 g/l (12). At KHH, the mean WBC count was 15.6 x 10^9/l with mean hemoglobin of 8.0 g/l (24). These values are comparable with the ones of O'Driscoll et al. who found mean hemoglobin of 7.8 g/l (6). All these studies reported platelets counts within reference range: 150-450 x 10^9/l in this study the mean platelet count was 347 x 10^9/l. On correlation with the serum LDH level, a significant correlation was found with the hemoglobin. This was an inverse correlation that translated into a P-value of 0.014, thus demonstrating that the elevation of serum LDH in SCD is likely to be associated with a low level of hemoglobin (anemia). These findings were similar to the ones documented by Burns et al. (34).

Another interesting finding was the relationship between gender and high serum LDH level where male participants were more likely to have an elevated serum LDH level than the female participants. This correlation was demonstrated by a P-value of 0.025. This could be related to the findings of Dover et al. who reported that female SCD patients have a higher number of RBC with HbF and their RBCs less likely to sickle compared to their male counterparts and were less likely to get sickling of RBCs that is the basis of vaso-occlusion and hemolysis. This makes the female SCD less likely prone to SCD complications (27).

Serum LDH levels as a marker of hemolysis in SCD reflects a combinational impact of at least ten components: intravascular hemolysis, ineffective erythropoiesis and extra vascular hemolysis among others observed in SCD. These components are characterized by inter-individual variability that has not been defined for SCD. From this observation, Hebbel in his study

recommended the use of serum LDH level in SCD as a longitudinal marker for a single patient rather than as a discriminator between patients (49). The design and size of this study is a limitation as some parameters cannot be discussed conclusively.

Serum LDH level is a reliable marker of hemolysis. In this study, it did not correlate with the reticulocyte count that was used as a surrogate marker of hemolysis. From the findings of this study, the use of serum LDH levels in predicting for SCD severity or occurrence of complications is not recommended. This could be explained by the presence of co morbidities affecting various organs and tissues among SCD patients leading to an elevated total serum LDH level not necessary of a hematological origin. However, a high serum LDH was found to be a predictor of severe anemia.

9. CONCLUSIONS

1. The mean serum LDH level among children with SCD attending KNH and aged 1- 13 years is 628.8 IU/L.
2. Thrombotic events as well as infections were the most common SCD related complications diagnosed in the course of this study and these were documented in 24 participants (47.4%).
3. A significant correlation between the elevated serum LDH level and the low hemoglobin level was demonstrated by the findings of this study.
4. No relationship was established between the elevation of serum LDH levels with the presence of complaints or complications except in case of severe anemia.
5. Elevated serum total LDH levels significantly correlate with gender with higher values found in males than in females.
6. There was no statistically significant correlation between the serum LDH levels and the leukocyte count, the platelets count or the reticulocyte count.
7. This study did not establish a correlation between total serum LDH levels and hydroxyurea therapy.

10. STUDY RECOMMENDATIONS

1. From the findings of this study, it is recommended that future studies should use the LDH iso-enzymes instead of the total serum LDH level as this will confirm that the serum LDH level elevation in children with SCD is of hematological origin.

2. Further studies to look into the role of gender in elevation of serum LDH levels in the studied population are encouraged.

11. STUDY LIMITATIONS

1. Locally generated data on reference ranges of hematological parameters are not available for comparison due to limited resources.

2. Some of the required data on past laboratory results and blood transfusions could not be authenticated as it was missing from the patients records at KNH.

12. REFERENCES

1. Okpala, E., Thomas, V., Westerdale, N., *et al*. The comprehensive care of sickle cell disease. *Eur J Haematol*. 2002; 68 (3): 157-162.

2. World health Organization (2006). *Report by the Secretariat of the Fifty-ninth World Health Assembly*. A 50 / 9.

3. Green, J. P., Foerster, J., Rodgers, G. M., *et al*. Winthrobe's clinical hematology. Lippincott Williams and Wilkins. 2006: 12th edition. Vol1: 1038-1082.

4. Steinberg, M. H. Pathophysiology of sickle cell disease. *Bailliere's Clin Haematol*. 1998; 11(1): 163-184.

5. Vilpo, J. A., Talvensaari, K. K., Mortensen, E., *et al*. Hemolysis: Which laboratory investigations and when? *Scand. J. Clin. Lab. Invest. Suppl*. 1990; 200: 10-19.

