GHANA COLLEGE OF PHYSICIANS AND SURGEONS

FACULTY OF LABORATORY MEDICINE

(HAEMATOLOGY)

EFFECTS OF HYPERHAEMOLYSIS ON FETOMATERNAL OUTCOMES IN PREGNANT WOMEN WITH SICKLE CELL DISEASE

SUBMITTED TO THE FACULTY OF LABORATORY MEDICINE, IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE CONFERMENT OF A FELLOW OF THE GHANA COLLEGE OF PHYSICIANS (FGCP)

DR EUGENIA VICKY NAA KWARLEY ASARE, *MGCP, MBCHB, BSC.*

LM/06/15/001/F

SEPTEMBER, 2021

DECLARATION

I declare that this dissertation has been composed solely by myself. It has not been submitted, in whole or in part, in any previous application or examination for the award of a degree. Unless stated otherwise, by reference or acknowledgement, this work presented is entirely my own.

……..

DR. EUGENIA VICKY N.K. ASARE
LABORATORY MEDICINE (HAEMATOLOGY)
Korle-Bu Teaching Hospital
P.O. BOX KB 77
ACCRA

+233 206 301 166
eugquart@gmail.com

CERTIFICATION

Principal Supervisor:

Prof Edeghonghon Olayemi, *FGCPS, FWACP, MBBS, MSc*

Position: Consultant Haematologist, Department of Haematology, Korle Bu Teaching Hospital

.....

Co- supervisor:

Dr Samuel Antwi Oppong, *FGCPS , FWACS, MBChB*

Position: Consultant Obstetrician- Gynaecologist, Department of Obstetrics and Gynaecology, Korle Bu Teaching Hospital

.....

ACKNOWLEDGEMENTS

I would first like to acknowledge the massive support given to me by my supervisors (Prof. E. Olayemi and Dr Samuel A. Oppong) in fine tuning this work. Their criticism, suggestions and encouragement helped formulate this work.

Prof. J.K Acquaye, Dr. Yvonne Dei- Adomakoh, Dr Amma A. Benneh- Akwasi Kuma, your direction and remarks have been inspiring in completing this work. I am most grateful.

My deepest gratitude goes to my husband Dr. Amgbo Asare who is my rock and continues to spur me on.

Dr Ben Mensah (Korle-Bu Polyclinic Laboratory), Mr Joshua Asare (Korle-Bu Polyclinic Laboratory), Ms. Rahina Swalah (Korle-Bu Polyclinic laboratory) and Mr Enoch A. Mensah (Department of Haematology Research Laboratory), you have been reliable in assisting me throughout this process.

To Reverend Thomas Ndanu, Mr. Jerry Sifa (Sifa Research Consult) and John Ayete- Nyampong, I am grateful for the support with the statistics and data analysis.

To all the residents, Department of Haematology, I would like to say thank you for giving me all the support.

I would also like to thank all my study participants for their dedication and retention in the study. Without them, this study would not have been possible.

My eternal thanks to the Almighty God for life and fortitude to do this work despite the trials

ABSTRACT

Background: Sickle cell disease (SCD) is a public health problem in sub-Saharan Africa. Recent improvement in healthcare has ensured significant increase in survival and increased chance of getting pregnant. Chronic haemolysis is a feature of SCD; haemolysis has been linked to chronic uncompensated anaemia, pulmonary hypertension, chronic leg ulcers, priapism, renal impairment, acute vaso-occlusion, venous thromboembolism and death in SCD. Hyperhaemolysis occurs in stressful conditions, and pregnancy can be stressful. Pregnancy in SCD, can be life-threatening and may be associated with poor fetomaternal outcome. Few studies, have looked at hyperhaemolysis during pregnancy and its association with fetomaternal outcome.

Methodology: A prospective cohort study with 25 pregnant women with SCD, and their 2 sets of matched controls (23 pregnant women without SCD; 25 non-pregnant women with SCD) was conducted at the Obstetrics department, Korle-Bu Teaching Hospital, and the adult sickle cell clinic, Ghana Institute of Clinical Genetics, Korle-Bu. The pregnant women with and without SCD were matched for gestational age (±2weeks) and enrolled during the second trimester. Most pregnant women presenting to the clinic for their booking visit are unsure of the date of their last menstrual period. As such, the gestational age is determined by an obstetrics scan. During the second trimester an obstetrics scan gives an error margin of 10 to 14 days; hence there will be no difference in the gestational age match using ±2weeks. The SCD group (pregnant/non-pregnant) were matched for age (±2years) and phenotype. The age match was based on convenience. All pregnant women with and without SCD were followed up prospectively till six weeks postpartum. The non-pregnant women with SCD had only baseline samples drawn. The primary outcome of the study was to determine the association between hyperhaemolysis and fetomaternal outcome in pregnant women with SCD using selected markers of haemolysis- haemoglobin, absolute reticulocyte count, lactate dehydrogenase (LDH), aspartate aminotransferase (AST), serum bilirubin and urobilinogen. The maternal outcomes were acute pain, ACS and anaemia. The fetal outcomes were spontaneous abortion, intrauterine fetal death (IUFD), preterm birth and low birthweight. Data was extracted using Excel and analysed by the use of means, standard deviation, analysis of variance and logistic regression using STATA corps version 14.

Results: The mean age of the study participants was 30.3 ± 5.3 years. The mean gestational age at enrollment for the pregnant women with and without SCD was 19.4 ± 3.7 weeks. There were 13 acute pain episodes in nine pregnant women with SCD with a pain incidence rate of 1.23 events per patient-years. There was no admission for ACS during the study period. Using the criteria for defining

hyperhaemolysis, only one patient each had a drop in Hb ≥20% from baseline at 28 and 36 weeks' gestation and six weeks postpartum; with no corresponding 25% increase in reticulocyte count and an increase in LDH, indirect bilirubin and AST. There were no significant differences in the concentrations of the haemolytic markers during study follow-up. Compared to the pregnant women without SCD, the pregnant women with SCD had more caesarean deliveries [4(19.0%) vrs 18(72.0%);p=0.01]; preterm births [5(27.8%) vrs 10(41.7%);p=0.35], low birthweight babies [3(16.7%) vrs 7(29.2%); p=0.74] and IUFD [0 (0.0%) vrs 2 (8.0%)] respectively.

Conclusion: This study did not demonstrate hyperhaemolysis in pregnant women with SCD; hence no association between hyperhaemolysis and fetomaternal outcome could be determined. There was no difference in steady state laboratory parameters of the selected markers of haemolysis between the SCD cohorts (both pregnant and non-pregnant).

CONTENTS

DECLARATION ii
CERTIFICATION iii
ACKNOWLEDGEMENTS iv
ABSTRACT v
CONTENTS vii
LIST OF TABLES x
LIST OF FIGURES xi
LIST OF ABBREVIATIONS xii
CHAPTER 1: BACKGROUND 1
1.1 Introduction 1
1.2 Problem Statement 2
1.3 Justification / Relevance 3
1.4 Hypothesis 3
CHAPTER 2: AIM AND SPECIFIC OBJECTIVES 4
2.1 Aim 4
2.2 Specific Objectives 4
CHAPTER 3: LITERATURE REVIEW 5
3.1 Haemoglobin 5
3.2 Sickle Cell Disease 7
3.3 Haemolysis 10
3.3.1 Haemolysis in sickle cell disease 12
3.3.2 Markers of haemolysis 16
3.3.3 Haemolytic index in SCD 28
3.4 Sickle cell disease and pregnancy 29
3.4.1 SCD and adverse maternal outcomes 29
3.4.2 SCD and adverse fetal outcomes 39
3.4.3 Sickle cell disease, pregnancy and haemolysis 40
CHAPTER 4: METHODOLOGY 43
4.1 Study Design: 43
4.2 Study site: 43
4.3 Study population 44
4.3.1 Inclusion criteria 44

4.3.2 Exclusion criteria 45
4.4 Sample Size Determination 45
4.5 Study Procedure 46
4.5.1 Sampling Methods 47
4.5.2 Data collection 48
4.5.3 Data Handling 69
4.5.4 Statistical / Data analysis 69
CHAPTER 5: ETHICAL CONSIDERATIONS 71
CHAPTER 6: RESULTS 73
6.1 Socio-demographic characteristics of study participants at enrollment 74
6.2 Baseline study characteristics of the pregnant women with and without SCD 75
6.3 Frequency of hyperhaemolysis in pregnant women with SCD 75
6.4 Association between hyperhaemolysis and adverse maternal and fetal outcomes in women with SCD 79
6.5 Steady state values of the selected markers of haemolysis in non-pregnant women with SCD compared pregnant women with SCD 79
6.6 Additional results 81
6.6.1 Mean values of laboratory parameters of the pregnant women with and without SCD at baseline, 28 weeks and 36 weeks gestation 81
6.6.2 Trends in concentrations of laboratory parameters during follow up in pregnant women with SCD 85
6.6.3 Trends in concentrations of laboratory parameters during follow up in pregnant women with HbAA 86
6.6.4 Fetomaternal outcomes in pregnant women with and without SCD 87
6.6.5 Demographic characteristics of pregnant women with SCD according to phenotypes 90
6.6.6 Fetomaternal outcomes in pregnant women with SCD based on haemoglobin phenotypes 90
6.6.7 Fetomaternal outcomes in pregnant women with sickle cell anaemia compared to HbAA 92
CHAPTER 7: DISCUSSION 94
7.1 Hyperhaemolysis and fetomaternal outcome 94
7.2 Fetomaternal Outcomes In Women With And Without SCD: 97
7.3 Socio-demographic characteristics 99
CHAPTER 8: CONCLUSIONS AND RECOMMENDATIONS 102
8.2 CONCLUSIONS 102

8.2 RECOMMENDATIONS 102
CHAPTER 9: REFERENCES 103
APPENDIX I: ETHICAL APPROVAL 116
APPENDIX II: ELIGIBILITY FORM 117
APPENDIX III: CONSENT FORM FOR PREGNANT (BOTH SCD AND NON-SCD) PARTICIPANTS 118
APPENDIX IV: CONSENT FORM FOR NON- PREGNANT SCD PARTICIPANTS 123
APPENDIX V-A: QUESTIONNAIRE FOR PREGNANT (BOTH SCD AND NON-SCD) STUDY PARTICIPANTS: ENROLLMENT 128
APPENDIX V-B: QUESTIONNAIRE FOR PREGNANT (BOTH SCD AND NON-SCD) STUDY PARTICIPANTS: SUBSEQUENT VISITS 131
APPENDIX VI-A: QUESTIONNAIRE FOR NON- PREGNANT SCD STUDY PARTICIPANTS: ENROLLMENT 133
APPENDIX VII: CLINICAL ASSESSMENT FORM FOR STUDY PARTICIPANTS (BOTH PREGNANT AND NON- PREGNANT) 135
APPENDIX VIII: REFERENCE RANGES OF LABORATORY PARAMETERS USED IN DATA ANALYSIS 136
GLOSSARY (STUDY DEFINITIONS) 137

LIST OF TABLES

Table 1: Reference ranges of bilirubin during pregnancy 23
Table 2: Table comparing sensitivity and specificity of erythrocyte adenylate kinase to other markers of haemolysis(72) 27
Table 3: Maternal outcomes in pregnant women with sickle cell disease compared to pregnant women without sickle cell disease(10) 29
Table 4: Classification of public health significance of anaemia in populations on the basis of prevalence estimated from blood vessels of haemoglobin (90) 30
Table 5: Summary of haemostatic alterations in patients with sickle cell disease 37
Table 6: Fetal outcomes in pregnant women with and without sickle cell disease 39
Table 7: Steady state laboratory parameters of both pregnant and non-pregnant women with sickle cell disease 80
Table 8: Baseline laboratory parameters of pregnant women with HbSS and HbSC compared to HbAA 82
Table 9: Laboratory parameters of the pregnant women with HbSS and HbSC compared to HbAA at 28 weeks gestation 83
Table 10: Laboratory parameters of pregnant women with HbSS and HbSC compared to HbAA at 36 weeks gestation 84
Table 11: Trends in concentration of haemolytic markers in pregnant women with SCD during the study period 85
Table 12: Trends in concentrations of laboratory parameters in pregnant women without SCD during study period 86
Table 13: Fetomaternal outcomes in pregnant women with and without sickle cell disease 89

LIST OF FIGURES

Figure 1: Structure of adult haemoglobin(30) 5
Figure 2: Erythropoeisis during development: Gene expression of haemoglobin before and after birth(32) 6
Figure 3: Single gene amino acid substitution in sickle cell disease(38) 7
Figure 4: Pathophysiology of sickle cell disease related pathologies 9
Figure 5: Mechanisms and consequences of haemolysis(49) 13
Figure 6: Haemolysis associated nitric oxide bioactivity(23) 14
Figure 7: Processes involved in extravascular haemolysis(55) 16
Figure 8: Production and maturation of reticulocytes(59) 17
Figure 9: Bilirubin metabolism(71) 22
Figure 10: Processes involved in the hypercoagulable state in sickle cell disease (Ataga and Key, 2007)(145) 41
Figure 11: Process map showing recruitment and follow up in study participants 47
Figure 12: Unstained electrophoretic strips of study participants with SCD. 56
Figure 13: Stained electrophoretic strip of pregnant women without SCD (1st controls) 57
Figure 14: Schematic diagram demonstrating principle of flow cytometry for automated analyzers. 58
Figure 15: New methylene blue stain of reticulocytes (155) 61
Figure 16: Miller Ocular Disc Square Microscope 62
Figure 17: Urine dipstick and container(156) 66
Figure 18: Flowchart showing selection criteria of study participants included in the analysis 73
Figure 19: Phenotypic distribution of pregnant women with sickle cell disease 74
Figure 20: Pregnant women with SCD meeting criteria for defining hyperhaemolysis at 28 weeks gestation 76
Figure 21: Pregnant women with SCD meeting criteria for defining hyperhaemolysis at 36 weeks gestation 77
Figure 22: Pregnant women with SCD meeting criteria for defining hyperhaemolysis at 6 weeks postpartum 78

LIST OF ABBREVIATIONS

1. Ab……...Antibody
2. ACS……Acute Chest Syndrome
3. AFLP……..Acute fatty liver of pregnancy
4. ALP……Alkaline phosphatase
5. ANOVA…….Analysis of Variance
6. AST……...Aspartate aminotransferase
7. ALT……..Alanine aminotransferase
8. AVN……..Avascular Necrosis
9. BMI……...Body Mass Index
10. CD…….....Cluster of differentiation
11. CI……..Confidence interval
12. CS……..Caeserean Section or delivery
13. eDAMP…….Erythrocyte damage-associated molecular pattern
14. EDTA……Ethylene diamine-tetra-acetic acid
15. FBC……Full Blood Count
16. G6PD…….Glucose-6-Phosphate Dehydrogenase
17. GHS……...Ghana Health Service
18. GICG…….Ghana Institute of Clinical Genetics
19. Hb……..Haemoglobin
20. HbA……...Haemoglobin A
21. HbC……...Haemoglobin C
22. HbF……....Haemoglobin F or Fetal haemoglobin
23. HbS……....Haemoglobin S
24. Hct…….Haematocrit
25. HELLP……..Highly elevated liver enzymes and low platelets
26. HIV……....Human immunodeficiency virus
27. Hp……..Haptoglobin
28. Hx……..Haemopexin
29. ICAM-1……Intracellular adhesion molecule-1
30. IL-1……....Interleukin-1
31. IL-6……....Interleukin-6

32. IUFD……..Intrauterine Fetal Death
33. IUGR……Intrauterine growth restriction
34. KBTH……Korle-Bu Teaching Hospital
35. LDH……...Lactate dehydrogenase
36. mch……Mean corpuscular haemoglobin
37. mcv……Mean corpuscular volume
38. MP……Microparticles
39. NA……Not applicable
40. NAD…….. …...Nicotinamide adenine dinucleotide
41. NADH…….. …Reduced nicotinamide adenine dinucleotide
42. N**F**-kB……...Nuclear factor-kappa B Ligand
43. NO……Nitric oxide
44. ONOO-……..Perioxynitrite
45. PCV……...Packed Cell Volume
46. PKD……...Pyruvate kinase deficiency
47. PS……..Phosphatidylserine
48. RBC……..Red Blood Cell
49. RDW…….Red cell distribution width
50. RNA……..Ribonucleic acid
51. ROS……...Reactive Oxygen Species
52. SCA……...Sickle Cell Anaemia
53. SCD……...Sickle Cell Disease
54. SGOT……Serum glutamic-oxaloacetic aminotansferase
55. SGPT…….Serum glutamic pyruvic aminotransferase
56. Std dev……...Standard deviation
57. TAT……...Thrombin antithrombin complex
58. TF……..Tissue factor
59. UDP……...Uridyl diphosphate
60. USA……...United States of America
61. VCAM-1……...Vascular cell adhesion molecule-1
62. VTE……...Venous Thromboembolism
63. WBC……White blood cell
64. WHO……World Health Organization

CHAPTER 1: BACKGROUND

1.1 Introduction

Sickle cell disease (SCD) is a chronic haemolytic disorder. It involves the inheritance of an abnormal gene which codes for valine at position 6 of the β- globin chain resulting in HbS. Homozygous form of the disease (HbSS) is known as sickle cell anaemia (SCA); whereas double heterozygotes include HbSC, Hb S- beta thalassemia and HbSD, the commonest in Ghana being HbSC,[1] HbS/β^+-thalassemia and HbS/β^0-thalassemia.[2] In SCD, the abnormal genotypes are associated with a wide range of clinical phenotypes: anaemia, chronic haemolysis, vaso-occlusive events and end organ failure. The disease is most prevalent in sub-Saharan Africa with approximately 200,000 SCD-related births per year.[3] In Ghana, 15 to 30% of newborns are delivered with sickle cell trait (HbAS) while 2% are born with SCD each year.[4–6] Improvements in public awareness, technology and medical care, have significantly reduced SCD-related mortality.[7] Currently, more women with SCD are living to their reproductive ages,[8] and getting pregnant.[9]

Pregnancy in SCD has become an emerging life-threatening condition in Africa. A recent meta-analysis found that pregnant women with SCD in both high- and low-income countries, had increased odds of maternal and perinatal morbidity and mortality compared to pregnant women without SCD.[10] The reported adverse maternal outcomes included increased occurences of antenatal hospitalizations, acute vaso-occlusive events [acute pain and acute chest syndrome (ACS)],[11–14] severe anaemia and venous thromboembolism (VTE).[15] Pulmonary complications are the commonest cause of death.[16] The reported adverse fetal outcomes in pregnant women with SCD compared to the outcomes in the non- SCD population included spontaneous abortions, low birthweight, intrauterine growth restriction (IUGR), preterm birth, intrauterine fetal death/ stillbirth, perinatal and neonatal mortality.[10]

Haemolysis in SCD and other haemoglobinopathies, is a pathologic event with known cardiovascular, pulmonary, gastrointestinal and renal manifestations.[17] It results from the early destruction of sickled red blood cells (RBCs). It can be either intravascular or extravascular. In SCD, the average red cell lifespan is less than 20 days;[18,19] as compared to the average red cell lifespan in the normal population of 90 to 120 days.[18,19] The rate of destruction of these sickled RBCs far exceeds the rate of production of RBCs, hence a chronic uncompensated anaemia results.

The chronic haemolysis in SCD adversely affects the bioavailability of nitric oxide, leading to pulmonary hypertension,[17] chronic leg ulcers, priapism, renal impairment, acute pain episodes, ACS, VTE, and death.[20–26] Markers of haemolysis have been correlated with the consumption of nitric oxide, the development and severity of chronic end organ damage.[20,21]

1.2 Problem Statement

Sickle cell disease patients have an ongoing chronic haemolysis. However, increased haemolysis (hyperhaemolysis), is known to occur in stressful conditions in these patients.[27] Pregnancy, is a stressful condition.

A retrospective study looking at the causes of maternal deaths in 44 pregnant women with SCD at the Korle-Bu Teaching Hospital, Accra from January 2010 to December 2016 reported a worrying trend.[28] Thirty-three (75.0%) of the women were hospitalized for acute pain episodes with approximately 80% of them having a sequelae of ACS. There were also 2(4.5%) spontaneous abortions and 12(27.3%) intrauterine fetal deaths.[28] A prospective cohort study was conducted in the same setting with 90 subjects from May 2015 to April 2016. During the study period 40(44.4%) pregnant women with SCD were hospitalized for acute pain episodes with 14(16.0%) of them developing a sequelae of ACS.[29] There were also 3(3.3%) spontaneous abortions and 2(2.2%) perinatal deaths.[29]

Of note however, is the fact that only 8(18%) cases in the retrospective cohort study, and none of the patients in the prospective cohort study had documented evidence of preceding hyperhaemolysis. The hyperhaemolytic states may have been missed, because markers of haemolysis were not systematically evaluated in both cohorts. The association if any, between hyperhaemolysis and fetomaternal outcome was therefore not established.

It is clear from the above studies that pregnant SCD women face a significant risk of adverse fetomaternal outcomes. Establishing the effect of hyperhaemolysis on their fetomaternal outcomes will enable healthcare providers identify and intervene early during increased haemolytic episodes in these women.

Additionally, the steady state mean values of the markers of haemolysis in both non-pregnant and pregnant women with SCD are currently not known. The frequency of hyperhaemolytic episodes during pregnancy

and the association between hyperhaemolysis and adverse fetomaternal outcomes amongst women with SCD have also not yet been established. These are the answers that this study sought to elucidate.

1.3 Justification / Relevance

This study has the potential to provide baseline data that will establish the association between hyperhaemolysis and fetomaternal outcome in pregnant women with SCD. It will also provide an estimate of the steady state of some selected markers of haemolysis in both pregnant and non-pregnant women with SCD.

Due to the absence of systematic evidence and protocols to guide Clinicians on good clinical practice, this study will help develop guidelines to improve the management of women with SCD during pregnancy in Ghana. Monitoring of women with SCD during pregnancy for hyperhaemolysis, might help improve fetomaternal outcomes associated with these patients.

1.4 Hypothesis

Hyperhaemolysis in pregnant women with SCD results in poor fetomaternal outcome.

CHAPTER 2: AIM AND SPECIFIC OBJECTIVES

2.1 Aim

To determine the association between hyperhaemolysis and fetomaternal outcomes in pregnant women with SCD at the Korle-Bu Teaching Hospital.

2.2 Specific Objectives

- To determine the frequency of hyperhaemolysis in pregnant women with SCD
- To determine the association between hyperhaemolysis (if any) and acute maternal events in women with SCD
- To determine the association between hyperhaemolysis (if any) and adverse fetal outcomes in women with SCD
- To compare the steady state values of the selected markers of haemolysis in non-pregnant women with SCD and pregnant women with SCD

CHAPTER 3: LITERATURE REVIEW

3.1 Haemoglobin

Haemoglobin is an oxygen binding protein of red blood cells (RBCs), weighs 68 Kilo Daltons and is made up of haem (an iron-porphyrin complex called Iron-Protoporphyrin IX) with an attached globin protein. It consists of four polypeptide subunits (tetramer) of two identical alpha-like globin chains [alpha (α) and zeta (ζ)] and two identical beta-like globin chains [gamma (γ), delta (δ), epsilon (ε) or beta (β)]. Each alpha polypeptide unit has 141 amino acid residues and each beta polypeptide unit has 146 amino acid residues. The alpha-like globin genes (α) are on the short arm of chromosome 16 while the beta-like globin genes (γ, δ, ε or β) are on the short arm of chromosome 11. The four subunits are bound as one unit by non-polar interactions and hydrogen bonds. Each haemoglobin subunit has one polypeptide unit and an attached cofactor - haem group; that has a ferrous iron atom centre for binding reversibly to oxygen. Each haemoglobin molecule therefore contains four iron atoms which carry four oxygen molecules. The functions of the haemoglobin molecule include transport of oxygen from the lungs to the peripheral tissues, transport of carbon dioxide to lungs, as well as contributing to acid-base balance.

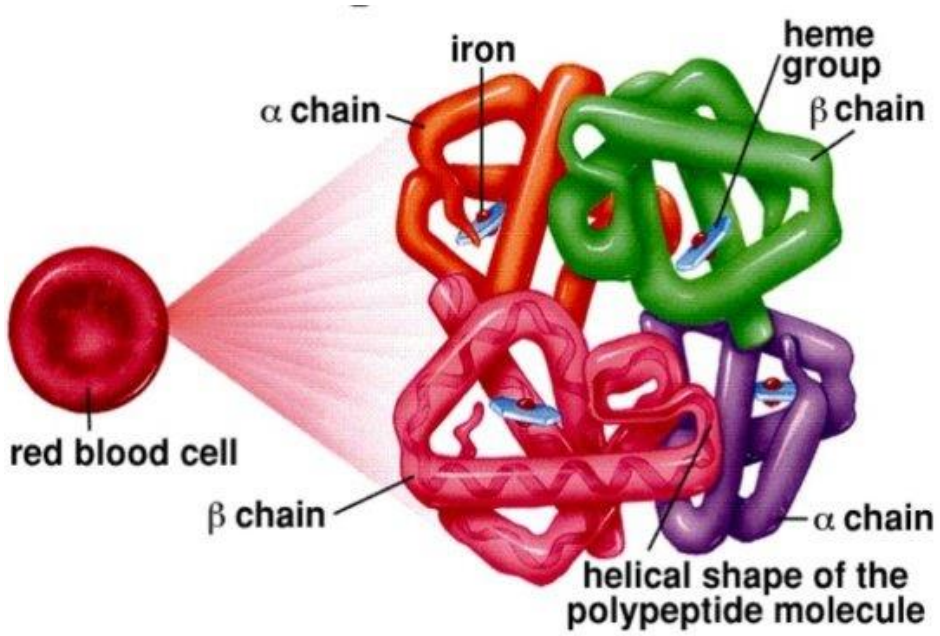

Figure 1: Structure of adult haemoglobin[30]

Different haemoglobin molecules are expressed **at** the different developmental stages of life. The types of haemoglobin include the embryonic, fetal and adult haemoglobins. In embryonic development there are

four different kinds of haemoglobins: $\zeta_2\varepsilon_2$ (Hb Gower-1), $\alpha_2\varepsilon_2$ (Hb Gowler-2), $\zeta_2\gamma_2$ (Hb Portland-1) and $\zeta_2\beta_2$ (Hb Portland-2).[31] These embryonic haemoglobins are found within the first few months of life. At conception, haematopoiesis occurs within the yolk sac. The major haemoglobins synthesized include Hb Gower-1 ($\zeta_2\varepsilon_2$) and Hb Gower-2 ($\alpha_2\varepsilon_2$) with approximately 32% ζ globin chains, 34% ε globin chains and 24% α globin chains. At approximately 3 weeks gestation, whilst a slight reduction in production of the ζ and ε globin chains occurs, approximately 20% and < 5% of the γ and β globin chains respectively, are produced in addition in the yolk sac which combine with the existing ζ globin chain to produce Hb Portland-1 ($\zeta_2\gamma_2$) and Hb Portland-2 ($\zeta_2\beta_2$). Once the embryo develops into a fetus, the haemoglobins are replaced by fetal Hb. At approximately six weeks gestation, the liver takes over the production of the globin chains (α, γ and β) with a drastic reduction in the production of the ζ and ε chains. From 24 weeks gestation, haematopoiesis occurs in the liver, spleen as well as the bone marrow. At birth, while production of the γ globin chain is reducing, production of the β and δ globin chain increases; with most of haematopoiesis occurring in the bone marrow. In fetal life, the bulk of the haemoglobin synthesized is HbF ($\alpha_2\gamma_2$). The fetal haemoglobin (HbF) has a higher affinity for carrying oxygen as compared to the adult haemoglobin, and remains in the child's blood till six months of life where there is a switch to the adult Hb ($\alpha_2\beta_2$). In normal adults, the bulk of the haemoglobin produced is HbA ($\alpha_2\beta_2$): 95-98% with trace amounts of $\alpha_2\delta_2$ (HbA2): 2-3%, and HbF ($\alpha_2\gamma_2$): 0.8-2%, *Figure 2*.

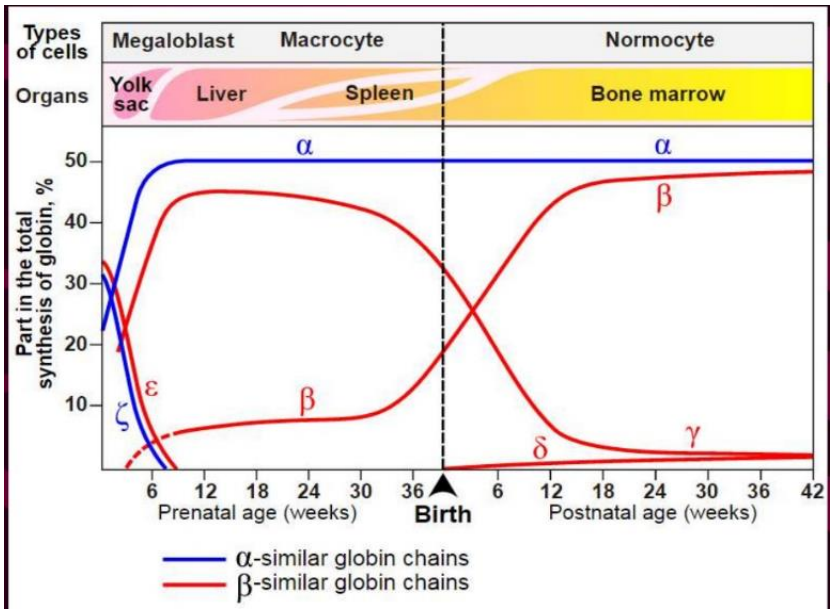

Figure 2: Erythropoeisis during development: Gene expression of haemoglobin before and after birth[32]

Inherited haemoglobin disorders: These can be classified as either structural haemoglobin abnormalities [haemoglobin S (HbS), other haemoglobins with altered oxygen affinity, unstable haemoglobins and HbM][33] or haemoglobin disorders resulting from an imbalanced (reduced or absent) globin chain production (for example, thalassemia).

3.2 Sickle Cell Disease

Sickle cell disease is recognised as one of the major public health problems by the World Health Organization.[34] It is most prevalent in the sub-Saharan region of Africa with approximately 200,000 SCD births per year.[3] Currently, it has been estimated that, yearly there are more than 300,000 births with sickle cell anaemia worldwide; of which approximately 85% of these births are in sub- Saharan Africa.[35,36] In Ghana each year, 15 – 30% of newborns have sickle cell trait (HbAS) whilst 2% of neonates are delivered with SCD.[4–6]

Sickle cell disease (SCD) is a chronic haemolytic disorder involving the inheritance of an abnormal gene which codes for valine at position 6 of the β- globin chain resulting in HbS. Haemoglobin S (HbS) is a result of a single amino acid substitution in the Hb molecule. The base change in gene coding for *β*-globin chain of adult haemoglobin involves the substitution of glutamic acid (coded by GAG) for valine (coded by GTG) at codon 6 of the gene (Glu6Val).[37]

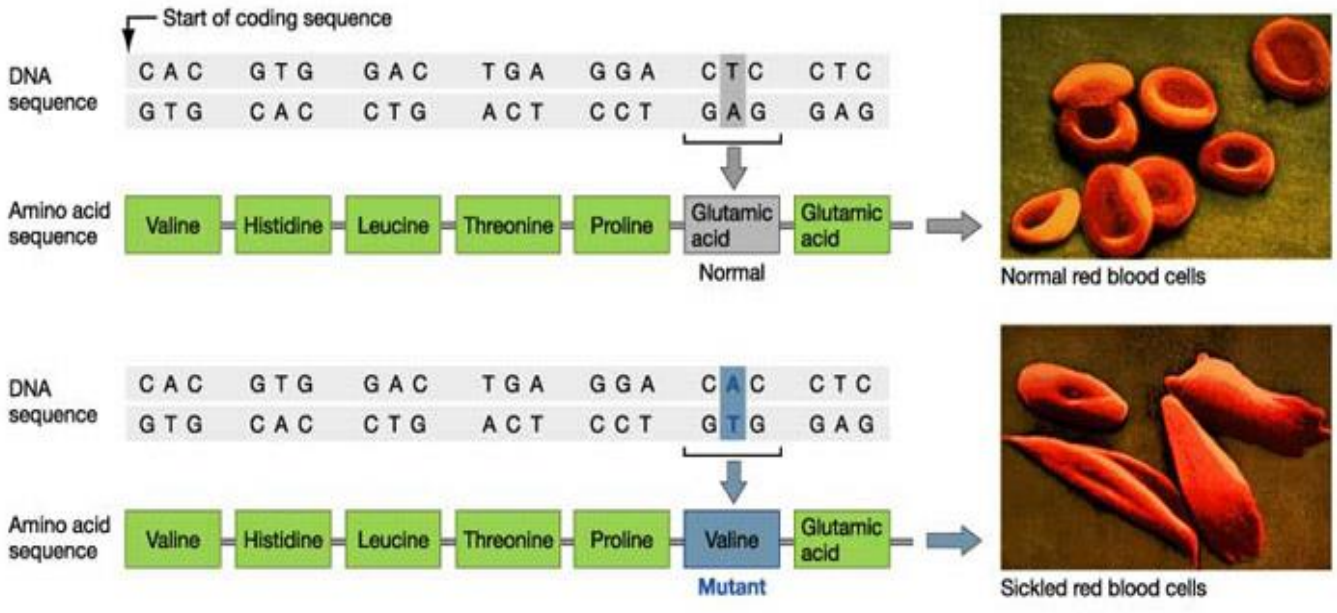

Figure 3: Single gene amino acid substitution in sickle cell disease[38]

Homozygous mutation is known as sickle cell anaemia (SCA). Double heterozygosity occurs by the inheritance of dual mutations in the two β-globin alleles (that is, combination of HbS and another β-globin mutation), leading to the formation of compound heterozygous groups with subsequent reduction or absence in the expression of a normal β-globin chain. The most common heterozygous groups are HbSC [where there is glutamic acid to valine substitution (HbS) and glutamic acid to lysine substitution (HbC)],[1] HbS/β^+-thalassemia and HbS/β^0-thalassemia.[2]

The surface of the HbS molecule has mainly hydrophilic amino acid side chains that are held together with smaller hydrophobic side chains. The abnormal haemoglobin (HbS) in the deoxygenated state, has the beta-6-valine becoming buried inside a hydrophobic pocket on an adjacent beta-globin chain, which joins all the molecules together to form insoluble polymers. These polymerize, aggregate and lead to the formation of rigid, adherent sickled RBCs that become trapped in the microcirculation causing vaso-occlusion as well as ischemia-reperfusion injury in various organs.[39] These abnormal sickled shaped RBCs are also prone to haemolysis as a result of mechanical shearing of the RBC membrane by intracellular HbS crystal polymers, oxidative and metabolic stress.[40,41] Haemolysis leads to exposure of surface phospatidylserine residues and a reduction in membrane fluidity. The pathophysiology of SCD involves the vascular endothelium, neutrophils, coagulation mechanism and inflammatory response triggered by the abnormality in the red cells,[40,42,43] *Figure 4*.

