

**Maternal DNA Contamination of Chorionic Villi and  
Population Genetics study in Sickle Cell  
Anaemia**

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# TABLE OF CONTENTS

Declaration

Dedication

Acknowledgement

List of Tables

List of Figures

List of Abbreviations

Definition of terms

Abstract

## **1.0. CHAPTER ONE: INTRODUCTION**

1.1. Background of Study

1.2. Problem Statement

1.3. Research Questions

1.4. Objectives

1.4.1 General Objectives

1.4.2 Specific Objectives

1.5. Rationale for Study

## **2.0. CHAPTER TWO: LITERATURE REVIEW**

2.1. The Human Haemoglobin (Genotype): General Characteristics

2.1.1. Molecular Genetics of Normal and Abnormal Haemoglobins

2.2. Mode of transmission and inheritance of Sickle Cell Anaemia and traits

2.3. History and Evolution of Sickle Cell Anaemia

2.4. Epidemiology of Sickle Cell Disease

2.4.1. Global prevalence of Sickle Cell Anaemia

2.4.2. Status of Sickle Cell Anaemia in Nigeria

- 2.4.3. Factors influencing the distribution of SCA
- 2.5. Pathophysiology and Phenotype of Sickle Cell Anaemia
- 2.6. Variability of Phenotypic Expression
- 2.7. Gene Frequency Distribution
- 2.8. The Placenta
- 2.8.1 The Chorion Villi
- 2.8.2 Maternal Uterine Decidua
- 2.9. Maternal Uterine Decidua Cell Contamination
- 2.10 Prenatal Diagnosis Sampling Procedures
- 2.11. Principles of Molecular diagnosis
- 2.11.1. DNA Extraction
- 2.11.2. Molecular Analysis Techniques
- 2.12. Awareness and Utilization of Prenatal Diagnosis
- 2.13. Ethical Issues

### **3.0. CHAPTER THREE: METHODOLOGY**

- 3.1. Study Design
- 3.2. Research Centres
- 3.3. Sample size Determination
- 3.4. Subjects' Recruitment
- 3.5. Chorionic Villous Sampling
- 3.6. Villi dissection/Removal of Maternal Decidua Cell
- 3.7. Deoxyribose Nucleic Acid (DNA) Extraction
- 3.8. Amplification Refractory Mutation System–Polymerase Chain Reaction (ARMS PCR) Analysis
- 3.9. Gel documentation and Ultraviolet transillumination

### **3.9. Questionnaire administration**

#### **3.10. Data Collection and Analysis**

## **4.0. CHAPTER FOUR: RESULTS**

### **4.1. General**

#### **4.2. Bio Social and Obstetric Characteristics**

#### **4.3. Maternal DNA in Chorionic Villi**

#### **4.4. Distribution of Fetal Haemoglobin Genotypes based on Mendelian Ratio**

#### **4.5. Frequency Distribution of Fetal Haemoglobin Genotypes and Alleles**

#### **4.6. Distribution of Haemoglobin Genotypes Fetal, Neonatal and Adolescent Population**

#### **4.7. CVS Complications**

#### **4.8. Awareness and Acceptance of (Early) Prenatal Diagnosis**

## **5.0. CHAPTER FIVE: DISCUSSION**

## **6.0. CHAPTER SIX: SUMMARY OF FINDINGS AND CONTRIBUTION TO KNOWLEDGE**

### **6.1. Summary of findings**

### **6.2. Contribution to knowledge**

### **6.3. Works yet to be done**

## **Conclusion**

## **REFERENCES**

## **LIST OF TABLES**

Table 1: Methods of SCA analysis

Table 2: Baseline Social and Obstetric parameters

Table 3: Complications of Chorionic Villous Sampling

Table 4: Fetal Haemoglobin Genotype results

Table 5: Results/outcome with serial concentrations of maternal DNA

Table 6: Analysis of Fetal Haemoglobin Genotype

Table 7: Distribution of Haemoglobin Genotypes In-utero, Neonatal and Adulthood

Table 8: Awareness.....

## **LIST OF FIGURES**

Figure 1: Matrix diagram showing the possible tetrameric products of the  $\alpha$  -globin cluster on chromosome 16 and the  $\beta$  - globin cluster on chromosome 11

Figure 2: Haemoglobin sequences in adult haemoglobin A and S

Figure 3: Pattern of inheritance

Figure 4: Global distribution of pathological Haemoglobin disorders.

Figure 5: Primary villi

Figure 6: Secondary villi

Figure 7: Tertiary villi

Figure 8: Placenta anatomy

Figure 9: Placenta and Fetus at 11-13<sup>+6</sup> weeks

Figure 10: CVS needle in placenta tissue

Figure 11: Chorionic villi in EDTA

Figure 12: Photographic documentation for weight estimation

Figure 13: CVS Needles

Figure 14: Haemoglobin genotype AA band

Figure 15: Haemoglobin genotype AS band

Figure 16: Haemoglobin genotype SS band

Declaration

Dedication

Acknowledgement

### **List of Abbreviations**

ASO	Allele Specific Oligonucleotide
ARMS	Allele Refractory Mutation System
RFLP	Restriction Fragment Length Polymorphism
PCR	Polymerase Chain Reaction
EDTA	Ethylene Diamine Tetra-acetic Acid
CVS	Chorionic Villous Sampling
SCA	Sickle Cell Anaemia
SCD	Sickle Cell Disease
SCT	Sickle Cell Trait
WHO	World Health Organization
AR	Autosomal Recessive
AD	Autosomal Dominant

### **Definition of Operational terms**

**Haemoglobin:** It is the normal colouring matter of the red blood cells of vertebrate animals. It has haematin and globulin and is occasionally referred to as haematoglobulin. In arterial blood it is combined with oxygen and is called oxyhaemoglobin. However, in different animals, it crystallizes into different forms where it is called haemocytocrystallin.

**Sickle Cell Anaemia:** Is a group of inherited red blood cell disorders, or a collection of recessive genetic disorders characterised by a haemoglobin variant called haemoglobin S in homozygous state. Sickle cell anaemia is at times used interchangeably with sickle cell disease.

**Fetal Period:** Refers to the interval between conception and

**Infancy:** Refers to the interval between

**Adolescence:**

**Prenatal Counselling:** Is defined as the process of communicating information to the client as a basis for an informed opinion about all aspects of prenatal diagnosis.

**Prenatal Screening:** Refers to steps or procedures done during pregnancy, to select the group of pregnant women in the general population that is at a higher risk of a particular congenital abnormality.

**Prenatal Diagnosis:** Refers to the processes or steps involved in the screening and confirmation of fetal disorders arising from genetic, environmental or combination of both factors

**Frequency:** Is the measure of the occurrence of a disease in the population.

**Prevalence:** This is the proportion of the population that are cases of a disease. It includes both new and existing cases.

**Incidence:** This is a rate at which new cases of a disease occur in a population during a specific period of time.

**Sample:** Is a sub-set of a population observed for the purposes of making inferences about the nature of the total population.

**Sampling Frame:** Is actual or quasi-list of sampling units from which the sample or some stage of sampling is selected. The representativeness of the sample depends directly on the

extent to which a sampling frame contains all the members of the total population which the sample is meant to represent.

**Cross Sectional Study:** This is a study in which a representative sample of study subjects from the population is obtained regardless of exposure or outcome status.

**Sensitivity:** Is the ability of a test to correctly detect those who have a disease among persons with the disease. A test with high sensitivity will have few false negatives.

**Specificity:** It is the ability of a test to correctly detect those who do not have a disease among persons without the disease. A test with high specificity will have few false positives.

**Predictive Value:** This measures whether an individual actually has a disease or not given the results of a screening test. There is positive predictive value and negative predictive value.

**Positive Predictive value:** Is defined as a proportion of persons who actually have the disease (true positive) among all the test positive results of the screening test.

**Negative Predictive Value:** Is the proportion of individuals who actually do not have the disease (true negative) among all the test negative results of the screening test.

**Confidence Interval:** Defines the variability of the estimate of a parameter or event (e.g. disease) in specific samples. It specifies how likely the disease occurs in certain samples. It uses upper and lower limits

**Confidence Limits:** These are upper and lower levels (ends) of confidence interval.

**Odds Ratio:** Is the ratio of the chance or likelihood of the occurrence of the disease to the chance of it not occurring.

**Hereditary Persistence of Sickle Cell Haemoglobin:** Is an inherited condition in which fetal haemoglobin levels persistently remain higher than 2% of the total haemoglobin.

## **Abstract**



Sickle cell anaemia is the most common single gene defect among black population. The two main levels of control are early prenatal diagnosis and premarital counselling and screening. Early prenatal diagnosis is performed on the chorionic villi, which is usually contaminated with DNA from the maternal decidua cells during sampling. Co- amplification of the maternal and fetal DNA is a major challenge during molecular diagnosis using the ARMS PCR protocol. The outcome would be laboratory misdiagnosis.

In this study, the ARMS PCR protocol was used for **the** molecular diagnosis, **followed by** analysis of genotypes and alleles in the fetal population **resulting from following** assortative mating between the genotype groups that would result in the delivery of haemoglobin genotype SS progeny. The effect of maternal DNA contamination of chorionic villi was indirectly studied using DNA extracted from maternal venous blood. The study analysed the frequencies of genotypes and alleles in the fetal population, as well as the general population, with the aim of establishing the trend and deviations from expectations following assortative mating pattern and the fitness of the various haemoglobin genotypes. The frequency of abortion, as well as awareness and acceptance of the procedure among women were also studied.

**Methodology:** Seventy four pregnancies from AS and AS union had chorionic villous sampling and molecular diagnosis between 11<sup>+0</sup> – 13<sup>+6</sup> weeks of pregnancy. Homozygous haemoglobin genotype AA or SS were further analysed for the effect of adding different amounts of heterozygous maternal DNA to fixed amount of fetal DNA. Another 74 couples completed questionnaire to determine the gene frequencies among their offspring between the neonatal periods to adulthood.

**Results:** More than a third was between 26-30 years and Para 3-4. The majority were Christian with secondary or tertiary education and in the middle or higher socio economic group. Miscarriage was not recorded in any case.

Fifty percent (37 fetuses) fetal haemoglobin genotype were HbA<sub>1</sub>HbS, and remaining 50% were either HbS<sub>1</sub>HbS (15 cases: 18.0%) or HbA<sub>1</sub>HbA (22 cases: 32.0%). **More )X vs X Chi square) of HbX<sub>1</sub>HbX than HbX<sub>1</sub>HbX changed with maternal DNA changed with maternal DNA contamination.**

Analysis of Thirty seven foetuses with either homozygous haemoglobin genotype AA or SS after the addition of between 0.25 – 1.0ul of maternal DNA to 4.0ul of fetal DNA showed a greater statistically significant change to AS occurred in fetal haemoglobin AA than SS (37.5% vs 11.1%). Both produced statistically significant change at maternal DNA concentration  $\geq 1.0$ ul (77.5% - 81.2%).

The trend of distribution of genotype in the various population groups **showed a general decline in the frequency of haemoglobin XX from fetal to infancy and adulthood. Statistical difference between observed and expected frequencies was observed in the adulthood population.....**

The study demonstrates the feasible (application) of ARMS-PCR for molecular diagnosis of fetal haemoglobin genotype and the distribution of haemoglobin genotype in pregnancy.