6. O'Driscoll, S., Height, S. E., Dick, M. C., *et al*. Serum lactate dehydrogenase as a biomarker in children with sickle cell disease. *Br. J. Haematol*. 2007; 140: 206-209.

7. Conran, N., Franco-Penteado, C. F., Costa, F.F., *et al*. Newer aspects of the pathophysiology of sickle cell Vaso - occlusion. *Hemoglobin*. 2009; 33 (1): 1-16.

8. Rodgers, P. G. and Young, N. S. Bethesda. Handbook of clinical hematology. Lippincott Williams and Wilkins. 2005; 1st edition: 36-60.

9. Desai, D. V. and Dhani, H. Sickle cell disease: history and origin. *The internet journal of hematol*. 2004; 1 (2): 1540-2649.

10. Diallo, D. and Tchernia, G. L. Sickle cell disease in Africa. *Curr. Opin. Hematol*. 2002; 9111-9116.

11. Foy, H., Kondi, A., Timm, G. L., *et al*. Sickle cell rates in Kenya and the Southern Sudan. *Br. Med. J*. 1954; 1 (4857): 294-297.

12. Makani, J., Kirkham, F. J., Komba, A., *et al*. Risk factors for high cerebral blood flow velocity and death in Kenyan children with sickle cell anemia: role of haemoglobin oxygen saturation and febrile illness. *Br. J. haematol*. 2009; 145: 529-532.

13. Thomsen, T. W. and Setnik, G.S. Elderly survivors with homozygous sickle cell disease. *N. Engl. J. Med*. 2007; 3566(6): 1-2.

14. Frenette, P. S. Sickle cell vaso - occlusion: multistep and multicellular paradigm. *Curr. Opin, Hematol*. 2002; 9100-9106.

15. Okpala, I. Leukocytes adhesion and the pathophysiology of sickle cell disease. *Curr. Opin, Hematol.* 2006; 13 (1): 40-44.

16. Gill, B. F. M., Sleeper, L.A., Weiner, S. J., *et al.* Events in the first decade in a cohort of infants with sickle cell disease. *Blood.* 1995; 86: 776-783.

17. Babhulkar, S. S., Ketan Pande and Babhulkar Sushrut. The hand-foot syndrome in sickle-cell haemoglobinopathy. *J Bone Joint Surg (Br).* 1995; 77-B: 310-312.

18. Donald R. Harkness. Hematological and clinical features of sickle cell disease. *Rev. Hematol.* 1980; 4 (3&4): 313-334.

19. Antonio Almeida and Irene Roberts. Bone involvement in sickle cell disease. *Br J Haematol.* 2005; 129 (4): 482-490.

20. Makani, J., Williams, T. N. and Marsh, K. Sickle cell disease in Africa: burden and research priorities. *Ann. Trop. Med & Parasitol.* 2007; 101 (1): 3-14.

21. Fosdal, M. B. and Wojner- Alexandrov, A. W. Events of hospitalization among children with sickle cell disease. *J. Paed. Nursing.* 2007; 22 (4): 342-346.

22. Radaelli, F., Calori, R., Faccini, P., *et al.* Early cutaneous lesions secondary to hydroxyurea therapy. *Am. J. Hematol.* 1998; 83: 82-83.

23. Smith, J. A., Espeland, M., Bellevue, R., *et al.* Pregnancy in sickle cell disease. *Obstet. Gynecol.* 1996; 87 (2): 199-204.

24. Odero, N. Agai. The prevalence of pulmonary hypertension in children with sickle cell disease at Kenyatta National Hospital. Masters of Medicine Dissertation (Paediatrics). 2009.

25. Raghupathy, R., Manwani, D., Little, J. A., *et al.* Iron overload in sickle cell disease. *Adv. Hematol.* 2010; Article 1D, 272940.

26. Gabriel, A. and Przybylski, J. Sickle-cell anemia: A look at global haplotype distribution. *Nat. Ed.* 2010; 3 (3): 2-5.