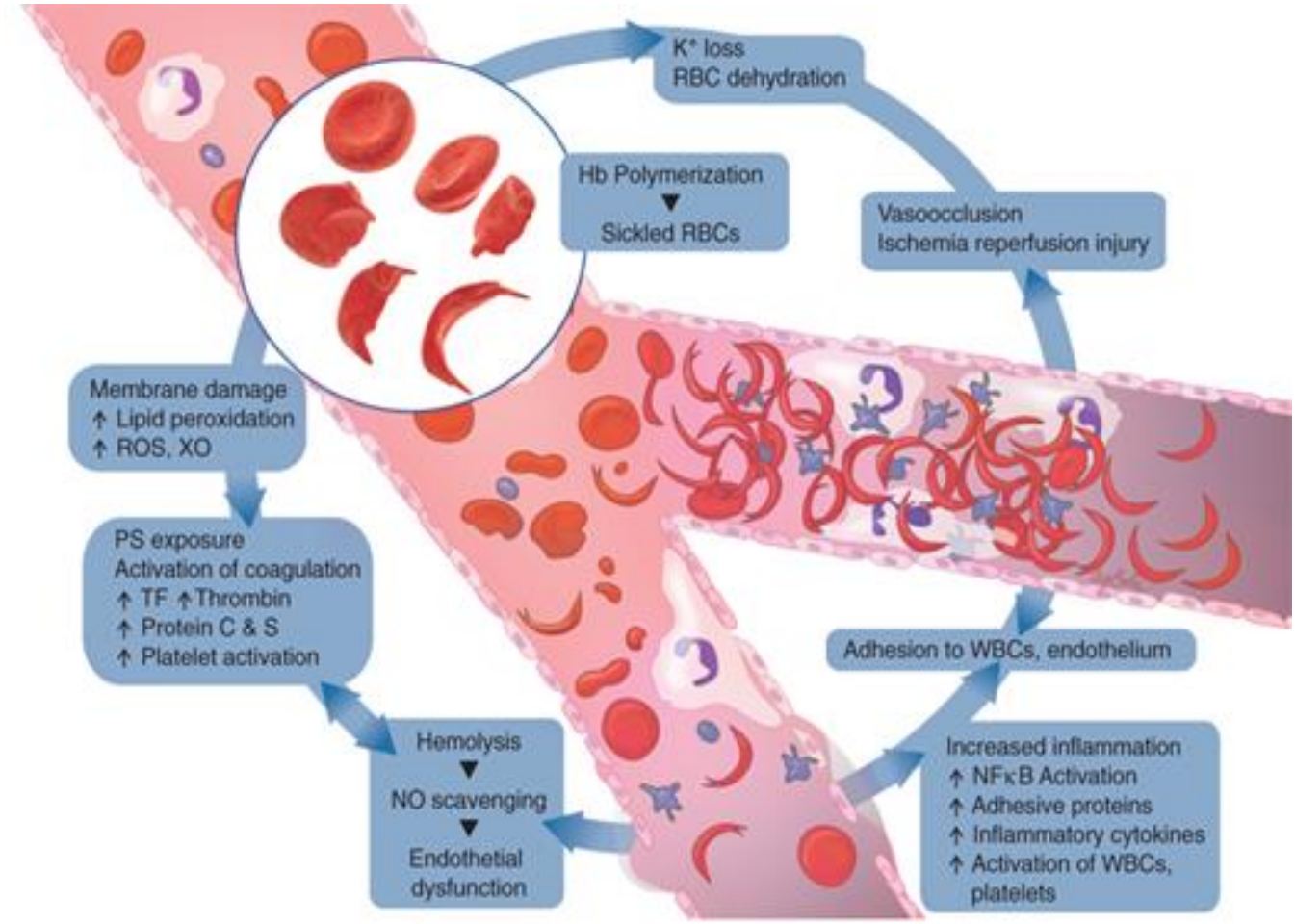

Figure 4: Pathophysiology of sickle cell disease related pathologies

The abnormal genotypes are hence correlated to a fairly wide range of clinical phenotypes such as anaemia, chronic haemolysis, vaso-occlusive events [such as acute pain episode in the chest, abdomen and limbs, acute chest syndrome and venous thromboembolism] as well as end organ damage resulting in autosplenectomy, cerebral infarction and childhood cerebral atrophy. Other clinical manifestations during adulthood include intracranial haemorrhage, retinopathy, sickle cell chronic lung disease, cardiomyopathy, splenic and hepatic sequestration, sickle cell nephropathy (glomerulosclerosis), osteonecrosis (avascular necrosis of femoral/ humeral head), gallstones (cholelithiasis) and chronic leg ulcers. Clinical symptoms of SCD can be precipitated by fever, infections, excessive physical activity or

exertion, temperature changes (cold weather leads to reflex vasospasm while warm weather predisposes to dehydration), hypoxia, high pH (which reduces HbS oxygen affinity) and stress.

3.3 Haemolysis

Haemolysis, is the destruction of RBCs from the peripheral blood circulation before they attain their normal lifespan of approximately 90 to 120 days in HbAA individuals.[18,19] Haemolysis usually occurs in a small percentage of RBCs as a mode of eliminating damaged RBCs. In disease however, haemolysis is usually associated with a haemolytic anaemia due to phagocytosis of deformed red cells, antibody-mediated destruction of RBCs through complement activation or phagocytosis, direct mechanical trauma, oxidative stress, direct RBC destruction (by trauma, toxins or lysis) or RBC fragmentation. Haemolytic anaemias can be chronic to life-threatening and can be classified as either immune or non-immune haemolytic anaemias, inherited or acquired haemolytic anaemias, or intravascular or extravascular haemolytic anaemias.

i. Immune or Non-immune Haemolytic anaemias

Immune haemolytic anaemias

Includes haemolytic anaemias that are mediated by antigen-antibody reactions with the RBC leading to complement mediated destruction or phagocytosis. These antibodies can either be self antibodies (autoimmune) or alloimmune; and the haemolysis involved here can be either extravascular or intravascular. Can be sub-classified into Autoimmune haemolytic anaemias and Alloimmune haemolytic anaemias.

Autoimmune haemolytic anaemias: can be further sub-classified into**:** Warm autoimmune haemolytic anaemia or Cold autoimmune haemolytic anaemia. Warm autoimmune haemolytic anaemia involves IgG antibodies, and accounts for most of the cases of autoimmune haemolytic anaemias (e.g Systemic Lupus Erythematosis). Cold autoimmune haemolytic anaemia involves IgM antibodies (e.g Cold Haemagglutin Disease).

Alloimmune haemolytic anaemias: include haemolysis caused by blood transfusion reactions.

Non-immune haemolytic anaemias

Includes haemolytic anaemias that are not mediated by immune causes. These haemolytic anaemias can be diagnosed by positive routine tests for haemolysis; and are characterized by a negative anti-human immunoglobulin or Coombs test. They can be further sub-classified as Hereditary or Acquired non-immune haemolytic anaemias.

Hereditary non-immune haemolytic anaemias: includes both qualitative haemoglobin disorders (e.g sickle cell disease) and quantitative haemoglobin disorders (e.g thalassemias), red cell membrane disorders and red cell enzymopathies.

Acquired non-immune haemolytic anaemias: includes haemolysis caused by infections, drug and metal intoxications and paroxysmal nocturnal.

ii. Inherited or Acquired Haemolytic Anaemias

Inherited or Intrinsic haemolytic anaemias can be further sub-classified as membranopathies (e.g. hereditary spherocytosis, hereditary elliptocytosis), haemoglobinopathies (e.g sickle cell disease and thalassemia) and enzymopathies (includes conditions such as pyruvate kinase deficiency (PKD) and Glucose-6-Phosphate Dehydrogenase (G6PD) deficiency). In SCD, there is the inheritance of an abnormal gene on the β-globin chain. The haemoglobin formed is hence abnormal and the RBC is prone to haemolysis. This haemolysis leads to exposure of phosphatidylserine on the RBC surface which leads to a myriad of complications.

Acquired or Extrinsic haemolytic anaemias are caused by factors outside the RBC. In this situation, there is normal haematopoiesis with normal RBC production in the bone marrow. These RBCs are later destroyed directly in the bloodstream from factors such as antibodies, or get trapped and recycled prematurely in the spleen. Examples include autoimmune haemolytic anaemias, alloimmune haemolytic anaemia, drug induced haemolytic anaemia, mechanical haemolytic anaemia and haemolysis associated with infections such as malaria.

iii. Haemolytic anaemias caused by either Extravascular or Intravascular haemolysis, or both

Intravascular haemolysis: Occurs when there is lysis of RBCs with sustained release of RBC microparticles and intracellular contents into the circulation. Conditions that can result in intravascular

haemolysis include prosthetic cardiac valves, Glucose-6-phosphate dehydrogenase (G6PD) deficiency, SCD and Thrombotic thrombocytopenic purpura.

Extravascular haemolysis: Occurs when abnormal or damaged RBCs with membrane alterations are removed from the blood circulation by the macrophages of the reticuloendothelial system of the spleen, liver, and bone marrow. This occurs in RBC membrane disorders like hereditary spherocytosis, and in haemoglobinopathies e.g SCD.

3.3.1 Haemolysis in sickle cell disease

Chronic haemolytic anaemia is an important pathology in SCD; and the amount of haemolysis varies between the genotypes of SCD. Generally, the lifespan of sickled RBCs is less than 20 days.[18,19] Most of the haemolysis in SCD is extravascular, with about one- third being intravascular.[39] This is because the pathology of SCD involves damaged or abnormal RBCs with membrane alterations or senescent RBCs which are removed from the blood circulation by the reticuloendothelial system of the spleen, liver, and bone marrow during extravascular haemolysis. CD163 and CD91 are scavenging macrophage receptors coexpressed on human dendritic cells and monocytes. Sickle cell disease patients are reported to have low serum levels of the anti-oxidant or scavenging molecules (haptoglobin and haemopexin)[44] used primarily in intravascular haemolysis probably due to the effect of repeated vaso-occlusive infarctions in the liver and spleen. These organs cannot hence synthesize more of these scavenging molecules. In SCD patients, the few remaining $CD163^{+}$ macrophages in the spleen and $CD91^{+}$ macrophages in the liver cannot effectively clear the haptoglobin-haemoglobin and haemopexin-haem complexes respectively, as they should. In other words, there appears to be increased catabolism without compensatory synthesis of these molecules.

3.3.1.1 Intravascular haemolysis in sickle cell disease

Haem (ferrous protoporphyrin IX) is an extremely important iron complex in the human body because of its responsibility in oxygen and electron transfer. This haem, is however highly toxic because it promotes oxidative damage through catalysing free radical reactions which cause covalent modification of protein, carbohydrates, lipids, as well as nucleotides that will result in tissue damage.[45,46]

In the event of red cell lysis from complement or shear mediation, cell-free haemoglobin is released into the circulation, with subsequent liberation of haem into the plasma[20]. Thus in chronic haemolysis, there are abnormally high levels of haem in the plasma.[20] In healthy non-SCD controls however, haem levels in plasma is undetectable due to the absence of haemolysis.[20] This excess amount of cell-free haemoglobin and haem are then bound and cleared rapidly by their respective scavenging or anti-oxidant molecules-haptoglobin and hemopexin (within 2 to 5 minutes for haem-hemopexin complex, and within 10 minutes for haemoglobin-haptoglobin complex.[47,48] Haptoglobin and haemopexin however, have already been reported to exist in reduced quantities in SCD patients;[44] with resultant impaired clearance of these damaging molecules.

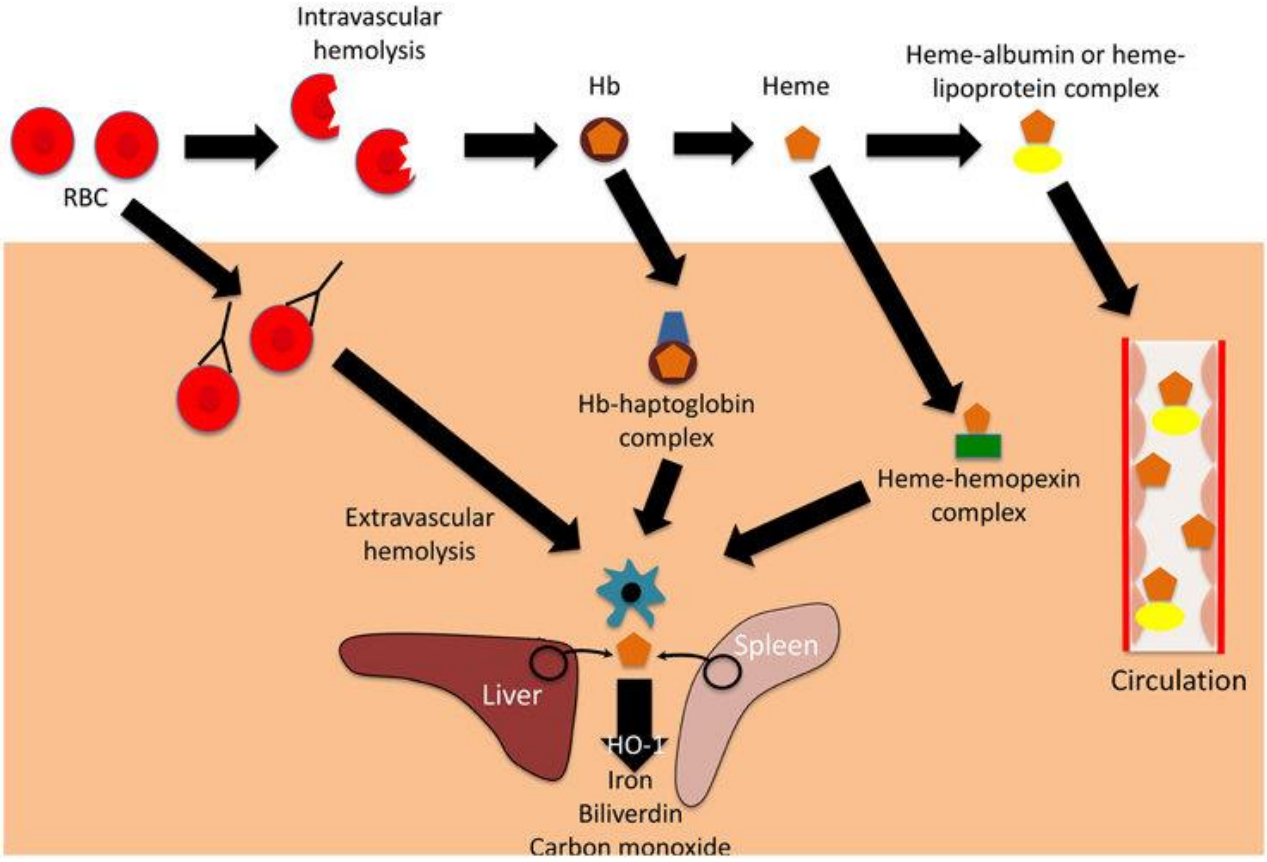

Figure 5: Mechanisms and consequences of haemolysis[49]

Both cell-free haemoglobin and haem consume nitric oxide (NO) to generate methaemoglobin and nitrate.[22] Further NO deficiency is caused by release of arginase-1 from sickled RBCs which degrades L-arginine, a key substrate in the formation of NO by endothelial nitric oxide synthase.[22,50] In addition, reactive oxygen species (ROS) reacts with NO via oxidative stress to produce peroxynitrite ($ONOO^{--}$)

which damages various proteins (by nitrosylation) leading to protein malfunction. By the action of all these molecules: eDAMPs (erythrocyte damage-associated molecular pattern molecules), cell-free haemoglobin, haem and arginase-1, the bioavailability of NO is very low. The low levels of NO negates its beneficial properties such as regulation of vascular tone,[23] inhibition of the expression of endothelial adhesion molecules,[23] potency of antithrombotic effects,[23] platelet activation,[26] increased platelet adhesion[51] and blockage of the progression of the cell cycle primarily at the G1/S phase. Thus, low NO leads to vasoconstriction, endothelial activation and cell proliferation with enhanced vaso-occlusive events.

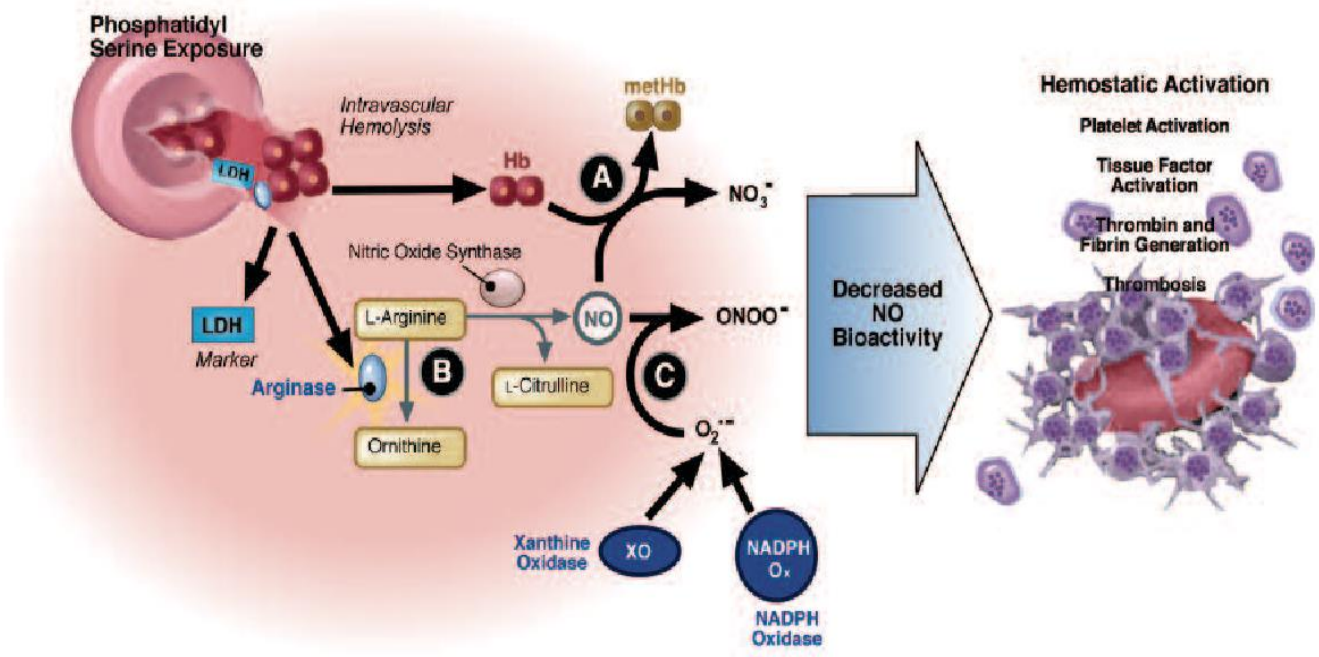

Figure 6: Haemolysis associated nitric oxide bioactivity[23]

Again, during intravascular haemolysis, a large proportion of extracellular haem is borne by erythrocyte-derived microparticles in the circulation, which then transfer their haem to endothelial cells.[26] This transfer, results in a higher expression of endothelial adhesion molecules [like vascular cell adhesion molecule-1 (VCAM-1) and intracellular adhesion molecule (ICAM-1)] which enhance leukocyte recruitment and binding between circulating cells and the vessel wall,[52] with a resultant poor vascularization and vaso-occlusion in SCD.[26]

Intravascular haemolysis is associated with an increased reticulocyte production due to a high red cell turnover. The reticulocytes have receptors and ligands eg. α4β1 and CD36,[53] which adhere to the endothelium and leukocytes, providing another link between haemolytic anaemia and vaso-occlusion.[54]

Intravascular haemolysis is further linked to red cell membrane damage. This results in the release of arginase and lactate dehydrogenase (LDH), as well as haemoglobin into plasma, and phosphatidylserine exposure on the surface of the red cell membrane. This results in the activation of tissue factor causing thrombosis (haemostatic activation).[23]

3.3.1.2 Extravascular haemolysis in sickle cell disease

Extravascular haemolysis happens when senescent, damaged or abnormal RBCs with membrane alterations are removed from the blood circulation by macrophages in the reticuloendothelial system. In extravascular haemolysis, RBCs with morphological abnormalities of the membrane surface and are unable to manoeuvre through splenic sinusoids, are immediately phagocytosed and destroyed by macrophages. This process also occurs in the liver. Haemoglobin is then degraded into haem and globin. The haem is further broken down into iron and protoporphyrin IX. This protoporphyrin IX is subsequently converted to biliverdin, and subsequently to unconjugated bilirubin via biliverdin reductase, with the release of carbon monoxide. The unconjugated bilirubin is then carried to the liver bound tightly to albumin. Here it is conjugated, and then excreted in the gastrointestinal tract as urobilinogen through the urine and stool. The globin is degraded to amino acids for the production of other proteins while the iron is bound to transferrin and transported into the circulation.

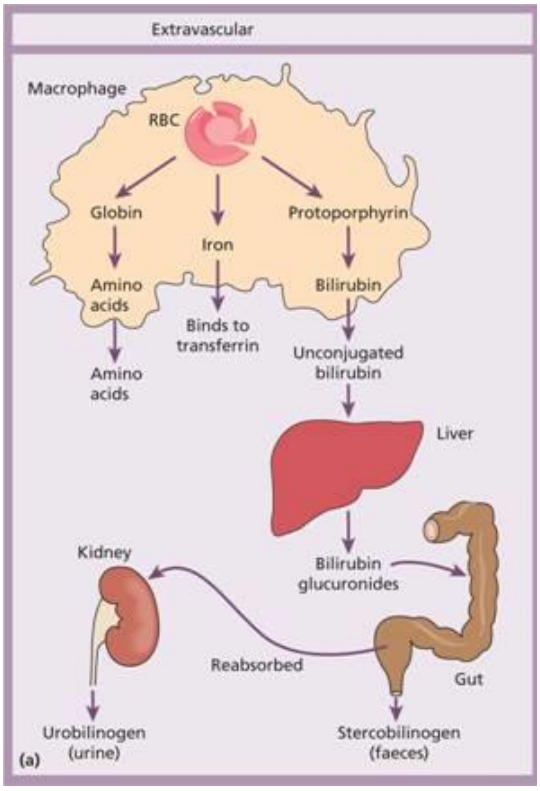

Figure 7: Processes involved in extravascular haemolysis[55]

Chronic haemolysis in SCD hence results in haem production and nitric oxide depletion leading to oxidative stress, endothelial cell activation and inflammation with resultant vaso-occlusion.[56,57]

3.3.2 Markers of haemolysis

The investigation of haemolytic anaemias involves recognizing the existence of increased products of haemoglobin catabolism, and signs of increased erythroid activity. The following markers of haemolysis can be used:

i. **Reticulocyte count**: Reticulocytes are non-nucleated direct precursors of RBCs and they contain residual RNA. This residual RNA exists in newly formed blood cells for 1 - 2 days before the cell reaches maturity.[58]

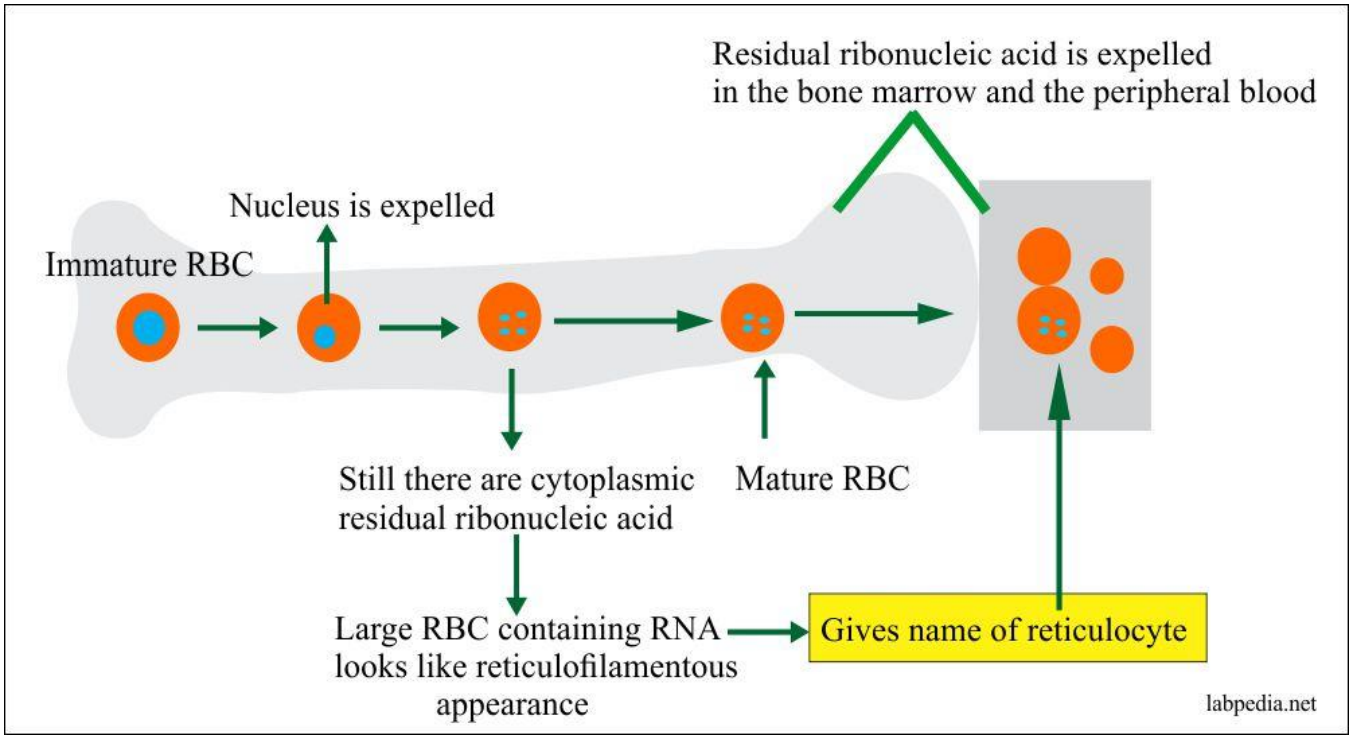

Figure 8: Production and maturation of reticulocytes[59]

Reticulocytes are reported to be approximately 8% larger compared to the mature RBC.[58] They are an index of haemopoietic activity in the bone marrow, and they are used to estimate the extent of effective erythropoiesis.

The reticulocyte count is a well documented and strong marker that correlates with the red cell lifespan because they represent a distinctive cohort of cells that have just been introduced into the peripheral blood.[58,60]

Clinical significance: It is elevated in haemolytic anaemias, and also in pathological and physiological conditions like haemorrhage and pregnancy; as well as following treatment of anaemias. Reticulocytosis is an important marker or pointer for monitoring recovery from haemolysis (that is, marrow compensation), or response to specific therapy for nutritional anaemia.[58] Reticulocytopenia usually occurs in conditions like aplastic anaemia, radiation therapy, untreated pernicious anaemia and bone marrow tumours where there is bone marrow suppression.

The number or proportion of reticulocytes **is** determined with the reticulocyte count. The reticulocyte count can be determined using either the manual or automated method.

Manual method of reticulocyte count

Advantages for the manual reticulocyte count include the fact that it is useful in smaller or medium sized low-resourced laboratories and has a lower cost compared to the automated method. This method however is tedious, and is prone to random errors such as intraobserver and interobserver variability. A study by Simionatto et al in Brazil which was translated and published in English language, reported a random error margin of between 7% and 40%. [61]

Automated method of reticulocyte count

The automated method is cost-effective, has much greater precision as well being more accurate in determining maturation levels of the reticulocytes as expressed by the immature reticulocyte proportion and also by other important factors, including the volume and concentration of haemoglobin.[62] It however demands special precautions such as avoiding formation of bubbles when agitating samples within the reagent bottle; ensuring that the sample-stain diluent suspensions are incubated at room temperature; protecting the sample from exposure to light to ensure specimen integrity by avoiding haemolysis with subsequent erroneously low results; and also protecting the reagents since they are also light sensitive.[63]

Simionatto et al in Brazil in 2010 concluded that variation between the manual and automated methods was very low. They confirmed that, if the execution criteria are strictly followed and quality control maintained, identification and evaluation of reticulocyte count either manually or by the use of automated method would be precise and trustworthy.[63]

The reticulocyte count is reported as either an absolute reticulocyte count or as reticulocyte percentage, depending on the presence or absence of anaemia. If anaemia is present, the reticulocyte percentage has to be corrected; hence it is best to use the absolute reticulocyte count which doesn't require adjustment. It is assumed that in the normal subject in a steady physiological state, the absolute number or percentage of reticulocytes found will theoretically be 1/120 or 0.8% of all red cells (representing the proportion of the reticulocyte lifespan in the peripheral blood to the normal red cell lifespan).[58]

Considering the fact that the normal red cell lifespan may have a 10% variation, this would in theory, give a reticulocyte range of 0.7% to 0.9% of all red cells.[58] This range would however depend on observer variability in reporting what is positively stained. Hence reference ranges in various laboratories may differ.[58]

The Reticulocyte Percentage is the percentage of reticulocytes per 1000 RBCs

$$\text{Reticulocyte percentage} = \frac{\text{number of reticulocytes per 1000 RBCs}}{1000} \times 100\%$$

The correction of the reticulocyte count can be done using these formulas

$$\textit{Corrected reticulocyte count (\%)} = \text{reticulocyte count} \times \frac{\text{patient's Hb or haematocrit (hct)}}{\text{normal Hb or haematocrit (hct)}}$$

The mean normal Hb concentration for males is 14g/dL with a Hct of 45%; whereas in females it is 13g/dL with a Hct of 42%.

The normal reference range of the corrected reticulocyte percentage varies with age as well as laboratories.[58,63] For adults (irrespective of the gender), the normal value ranges between 0.5 to 2.5%. In newborns (0 – 2 weeks) the value ranges from 1.8% to 8.0%; whereas in infants the value ranges from 2.5% to 6.0%. In children up to 5 years, the normal reference range is 0.5% to 5.0%.

The Reticulocyte Production Index (RPI), is used to assess whether the bone marrow has adequate production compared to the anaemic condition. The absolute number of reticulocytes is determined as an index called the reticulocyte production index.

$$\text{Reticulocyte Production Index (RPI)} = \frac{\text{Corrected Retic count (\%)}}{\text{Correction factor (}\sim 2\text{)}}$$

Where correction factor is number of days of maturation or maturation time

$$\text{Alternatively, RPI} = \frac{\text{patient's haematocrit}}{\text{normal haematocrit}} \times \frac{\text{patient's reticulocyte count}}{\text{maturation time}}$$

Hutchinson et al (1996) defined Maturation time as a representation of the maturation time of RBC's (in days) at various levels of anaemia[58,64]. For a Hct value of ≥40%, the normal maturation time is 1.0 and a Hct value of 30% – 39.9% will yield a maturation time of 1.5. A Hct of 20-29.9% will yield a maturation time of 2.0. If a Hct <20% then the maturation time is expected to be 2.5.

The normal reference range for the RPI is 1 to 3. An RPI of greater than 3 shows a normal bone marrow response to anaemia whereas an RPI of less than 2 shows inadequate response to anaemia.

The Absolute Reticulocyte Count (ARC) is the actual number of reticulocytes in 1L of whole blood

$$\text{Absolute reticulocyte count} = \frac{\text{reticulocyte count (\%)}}{100} \times \text{RBC count } (10^{12}/\text{ L})$$

The reference range of the absolute reticulocyte count varies depending on the laboratory but usually found to be between 50 x 10^9/L to 100 x 10^9/L.[65,66]

For this study, the absolute reticulocyte count was used. This is because the absolute reticulocyte count would not require adjustment if anaemia is present. Most SCD patients have chronic anaemia.

i. **Whole blood haemoglobin (Hb):** Haemoglobin, is the oxygen carrying molecule of the RBC. In haemolytic anaemias, this is considered the most direct indicator of clinical severity and outcome.[67] This is considered a weak correlate of haemolytic rate because it does not directly relate to RBC survival.[41]

The normal reference range for non-pregnant females is 12 – 15.8g/dL.[68] Reference ranges in pregnancy however, vary according to trimesters.[68–70] The reference ranges for the first and second to third trimesters vary between 11.6 – 13.9 g/dL and 9.5 – 15 g/dL respectively.

ii. **Bilirubin and urobilinogen:** Bilirubin can be derived from either the catabolism of the protoporphyrin ring of haem by the action of microsomal haem-oxygenase or from the catabolism of haemoglobin in

the reticuloendothelial system (RES). Majority (85%) of bilirubin is however derived from catabolism of Hb from RES.

During the catabolism of Hb, haem is released from phagocytosed senescent red blood cells in the spleen and liver. The haem released is taken up by the cells of the RES including phagocytes, the Kupffer cells of the liver, and cells in the spleen and bone marrow. This haem is then acted on by haem-oxygenase with the release of iron and carbon monoxide which is excreted by the lungs. The reaction produces a green pigment. Biliverdin is acted on by biliverdin reductase with the release of carbon monoxide and bilirubin (a yellow pigment). The unconjugated bilirubin produced is highly insoluble in aqueous solutions at neutral pH due to its compact hydrogen bonds. Unconjugated bilirubin in the periphery is then transported to the liver tightly bound to albumin. In the liver, this bilirubin is converted to bilirubin mono- and diglucuronides through the action of a microsomal enzyme known as bilirubin uridyl diphosphate (UDP)- glucuronosyltransferase. This then travels through the biliary tree and ultimately into the duodenum and excreted with bile as conjugated bilirubin. The normal gastrointestinal bacterial flora then convert majority of the conjugated bilirubin into urobilinogen and a small amount of urobilin. Urobilinogen is a colourless pigment; most of it is excreted with the faeces (about 90%), and the rest re-absorbed by the gastrointestinal mucosa back into the bloodstream where it is recaptured by the hepatocytes and re-excreted in the bile; as well as excreted by the kidneys and expelled in urine. Figure 9 on the next page shows the metabolism of bilirubin.

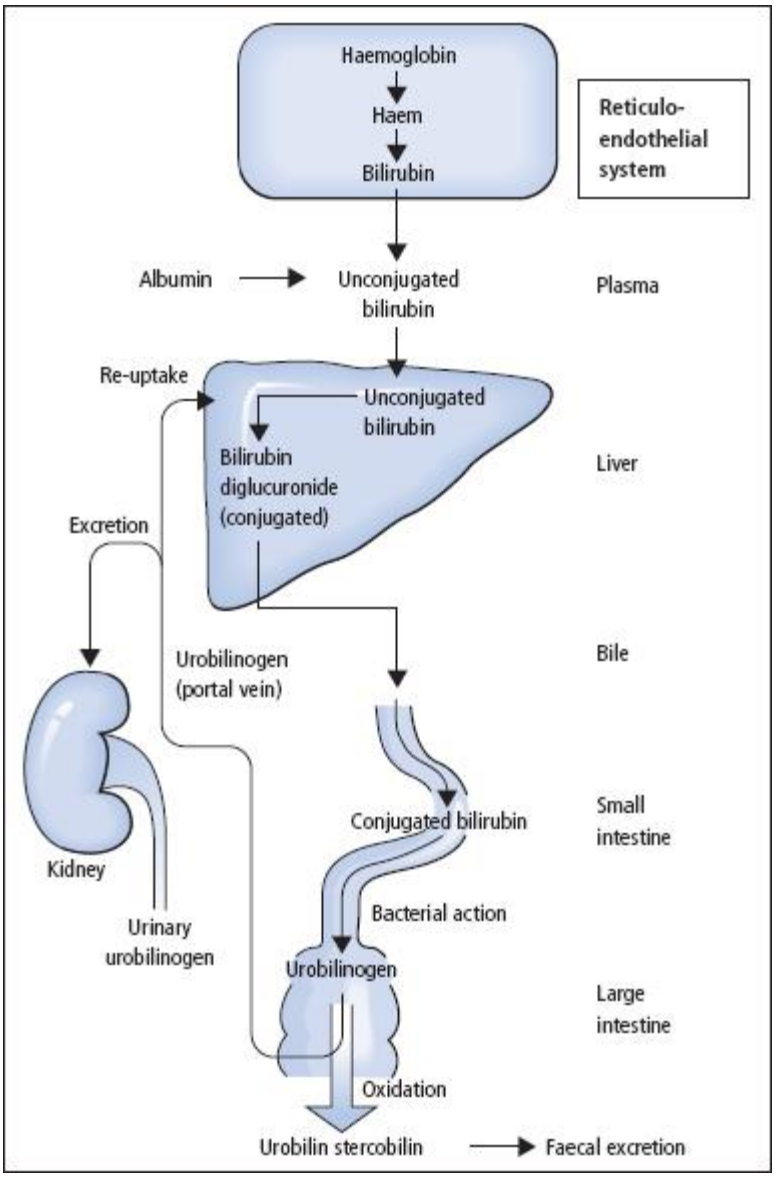

Figure 9: Bilirubin metabolism[71]

Clinical Significance of bilirubin: Unconjugated bilirubin is an excellent marker for extravascular haemolysis and to a lesser degree, intravascular haemolysis. Bilirubin is an early marker of response to therapy as its value returns to normal or within 10% of normal values in up to 4 hours after haemolysis has stopped. The main disadvantage is that, it is influenced by processes independent of haemolysis such as hepatitis, biliary obstruction, prolonged fasting and cirrhosis, and by drugs like erythromycin and penicillin, oral contraceptives, methyldopa, sulphonamides and indomethacin.[72]

Clinical Significance of urine urobilinogen: Levels of urine urobilinogen vary with the degree of haemolysis, and may be increased in haemolytic anaemias, or in the case of infectious hepatitis and cirrhosis.

The interpretation of the urine urobilinogen test include false positive and false negative results. The False positive results include use of substances that will react with the Erlich's reagent (this forms the basis for the reaction) as well as use of drugs containing Azo dyes such as sulphadiazine (due to the pink or red colour in positive reactions). False negative results will be obtained if stale urine that has been exposed to light is used.

Normal reference range for total bilirubin, unconjugated bilirubin and conjugated bilirubin varies in the non-pregnant and pregnant adult[70] and with laboratories. Reference ranges for total bilirubin, indirect bilirubin and direct bilirubin in the non-pregnant adult are 5.1 – 22.2 umol/L, 1.7 – 8.6 umol/L and 1.7 – 6.8 umol/L respectively.

During pregnancy the following reference ranges in Table 1 are used[68–70]

Table 1: Reference ranges of bilirubin during pregnancy

Period of pregnancy	**Total bilirubin (umol/L)**	**Unconjugated (indirect) bilirubin (umol/L)**	**Conjugated (direct) bilirubin (umol/L)**
First trimester	1.7 – 6.8	1.7 – 8.6	0 – 1.7
Second trimester	1.7 – 13.7	1.7 – 8.6	0 – 1.7
Third trimester	1.7 – 18.8	0 – 8.5	0 – 1.7

iii. **Aspartate aminotransferase (AST):** This enzyme facilitates the reversible transfer of an α-amino group between aspartate and glutamate. It is therefore an important enzyme in amino acid metabolism. This enzyme was formerly called serum glutamic-oxaloacetic transaminase (SGOT). It is found in various cells within the body but the majority are in the cells of the heart and liver and, to a lesser

proportion, in the cells of the kidneys, muscles, brain and RBCs. It has two isoenzymes [GOT1/ cAST (cytosolic isoenzyme derived mainly from RBCs and heart) and GOT2/mAST (mitochondrial isoenzyme present predominantly in the liver).