Nucleic acid samples can be readily checked for concentration and quality using the NanoDrop 1000 Spectrophotometer.

**260/280:** ratio of sample absorbance at 260 and 280 nm. The ratio of absorbance at 260 and 280 nm is used to assess the purity of DNA and RNA. A ratio of  $\sim 1.8$  is generally accepted as “pure” for DNA; a ratio of  $\sim 2.0$  is generally accepted as “pure” for RNA. If the ratio is

appreciably lower in either case, it may indicate the presence of protein, phenol or other contaminants that absorb strongly at or near 280 nm. See “260/280 Ratio” in the Troubleshooting section for more details on factors that can affect this ratio.

**260/230:** ratio of sample absorbance at 260 and 230 nm. This is a secondary measure of nucleic acid purity. The 260/230 values for “pure” nucleic acid are often higher than the respective 260/280 values. They are commonly in the range of 1.8-2.2. If the ratio is appreciably lower, this may indicate the presence of co-purified contaminants.

**ng/ul:** sample concentration in ng/ul based on absorbance at 260 nm and the selected analysis constant. See the “Concentration Calculation (Beer’s Law)” in the appendix for more details on this calculation.

#### ....**Nucleotide mix in your sample**

The five nucleotides that comprise DNA and RNA exhibit widely varying 260:280 ratios<sup>\*\*</sup>. The following represent the 260:280 ratios estimated for each nucleotide if measured independently:

Guanine: 1.15

Adenine: 4.50

Cytosine: 1.51

Uracil: 4.00

Thymine: 1.47

The resultant 260:280 ratio for the nucleic acid being studied will be approximately equal to the weighted average of the 260:280 ratios for the four nucleotides present. It is important to note that the generally accepted ratios of 1.8 and 2.0 for DNA and RNA are “rules

**17-12** *Section 17- Troubleshooting*

of thumb". The actual ratio will depend on the composition of the nucleic acid. Note: RNA will typically have a higher 260:280 ratio due to the higher ratio of Uracil compared to that of Thymine.

.. Leninger, A. L. *Biochemistry*, 2<sup>nd</sup> ed., Worth Publishers, New York, 1975

#### 260/280 Ratio

Many researchers encounter a consistent 260/280 ratio change when switching from a standard cuvette spectrophotometer to the

**17-11** *Section 17- Troubleshooting*

NanoDrop Spectrophotometer. The three main causes for this are listed below:

#### **Change in sample acidity**

Small changes in solution pH will cause the 260:280 to vary<sup>\*\*</sup>. Acidic solutions will under-represent the 260:280 ratio by 0.2-0.3, while a basic solution will over-represent the ratio by 0.2-0.3. If comparing the NanoDrop 1000 Spectrophotometer to other spectrophotometers, it is important to ensure that the pH of an undiluted sample measured on the NanoDrop 1000 Spectrophotometer is at the same pH as the diluted sample measured on the second spectrophotometer.

<sup>\*\*</sup> William W. Wilfinger, Karol Mackey, and Piotr Chomczynski, Effect of pH and Ionic Strength on the Spectrophotometric Assessment of Nucleic Acid Purity: *BioTechniques* 22:474-481 (March 1997)

#### **Wavelength accuracy of the spectrophotometers**

Although the absorbance of a nucleic acid at 260nm is generally on a plateau, the absorbance curve at 280nm is quite steeply sloped. A slight shift in wavelength accuracy will have a large effect on 260:280 ratios. For example, a +/- 1 nm shift in wavelength accuracy will result in a +/- 0.2 change in the 260:280 ratio. Since many spectrophotometers claim a 1 nm accuracy specification, it is possible to see as much as a 0.4 difference in the 260:280 ratio when measuring the same nucleic acid sample on two spectrophotometers that are both within wavelength accuracy specification.

#### **Nucleotide mix in your sample**

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# CHAPTER ONE

## INTRODUCTION

### 1.1 Background of Study

Sickle cell anaemia is the commonest single gene defect defects among blacks and the first molecular disease diagnosed through the application of molecular technology(Nietert, Silverstein andAbboud, 2002; Pauling, Itano and Singer *et. al.*, 1949; Gelerhter and Collins, 1990). The term Sickle cell anaemia (SCA) is sometimes used interchangeably with Sickle cell disease (SCD), especially in regions where it is the most predominant form of the disease(Mpalampa, *et al.*, 2012).Sickle cell anaemia is a genetic disease caused bymutation in  $\beta$ -globin gene located on the short arm of human chromosome 11 (Ashley-Koch *et al.* 2000; Gelerhter and Collins, 1990).The mutation is characterized by a single nucleotide substitution in the sequence that codes for the Amino Acidin position 6 of the globin chain. This polymorphism involves the substitution of Adenine by Thymine in the triplet base codon. Consequently, the polar Glutamic acid, produced by this triplet codon (GAG) in Haemoglobin A is replaced by the neutral Valine in Haemoglobin S produced by the triplet codon (GTG) (Gelehrter andCollins, 1990).

Sickle cell anaemia is associated with severe morbidity and high mortality among those affected in developing countries (WHO, 2006). It is associated with varying clinical, psychological and economic challenges that puts enormousburden on the affected individual, family and society at large. In state of deoxygenation, affected individuals suffers from the effects of polymerization,vascular occlusion and haemolysis of red blood cells. The quality of lives is often impaired due to recurrence of ill health and mortality results from

complications. In developed countries, the severity of the morbidity and mortality is positively modified by the availability and utilization of specialised comprehensive care (Mpala *et. al.*, 2001). On the contrary, Nigeria as well as many other developing countries in Africa, still lack coordinated neonatal screening program and care service that can modify outcome in affected individuals (Lane, *et. al.*, 2001). Hence it is associated with high morbidity and mortality. In Africa, SCA is estimated to contribute to between 5- 16% of under-five deaths and only about 50% of the affected children live beyond the fifth birthday (WHO, 2006). In Uganda, about 70 – 80% of children with sickle cell disease die before the age of two years (Ndugwa and Serjeant, 2003).

The two main methods to control the prevalence of sickle cell anaemia are prenatal diagnosis and premarital genetic counselling and screening. In 1987, the World Health Organization Informal Consultative Group on Hereditary Diseases Programme proposed early prenatal screening and diagnosis through polymerase chain reaction based technology as a feasible way of reducing the burden of sickle cell diseases in Africa (WHO, 1987; Akinyanju, *et al.*, 1999). Molecular protocol evolved from Restriction fragment length polymorphism to RFLP-PCR, which was used for the first 50 samples in Nigeria (Adewole, *et. al.*, 1999). The major drawbacks of any protocol involving RFLP include need for larger sample for analysis, involvement of numerous steps and reagents and the high cost of analysis arising from these factors. Some of the steps may also involve the use of toxic radioactive reagents. Several innovations aimed at improving on these drawbacks have evolved over time, resulting in one of the newer molecular protocols referred to as amplification refractory mutation system-polymerase chain reaction (ARMS PCR). In all of these protocols, the fetal tissue required for diagnosis is the chorionic villus, obtained through a specialized procedure referred to as Chorionic villous sampling. Fundamental to all molecular diagnosis done on the chorionic

villi is sample dissection and cleaning. This step is aimed at eliminating maternal DNA derived from the maternal decidua cells present in the sample. The most important sequel of inadequate isolation of maternal cells from fetal sample is misdiagnosis, a phenomenon that is particularly associated with PCR based protocols that amplify DNA (Cao and Rosatelli, 1993; Oehme, Jonatha and Horst, 1986). Effective methods to evaluate the effect of maternal DNA in maternal decidua on the reliability of homologous fetal haemoglobin genotype result are not readily available. An indirect way of assessing the influence of maternal DNA in fetal sample is through using DNA extracted from maternal venous blood. This correlation between DNA and maternal decidua could be subsequently evaluated.

The number of aspiration attempts determines the quantity of chorionic villi and also influences the risk of miscarriage. The weight of villi sample that will yield adequate quantity of DNA without compromising pregnancy is usually small compared with other sources of DNA such as blood (Lilford, 1990; Oloyede and Akinde, 2004). Miscarriage remains the most worrisome risk to most women. Consequently, the risk would also be an important counselling point. In addition to the risk of miscarriage, other factors such as education level, religion, socio-cultural background of the prospective parents and the experience of having cared for or lost a child with SCA may influence the acceptance and utilization of prenatal diagnosis (Oloyede *et al.*, 2005). Other important influencing factors are gestational age of screening/diagnosis and safety of invasive procedures (Oloyede, 2008).

The burden of sickle cell among black population could be explained by two main factors. These are ineffective premarital genetic counselling and screening and high prevalence of AS that is encouraged by heterozygote advantage of haemoglobin AS, secondary to malaria endemicity in the population, (Taiwo *et al.*, 2011). The high prevalence of haemoglobin AS has contributed to marriages between heterozygous individuals in the population and also



continue to produce genotypes and alleles in the gene pool. The expectations from assortative mating include haemoglobin genotypes in offsprings according to the Mendelian ratio of 1:2:1 (AA, AS and SS respectively), without any significant alteration in the allele frequencies. **(but produces increased frequency of recessive homozygotes).**

Studies on haemoglobin genotype frequencies and distribution in the extra uterine (neonatal and adolescent) populations are usually inclusive of all mating types, without focussing on haemoglobin genotypes resulting from heterozygous marriages, which are main genotype group that would produce the SS off spring. Similarly, there is no study on the genotype and allele distribution in the fetal population, from which the neonatal and adolescent populations derive their gene pool. A study on the gene frequencies in fetal, neonatal and adolescent population would provide a global picture and assist the design of more effective counselling and screening strategies (based on the prevalence of heterozygotes). It would also demonstrate the fitness potentials of the various genotypes by comparing data trends in all population groups. Indirectly, this points towards the factors that influence survival (fitness of the genotypes).

The research was designed to demonstrate the influence of maternal DNA contamination of chorionic villi, on the fetal haemoglobin genotype, using the ARMS PCR protocol. It shall also evaluate the trend in gene frequencies and distribution in fetal, neonatal and adult population from heterozygous matings and determine deviations from the expected Mendelian ratio. Other expectations from the study include miscarriage frequency and awareness of procedure among women. It is expected that the outcome of the research will be the first to establish data on in utero gene frequencies and be a pointer towards possible

factors that affect genotype survival. It shall also strengthen more purposeful awareness campaign, and genetic counselling and screening for the control of the condition.

## **1.2. Problem Statements**

Sickle cell anaemia is a genetic problem with severe and multidimensional consequences.

The challenges associated with molecular methods of prenatal diagnosis especially ARMS-PCR, is the possibility of laboratory diagnosis error from sample contamination by maternal decidua DNA. This could have serious adverse consequences on the overall acceptance and utilization of molecular diagnosis. The magnitude of the influence is yet to be appropriately defined.

The study of genotypes and allele frequencies are essential to the effectiveness of a genetic counselling and screening programme. Determination of the data in pregnancy would be acceptable to compliment the existing pool of data.