27. Dover, G. J., Smith, K. D., Chang, Y. C., *et al.* Fetal hemoglobin level in sickle cell disease and normal individuals are partially compiled by an X-linked gene located at Xp 22.2. *Blood.* 1992; 80 (3): 816-824.

28. Rees, D. C., Williams, T. N., Gladwin, M.T., *et al.* sickle - cell disease. *Lancet.* 2010; 376: 2018-2041.

29. Weatherall, D. J. and Clegg, J. B. Inherited hemoglobin disorders: an increasing global health problem. *Bulletin of the World Health Organization.* 2001, 79 (8).

30. Hoffbrand, A. V., Catovsky, d. and Tuddenham, E. G. D. Postgraduate haematology. Blackwell publishing. 5th Edition. 2005; 104-118.

31. Ojwang, P. J., Ogada, T., Beris, P., *et al*. Haplotypes and globin genes analyses in sickle cell anemia patients from Kenya. *Br J Haematol.* 1987; 65: 211-215.

32. Barbara J. Bain. Hemoglobinopathy diagnosis. Blackwell science. 2nd edition. 2001; 113-154.

33. Harkness, D. R. Hematological and clinical features of sickle cell disease: A review. *Hemoglobin*. 1980; 4 (3&4): 313-334.

34. Burns, E. R., Kale, A., Murthy, V. V., *et al*. Diagnosis of the hemolytic state using serum levels of erythrocyte adenylate kinase. *J. Clin. Lab. Analysis*. 2000; 183: 180-183.

35. Steinberg, M. H. Predicting clinical severity in sickle cell anemia. *Br. J. Haematol*. 2005; 129 (4): 465-481.

36. Kato, M. G., Machado, R. F., Little, J. L., *et al*. Lactate dehydrogenase as a biomarker of hemolysis associated nitric oxide resistance, priapism, leg ulceration, pulmonary hypertension and death in patients with sickle cell disease. *Blood*. 2006; 107: 2279-2285.

37. Miller, S. T., Sleeper, L.A., Pegelow, C. H., *et al*. Prediction of adverse outcomes in children with sickle cell disease. *N. Engl. J. Med.* 2000; 42 (2): 83-89.

38. Burtis, A.C., Ashwood, E.R. and Bruns, D. E. TIETZ Fundamentals of clinical chemistry. Elsevier 2008; 6th edition: 321-322.

39. Stuart, M. J. and Nagel, R. L. Sickle cell disease. *Lancet*. 2004; 364: 1343-1360.

40. Lane, P. A., Buchanan, G. R., Hutter, J. J., *et al*. Sickle cell disease in children and adolescents: diagnosis, guidelines for comprehensive care, care paths and protocols for management of acute and chronic complications. *Annual meeting of the sickle cell disease care consortium, Sedona, AZ.* November 10-12, 2001.

41. Amrolia, P.J., Almeida, A., Halsey, C., *et al*. Therapeutic challenges in childhood sickle cell disease. Part I: Current and future treatment options. *Br. J. Haematol*. 2003; 120: 725 -736.

42. Sumba, O. J. Correlation between serum ferritin and transfusion and other variables in children with sickle cell disease. Masters of medicine dissertation (Human pathology). 2004.

43. Brawley, O. W., Cornellus, L. J., Edwards, L. R., *et al*. National institute of health consensus development conference statement: Hydroxyurea treatment for sickle cell disease. *Ann. Intern. Med*. 2008; 148: 932-938.

44. Strousse, J. J., Lanzkron, S., Beach, M. C., *et al.* Hydroxyurea for sickle cell disease: A systematic review for efficacy and toxicity in children. *Pediatrics*. 2008; 122: 1332-1342.

45. Russell E. Ware., How I use hydroxyurea to treat young patients with sickle cell anemia. *Blood*. 2010; 115 (26): 5300- 5311.

46. Lewis, S. M., Bain, B. J. and Bates, I. Dacie and Lewis practical haematology. Elsevier 2006; 10th edition: 11-24.

47. Aliyu, Z., Gordeuk, V., Sachdev, V., *et al*. Prevalence and risk factors for pulmonary artery systolic hypertension among sickle cell disease patients in Nigeria. *Am. J. Hematol*. 2008; 83: 485-490.