Clinical Significance: Healthy individuals show low levels of AST in blood. Levels may rise in acute haemolytic anaemia, viral hepatitis, cholecystitis, acute fatty liver of pregnancy (AFLP), preeclampsia, highly elevated liver enzymes and low platelets (HELLP) syndrome, intrahepatic cholestasis, hyperemesis gravidarum, acute pyelonephritis, drugs (including heparin, isoniazid, acetaminophen, methyldopa) and pulmonary embolism,.

The normal concentrations in non-pregnant adults ranges from 12 to 38 U/L;[68] whereas the normal concentrations in the pregnant woman varies with trimesters.[68–70] The normal concentrations for the first and second to third trimesters are 3 – 23 U/L and 4 - 32 U/L respectively.

iv. **Alanine aminotransferase (ALT):** This enzyme converts alanine into pyruvate as an intermediate for cellular energy production. It is found primarily in the liver and kidney, but can be found in smaller amounts in the muscles and heart. It was formerly called serum glutamic pyruvic transaminase (SGPT). It is usually measured as a component of the liver function panel concurrently with AST to determine organ damage.

Clinical significance: This enzyme is more specific for the liver; hence levels are increased with liver damage, and it is used primarily to screen for liver damage, as well as monitor liver disease. Elevated levels of this enzyme can be seen in viral hepatitis, fatty liver of pregnancy, autoimmune hepatitis, HELLP syndrome, preeclampsia and chronic liver disease.

Its disadvantage is that, even though levels rise with liver damage, it cannot show the extent of damage to the liver or the amount of fibrosis involved.

The normal concentrations in the non-pregnant adult is 7 to 41 U/L.[68] Again, the normal concentrations in the pregnant woman varies with trimesters.[68–70] The reference ranges for the first to second and third trimesters are 2 - 33 U/L and 2 - 25 U/L respectively.

v. **Serum LDH**: It is an enzyme in the glycolytic pathway which catalyzes lactate conversion into pyruvic acid. It is found in the cytoplasm of cells, and is widely and unevenly distributed in the body with 5 iso-enzymes- LDH-1 and LDH-2 (71% in the RBCs), LDH-3: lungs, LDH-4: kidneys, placenta and pancreas, LDH-5: skeletal muscle. Physiologically it is measurable within serum due to normal physiological cellular turnover.

Clinical Significance: During haemolysis, LDH-1 and LDH-2 are often increased and may be useful in differentiating between intravascular haemolysis and extravascular haemolysis. In intravascular haemolysis, the serum LDH levels are increased 4- to 5- fold above the upper limit of normal because the enzyme is contained in the RBC. In extravascular haemolysis however, its levels are mildly increased.[73]

The main advantage of using serum LDH as a haemolytic marker is that it is useful in assessing response to therapy because its level declines along with the reduction of the rate of haemolysis. The disadvantages of the test however, include the fact that its levels can be elevated in several conditions other than haemolysis, especially those conditions that involve cellular necrosis, apoptosis and high cellular or tissue turnover.

The normal reference range in the non-pregnant adult is 115 – 211 U/L.[68] During pregnancy, the LDH levels vary according to trimester and with laboratories.[68–70] For the first and second trimester, the reference range varies between 78 – 433 U/L. whereas in the third trimester, it ranges between 82 - 524 U/L.

vi. **Haptoglobin (Hp)**: It is an alpha-2-glycoprotein synthesized primarily in the liver. It can be detected in cells of the lungs, thymus, kidneys, spleen, and heart.[74,75] It has two major alleles (Hp1 and Hp2) with three possible variants/ genotypes (Hp1-1, Hp2-2 and Hp2-1) with different binding strength, anti-

oxidative capability, clearance rate and molecular weights distinguishable at high resolution electrophoresis.[76]

Clinical Significance: Functionally, it is important for binding cell-free haemoglobin. Haemoglobin-bound Hp targets a specific cellular pathway of clearance via the monocyte/macrophage surface receptor CD163.[77] There are aberrantly low levels of haptoglobin in SCD;[44] and this has been confirmed and reported by Allotey et al in a study conducted in adult SCD patients attending the sickle cell clinic, GICG in 2012 (unpublished data). Haptoglobin is also documented to be associated with inflammatory diseases like infections (malaria, HIV, tuberculosis) and non-infectious diseases like obesity, diabetes and cardiovascular disease. Another study conducted at the adult sickle cell clinic, GICG in Korle-Bu reported that Hp2-2 phenotype has been linked to phenotype-dependent poor disease outcomes.[78]

Haptoglobin is significantly decreased during intravascular haemolysis[79] due to an increased plasma cell-free Hb and altered free or complexed haptoglobin balance. It is also decreased during extravascular haemolysis where some structurally abnormal or altered RBCs escaped from the reticulo-endothelial system into the circulation.[80]

The methods for testing include spectrophotometry, immunoreactive methods, and gel electrophoresis.[79]

The disadvantages associated with use of the haptoglobin test include the fact that it is considered as an acute phase reactant. Only a few studies have also validated its use as a marker for haemolysis[79]. Haptoglobin is dynamic hence it is influenced by the Hb load from haemolysis, conditions that will impair synthesis (eg. liver impairment, malnutrition), inflammatory mediators (IL-1 and IL-6)[81] and genetically determined Hp isotypes (eg. Congenital Ahaptoglobinemia).

vii. **<u>Hemopexin (Hx)</u>**: It is a single chain glycoprotein produced by the liver. It binds to haem; and haem-bound Hx complexes are cleared by the CD91 pathway. It mediates haem-iron recovery into hepatocytes for catabolism and excretion, and prevents the pro-oxidant and pro-inflammatory effects of haem. It also promotes detoxification of haem, especially when haptoglobin concentrations are very

low or depleted.[79] Haemopexin however, is influenced by the kinetics of the Hb load, are found in aberrantly low levels in SCD[44] and it is considered as an acute phase reactant/ protein.

viii. **Erthrocyte adenylate kinase (EAK)**: The enzyme adenylate kinase is involved in the phosphorylation transfer between adenosyl- triphosphate (ATP) and adenosyl- monophosphate (AMP) in potassium sensitive ATP channels. Just like LDH, it has at least five isenzymes, and can be found in the blood, brain, skeletal and cardiac muscles. Erythrocyte adenylate kinase is a red cell specific enzyme released from damaged red cells. It is a highly sensitive and specific marker for erythrocyte haemolysis compared to LDH and reticulocyte count, and can be used to differentiate between disorders of red cell destruction (such as occurs in SCD) and disorders of inadequate red cell production or blood loss. . Its activity can be measured in serum by rapid electrophoresis or immunological methods and correlates linearly with the degree of hemolysis in vitro.[72]

Table 2: Table comparing sensitivity and specificity of erythrocyte adenylate kinase to other markers of haemolysis[72]

	Sensitivity	Specificity	Likelihood ratio	-Likelihood ratio
EAK	96	97	35.5	0.04
LDH	100	59	2.42	0
Total bilirubin	39	50	0.78	1.22
% Reticulocytes	100	85	6.8	0

Unfortunately, the haptoglobin and hemopexin tests, as well as the erythrocyte adeylate kinate test are reported to be expensive, and not routinely run in the clinical laboratories. They are used for research purposes.

3.3.2.1 Effect of SCD on selected markers of haemolysis

Sickle cell disease is a chronic haemolytic disorder hence there is constant lysis of red cells leading to changes in the concentrations of markers of haemolysis. The effect of these changes on the well-being of the patient varies from person to person and can change over time with change in conditions.[82,83] Exacerbated haemolysis will result in hyperbilirubinaemia, increased urine urobilinogen, elevated liver

enzymes and serum LDH, as well as reticulocytosis. Sickle cell disease patients are known to have steady state blood Hb levels. Blood Hb levels however are known to drop further from baseline concentrations during complications such as exacerbated haemolysis, SCD nephropathy, aplastic crisis or acute sequestration crisis.

Two studies demonstrated a relationship between markers of haemolysis in the steady state and during acute pain episodes. The first study published by Ballas and Marcolina in 2006 looked at adult African-Americans with HbSS followed prospectively and longitudinally at the Sickle Cell Center of the Cardeza Foundation of Jefferson Medical College and Thomas Jefferson University Hospital over 15 years in Pennsylvania, USA. This study reported increased haemolysis evidenced by a drop in Hb with associated increase in reticulocyte count, bilirubin, LDH and AST during the evolution of acute painful episode.[84] The reticulocyte count increased from 9.5 ± 5.44 (%) in the steady state to 20.9 ± 8.14 (%) during acute painful episodes.[84] The second study, a retrospective study published by Gardner et al in 2013 was conducted at King's College Hospital, London, UK. This study looked at clinical and laboratory profiles of sickle-related VOCs over a ten-year period (2004–2013 inclusive) and involved 751 adult patients with 4,136 patient-years of observation. Study reported a 28% percentage LDH increase (from 379 +/- 155 IU/L in steady state to 507+/-272 IU/L in VOC) during haemolysis.[85]

3.3.3 Haemolytic index in SCD

This reflects direct markers of intravascular haemolysis (plasma red blood cell microparticles and cell-free haemoglobin) in patients with SCD and allows for adjusted associations between haemolytic severity and clinical outcomes.[86] It can however be calculated from the values of the indirect markers of intravascular haemolysis (LDH, total bilirubin, reticulocytes and aspartate transaminase) using the principal component analysis.[87] The principal component analysis is a long established statistical method useful for studying underlying mechanisms reflected in individual biologic or physical measurements.[88,89] The haemolytic index is useful when the objective of the study is to investigate the role of an underlying mechanism.

3.4 Sickle cell disease and pregnancy

3.4.1 SCD and adverse maternal outcomes

In SCD, the commonly recognised adverse maternal outcomes include an increased odds of antenatal hospitalizations, acute vaso-occlusive events including acute pain and ACS,[11–14] and severe anaemia. Pulmonary complications are a common cause of death in this group of patients.[16] The odds ratio of some maternal outcomes are listed in Table 3.

Table 3: Maternal outcomes in pregnant women with sickle cell disease compared to pregnant women without sickle cell disease[10]

Condition	**OR**	**95% CI**	**p-value**
Pre-eclampsia	2.05	1.47-2.85	<0.001
Eclampsia	3.02	1.20-7.58	0.019
Recurrent infections	2.48	1.23-5.01	0.011
Caesarean delivery	1.42	1.04-1.93	Not documented
Maternal mortality	10.91	1.83-65.11	0.009

3.4.1.1 Sickle cell disease, pregnancy and anaemia

Anaemia in pregnancy is considered a major public health problem worldwide.[90,91] Globally, anaemia affects 32.4 million (38.2%) pregnant women, and it is a severe public health problem in Africa affecting 46.3% of pregnant women.[92] In tropical countries, the incidence of anaemia in pregnant women is 40% - 80% whereas in developed countries, it ranges between 10% – 20%.[93] A high incidence and severity of anaemia has been reported to occur in primigravidae living in malaria endemic areas.[94] In Ghana, a baseline study conducted in 2005 by the Ghana Health Service (GHS) on the prevalence and aetiology of anaemia in Ghana, put the prevalence of anaemia in pregnancy at approximately 36% at registration and 25% at term.[95] A cross-sectional study conducted on 214 pregnant women who were attendants at KBTH, Accra, Ghana; and published by Dei-Adomakoh et al in 2014 reported the prevalence of anaemia in pregnant Ghanaians in their second trimester as 70%.[96]

In pregnancy, anaemia has a significant impact on the health of the fetus as well as that of the mother. It is associated with preterm deliveries, low birthweight, morbidity and perinatal mortality due to the impairment of oxygen delivery to the placenta and fetus.[97]

Classification of anaemia in pregnancy:

Blood haemoglobin concentrations during pregnancy have been detected to be generally lower than the non-pregnant state due to the maternal plasma expansion, along with the growing fetus's use of the maternal iron stores. Generally, the cut-offs for diagnosing anaemia in pregnancy have remained unchanged since 1968. Recent standard guidelines laid down by WHO, still maintain that anaemia in pregnancy is diagnosed when the haemoglobin (Hb) concentration in the peripheral blood is 11.0 g/dl or less.[90,91] In postpartum mothers, anaemia is when blood Hb concentration is less than or equal to 10.0g/dL.[98] According to the WHO report on anaemia in 2011, "anaemia can generally be classified based on the public health significance to the population, and on the blood Hb concentration.

i. ***Classification of anaemia in pregnancy based on the public health significance to the population***: Table 4 outlines this classification system

Table 4: Classification of public health significance of anaemia in populations on the basis of prevalence estimated from blood vessels of haemoglobin [91]

Category of public health significance	**Prevalence of anaemia (%)**
Severe	40 or higher
Moderate	20.0 – 39.9
Mild	5.0 – 19.9
Normal	4.9 or lower

ii. ***Classification of anaemia in pregnancy based on blood Hb concentration:*** Anaemia in pregnancy can be classified as "either mild, moderate or severe based on the blood Hb concentration. In pregnant women, an Hb level <7g/dL is considered as severe anaemia; an Hb level between 7.0g/dL - 9.9g/dL is considered moderate anaemia; while an Hb level ranging between 10.0g/dLl - 10.9g/dL is considered mild anaemia."[91]

Aetiopathogenesis of anaemia in pregnancy:

The aetiopathogenesis of anaemia in pregnancy can broadly be classified as either Physiological or Pathological.

i. ***Physiological anaemia in pregnancy***: During pregnancy, there is a disproportionate increase in the plasma volume and the RBC mass. In the first two weeks of pregnancy, the earliest changes that occur include adaptations to the cardiovascular and haematological systems. In the first month of pregnancy the heart rate and stroke volume increase, resulting in a proportional increase in cardiac output by 30-50%. Additionally, there is a 25% - 50% increase in plasma volume, plus excessive erythropoietin production from the kidneys resulting in an increase in RBC mass by 20-30%.[99] This increase progresses until the 32nd to 34th week of pregnancy. The consequence of these changes, is a 1-2 g/dL decrease in blood haemoglobin value creating a haemodilutional effect with dilutional anaemia.[100]

   Another physiological adaptation resulting in the anaemia in pregnancy, is the marked demand for extra iron especially in the second trimester.

ii. ***Pathological anaemia in pregnancy***, is anaemia resulting from any other cause apart from the normal physiological cause of anaemia. Examples of pathological causes of anaemia in pregnancy include deficiency anaemias and anaemia due to haemolysis, haemorrhage or malaria infection**.**

   Clinical significance of deficiency anaemias: Deficiency or nutritional anaemias are defined as a condition in which the haemoglobin content of blood is lower than normal as a result of deficiency of one or more essential nutrients, regardless of causes of the deficiency.[101] The aetiopathogenesis of these anaemias include high demand, inadequate intake, malabsorption or excess loss. Deficiency anaemias include iron deficiency anaemia (the commonest) and megaloblastic anaemia. Deficiency anaemia may contribute to maternal and fetal morbidity and mortality through effects on the maternal immune system leading to an increased susceptibility to infections,[102] decreased performance and work capacity,[103] as well as mental disturbances (cognition, emotions and psychomotor) during the postpartum period[104] and infant life.[105] It has also been associated with preterm delivery,[106] low birthweight[107] and increased blood loss during the peripartum period.[108]

Worldwide, iron deficiency anaemias is the most common type of anaemia in pregnancy, representing 75% of all anaemia in pregnancy.[109] The high prevalence of iron deficiency anaemia is as a result of the high demand and negative balance (especially during menstruation) in the non-pregnant state which is carried forward into pregnancy.

A study by Dei-Adomakoh et al at KBTH and published in 2014 reported that twenty seven (18%) of the anaemic pregnant subjects in the second trimester had low serum iron and six (4%) had low serum folate levels.[96]

Clinical significance of haemolysis: Haemolysis during pregnancy may result from haemoglobinopathies such as SCD, and in rare cases, autoimmune haemolytic anaemia which resolves after pregnancy. Haemolysis will result in increased breakdown and rapid turn over of red blood cells with a resultant anaemia.

Clinical significance of malaria infection: Malaria infection in pregnancy contributes significantly to both maternal and perinatal morbidity and mortality. In the mother, infection has been associated with severe anaemia, haemolysis, renal failure, thrombocytopenia, pulmonary oedema and maternal death.[110] In the fetus, it has been associated with higher rates of miscarriage, intrauterine fetal death, premature delivery, low birthweight neonates, and neonatal death due to placental sequestration of infected RBCs.[110] Pregnant women are prone to more severe infection due to their immunocompromised state; and this risk is higher in primigravidas, adolescents and during the second trimester. Pregnant women suffer disproportionately from severe anaemia as a result of malarial infection. Malaria accounts for 25% of severe anaemia in pregnancy;[110] and the aetiology is multifactorial. The commonest being folic acid deficiency and microcytic anaemia. In addition, sequestration of malaria infected RBCs in the placenta, as well as haemolysis from complicated malaria infection can result in severe anaemia.[110] Current strategies in the prevention of malaria disease in pregnancy include use of insecticide-treated bed nets (ITN) and intermittent presumptive treatment (IPT) with antimalarial medications. IPT involves administration of two or more doses of chemoprophylaxis after 20 weeks of gestation in an attempt to reduce subclinical malarial load.
During pregnancy, anaemia is the most common complication; even more so in women with sickle cell disease. In SCD, there is an existing chronic uncompensated anaemia due to the chronic haemolytic

process. Together, these two processes (anaemia in pregnancy and chronic anaemia in SCD) lead to a drop in the steady state blood Hb values in pregnant women with SCD with resultant increased anaemia. Hyperhaemolysis in pregnant women with SCD will further worsen anaemia with a resultant increased risk of both maternal and perinatal morbidity.[111–114]

3.4.1.2 Sickle cell disease, pregnancy and acute vaso- occlusive events

Acute vaso-occlusive events in SCD include acute pain episode, acute chest syndrome (ACS) and venous thromboembolism (VTE).[115] During pregnancy in SCD, there is an increase in metabolic demand and an increase in blood viscosity as a result of the hypercoagulable state as well as increased red blood cell sickling.[116] This effect is associated with an increase in occurrence of acute pain episodes, ACS, osteonecrosis, hepatic necrosis, leg ulcers and thromboembolic events.[116]

1. ***Acute pain episode***: In pregnant women with SCD, acute pain episode is said to be the most frequent complication and the most common cause of hospital admission.[13,14] Between 27% - 50% of pregnant women with SCD have acute pain episodes at sites such as the lower limbs, waist, chest and ribs.[13,14]

   Acute pain episode has been noted to evolve through four phases; notably, the prodromal, initial, established and resolving phases.[117] During the prodromal phase of acute painful events, vaso-occlusion, inflammation and nociception occur concurrently. The prodromal phase is usually associated with numbness, paraesthesia or aches; and it usually lasts up to 1 or 2 days without medical intervention. Pain becomes most severe by day 3 of the crisis and starts decreasing by day 6 or 7. Serious complications of acute painful crisis like ACS, multi-organ failure and sudden death can occur between 1 to 5 days after start of acute pain without medical intervention.[117] The vaso-occlusion that occurs during the prodromal phase of acute painful events leads to hypoxia, ischaemia and eventually tissue damage. This is followed by chronic vascular inflammation which worsens with recurrent painful episodes. During the inflammation, there is release of inflammatory mediators from the injured cells, macrophages, mast cells and platelets;[117] this activates nociceptors on the peripheral afferent nerves with a resultant nociceptive effect.

   The pathophysiology of acute pain and ACS in SCD arises from a cascade of interactions between the RBCs, vascular endothelium, neutrophils, coagulation mechanism and inflammatory response.[40,42,43] Neutrophils are an important component of the innate immune system, providing immunity against

invading pathogens, and have also been reported to be pro-inflammatory.[118] When endothelial cells are activated, there is recruitment of neutrophils initiated by rolling of neutrophils on endothelial selectins and adhesion mediated by integrins. Adherent neutrophils through E-selectin, then activate Mac-1 (an αMβ2 integrin) which in turn captures circulating sickle RBCs with a resultant prolonged obstruction of blood flow in the veins.[118]

Leukocytosis with a predominance in neutrophils is a common physiological adaptation to stress during pregnancy;[100] hence this might explain why pregnant women with SCD have more pain during pregnancy.[119] Additionally, vascular occlusion may occur in the placenta with a resultant necrosis, infarction and villous fibrosis , consequently leading to decreased utero-placental circulation and eventual fetal demise due to chronic fetal hypoxia.[120]

2. ***Acute chest syndrome*** is the second most common cause of hospitalization and complication, reported in 7–20% of pregnancies.[11,12] Acute chest syndrome is defined as the presence of a new pulmonary infiltrate on chest radiograph along with fever, chest pain, respiratory distress, or new onset hypoxemia (PaO2 of 71mmHg; 9.5KPa on room air).[121] The aetiopathogenesis is incompletely understood, and may be multifactorial in nature, and not identifiable in 46% of cases.[121] It includes pulmonary infections, atelectasis, infarctions (from intrapulmonary sickling and fat embolism) and venous thromboembolism.[122]

The predisposing or risk factors to developing an ACS in pregnancy include acute pain episode,[28,123] pulmonary infection, thromboembolism and caesarean delivery. Management of acute pain in SCD often requires the use of opioid analgesics and sometimes intravenous fluids. Opioid analgesics such as morphine have been known to cause respiratory depression, hypoventilation and then ataelectasis; this coupled with the fluid overload/ pulmonary oedema from excessive use of intravenous fluids will predispose to ACS.[124] Vaso-occlusion in the bone will lead to marrow infarction, fat embolism and then ACS. Surgery (especially abdominal which includes caesarean delivery) has also been documented to predispose SCD patients to ACS. After abdominal surgery, patients tend to have abdominal pain if not given adequate analgesics. Any situation that will result in an increased abdominal pressure tends to aggravate this pain; hence patients tend to splint as a defence mechanism

to avoid pain. In cases where patients are given general anaesthesia, mucous secretions tend to accumulate in the airway, thereby resulting in atelectasis which predisposes to ACS.

The documented mechanisms of vascular/ pulmonary damage in ACS includes the release of haem and reduced bioavailability of nitric oxide (NO) secondary to haemolysis (results in vasoconstriction); increased endothelin 1 (a potent vasoconstrictor) leading to increased vaso-occlusion;[125] activation of second messengers of NO such as nuclear factor- kappa B (NF-kB) ligand with a resultant upregulation of endothelial adhesion molecules such as VCAM-1 and ICAM-1 which facilitate the binding of sickle RBCs, white blood cells and platelets to the vascular endothelium. These processes would lead to a damage to vascular endothelium, worsened vaso-occlusion and hypoxia.[118]

Acute chest syndrome is the most common manifestation of cardiopulmonary disease and the leading cause of death in patients with SCD.[28] It is characterized by fever, increased respiratory effort, chest pain, drop in haemoglobin oxygen saturation and new radiodensity on chest x-ray.[29] In a case series looking at the causes of maternal deaths in women with SCD at KBTH (2010-2016), the multidisciplinary SCD obstetrics team reported that approximately 87% of all the maternal deaths was due to an ACS, with almost 80% of deaths having a preceding acute pain episode.[28] Most of the ACS cases and deaths occurred during the third trimester and puerperium.[28,123]

3. ***Venous thromboembolism***: Venous thromboembolism (VTE) constitutes deep venous thrombosis (DVT) and pulmonary embolism (PE). Both pregnancy and SCD are considered as hypercoagulable states. Whilst pregnancy increases the risk of venous thromboembolism (VTE) 4- to 5- fold over that in the non-pregnant state,[126,127] SCD is considered an independent risk factor for pregnancy-related VTE with an odds ratio of 6.7 (95% CI, 4.4-10.1).[128] Pregnant women with SCD are 2.5 times more likely to experience deep vein thrombosis compared to healthy age matched pregnant controls.[129] Similarly, cerebral-vein thrombosis is reported to be 5 times more likely to occur in pregnant women with sickle cell disease compared to pregnant women without sickle cell disease.[129]

   The obstetric and gynaecological risk factors associated with VTE are discussed in this paragraph. Pregnancy, is considered an acquired hypercoagulable state due to the increased oestrogen activity coupled with multiple changes in the coagulation system - such as an increase in Factors I, II, VII, VIII,

IX and XII; associated with an increased resistance to anti-thrombotic factors such as Protein S and activated Protein C deficiency (as gestation progresses) and inhibition of fibrinolysis.[130] While these changes are good to help secure haemostasis and minimize intrapartum bleeding, they also predispose the pregnant woman to VTE. In pregnancy, there might also be a weight gain with an increase in body mass index (BMI); and this has been shown to be a risk factor for VTE.[131] Pregnancy, is associated with a risk of developing pregnancy-induced hypertension; and hypertension has been linked to endothelial dysfunction. The normal endothelium is antithrombotic; but in endothelial dysfunction, there is a balance shift between clot generation and breakdown towards thrombosis due to decreased synthesis of nitric oxide and prostacyclin, and increased endothelin-1. A retrospective study done by Lindqvist P et al in 1999 revealed that women with pre-eclampsia were at a 3-fold higher risk of getting a VTE postpartum.[132] As pregnancy progresses, there is mechanical venous compression of the inferior vena cava and pelvic veins by the enlarging uterus with resultant stasis; and this can predispose to a VTE. Again, with increasing reduction in mobility as pregnancy progresses (especially in the third trimester), during early postpartum period and during hospitalizations for pregnancy- related complications, stasis results; and this can also predispose to a VTE. Caesarean delivery (CS) has been associated with an increased risk of developing VTE. Compared to vaginal delivery, a meta- analysis by Blondon et al reported that the relative risk of VTE following caesarean delivery ranged from 1 to 22, with a meta-analytic odds ratio of 3.7 (95% CI, 3.0-4.6).[133] This same study also reported a pooled incidence of 2.6 VTE per 1,000 CS (95% CI, 1.7-3.5), with an increased incidence in studies with a longer and better follow-up in the postpartum period (4.3 per 1,000 CS).[133] Most studies done on VTE in pregnancy suggests that VTE is more common in the postpartum period, with a 5- fold increased risk of DVT as compared to during pregnancy.[126,127] Maternal age has also been implicated in VTE. In a retrospective study done in the US, women aged 35 years and older were found to have 38% higher risk for VTE.[128] Finally, race or ethnic background has also been implicated in the development of a VTE during pregnancy; with Blacks or African-Americans having a higher incidence. James et al, reported a 64% higher risk of VTE in black women during pregnancy.[128]

The hypercoagulable state in SCD and its subsequent predisposition to thromboembolic phenomena is attributed to several factors including the fact that components of haemostasis are altered in the direction of a procoagulant phenotype;[134] there is a problem with platelet function,[135,136], natural/

physiologic anticoagulants as well as the fibrinolytic systems[135] and there is loss of normal membrane phospholipid asymmetry with abnormal exposure of phosphatidylserine on red cell surface.

In SCD, there are also low levels of protein S and C, increased expression of tissue factor on blood monocytes and endothelial cells,[128] raised levels of factor VIII, the generation of hydroxyl radicals and liberation of fibronectin and thrombospondin. Table 5 summarizes the haemostatic alterations in patients with SCD.

Table 5: Summary of haemostatic alterations in patients with sickle cell disease

INCREASED LEVELS		**DECREASED LEVELS**
Platelet activation	Fibrinopeptide A	Factor V
Platelet aggregation	D- dimer	Factor XII
Phosphatidylserine-rich platelets and red blood cells	Plasminogen activator inhibitor	Factor IX
Thrombin- antithrombin complexes		Protein C
Prothrombin fragment F1.2		Protein S
Plasmin- antiplasmin complexes		
Fibrinogen and fibrin- fibrinogen complex		

3.4.1.3 Sickle cell disease, pregnancy and end organ damage

Patients with SCD develop accumulating end organ damage as a result of both micro- and macrovascular vaso-occlusion and chronic haemolytic anaemia. The vaso-occlusive related end organ damage includes avascular osteonecrosis and retinopathy; while the haemolysis-related organ damage include renal failure/ nephropathy, pulmonary hypertension with pulmonary thrombosis, chronic leg ulcers, venous thromboembolism (stroke) and cholelithiasis (gallstones). The haemolysis-related organ damage is linked to the reduction in nitric oxide bioavailability and pathologic effects of erythrocyte damage-associated molecular pattern molecules (eDAMPs) made up of plasma cell free haemoglobin, heme and arginase 1. These disrupt endothelial function, drive oxidative and inflammatory stress with enhanced vasoconstriction and vasculopathy. The physiological changes or adaptations that occur during pregnancy, may overburden organs that already have chronic injuries secondary to SCD, thereby worsening end organ damage.

Sickle cell nephropathy is a common chronic complication in adults with sickle cell disease.[137,138] The major manifestations include albuminuria, acute tubular necrosis, and renal failure. During pregnancy, the physiological adaptation of many organ systems including increased plasma volume with accompanying increase in renal blood flow, increased glomerular filtration rate and solute delivered to the kidney may worsen any such pre-existing disease of the kidney. In the general population during pregnancy, glomerular filtration rate increases approximately 50% with a concordant decrease in serum creatinine; urea, and uric acid values and a drop in blood pressure levels by approximately 10mmHg.[139]

Pulmonary hypertension, is another complication associated with chronic end organ damage in SCD. Pulmonary hypertension is defined as an increase in mean pulmonary arterial pressure (mPAP) ≥25 mmHg at rest as assessed by right heart catheterisation.[140] Using Doppler echocardiographic screening alone, approximately 30% of adults with SCD are diagnosed with Pulmonary hypertension.[141,142] Doppler echocardiographic screening with right heart catheterization however, have confirmed that 6 – 10% of adult SCD patients have a mean pulmonary artery pressure of ≥25 mmHg with a high mortality.[143,144] The potential aetiologies reported include reduced biaoavailability of NO and vasculopathy secondary to intravascular haemolysis; upregulated hypoxic responses as a result of anaemia, microvascular obstruction and low oxygen saturation and chronic pulmonary VTE.[83] Pulmonary hypertension is associated with multiple clinical conditions, and is characterized by endothelial dysfunction and imbalance between vasodilators and vasoconstrictors within the pulmonary circulation, followed by progressive smooth

muscle and neointimal hyperplasia and physical obliteration of the arteriolar circulation.[145] The physiological adaptation of pregnancy includes an increased plasma volume with a decrease in systemic vascular resistance resulting in an increased cardiac output. During pregnancy, women with pulmonary hypertension are reported to have a high risk of morbidity and mortality.[146] This is because in the presence of pulmonary hypertension, pulmonary vascular disease prevents the fall in pulmonary vascular resistance and this results in an increase in pulmonary artery pressure and cardiac output. Progressive increases in pulmonary vascular resistance (PVR) will result in right ventricle (RV) failure and reduced cardiac output. Again there is increased risk of pulmonary emboli and pulmonary arterial thrombosis as a result of the hypercoagulability in pregnancy.

3.4.2 SCD and adverse fetal outcomes

There are recognized adverse fetal outcomes related to pregnancy in SCD compared to the non-SCD population. Adverse fetal outcomes include an increased odds of spontaneous abortions, intrauterine growth restriction,[10] low birth weight,[10] preterm birth/ prematurity,[10] stillbirth,[10] perinatal mortality,[10] and neonatal mortality.[10] The increased odds ratio are documented in Table 6. These increased odds of adverse fetal outcomes, may be attributed to the frequent maternal vaso-occlusive events with resultant micro-infarcts in the placenta which compromises fetal growth.

Table 6: Fetal outcomes in pregnant women with and without sickle cell disease

Condition	**OR**	**95% CI**	**p-value**	**Reference**
Intrauterine growth restriction	2.79	1.85– 4.21	Not documented	10
Low birthweight	2.00	1.42–2.83	Not documented	10
Preterm birth/ prematurity	2.14	1.56-2.95	<0.001	10
Stillbirth	4.05	2.59-6.32	<0.001	10
Perinatal mortality	3.76	2.34–6.06	Not documented	10
Neonatal mortality	2.71	1.41-5.22	0.003	10

3.4.3 Sickle cell disease, pregnancy and haemolysis

Chronic haemolytic anaemia is an important pathology in SCD; and haemolysis varies in intensity among the genotypes of SCD. Generally, the lifespan of sickled RBCs is less than 20 days.[18,19] As previously mentioned, the haemolytic process in SCD, has been linked to many of the pathophysiological processes of complications in SCD such as anaemia, acute vaso-occlusive events (acute pain, ACS, VTE) and end organ damage.

In SCD there is chronic haemolysis which results in a decreased bioavailability of nitric oxide. Low levels of NO adversely affects its positive properties such as regulating the vascular tone,[23] inhibiting endothelial adhesion molecule expression,[23] triggering platelet activation,[26] increasing platelet adhesive properties,[51] and potent antithrombotic effects.[23] This imbalance is skewed towards vasoconstriction, endothelial activation and proliferation, with enhanced vaso-occlusive events. The decreased nitric oxide bioavailability coupled with the pathologic effects of plasma cell free haemoglobin with enhanced vasoconstriction and vasculopathy result in haemolysis-related organ damage such as renal failure/ nephropathy, pulmonary hypertension with pulmonary thrombosis, chronic leg ulcers, venous thromboembolism (stroke) and cholelithiasis (gallstones).

Again, during intravascular haemolysis, a large fraction of extracellular haem is carried by erythrocyte-derived microparticles in the circulation, which then transfer their haem to endothelial cells.[26] This transfer, results in an increased expression of endothelial adhesion molecules [like vascular cell adhesion molecule-1 (VCAM-1) and intracellular adhesion molecule (ICAM-1)] which promote leukocyte recruitment and binding between circulating cells and the vessel wall,[52] with a resultant vascular dysfunction and vaso-occlusion in SCD.[26]

Haemolysis also results in an increased number of reticulocytes due to a high cell turnover. The reticulocytes have receptors and ligands eg. $\alpha 4\beta 1$ and CD36,[53] which adhere to the endothelium and leukocytes, providing another link between haemolytic anaemia and vaso-occlusion.[54]

Intravascular haemolysis is further associated with red cell membrane damage. This leads to the release of arginase and lactate dehydrogenase (LDH), as well as haemoglobin into plasma, and phosphatidylserine

exposure at the red cell membrane surface. This results in the activation of tissue factor causing thrombosis (haemostatic activation).[23]

Gladwin et al reported that "measures of haemolytic rate (haemoglobin, total and indirect bilirubin, reticulocyte count and lactate dehydrogenase) correlated with indices of hypercoagulability. In fact, hemoglobin levels were inversely correlated and lactate dehydrogenase values directly correlated with all measures of haemostatic activation, including thrombin-antithrombin complex (TAT), prothrombin fragment F1+2, D-dimer, sCD40L (a marker of platelet activation), and soluble VCAM-1 (a marker of endothelial activation)."[23]

The ischaemia- reperfusion injury, increased plasma levels of sCD40L and increased haemolysis are all potential mechanisms for increased tissue factor (TF) expression on monocytes and leucocytes;[147] and this triggers the coagulation cascade via the extrinsic pathway.