## **1.3. Research Questions**

- i. What is the performance of a molecular method (ARMS-PCR) for prenatal diagnosis of SCA
- ii. What is the effect of heterozygous maternal DNA contamination of CV on fetal haemoglobin genotype result
- ii. Is the Haemoglobin genotype distribution expected from HbA<sup>HbS</sup> and HbA<sup>HbS</sup> mating pattern, maintained in the fetal population
- iii. Is the allelic frequencies in the fetal population in accordance with expectation from heterozygous mating pattern
- iv. What is the trend in the genotype and allele frequencies and distribution from fetal live to infancy and finally adulthood from heterozygous mating types.

- v. What is the risk of miscarriage attributable to chorionic villous sampling
- vi. Is early prenatal diagnosis acceptable among Nigerian women
- vi. Is the awareness of molecular diagnosis of SCA adequate in the study population

## **1.4. Objectives**

### **1.4.1 General Objective**

To evaluate the effect of maternal venous blood DNA on fetal haemoglobin genotype results as an indirect method of analysing effect of decidua DNA using ARMS PCR molecular technique.

The secondary goal is to determine the fetal haemoglobin genotypes and allele frequencies in relation to assortative mating and their trend during infancy and adolescence.

### **1.4.2. Specific Objectives**

- i. To determine the effect of different concentrations of heterozygous maternal DNA contamination of chorionic villi on fetal haemoglobin genotype result (using indirectly maternal venous blood DNA)
- ii. To determine the frequency and distribution of fetal haemoglobin genotypes and establish any deviations from the expectation from heterozygous mating pattern
- iii. To determine the allele frequencies in pregnancy and establish any deviations from the expected frequencies in heterozygous mating pattern
- iv. To determine the trend in the frequencies and distributions of genotypes and alleles in the fetal, infancy and adolescence from heterozygous mating
- v. To determine the prevalence of procedure related miscarriage and compare with world wide established rates
- vi. To evaluate the level of awareness about molecular diagnosis of SCA and its

acceptance in the study population

### **1.5. Rationale for Study**

Despite advances in medical care and interventions for sickle cell anaemia, the condition has remained a major cause of morbidity and mortality, with enormous impact on health system (Bunn, 1997; Gilman, McFarlane and Huisman, 1976; WHO, 2006).

With the introduction of a PCR based method that co amplifies DNA from all sources, one of the most challenging possibility is that of maternal DNA contamination of chorionic villi and the effect this could have on fetal haemoglobin genotype results. This provides the rationale for the evaluation of effect of maternal DNA in fetal sample.

The study of genotypes and allele frequencies are essential to the effectiveness of a genetic counseling and screening programme. Studies have demonstrated the distribution of haemoglobin genotypes and allele in adult population, where both random and assortative matings occur and has been used to design counseling and screening programmes. A study that focuses on the frequencies and distribution from assortative mating is more appropriate, because they contribute significantly to the pool of haemoglobin genotype AS that are to be screened and haemoglobin genotype SS that are affected by severe morbidity and mortality. Such data in would be acceptable to compliment the existing pool of data, because Hardy Weinberg conditions are rarely strictly fulfilled in human population.

## **CHAPTER TWO**

### **LITERATURE REVIEW**

#### **2.1. The Human Haemoglobin (Genotype): General Characteristics**

Sickle cell anaemia refers to the occurrence of homozygous HbS genotype (HbSHbS; one  $\beta^S$  gene on each chromosome). It is the commonest component of the sickle cell disease entity, the others made up of sickle cell-haemoglobin C (SC) disease, sickle cell- $\beta^+$  thalassaemia, and sickle cell- $\beta^0$  thalassaemia (Serjeant, 1981). It results from a genetically determined abnormality of the haemoglobin structure. The haemoglobin is the most predominant protein in the red blood cell, being about 70% (Gelehrter and Collins, 1990).

### **2.1.1. Molecular Genetics of Normal and Abnormal Haemoglobins**

The normal haemoglobin molecule consists of an iron protoporphyrin (haem) attached to two pairs of polypeptide (globin) chains. Studies have shown that human haemoglobins are derived from zeta ( $\epsilon$ ), epsilon ( $\zeta$ ), alpha ( $\alpha$ ), beta ( $\beta$ ), delta ( $\delta$ ) and gamma ( $\gamma$ ) chains (Robert and French, 1978; Perutz, 1976). Together, these are known as globin chains and are made up of different number of amino acids. The main determinants are the alpha and beta gene clusters located on the short arms of chromosomes 16 and 11 respectively (Gelehrter and Collins, 1990; Ashley-Koch, Yang and Olney, 2000).

#### ***The $\alpha$ -globin gene cluster (5'- $\zeta$ - $\psi\alpha$ - $\alpha 2$ - $\alpha 1$ -3')***

The gene cluster of alpha globin family chain is located on the short arm of chromosome 16, on the 25 kilo-base (kb) region and has 141 amino acids.

The alpha globin locus contains four alpha globin genes for the synthesis of the alpha globin proteins. The alpha gene cluster also has epsilon gene which is only expressed during the early weeks of embryogenesis for the synthesis of epsilon chains needed for production of embryonic haemoglobins called Gower 1 and Portland 2 but are later on replaced by the production of alpha globin 5'- $\alpha 2$ - $\alpha 1$ -3', as its major globin. Gower 1 has two epsilon and two zeta chains ( $\zeta_2\epsilon_2$ ) while Portland 2 has two epsilon and two gamma chains ( $\zeta_2\gamma_2$ ).

***Non-α globin gene cluster(5'-ε-Gγ-Aγ- ψβ-δ-β-3' genes).***

The gamma, beta and delta genes are located within the beta gene cluster located on the short arm of chromosome 11 on 60 kilo base region and have 146 amino acids(Robert and French, 1978). Within the beta gene cluster there is zeta gene which is synthesized only during embryogenesis for the production of Gower 1 and 2 haemoglobins. Gower 2 has two alpha chains and two zeta chains ( $\alpha_2\epsilon_2$ ). The gamma gene is required mostly during foetal (HbF) hemoglobin formation after which its synthesis reduces significantly. The beta gene cluster also begins the transcription of the beta globin as minor till shortly before delivery, when it becomes the predominant globin chain.

**CHROMOSOME 11**

**EMBRYONIC**

**FETAL**

**ADULT**

$\epsilon G\gamma-A\gamma\delta\beta$

**Hb Gower IHb Portland**

**EMBRYONIC**  $\zeta\zeta_2 \epsilon_2\zeta_2 G\gamma_2$

$\zeta_2 A\gamma_2$

**FETAL**  $\alpha_2$  **Hb Gower IIHb FHbA<sub>2</sub>HbA**

**AND**

$\alpha_2 \alpha_2 G\gamma_2\alpha_2 \delta_2\alpha_2\beta_2$

**ADULT**  $\alpha_1\alpha_2 A\gamma_2$

Figure 1: A matrix diagram of possible tetrameric products of the α-globin cluster on chromosome 16 and the β-globin cluster on chromosome 11, with their historical names.

Source: Principles of Medical Genetics; Thomas D. Gelehrter and Francis S. Collins.

The expression of  $\alpha_1$  and  $\alpha_2$  globin genes located on chromosome 16p13.3 and the β globin gene located on chromosome 11p15.5, provide α and β globin polypeptides. Together with the co-ordinated production of haem, the non-protein portion of Hb chains, results in the formation of HbA, in normal individuals(Ashley-Koch, Yang and Olney, 2000; Angastiniotis

and Modell, 1998). The normal detectable haemoglobins in an adult have been found to be hemoglobin A (HbA-95%), haemoglobin A<sub>2</sub> (HbA<sub>2</sub> -2-3.5%) and haemoglobin F (HbF-1.5-3.0%). The haemoglobin A has two  $\alpha$  chains and two  $\beta$  helix chains ( $\alpha_2\beta_2$ ), haemoglobin HbA<sub>2</sub> has two  $\alpha$  chains and two delta ( $\alpha_2\delta_2$ ) while haemoglobin F has two  $\alpha$  chains and two gamma ( $\alpha_2\gamma_2$ ) chains (Gelehrter and Collins, 1990). Usually the level is < 2%, but may be more in beta thalassemia and inherited condition known as hereditary persistence of foetal haemoglobin (HPFH) which is common among Kuwaitis (Marouf, 2003).

There are numerous structural variants of the normal haemoglobin A caused by mutation. Mutation is defined as a heritable change in the sequence of genomic DNA (Gelehrter and Collins, 1990). It produces polymorphism in a population. Although mutation is a constant event, it often does not produce serious phenotypic phenomenon in humans. Over half of the amino acids produced from alpha and beta genes result from missense mutation, with the beta gene having the highest frequencies (Gelehrter and Collins, 1990; Clarke and Higgins, 2002). Among the various forms of mutation, the missense mutation is the most relevant to the development of sickle cell anaemia.

Haemoglobin S is a structural variant of normal haemoglobin A. It is due to a mutational base change resulting in the substitution of polar glutamic amino acid by non polar valine amino acid in the 6<sup>th</sup> position of beta globin chain ( $\beta 6$  Glu  $\rightarrow$  Val) (Clarke and Higgins, 2002; Mohsen, *et. al.*, 2011). Gene sequencing has shown that amino acids in the haemoglobin are coded by three base nucleotides called the codon. In haemoglobin A, the nucleotide sequence GAG produces Glutamic acid in position 6 of the beta globin chain, while in haemoglobin S the nucleotide sequence is GTG produces Valine. This substitution affects the 6<sup>th</sup> codon of the  $\beta$  globin gene. Individuals homozygous to HbS gene have only HbS in place of Hb A (HbSHbS), with concomitant production of Hb F and Hb A<sub>2</sub>.

HBB Sequence in Normal Adult Hemoglobin (Hb A):							
Nucleotide	CTG	ACT	CCT	GAG	GAG	AAG	TCT
Amino Acid	Leu	Thr	Pro	Glu	Glu	Lys	Ser
	3			6			9
HBB Sequence in Mutant Adult Hemoglobin (Hb S):							
Nucleotide	CTG	ACT	CCT	GTG	GAG	AAG	TCT
Amino Acid	Leu	Thr	Pro	Val	Glu	Lys	Ser
	3			6			9

Figure 2: Haemoglobin sequences in adult haemoglobin A and S

Source:

Linus Pauling, *et al.*,**(X)** was the first scientist to postulate the molecular abnormality in sickle cell anaemia. Vernon Ingram in 1957, used the peptide mapping analysis to demonstrate that in sickle cell anaemia, the beta globin gene differs by a single amino acid at position 6 (Ingram, 1957). The beta globin chain contains 146 amino acids arranged in sequence. In the HbA, the amino acid residue in position 6 is Glutamic acid (negatively charged), while it is Valine (neutral) in HbS.

## **2.2. Mode of Transmission and Inheritance of Sickle Cell Anaemia and its traits**

The abnormal haemoglobins are inherited as autosomal recessive (AR) disorders. The usual mating pattern is between two carriers of the trait, referred to as heterozygotes HbAHbS. There is a 25 per cent chance of having a homozygous HbSHbS (Sickle cell anaemia, SCA) child, 25% chance of homozygous HbAHbA and 50% chance of heterozygote HbAHbS . Rarely transmission occurs from union between homozygote HbSHbS and homozygote HbAHbA, producing a 50% chance of chance of HbAHbS and HbSHbS in the offspring.