48. Platt, O. S., Brambilia, D, J., Rosse, W. F., *et al*. Mortality in sickle cell disease. Life expectancy and risk factors for early death. *New Engl J Med*. 1994; 330: 1639-1644.

49. Hebbel, J. R., Reconstructing sickle cell disease: A data-based analysis of the "hyperhemolysis paradigm" for pulmonary hypertension from the perspective of evidence - based medicine. *Am. J. Hematol.* 2011; 86: 123-154.

13. APPENDICES

APPENDIX I: DATA SHEET AND QUESTIONNAIRE.

SERUM LACTATE DEHYDROGENASE LEVEL IN CHILDREN WITH SICKLE CELL DISEASE AT K.N.H.

Date:

BIODATA:

- Study number:

-IP number:

-Name:

-Age (years):

-Sex: 1. [   ] F

             2.   M [   ]

MEDICAL HISTORY:

1-Presenting complaint:

Complaint:	Intensity:		
	Mild	Moderate	Severe
-Pain:			
-Fever:			
-Cough:			
-Others (specify):			

2-Admission in the last 1 year: Yes   No

    Diagnosis:

    Number of admissions:

3-Transfusion in last 1 year: Yes   No

        Number:

4-Hydroxyurea treatment: Yes   No

            (Continuous use for 6 months) Dose (mg):

5-Duration of SCD since diagnosis: <1 year   >1 year (number of years)

                Months   Years

6-Diagnosis:

7-Complications (Newly diagnosed):

LABORATORY RESULTS:

1-Haemogram:

Parameters:	Participants results:	Units:	Reference ranges*:
1. Hemoglobin level	……………………………..	g/l	110 - 155
2. Red blood cells	……………………………..	$X10^{12}$/l	4.0 - 5.2
3. **White blood cells** (Total count)	……………………………..	$X10^{9}$/l	5 - 15
Differential count (WBC) :			
-Neutrophils:	……………………………..	$X10^{9}$/l	1.5- 8
-Lymphocytes:	……………………………..	$X10^{9}$/l	1 - 9
-Monocytes:	……………………………..	$X10^{9}$/l	0.2 - 1
-Eosinophils:	……………………………..	$X10^{9}$/l	0.1 - 1
-Basophils:	……………………………..	$X10^{9}$/l	0.02 - 0.1
4. Platelets	……………………………..	$X10^{9}$/l	170 - 490

*Reference ranges established by Dacie and Lewis (46).

2-Reticulocytes:

Participant results:	Units	Reference range:
……………………………	%	0.5- 2.5
……………………………	$x10^9/l$	30- 100

3-PBF (peripheral blood film):

Erythrocyte:

Leucocytes:

Platelets:

Others:

4-Serum total LDH level (IU/L):

APPENDIX II: CONSENT FORM

INTRODUCTION TO THE STUDY:

SERUM LACTATE DEHYDROGENASE LEVEL IN CHILDREN WITH SICKLE CELL DISEASE AT KENYATTA NATIONAL HOSPITAL.

Introduction

I am DR Germaine Serubuga Makory, currently a Masters student in Human Pathology at the University of Nairobi and conducting a study on use of serum lactate dehydrogenase (LDH) level in Children with Sickle Cell disease at Kenyatta National hospital (0-13 years).

Purpose

In the current study, a research will be done in order to determine the correlation of the serum LDH level with the severity of the disease and occurrence of the complications in SCD.

Description of the study

A dully signed informed consent from the parents/guardians to the enrollment of their dependents will be first obtained by the principal investigator. These will be until a study size of 139 children with SCD attending KNH and aged 1- 13 years will be achieved. A short questionnaire will be administered by the investigator and any other necessary information shall be drawn from the participant's medical records. From each participant, samples for the tests of serum LDH level as well as the total blood count and reticulocyte count shall be obtained. The results of these tests shall be used to establish a correlation between the serum LDH level, the disease severity and the occurrence of SCD complications.

Procedures

Using a sterile needle and syringe, a total of 4milliliters of blood shall be collected from one of the veins of the arms through venous puncture.

Potential risks

During blood sample collection, some discomfort is expected but this will not worsen the condition of the participant or cause any other illness. All care shall be taken to minimize the risk that may arise from a venous puncture.