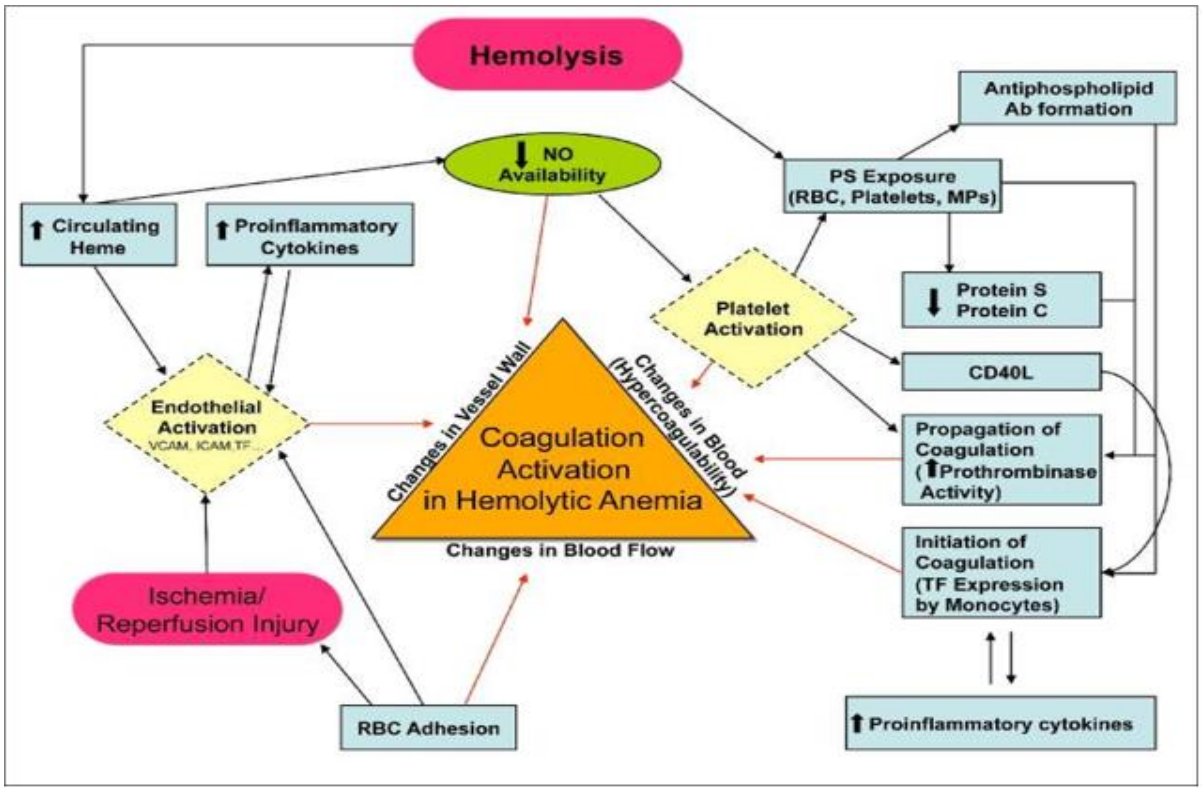

Figure 10: Processes involved in the hypercoagulable state in sickle cell disease (Ataga and Key, 2007)[148]

KEY: Ab: antibody; NO:nitric oxide; PS: phosphatidylserine; RBC: red blood cell; MPs: microparticles; TF: tissue factor

Hyperhaemolysis in pregnant women with SCD may further worsen anaemia, vaso-occlusion and end organ damage with a resultant increased risk of both maternal and perinatal morbidity.[111–114] The heterogeneity of the pathophysiology in SCD, however makes it unclear whether the presence of hyperhaemolysis (acute or exacerbated haemolysis) contributes to the adverse pregnancy complications observed in these patients. An increase in reticulocyte count and LDH during haemolysis have been associated with acute pain episodes.[84,85]

Contrary to this however, a study by van Tuijn et al in Netherlands (2010) reported that pregnancy complications in SCD were more prevalent in women with vaso-occlusion related organ damage than haemolysis related organ damage.[149]

CHAPTER 4: METHODOLOGY

4.1 Study Design:

This was a prospective cohort study conducted from 18th February 2019 to 30th December 2019 in pregnant women with and without SCD who received obstetric care at the Korle-Bu Teaching Hospital (KBTH); and non- pregnant women with SCD who received care at the adult sickle cell clinic of the Ghana Institute of Clinical Genetics (GICG), Korle-Bu.

4.2 Study site:

The study sites were the Obstetrics Department, KBTH, the adult sickle cell clinic of the Ghana Institute of Clinical Genetics and the Korle-Bu Polyclinic Laboratory.

Korle-Bu Teaching Hospital: The KBTH is the largest tertiary hospital in Ghana located in the national capital, Accra. The Accra sub- metropolitan area has an estimated population of about 4 million. The hospital serves as the major tertiary referral hospital for most of southern Ghana.

Obstetrics Department, KBTH: The department runs for 24hours every day, and has both outpatient and in-patient services. The inpatient services include two labour wards and six lying-in wards. The maternity unit has a total bed capacity of 261, and receives referral cases from both primary and secondary care facilities. Over 80% of the unit's 9,000 - 10,000 deliveries a year are referred from these facilities. The outpatient services include antenatal clinics; high-risk clinics like the SCD Obstetrics clinic; a Fetomaternal Unit with ultramodern ultrasound scans and a Public Health Unit.

SCD Obstetrics Clinic, KBTH: The SCD obstetrics clinic is held once a week at the Obstetrics Department, KBTH. Each year, approximately 150 to 200 pregnant women with SCD attend the antenatal and postnatal clinic. Majority of the pregnant women with SCD are referred from the Ghana Institute of Clinical Genetics (GICG). This clinic is run by a multi-disciplinary SCD obstetrics team including obstetricans, haematologists, anaesthesiologist with an interest in pulmonology, a midwife and public health nurses. Each pregnnat woman with SCD is reviewed by both an obstetrician and a haematologist on outpatient basis and during admissions for acute events.

Ghana Institute of Clinical Genetics: The GICG runs a daily outpatient clinic with urgent daycare beds. It is located on the KBTH campus. Approximately 2,500 adolescents and adults with SCD are attended to each year at the clinic, of which approximately 1,400 are women of reproductive age.[150]

Korle-Bu Polyclinic: The Korle-Bu Polyclinic is a 42-bed facility that offers primary health care to the Korle-Bu community, its environs and the city of Accra as a whole. It was established primarily to serve as a service delivery facility in the catchment area, but has grown over the years into a sub-BMC under the Korle-Bu Teaching Hospital. It has been accredited both by the Ghana College of Physicians and Surgeons and the West African College of Physician to train Family Physicians. They have a range of services which include a Laboratory. The laboratory is well resourced tailor made to meet the demands of the clinicians for ultimate patient care. The Biomedical scientists with the assistance of Medical Laboratory Technicians provide expert services in all departments of the laboratory. The laboratory services run are Microbiology, Chemical pathology, Immunology and Haematology, The haematology unit of the laboratory performs investigations, including Full blood count, Reticulocyte count, Sickling, Blood film for malaria parasites and Haemoglobin electrophoresis. The Haematology analyser in use is the Mindray BS5300 which is a five-part analyser and the Chemistry analyser is Mindray BC200E. The site was chosen due to cost effectiveness.

4.3 Study population

The study population included 25 pregnant women with SCD, 23 pregnant women without SCD (1st control) and 25 non- pregnant women with SCD (2nd control). Pregnant women without SCD were chosen as 1st controls to eliminate possible bias from pregnancy related events. The non-pregnant women with SCD were chosen as 2nd controls to eliminate possible bias from SCD related events as well as reduce confounders. Since the non-pregnant population also have SCD they are expected to have an ongoing chronic haemolysis which is part of the disease pathophysiology. We do not know the steady state values of the selected markers of haemolysis in the pregnant women with SCD prior to they becoming pregnant; hence these controls will provide an estimate of their distribution in the non-pregnant SCD steady state.

4.3.1 Inclusion criteria

1. All study participants were adults within the reproductive ages of 18 to 45 years as defined by World Health Organization (WHO).[151]
2. Pregnant women confirmed by a pregnancy test or/ and pelvic ultrasound scan.

3. Pregnant women in their second trimester with a confirmed diagnosis of SCD (HbSS, HbSC) by haemoglobin (Hb) electrophoresis.
4. Pregnant women with HbAA confirmed by haemoglobin (Hb) electrophoresis were chosen as 1st controls.
5. Non-pregnant women with a confirmed diagnosis of SCD (HbSS, HbSC) by haemoglobin (Hb) electrophoresis as 2nd controls.
6. Pregnant and non-pregnant women with SCD in steady state at enrollment/ baseline visit. That is, "should not have had any acute painful episode that required treatment in the emergency department or in the hospital for at least four consecutive weeks; no history of blood transfusion during the previous four months prior to enrollment; and no history of intercurrent illness such as infection or inflammation during the previous four weeks."[152]
7. Pregnant study participants should have at least two laboratory reports for each laboratory test (blood Hb, absolute reticulocyte count, serum bilirubin, serum LDH, AST, ALT and urine urobilinogen).
8. Non-pregnant women with SCD should have at least one laboratory report for each laboratory test (blood Hb, absolute reticulocyte count, serum bilirubin, serum LDH, AST, ALT and urine urobilinogen).

4.3.2 Exclusion criteria

1. Pregnant and non-pregnant women with SCD with other phenotypes on Hb electrophoresis.
2. Patients with sickle cell trait
3. Pregnant women who plan to deliver outside KBTH.
4. Non-pregnant women with SCD (2nd control) who subsequently became pregnant during the study period.

4.4 Sample Size Determination

The primary outcome of the study, is to establish the relationship between hyperhaemolysis (evidenced by increased markers of haemolysis) and fetomaternal outcome in pregnant women with SCD. An increase in reticulocyte count will be used as the marker for haemolysis. Since acute pain episode is the most

common cause of hospitalization in pregnant women with SCD,[13,14] it will be used as the main maternal outcome measure to calculate the sample size.

To determine the sample size required for this study, a statistical tool comparing the mean values of the reticulocyte counts during the steady state and acute painful episodes was used. Based on findings from the Ballas and Marcolina study,[84] the formula below was used in calculation of the sample size

$$N=[4\sigma^2(Z_{crt}+Z_{pwr})^2] / D^2$$ (Pagano et al. 2000)[153]

Where, $\sigma = 8.14$

Z_{crt} =1.96, the z-score at 95% confidence levels

Z_{pwr} = 0.842, the Z-value for statistical power of 0.8

D= minimum mean difference between the two states = 20.9 – 9.5 = 11.4

$N= 4 \times 8.14^2 (1.96+0.842)^2 / 11.4^2$

N= 16.23 = 17 per group; so three groups will be 51

Since study involved recruiting pregnant women, some of whom may not have delivered in KBTH e.g. if they went into labour at night**,** adding 30% non response or possible loss to follow up gave a total sample size of 66 for all the three groups**.** Rounding it up gave a sample size of 25 per group**.**

4.5 Study Procedure

The median gestational age at enrollment of pregnant women with SCD attending antenatal clinic at KBTH is 24.3 (range 7-40) weeks.[29] Hence, recruitment for the pregnant study participants started in the second trimester (13 weeks 1 day to 26 weeks gestation).

An Eligibility criteria form was used as a checklist for ease of recruiting all eligible patients ***(Appendix II)***; and unique study identification numbers given to all consented participants.

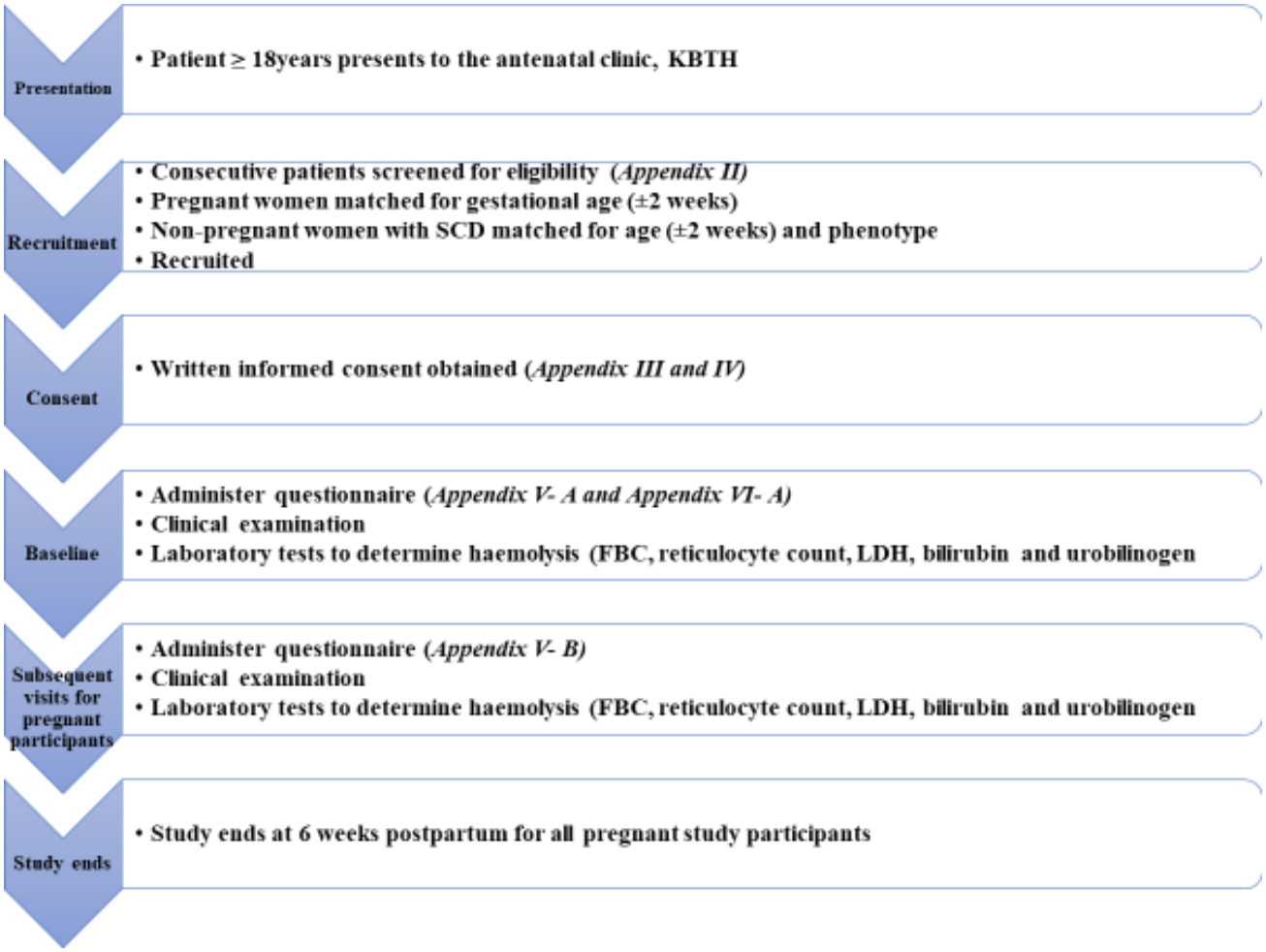

Figure 11: Process map showing recruitment and follow up in study participants

In addition to the above processes, all pregnant SCD study participants who were admitted with acute events, had some of the specified laboratory tests done at their own cost as part of standard care protocol of the SCD Obstetrics multidisciplinary care.

All non-pregnant women with SCD had only baseline laboratory samples (blood and urine) taken and processed for blood Hb, absolute reticulocyte count, serum bilirubin, serum LDH, AST, ALT and urine urobilinogen .

4.5.1 Sampling Methods

Using the attendance list for the day, consecutive patients [pregnant women with and without SCD in their 2nd trimester (gestational ages between 13 weeks 1 day to 26 weeks) attending antenatal care at KBTH;

and non-pregnant women with SCD receiving care at GICG] who met the inclusion criteria, were recruited. The pregnant women were matched for gestational age (± 2 weeks) but not age. Most pregnant women presenting to the clinic for their booking visit are unsure of the date of their last menstrual period. As such, the gestational age is determined by an obstetrics scan. During the second trimester an obstetrics scan gives an error margin of 10 to 14 days; hence there will be no difference in the gestational age match of ±2weeks. The non-pregnant women with SCD were matched for age (± 2 years) and phenotype to the pregnant women with SCD.

In order to ensure retention and avoid loss to follow up:

1. Details of all study procedures was explained to all individuals in their local language by study personnel (either study nurse, research assistants or principal investigator)
2. A summary of the study procedures was provided in the informed consent for them to read and understand**.**
3. All study procedures were done during the antenatal, postnatal or routine clinic visits to avoid loss to follow up.
4. Study participants were sent reminders a day prior to their intended follow up visit.

4.5.2 Data collection

The questionnaire (which took approximately 10 minutes to fill) was pretested on 4 patients (5% of the study population) before the study commenced. Two research assistants were trained to help to administering the questionnaire.

Consenting study participants, after having had the study thoroughly explained to them, filled out structured questionnaires at baseline ***(Appendix V- A and VI- A)*** and during follow up visits ***(Appendix V- B)*** which were used to assess participants' demographic characteristics and medical history. Each patient had a brief clinical examination after filling out the questionnaire.

The main maternal outcomes collected included:

a) Incidence of acute pain episodes
b) Incidence of acute chest syndrome (ACS)
c) Incidence of severe anaemia (evidenced by a drop in baseline Hb concentration by ≥2g/dL)

The main fetal outcomes collected included incidence of:

a) Spontaneous abortions
b) Preterm births
c) Low birthweight infants
d) Stillbirth infants/ Intrauterine fetal death (IUFD)

The following variables were recorded;

a) **Demographic data**: Age, education and occupation
b) **Haemoglobin phenotype**: SS, SC, AS and AA were recorded
c) **Obstetrics and Gynaecological history**: Gravidity, parity, gestational age at recruitment, history of abortions or stillbirths
d) **Clinical/ Medical history**: History of chronic illness; steady state haemoglobin; frequency of sickle cell crisis (acute pain episode and ACS episodes) during current pregnancy; number of hospitalizations/ admissions during current pregnancy (including reason and duration of admission); number of blood transfusions during current pregnancy; history of SCD chronic complications (stroke, nephropathy, gallstones. avascular necrosis (AVN), pulmonary hypertension, chronic leg ulcers).
   For clinical evidence of haemolysis, participants were also asked to state their urine colour, verify if they had noticed any progressive jaundice or increased yellow colouration of the eye, as well as state whether they had symptoms of anaemia.
e) **Clinical measures**: Blood pressure, pulse rate, oxygen saturation, respiratory rate, temperature.
   - Blood pressure and pulse rate were obtained using an OMRON digital BP machine (Intelli sense R) and measurement were done in the sitting position using the left arm
   - Oxygen saturation was obtained using a pulse oximeter
   - Respiratory rate was counted manually over 1 minute
   - Temperature was obtained using a digital thermometer
   - Weight was obtained using a standing scale
f) **Laboratory tests**: Samples were taken for Hb electrophoresis**,** FBC, manual reticulocyte count, serum LDH, serum bilirubin (total, direct and indirect), AST, ALT and urine urobilinogen.

4.5.2.1 Sample collection

Specimen required: Venous blood and fresh urine samples

Patient preparation: No special patient preparation was required before samples were taken.

Time periods of sample collection: Study samples were collected at convenience (between 8am and 12pm) on the clinic day when study participants presented at enrollment/ baseline and during the specified serial follow up visits- 28 and 36 weeks gestation; and six weeks postpartum for pregnant women with and without SCD).

In addition, laboratory results were obtained from the medical records of the pregnant women with SCD during acute events as part of standard care protocol of the multidisciplinary sickle cell obstetrics clinic.

For the non-pregnant control study participants laboratory samples were collected from 8am to 12pm at baseline/ enrollment during clinic hours at the adolescent and adult sickle cell clinic of the GICG.

The aim was to have at least two laboratory samples for all pregnant study participants with and without SCD, and at least one laboratory sample for non-pregnant women with SCD.

4.5.2.2 Method of sample collection:

- **Venous blood**: In total, 9mL of venous blood sample was drawn from each study participant at each study visit.
  - 4 mL of this venous blood was put into a bottle with ethylene diamine-tetra-acetic acid (EDTA) anticoagulant for haemoglobin (Hb) electrophoresis, FBC and reticulocyte count. The blood in the EDTA tube was then inverted 8 – 10 times to ensure adequate mixing.[154]
  - 5 mL of venous blood was put into a gel separator bottle for serum lactate dehydrogenase (S- LDH), serum bilirubin (direct and indirect bilirubin), AST and ALT tests. The blood in the gel separator bottle was allowed to clot.[154]
- **Urine sample**: At least 10 mL of fresh urine specimen was collected into a clean, dry, detergent-free urine container to test for urobilinogen.

4.5.2.3 Precautions taken during sample collection[154]:

The following precautions were adhered to;

- Sample collection was done under aseptic techniques.
- Sample collection was done in a quiet, well-lit area to prevent distraction and multiple or traumatic venepunctures.
- To avoid haemolysis which would interfere with analysis, the following precautions were taken:
  - Tourniquets were tied for short periods during sample collection
  - Mixing of blood samples in the tube was done by inverting the sample bottles gently eight times
- The venepuncture procedure was explained to each study participant before actual sample collection. This was to prevent anxiety attacks during blood sample collection.
- The samples from each subject were duly labelled with unique study identification numbers. This was done systematically to avoid errors.

4.5.2.4 Handling of sample:

- All laboratory samples were collected and labelled at the study sites- Obstetrics clinic, KBTH (for pregnant women) and GICG (for non-pregnant women).
- **Sample Transportation**: At the end of recruitment and follow-up visit for the day, the laboratory samples were transported to the laboratory at Korle-Bu Polyclinic(within the premises of Korle-Bu) in routine containers (for urine) and cryobox (for blood) at room temperature.
- **Receipt of specimen at the Laboratory**: Samples were visually checked or inspected for sufficient volume as well as for features of haemolysis before analysis.

4.5.2.5 Mode of sample and reagent storage

Haematology laboratory tests:

The RBCs, white blood cell count (WBC), platelet count, and red cell indices are documented to be stable for 8 hours after blood collection. Reticulocytes are considered relatively unstable after 4 hours especially at room temperature as a consequence of possible in vitro maturation to RBCs.[155] In view of the above, samples for Hb electrophoresis, FBC and absolute reticulocyte count were kept on the bench and laboratory tests run/ analysed within 4 hours after collection.

Biochemistry laboratory tests:

Storage of Reagents

Reagents were stored at 2 to 8°C strictly in an uninverted position and away from light. The reagent is stable until the expiration date appearing on the label when stored at this temperature. Once uncapped and on board the analyzer, reagents were stable for up to 23 days.

Storage of Specimen

Venous blood (5ml) was collected by standard venipuncture techniques into gel blood collection tubes (with inert gel barriers). Blood was allowed to clot adequately and centrifugation was effected at 5000 revolutions per minute for 15 minutes to harvest adequate serum for analysis. Serum was immediately separated from red cells and gel and kept in another test tube ready for analysis. It was ensured that the separated serum was entirely free of any clots. For serum specimen which were not run immediately after harvesting, samples were stored at 2 to 8ºC and run within the next 3 days. Serum samples are reported stable for ten days when refrigerated (2 to 8°C), two weeks when frozen (-20°C), and four days when stored at room temperature (15 to 30°C). Non-haemolysed serum was strictly ensured.

Sample for urinalysis: Urine samples were analysed within 4 hours after collection.

4.5.2.6 Laboratory analysis:

- The procedure for Haemoglobin (Hb) Electrophoresis is described in the next pages.
- Full blood count (FBC) was carried out using the Mindray BC5300 Automated Haematology Analyser. The principle and procedure for FBC by the manufacturers were followed.
- A manual reticulocyte count was carried out instead of the automated reticulocyte count due to cost. The procedure for manual reticulocyte count is described in the next pages. As part of Quality Control, random samples were sent to the Central Laboratory, KBTH and MDS Lancet (a private laboratory outside Korle-Bu) for analysis. Analysis on these samples were done using the Sysmex Automated Haematology Analyser, and results compared with the manual count.
- Blood biochemistry (serum LDH; total, direct and indirect serum bilirubin; AST and ALT) were carried out using the Mindray BS200E Fully Automated Biochemistry Analyzer. The principle and procedure for biochemistry laboratory tests by the manufacturers were followed.

- Test for urine urobilinogen
  - A urobilinogen test was carried out using the Combi 10 urine dipstick.
  - The principle and procedure for urobilinogen test by the manufacturers, were followed.
  - Before use, strips were examined for discolouration and expiry date.

4.5.2.6.1 Method for Haemoglobin (Hb) Electrophoresis

Principle of Hb Electrophoresis

At alkaline pH, Hb is a negatively charged protein and when subjected to a direct power supply from an electrical field will migrate toward the anode (+). Separation of the haemoglobins also depends on the size or molecular mass of the molecules, shape of the molecule (whether it is hydrophobic), the frictional effects of the medium, the strength of the electric field, the ionic strength of the buffer and the temperature of the buffer. Structural variants that have a change in the charged amino acids on the surface of the molecule at alkaline pH separate from Hb A eg. HbF, HbS, HbC, HbD and HbE. Haemoglobin variants that have an amino acid substitution that is sited internally (for example, haemoglobins with reduced oxygen affinity) may not separate from the haemoglobin variants of clinical importance, and those that have an amino acid substitution that has no effect on overall charge will not separate by electrophoresis.

Reagents and equipment

Tris-EDTA-Borate

Ponceau S Stain

5% Glacial acetic acid

Cellulose acetate membranes

Electrophoretic tank and power pack

Blotting paper

Applicators

Preparation of Haemolysate and Control

1. Blood samples of each patient, as well as the control sample were washed independently, four times in physiological saline (0.85%) by centifuging the blood sample at 3500 rpm for 5 minutes. This was to allow removal of plasma and denatured haemoglobins from the sample.
2. The washed red blood cells of each blood sample were then frozen at -80^0 C and thawed for 10 minutes to lyse the red cells.
3. A quarter volume carbon tetra chloride (CCl4) was then added to ensure further adequate removal of all red cell waste after centrifugation.
4. The mixture was vortexed and centrifuged to separate the haemoglobin solution.

5. In preparing controls, equal volumes of Hb AA, AS, and AC blood samples were mixed. This was to ensure a good representation of the haemoglobin bands A, S and C during separation.
6. Few drops of 0.2M KCN was added for elution.
7. A 20% concentration of haemolysate was then prepared.
8. The quality of the control was assessed by electrophoresis to ensure that three bands were present; bands A, S and C.

Storage of reagents:

The reagents were stored at 15 to 30^{o}C in a tightly closed bottle to prevent evaporation

Procedure for the Hb Electrophoresis

- The electrophoretic tank was filled with Tris-EDTA–Borate (TEB) buffer at a pH of 8.4 to a depth of about 2.5cm.
- The cellulose acetate membrane was then impregnated with the TEB buffer.
- 20µl of each test haemolysate was pipetted into the appropriate wells with a constant distance from one bridge to the other.
- The cellulose acetate membrane that was previously impregnated with the TEB buffer was then blotted with gauze to remove any obvious surface fluid.
- Tha applicator was then loaded by depressing the tips into the sample wells twice. The first load was applied to clean blotting paper while the second load was applied to the cellulose acetate membrane.
- By means of an applicator, the control haemolysate and test haemolysates are applied on the same line of origin on the cellulose acetate membrane.
- The cellulose acetate membrane was then positioned flat with the plastic side uppermost on the frame of the electrophoretic tank with the two ends in contact with the TEB buffer.
- The tank was subsequently covered and a a current of 50mA with voltage of 350V allowed to run for 30 min.
- The membrane was removed from the electrophoretic tank immediately after the run of 30 min and stained with Ponceau-S for about 5 min
- It was then de-stained with 2-5% glacial acetic acid in three successive washes, with each wash lasting 2 minutes. This was to ensure a clear or white background
- The cellulose acetate papers were then air-dried.

- The membranes were then labelled
- The Hb phenotypes were then read and recorded. The figure below demonstrates the relative mobility of some of the abnormal haemoglobins.

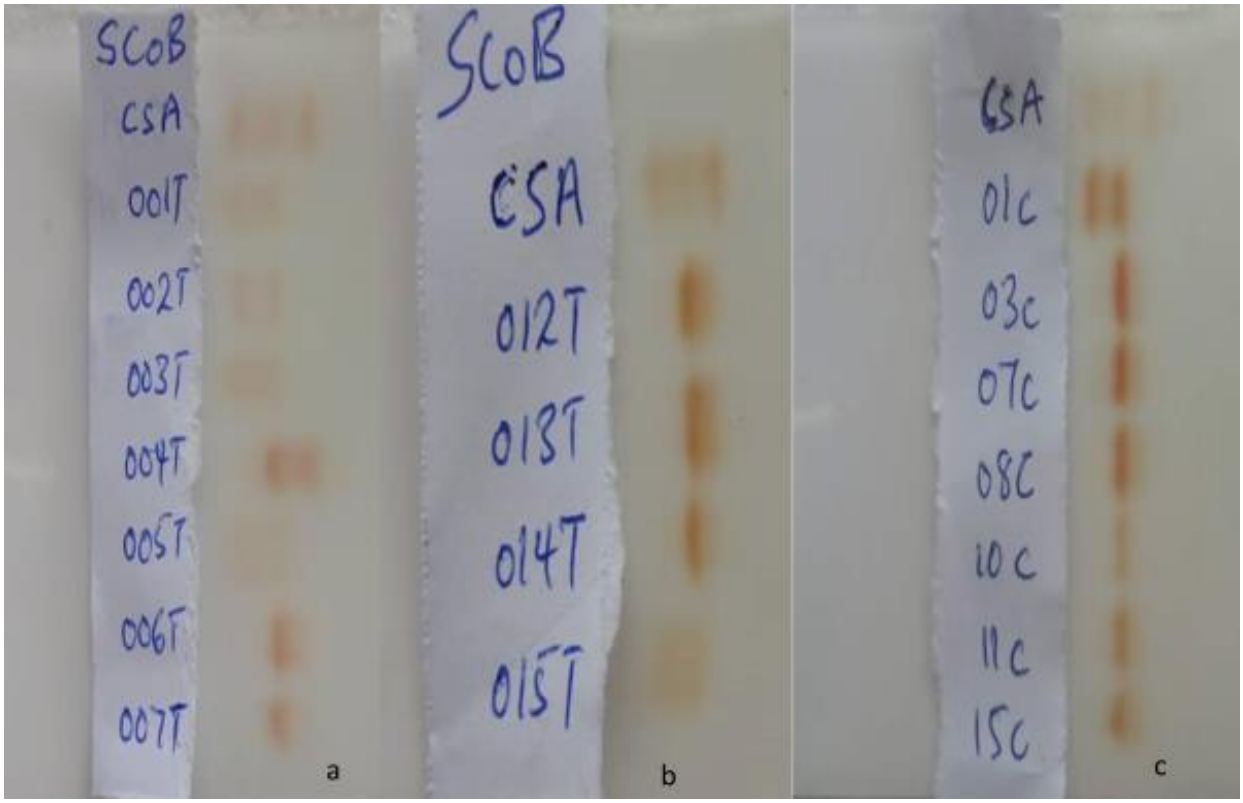

Figure 12: Unstained electrophoretic strips of study participants with SCD.

Figures (a) and (b) show electrophoretic patterns in pregnant women with SCD; Figure (c) shows electrophoretic patterns in non-pregnant women with SCD (2nd controls)

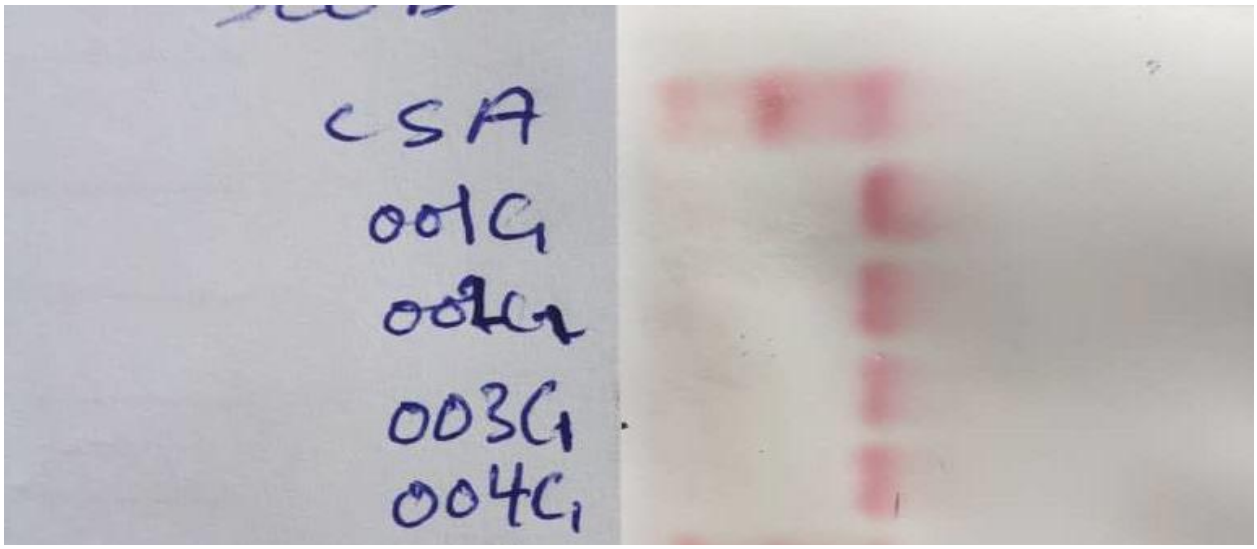

Figure 13: Stained electrophoretic strip of pregnant women without SCD (1st controls)

Precautions taken:

1. Reagents that showed signs of deterioration like dampness, discolouration or bacterial contamination were discarded
2. Ponceau S stain that had large amounts of precipitate were discarded
3. Freshly venepunctured whole blood samples collected into EDTA bottles were inverted eight times to ensure good mixing
4. Samples were washed with physiological saline four times
5. Samples were stored at -80^{o}C to ensure preparation of good haemolysates by the freeze and thaw method.
6. The quality of separation was affected primarily by both the amount of haemoglobin applied and the positioning of the origin.

4.5.2.6.2 Methodology for Full Blood Count (FBC) using Mindray BC5300 Automated Haematology analyser

Test Principle

The principle for the test is based on the coulter principle. Two electrodes placed in isotonic solutions are separated by a glass tube having a small aperture. A vacuum is applied and as a cell passes through the aperture, flow of current is impeded and a voltage pulse is generated. The requisite condition for cell counting by this method is high dilution of sample so that minimal numbers of cells pass through the aperture at one point of time. There are two electrodes on either side of the aperture; as the solution in which the cells are suspended is an electrolyte solution, an electric current is generated between the two electrodes. When a cell passes through this narrow aperture across which a current is flowing, change in electrical resistance (i.e. momentary interruption of electrical current between the two electrodes) occurs. A small pulse is generated due to a temporary increase in impedance. This pulse is amplified, measured, and counted. The height of the pulse is proportional to cell volume. The width of the pulse corresponds with the time required for the cell to traverse the aperture. Cells that do not pass through the center of the aperture generate a distorted pulse that is not representative of the cell volume.

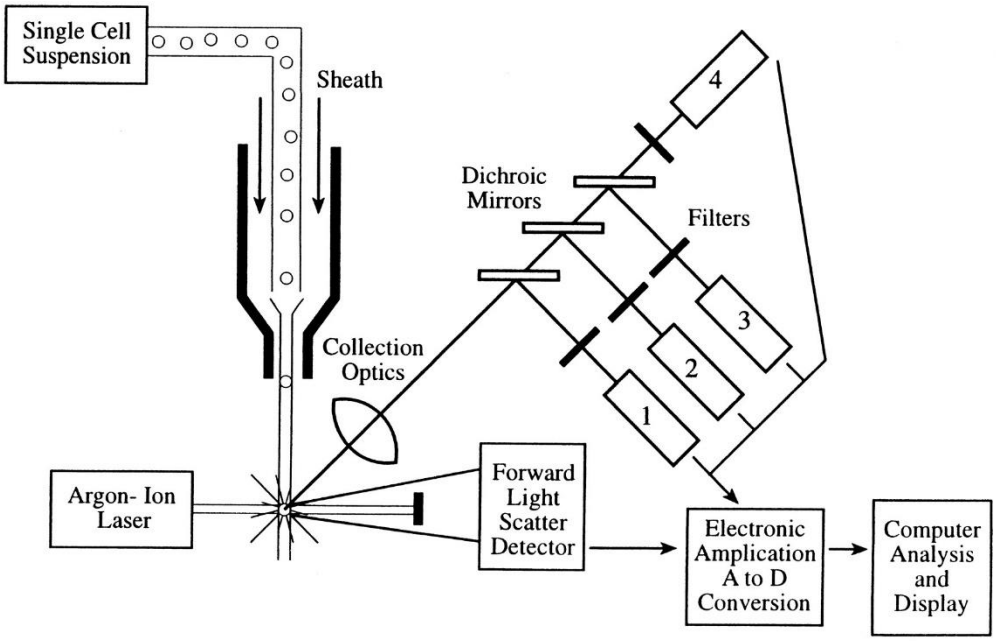

Figure 14: Schematic diagram demonstrating principle of flow cytometry for automated analyzers

The haemoglobin concentration was determined using the imidazole-haemoglobin complex measured at 540nm.

Procedure

The Mindray BC5300 Haematology analyser was calibrated using an International calibrant for which specific blood cell count values had been assigned. Commercial controls were run alongside the batch of each day's cell count on study samples. 4mL of freshly venepunctured whole blood specimen was collected into potassium ethylene diamine tetra acetic acid (EDTA) sample collection tubes. Sample was inverted eight times to ensure good mixing. Cell counts were performed by aspirating 25 µl of the freshly well mixed EDTA blood sample into the haematology analyser within 4 hours of sample collection. Results were displayed on the LED monitor. After results had been validated based on the clinical summary of the study participants, results were printed and entered into the database for data analysis.

4.5.2.6.3 Methodology for manual reticulocyte count

Principle

Using a supravital stain (new methylene blue), residual ribosomal ribonucleic acid (RNA) within the reticulocytes is precipitated. An equal volume of stain is then added to EDTA-anticoagulated blood, the dilution mixture incubated, and a smear is prepared. The smear is examined to determine the number of reticulocytes present. An erythrocyte containing two or more particles of blue-stained material is a reticulocyte. The number of reticulocytes is expressed as a percentage of the total number of erythrocytes counted.[156]

Specimen and storage

4mL of whole blood specimen in potassium EDTA sample collection tubes. Analysis was performed within 6 hours after sample collection.