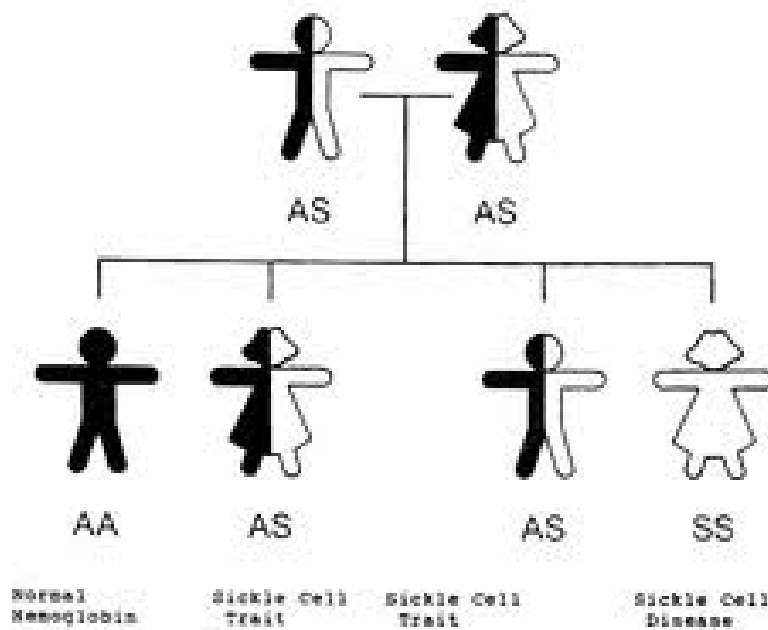


Figure 3: Pattern of inheritance

Source: [http/ diaryofastethoscope.wordpress.com](http://diaryofastethoscope.wordpress.com)

### 2.3. History and Evolution of Sickle Cell Anaemia

The first documented data on sickle cell anaemia was published by Herrick in the United States in 1910 (Curt, 1973). The first diagnosis of SCA was made from a dental student whose blood film was shown to contain irreversibly sickled red blood cells. There are numerous contradictory reports on the exact origin of sickle cell anaemia. While some researchers trace the origin to the Venddoits in Middle East, others have postulated its origin from Africa (Desai and Hiren, 2004). Among Africans, the major controversy centres around its origin either from Ghana in 1670 or Nigeria in 1874 or Sudan in 1925 (Desai, and Hiren, 2004; Iheanyi, 2005; Walter, 1927).

The association between sickle cell anaemia and abnormal haemoglobin was reported in 1945, following observation of abnormality within the haemoglobin molecules of sickle cell individuals and which could be separated from the normal haemoglobin using gel

electrophoresis. Later studies found that sickle cell anaemia was due to substitution of Glutamic amino acid by Valine amino acid at the sixth position of the beta globin chain (Robert and French, 1978; Clarke and Higgins, 2002).

## **2.4. Epidemiology of Sickle Cell Anaemia**

### **2.4.1. Global Prevalence**

Sickle cell disease is the most commonly inherited blood disorder on the globe affecting an estimated 100 million people world-wide with particularly high prevalence among the black races in Africa, India, the Caribbean, the Middle East and persons of Mediterranean origin. (Ohaeri and Shokundi, 2001; Nietert, Silverstein and Abboud, 2002; Serjeant, 1981; Mohsen, El-Hazmi and Arjumand, 2011). The term is used interchangeably with SCA and is reported to be the commonest type of sickle cell disease (Serjeant, 1981).

Approximately 5% of the world population is believed to be carrying the genes responsible for the different haemoglobinopathies and about 300,000 infants are born annually with major haemoglobin disorders including sickle cell disease (WHO, 2006). In the United States of America (USA), sickle cell anaemia has been found to be the most frequent autosomal recessive gene disorder affecting approximately 1:375-500 persons of African ancestry, with a prevalence of 0.2% (Doris and Wetherland, 2000).

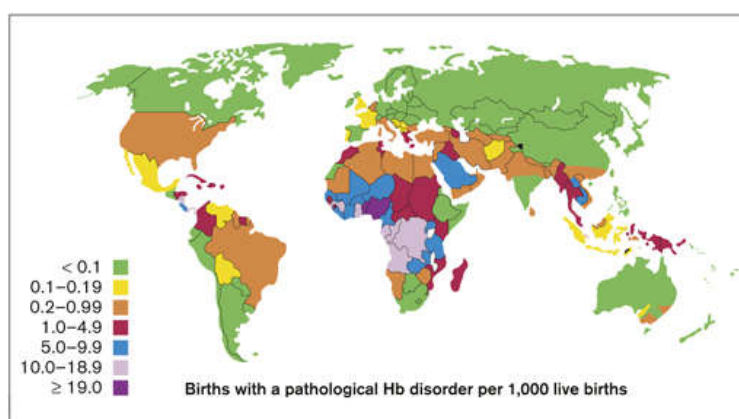


Figure 4: Global distribution of pathological Hb disorders.

Source: WHO. WHO Technical Report Series 865. 1996. WHO, Geneva, Switzerland

In addition, other sickle cell variants such as haemoglobin S, alpha and beta thalassemia have been found to be highly prevalent among native populations in some of the European countries such as Turkey, Italy, Cyprus and Greece (Nietert, Silverstein and Abboud, 2002). Studies among the Kuwaitis, showed that 23.5 per cent had abnormal haemoglobin genotypes, with HbAHbS being 6% and SCA being 0.9% (Mohsen, El-Hazmi and Arjumand, 2011; Marou, D'souza and Adekile, 2002).

In United Arab Emirates (UAE), among the UAE nationals, the frequency of SCD was reported as between 1.9- 4.6% in a major studies from three major Peninsular Arab States involving pre-school children and adults (White, *et. at.*, 1986; Miller, *et al.*, 2003). In a more recent survey, the overall incidence of SCD among 22,200 screened neonates as 0.04 per cent (0.07% for UAE citizens and 0.02% for non-UAE citizens), where the incidence of HbAHbS was overall 1.1 per cent (1.5% for UAE citizens and 0.8% for non-UAE citizens) (Al Hosani, *et al.*, 2005). In these studies, wide variations were reported in the clinical features ranging from moderate to a severe disease

In a study on 5000 Oman subjects derived from three States of Arabian Peninsula, the frequency of SCD was reported as 3.8 per cent (White, *et. at.*, 1986). Another study reported the birth prevalence of symptomatic haemoglobinopathies in 23 Omani tribes through screening of a national register, as 1 in 323 live births or 3.1 per 1000 live births during 1989-1992, which included 2.7 per 1000 live births of homozygous SCD (Rajab, Patton and Modell, 2000). It was calculated that each year, 118 new cases of SCD were expected to be born and HbAHbS frequency was 10 per cent (Rajab, Patton and Modell, 2000). In this country also, variation in clinical severity was reported. The overall prevalence of HbS as 5.8

per cent was reported in a study, though there were significant regional variations (Al-Riyami,*et.al.*, 2001). In India, the frequency of sickle cell gene has been found to be as high as 0.31 in some parts of the country. Studies in the Tuluka district of the Indian State of Maharashtra showed that out of 4116 persons screened for sickle cell anaemia, 814 (19.8%) were HbAHbS and 44 (1.07%) were HbSHbS (Kate, 2000). In this country, there was variation in prevalence of SCT and SCA among tribes (Babu, Leela and Krishna-Kusuma, 2002). In Iran the prevalence of sickle cell trait has been estimated to be 1.43% while that of HbSHbS is 0.1% (Habibzadeth, *et. al.*, 1999).

The African continent is described as the epicenter of sickle cell disease with an annual estimated number of 200,000 new born affected by sickle anaemia (Diallo and Tchernia, 2002; Weatherall and Clegg, 2001). This constitutes two third (66.7 %) of the children born with the haemoglobin disorders in the whole world. Nigeria accounts for about 150,000 out of these cases. In some parts of the African continent, sickle cell anaemia has been found to affect 1 in 60 newborn infants (Lees and Davies, 2005), giving a prevalence of about 2%, whilst the sickle cell trait ranges between 10% to 40% across wet equatorial Africa, decreasing to less than 2% in the dry areas of Northern and Southern Africa. About 60% of sickle cell anaemia infants in Africa die yearly, with Nigeria accounting for about 80% of total mortality (WHO, IRIN, 2013, Akinyanju, *et al.*, 1999; Olatunji, 2003; Oluwagbemiga, 1998).

Several studies conducted estimated that 20% of the Ugandan population had sickle cell trait, with wide variations among tribes (Lehmann and Raper, 1949). The Karimojong, Bakiga, Banyakole and Bahima recording the lowest frequency of 1-4%, Baganda, Iteso, Acholi and Banyoro recording 16-20%, Basoga, Bagisu and Lugbara recording 20-28% and Bamba

recording the highest frequency of 40% which is believed to be the highest in the whole world (Trowell, 1945; Charache, 1990). A more recent statistic shows this to be inaccurate (Okwiet, *et al.*, 2010). Of the 900,000 thousand children born annually in Uganda approximately 2.8% have sickle cell anaemia and about 20,000 (70-80%) of sickle cell anaemia patients possibly die before their 5<sup>th</sup> birth day (MFEP., 1992; N H S., 2004).

#### **2.4.2. Status of Sickle Cell Anaemia in Nigeria**

Up to date reports estimated that around 2-3% of newborns in Nigeria were affected by sickle cell anaemia, giving a total of 100,000-150,000 affected children born every year in the country (WHO, 2006). Over 80% of these children die before they celebrate their fifth birth day. About 25-30% of the Nigerian population carries the trait and about 2-3% has the disease (Akinyanju, *et al.*, 1999, Olatunji, 2003, Oluwagbemiga, 1998). The implication is that majority of cases in Africa and West Africa is found in Nigeria.

#### **2.4.3. Factors Influencing the Distribution of SCA**

The distribution of SCA in different parts of the world is defined by three major factors. These are population of indigenous blacks, population migration and malaria endemicity.

##### **Population Structure of Indigenous Population**

SCA is generally regarded as a disease of the blacks and consequently found more in regions inhabited by the black race.

The number of sickle cell disease in the USA, is projected to reach 10,000 in near future (Hassel, 2010). This consists of about 1 in 500 African-American children and 1 in 36,000 Hispanic – American children born will have sickle cell anaemia (Hassel, 2010)

## **Migration**

Migration of substantial populations from the high prevalence areas of Africa to low prevalence countries in Europe has dramatically increased in recent decades and in some European countries, the prevalence of sickle cell disease is on the increase (Roberts and de Montalembert, 2007). In the United Kingdom, about 200 children are born annually with sickle cell disease, while the highest prevalence has been observed in France. The prevalence far outweighs the birth prevalence of other genetic conditions such as cystic fibrosis (Bardakdjian and Wajcman, 2004).

## **Malaria Endemicity**

The sickle haemoglobin (HbS) mutation confers a genetic advantage against malaria in areas where malaria is endemic. The carriers of HbS have a selective natural resistance against *P. falciparum* which causes malaria development and this is a major advantage to survival in adverse conditions. Several reports in the Middle Eastern Arab, European, African and American countries validated the “malaria hypothesis” by showing a close correlation between the frequencies of the abnormal gene and past and present history of malaria endemicity (Sickle Cell Society, 2008; Weatherall and Clegg 2001; Modell and Darlison, 2008; WHO, 1996). Therefore, in those geographic areas where malaria is endemic, carriers of HbAS are more likely to pass their genes on to the next generation.