Benefits

The participation of your child in this study will help in demonstrating that the elevation of serum LDH level in children with SCD correlates with the occurrence of complications and the disease severity. Multiple laboratory parameters will be used for comparison and the study outcomes will serve as a basis of recommendation on use of serum LDH level in the routine follow up of children with SCD. This study aims at achieving a reduction in SCD related complications.

Confidentiality

All collected information shall be kept confidential, only abnormal results will be disclosed to the attending clinician for treatment purposes.

You are free to ask any question related to the study at any time.

I humbly request you to give your consent for enrollment of your dependant into the study.

Dr. G. S. Makory, tel. no: 0723868660.

CONSENT FORM

SERUM LACATE DEHYDROGENASE LEVEL IN CHILDREN WITH SICKLE CELL DISEASE AT KENYATTA NATIONAL HOSPITAL.

I……………………………….declare that I have read as well as understood the study being conducted by DR G. S. Makory and do hereby give an informed consent for my dependant……………………………………to be enrolled.

I am aware that I can withdraw from this study at any time without compromise to his/her medical care.

Signed: …………………………….

Thumb print: ………………………..   Date: …………

Signature of questionnaire administrator (DR. Makory)………………….

Witness: ……………………………..   Date: ……........

APPENDIX III: LDH METHODOLOGY

For the serum LDH measurement a two parts liquid reagent was used in a kinetic method. For enzyme activity pyruvate will was used as a substrate.

The kit used in this study was the "LDH SCE Olympus® Liquid reagent " from Audit Diagnostics. This is an in Vitro Diagnostic reagent pack made up of 2reagents for the quantitative determination of LDH in serum and plasma on Olympus® automated analyzers.

The principle of the test involves the conversion of pyruvate to lactate with the subsequent oxidation of NADH to NAD.

The reaction is shown in the next equation:

$$\text{Pyruvate} + \text{NADH} + \text{H}^+ \xrightarrow{\quad\text{LDH}\quad} \text{L- Lactate} + \text{NAD}^+$$
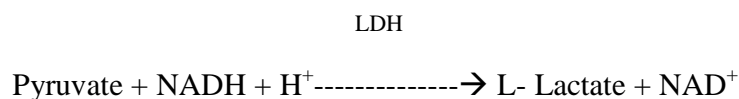
LDH catalyses the oxidation of the lactate to pyruvate reducing the nicotinamide adenine dinucleotide (NAD) to NADH. As NADH is produced, the activity of LDH is determined by the rate of increase in absorbance at 340 nm.

The reagents for this test are delivered ready for use and must be stored at a recommended temperature of $2 - 8^0$C until expiry date indicated on the bottles.

Factors affecting the serum LDH level: haemolysis of the sample gives an erroneous high result due to the leakage of LDH from the RBC's. The same happens if Plasma is used instead of Serum as Platelets contain high amount of LDH. The samples will be examined using a clinical chemistry analyzer at a constant temperature of 37^0C.

On performance characteristic, the assay is linear within a range of 2779 IU/L as the lowest detectable level has been estimated at 2.1 IU/L.

The results were calculated automatically by the instrument as follow:

$$\text{Activity in } U/L = \frac{\Delta \text{ Abs/min x Factor}}{SV \text{ x } 6.3 \text{ x } P}$$

With:

$$\text{Factor} = \frac{TV \text{ x } 1000}{SV \text{ x } 6.3 \text{ x } P}$$

Where: TV= Total reaction volume in ml.

SV= Sample volume in ml.

6.3= Millimolar absorption coefficient of NADH at 340 nm.

P= Cuvette path length in sm.

A serum LDH level above 360U/L was considered high.

APPENDIX IV: HEMATOLOGY LABORATORY TECHNIQUES

Phlebotomy Procedure:

- This started by confirmation the patient particulars: name, age, hospital number and department that must appear on both the request form and the specimen containers.
- The skin at the site of phlebotomy was   disinfected using 70% Spirit or Chlorohexidine 0, 5% and allowed to dry spontaneously.
- Using a sterile needle and a sterile syringe with gloved hands for safety measures, blood was drawn from the antecubital vein, other visible vein in the forearm or dorsal aspect of the hand in children below 4 years in the amount of 4 milliliters.
- The blood drawn was distributed into two tubes: one with EDTA (ethylenediaminetetra-acetic acid: anti-coagulant) and the other without an anticoagulant (plain).
- The plain sample was left at room temperature for the blood to clot and separation was done to collect the serum for LDH Assay.