Reagents

One gram (1.0 g) of New methylene blue (CI 52030) was dissolved in 100 ml of iso-osmotic phosphate buffer pH 6.5 to form the reticulocyte staining preparation.

Staining and slide preparation procedure

1. Using a plastic Pasteur pipette, 2 to 3 drops of the New Methylene blue dye solution were pipetted into a 75 × 10 mm plastic tube.
2. Two to 4 drops of the patient's thoroughly mixed EDTA anticoagulated blood was added to the dye solution.
3. The contents of the plastic tube were then mixed gently by using a Pasteur pipette to avoid haemolysis
4. The whole mixture was then incubated at room temperature (25°C) for 15–20 minutes.
5. At the end of the incubation time, the red cells sediment was resuspended by gentle mixing again with the Pasteur pipette.
6. Using a capillary tube, a drop of the resuspended mixture was placed near the frosted edge of three glass slides, and thin peripheral smears prepared.
7. The slides were then labelled with the patients' unique identification number and allowed to air dry.
8. After drying, the films were examined without fixing or counterstaining.

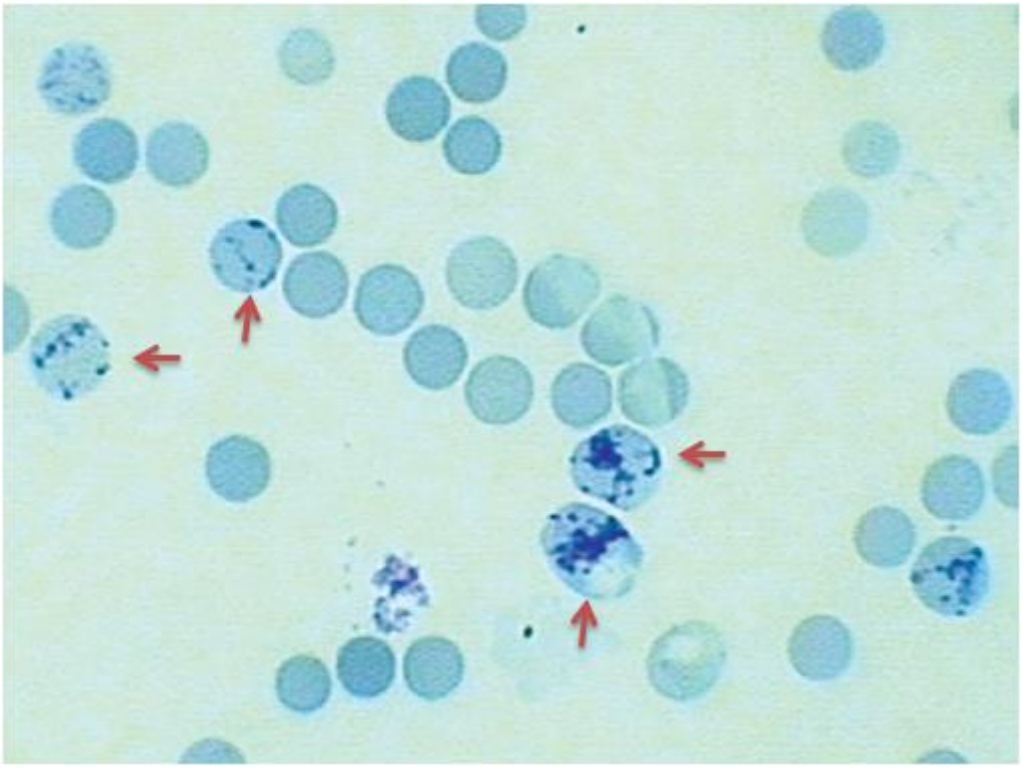

Figure 15: New methylene blue stain of reticulocytes [157]

Counting Procedure

1. The slide was placed on the microscope stage, and using the low power objective (x10), an area in the thin portion of the smear where the red cells were evenly distributed, undistorted, not touching each other and where the staining was optimum was chosen for examination.
2. The objective was carefully changed to the oil-immersion objective (x100); and an area in which there were approximately 100 red cells per oil immersion field was chosen.
   NB: This was done by mentally dividing the field into 4 quadrants. Cells in one quadrant were counted. If there were approximately 25 cells in one quadrant, then the field was good for counting.
3. The Miller Ocular eyepiece was used to count the cells with blue staining filaments or at least two or more discrete blue aggregates of reticulum in the erythrocyte.
   NB: The Miller disc imposes two squares (one 9 times the area of the other) onto the field of view. A good area for counting will show 3 – 10 RBCs in the smaller square of the Miller disc

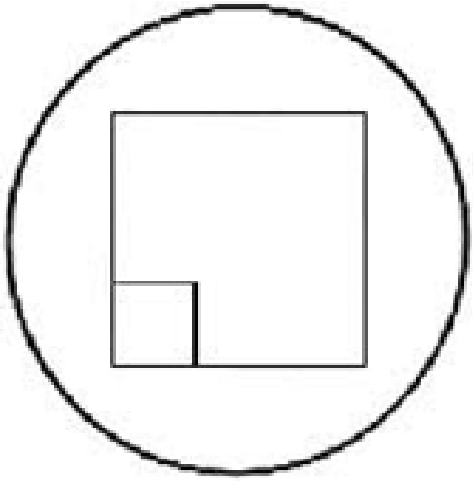

Figure 16: Miller Ocular Disc Square Microscope

4. The reticulocytes within the entire large square including those touching the lines on the left and bottom of the ruled area were counted.
5. RBCs in the smaller square whether they contained stained RNA or not were also counted.
6. All prepared 3 slides were counted and an average count used

Counting was continued until a minimum of 111 RBCs were observed between 15 – 20 fields. This corresponded to 1000 RBCs.

Calculation of reticulocytes:

Number of reticulocytes in n fields = x

Average number of red cells per field = y

Total number of red cells in n fields = $n \times y$

Reticulocyte percentage = $[x \div (n \times y)] \times 100\%$

Absolute reticulocyte count = % × RBC count

Results were then entered into the database.

At the Korle-Bu Polyclinic Laboratory where the samples were run, the normal reference range for the percentage reticulocyte count for adult females ranged between 0.5% to 4.0%; whereas the absolute reticulocyte count for adult females ranged between 18 x 10^9/L to 158 x 10^9/L.

Precautions taken:

1. Resuspension of the whole blood-stain mixture prior to preparing smears was done to avoid the reticulocytes from remaining on top of the mixture during incubation. Reticulocytes have a lower density than mature red blood cells.
2. The incubation time was not allowed to exceed the stipulated time of 15 – 20 minutes. This was to prevent the erroneous results of the new methylene blue dye adhering to mature red cells.
3. To decrease the error of imprecision between reticulocyte counts performed on the same blood sample
   a. Three slides were prepared for each sample, and an average of the reticulocyte count used
   b. An experienced biomedical scientist also performed counts on each patient independently, and counts compared. In the case of discrepancies, manual counts were repeated and counts were confirmed using the Sysmex automated haematology analyser from the Central Laboratory, KBTH or MDS_Lancet (a private laboratory).
   c. Random samples were sent to the Central Laboratory, KBTH for reticulocyte counts to be performed with the automated analysers. In the case of discrepancies, trouble-shooting for staining and slide preparation procedure as well as counting procedure was done, and technique improved upon.

Sources of error

a. The difference between a refractile appearance of erythrocytes and reticulocytes should be known
b. Filtration of the stain is required for precipitated material.
c. The difference between erythrocyte inclusions and reticulocytes should be known
d. Decreased reticulocyte counts may occur as a result of under staining of blood with New Methylene Blue thus incubation must be done within a specified time.
e. High glucose levels can cause reticulocytes to stain
f. Where no reticulocytes are observed after scan of at least two slides, report, “none seen”.

4.5.2.6.4 Methodology for blood biochemistry using Mindray BC200E Fully Automated Analyser

Test Principles

Serum Bilirubin Test

Total (conjugated and unconjugated) bilirubin couples with a diazo reagent in the presence of a surfactant to form azobilirubin. The diazo reaction is accelerated by the addition of surfactant as a solubilizing agent. The increase in absorbance at 548 nm due to azobilirubin is directly proportional to the serum bilirubin concentration.

Serum *Aspartate Aminotransferase (AST) Test*

Aspartate aminotransferase (AST) catalyzes the transfer of the amino group from L-aspartate to α-Ketoglutarate to yield oxalacetate and L-glutamate. The oxalacetate undergoes reduction with simultaneous oxidation of nicotinamide adenine dinucleotide + hydrogen (NADH) to nicotinamide adenine dinucleotide (NAD) in the malate dehydrogenase (MDH) catalyzed indicator reaction. The resulting rate of decrease in absorbance at 340nm is directly proportional to the AST activity. Lactate dehydrogenase (LDH) is added to prevent interference from endogenous pyruvate which is normally present in serum.

Serum *Alanine Aminotransferase (ALT) Test*

Alanine pyruvatetransferase (ALT) or Glutamate pyruvate transaminase (GPT) catalyses the reversible transfer of an amino group from alanine to ketoglutarate forming glutamate and pyruvate. The pyruvate produced is reduced to lactate by lactate dehydrogenase (LDH) and NADH: The rate of decrease in concentration of NADH, measured photometrically, is proportional to the catalytic concentration of ALT present in the sample.

Serum *Lactate Dehydrogenase (LDH) Test*

Lactate Dehydrogenase (LDH) catalyzes the conversion of lactate to pyruvate, the forward reaction and the conversion of pyruvate to lactate, the reverse reaction. The enzyme may be assayed using either material as a substrate; however, the enzyme activities obtained by the two methods are not directly comparable. The forward reaction does not require preincubation to exhaust endogenous α-keto acids and displays linearity over a wider range of activity in patient samples. This LDH method utilizes the forward

reaction. Lactate and NAD^+ are converted to pyruvate and NADH by the action of LDH. NADH strongly absorbs light at 340 nm, whereas NAD^+ does not. The rate of increase in absorbance at 340 nm is directly proportional to the LDH activity in the sample.

Procedure for serum biochemistry

The Mindray BC200E Fully Automated analyser was switched on and start-up effected for about 10 minutes for the software to synchronize with the fluidics and hydraulics of the analyser. The reagent mixture (ready to use) for the specific chemistry laboratory test was placed on board the analyser. Chemistry calibrator catalog number C7506-50 and control catalog number 12-C7592-50 were placed on board and calibration run for all serum biochemistry laboratory tests. Calibration for serum bilirubin was stable for approximately 14 days (336 hours) when run while that of AST and ALT was stable for approximately 23 days (552 hours). Calibration for LDH was also stable for approximately 30 days (720 hours) when run. Calibration was required with each change in reagent lot number. Calibration was verified with at least two levels of controls according to the established quality control requirements for the laboratory. Control solutions were run prior to specimen runs. On any occasion that control results fell outside acceptable ranges, recalibration was effected. Serum samples for study participants were thus run after calibration and controls and results displayed on the LED screen. After results had been validated based on the clinical summary of the study participants, results were printed and entered into the database for data analysis.

At the Korle-Bu Polyclinic Laboratory where the samples were run, the normal reference range for the total serum bilirubin, direct serum bilirubin and indirect serum bilirubin were 3.4 to 22.0 umol/L, $\leq$ 5.13 umol/L and $\leq$10.0 umol/L respectively. The normal reference range used for AST and ALT was 0 – 35 U/L. The normal reference range used for LDH was 100 – 190 U/L.

4.5.2.6.5 Methodology for testing urine urobilinogen

Test Principle

Urobilinogen and diazonium produce pink azo dyes under the function of a strong acid medium.

Specimen and storage

Fresh urine specimen were voided into clean, dry, leak-proof wide-mouthed screw capped containers. Specimen was mixed well prior to analysis within 4 hours of collection.

Procedure

1. The reagent test strip was taken from the canister and the cap promptly replaced after taking out strip.
2. Strips were then dipped briefly in the urine making sure that the test areas were fully immersed.
3. Excess urine was removed from the strip by running the edge of the strip along the rim of the urine container.
4. The strip was held up horizontally and reaction was then read in good light at exactly the time stated by the manufacturer.
5. Reactions were compared by holding the strip close to the colour chart on the container label and results recorded.

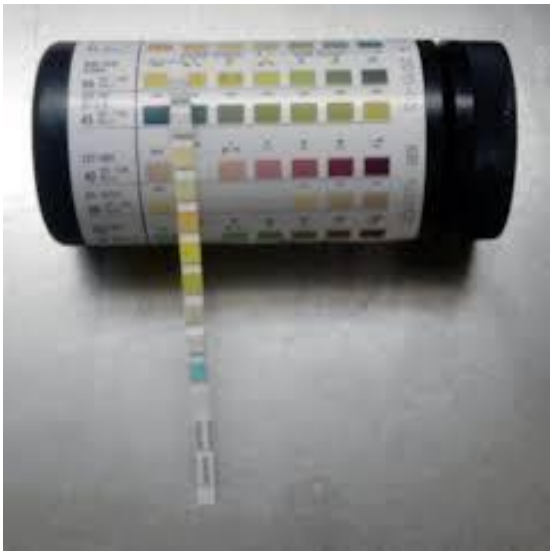

Figure 17: Urine dipstick and container[158]

Precautions

The strip was read between 1-2 minutes after dipping.

For positive results the test was repeated and compared with the colour chart at the time specified.

Colour changes beyond 2 minutes were not recorded.

Controls were read before test specimen were analyzed.

4.5.2.7 Use of controls

Controls were run daily on both the haematology and chemistry analyzers before samples were run.

How controls were obtained:

- Controls for the haematology and blood biochemistry analysers were obtained from the manufacturers.
- For Hb electrophoresis, controls were obtained from individuals with HbAA, HbAS and HbAC phenotypes and prepared at the beginning of each week.
- Control reference solutions for the urinalysis were obtained from the manufacturers.

Storage of controls:

- ***Haematology and blood biochemistry analysers***:
  - The storage instructions supplied with the controls by the manufacturer were adhered to.
  - The control samples were kept refrigerated at 2-8^{0}C.
- ***Urine dipsticks/ strips:***
  - To prevent deterioration of the reagent urine strips, the following were adhered to:
  - The urine strips were protected from moisture and excessive heat and light.
  - The urine strips were not refrigerated.
  - The urine strips were removed only as required.

4.5.3 Data Handling

All data collected for this study were used for research purposes only. The data collected from patient's medical record were limited to only what is necessary to carry out the study as outlined. No data from the study was reported on an individual basis; all findings from this study were reported in statistical summary form only. All data were treated with strict confidentiality. Each participant was assigned a unique study code number that was used on all data forms; patient names were not used. A list of patient names and study code numbers were maintained separately from the data extraction forms, under secured lock. The Principal Investigator was the only one who had access to this information. Access to the files were logged electronically. Data will be stored for two years after the study (from December 2019 to 31st December 2021) after which data will be discarded.

4.5.4 Statistical / Data analysis

The study sought to determine the association between increased haemolysis and fetomaternal outcome. Analysis was done using Stata statistical software package (StataCorp.2007. Stata Statistical Software. Release 14. StataCorp LP, College Station, TX, USA) and significant levels was taken as $\alpha < 0.05$. Data was presented in tables and charts.

Categorical variables such as the phenotype, parity, education and occupation were summarised by frequencies and proportions. Continuous variables such as age, weight, gestational age (at enrollment and delivery), total duration of follow up and selected markers of haemolysis were summarised by means and standard deviation (std dev).

The student t- test was used to compare the means of the gestational age at enrollment, delivery and total follow up time between the pregnant women with and without SCD; and between the pregnant women with HbSS and HbSC phenotypes.

One-way analysis of variance (ANOVA) was used to compare the means of the markers of haemolysis between pregnant women with and without SCD. A one- way analysis of variance (ANOVA) test was also used to compare the means of the selected markers of haemolysis in the pregnant women with SCD at enrollment (baseline) and during the follow up visits (28 weeks, 36 weeks and 6 weeks postpartum).

For the first objective, which was to determine the frequency of hyperhaemolysis in pregnant women with SCD, the criteria for defining hyperhaemolysis in SCD (***see Glossary***) was used to determine the number of pregnant women with SCD who met the criteria.

The second and third objectives were to determine the association between hyperhaemolysis during pregnancy and acute maternal outcomes in women with SCD; and to determine the association between hyperhaemolysis during pregnancy and adverse fetal outcomes in women with SCD. There was no increased frequency of haemolysis (hyperhaemolysis) hence no logistic regression was run.

The fourth objective was to compare the mean steady state values of the selected markers of haemolysis in non- pregnant women with SCD and pregnant women with SCD. Comparison was made using the t-test with a p value of less than 0.05.

Additionally, the Fisher's exact test was used to compare the maternal outcomes such as mode of delivery; and the fetal outcomes such as preterm delivery, birthweight and perinatal outcome between the pregnant women with and without SCD. The significant level was set at a p value less than 0.05.

The pain rate was defined and calculated as the sum of the number of pain episodes requiring hospitalization during the study period per the number of patient- years; with the number of patient-years being calculated as the sum of duration of follow up during and after pregnancy.

The ACS rate was defined and calculated as the sum of the number of ACS episodes requiring hospitalization during the study period per the number of patient- years; with the number of patient-years being calculated as the sum of duration of follow up during and after pregnancy.

CHAPTER 5: ETHICAL CONSIDERATIONS

This study was approved by the Scientific and Technical Committee and the Institutional Review Board of the Korle-Bu Teaching Hospital (approval number: KBTH-IRB/000100/2018)- ***Appendix I***. In addition, Institutional approval was granted by the Department of Obstetrics, KBTH and the Ghana Institute of Clinical Genetics (GICG), Korle-Bu.

The study was fully and clearly explained to all eligible participants in a language understood by them, using the study information leaflet provided. Acceptance was confirmed by signing of the consent form by the participant. All consent forms were countersigned by the Principal Investigator.

All data collected for this study were used for research purposes only. The data collected from the medical record were limited to only what was necessary to carry out the study as outlined. No data from the study were reported on an individual basis; all findings from this study were reported in statistical summary form only.

Patient confidentiality was assured and study documents were safely locked and kept by the Principal Investigator. The Principal Investigator was the only one who had assess to the documents.

The participants were informed of the voluntary nature of their participation and their liberty to withdraw from the study at any point should they wish to do so, without affecting their care subsequently.

All cost of treatment (including blood transfusions and intravenous fluids) were borne by the study participants since it is the normal routine management procedure. There was no extra cost to the study participants beyond their routine follow up, investigations and procedures as part of their standard of care.

Possible Benefits: The direct benefits to the study participants with SCD from this study were that they were referred to the adult sickle cell clinic (Ghana Institute of Clinical Genetics, GICG) to continue management at the end of the study. The indirect benefits to the study participants with SCD were that, participation may help develop new and more effective preventive measures as well as better understanding and treatment modalities for the management of pregnant women with sickle cell disease.

Possible Risks: This research presented minimal risk to participants and their babies. The main risks included, psychological distress from discussing upsetting issues such as hospital admissions, treatment options and adverse fetal outcomes e.g. stillbirths and intra-uterine growth restriction. Confidentiality was protected as described in the section below. There was also some discomfort in drawing blood sample, even though, every effort was made to reduce such experience to the barest minimum.

Participants were not paid for their participation in the study; however, in order to ensure retention, pregnant study participants were given transportation fare for their follow- up visits.

CHAPTER 6: RESULTS

Data for 73 study participants (25 pregnant women with SCD, 23 pregnant women without SCD and 25 non-pregnant women with SCD) were used for analysis. Figure 23 below describes the selection criteria.

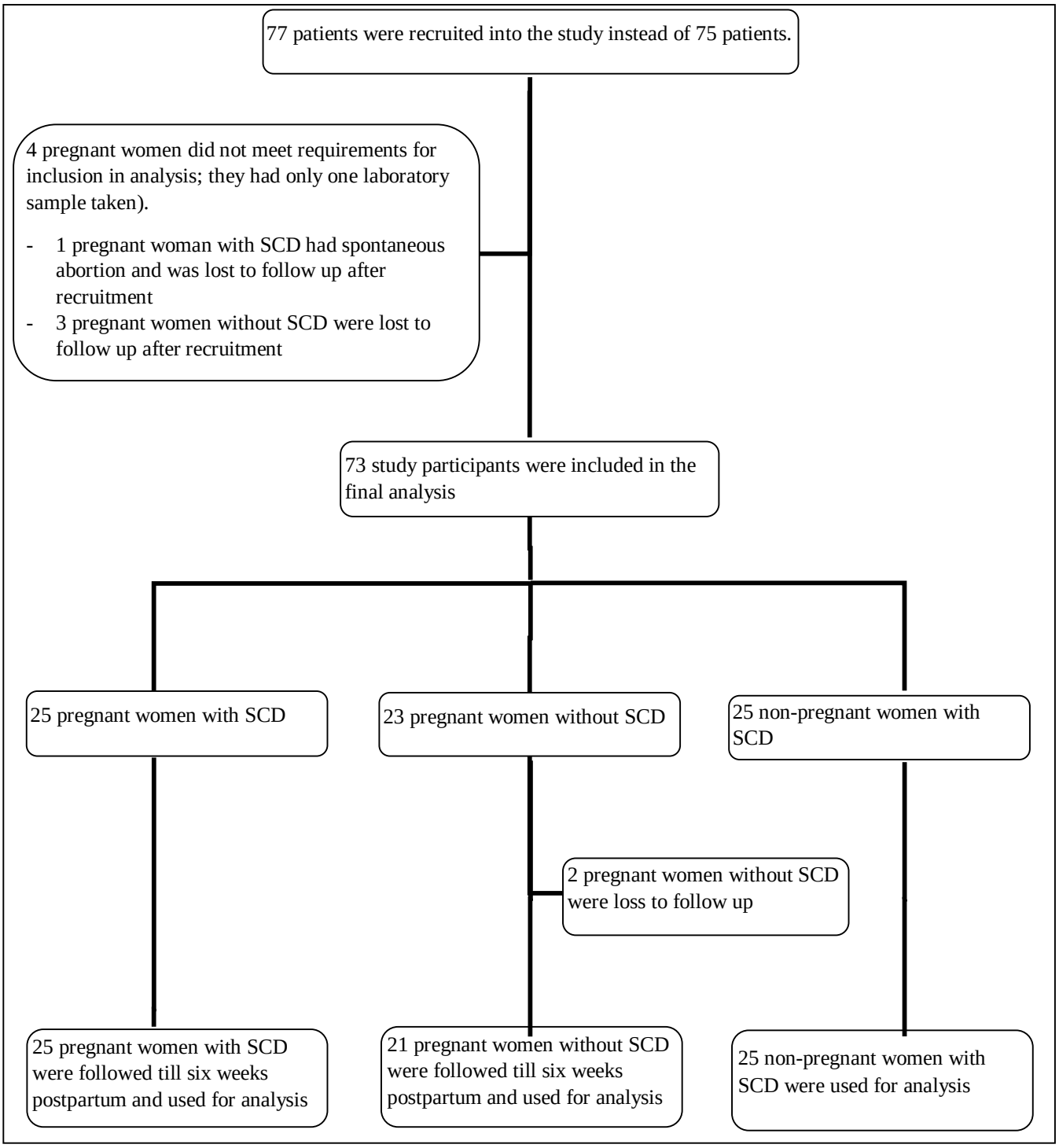

Figure 18: Flowchart showing selection criteria of study participants included in the analysis

6.1 Socio-demographic characteristics of study participants at enrollment

The 73 study participants were aged between 21 and 42 years with a mean age of 30.3 ± 5.3 years. Although the pregnant women with and without SCD were not matched for age, there was no significant difference in their mean ages (29.8 ± 5.2 years vrs 31.7 ± 5.4 years respectively; p=0.42). The mean weight of the pregnant women with SCD was 62.8 ± 8.4 kg; while the mean weight of the pregnant women without SCD and non-pregnant women with SCD were 76.7 ± 18.1 kg and 59.8 ± 11.3 kg, respectively. Almost all the study participants had some level of formal education, with more than 60.0% having acquired a secondary education. About two-thirds each of the SCD cohort [15 (60.0%) pregnant women with SCD and 17 (68.0%) non-pregnant women with SCD], and three-quarters of the pregnant women without SCD [18 (78.3%)] had an informal occupation such as petty trading..

Among the pregnant women with SCD, 16 (64.0%) had sickle cell anaemia while 9 (36.0% had HbSC phenotype as shown in Figure 21 below

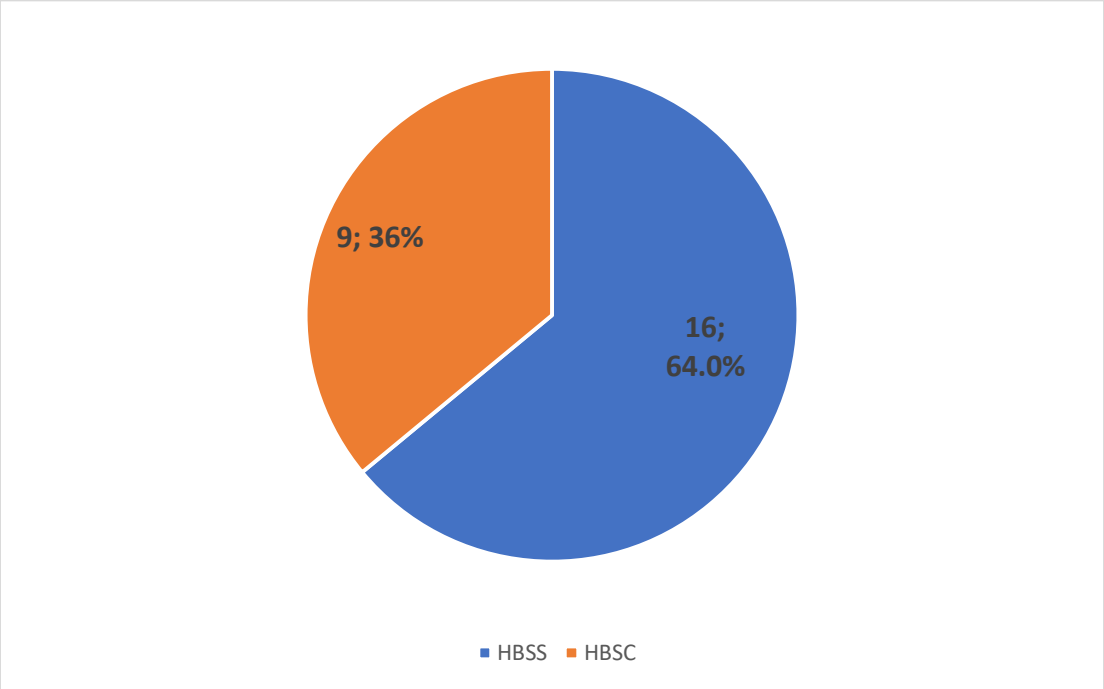

Figure 19: Phenotypic distribution of pregnant women with sickle cell disease

6.2 Baseline study characteristics of the pregnant women with and without SCD

The mean gestational age at enrollment for both the pregnant women with and without SCD (n=48) was 19.4 ± 3.7 weeks (SCD: 19.1 ± 3.8 weeks; non-SCD: 19.7 ± 3.7 weeks; p=0.58). There was no significant difference in the mean gestational age at delivery between the pregnant women with and without SCD (37.0 ± 2.8 weeks vrs 36.2 ± 5.4 weeks respectively; p=0.57). The mean duration of follow-up for both the pregnant women with and without SCD was 22.6 ± 5.4 weeks (23.8 ± 5.4 weeks vrs 21.3 ± 5.6 weeks respectively; p=0.11). All the pregnant women with SCD had had between zero and two births at enrollment. In contrast, 20 (87.0%) of the pregnant women without SCD had zero to two births. There was however no significant difference in the parity between the pregnant women with and without SCD (p=0.26).

6.3 Frequency of hyperhaemolysis in pregnant women with SCD

The follow up times in the pregnant women with SCD were categorized as enrollment, 28 weeks gestation, 36 weeks gestation and six weeks postpartum.

Using the criteria for defining hyperhaemolysis (***see Glossary)***, half of the women at 28 weeks gestation, and a third at 36 weeks gestation and six weeks postpartum respectively, had a drop in Hb from baseline; with only one of them in each category having a decrease in Hb ≥ 20% from baseline. In the pregnant women with SCD who had a decrease in Hb ≥ 20% from baseline, there was no corresponding increase in reticulocyte count by ≥25% from baseline. None of the pregnant women with SCD met the diagnostic criteria for defining hyperhaemolysis (Figures 22, 23 and 24).

This study hence did not demonstrate hyperhaemolysis in pregnant women with SCD.

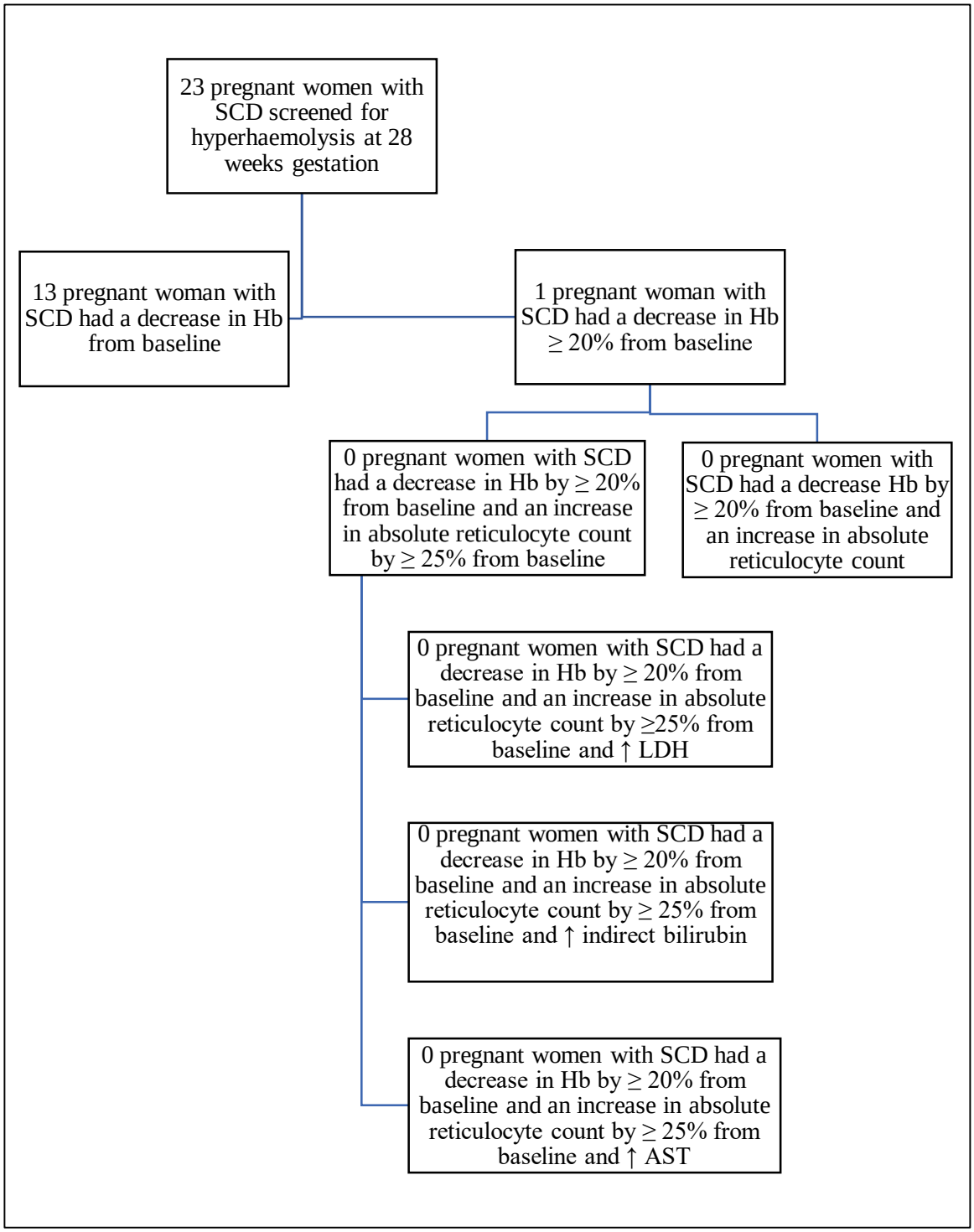

Figure 20: Pregnant women with SCD meeting criteria for defining hyperhaemolysis at 28 weeks gestation

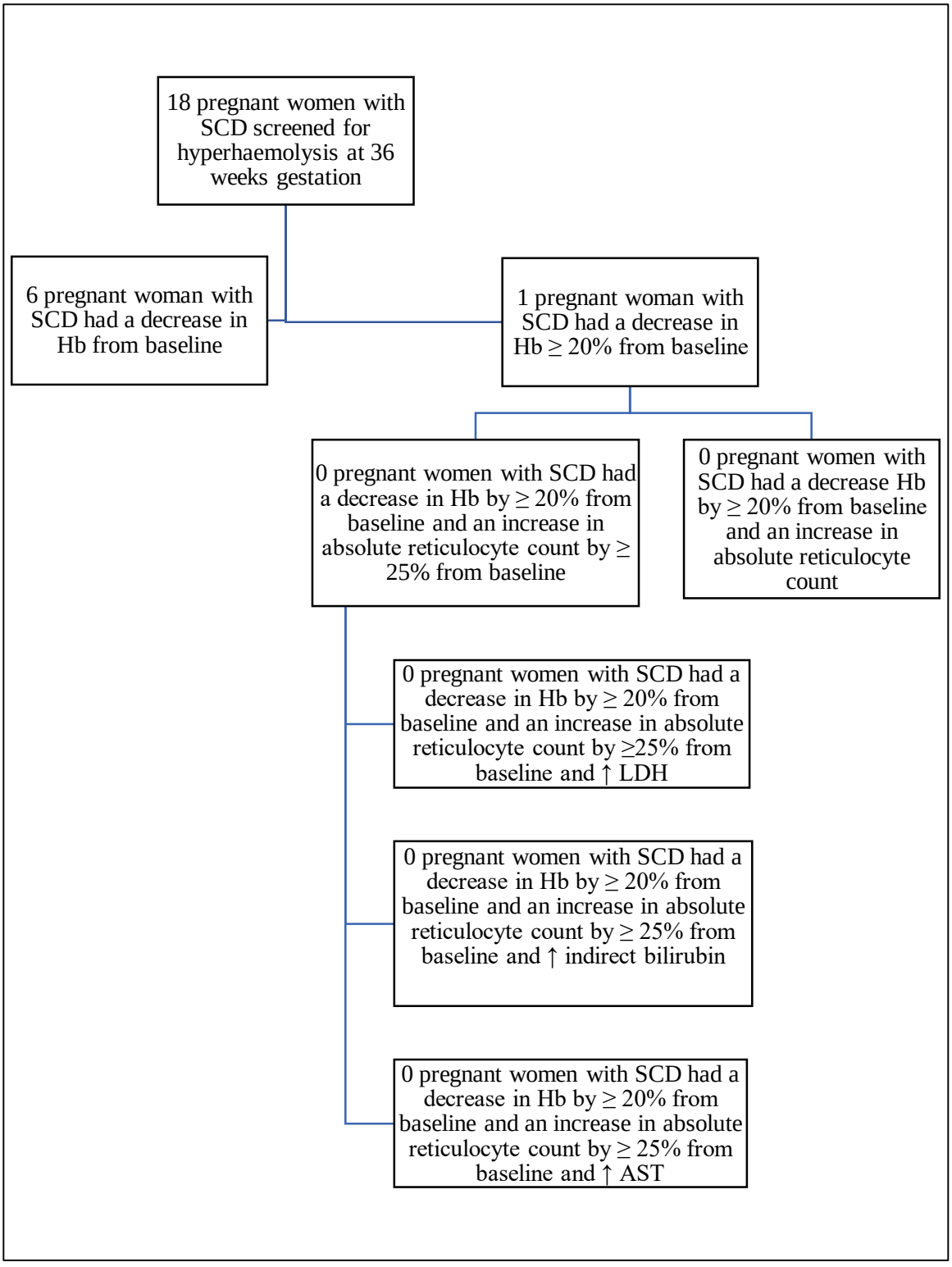

Figure 21: Pregnant women with SCD meeting criteria for defining hyperhaemolysis at 36 weeks gestation

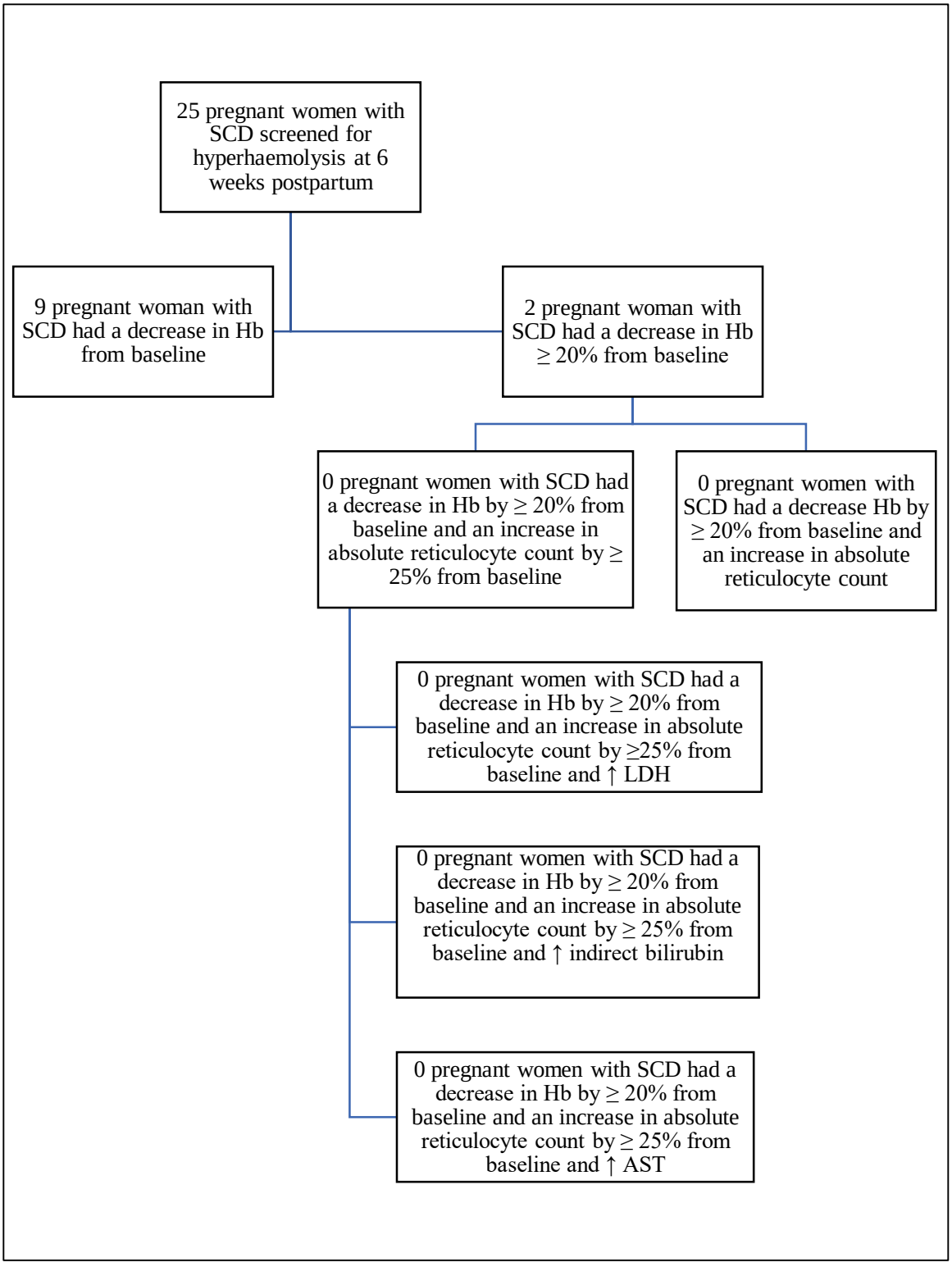

Figure 22: Pregnant women with SCD meeting criteria for defining hyperhaemolysis at 6 weeks postpartum

6.4 Association between hyperhaemolysis and adverse maternal and fetal outcomes in women with SCD

The aim of this study was to determine the association between hyperhaemolysis and fetomaternal outcomes in pregnant women with SCD. Using the criteria for defining hyperhaemolysis in SCD (***see Glossary***), none of the pregnant women with SCD met this criteria as shown in Figures 22, 23 and 24. This study therefore did not demonstrate hyperhaemolysis in pregnant women with SCD; hence no association between hyperhaemolysis and fetomaternal outcome could be determined.