Several mechanisms are believed to be contributory. It has been thought that accelerated acquisition of malaria specific immunity could confer on the sickle cell traits, resistance to malaria. This is probably due to the ability of the ring forms of *P. falciparum* developing in infected red blood cell to stimulate the production and expression of hemichromes associated with enhanced oxidant membrane damage, which results into an aggregation of band 3 protein and binding of immunoglobulin G (IgG) and complement C3c. This process culminates

in enhanced phagocytosis and clearance of the sickled HbAHbS red blood cells infected with ring shape *P.falciparum* parasites(Ayi, *et al.*, 2004).Other studies from Gabon have also postulated that increase in number of *P.falciparum* strains in HbAHbS individuals could be exposing these persons to a plethora of *P.falciparum* antigens capable of inducing malaria specific immunity (Ntoumu, Mercereau and Ossari, *et al*, 1997). According to studies from Gambia, it was found that the expression of the variant antigen called *P.falciparum* erythrocyte membrane protein-1 on the surface of malaria infected red blood cell caused the increased production of antibodies towards *P.falciparum* antigens leading to the destruction of the malaria infected red blood cell (Marsh, Otoo and Hayes *et al.*, 1998). Other studies also noted that the production of peripheral blood mononuclear cells (PBMC) against *P.falciparum* circulating soluble antigens in AS children was higher than in HbAHbA and HbSHbS children and were therefore protected against malaria (Abu-Zeid, Abdulhadi, Theander, *et al.*, 1992).

## **2.5. Pathophysiology and Phenotype of Sickle Cell Anaemia**

The main function of haemoglobin is to transfer oxygen to peripheral cells. This is achieved by selective binding to oxygen in the lungs and its release in the body tissues. In the capillaries, carbon dioxide is converted to carbonic acid as the product of body metabolism. This resulting acidic pH, (low pH) reduces the affinity of haemoglobin for oxygen and facilitates its release into the tissues. In the lungs, release of CO<sub>2</sub> resulting in high pH, promotes oxygen binding capacity. This process of oxygen binding and transport is impaired in the presence of haemoglobin S. Consequently, the peripheral tissues are in constant state of low oxygenation.

The HbS is soluble in the oxygenated state, as that encountered in the lungs, but once the haemoglobin delivers the oxygen to the tissues, the HbS in the deoxygenated form undergoes

a major conformational change, which leads to the formation of long fibrous aggregates (polymers or insoluble tetramers) due to hydrophobic interactions between the Valines in the adjacent HbS molecules. Hydrophobic interactions are promoted by the loss of the negative charge due to substitution of Glutamic acid by Valine and are most pronounced in state of reduced oxygenation, but reversible in state of oxygenation (Barbara and Barbara, 2001). The hydrophobic interactions between the valines in the adjacent HbS molecules results in the formation of sticky patches on the surface of the beta chains. The insoluble tetramers that polymerizes in the state of the low oxygenation leads to a long fibrous precipitates that distort its shape from normal spherical biconcave disc to the characteristic sickle shape, causing erythrocyte rigidity and inability to pass through the fine capillary beds which have smaller diameters than the cells diameter.

The haemoglobin polymerisation is central mechanism to the pathophysiology of SCD .and may produce complications such as bone pain/vasoocclusive crisis, hemolytic crisis or sequestration crisis. Recurrent sickling and desickling in the tissues and the lungs respectively increase the fragility of the red cells leading to haemolysis and hence chronic anaemia (GeletherandCollins, 1990).Vaso occlusion is believed to result from high affinity interaction of rigid polymers with vascular endothelium membrane and leucocytes through adhesion process involving the sub-endothelial extracellular matrix molecules such as intergrins(Swerlick, 1993). It results from blockage of the micro vascular vessels by the rigid sickled red cells, leading to the development of painful crises, hand-foot syndrome, inflammation, cerebrovascular disease and cognitive impairment (Stinson and Nasser, 2003; Rodgera, 1997; Howlett, *et. al.*, 1997).The process of vaso occlusion is further enhanced by adhesion between sickled red blood cells and activated endothelium which prolong their transit time leading to further polymerization.



Recurrent episodes of vaso-occlusion and inflammation lead to vasculopathy which further results in progressive damage to vital organs, including the brain, kidneys, lungs, bones, and cardiovascular system obstructs microcirculation, and causes tissue infarction. These may present as hand-foot syndrome in children, fatigue, anaemia, and shortness of breath, pain that occurs unpredictably in any body organ or joints, jaundice, delayed growth and puberty in children. In addition, infections, stroke, and acute chest pain are some of the major complications. These phenotypes may be expressed from early life and become more apparent with increasing age.

Several factors such as infections, dehydration, fever, cold weather and stress precipitate the formation of sickling and its sequelae. Other factors such as haemoglobin concentration and acidity may also be linked to sickling. Increased acidity has been shown to stimulate the release of potassium ions from the cell resulting into increased concentration of calcium ions in the cell. The loss of potassium ions affects the normal function of the cell membrane pump resulting into the failure of the Gardos channels to close leading to excess release of water from the cell. This process finally causes dehydration which increases the density of the haemoglobin S within the cell, thereby accelerating the sickling process leading to excessive disruption of red blood cells which is characteristic of anaemia in sickle cell patients (Stone, Stuart and Nash, 1996).

## 2.6. Variability of Phenotypic Expression

### Genetic Factors Contributing to the Variability of SCA Phenotype

Although the underlying genetic mechanism for SCA is the uniform substitution mutation of Adenine(GAG)with Thymine in(GTG), in all cases, yet to be fully explained are the factors responsible for the diversity in magnitude of phenotypic presentations. This is because of the wide spectrum of phenotype, ranging from a benign and almost asymptomatic phenotype to a severe and life threatening state. For example, while the Saudi SCA patients in the eastern province exhibit mild forms of the phenotype including vaso-occlusive complications, persistence of splenic functions, lower morbidity due to other complications and lower risk during pregnancy, those of African origin are usually more severe (El-Hazmi, Al-Hazmi and Warsy, 2011).

Several genetic factors have been strongly implicated in modulating the phenotype, with some ameliorating the disease while others have an augmenting influence. Factors believed to ameliorate the phenotypic expression include co-existing genetic abnormalities, such as G-6-PD deficiency, thalassemia or other abnormal Hb variants, thus producing a benign form of the disease (Weatherall *et. al.*, 1969; Gelpi, 1970). The influence of elevated HbF is controversial, with some studies reporting beneficial effect and others reporting adverse effect (Pembrey, *et. al.* 1975; Pembrey, *et. al.* 1978; Perrine, *et. al.*, 1972; El-Hazmi, 1990; El-Hazmi and Warsy, 2011; El-Hazmi, 1992). *The  $\beta$ - globin gene haplotypes also influences the SCA clinical presentation of SCA. If the HbS mutation takes place on a chromosome carrying the Saudi-Indian haplotype, the HbS generally gives rise to a mild form mostly with an elevated HbF. The same mutation, if occurs on a chromosome carrying a Benin haplotype, is generally associated with lower HbF levels and a severe disease (El-Hazmi, Bahakim and Warsy, 1992; El-Hazmi, 1986).* Elevated Hb F levels clearly play a role in reducing severity of phenotype, possibly through interfering with HbS sickling process. Associated  $\alpha$ -

thalassemia also influences the severity of the disease and ameliorates the disease, but this depends also on the number of  $\alpha$ -gene deleted or on the type of mutation producing the thalassemia state (El-Hazmi, Al-Hazmi and Warsy, 2011). Presence of associated  $\beta$ -thalassemia influences the clinical presentation and is dictated by the nature of  $\beta$ -thalassemia mutation.  $\beta^+$  mutations producing HbS/ $\beta^+$  thalassaemia state have an ameliorating effect, while  $\beta^0$  mutations resulting in HbS/ $\beta^0$ thalassaemia state may be equally severe as SCA. Studies on the effect of HbF and G $\gamma$ /A $\gamma$  ratio have demonstrated that patients with a mild disease generally have a high ratio, while the reverse is true in patients with a severe disease (El-Hazmi *et. al.*, 1994; El-Hazmi, 1995; El-Hazmi, 1990; El-Hazmi, 1989; El-Hazmi and Warsy, 1993). Contradictions are frequent when it comes to associated G-6-PD deficiency, where both ameliorating effects and adverse effects have been reported in studies reported from the Middle Eastern Arab countries (El-Hazmi, Al-Hazmi and Warsy, 2011).

## 2.7. Gene Frequencies

Study of gene frequencies includes genotypic and allelic frequencies are necessary in the control of SCA. This is however dependent on some factors such as random mating pattern and epidemiological factors. In assortative mating pattern, as in the study population, it would influence the outcome of the results. *Any influence on HWE?*

In a study in south western, the normal and sickle cell haemoglobin genotypes distribution was such that 366 (73.1%) had HbAHbA genotype, 123(24.5%) had HbAHbS, while 12 (2.4%) had HbSHbS giving genotypic frequencies that were not significantly different from Hardy–Weinberg expectations ( $P>0.05$ ) (Taiwo *et. al.*, 2011). The study shows that the needs for improved awareness drive and coordinated genetic counselling for sickle cell disease in Nigeria. This is particularly important in adolescents, who constitute the majority of intending families that will produce genes that makes up the gene pool that would be found in

the foetuses (future generations). Malaria endemicity which is one of the major determinants of gene frequency distribution could also play a role in utero through the occurrence of placenta malaria.

### **Gene frequency – in utero, infancy and adulthood**

## **2.8. The Placenta**

The term placenta is derived from the Greek word, *plakuos*, meaning '*flat cake*', on the basis of its gross anatomical appearance. The placenta is a materno-fetal organ which began its development with the implantation of the blastocyst. It allows nutrient, gas, and waste exchange along with providing a protective barrier to the developing embryo and fetus and is delivered with the fetus at birth. The functional unit of the placenta is cotyledons, which are further grouped into visible lobes (Baker, 2006). Morphologically, the placenta is derived partly from fetal origin (the trophoblast) and partly from maternal origin (the transformation of the uterine endometrium)

### **2.8.1 The Chorion Villi**

The chorion villi are the fetal portion of the placenta, the other being the decidua basalis from the mother. It forms the bulk of the placenta tissue and the term is often used interchangeably with the term 'placenta' in prenatal diagnosis. Chorion villi are the sine qua non of pregnancy from a histomorphologic perspective and are by definition, products of conception.

The chorion is the protective and nutritive membrane that attaches fetus to the uterus. From it, the villi sprout out to provide a maximum area of contact with maternal blood. Chorion villi invade the uterine decidua and absorb nutritive materials from it to support the growth of the embryo. The villi begin primary development in the fourth week, becoming fully vascularized between the fifth and sixth weeks.

## Primary Villi

The villi that buds from chorionic plate, is referred to as primary villi and is contained in each cotyledon. They are made up of trophoblast derived cells namely the inner cytotrophoblast and outer syncytiotrophoblast forming finger like projections into the maternal decidua. They are non-vascular.