Blood film preparation:

Using fresh blood or EDTA anticoagulated blood, one drop of blood was put in the middle of a clean slide and using a clean glass spreader with a rapid movement a thin spread of the blood was done and the films were left to dry in the air. For evaluation, the blood films were stained with a standardized Romanovsky stain: Leishman stain.

Romanovsky stain:

Fixative: One volume of Stock solution of azure B-eosin Y was mixed with 14 volumes of methanol.

Staining solution: Was done using a dilute Stock's solution: 1 unit to 14 volumes of HEPES (N-2-hydroxyethylpiperazine-N'- 2-ethane-sulfonic acid) buffer at pH 6.6.

The dried films were left for 3 minutes in the fixative than in the diluted staining solution for 15 minutes, rinsed in phosphate buffer solution, pH 6.8 for 1 minute, rinsed with water, air dried and mounted.

Reticulocyte stain:

Staining solution: Was done by dissolving 1.0 g of new methylene blue or azure B in 100ml of Iso-osmotic phosphate buffer pH 6.5.

Method: Two or 3 drops of the dye solution were delivered into a 75-x10mm plastic tube by means of a plastic Pasteur pipette. Two - 4 volumes of the patient's EDTA anticoagulated blood were added to the dye solution and mixed. The mixture was kept at 37^0C for 15-20 minutes. The red cells were resuspended by gentle mixing and films were made on glass slides. Once dried, the films were examined without fixing or counterstaining under the microscope.

Complete blood count:

This was be done by automation using an anticoagulated blood sample with EDTA. The machine that was used is the "CELL DYN 3200". This is a multiparameter hematology analyzer whose principle is based on 2 independent measurement methods:

1. Impedance method: Determines the counts of WBC, RBC and platelets. The blood sample is suspended in a highly conductive diluents solution and made to pass through an aperture between 2 electrodes. This causes a change in the impedance between the electrodes corresponding to the value and the number of the cells in the blood. The cells are determined by the number of the pulses produced as the impedance changes and their size is estimated by the height of the pulses.

2. Modified cyanmethemoglobin method: For determination of Hb concentration, the "CELL DYN 3200" has a hemolysate amongst its reagents and when it is added to the blood, it leases the red blood cells and the hemoglobin is released. The cyan in the reagent reacts with the released Hb to form cyanhemoglobin. This cyanhemoglobin absorbs green light well at 540 nm. The Hb concentration is determined from the absorbed light by photometry.

The machine is fitted with a software to calculate the RBC indices: Hct, MCV, MCH and MCHC.

APPENDIX V: ETHICAL CLEARANCE

Ref: KNH-ERC/ A/404

18th February 2010

Dr. Germaine S. Makory
Dept.of Human Pathology
School of Medicine
University of Nairobi

Dear Dr. Makory

RESEARCH PROPOSAL: "SERUM LACTATE DEHYDROGENASE LEVEL IN CHILDREN WITH SICKLE CELL DISEASE AT KENYATTA NATIONAL HOSPITAL"   (P363/12/2009)

This is to inform you that the KNH/UON-Ethics & Research Committee has reviewed and **approved** your above revised research proposal for the period 18th February 2010 – 17th February 2011.

You will be required to request for a renewal of the approval if you intend to continue with the study beyond the deadline given. Clearance for export of biological specimens must also be obtained from KNH/UON-Ethics & Research Committee for each batch.

On behalf of the Committee, I wish you a fruitful research and look forward to receiving a summary of the research findings upon completion of the study.

This information will form part of the data base that will be consulted in future when processing related research study so as to minimize chances of study duplication.

Yours sincerely

DR. L. W. MUCHIRI
AG. SECRETARY, KNH/UON-ERC
c.c.   Prof. K. M. Bhatt, Chairperson, KNH/UON-ERC
        The Deputy Director CS, KNH
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