6.5 Steady state values of the selected markers of haemolysis in non-pregnant women with SCD compared pregnant women with SCD

Generally, the mean steady state concentrations of the markers of haemolysis in the non-pregnant women with SCD were relatively higher compared to the laboratory values in the pregnant women with SCD. There was no statistically significant difference in the Hb ($p= 0.89$), absolute reticulocyte count ($p= 0.27$), direct bilirubin ($p= 0.05$), indirect bilirubin ($p= 0.05$), LDH ($p= 0.14$), AST ($p= 0.05$) and ALT ($p= 0.24$). There was however a statistical difference in the steady state total bilirubin in non-pregnant women with SCD compared to the pregnant women with SCD ($p= 0.04$).

Table 7: Steady state laboratory parameters of both pregnant and non-pregnant women with sickle cell disease

Laboratory parameters (baseline)	Non-pregnant women with SCD (N= 25)	Pregnant women with SCD (N= 25)	p-value
Mean Hb, (std. dev.) (g/dL)	8.7 (1.8)	8.8 (1.8)	0.89
Mean absolute reticulocyte count, (std. dev.)(x 10^9/L)	103.0 (32.4)	122.7 (80.7)	0.27
Mean total bilirubin, (std. dev.) (umol/L)	43.0 (36.3)	26.2 (13.5)	0.04
Mean direct bilirubin, (std. dev.) (umol/L)	8.9 (7.9)	5.5 (3.4)	0.05
Mean indirect bilirubin, (std. dev.) (umol/L)	34.1 (31.8)	20.6 (11.3)	0.05
Mean LDH, (std. dev.) (IU/L)	373.2 (152.3)	322.1 (73.3)	0.14
Mean AST, (std. dev.) (IU/L)	42.7 (25.2)	30.5 (15.9)	0.05
Mean ALT, (std. dev.) (IU/L)	35.1 (20.7)	27.9 (22.1)	0.24

6.6 Additional results

6.6.1 Mean values of laboratory parameters of the pregnant women with and without SCD at baseline, 28 weeks and 36 weeks gestation

Table 8, 9 and 10 compares the laboratory parameters between the pregnant women without SCD and the two predominant SCD phenotypes (HbSS and HbSC).

At baseline, the pregnant women with sickle cell anaemia had relatively higher mean concentrations of the markers of haemolysis with the exception of the mean Hb. The absolute reticulocyte count, total bilirubin, direct bilirubin, indirect bilirubin, AST and ALT values were not statistically different compared to both the pregnant women with HbSC and HbAA at p=0.27, p=0.11, p=0.07, p=0.17, p=0.14 and p=0.83 respectively. The mean Hb and mean LDH were however elevated, and statistically different compared to both the pregnant women with HbSC and HbAA (p=0.01; p=0.01 respectively), *Table 8*.

Similarly, the pregnant women with HbSS had relatively higher mean concentrations of the markers of haemolysis with the exception of the mean Hb at 28 weeks gestation. The mean Hb and mean LDH were statistically different compared to both the pregnant women with HbSC and HbAA (p=0.00; p=0.01 respectively). All the other haemolytic markers were not statistically significant when the three groups were compared- absolute reticulocyte count (p=0.71), total bilirubin (p=0.41), direct bilirubin (p=0.13), indirect bilirubin (p=0.37), AST (p=0.37) and ALT (p=0.07), *Table 9*.

At 36 weeks gestation, the markers of haemolysis were elevated in the pregnant women with SCA compared to the pregnant women with HbSC and HbAA, but the absolute reticulocyte count, total bilirubin and indirect bilirubin were not statistically significant at p=0.13, p=0.10 and p=0.46 respectively. The Hb, direct bilirubin, LDH, AST and ALT were significantly elevated at p=0.00, p=0.00, p=0.02, p=0.03 and p=0.02 respectively, *Table 10*.

Table 8: Baseline laboratory parameters of pregnant women with HbSS and HbSC compared to HbAA

		Pregnant women with HbSS (n = 16)	**Pregnant women with HbSC (n = 9)**	**Pregnant women with HbAA (n=23)**	**p value**
Mean Hb, (std dev) (g/dL)		8.1 (1.2)	9.9 (2.1)	10.4 (1.2)	0.01
Mean absolute reticulocyte count, (std dev) (x 10^9/L)		136.4 (96.2)	98.3 (33.2)	115.5 (22.6)	0.27
Mean total bilirubin, (std dev) (umol/L)		29.4 (14.3)	20.3 (10.4)	23.7 (11.3)	0.11
Mean direct bilirubin, (std dev) (umol/L)		6.5 (3.7)	3.9 (1.9)	1.7 (1.4)	0.07
Mean indirect bilirubin, (std dev) (umol/L)		23.0 (12.1)	16.4 (8.8)	19.0 (10.7)	0.17
Mean AST, (std dev) (IU/L)		34.1 (18.1)	34.2 (6.5)	15.6 (5.9)	0.14
Mean ALT, (std dev) (IU/L)		28.6 (25.1)	26.6 (16.9)	18.0 (7.1)	0.83
Mean LDH, (std dev) (IU/L)		351.0 (67.4)	270.7 (54.4)	185.9 (99.3)	0.01
Urobilinogen test, n (%)	Normal	16 (100.0)	9 (100.0)	23 (100.0)	0.23
	Increased	0 (0.0)	0 (0.0)	0 (0.0)	
Bilirubin, n (%)	Neg	16 (100.0)	9 (100.0)	23 (100.0)	0.24
	Pos	0 (0.0)	0 (0.0)	0 (0.0)	

Table 9: Laboratory parameters of the pregnant women with HbSS and HbSC compared to HbAA at 28 weeks gestation

		Pregnant women with HbSS (n = 14)	**Pregnant women with HbSC (n = 9)**	**Pregnant women with HbAA, n=17**	**p value**
Mean Hb, (std dev) (g/dL)		8.0 (0.9)	9.8 (0.8)	10.7 (1.2)	0.00
Mean absolute reticulocyte count, (std dev) (x 10^9/L)		121.9 (76.5)	109.4 (55.6)	128.8 (34.7)	0.71
Mean total bilirubin, (std dev) (umol/L)		32.8 (16.6)	20.9 (7.0)	20.2 (9.4)	0.41
Mean direct bilirubin, (std dev) (umol/L)		10.5 (10.9)	4.6 (2.9)	4.5 (5.6)	0.13
Mean indirect bilirubin, (std dev) (umol/L)		22.3 (10.2)	16.3 (5.6)	15.5 (8.2)	0.37
Mean AST, (std dev) (IU/L)		44.7 (66.1)	25.5 (15.8)	21.3 (7.7)	0.37
Mean ALT, (std dev) (IU/L)		22.6 (13.5)	21.2 (7.1)	24.4 (9.5)	0.07
Mean LDH, (std dev) (IU/L)		358.6 (145.9)	306.6 (137.1)	210.3 (81.9)	0.01
Urobilinogen test, n (%)	Normal	14 (100)	9 (100)	17 (100)	0.20
	Increased	0	0	0	
Bilirubin, n (%)	Neg	14 (100)	9 (100)	17 (100)	0.34
	Pos	0	0	0	

Table 10: Laboratory parameters of pregnant women with HbSS and HbSC compared to HbAA at 36 weeks gestation

		Pregnant women with HbSS (n = 9)	**Pregnant women with HbSC (n = 9)**	**Pregnant women with HbAA, n=14**	**p value**
Mean Hb, (std dev) (g/dL)		8.1 (1.0)	10.0 (0.9)	11.0 (1.0)	0.00
Mean absolute reticulocyte count, (std dev) (x 10^9/L)		136.8 (48.6)	101.2 (30.9)	118.4 (29.8)	0.13
Mean total bilirubin, (std dev) (umol/L)		56.4 (30.3)	28.9 (18.1)	16.9 (7.2)	0.10
Mean direct bilirubin, (std dev) (umol/L)		16.2 (6.6)	5.6 (3.7)	5.3 (5.4)	0.00
Mean indirect bilirubin, (std dev) (umol/L)		38.1 (31.8)	23.2 (17.7)	13.4 (5.4)	0.46
Mean AST, (std dev) (IU/L)		44.6 (30.5)	24.6 (17.0)	20.7 (6.5)	0.02
Mean ALT, (std dev) (IU/L)		44.3 (19.6)	25.5 (17.6)	26.5 (12.3)	0.03
Mean LDH, (std dev) (IU/L)		387.0 (154.2)	205.6 (40.5)	223.8 (173.9)	0.02
Urobilinogen test, n (%)	Normal	9 (100)	9 (100)	14 (100)	0.64
	Increased	0	0	0	
Bilirubin, n (%)	Neg	9 (100)	9 (100)	14 (100)	0.71
	Pos	0	0	0	

6.6.2 Trends in concentrations of laboratory parameters during follow up in pregnant women with SCD

The means of the laboratory parameters in the pregnant women with SCD showed no statistically significant change during follow up through to six weeks postpartum: Hb (p=0.88), absolute reticulocyte count (p=0.87), total bilirubin (0.53), direct bilirubin (p=0.46), indirect bilirubin (p=0.63), LDH (p=0.82), AST (p=0.97) and ALT (p=0.66), Table 11.

Table 11: Trends in concentration of haemolytic markers in pregnant women with SCD during the study period

	Enrollment (n=25)	**28 weeks gestation (n=23)**	**36 weeks gestation (n=18)**	**6 weeks postpartum (n=25)**	**P value**
Mean Hb, (std. dev.) (g/dL)	8.8 (1.8)	8.7 (1.2)	9.1 (1.3)	9.2 (1.8)	0.88
Mean absolute reticulocyte count, (std. dev.) (x 10^9/L)	122.7 (80.7)	117.0 (68.0)	119.9 (43.5)	139.3 (58.6)	0.87
Mean total bilirubin, (std. dev.) (umol/L)	26.2 (13.5)	28.1 (14.7)	42.7 (28.1)	29.4 (20.4)	0.53
Mean direct bilirubin, (std. dev.) (umol/L)	5.5 (3.4)	8.2 (9.1)	10.9 (7.5)	6.0 (4.7)	0.46
Mean indirect bilirubin, (std. dev.) (umol/L)	20.6 (11.3)	19.9 (9.0)	30.7 (26.1)	23.3 (19.0)	0.63
Mean LDH, (std. dev.) (IU/L)	322.1 (73.3)	328.3 (141.8)	296.3 (143.8)	351.6 (139.6)	0.82
Mean AST, (std. dev.) (IU/L)	30.5 (15.9)	37.2 (52.6)	34.6 (26.1)	35.2 (23.4)	0.97
Mean ALT, (std. dev.) (IU/L)	22.1 (11.2)	27.9 (22.1)	34.9 (20.5)	29.7 (22.9)	0.66

6.6.3 Trends in concentrations of laboratory parameters during follow up in pregnant women with HbAA

The means of the laboratory parameters in the pregnant women with HbAA showed no statistically significant change during follow up through to six weeks postpartum: Hb (p=0.29), absolute reticulocyte count (p=0.53), total bilirubin (0.31), direct bilirubin (p=0.18), indirect bilirubin (p=0.25), LDH (p=0.68), AST (p=0.06) and ALT (p=0.07) , Table 12.

Table 12: Trends in concentrations of laboratory parameters in pregnant women without SCD during study period

	Enrollment, n= 23	**28 weeks gestation, n= 17**	**36 weeks gestation, n= 14**	**6 weeks postpartum, n= 21**	**p value**
Mean Hb, (std. dev.) (g/dL)	10.4 (1.2)	10.7 (1.2)	11.0 (1.0)	11.1 (1.5)	0.29
Mean absolute reticulocyte count, (std. dev.) (x 10^9/L)	115.5 (22.6)	128.8 (34.7)	118.4 (29.8)	130.7 (53.1)	0.53
Mean total bilirubin, (std. dev.) (umol/L)	23.7 (11.3)	20.2 (9.4)	16.9 (7.2)	21.5 (16.1)	0.31
Mean direct bilirubin, (std. dev.) (umol/L)	1.7 (1.4)	4.5 (5.6)	5.3 (5.4)	3.6 (6.5)	0.18
Mean indirect bilirubin, (std. dev.) (umol/L)	19.0 (10.7)	15.5 (8.2)	13.4 (5.4)	17.9 (13.2)	0.25
Mean AST, (std. dev.) (IU/L)	15.6 (5.9)	21.3 (7.7)	20.7 (6.5)	20.3 (9.9)	0.06
Mean ALT, (std. dev) (IU/L)	18.0 (7.1)	24.4 (9.5)	26.5 (12.3)	20.3 (10.9)	0.07
Mean LDH, (std. dev.) (IU/L)	185.9 (99.3)	210.3 (81.9)	223.8 (173.9)	248.6 (117.1)	0.68

6.6.4 Fetomaternal outcomes in pregnant women with and without SCD

Maternal outcomes

Hypertensive disorders in pregnancy:

Three (12.0%) of the pregnant women with SCD (all with HbSS) were diagnosed and had hospital admissions for hypertensive disorders in pregnancy- two during prepartum (with one complicated by sickle cell nephropathy); and the last at two weeks postpartum. There were however more pregnant women without SCD diagnosed with hypertensive disorders in pregnancy than pregnant women with SCD [7 (33.3%) vrs 3 (12.0%); p=0.03], *Table 13.*

Mortality:

All the pregnant women with and without SCD were still alive at the end of the study. This put the maternal case fatality rate at 0%, *Table 13.*

Delivery

There were 24 (96.0%) deliveries among the pregnant women with SCD compared to 18 (85.7%) deliveries among the pregnant women without SCD. Three of the participants without SCD had spontaneous abortions. In contrast, only one pregnant woman with SCD had a spontaneous abortion. The pregnant women with SCD had more caesarean deliveries compared to vaginal deliveries [18 (72.0%) vrs 4 (19.0%)]. In all, there was a significant difference in the mode of delivery between the pregnant women with SCD and the pregnant women without SCD (p=0.01), *Table 13.*

Perinatal outcomes

Time of delivery: None of the pregnant women with and without SCD had very preterm births (at 28 to 32 weeks gestation). There were 10 (41.7%) moderate to late preterm births (32 weeks to 36 weeks + 6 days gestation) in the pregnant women with SCD compared to five (27.8%) moderate to late preterm births in the pregnant women without SCD. Most of the deliveries in both the pregnant women with and without SCD were term deliveries [14 (58.3%) vrs 13 (72.2%)] respectively. There was however no significant difference in the number of preterm and term births between the pregnant women with and without SCD (p=0.35), *Table 13.*

Birthweight: None of the pregnant women with and without SCD had very low birthweight babies (< 1,500g). There were seven (29.2%) low birthweight babies (< 2,500g) in the pregnant women with SCD compared to three (16.7%) low birthweight babies in the pregnant women without SCD. Most of the babies in both the pregnant women with and without SCD had normal weight (2,500g to 4,000g) at birth [16 (66.7%) vrs 14 (77.8%)] respectively. Two pregnant women with and without SCD had big babies (> 4,000g) each. Though the rate of LBW babies almost doubled in women with SCD compared to those without SCD, 29.2% and 16.7% respectively, the difference was not significant when the frequencies were compared (p=0.74). There was also no significant difference in the mean birthweights of babies born to the pregnant women with SCD compared to pregnant women without SCD (2.9 ± 0.7 kg vrs 3.3 ± 0.4 kg respectively; p=0.21), *Table 13.*

Fetal outcome: There was only one (4.0%) spontaneous abortion in the pregnant women with SCD compared to three (14.3%) spontaneous abortions in the pregnant women without SCD. Two (8.0%) pregnant women with SCD had intra-uterine fetal deaths (IUFD) whereas no pregnant woman without SCD had IUFD. In all 22 (88.0%) pregnant women with SCD and 18 (85.7%) of the pregnant women without SCD had live births. There was no statistical difference in the fetal outcome in the pregnant women with SCD compared to the pregnant women without SCD (p=0.28)**,** *Table 13.*

Perinatal deaths: There was a higher perinatal death rate in the pregnant women with SCD compared to the pregnant women without SCD (83.3 vrs 0 per 1,000 total births respectively)**,** *Table 13*.

Table 13: Fetomaternal outcomes in pregnant women with and without sickle cell disease

Characteristic		Pregnant women with SCD (n = 25)	Pregnant women with HbAA (n = 21)	P value
MATERNAL OUTCOME				
Number of patients diagnosed with Hypertensive disorders in pregnancy, n (%)		3 (12.0%)	7 (33.3%)	0.03
Mean gestational age at delivery (std. dev)		37.0 (2.8)	36.2 (5.4)	0.57
Mode of delivery, n (%)	Caesarean	18 (72.0)	4 (19.0)	0.01
	Vaginal	6 (24.0)	14 (66.7)	
	Not delivered	1 (4.0)	3 (14.3)	
Maternal outcome, n (%)	Alive	25 (100.0)	21 (100.0)	
	Deaths	0 (0.0)	0 (0.0)	
PERINATAL OUTCOME				
Time of delivery, n (%)	Very preterm (28 to 32 weeks)	0 (0.0)	0 (0.0)	0.35
	Moderate to Late preterm (32 weeks to 37 weeks + 6 days)	10 (41.7)	5 (27.8)	
	Term delivery (>37 weeks to 40 weeks)	14 (58.3)	13 (72.2)	
Mean birthweight (std. dev)		2.9 (0.7)	3.3 (0.4)	0.21
Birthweight, n (%)	Very low birthweight (<1,500g)	0 (0.0)	0 (0.0)	0.74
	Low birthweight (<2,500g)	7 (29.2)	3 (16.7)	
	Normal weight (2,500g to 4,000g)	16 (66.7)	14 (77.8)	
	Big baby (>4,000g)	1 (4.2)	1 (5.6)	
Fetal outcome, n (%)	Spontaneous abortions	1 (4.0)	3 (14.3)	0.28
	Intra-uterine fetal death (IUFD)	2 (8.0)	0 (0.0)	
	Alive	22 (88.0)	18 (85.7)	
Perinatal deaths, n (%)		2 (8.0)	0 (0.0)	-
Perinatal deaths per 1,000 total births		83.3	0	-

Not delivered includes spontaneous abortions (< 28 weeks gestation).

6.6.5 Demographic characteristics of pregnant women with SCD according to phenotypes

Comparing the pregnant women with HbSS to HbSC, there was no difference in their mean age (30.0 ± 5.5 years vrs 29.4 ± 4.7 years; p=0.80), mean gestational age at enrollment (19.2 ± 3.3 weeks vrs 19.0 ± 4.8 weeks; p=0.90) and the mean gestational age at delivery (37.0 ± 1.9 weeks vrs 36.8 ± 4.1 weeks; p=0.90).

6.6.6 Fetomaternal outcomes in pregnant women with SCD based on haemoglobin phenotypes

Maternal outcome

Hospital admissions:

Thirteen (52.0%) of the pregnant women with SCD had 20 admissions for acute events (acute pain episode, anaemia and haemolysis) and for hypertensive disorder in pregnancy. Of the 13 pregnant women with SCD who were admitted, five (38.5%) were admitted for acute pain episode only, two (15.4%) for acute pain with anaemia, and one (7.7%) each for anaemia only; anaemia with haemolysis; acute pain with haemolysis; and acute pain with hypertensive disorder in pregnancy. Two (15.4%) of the pregnant women with SCD were also admitted for hypertensive disorder in pregnancy alone. Almost all of the patients who were admitted had HbSS. Only one pregnant woman with HbSC disease had an admission for acute pain with haemolysis.

Acute pain: Nine pregnant women with SCD (HbSS: 8; HbSC: 1) were admitted for acute pain episodes during the study visit. There were however 13 admissions for acute pain episode among these nine pregnant women with SCD; with HbSS patients having 12 admissions for acute pain compared to pregnant women with HbSC phenotype [12 (75.0%) vrs 1 (11.0%); p= 0.01]. The pain incidence rate was 1.23 events per patient-years in the pregnant women with SCD (HbSS: 1.14 events per patient-years; HbSC: 0.09 events per patient-years). Most of the acute pain events occurred during the third trimester (8/13; 61.5%) with five (38.5%) of them occurring in the second trimester.

Acute chest syndrome: There were no admissions for acute chest syndrome in neither the pregnant women with sickle cell anaemia (HbSS) nor the pregnant women with HbSC disease.

Delivery:

Even though more pregnant women with HbSS phenotype had more caesarean deliveries compared to the pregnant women with HbSC disease [13 (81.3%) vrs 5 (55.6%), there was no significant difference in their mode of delivery (p=0.14).

Perinatal outcome:

Time of delivery: There were seven (46.7%) moderate to late preterm births (32 to 37 weeks gestation) in the pregnant women with HbSS compared to three (33.3%) moderate to late preterm births in the pregnant women with HbSC. Most of the deliveries in both the pregnant women with sickle cell anaemia and with HbSC disease were term deliveries [eight (53.3%) vrs six (66.7%)] respectively. There was no significant difference in number of babies born preterm between the HbSS and HbSC women (p=0.68).

Birthweight: There were seven (46.7%) low birthweight babies (<1,500g) delivered to mothers with HbSS. In contrast, none of the pregnant women with HbSC had low birthweigh babies. Most of the babies delivered to both the pregnant women with sickle cell anaemia and with HbSC disease had normal birthweights [eight (53.3%) vrs eight (88.9%)] respectively. Only one (11.1%) woman with HbSC disease had a big baby. There was a significant difference in the frequencies of birthweights in babies born to pregnant women with HbSS compared to the pregnant women with HbSC phenotype (p=0.03). Similarly, there was a significant difference in the mean birthweights of the pregnant women with SS compared to SC; 2.7 ± 0.5 and 3.3 ± 0.4 respectively (p<0.01).

Fetal outcome: The pregnant women with HbSS had one (6.3%) spontaneous abortion and two (12.5%) intra-uterine fetal deaths (IUFD) whereas no pregnant woman with HbSC had a spontaneous abortion or IUFD. There were 13 (93.8%) live births in the pregnant women with HbSS and 9 (100.0%) livebirths in the pregnant women with HbSC. There was no statistical difference in the fetal outcome in the pregnant women with SCD compared to the pregnant women without SCD (p=0.69).

Perinatal deaths: **Both** perinatal deaths (IUFD: 2) were in the pregnant women with HbSS phenotype compared to HbSC (2 perinatal deaths compared to 0 perinatal deaths respectively).

6.6.7 Fetomaternal outcomes in pregnant women with sickle cell anaemia compared to HbAA

Maternal outcomes:

Hypertensive disorders in pregnancy:

Three (12.0%) of the pregnant women with HbSS were diagnosed and had hospital admissions for hypertensive disorders in pregnancy- two during prepartum (with one complicated by sickle cell nephropathy); and the last at two weeks postpartum. More pregnant women without SCD were diagnosed with hypertensive disorders in pregnancy than pregnant women with SCD but this was not statistically significant (p=0.14].

Mortality:

All the pregnant study subjects were still alive at the end of the study. This put the maternal case fatality rate at 0%.

Delivery:

The mean gestational age at delivery in the pregnant women with sickle cell anaemia compared to pregnant women with HbAA was 37.0 ± 1.9 weeks vrs 37.0 ± 1.9 weeks respectively (p=0.57). There were 16 deliveries among the pregnant women with HbSS compared to 18 (85.7%) deliveries among the pregnant women without SCD. Three (14.3%) of the participants with HbAA had spontaneous abortions. In contrast, only one pregnant woman with HbSS had a spontaneous abortion. The pregnant women with HbSS had more caesarean deliveries compared to vaginal deliveries [13 (81.3%) vrs 4 (19.0%)]. In all, there was a significant difference in the mode of delivery between the pregnant women with HbSS and the pregnant women without SCD (p=0.01).

Perinatal outcomes

Time of delivery: None of the pregnant women with HbSS and HbAA had very preterm births (28 to 32 weeks gestation). There were 7 (46.7%) moderate to late preterm births (32 weeks to 36 weeks + 6 days gestation) in the pregnant women with sickle cell anaemia compared to five (27.8%) moderate to late preterm births in the pregnant women without SCD. Most of the deliveries in both the pregnant women with HbSS and HbAA were term deliveries [8 (53.3%) vrs 13 (72.2%)] respectively. There was however

no significant difference in the number of preterm and term births between the pregnant women with and without SCD (p=0.26).

Birthweight: The mean birthweight in the pregnant women with sickle cell anaemia compared to pregnant women with HbAA was 2.7 ± 0.5kg vrs 3.0 ± 0.8kg respectively (p=0.04). None of the pregnant women with HbSS and HbAA had very low birthweight babies (< 1,500g). There were seven (46.7%) low birthweight babies (< 2,500g) in the pregnant woman with HbSS compared to three (16.7%) low birthweight babies in the pregnant women without SCD. Most of the babies in both the pregnant women with sickle cell anaemia and without SCD had normal weight (2,500g to 4,000g) at birth [8 (53.3%) vrs 14 (77.8%)] respectively. One pregnant woman without SCD had a big baby (> 4,000g). Though the rate of LBW babies more than doubled in women with HbSS compared to those with HbAA, 46.7% and 16.7% respectively, the difference was not significant when the frequencies were compared (p=0.14).

Fetal outcome: There was only one (6.3%) spontaneous abortion in the HbSS group compared to three (14.3%) spontaneous abortions in the HbAA group. Two (12.5%) pregnant women with HbSS had intra-uterine fetal deaths (IUFD) whereas no pregnant woman without SCD had IUFD. In all 13 (81.3%) pregnant women with HbSS and 18 (85.7%) of the pregnant women without SCD had live births. There was no significant difference comparing the fetal outcomes in the women with HbSS compared to those with HbAA (p=0.20).

Perinatal deaths: There were more perinatal deaths in the HbSS group compared to the HbAA group (2 vrs 0 respectively).

CHAPTER 7: DISCUSSION

7.1 Hyperhaemolysis and fetomaternal outcome

Chronic haemolysis, is an ongoing feature and an important pathology of SCD. Haemolysis varies in intensity among the genotypes of SCD;[18,19] and increased haemolysis (hyperhaemolysis) is known to occur in stressful conditions. Pregnancy, is a stressful condition. The frequency of hyperhaemolytic episodes during pregnancy and the association between hyperhaemolysis and adverse fetomaternal outcomes amongst women with SCD have not yet been established. The aim of this study was to determine the association between hyperhaemolysis and fetomaternal outcomes in pregnant women with SCD.

This study reports novel findings in pregnant women with SCD. Contrary to what has been postulated that pregnancy is stressful, and stressful conditions result in increased haemolysis in SCD patients,[27] the mean markers of haemolysis during follow up were not statistically different. Similarly, using the criteria for defining hyperhaemolysis- "marked drop in Hb determined in the steady state with evidence of increased red blood cell destruction in the absence of other unidentifiable causes such as splenic and hepatic sequestration" (***see Glossary***), none of the pregnant women with SCD met this criteria during follow up. Haemolysis was hence not increased in the pregnant women with SCD in this study. Hyperhaemolysis may therefore not be a significant complication of pregnancy in SCD.

Haemolysis is a complex process that can occur intravascularly, extravascularly or both. It can be diagnosed by a combination of non-specific laboratory tests including serum LDH, reticulocyte count and serum bilirubin. The site of haemolysis may differentially affect the LDH and bilirubin levels. With the exception of an elevated mean serum bilirubin and mean serum LDH throughout pregnancy, all the other markers of haemolysis for the pregnant women with SCD remained within the normal reference range in this study. Most of the pregnant women with SCD had hyperbilirubinaemia (both conjugated and unconjugated) and an elevated serum LDH during follow up. The elevated mean direct and indirect serum bilirubin and serum LDH supports the evidence that the haemolysis in SCD is both intravascular and extravascular; but largely extravascular.[39] Unfortunately, the highly sensitive and specific markers of intravascular haemolysis such as haptoglobin, haemopexin, erythrocyte adenylate kinase and

haemosiderin in urine were not tested; hence a conclusion cannot be made about intravascular haemolysis in pregnant women with SCD.

Haemolysis in general, has been associated with acute pain episodes.[84,85] Even though the baseline serum bilirubin and serum LDH values of the pregnant women with SCD were elevated albeit of a lower value compared to the non-pregnant women with SCD, the selected markers of haemolysis in this study were not associated with acute pain episodes in this cohort. This contradicts findings from other studies where haemolysis evidenced by an increase in reticulocyte count[84] and an increase in serum LDH[85] have been associated with acute pain events. In this study, the serum LDH value in the nine pregnant women with SCD who had acute pain was increased by approximately 9.9% from the baseline or steady state value at the time of acute pain; and this is low compared to the percentage increase of 28% reported by Gardner et al where LDH increased from 379 +/- 155 IU/L in steady state to 507+/-272 IU/L in VOC as a result of haemolysis.[85]

This study reported a third of the pregnant women with SCD experiencing a stressful pregnancy with more admissions for acute maternal events (acute pain and severe anaemia). Most of the acute pain episodes occurred in the third trimester. This finding is similar to studies conducted in both low-resource setting by Asare et al in 2018,[28,119] and high resource settings by Cardoso et al. in 2014.[123] During pregnancy, the major haematological changes are physiologic anaemia, mild thrombocytopenia and a hypercoagulable state (increased procoagulant factors, low fibrinolysis). There is also an increase in metabolic demand as well as increased red blood cell sickling.[116] Another common adaptation during pregnancy, is leukocytosis, with a predominance in neutrophils, due to the physiological stress of pregnancy.[100] The neutrophil count begins to increase in the second month of pregnancy and plateaus in the second or third trimester.[100] Both pregnancy and SCD are hypercoagulable states. The pathophysiology of acute pain and ACS in SCD arises from a cascade of interactions between the RBCs, vascular endothelium, neutrophils, coagulation mechanism and inflammatory response.[40,42,43] These effects are associated with an increase in occurrence of acute pain episodes, ACS, osteonecrosis, hepatic necrosis, leg ulcers and thromboembolic events.[116] Other factors that could have played a role in the frequency of acute pain episodes include rainy or cold climate,[150] and the increased risk of recurrent infections in this cohort.[10,15,159]

The pregnant women with SCD as expected had a lower mean Hb with a higher reticulocyte count as compared to the pregnant women without SCD. This could be explained by the fact that patients with

SCD have chronic haemolysis with a resultant chronic anaemia and increased bone marrow turnover.[115,160] The elevated reticulocyte count could provide another link between haemolytic anaemia and vaso-occlusion[54] since reticulocytes have receptors and ligands eg. α4β1 and CD36,[53] which adhere to the endothelium and leukocytes during vaso-occlusion.

It is widely reported that HbSS is a more severe phenotype than HbSC,[161] with more SCD-related complications in the non-pregnant state. Pregnant women with SCA in this study had lower blood Hb concentration with marked elevation of absolute reticulocyte count, serum bilirubin and serum LDH, and mild elevation of serum AST and ALT compared to the HbSC group. It is worth noting however that the mean Hb concentrations in the HbSS group were within the normal for steady state compared to the non-pregnant group and did not fall during pregnancy. This may be attributed to the regular use of haematenics (oral iron supplements, folic acid and multivitamin) during pregnancy to prevent severe anaemia. The afore mentioned laboratory markers seem to translate the pathophysiological mechanism underlying SCD where acute pain episodes are heightened by persistent haemolysis. The SS group had more admissions for acute pain episodes compared to the SC group. Despite the fact that pregnant women with HbSS had higher frequencies of moderate-to-late preterm birth, low birthweight babies and perinatal deaths, findings from this study showed that pregnancy outcomes in both groups were comparable. These findings are similar to those reported by Oppong et al at KBTH in 2018.[159] Generally, it is well known that sickle cell anaemia runs a severe but markedly variable clinical course compared to HbSC disease which is milder and may be diagnosed in adulthood.[8] In sub-Saharan African countries like Ghana and Nigeria where SCD is most prevalent, the homozygous SS form is more prevalent at birth and during adolescence.[6,150] However, due to anecdotal evidence of a high mortality rate in HbSS patients, more HbSC patients are seen during adulthood;[150] and pregnancy is seen more frequently among patients with HbSC disease.[29,159] A previous study by Serjeant et al conducted in Jamaica in 2005, reported a relatively benign pregnancy outcome in women with HbSC disease compared to HbSS.[8] Findings from this study and from other studies however, have reported statistically similar obstetric and SCD-related outcomes (including acute pain rates) in both pregnant women with HbSS and HbSC.[28,119,159,162] Pregnant women with HbSC therefore need to be managed with the same aggressive multidisciplinary approach to care of pregnant women with sickle cell anaemia.

The non-pregnant women with SCD were chosen as 2nd controls to eliminate possible bias from SCD related events as well as reduce confounders. Since the non-pregnant population also have SCD; they are expected to have an ongoing chronic haemolysis which is part of the disease pathophysiology. We however did not know the steady state values of the selected markers of haemolysis in the pregnant women with SCD prior to them becoming pregnant. The markers of haemolysis evaluated in the non-pregnant women were hence to provide an estimate of the steady state distribution of these markers in the pregnant women with SCD. Generally, the mean concentrations of serum bilirubin, serum LDH, serum AST and ALT were higher confirming the chronic haemolytic process of SCD. It is worth noting that the steady state whole Hb concentrations were comparable among the pregnant and non-pregnant SCD cohorts and did not fall during pregnancy. This may be attributed to the regular use of haematenics (oral iron supplements, folic acid and multivitamin) during pregnancy to prevent severe anaemia. The absolute reticulocyte count values also increased marginally throughout the trimesters of pregnancy even though they were still within the normal reference range. The increase in the absolute reticulocyte count coupled with the maintenance of the blood Hb concentration with increasing trimesters of pregnancy further validates the employed strategy of using haematenics to prevent severe anaemia during pregnancy. As expected, changes in value of certain serum liver function tests occur during normal pregnancy.[68–70] In this study, the serum bilirubin, AST and ALT concentrations in the pregnant women with SCD during all three trimesters of pregnancy were lower compared to the non-pregnant SCD controls. These findings are comparable to published studies on this topic.[68–70] Contrary to this, Bacq reported that serum ALT and AST activity levels during pregnancy remain within the normal limits established for non-pregnant women.[163]

7.2 Fetomaternal Outcomes In Women With And Without SCD:

Pregnant women without SCD were chosen as 1st controls to eliminate possible bias from pregnancy related events.