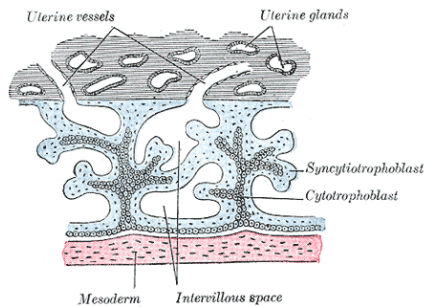


Figure 5: Primary villi

Source: <http://php.med.unsw.edu.au/embryology/index.php?title=File:Gray0036.gif>

## Secondary Villi

The primary villi increase in size and ramify, while the mesoderm core grows into them at about the 5<sup>th</sup> week. The secondary villi are completely formed by the 8<sup>th</sup> week, made up of the mesoderm covered by a double layer of epithelium namely, inner cytotrophoblast and outer cellular syncytiotrophoblast. The mesoderm is one of the three tissue layers in the embryo of a metazoan animal. Through embryonic development, it will produce many internal organs of the adult, e.g. muscles, spine, and circulatory system.

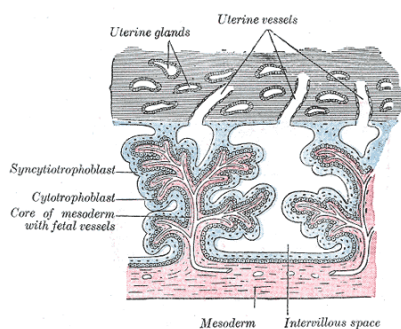


Figure 6: Secondary Chorionic villi

Source: <http://php.med.unsw.edu.au/embryology/index.php?title=File:Gray0037.gif>

### Tertiary Villi

During the tertiary stage (fifth to sixth week), the branches of the umbilical vessels grow into the mesoderm; in this way, the chorionic villi are vascularized. At this point, the villi contain trophoblast, mesoderm, and blood vessels. The blood vessels developed from differentiation of the mesenchyme forming arteriocapillary network, fuse with placental vessels, developing in connecting stalk.

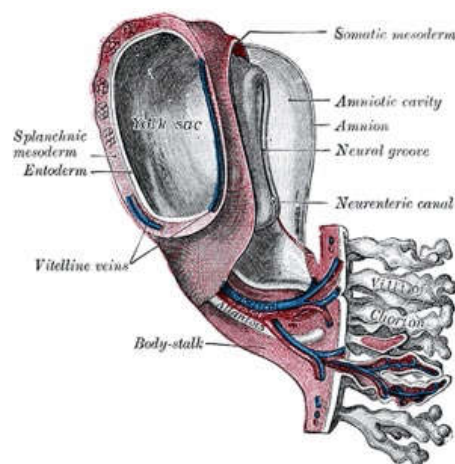


Figure 7: Tertiary Chorionic Villi

Source: <http://php.med.unsw.edu.au/embryology/index.php?title=File:Gray0031.jpg>

The number of stem villi does not increase after the 12th week, because the number of lobules has become fixed and subsequent growth is by proliferation and growth of peripheral villi (Diana Hamilton-Fairley, 2004). Villi appear as fronds of seaweed under water as the maternal blood circulates around them (Diana Hamilton-Fairley, 2004). In vitro, the chorionic

villi has a characteristic fluffy white appearance and floats on the surface of the dissecting medium or saline (Oloyede *et al.* 2002). Bush like villi are generally better than root like villi. Of particular significance is the fact that the villi being of fetal origin reflects the genetic makeup of the fetus in its entirety. It therefore provides acceptable reflection of fetal genetic identity.

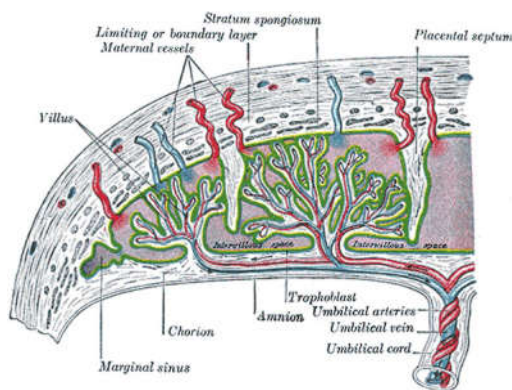


Figure 8: Placental anatomy

Source: [http://php.med.unsw.edu.au/embryology/index.php?title=Placenta\\_Development](http://php.med.unsw.edu.au/embryology/index.php?title=Placenta_Development)

### 2.8.2. Maternal Uterine Decidua

Uterine decidua refers to the uterine lining (endometrium) during a pregnancy, which forms the maternal part of the placenta. At implantation, the maternal endometrium is changed by the decidua reaction (epithelial transformation of the fibroblasts of the uterine stroma with lipid and glycogen accumulation) into what is called the decidua. Under the influence of progesterone, it forms highly-characteristic cells. The various parts are (i). decidua basalis, where implantation takes place and basal plate formed, (ii). decidua capsularis, the part that surrounds the chorion and (iii). decidua parietalis, the part that is on the opposite uterine wall. Of these, the basalis is the most relevant part in prenatal diagnosis.

## 2.9. Maternal Decidua Cell Contamination

The maternal decidua is the uterine endometrial cells and is therefore maternal in origin. It often accompanies the chorion villi during CVS, because of their close relationship during the formation of the placenta. The maternal decidua is difficult to identify separately from the chorion villi on ultrasonography. Its presence and inadequate isolation from fetal chorion villi is the potential cause of diagnostic errors which can occur after CVS (Cao and Rosatelli, 1993; Sonja Pop-Trajković, 2012). This is particularly so with PCR amplified DNA (Cao and Rosatelli, 1993).

The frequency and amount of maternal decidua cells in a given amount of chorionic villi that will produce diagnostic error has not been well established. While contamination of samples with significant amounts of maternal decidua cell is almost always associated with small sample size, it has also been reported that in centres, high on the learning curve, the problem of contamination has reduced significantly (Sonja Pop-Trajković, 1993). The potential pitfall resulting from maternal decidua cell contamination can be avoided by careful dissection of the maternal decidua from the chorion villi and by the simultaneous amplification of a suitable polymorphism (Cao and Rosatelli, 1993). The former is most relevant in poor resource settings. In addition, most laboratories use a small amount of DNA (less than 3-4 micrograms) and limit the number of thermal cycles to minimize undesirable co-amplification of traces of maternal DNA that might be present in the sample (Cao and Rosatelli, 1993; Renate Oehme, and Jonatha, 1986).

## **2.10. Prenatal Diagnosis Sampling Procedures**

The gestational age at which prenatal diagnosis is performed determines the method used. Initially, prenatal diagnosis of SCD was performed on a small amount of fetal blood aspirated from the placenta, umbilical cord, or even fetal heart, either blindly, with ultrasound



guidance, or through a fetoscope at the 20<sup>th</sup> week of pregnancy (Kan *et al*, 1975). The procedure was associated with 1-2% fetal loss and other complications. Also, abortion in midtrimester could be painful and complicated. These drawbacks discourage its wide scale implementation and are also in part responsible for the development of newer techniques that could be implemented at earlier gestational ages. The gold standard in modern prenatal diagnosis is chorionic villous sampling (CVS), performed between 11<sup>th</sup> - 13<sup>th</sup> weeks (Brambati, *et. al*, 1988; Horwell, *et al.*, 1983; Oloyede and Akinde, 2004). Before this gestation age, there is rapid embryonic development and organogenesis and a higher background risk of spontaneous abortion and limb deformities (Lilford, 1990; Stoler, 1999). After 13 weeks, termination of pregnancy is associated with relatively higher risk of complications as mentioned previously. The risk of procedure related abortion is associated with this method is of the order of 1-2% ((Lilford, 1990; Jauniaux, 2003; Oloyede, 2009). Chorion villi may be obtained transcervically or transabdominally. The transabdominal approach is preferred because of its several advantages, including lower infection rate, incidence of amniotic fluid leakage, and also because it is largely preferred by pregnant women (Antonio Cao, 1999; Oloyede, 2006). The overall success of CVS depends on several factors such as, the skill of the operator, type of instruments, route of sampling and number of aspirations (Alfirevic, 2000). Another first trimester option is early amniocentesis (EA). It is however associated with a far higher risk of complications such as miscarriage, respiratory distress and fetal limb deformities compared with CVS ((Lilford, 1990; Jauniaux, 2003; Oloyede and Akinde, 2004). The most recent development involves the use of cell free fetal DNA, which is non-invasive.

**Table 1: Methods of SCA Analysis**

<u>Sample</u>	<u>Procedure</u>	<u>Abortion risk (%)</u>	<u>Method of analysis</u>	<u>Remarks</u>
---------------	------------------	--------------------------	---------------------------	----------------

Fetal Blood	Cordocentesis	1-2	HB electrophoresis	Obsolete
Amniotic cells	Amniocentesis	< 0.5	DNA	In use
Chorionic villi	Chorion villous Sampling	< 0.5	DNA	In use
Cell free fetal DNA	Phlebotomy	Nil	DNA	Experimental

## 2.11. Principles of Molecular Diagnosis

The two main aspects of molecular diagnosis of SCA are (i) DNA extraction and (ii) Molecular analysis. These steps are followed by gel electrophoresis and UV documentation

### 2.11.1 DNA Extraction

DNA extraction from tissues can be achieved using different methods and protocols. In all these protocols used, few steps are fundamental and required for a successful extraction, while some may be optional depending on the source of sample (Oloyede, 2011). The disruption or lysis of cell membrane, which is the initial process, could be achieved using one of several methods such as osmosis based method, enzymatic method, bead method, sonication, detergent method, solvent use or mechanical method (Lahiri, *et al.*, 1993; Nasiri, *et al.*, 2005; Holgado, *et al.*, 2008). The enzymatic method is widely used in the process of cell membrane disruption, with the precise role of the enzyme being to prepare the cell for the actual disruption. Various classes of enzymes could be utilized for this purpose. They include proteases, zymolase, cellulose, lysozyme, lysostaphin and glycanases. Among these groups, the proteases are the most commonly used enzyme, with proteinase K and trypsin having been used in many DNA extraction protocols (Hanson, *et al.*, 2008; Lia, 2009). Potential problems with the use of enzymes include enzyme stability and the possibility that the enzyme could affect other materials after achieving cell membrane lysis. The actual disruption of the cell wall requires the dissolution of membrane lipid. Generally, animal cells

are dense and complex and therefore require combination of steps such as enzymatic digestion and mechanical agitation to achieve cell wall disruption.

The bead method is another common laboratory based method of achieving cell membrane lysis. Otherwise referred to as bead beating, it utilizes either of small glass, ceramic, zirconium or steel beads combined with a high level of agitation or shaking and can be used for all types of cellular materials including spores, plant and animal tissues. Cell membrane disruption by mechanical agitation or homogenization involves the use of either the vortex mixer or centrifuge (Oloyede, 2011). This method has been successfully used for DNA extraction from the chorionic villi (Holgado, *et al.*, 2008). Sonication by applying ultrasound to the sample to electronically generate high frequency mechanical energy, which is transmitted to the sample through the metal probe, can also be used for mechanical cell membrane disruption. The major disadvantage is the high noise level that it generates and yield variability. Detergent based method of causing cell lysis has been used in many protocols as an alternative to physical lysis even though it has not been widely used in DNA extraction from human tissues. It acts by disrupting the lipid-lipid, lipid-protein and protein-protein interaction in the lipid barrier surrounding the cells. The choice of either ionic or non ionic detergent such as SDS or Triton X detergents respectively depends on a number of factors, most important of which is the desire to maintain the protein function or interactions after disruption or otherwise.