Maternal outcomes

Pregnant women with SCD had more admissions to hospital for acute events. This shows that SCD is associated with increased morbidities and a risk factor for hospital admission during pregnancy.[10,15,159]

There were more caesarean (CS) deliveries (72%) in the cohort of pregnant women with SCD compared to pregnant women without SCD, even though it has not been shown that SCD is a risk factor for caesarean

delivery. This finding compares similarly to the study by Oppong et al (2018) in the same setting who also observed a higher caeserean delivery rate in this group.[159]

There was no mortality in this cohort. This can be attributed to better healthcare delivery by a multidisciplinary SCD obstetrics team during pregnancy with a resultant reduction in materal mortality to similar levels in pregnant women with SCD.[159]

Fetal outcomes

The pregnant women with SCD had relatively more moderate-to-late pre-term deliveries compared to the pregnant women without SCD. This could be related to the increased morbidities of acute pain episode.[10]

Low birthweight (<2500g) was observed in pregnant women with SCD (29.2%) compared to pregnant women with HbAA (16.7%) and this finding was comparable to findings reported by Oppong et al (2018) where 25.2% of babies delivered to the pregnant women with SCD had a low birthweight.[159] The high number of low birthweight babies could be as a result of maternal anaemia, intrauterine growth restriction and preterm delivery.

This study found no difference in the fetal outcome and perinatal deaths comparing both pregnant women with SCD and pregnant women without SCD. Contrary to this, Boafor et al in 2016 reported in a meta-analysis, an increased adverse fetal outcome in pregnant women with SCD compared to those without SCD.[10] Our finding however corroborates with findings by Oppong et al where a multidisciplinary approach to care of pregnant women with SCD reduced perinatal deaths in pregnant women with SCD to levels comparable with pregnant women without SCD.[159] A multidisciplinary approach to care of pregnant women with SCD should hence be the gold standard to reduce adverse fetomaternal outcomes.

7.3 Socio-demographic characteristics

The 73 participants had a mean age of 30.3 ± 5.3 years which was within the reproductive age using the WHO criteria (15 to 44 years).[151] This compares to a study done in the same setting (KBTH) by Oppong et al (2018) where the pregnant women with and without SCD had a mean age of 29.5 ± 4.8 years.[159]

Nineteen (76%) of the study participants were within the ages of 20 to 34 years. This compares similarly to a study published by Asare et al in the same setting in 2018 where a high proportion (81.1%) of the pregnant women were young, and of the same age group.[28] In general, it was observed that pregnant women with SCD are likely to be younger females aged less than 35 years. The contributing factors include the fact that there is improvement in their healthcare with an increased survival beyond childhood,[7] and a resultant increased chance of getting pregnant.[8] The young age seen at the antenatal clinic may also be attributed to the high mortality rate as a result of high incidence of chronic complications and multi-organ failure seen in older adults with SCD.[164] Recently, a study published by Vichinsky et al in 2017 in a high resource setting reported that "the National Center for Health statistics in the US published population-based surveillance data for causes of death among 12,000 patients with SCD; and found an overall age of death for women with SCD as 43 years."[164] One of the causes of multi-organ failure, is chronic renal disease. A study published by Sharpe et al in 2014 reported that "at least 25% of older sickle cell adults have chronic kidney disease."[165] In Ghana, unpublished data from a study conducted at the adult sickle cell clinic, GICG in Korle-Bu over a period of five years, found a high prevalence (67%) of microalbuminuria (one of the earliest signs of renal impairment) in the adult population. Another contributing factor could be societal expectations for young females to start bearing children early; more so in patients with SCD whose life expectancy is shorter than the average female population. Elleamoh et al in 2019 reported that "marriage and childbearing are integral components of cultural and social processes in Africa."[166]

Pregnant women without SCD had a higher mean weight compared to both pregnant women with SCD and non-pregnant women with SCD. Prior studies have shown that, individuals with SCD have a low body mass index (BMI; calculated with weight and height) when compared with individuals without SCD due to the increased resting metabolic rate. According to Khan et al, the proposed mechanisms for the increased metabolic rate include "hyper protein metabolism, increased haemolysis, increased red cell turnover, increased cardiac output, and appetite suppression due to enhanced cytokine production."[167]

All the pregnant women with SCD had a maximum parity of two at enrollment. This may be due to the increased maternal and fetal complications associated with SCD such as spontaneous abortions, IUFD, and perinatal death.[10] This group also have a lower fecundity rate compared to pregnant women without SCD due to their peculiar inherent haematological condition.[8] Fourty-eight percent of the pregnant women with SCD were nulliparous at enrollment; and this compares with Oppong et al (2018) where 45% of pregnant women with SCD were nulliparous;[159] but Asare et al had a higher proportion of 71.5% pregnant women with SCD who were nulliparous.[28]

Pregnant women with SCD enrolled at the antenatal clinic relatively early compared to the pregnant women without SCD. This finding can be explained by the proximity and prompt referral of pregnant SCD women seen at the adult Sickle Cell Clinic (GICG) to the Obtetric Clinic in KBTH, as they are considered a high-risk group of pregnant women.[10,28,159] The Obstetric Clinic also runs a high-risk multidisciplinary obstetrics clinic where prompt referrals irrespective of gestational age are attended to.

Both the pregnant women with and without SCD had comparable gestational ages at delivery [37.0 ± 2.8 weeks for the former, 36.2 ± 5.4 weeks (p=0.57) for the latter]. A gestational age of birth at 37 weeks is described by WHO as "late preterm birth"; [91] which was observed in both the pregnant women with and without SCD. In SCD there is increased risk of maternal morbidities such as acute pain episodes with a resultant reduced blood flow to the placenta. Pregnant women with SCD also have an increased risk of adverse fetal outcomes including preterm birth.[15,28,123] These two findings were observed in this cohort.

Even though both the pregnant women with and without SCD had a similar gestational age at enrollment, the pregnant women with SCD had a longer duration of follow-up than the pregnant women without SCD at 23.8 ± 5weeks vrs 21.3 ± 5.6 weeks respectively (p=0.11). This could be explained by the fact that the pregnant women with SCD had a relatively longer mean gestational age at delivery and more compliant to both antenatal and postnatal care at the hospital.

Study Limitations

The limitations of this study include:

- Medications (for example suphathiazine and methyldopa) which might influence laboratory parameters of the markers of haemolysis were not eliminated during the recruitment of controls.
- There was no laboratory data on the pregnant women with SCD before they became pregnant. This would have been the ideal baseline.
- Study was unable to determine if the haemolysis in the pregnant women with SCD was more intravascular or extravascular since this was not systematically evaluated.
- Study was not designed or powered enough to compare differences between HbSS and HbSC
- It was difficult to match the pregnant participants for both age and gestational age

CHAPTER 8: CONCLUSIONS AND RECOMMENDATIONS

6.2 CONCLUSIONS

This study aimed at finding the association between hyperhaemolysis and fetomaternal outcome in pregnant women with SCD.

The findings from this study have demonstrated that:

1. There was no increased haemolysis (hyperhaemolysis) during pregnancy in women with SCD.
2. There was no significant difference in the steady state values of the selected markers of haemolysis in the pregnant and non-pregnant SCD cohorts.
3. At a significant level of 5%, there is sufficient evidence that haemolysis was not associated with adverse fetomaternal outcomes in pregnant women with SCD.

8.2 RECOMMENDATIONS

- A longitudinal study with a high power to compare differences between HbSS and HbSC be conducted in women with SCD from preconception through to conception, delivery and postpartum at the adolescent and adult sickle cell clinic of the Ghana Institute of Clinical Genetics, Korle-Bu. This would help provide baseline and follow up data as well as help to compare differences between HbSS and HbSC.
- Other markers of haemolysis with a high sensitivity and specificity index such as haptoglobin, haemopexin, erythrocyte adenylate kinase and haemosiderin in urine be used to determine haemolysis in this cohort; and subsequently their association with fetomaternal outcome.
- All pregnant women with SCD be managed the same despite the different phenotypes since there was no difference in the acute pain rate between the HbSS and HbSC phenotypes.

CHAPTER 9: REFERENCES

1. Nagel RL, Steinberg MH, Fabry ME. The paradox of hemoglobin SC disease. Blood Rev. 2003;17(3):167–78.

2. Serjeant GR, Serjeant BE, Fraser RA, Hambleton IR, Higgs DR, Kulozik AE, et al. Hb S-β-Thalassemia: Molecular, Hematological and Clinical Comparisons. Hemoglobin. 2011;35(1):1–12.

3. Diallo D, Tchernia G. Sickle cell disease in Africa. Curr Opin Hematol. 2002;9(2):111.

4. Acquaye CT, Oldham JH. Variants of haemoglobin and glucose-6-phosphate dehydrogenase-I. distribution in Southern Ghana. Ghana Med J. 1973;12(4):412–8.

5. Konotey-Ahulu FID. The Sickle Cell Disease Patient. London and Basingstoke: Macmillan Publishing Company; 1991.p.xv.

6. Ohene-Frempong K, Oduro J, Tetteh H, Nkrumah F. Screening newborns for sickle cell disease in Ghana. Pediatrics. 2008;121(Supplement 2):S120–1.

7. Quinn CT, Rogers ZR, McCavit TL, Buchanan GR. Improved survival of children and adolescents with sickle cell disease. Blood. 2010;115(17):3447–52.

8. Serjeant GR, Hambleton I, Thame M. Fecundity and pregnancy outcome in a cohort with sickle cell-haemoglobin C disease followed from birth. BJOG Int J Obstet Gynaecol. 2005;112(9):1308–14.

9. Rogers DT, Molokie R. Sickle Cell Disease in Pregnancy. Obstet Gynecol Clin North Am. 2010;37(2):223–37.

10. Boafor TK, Olayemi E, Galadanci N, Hayfron-Benjamin C, Dei-Adomakoh Y, Segbefia C, et al. Pregnancy outcomes in women with sickle-cell disease in low and high income countries: a systematic review and meta-analysis. BJOG Int J Obstet Gynaecol. 2016;5(123):691–8.

11. Smith JA, Espeland M, Bellevue R, Bonds D, Brown AK, Koshy M. Pregnancy in sickle cell disease: experience of the Cooperative Study of Sickle Cell Disease. Obstet Gynecol. 1996;87(2):199–204.

12. Serjeant GR, Loy LL, Crowther M, Hambleton IR, Thame M. Outcome of pregnancy in homozygous sickle cell disease. Obstet Gynecol. 2004;103(6):1278–85.

13. Rajab KE, Issa AA, Mohammed AM, Ajami AA. Sickle cell disease and pregnancy in Bahrain. Int J Gynaecol Obstet Off Organ Int Fed Gynaecol Obstet. 2006;93(2):171–5.

14. Al Jama FE, Gasem T, Burshaid S, Rahman J, Al Suleiman SA, Rahman MS. Pregnancy outcome in patients with homozygous sickle cell disease in a university hospital, Eastern Saudi Arabia. Arch Gynecol Obstet. 2009;280(5):793–7.

15. Oteng-Ntim E, Chase AR, Howard J, Khazaezadeh N, Anionwu EN. Sickle cell disease in pregnancy. Obstet Gynaecol Reprod Med. 2008;18(10):272–8.

16. Asnani MR, McCaw-Binns AM, Reid ME. Excess Risk of Maternal Death from Sickle Cell Disease in Jamaica: 1998–2007. PLOS ONE. 2011;6(10):e26281.

17. Rother RP, Bell L, Hillmen P, Gladwin MT. The Clinical Sequelae of Intravascular Hemolysis and Extracellular Plasma Hemoglobin: A Novel Mechanism of Human Disease. JAMA. 2005;293(13):1653–62.

18. Milner PF, Charache S. Life span of carbamylated red cells in sickle cell anemia. J Clin Invest. 1973;52(12):3161–71.

19. Franco RS, Yasin Z, Palascak MB, Ciraolo P, Joiner CH, Rucknagel DL. The effect of fetal hemoglobin on the survival characteristics of sickle cells. Blood. 2006;108(3):1073–6.

20. Reiter CD, Wang X, Tanus-Santos JE, Hogg N, Cannon Iii RO, Schechter AN, et al. Cell-free hemoglobin limits nitric oxide bioavailability in sickle-cell disease. Nat Med. 2002;8(12):1383–9.

21. Kato GJ. 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(6):2279–85.

22. Kato GJ, Gladwin MT, Steinberg MH. Deconstructing sickle cell disease: Reappraisal of the role of hemolysis in the development of clinical subphenotypes. Blood Rev. 2007;21(1):37.

23. Gladwin MT, Kato GJ. Hemolysis-associated hypercoagulability in sickle cell disease: the plot (and blood) thickens! Haematologica. 2008;93(1):1–3.

24. Buehler PW, Baek JH, Lisk C, Connor I, Sullivan T, Kominsky D, et al. Free hemoglobin induction of pulmonary vascular disease: evidence for an inflammatory mechanism., Free hemoglobin induction of pulmonary vascular disease: evidence for an inflammatory mechanism. Am J Physiol Lung Cell Mol Physiol Am J Physiol - Lung Cell Mol Physiol. 2012;303, 303(4, 4):L312, L312-26.

25. Chen G, Zhang D, Fuchs TA, Manwani D, Wagner DD, Frenette PS. Heme-induced neutrophil extracellular traps contribute to the pathogenesis of sickle cell disease. Blood. 2014;123(24):3818–27.

26. Camus SM, De Moraes JA, Bonnin P, Abbyad P, Le Jeune S, Lionnet F, et al. Circulating cell membrane microparticles transfer heme to endothelial cells and trigger vasoocclusions in sickle cell disease. Blood. 2015;125(24):3805–14.

27. Patel B, Coviello E, Shenoy A. Pregnancy in Women with Sickle Cell Disease. Blood. 2017;130(Suppl 1):4801–4801.

28. Asare EV, Olayemi E, Boafor T, Dei-Adomakoh Y, Mensah E, Osei-Bonsu Y, et al. A case series describing causes of death in pregnant women with sickle cell disease in a low-resource setting. Am J Hematol. 2018;93(7):E167–70.

29. Asare EV, Olayemi E, Boafor T, Dei-Adomakoh Y, Mensah E, Ghansah H, et al. Implementation of multidisciplinary care reduces maternal mortality in women with sickle cell disease living in low-resource setting. Am J Hematol. 2017;92(9):872–8.

30. Saxena R, Banerjee T, Aniyery R. Thalassemia and its Management during Pregnancy. World J Anemia. 2017;1:5–17.

31. Structural Biochemistry/Protein function/Hemoglobin - Wikibooks, open books for an open world [Internet]. [cited 2020 Jan 11]. Available from: https://en.wikibooks.org/wiki/Structural_Biochemistry/Protein_function/Hemoglobin

32. Wood WG. Haemoglobin Synthesis During Human Fetal Development. Br Med Bull. 1976;32(3):282–7.

33. Marengo-Rowe AJ. Structure-function relations of human hemoglobins. Proc Bayl Univ Med Cent. 2006;19(3):239–45.

34. Press Conference On Raising Awareness Of Sickle-Cell Anaemia | Meetings Coverage and Press Releases [Internet]. [cited 2018 Feb 20]. Available from: http://www.un.org/press/en/2009/090619_Anaemia.doc.htm

35. Piel FB, Patil AP, Howes RE, Nyangiri OA, Gething PW, Dewi M, et al. Global epidemiology of sickle haemoglobin in neonates: a contemporary geostatistical model-based map and population estimates. The Lancet. 2013;381(9861):142–51.

36. Piel FB, Hay SI, Gupta S, Weatherall DJ, Williams TN. Global Burden of Sickle Cell Anaemia in Children under Five, 2010–2050: Modelling Based on Demographics, Excess Mortality, and Interventions. PLOS Med. 2013;10(7):e1001484.

37. Murayama M. Structure of Sickle Cell Hemoglobin and Molecular Mechanism of the Sickling Phenomenon. Clin Chem. 1967;13(7):578–88.

38. Medical Home Portal - Sickle Cell Disease [Internet]. [cited 2020 Nov 18]. Available from: https://www.medicalhomeportal.org/diagnoses-and-conditions/sickle-cell-disease

39. Kato GJ, Gladwin MT. Mechanisms and clinical complications of hemolysis in sickle cell disease and thalassemia. Disord Hemoglobin Genet Pathophysiol Clin Manag Second Ed. 2009;201–24.

40. Frenette PS, Atweh GF. Sickle cell disease: old discoveries, new concepts, and future promise. J Clin Invest. 2007;117(4):850–8.

41. Hebbel RP. 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(2):123–54.

42. Hebbel RP, Vercellotti GM, Nath KA. A Systems Biology Consideration of the Vasculopathy of Sickle Cell Anemia: The Need for Multi-Modality Chemo-Prophylaxis. Cardiovasc Haematol Disord - Drug Targetsrug Targets - Cardiovasc Hematol Disord. 2009;9(4):271–92.

43. Rees DC, Williams TN, Gladwin MT. Sickle-cell disease. The Lancet. 2010;376(9757):2018–31.

44. Muller-Eberhard U, Javid J, Liem HH, Hanstein A, Hanna M. Brief Report: Plasma Concentrations of Hemopexin, Haptoglobin and Heme in Patients with Various Hemolytic Diseases. Blood. 1968;32(5):811–5.

45. Wagener FADTG. Different Faces of the Heme-Heme Oxygenase System in Inflammation. Pharmacol Rev. 2003;55(3):551–71.

46. Kumar S, Bandyopadhyay U. Free heme toxicity and its detoxification systems in human. Toxicol Lett. 2005;157(3):175–88.

47. Smith A, McCulloh RJ. Hemopexin and haptoglobin: allies against heme toxicity from hemoglobin not contenders. Front Physiol [Internet]. 2015 [cited 2020 Jan 12];6. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4485156/

48. Thorell SE, Parry-Jones AR, Punter M, Hurford R, Thachil J. Cerebral venous thrombosis—A primer for the haematologist. Blood Rev. 2015;29(1):45–50.

49. Orf K, Cunnington A. Infection-related hemolysis and susceptibility to Gram-negative bacterial co-infection. Front Microbiol. 2015;6:666.

50. Morris CR, Kato GJ, Poljakovic M, Wang X, Blackwelder WC, Sachdev V, et al. Dysregulated Arginine Metabolism, Hemolysis-Associated Pulmonary Hypertension, and Mortality in Sickle Cell Disease. JAMA. 2005;294(1):81–90.

51. Proença-Ferreira R, Franco-Penteado CF, Traina F, Saad STO, Costa FF, Conran N. Increased adhesive properties of platelets in sickle cell disease: roles for αIIbβ3-mediated ligand binding, diminished cAMP signalling and increased phosphodiesterase 3A activity. Br J Haematol. 2010;149(2):280–8.

52. Wagener FADTG, Eggert A, Boerman OC, Oyen WJG, Verhofstad A, Abraham NG, et al. Heme is a potent inducer of inflammation in mice and is counteracted by heme oxygenase. Blood. 2001;98(6):1802–11.

53. Hebbel RP. Perspectives series: cell adhesion in vascular biology. Adhesive interactions of sickle erythrocytes with endothelium. J Clin Invest. 1997;99(11):2561–4.

54. Steinberg MH. Disorders of hemoglobin: genetics, pathophysiology, and clinical management. Cambridge [etc.: Cambridge University Press; 2010.

55. Processes involved in Extravascular haemolysis. [cited 2020 Nov 19]. Available from: http://www.pitt.edu/~super1/lecture/lec35371/011.htm

56. Belcher JD. Transgenic sickle mice have vascular inflammation. Blood. 2003;101(10):3953–9.

57. Belcher JD, Mahaseth H, Welch TE, Vilback AE, Sonbol KM, Kalambur VS, et al. Critical role of endothelial cell activation in hypoxia-induced vasoocclusion in transgenic sickle mice. Am J Physiol-Heart Circ Physiol. 2005;288(6):H2715–25.

58. Bessman JD. Reticulocytes. In: Walker HK, Hall WD, Hurst JW, editors. Clinical Methods: The History, Physical, and Laboratory Examinations [Internet]. 3rd ed. Boston: Butterworths; 1990 [cited 2020 Jan 12]. Available from: http://www.ncbi.nlm.nih.gov/books/NBK264/

59. Reticulocyte Count (Retic count), Interpretations – Labpedia.net [Internet]. [cited 2020 Nov 18]. Available from: https://www.labpedia.net/reticulocyte-count-retic-count/

60. Powars DR. Sickle cell anemia and major organ failure. Hemoglobin. 1990;14(6):573–98.

61. Simionatto M, Paula JP, Nascimento AJ, Leonart MSS, Cicchetti D. Analysis of manual reticulocyte counts in the clinical laboratories of Ponta-Grossa and Campos Gerais, PR, Brazil. Rev Bras Hematol E Hemoter. 2009;31(5):315–20.

62. Zandecki M, Genevieve F, Gerard J, Godon A. Spurious counts and spurious results on haematology analysers: a review. Part II: white blood cells, red blood cells, haemoglobin, red cell indices and reticulocytes. Int J Lab Hematol. 2007;29(1):21–41.

63. Simionatto M, De JP, Chaves MA, Bortoloso M, Cicchetti D, Leonart MS, et al. Manual and automated reticulocyte counts. Hematol Amst Neth. 2010;15(6):406–9.

64. Reticulocyte Production Index [Internet]. [cited 2019 Nov 9]. Available from: https://www.merckmanuals.com/medical-calculators/ReticProdIndex.htm

65. Piva E, Brugnara C, Chiandetti L, Plebani M. Automated reticulocyte counting: state of the art and clinical applications in the evaluation of erythropoiesis. Clin Chem Lab Med. 2010;48(10):1369–80.

66. Means RT, Glader B. General Considerations. Greer JP, Foerster J, Rodgers GM, eds. Anemia: Wintrobe's Clinical Hematology. 12th ed. Philadelphia, PA: Lippincott Williams and Wilkins; 2009. Vol. 1: Chapter 26.

67. Barcellini W, Fattizzo B, Zaninoni A, Radice T, Nichele I, Di Bona E, et al. Clinical heterogeneity and predictors of outcome in primary autoimmune hemolytic anemia: a GIMEMA study of 308 patients. Blood. 2014;124(19):2930–6.

68. Abbassi-Ghanavati M, Greer LG, Cunningham FG. Pregnancy and laboratory studies: a reference table for clinicians. Obstet Gynecol. 2009;114(6):1326–31.

69. Normal Reference Ranges and Laboratory Values In Pregnancy [Internet]. [cited 2019 Nov 2]. Available from: http://perinatology.com/Reference/Reference%20Ranges/Reference%20for%20Serum.htm

70. Manual Of Laboratory And Diagnostic Tests 7th Fischbach [Internet]. [cited 2019 Nov 9]. 755 p. Available from: http://archive.org/details/Manual_Of_Laboratory_And_Diagnostic_Tests_7th_Fischbach

71. Themes UFO. Liver Disease [Internet]. Basicmedical Key. 2016 [cited 2020 Nov 18]. Available from: https://basicmedicalkey.com/liver-disease-4/

72. Burns ER, Kale A, Murthy VV. Diagnosis of the hemolytic state using serum levels of erythrocyte adenylate kinase. Am J Hematol. 2000;64(3):180–3.

73. Kato GJ. 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(6):2279–85.

74. Kalmovarin N, Friedrichs WE, O'Brien HV, Linehan LA, Bowman BH, Yang F. Extrahepatic expression of plasma protein genes during inflammation. Inflammation. 1991;15(5):369–79.

75. Yang F, Friedrichs WE, Navarijo-Ashbaugh AL, deGraffenried LA, Bowman BH, Coalson JJ. Cell type-specific and inflammatory-induced expression of haptoglobin gene in lung. Lab Investig J Tech Methods Pathol. 1995;73(3):433–40.

76. Ratanasopa K, Chakane S, Ilyas M, Nantasenamat C, Bulow L. Trapping of Human Hemoglobin by Haptoglobin: Molecular Mechanisms and Clinical Applications. Antioxid Redox Signal. 2013;18(17):2364–74.

77. Kristiansen M, Graversen JH, Jacobsen C, Sonne O, Hoffman H-J, Law SKA, et al. Identification of the haemoglobin scavenger receptor. Nature. 2001;409(6817):198–201.

78. Quaye IK. Haptoglobin, inflammation and disease. Trans R Soc Trop Med Hyg. 2008;102(8):735–42.

79. Shih AWY, McFarlane A, Verhovsek M. Haptoglobin testing in hemolysis: Measurement and interpretation. Am J Hematol. 2014;89(4):443–7.

80. Körmöczi GF, Säemann MD, Buchta C, Peck-Radosavljevic M, Mayr WR, Schwartz DWM, et al. Influence of clinical factors on the haemolysis marker haptoglobin. Eur J Clin Invest. 2006;36(3):202–9.

81. Mitani K, Fujita H, Kappas A, Sassa S. Heme oxygenase is a positive acute-phase reactant in human Hep3B hepatoma cells. Blood. 1992;79(5):1255–9.

82. Taylor JG, Nolan VG, Mendelsohn L, Kato GJ, Gladwin MT, Steinberg MH. Chronic hyper-hemolysis in sickle cell anemia: association of vascular complications and mortality with less frequent vasoocclusive pain. PloS One. 2008;3(5):e2095.

83. Gordeuk VR, Minniti CP, Nouraie M, Campbell AD, Rana SR, Luchtman-Jones L, et al. Elevated tricuspid regurgitation velocity and decline in exercise capacity over 22 months of follow up in children and adolescents with sickle cell anemia. Haematologica. 2011;96(1):33–40.

84. Ballas SK, Marcolina MJ. Hyperhemolysis during the evolution of uncomplicated acute painful episodes in patients with sickle cell anemia. Transfusion (Paris). 2006;46(1):105–10.

85. Gardner K, Drasar E, Awogbade M, Allman M, Thein SL. Super-Elevated LDH and Thombocytopenia Are Markers Of An Unusual Sickle Phenomenon. Blood. 2013;122(21):2220–2220.

86. Nouraie M, Lee JS, Zhang Y, Kanias T, Zhao X, Xiong Z, et al. The relationship between the severity of hemolysis, clinical manifestations and risk of death in 415 patients with sickle cell anemia in the US and Europe. Haematologica. 2013;98(3):464–72.

87. Minniti CP, Sable C, Campbell A, Rana S, Ensing G, Dham N, et al. Elevated tricuspid regurgitant jet velocity in children and adolescents with sickle cell disease: association with hemolysis and hemoglobin oxygen desaturation. Haematologica. 2009;94(3):340–7.

88. Genser B, Cooper PJ, Yazdanbakhsh M, Barreto ML, Rodrigues LC. A guide to modern statistical analysis of immunological data. BMC Immunol. 2007;8:27.

89. Belle G van, Fisher LD, Heagerty PJ, Lumley T. Biostatistics: A Methodology For the Health Sciences. John Wiley & Sons; 2004. 895 p.

90. DeMaeyer EM, Dallman P, Gurney JM, Hallberg L, Sood SK, Srikantia SG, et al. Preventing and controlling iron deficiency anaemia through primary health care : a guide for health administrators and programme managers [Internet]. World Health Organization; 1989 [cited 2019 Nov 9]. Available from: https://apps.who.int/iris/handle/10665/39849

91. WHO_NMH_NHD_MNM_11.1_eng.pdf [Internet]. [cited 2020 Jan 12]. Available from: https://apps.who.int/iris/bitstream/handle/10665/85839/WHO_NMH_NHD_MNM_11.1_eng.pdf?ua=1

92. Stevens GA, Finucane MM, De-Regil LM, Paciorek CJ, Flaxman SR, Branca F, et al. Global, regional, and national trends in haemoglobin concentration and prevalence of total and severe anaemia in children and pregnant and non-pregnant women for 1995–2011: a systematic analysis of population-representative data. Lancet Glob Health. 2013;1(1):e16–25.

93. Milman N. Prepartum anaemia: prevention and treatment. Ann Hematol. 2008;87(12):949–59.

94. Matteelli A, Donato F, Shein A, Muchi JA, Leopardi O, Astori L, et al. Malaria and anaemia in pregnant women in urban Zanzibar, Tanzania. Ann Trop Med Parasitol. 1994;88(5):475–83.

95. GHS_ANNUAL_REPORT_2016_n.pdf [Internet]. [cited 2020 Jan 12]. Available from: https://www.ghanahealthservice.org/downloads/GHS_ANNUAL_REPORT_2016_n.pdf

96. Dei-Adomakoh Y, Acquaye JK, Ekem I, Segbefia C. Second Trimester Anaemia in Pregnant Ghanaians. West Afr J Med. 2014;33(4):229–33.

97. Okunade KS, Adegbesan-Omilabu MA. Anaemia among pregnant women at the booking clinic of a teaching hospital in south-western Nigeria. Int J Med Biomed Res. 2014;114–20.

98. Pavord S, Myers B, Robinson S, Allard S, Strong J, Oppenheimer C. UK guidelines on the management of iron deficiency in pregnancy. Br J Haematol. 2012;156(5):588–600.

99. Bernstein IM, Ziegler W, Badger GJ. Plasma volume expansion in early pregnancy. Obstet Gynecol. 2001;97(5 Pt 1):669–72.

100. Chandra S, Tripathi AK, Mishra S, Amzarul M, Vaish AK. Physiological Changes in Hematological Parameters During Pregnancy. Indian J Hematol Blood Transfus. 2012;28(3):144–6.

101. Goonewardene M, Shehata M, Hamad A. Anaemia in pregnancy. Best Pract Res Clin Obstet Gynaecol. 2012;26(1):3–24.

102. Ekiz C, Agaoglu L, Karakas Z, Gurel N, Yalcin I. The effect of iron deficiency anemia on the function of the immune system. Hematol J Off J Eur Haematol Assoc. 2005;5(7):579–83.

103. Iron Deficiency and Reduced Work Capacity: A Critical Review of the Research to Determine a Causal Relationship | The Journal of Nutrition | Oxford Academic [Internet]. [cited 2020 Jan 12]. Available from: https://academic.oup.com/jn/article/131/2/676S/4686866

104. Maternal Iron Deficiency Anemia Affects Postpartum Emotions and Cognition | The Journal of Nutrition | Oxford Academic [Internet]. [cited 2020 Jan 12]. Available from: https://academic.oup.com/jn/article/135/2/267/4663632

105. Beard JL. Why Iron Deficiency Is Important in Infant Development. J Nutr. 2008;138(12):2534–6.

106. Scholl TO, Hediger ML. Anemia and iron-deficiency anemia: compilation of data on pregnancy outcome. Am J Clin Nutr. 1994;59(2):492S-501S.

107. Cogswell ME, Parvanta I, Ickes L, Yip R, Brittenham GM. Iron supplementation during pregnancy, anemia, and birth weight: a randomized controlled trial. Am J Clin Nutr. 2003;78(4):773–81.

108. Arnold DL, Williams MA, Miller RS, Qiu C, Sorensen TK. Iron deficiency anemia, cigarette smoking and risk of abruptio placentae. J Obstet Gynaecol Res. 2009;35(3):446–52.

109. Imdad A, Bhutta ZA. Routine Iron/Folate Supplementation during Pregnancy: Effect on Maternal Anaemia and Birth Outcomes. Paediatr Perinat Epidemiol. 2012;26(s1):168–77.

110. Schantz-Dunn J, Nour NM. Malaria and Pregnancy: A Global Health Perspective. Rev Obstet Gynecol. 2009;2(3):186–92.

111. Steer PJ. Maternal hemoglobin concentration and birth weight. Am J Clin Nutr. 2000;71(5):1285S-1287S.

112. Ezzati M. Comparative Quantification of Health Risks: Sexual and reproductive health. World Health Organization; 2004. 2282 p.

113. Zhang Q, Ananth CV, Rhoads GG, Li Z. The Impact of Maternal Anemia on Perinatal Mortality: A Population-based, Prospective Cohort Study in China. Ann Epidemiol. 2009;19(11):793–9.

114. Kozuki N, Lee AC, Katz J. Moderate to Severe, but Not Mild, Maternal Anemia Is Associated with Increased Risk of Small-for-Gestational-Age Outcomes. J Nutr. 2012;142(2):358–62.

115. Chiang EY, Frenette PS. Sickle cell vaso-occlusion. Hematol Oncol Clin North Am. 2005;19(5):771–84, v.

116. Jain D, Lodha P, Colah R, Atmapoojya P, Atmapoojya P. SICKLE CELL DISEASE AND PREGNANCY. Mediterr J Hematol Infect Dis. 2019;11(1):e2019040–e2019040.

117. Ballas SK, Gupta K, Adams-Graves P. Sickle cell pain: a critical reappraisal. Blood. 2012;120(18):3647–56.

118. Zhang D, Xu C, Manwani D, Frenette PS. Neutrophils, platelets, and inflammatory pathways at the nexus of sickle cell disease pathophysiology. Blood. 2016;127(7):801–9.

119. Asare EV, Olayemi E, Boafor T, Dei-Adomakoh Y, Dip EM, Benjamin CH, et al. Third trimester and early postpartum period of pregnancy have the greatest risk for ACS in women with SCD. Am J Hematol. 2019;94(12):E328–31.

120. Meeks D, Robinson SE, Macleod D, Oteng-Ntim E. Birth Weights in Sickle Cell Disease Pregnancies: A Cohort Study. PLOS ONE. 2016;11(10):e0165238.

121. Vichinsky EP, Neumayr LD, Earles AN, Williams R, Lennette ET, Dean D, et al. Causes and Outcomes of the Acute Chest Syndrome in Sickle Cell Disease [Internet]. 2009 [cited 2020 Jan 12]. Available from: https://www.nejm.org/doi/10.1056/NEJM200006223422502?url_ver=Z39.88-2003&rfr_id=ori%3Arid%3Acrossref.org&rfr_dat=cr_pub%3Dwww.ncbi.nlm.nih.gov

122. Siddiqui AK, Ahmed S. Pulmonary manifestations of sickle cell disease. Postgrad Med J. 2003;79(933):384–90.

123. Resende Cardoso PS, Lopes Pessoa de Aguiar RA, Viana MB. Clinical complications in pregnant women with sickle cell disease: prospective study of factors predicting maternal death or near miss. Rev Bras Hematol E Hemoter. 2014;36(4):256–63.

124. Kopecky EA, Jacobson S, Joshi P, Koren G. Systemic Exposure to Morphine and the Risk of Acute Chest Syndrome in Sickle Cell Disease. Clin Pharmacol Ther. 2004;75(3):140–6.

125. Ergul S, Brunson CY, Hutchinson J, Tawfik A, Kutlar A, Webb RC, et al. Vasoactive factors in sickle cell disease: In vitro evidence for endothelin-1-mediated vasoconstriction. Am J Hematol. 2004;76(3):245–51.

126. Pomp ER, Lenselink AM, Rosendaal FR, Doggen CJM. Pregnancy, the postpartum period and prothrombotic defects: risk of venous thrombosis in the MEGA study. J Thromb Haemost. 2008;6(4):632–7.

127. Heit JA, Kobbervig CE, James AH, Petterson TM, Bailey KR, Melton LJ. Trends in the incidence of venous thromboembolism during pregnancy or postpartum: a 30-year population-based study. Ann Intern Med. 2005;143(10):697–706.

128. James AH, Jamison MG, Brancazio LR, Myers ER. Venous thromboembolism during pregnancy and the postpartum period: Incidence, risk factors, and mortality. Am J Obstet Gynecol. 2006;194(5):1311–5.

129. Villers MS, Jamison MG, Castro LMD, James AH. Morbidity associated with sickle cell disease in pregnancy. Am J Obstet Gynecol. 2008;199(2):125.e1-125.e5.

130. Bremme KA. Haemostatic changes in pregnancy. Best Pract Res Clin Haematol. 2003;16(2):153–68.

131. Jacobsen AF, Skjeldestad FE, Sandset PM. Ante- and postnatal risk factors of venous thrombosis: a hospital-based case–control study. J Thromb Haemost. 2008;6(6):905–12.

132. Lindqvist P, Dahlbäck B, Marsál K. Thrombotic Risk During Pregnancy: A Population Study. Obstet Gynecol. 1999;94(4):595.

133. Blondon M, Casini A, Hoppe KK, Boehlen F, Righini M, Smith NL. Risks of Venous Thromboembolism After Cesarean Sections: A Meta-Analysis. CHEST. 2016;150(3):572–96.

134. Ataga KI. Hypercoagulability and thrombotic complications in hemolytic anemias. Haematologica. 2009;94(11):1481–4.

135. Tomer A, Harker LA, Kasey S, Eckman JR. Thrombogenesis in sickle cell disease. J Lab Clin Med. 2001;137(6):398–407.

136. Villagra J, Shiva S, Hunter LA, Machado RF, Gladwin MT, Kato GJ. Platelet activation in patients with sickle disease, hemolysis-associated pulmonary hypertension, and nitric oxide scavenging by cell-free hemoglobin. Blood. 2007;110(6):2166–72.

137. Ataga KI, Orringer EP. Renal abnormalities in sickle cell disease. Am J Hematol. 2000;63(4):205–11.

138. Drawz P, Ayyappan S, Nouraie M, Saraf S, Gordeuk V, Hostetter T, et al. Kidney Disease among Patients with Sickle Cell Disease, Hemoglobin SS and SC. Clin J Am Soc Nephrol CJASN. 2016;11(2):207–15.

139. Cheung KL, Lafayette RA. Renal physiology of pregnancy. Adv Chronic Kidney Dis. 2013;20(3):209–14.

140. Galiè N, Hoeper MM, Humbert M, Torbicki A, Vachiery J-L, Barbera JA, et al. Guidelines for the diagnosis and treatment of pulmonary hypertensionThe Task Force for the Diagnosis and Treatment of Pulmonary Hypertension of the European Society of Cardiology (ESC) and the European Respiratory Society (ERS), endorsed by the International Society of Heart and Lung Transplantation (ISHLT). Eur Heart J. 2009;30(20):2493–537.

141. Castro O, Hoque M, Brown BD. Pulmonary hypertension in sickle cell disease: cardiac catheterization results and survival. Blood. 2003;101(4):1257–61.

142. Gladwin MT, Sachdev V, Jison ML, Shizukuda Y, Plehn JF, Minter K, et al. Pulmonary Hypertension as a Risk Factor for Death in Patients with Sickle Cell Disease. N Engl J Med. 2004;350(9):886–95.

143. Fonseca GHH, Souza R, Salemi VMC, Jardim CVP, Gualandro SFM. Pulmonary hypertension diagnosed by right heart catheterisation in sickle cell disease. Eur Respir J. 2012;39(1):112–8.

144. Mehari A, Gladwin MT, Tian X, Machado RF, Kato GJ. Mortality in Adults With Sickle Cell Disease and Pulmonary Hypertension. JAMA. 2012;307(12):1254–6.

145. Potoka KP, Gladwin MT. Vasculopathy and pulmonary hypertension in sickle cell disease. Am J Physiol - Lung Cell Mol Physiol. 2015;308(4):L314–24.

146. Pieper PG, Hoendermis ES. Pregnancy in women with pulmonary hypertension. Neth Heart J. 2011;19(12):504–8.

147. Solovey A, Gui L, Key NS, Hebbel RP. Tissue factor expression by endothelial cells in sickle cell anemia. J Clin Invest. 1998;101(9):1899–904.

148. Ataga KI, Key NS. Hypercoagulability in sickle cell disease: new approaches to an old problem. Hematol Am Soc Hematol Educ Program. 2007;91–6.

149. van Tuijn CFJ, van der Stap RG, van der Post JAM, Peters M, Biemond BJ. Pregnancy Complications In Sickle Cell Disease Are More Prevalent In Women with Vaso-Occlusion Related Organ Damage Than Hemolysis Related Organ Damage. Blood. 2010;116(21):2661–2661.

150. Asare EV, Wilson I, Benneh-Akwasi Kuma AA, Dei-Adomakoh Y, Sey F, Olayemi E. Burden of Sickle Cell Disease in Ghana: The Korle-Bu Experience [Internet]. Advances in Hematology. 2018 [cited 2020 Jan 13]. Available from: https://www.hindawi.com/journals/ah/2018/6161270/

151. World Health Organization, editor. Reproductive health indicators: guidelines for their generation, interpretation and analysis for global monitoring. Geneva: World Health Organization; 2006. 63 p.

152. Ballas SK. More definitions in sickle cell disease: Steady state v base line data. Am J Hematol. 2012;87(3):338–338.

153. Pagano M GK. Principles of Biostatistics - eBook [Internet]. [cited 2018 Feb 20]. Available from: http://it-book.org/pdf/principles-of-biostatistics

154. Dhingra N, Safe Injection Global Network, World Health Organization. WHO guidelines on drawing blood: best practices in phlebotomy [Internet]. 2010 [cited 2018 Jul 31]. Available from: http://www.ncbi.nlm.nih.gov/books/NBK138650/

155. Lombardi G, Colombini A, Lanteri P, Banfi G. Reticulocytes in sports medicine: an update. Adv Clin Chem. 2013;59:125–53.

156. Newhouse D. Reticulocyte Count. :4. [cited 2020 Jan 12]. Available from: https://www.healthline.com/health/reticulocyte-count#results.

157. Giri D. Reticulocyte Count : Principle, Procedure, Calculations and Clinical Significances [Internet]. LaboratoryTests.org. 2018 [cited 2020 Nov 18]. Available from: http://laboratorytests.org/reticulocyte-count/

158. Urine test strip. In: Wikipedia [Internet]. 2020 [cited 2020 Nov 19]. Available from: https://en.wikipedia.org/w/index.php?title=Urine_test_strip&oldid=985996143

159. Oppong SA, Asare EV, Olayemi E, Boafor T, Dei-Adomakoh Y, Swarry-Deen A, et al. Multidisciplinary care results in similar maternal and perinatal mortality rates for women with and without SCD in a low-resource setting. Am J Hematol. 2019;94(2):223–30.

160. Ballas SK, Gupta K, Adams-Graves P. Sickle cell pain: a critical reappraisal. Blood. 2012;120(18):3647–56.

161. Guarda CC da, Yahouédéhou SCMA, Santiago RP, Neres JS dos S, Fernandes CF de L, Aleluia MM, et al. Sickle cell disease: A distinction of two most frequent genotypes (HbSS and HbSC). PLOS ONE. 2020;15(1):e0228399.

162. Thame MM, Singh-Minott I, Osmond C, Melbourne-Chambers RH, Serjeant GR. Pregnancy in Sickle Cell-Haemoglobin C (SC) Disease, A Retrospective Study of Birth Size and Maternal Weight Gain. Eur J Obstet Gynecol Reprod Biol. 2016;203:16–9.

163. Bacq Y. The Liver in Normal Pregnancy [Internet]. Madame Curie Bioscience Database [Internet]. Landes Bioscience; 2013 [cited 2021 Mar 31]. Available from: https://www.ncbi.nlm.nih.gov/books/NBK6005/

164. Vichinsky E. Chronic organ failure in adult sickle cell disease. Hematol Am Soc Hematol Educ Program. 2017;2017(1):435–9.

165. Sharpe CC, Thein SL. How I treat renal complications in sickle cell disease. Blood. 2014;123(24):3720–6.

166. Elleamoh GE, Dake FAA. “Cementing” marriages through childbearing in subsequent unions: Insights into fertility differentials among first-time married and remarried women in Ghana. PLOS ONE. 2019;14(10):e0222994.

167. Khan SA, Damanhouri G, Ali A, Khan SA, Khan A, Bakillah A, et al. Precipitating factors and targeted therapies in combating the perils of sickle cell disease--- A special nutritional consideration. Nutr Metab [Internet]. 2016 [cited 2020 Jan 16];13. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4977632/

168. Yawn BP, Buchanan GR, Afenyi-Annan AN, Ballas SK, Hassell KL, James AH, et al. Management of Sickle Cell Disease: Summary of the 2014 Evidence-Based Report by Expert Panel Members. JAMA. 2014;312(10):1033–48.

169. Ballas SK, Kesen MR, Goldberg MF, Lutty GA, Dampier C, Osunkwo I, et al. Beyond the Definitions of the Phenotypic Complications of Sickle Cell Disease: An Update on Management [Internet]. The Scientific World Journal. 2012 [cited 2018 Feb 19]. Available from: https://www.hindawi.com/journals/tswj/2012/949535/

170. World Health Organization, editor. The WHO application of ICD-10 to deaths during pregnancy, childbirth and the puerperium, IDC MM. Geneva: World Health Organization; 2012. 68 p.

171. Howson CP, Kinney MV, McDougall L, Lawn JE, the Born Too Soon Preterm Birth Action Group. Born Too Soon: Preterm birth matters. Reprod Health. 2013;10(1):S1.

APPENDIX I: ETHICAL APPROVAL

In case of reply the number
And the date of this
Letter should be quoted

My Ref. No. KBTH/MD/C3/19

Your Ref. No......

KORLE BU TEACHING HOSPITAL
P. O. BOX KB 77,
KORLE BU, ACCRA.

Tel: +233 302 667759/673034-6
Fax: +233 302 667759
Email: Info@kbth.gov.gh
pr@kbth.gov.gh
Website: www.kbth.gov.gh

21st January, 2019

DR EUGENIA VICKY NAA KWARLEY ASARE
DEPARTMENT OF HAEMATOLOGY
KORLE BU

EFFECTS OF INCREASED HAEMOLYSIS ON FOETO-MATERNAL OUTCOMES IN PREGNANT WOMEN WITH SICKLE CELL DISEASE

KBTH-IRB /000100/2018

Investigator: Dr. Eugenia Vicky Naa Kwarley Asare

The Korle Bu Teaching Hospital Institutional Review Board (KBTH IRB) reviewed and granted approval to the study entitled "Effects of increased haemolysis on foeto-maternal outcomes in pregnant women with sickle cell disease"

Please note that the Board requires you to submit a final review report on completion of this study to the KBTH-IRB.

Kindly, note that, any modification/amendment to the approved study protocol without approval from KBTH-IRB renders this certificate invalid.

Please report all serious adverse events related to this study to KBTH-IRB within seven days verbally and fourteen days in writing.

This IRB approval is valid till 30th December, 2019. You are to submit annual report for continuing review.

Sincere regards,

MR OKYERE BOATENG
CHAIR (KBTH-IRB)

Cc: The Chief Executive Officer
Korle Bu Teaching Hospital

APPENDIX II: ELIGIBILITY FORM

1. Is the patient pregnant? ☐ Yes ☐ No

2. Does patient have sickle cell disease? ☐ Yes ☐ No

3. Is there documented evidence of the Hb phenotype? ☐ Yes ☐ No
   If yes, what is the phenotype ☐ HbSS ☐ HbSC ☐ HbAA

   If the patient has sickle cell disease,

4. Has the patient had any acute painful episode that required treatment in the emergency department or in the hosiptal for at least four consecutive weeks after a previous painful crisis?
   Yes ☐ No ☐

5. Has the patient had any blood transfusion in the last four months?
   Yes ☐ No ☐

6. Has the patient had any intercurrent illness such as infection or inflammation during the last four weeks? Yes ☐ No ☐

APPENDIX III: CONSENT FORM FOR PREGNANT (BOTH SCD AND NON-SCD) PARTICIPANTS

Title: Effects of Increased Haemolysis on Foeto-maternal Outcome In Pregnant Women With Sickle Cell Disease at the Korle-Bu Teaching Hospital

Principal Investigator: Dr. Eugenia Vicky N.K. Asare. Department of Haematology, Korle- Bu Teaching Hospital, P.O.Box KB77, Korle- Bu, Accra

Information: (To be read or translated to patients that are 18 years of age and older that meet study criteria in their own mother tongue by study personnel (Principal Investigator or research nurse)

Dear Volunteer,

This consent form contains information about the research titled **"Effects of hyperhaemolysis on Feto-maternal Outcomes In Pregnant Women With Sickle Cell Disease at the Korle-Bu Teaching Hospital."** In order to be sure that you are duly informed about your participation in this research, I am asking you to read (or have read to you) this Consent Form. You will also be asked to sign it (or thumbprint) in front of a witness. I will give you a copy of this form. This consent form might contain some words that are unfamiliar to you. Please ask me to explain anything you may not understand.

Why is this study planned?

You are being asked to participate in the above study in order to look at the steady state/ baseline level of some laboratory tests used to detect haemolysis (increased breakdown of red blood cells) as well as to determine whether pregnancy is a cause of further increased breakdown of red blood cells. This study also seeks to establish a relationship between some laboratory tests used to detect haemolysis and pregnancy outcome in women with sickle cell disease followed up in a systematic manner throughout their pregnancy till six weeks postpartum. Such a follow up will allow me to record all events that occur during the pregnancy period. I am interested in your opinion because you are receiving care at the Korle-Bu Teaching Hospital.

What does this study entail?

This study entails that the Investigator follows you up from enrollment till end of study (which is six weeks postpartum). Study time periods are enrollment (in the second trimester), 28 and 36 weeks gestation

and six weeks postpartum for pregnant women with and without SCD. In addition, pregnant women with SCD on admission will be followed up during acute events.

Each study visit will require you filling out a questionnaire which will take approximately 10 minutes; after which you will have a brief clinical examination with the measurement of blood pressure, pulse rate, oxygen saturation, respiratory rate, and temperature. You will also have 7mLs of venous blood and 10mLs of fresh urine sample collected during each study visit.

Possible Benefits:

The direct benefit to you from this study will be that haemolytic events will be detected and treatment initiated early. Additionally, your participation may help develop new and more effective preventive measures as well as enable better understanding and treatment plans/ modalities for the management of pregnant women presenting with sickle cell disease. You will not be paid for participation in this study and you are also not expected to pay anything. Cost of treatment (including blood transfusions and intravenous fluids) which fall under normal routine management procedure for patients will be covered under NHIS. All patients will be required to enroll on NHIS. There will be no extra cost to the study participants beyond their routine follow up, investigations and procedures as part of their standard of care. You will be given hardcopies of your study laboratory results as baseline laboratory results.

Possible Risks:

This research presents minimal risk to you or your baby. The main risks include, psychological distress from discussing potentially upsetting issues such as hospital admissions treatment options and adverse foetal outcomes e.g. stillbirths and intra-uterine growth restriction. Confidentiality will be protected as described in the section below.

Withdrawal from study:

I would like to stress that this study is strictly voluntary. Should you decide not to participate in the study, it will have no consequences for you. Should you, at any point during the study, decide that you do not wish to participate any further, you are free to terminate/ stop the participation, effective immediately. Any such decision will be respected without any further discussion. Your decision will not affect the health care you would normally receive.

Confidentiality:

All efforts, within reason, will be made to keep your protected health information (PHI) private. PHI is your health information that is, or has been gathered or kept by Korle-Bu Teaching Hospital, as a result of your healthcare. This includes data gathered for research studies that can be traced back to you. Using or sharing such data will follow ethical privacy rules by research personnel. By signing the consent for this study, you are agreeing to the use and likely sharing of your PHI. If you decide to be in this research study, you are also agreeing to let me use and share you and your child's PHI as described below.

As part of the study, I may share the results of you and your child's study and/or non-study linked laboratory results as well as parts of you or your child's medical record, with the personnel from Korle-Bu Teaching Hospital, and your doctor if a medical condition that needs urgent attention is discovered.

The study results will be kept in you and your child's research records for at least one year after the study is finished. At that time, the research data that has not been put in you or your child's medical records will be kept for an unknown length of time. Any research data that has been put into your medical record will be kept for an unknown length of time. Unless told otherwise, your consent to use or share PHI does not expire. If you change your mind, I ask that you contact me (Dr. Eugenia Vicky Asare) in writing and let me know that you withdraw your consent to participate in the study. My mailing address is Department of Haematology, P. O. Box KB77, Korle-Bu Teaching Hospital or Ghana Institute of Clinical Genetics, P. O. Box KB150, Korle- Bu, Accra. At that time, I will stop getting any more data about you. The health data I stored before you withdrew your consent will also not be reported on.

At any time, you may ask to have your data or samples destroyed. You should contact me (Dr. Eugenia Vicky Asare), at either Department of Haematology, P. O. Box KB77, Korle-Bu Teaching Hospital or Ghana Institute of Clinical Genetics, P. O. Box KB150, Korle- Bu, Accra. (Telephone number 0206301166) to have your sample destroyed and no longer used for research. I will not be able to destroy research data that has already been gathered using your sample. Also, if your identity was removed from the samples, I will not be able to locate and destroy them. There will be no costs to you for any of the tests done on your samples. You will not be paid for the use of your samples.

Contacts:

If you ever have any questions about the research study or study-related problems, you may contact me (Dr. Eugenia Vicky Asare), at either Department of Haematology, P. O. Box KB77, Korle-Bu Teaching

Hospital or Ghana Institute of Clinical Genetics, P. O. Box KB150, Korle- Bu, Accra. (Telephone number 0206301166). For questions about the ethical aspects of this study or your rights as a volunteer, you may contact the Office of the Institutional Review Board, Korle-Bu Teaching Hospital (Telephone number 0302667759).

Your Rights As A Participant:

This research has been reviewed and approved by the Institutional Review Board, Korle-Bu Teaching Hospital. An Institutional Review Board is a committee that ONLY reviews research studies in order to help protect participants. If you have any questions about your rights as a research participant, you may contact the Chairman of the Institutional Review Board, Korle-Bu Teaching Hospital.

VOLUNTEER AGREEMENT:

The above document describing the benefits, risks and procedures for the research titled: "***Effects of Hyperhaemolysis on Fetomaternal Outcomes in Pregnant Women with Sickle Cell Disease at Korle-Bu Teaching Hospital***", has been read and explained to me. I have been given an opportunity to have any questions about the research answered to my satisfaction. I agree to participate as a volunteer.

---------- ----------

Date (Day/Month/Year) Name of Volunteer/Study Participant (PRINT)

Signature or Thumbprint of Volunteer/Study Participant

If volunteer/study participant cannot read the form themselves, a witness must sign here:

I was present while the benefits, risks and procedures were read to the volunteer. All questions were answered and the volunteer has agreed to take part in the research.

---------- ----------

Date (Day/Month/Year) Name of Witness (PRINT)

Signature or Thumbprint of witness

I certify that the nature and purpose, the potential benefits, and possible risks associated with participating in this research have been explained to the above individual.

| --- | --- | --- |
| Date | Time of Consent (HH; MM) | Name of Study Personnel (PRINT) |

Signature of Principal Investigator

APPENDIX IV: CONSENT FORM FOR NON- PREGNANT SCD PARTICIPANTS

Information: (To be read or translated to patients that are 18 years of age and older that meet study criteria in their own mother tongue by study personnel (Principal Investigator or research nurse)

Dear Volunteer,

This consent form contains information about the research that aims at **"Determining The Steady State of Some Selected Markers of Haemolysis In Non-Pregnant and Pregnant Women With Sickle Cell Disease."** In order to be sure that you are duly informed about your participation in this research, I am asking you to read (or have read to you) this Consent Form. You will also be asked to sign it (or thumbprint) in front of a witness. I will give you a copy of this form. This consent form might contain some words that are unfamiliar to you. Please ask me to explain anything you may not understand.

Why is this study planned?

You are being asked to participate in the above study in order to estimate the steady state of some laboratory tests used to detect haemolysis (increased breakdown of red blood cells) in non-pregnant women with sickle cell disease, by comparing them to the same laboratory tests in pregnant women with sickle cell disease followed up in a systematic manner throughout their pregnancy. You will be matched by phenotype and age to a pregnant woman with sickle cell disease. I will follow you up till your pregnant match delivers through six weeks postpartum. I am interested in your opinion because you are receiving care at the adult sickle cell clinic of the Ghana Institute of Clinical Genetics, Korle- Bu.

What does this study entail?

This study entails that the Investigator follows you up from enrollment till end of study (which is when your corresponding pregnant participant is six weeks postpartum). Study time periods are baseline/ enrollment and at the end of the study (when their corresponding pregnant cases are at six weeks postpartum).

Each study visit will require you filling out a questionnaire which will take approximately 10 minutes; after which you will have a brief clinical examination with the measurement of blood pressure, pulse rate,

oxygen saturation, respiratory rate, and temperature. You will also have 7mLs of venous blood and 10mLs of fresh urine sample collected during each study visit.

Possible Benefits:

The direct benefit to you from this study will be that haemolytic events will be detected and treatment initiated early. Additionally, your participation will help develop new and more effective preventive measures as well as better understanding and treatment modalities for the management of pregnant women presenting with sickle cell disease. You will not be paid for participation in this study and you are also not expected to pay anything. Cost of treatment (including blood transfusions and intravenous fluids) which fall under normal routine management procedure for patients will be covered under NHIS. All patients will be required to enroll on NHIS. There will be no extra cost to the study participants beyond their routine follow up, investigations and procedures as part of their standard of care. You will be given hardcopies of your study laboratory results as baseline laboratory results.

Possible Risks:

This research presents minimal risk to you. The main risks include, psychological distress from discussing potentially upsetting issues like hospital admissions and treatment measures. Confidentiality will be protected as described in the section below. There may also be some discomfort in drawing blood sample, however, every effort will be made to reduce such experience to the barest minimum.

Withdrawal from study:

I would like to stress that this study is strictly voluntary. Should you decide not to participate in the study, it will have no consequences for you. Should you, at any point during the study, decide that you do not wish to participate any further, you are free to terminate the participation, effective immediately. Any such decision will be respected without any further discussion. Your decision will not affect the healthcare you would normally receive.

Confidentiality:

All efforts, within reason, will be made to keep your protected health information (PHI) private. PHI is your health information that is, or has been gathered or kept by the Ghana Institute of Clinical Genetics, as a result of your healthcare. This includes data gathered for research studies that can be traced back to

you. Using or sharing such data must follow federal privacy rules by research personnel. By signing the consent for this study, you are agreeing to the use and likely sharing of your PHI. If you decide to be in this research study, you are also agreeing to let me use and share your child's PHI as described below.

As part of the study, I may share the results of your study and/or non-study linked laboratory results as well as parts of your medical record, with the personnel from Ghana Institute of Clinical Genetics, and your doctor if a medical condition that needs urgent attention is discovered.

The study results will be kept in your research records for at least one year after the study is finished. At that time, the research data that has not been put in your medical records will be kept for an unknown length of time. Any research data that has been put into your medical record will be kept for an unknown length of time. Unless told otherwise, your consent to use or share PHI does not expire. If you change your mind, I ask that you contact me (Dr. Eugenia Vicky Asare) in writing and let me know that you withdraw your consent to participate in the study. My mailing address is Department of Haematology, P. O. Box KB77, Korle-Bu Teaching Hospital or Ghana Institute of Clinical Genetics, P. O. Box KB150, Korle- Bu, Accra. At that time, I will stop getting any more data about you. The health data I stored before you withdrew your consent will also not be reported on.

At any time, you may ask to have your data or samples destroyed. You should contact me (Dr. Eugenia Vicky Asare), at either the Department of Haematology, P. O. Box KB77, Korle-Bu Teaching Hospital or Ghana Institute of Clinical Genetics, P. O. Box KB150, Korle- Bu, Accra. (Telephone number 0206301166) to have your sample destroyed and no longer used for research. I will not be able to destroy research data that has already been gathered using your sample. Also, if your identity was removed from the samples, I will not be able to locate and destroy them. There will be no costs to you for any of the tests done on your samples. You will not be paid for the use of your samples.

Contacts:

If you ever have any questions about the research study or study-related problems, you may contact me (Dr. Eugenia Vicky Asare), at either the Department of Haematology, P. O. Box KB77, Korle-Bu Teaching Hospital or Ghana Institute of Clinical Genetics, P. O. Box KB150, Korle- Bu, Accra. (Telephone number 0206301166). For questions about the ethical aspects of this study or your rights as a

volunteer, you may contact the Chairman of the Institutional Review Board, Korle-Bu Teaching Hospital (Telephone number 0302667759).

Your Rights As A Participant:

This research is part of a broader study, which has been reviewed and approved by the Institutional Review Board, Korle-Bu Teaching Hospital. An Institutional Review Board is a committee that ONLY reviews research studies in order to help protect participants. If you have any questions about your rights as a research participant, you may contact the Chairman of the Institutional Review Board, Korle-Bu Teaching Hospital.

VOLUNTEER AGREEMENT:

The above document describing the benefits, risks and procedures for the research has been read and explained to me. I have been given an opportunity to have any questions about the research answered to my satisfaction. I agree to participate as a volunteer.

---------- ----------

Date (Day/Month/Year) Name of Volunteer/Study Participant (PRINT)

Signature or Thumbprint of Volunteer/Study Participant

If volunteer/study participant cannot read the form themselves, a witness must sign here:

I was present while the benefits, risks and procedures were read to the volunteer. All questions were answered and the volunteer has agreed to take part in the research.

---------- ----------

Date (Day/Month/Year) Name of Witness (PRINT)

Signature or Thumbprint of witness

I certify that the nature and purpose, the potential benefits, and possible risks associated with participating in this research have been explained to the above individual.

| --- | --- | --- |
| Date | Time of Consent (HH; MM) | Name of Study Personnel (PRINT) |

Signature of Principal Investigator

APPENDIX V-A: QUESTIONNAIRE FOR PREGNANT (BOTH SCD AND NON-SCD) STUDY PARTICIPANTS: ENROLLMENT

Study ID number: **Visit number:**

Phenotype: Age:

Parity: Gestational age:

QUESTIONS

1. Do you have sickle cell disease (SCD)? □□Yes □□No □□Don't Know

   If yes, when were you diagnosed with SCD?.....

   If no, skip to question 3

2. Have you ever been diagnosed with any of these SCD co- morbidities?

   i. Acute chest syndrome □□Yes □□No □□Don't Know

   If yes, state how you were diagnosed…….

   If yes, when did it occur?.....

   Were you admitted? □□Yes □□No □□Don't Know

   If yes, state the duration you were admitted…..…….

   ii. Anaemia requiring blood transfusion in the last one year □□Yes □□No

   If yes, state how you were diagnosed…….

   If yes, how many times you have been transfused? □□1 □□2 □□3 □□4 □□5

   If yes, state the blood component you were given.……...

   How many units were you transfused?.....

   iii. Avascular necrosis of femoral or humeral head □□Yes □□No □□Don't Know

   If yes, when were you diagnosed? …….

   If yes, state how you were diagnosed…….

   iv. Chronic leg ulcers □□Yes □□No

   If yes, when were you diagnosed?.....

If yes, state how you were diagnosed…….

v. Pulmonary hypertension □□Yes □□No □□Don't Know

If yes, when were you diagnosed?.....

If yes, state how you were diagnosed……..

vi. Sickle cell kidney disease □□Yes □□No □□Don't Know

If yes, when were you diagnosed?.....

vii. Stroke □□Yes □□No □□Don't Know

If yes, when were you diagnosed?.....

If yes, state how you were diagnosed……..

viii. Gallstones □□Yes □□No □□Don't Know

If yes, when were you diagnosed?.....

If yes, state how you were diagnosed……..

3. Is this your first pregnancy? □□Yes □□No

If no,

Do you have a history of recurrent spontaneous abortions? □□Yes □□No

Do you have a history of stillbirths? □□Yes □□No

Do you have a history of preterm births? □□Yes □□No

Do you have a history of low birthweight infants? □□Yes □□No

4. Have you ever been diagnosed with hypertension □□ Yes □□No

a. If yes, when were you diagnosed with hypertension..…….....

APPENDIX V-B: QUESTIONNAIRE FOR PREGNANT (BOTH SCD AND NON-SCD) STUDY PARTICIPANTS: SUBSEQUENT VISITS

DEMOGRAPHICS

Study ID number: **Visit number:**

Phenotype: Age:

Parity: Gestational age:

QUESTIONS

Kindly jump to question 7 if you do not have SCD.

If you have SCD, have you ever been diagnosed with any of these SCD- related complications since your last antenatal visit?

1. Acute pain episode □□Yes □□No
   If yes, state the gestational age episode occured
   Were you admitted? □□Yes □□No
   If yes, state the date of admission…….…….
   If yes, state the duration you were admitted…..…….
2. Acute chest syndrome □□Yes □□No
   If yes, state the gestational age episode occured
   Were you admitted? □□Yes □□No
   If yes, state the date of admission…….…….
   If yes, state the duration you were admitted…..…….
3. Darkening of the urine (coca colour- like) □□Yes □□No
   If yes, state the gestational age episode occurred……
   Were you admitted? □□Yes □□No
   If yes, state the date of admission…….…….
   If yes, state the duration you were admitted..…….
4. Progressive yellowing of the eyes (jaundice) □□Yes □□No
   If yes, state the gestational age episode occured
   Were you admitted? □□Yes □□No
   If yes, state the date of admission…….…….

If yes, state the duration you were admitted..…….……

5. Anaemia requiring blood transfusion □□Yes □□No

   If yes, how many times you have been transfused? □□1 □□2 □□3 □□4 □□5

   If yes, state the reason for the transfusion…….……..

   If yes, state the blood component you were given…….……

   How many units were you transfused? □□1 □□2 □□3 □□4 □□5 □□6 □□>6

6. Gall bladder disease □□Yes □□No

   If yes, state the gestational age episode occured

   Were you admitted? □□Yes □□No

   If yes, state the date of admission…….……

   If yes, state the duration of admission…..…….……..

7. Have you had a spontaneous abortion since the last study visit? □□Yes □□No

8. Have you had a stillbirth since the last study visit? □□Yes □□No

9. Has the study participant delivered since the last study visit? □□Yes □□No

   If yes, what was the birth weight?.....

10. Have you had an early neonatal death (one week after delivery) since the last study visit?

    □□Yes □□No

APPENDIX VI-A: QUESTIONNAIRE FOR NON- PREGNANT SCD STUDY PARTICIPANTS: ENROLLMENT

Study ID number: **Visit number:**

Phenotype: Age:

QUESTIONS

1. Are you pregnant? □□Yes □□No
2. When was your last menstrual period (LMP)? **……..**
3. Do you have sickle cell disease (SCD)? □□Yes □□No

   If yes, when were you diagnosed with SCD?.....

4. Have you ever been diagnosed with any of these SCD co- morbidities?

   i. Acute chest syndrome □□Yes □□No □□Don't Know

   If yes, state how you were diagnosed…….

   If yes, when did it occur?.....

   Were you admitted? □□Yes □□No

   If yes, state the duration you were admitted…..……

   ii. Anaemia requiring blood transfusion □□Yes □□No

   If yes, state how you were diagnosed…….

   If yes, state the blood component you were given……...

   How many units were you transfused?.....

   iii. Avascular necrosis of femoral head □□Yes □□No □□Don't Know

   If yes, when were you diagnosed? …….

   If yes, state how you were diagnosed…….

   iv. Chronic leg ulcers □□Yes □□No

   If yes, when were you diagnosed?.....

   v. Pulmonary hypertension □□Yes □□No □□Don't Know

   If yes, when were you diagnosed?.....

If yes, state how you were diagnosed…….

vi. Sickle cell kidney disease □□Yes □□No □□Don't Know

If yes, when were you diagnosed?.....

If yes, state how you were diagnosed…….

vii. Stroke □□Yes □□No □□Don't Know

If yes, when were you diagnosed?.....

viii. Gallstones □□Yes □□No □□Don't Know

If yes, when were you diagnosed?.....

3. Have you ever been diagnosed with hypertension □□ Yes □□No

a. If yes, when were you diagnosed with hypertension..…….....

APPENDIX VII: CLINICAL ASSESSMENT FORM FOR STUDY PARTICIPANTS (BOTH PREGNANT AND NON- PREGNANT)

Study ID number : **Visit number :**

Phenotype: Age :

Parity: Gestational age :

Weight (kg) :

Height (cm) :

Body mass index (BMI) :

Blood pressure (mmHg) :

Pulse rate (per minute) :

SpO_2 measurement (%) :

Respiratory rate (per minute) :

Temperature (^0C) :

APPENDIX VIII: REFERENCE RANGES OF LABORATORY PARAMETERS USED IN DATA ANALYSIS

Reference range for anaemia in pregnancy[91]

	REFERENE RANGE
Severe anaemia	< 7.0 g/dL
Moderate anaemia	7.0 – 9.9 g/dL
Mild anaemia	10.0 – 10.9 g/dL
Normal	> 10.9 g/dL

Reference ranges used at the Korle-Bu Polyclinic Laboratory

	REFERENCE RANGE	**CUT OFF VALUE USED FOR DATA ANALYSIS**
Absolute reticulocyte count (x 10^9 /L)	18 – 158	158
Total bilirubin (umol/L)	3.4 – 22.0	22.0
Direct bilirubin (umol/L)	≤ 5.13	5.13
Indirect bilirubin (umol/L)	≤ 10.0	10.0
LDH (IU/L)	100 - 190	190
AST (IU/L)	0 - 35	35
ALT (IU/L)	0 - 35	35

GLOSSARY (STUDY DEFINITIONS)

For the purposes of this study, the following definitions were used.

Severe anaemia: Defined as a drop in haemoglobin (Hb) value by ≥ 2 g/dL from the steady state

Acute events: Defined as any acute event (acute pain episode, acute chest syndrome (ACS) or relative anaemia) requiring hospital admission and management, including blood transfusion.

Acute vaso-occlusive pain episode: Also known as acute pain episode was defined as acute sickle cell pain crisis requiring hospitalization and oral or intravenous opioid, distinguished from labour pains by absence of uterine contraction, labour progression, and delivery.

Acute chest syndrome (ACS): Per standard definition, ACS is defined as "the presence of a new radio density on chest radiograph accompanied by respiratory signs and symptoms."[168] A diagnosis of pneumonia in SCD is considered an ACS episode.[121]

"Due to the challenges of having a baseline chest x-ray for comparative purposes and in the absence of abdominal shields for chest x-rays during pregnancy, a diagnosis of ACS will be be made based on at least two of the following criteria: temperature >38^0C, respiratory rate >20 per minute, chest pain or abnormal pulmonary auscultatory findings, increased oxygen requirement (SpO_2 drop >3% from a documented steady-state value on room air) ± a new radio density on chest roentgenogram."[29] A chest x-ray will be done for only postpartum women. A diagnosis of pneumonia will be considered an ACS episode.

Hyperhaemolysis: Defined as "marked drop in Hb, determined in the steady state with evidence of increased red blood cell destruction in the absence of any other identifiable causes such as splenic or hepatic sequestration."[169]

Diagnostic criteria[169]

- Decrease in Hb ≥ 20% from baseline values
- Increase in reticulocyte count by 25% from baseline, or presence of nucleated red blood cells
- Evidence of haemolysis (e.g., increased LDH, unconjugated bilirubin, or AST) compared with baseline

- Without:
  - Resolving human parvovirus B19
  - Resolving folate deficiency
  - Resolving hydroxyurea toxicity
  - Blood loss/ haemorrhage
  - Splenic or hepatic sequestration, acute chest syndrome

Severity index

Severity is judged by percentage reduction in haemoglobin level

Classification

Transfusion related

- Innocent bystander mechanism
- Acute or delayed haemolytic transfusion reaction

Non-transfusion related

- Due to acute painful episodes, infection, drugs, G6PD deficiency, or autoimmune hyperhaemolysis

Second trimester: Period of gestation between 13 weeks 1 day and 26 weeks gestation

Third trimester: Period of gestation between 26 weeks 1 day and 40 weeks gestation

Hypertensive disorders of pregnancy using the WHO definition, includes pregnancy induced hypertension, preeclampsia and eclampsia[170]

- Pregnancy induced hypertension was defined as "a rise in blood pressure to 140mmHg systolic and or 90mmHg diastolic or more after 20 weeks gestation."
- Preeclampsia was defined as "a rise in systolic blood pressure to 140mmHg and or diastolic blood pressure of 90mmHg or more after 20 weeks with associated proteinuria 1+ on dipstick or 300mg in 24-hour urine collection or more."

Maternal mortality was defined using the WHO definition as "death of a woman during pregnancy and up to 42 days after termination of a pregnancy irrespective of the duration or site of the gestation

from any event related to the pregnancy or its management but excluding incidental or accidental causes."[170]

Spontaneous abortion: Defined as spontaneous termination of a non-viable fetus (before 28 weeks gestation)

Delivery was defined as birth (live birth or stillbirth) after 28 weeks of gestation.

Preterm delivery was defined and classified using the WHO criteria as "babies born alive before 37 weeks of pregnancy were completed[171]. There were sub-categories of preterm birth, based on gestational age: a) extremely preterm (less than 28 weeks); b) very preterm (28 to 32 weeks); c) moderate to late preterm (32 weeks to 36 weeks 1 day)."[171]

Low birthweight: Per WHO definition low birthweight was defined as "birthweights below 2,500 grams."[171]

Very low birthweight: Per WHO definition low birthweight was defined as "birthweights below 1,500 grams."[171]

Intrauterine fetal death: Defined as babies born detected dead in-utero after 28 weeks gestation.

Perinatal mortality: Per WHO was defined as "death of a fetus in-utero after 28 weeks and up to seven days after delivery."

Perinatal mortality rate: Per WHO was defined as "the number of stillbirths after 28 weeks gestation plus early neonatal death (newborn death within the first week of life) per 1000 total births."

Puerperium refers to "the period from delivery of the baby and placenta up to six weeks postpartum."

Adverse events and severe adverse events were defined as "adverse reactions that were expected complications in pregnant women with SCD, including acute pain, ACS, hyperhemolytic crisis,

hospitalization, and transfusion. We also defined severe adverse events as those adverse events that lead to prolonged hospitalization beyond 7 days, admission into the intensive care unit, and near death or death."