More than one extraction protocol could be combined to achieve the objective of high yield of pure DNA. While most were on blood and plant samples, few of them were conducted on the chorionic villi. These ones include the phenol-chloroform method, salting method, promega method, SDS-potassium-acetate method and sonicaid method (Dellaporta *et al.* 1983; Miller, *et al.*, 1988; Ahmad, *et al.*, 1995; Austin, *et al.*, 1997; Kazuyoshi, *et al.*, 2004). Phenol and chloroform are the standard solvents and one of the early methods used for

DNA extraction from the chorionic villi (Tilzer, *et al.*, 1989; Ausubel, 1987; Bakker, *et al.*, 1991). The application of phenol in purification of nucleic acid was first reported by Kirby (1956). Prior to this, was the report of the potency of phenol to extract proteins from aqueous solutions (Grassmann and Deffner, 1953). It is widely used in molecular biology for isolating DNA, RNA and proteins. During this process, it is often necessary to inactivate and/or remove enzymes or other proteins that derive from cell extracts or that were needed during a step of cloning procedure. The method relies on phase separation by centrifugation of a mix of the aqueous sample and a solution containing water-saturated phenol, chloroform and a chaotropic denaturing solution, resulting in an upper aqueous phase and a lower organic phase (mainly chloroform). Proteins removal is achieved by simply extracting the aqueous solution of nucleic acids with phenol, phenol/chloroform or phenol/chloroform/isoamyl alcohol. The major drawback is that phenol and chloroform as solvent are both hazardous and inconvenient and the extraction protocol is often more laborious and costlier (Tilzer, *et al.*, 1989). It however produces a higher yield DNA than most other protocol (Tilzer, *et al.*, 1989; Nasiri, *et al.*, 2005).

The salting method utilizes high salt concentrations to remove proteins. Described by Miller, it is rapid, safe and inexpensive method (Miller *et al.*, 1988). Several modifications of this original protocol have been used to obtain a higher and purer yield of DNA (Nasiri, *et al.*, 2005). Generally, most of these methods take several hours to days to complete and involve reagents that are more costly (Dellaporta *et al.* 1983; Nasiri, *et al.*, 2005). In Nigeria, DNA extraction in the first fifty samples was by the modified Miller method (Adewole, *et al.*, 1999).

The essential steps in the modified Miller protocol start with the use of proteinase K to achieve cell lysis. This is followed overnight incubation. The drawbacks of this method were

the long duration required to complete the process, usually overnight and the multiple reagents required. Additively, these factors contributed to the overall cost of the procedure.

### **2.11.2. Molecular Analysis Technique**

The goal of molecular analysis is to identify the B<sup>S</sup> mutation at the DNA level. DNA polymorphism can be detected through 2 main methods. These are DNA sequencing, which is laborious and expensive and application of DNA markers (Swarti, *et. al.*, 1999; Patricia, *et. al.*, 1998). The DNA molecular markers that are used to evaluate DNA polymorphism can be broadly classified as hybridization-based markers and polymerase chain reaction (PCR)-based markers (Swarti, *et. al.*, 1999).

#### **Polymerase Chain Reaction (PCR)**

The polymerase chain reaction (PCR) is a cell-free method of DNA cloning. PCR is a rapid and accurate in-vitro method for amplifying target DNA sequences present within a source of DNA. It is a method that is designed to permit selective amplification of the target DNA sequence(s) within a heterogeneous collection of DNA sequences (e.g. total genomic DNA). In order to achieve such selective amplification, some prior DNA sequence information from the target sequences is required. This information is used to design two oligonucleotide primers (amplimers) specifically for the target sequence. The primers are added to denatured template DNA, where they bind specifically to complementary DNA sequences at the target site. In the presence of a suitably heat-stable DNA polymerase and DNA precursors (4 deoxynucleotide triphosphates; dATP, dCTP, dGTP and dTTP), they initiate the synthesis of new DNA strands which are complementary to the individual DNA strands of the target DNA segment and the two will overlap each other.

The PCR is a chain reaction because newly synthesized DNA strands will act as templates for

further DNA synthesis in subsequent cycles. After about 25-30 cycles of DNA synthesis, the products of the PCR will include, in addition to the starting DNA, about 105 copies of the specific target sequence, an amount which is easily visualized as a discrete band of a specific size when submitted to agarose gel electrophoresis. A heat-stable DNA polymerase (the widely used Taq DNA polymerase is obtained from *Thermus aquaticus* and is thermostable up to 94°C) is used because the reaction involves sequential cycles composed of three steps:

i. Denaturation, typically at about 93-95°C for human genomic DNA. ii. Annealing at temperatures usually from about 50°C to 70°C depending on the  $T_m$  of the expected duplex (the annealing temperature is typically about 5°C below the calculated  $T_m$ ). iii. DNA synthesis, typically at about 70-75°C.

### **Specificity of Amplification and Primer design**

The specificity of amplification depends on the extent to which the primers can recognize and bind to sequences other than the intended target DNA sequences. For complex DNA sources, such as total genomic DNA from a mammalian cell, it is often sufficient to design two primers about 20 nucleotides long. This is because the chance of an accidental perfect match elsewhere in the genome for either one of the primers is extremely rare and for both sequences to occur by chance in close proximity in the specified direction is normally exceedingly low. Although conditions are usually chosen to ensure that only strongly matched primer-target duplexes are stable, spurious amplification products can nevertheless occur. This can happen if one or both chosen primer sequences contain part of a repetitive DNA sequence. Hence primers are usually designed to avoid matching to known repetitive DNA sequences, including large runs of a single nucleotide.

### **Restriction Fragment Length Polymorphism (RFLP)**

This is the earliest approach to direct DNA analysis. It involves the use of restriction enzyme to identify mutation by the absence of a restriction site, thus distinguishing between beta<sup>A</sup>-globin and beta<sup>S</sup>-globin genes. Examples of RE used include *HpaI*, *Mst II*, *BSU 36I* and *Dde* (Embury, *et al.*, 1987; Gurgey, *et al.* 1993; Husian, Kalavathi and Anandaraj, 1995). RE generate restriction fragments based on the absence of a restriction endonuclease site in genomic DNA and produce a larger restriction fragment (Chang and Kan, 1982; Orkin, *et al.*, 1982). For example, the enzyme *Mst II* involves codon 5, 6 and 7 and cuts the normal CCT-GAG-GAG in two DNA fragments, 228 and 202 bp long, but cannot fragment the mutant CCT-GTG-GAG and thus produces a 430 bp DNA fragment. The fragments are analysed by the southern blotting technique.

### **Restriction Fragment Length Polymorphism –Polymerase Chain Reaction (RFLP PCR)**

This is a modification of the original RFLP method. The fragments generated by the restriction enzyme are amplified to increase the quantity. The PCR amplification product may be analysed by southern blotting technique or ultraviolet documentation

### **Amplification Refractory Mutation System (ARMS-PCR) (Allele-Specific PCR)**

In this technique, oligonucleotide primers are designed to discriminate between target DNA sequences that differ by a single nucleotide in the region of interest. This is a form of allele-specific PCR, the PCR equivalent of the allele-specific hybridization which is possible with allele specific oligonucleotide (ASO) probes. In the case of allele-specific hybridization, alternative ASO probes are designed to have differences in a central segment of the sequence (to maximize thermodynamic instability of mismatched duplexes). However, in the case of allele-specific PCR, ASO primers are designed to differ at the nucleotide that occurs at the extreme 3' terminus. This is so because the DNA synthesis step in a PCR reaction is crucially

dependent on correct base-pairing at the 3' end. This method is used to type specific alleles at a polymorphic locus, and has particularly useful as a method for detecting a specific mutation, the so-called amplification refractory mutation system.

ARMS PCR involves the in vivo amplification of DNA sequences that contain the targeted DNA sequences. An oligonucleotide that is complementary to the DNA nucleotide sequences that flank the target sequence binds specifically to either the CCT-GTG-GAG sequence under stringent conditions, but not to the sequence CCT-GAG-GAG or vice versa (Swarti, *et. al.*, 1999). That means that the primers hybridizes the denatured single stranded genomic DNA. Each DNA is copied using a DNA polymerase to extend these primers in the 5' to 3' direction. With the aid of this PCR process, multiple copies of DNA are generated. The amplified fragments are separated by electrophoresis and banding patterns are detected by the use of methods such as dye staining or autoradiography.

ARMS primers are called Allele specific primers (ASP) 1 and 2 consisting of 30 base pairs each. The 2 primers are in the forward (F) direction, while the common C primer CON is in the reverse (R) direction of the mutant or normal allele. They are designed to correspond to specific base sequences with the difference occurring at the nucleotide at the extreme 3' terminus. One primer is for normal allele and the other for mutant allele. The primer for normal recognizes GAG and does not recognize GTG. The common C primer complements the remaining allele after separation of strands.

HbS M	CCCACAGGGCAGTAACGGCAGACTTCTGCA
HbS N	CCCACAGGGCAGTAACGGCAGACTTCTGCT
ComC	ACCTCACCTGTGGAGCCAC



## **2.12. Awareness and Utilization of Prenatal Diagnosis**

The awareness and utilization of health service is dependent on several factors. Most of the studies conducted in Nigeria on awareness and utilization about prenatal screening and diagnosis relates more to the use of ultrasound scan (Lamina *et al.*, 2004; Oloyede *et. al.*, 2006). There was an above average awareness and utilization because scan is widely available and offers sense of fulfilment to the women (Lamina *et al*, 2004). There was however no study that evaluated these parameters with respect to molecular laboratory technique. The gestational age of carrying out procedure, cost and reliability of results are important factors that could also influence acceptance and utilization. Termination of an affected pregnancy is less hazardous in the first trimester compared with second trimester procedures. A previous study showed that 70% of respondents would terminate an affected pregnancy after prenatal diagnosis (Oloyede, 2006).

A study in South western Nigeria on genotype and allele distribution showed that there is need for more awareness campaign and proper genetic counselling about sickle cell disease in Nigeria. This is particularly so for adolescents since they are likely to constitute the majority of intending couples and, therefore, those that will produce genes that will make up the gene pool for future generations (Taiwo *et. al.*, 2011).

## **2.13. Ethical Issues**

**Clearance:** The permission to carry out this study and use human materials such as blood samples and chorionic villi was sought from the Ethics and Research Committee (ERC) of the Olabisi Onabanjo University Teaching Hospital, Sagamu, Ogun State and the Management Board of High Rocks Fetal Medicine & Genetic Diagnosis Centre, Lagos.

**Consent:** Informed written consent was obtained directly from pregnant women that participated (consent form shown in appendix iii)

**Justice:** The miscarriages that may be caused by the procedure will be medically managed and all relevant information treated as confidential.

**Beneficence:** If the findings of this study were found suitable and adopted for policy formulations, it will guide laboratories in knowing the reliability of the result after maternal decidua contamination. Besides, Sickle cell anaemia would be diagnosed in early pregnancy and management instituted as early as possible thus minimizing child and maternal mortality due to the disease.

## **CHAPTER THREE**

### **METHODOLOGY**

#### **3.1. Study Design**

A descriptive cross sectional, laboratorybased study, to describe the effect of different concentrations of maternal DNA in fetal sample on the fetal haemoglobin genotype result using the ARMS PCR technique for the molecular diagnosis of SCA. It is also designed to evaluate the population genetics in the fetal, infancy and adolescent populations. The study was carried out over 12 month period. In the first phase, pregnant women were enrolled for chorionic villi sample in early pregnancy while the second phase, involves the molecular laboratory analysis. In the third phase, questionnaire was completed

Two sets of self-administered questionnaire were used. The first questionnaire was completed by each woman that had CVS, to collect data on their biosocial characteristics, awareness and acceptance of procedure. The second questionnaire was administered on married couples that had delivered children living up to adolescent age.

### 3.2. Research Centres

The first and second phases were conducted at High Rocks Fetal Medicine and Genetic Diagnosis Centre, Lagos, Nigeria and Medical Genetic Laboratory of Dr Zeinali, Iran.

The Medical Genetic Laboratory of Dr Zeinali, is dedicated fully to molecular and cytogenetic diagnosis, providing training and research opportunities to undergraduates and postgraduates students from various academic institutions across the Middle East and African countries. The centre provided the training in molecular techniques and analysis of test samples.

The High Rocks Fetal Medicine and Genetic Diagnosis Centre, Nigeria has a specialized unit that carries out invasive procedures such as chorionic villous sampling and a dedicated molecular diagnosis laboratory. All the pregnant women were enrolled at the centre, where the molecular diagnosis was also carried out. The second questionnaire was administered in five private medical centres.

### 3.3. Sample size Determination

The sample size was calculated using the standard formula:

$$n = \frac{z^2 pq}{d^2} \quad \text{where}$$

n = minimum sample size required for study

z = confidence level at 95% (standard value of 1.96) (corresponding to area under the normogram)

p = estimated prevalence rate of sickle cell anaemia (2-3%) in Nigeria.

$$p = 0.03$$

$$q = 1-p. \text{ In this case, } 1-0.03 = 0.97$$

d = margin of error at 5% (standard value of 0.05) or degree of accuracy required

$$\begin{aligned}
n &= \frac{(1.96)^2 \times 0.03 \times 0.97}{(0.05)^2} \\
&= \frac{3.8416 \times 0.03 \times 0.97}{0.0025} \\
&= 44.7
\end{aligned}$$

A minimum sample size of 45 is required in this study. The study however evaluated 74 women, which is in excess of the 5% attrition factored into the sample size.

Another 74 couples were recruited to complete the questionnaire to evaluate frequencies and distribution of genotypes and alleles and their trend in infancy and adolescence.

### **3.4. Subjects' Recruitment and Enrolment**

The subjects for CVS and molecular diagnosis were enrolled from the pregnant women attending the Fetal Medicine unit. The clinic undertakes antenatal diagnosis of various types of congenital malformations, some of which are of genetic origin such as Sickle cell anaemia. The clinic manages pregnant women from heterogeneous biosocial and obstetric background, attending to access prenatal diagnosis service.

Consecutive subjects that fulfilled the inclusion/exclusion criteria (discussed in the later section), were included in the study. Routinely, they all had preliminary abdominal ultrasound scan to confirm pregnancy, number of fetuses, viability of pregnancy and to also determine the accurate gestational age. Non directional counselling was provided before (pre) and after (post) chorionic villous sampling. In addition to this, request was made for enrolment into the study from those who eventually participated. Anonymity, as well as non-interference in clinical management, if and when they decide to opt out of study, were

guaranteed. All of them signed the informed consent form. An interviewer administered questionnaire was completed for each subject.

The following constituted guiding criteria for participation in the research.

#### **Inclusion criteria**

Confirmed viable intrauterine pregnancy between 11 and 13<sup>+6</sup> weeks

#### **Exclusion criteria**

- (i) Husband or wife with genotype other than AS
- (ii) Unexplained bleeding per vaginam.
- (iii) Co existing pelvic infection.
- (iv) Gestational age below 11 weeks or maximum placenta thickness below 11mm

Subjects for the evaluation of genes in infancy and adolescence were recruited from two private medical centres and Sickle cell clinics

### **3.5. Chorionic Villous Sampling (CVS)**

Routine abdominal ultrasound scanning was done prior to the procedure in all cases, to ascertain suitability for the procedure and determine the best path of needle introduction based on the location of the placenta. Following scanning, subjects were appropriately positioned in the supine position, cleaned with hibitane in spirit and draped. The chosen path of needle passage was infiltrated with 1% xylocaine under ultrasound guide. Aspirational biopsy was carried out by introducing the aspiration needles carefully into the placenta tissue. The stylet was withdrawn and replaced with a needle connected to a 10-20mls syringe that was pre-loaded with 2-4 mls of normal saline. Aspiration of villi was done under negative pressure created by the syringe. A maximum of 3 aspiration attempts was done in any case.

Figure 9: Fetus at 11 – 13<sup>+6</sup> weeks



Figure 10: CVS needle in placenta tissue

All subjects followed the routine advise of resting for 30 minutes after the procedure, avoiding strenuous activities for few days and sexual intercourse for 10-14 days, in order to the minimize risk of abortion (Akinyanju, *et. al.*,1999; Oloyede and Akinde, 2004). They were also counselled to report to their physician, any observed vaginal bleeding, passage of water per vaginam, fever or abdominal pain.

### **3.6. Dissection of Maternal Contaminants in Chorionic Villi**

The aspirated villi was flushed into a petri dish containing EDTA in saline and examined under low power inverted microscope for confirmation that the villi was obtained. Dissecting forceps was used to manually remove visible maternal decidua contaminants and the cleaner villi transferred into another petri dish and gently shaken to further separate chorionic villi from maternal decidua. This process was done several times, until a clean villi sample was obtained in each case. The final clean villi samples were compared with a photographic documentation of a reference standard, to determine the wet weight (Briambati,*et al*, 1987).

This final sample is transferred for immediate commencement of molecular analysis stored in the ultralow freezer at  $-20^{\circ}\text{C}$ .



Figure 11: Chorionic villi in petri dish

Figure 12: Photographic documentation for weight determination

### **3.7. DNA Extraction**

DNA extraction was carried out using boiling method. The steps involved are as follows:

#### **Chorionic Villi**

Trypsin (0.3mls) was added to 15mg of chorionic villi in eppendorf tube and the mixture centrifuged at 5000 rpm for 4 minutes. The supernatant was discarded leaving the residue, into which was added 100 u $\mu$ ls (0.1ml) of solution 1 (0.5gm NaOH), 250mls distilled  $\text{H}_2\text{O}$  at neutral pH), vortex and the tube firmly sealed with cellotape paper. The tube was then gently lowered and left in boiling water place on a Bunsen burner or sterilizing unit for 15-20 minutes. Next, 20 u $\mu$ ls (0.02mls) of solution 2 (6.02gm Tris and 250mls of distilled  $\text{H}_2\text{O}$  at pH 7.5) was added to the solution and centrifuged at 13000rpm for 2 minutes. The

supernatant containing the DNA was carefully pipetted into a new tube and the residue (protein and RNA) discarded.

### **Blood (Maternal and Paternal)**

R buffer (sucrose) 1000ul was added to 500ul of blood in eppendorf tube, mixed by vortexing and centrifuged at 1300 rpm for 4 minutes. The supernatant was discarded leaving the residue, a step that was repeated twice. To the residue was added 100 uLs (0.1ml) of Solution 1 (0.5gm NaOH, 250mls distilled H<sub>2</sub>O at neutral pH), vortexed and the tube firmly sealed with cellotape paper. The tube was then gently lowered and left in a boiling water placed on a Bunsen burner or sterilizing unit for 15 – 20 minutes. Next, 20 uLs (0.02mls) of solution 2 (6.05gm Tris and 250 mls of distilled H<sub>2</sub>O at pH 7.5) was added to the solution and centrifuged at 13000 rpm for 2 minutes. The supernatant containing the DNA was carefully pipette into a new tube and the residue (protein and RNA) discarded. The extracted DNA was evaluated for purity and quantity comparing with a band of known purity and quantity.

The purity of the extracted DNA was checked with nanodrop 1000, first by determining the ratio of absorbance at 260 and 280 nm. A ratio of ~1.8 was accepted as “pure” for DNA. An appreciably lower ratio may indicate the presence of protein, phenol or other contaminants that absorb strongly at or near 280 nm. A secondary check for purity was done measuring ratio of sample absorbance at 260 and 230 nm. Range between 1.8 -2.2 is accepted as pure DNA. An appreciably lower ratio, may indicate the presence of co-purified contaminants

### **3.8. ARMS PCR Analysis**

The second component is the amplification of DNA sequences in either the homozygous or heterozygous state that has been annealed by allele specific primers in combination with appropriate volumes of deoxynucleotides (dNTPs), distilled water (dH<sub>2</sub>O), buffers containing Mg<sup>2+</sup> and Taq DNA polymerase.



ARMS primers are called Allele specific primers (ASP) 1 and 2 and consist of 30 base pairs each and reverse primers of 20 base pairs. The 2 primers are in the forward direction, while the common C primer CON is in the reverse direction of the mutant or normal allele. They are designed to correspond to specific base sequence with the difference occurring at the nucleotide at the extreme 3' terminus. One primer is for normal and the other for mutant. The primer for normal recognises GAG and does not recognize GTG. The common C primer complements the allele after separation of strands.

<i>HbS M</i>	<i>CCCACAGGGCAGTAACGGCAGACTTCTGCA</i>
<i>HbS N</i>	<i>CCCACAGGGCAGTAACGGCAGACTTCTGCT</i>
<i>ComC</i>	<i>ACCTCACCCCTGTGGAGCCAC</i>

Master mix made up of the following constituents:

- A. PCR mix  $\beta$ {deoxynucleotides (dNTPs), distilled water (dH<sub>2</sub>O), MgCl<sub>2</sub>, 10X buffer}  
20ul x n (number of reaction sets)
- B. Primers {Forward (M&N) and Reverse (Com C)}  
Forwards 0.2ul x n (number of reaction sets)  
Reverse 0.1ul x n (number of reaction sets)
- C. Taq DNA polymerase enzyme  
0.2ul x n (number of reaction sets)

Into each reaction tube was transferred 4ul of extracted DNA and 20ul of master mix.

The first part of the molecular analysis was to determine the fetal haemoglobin genotype and identify foetuses that are haemoglobin genotypes AA and SS.

**Part A:**

<b><u>Tubes</u></b>	<b><u>DNA</u></b>	<b><u>Hb Genotype</u></b>
1 & 2	Control	N/N
3 & 4	Control	M/N
5 & 6	Control	M/M
7 & 8	Paternal	M/N
9 & 10	Maternal	M/N
11 & 12	Fetus	?
13 & 14	Fetus	?
15 & 16	Control Negative	-

The second part was to determine the effect of increasing concentration of maternal venous blood DNA in a fixed concentration of known fetal DNA would have on the fetal haemoglobin genotype HbA<sup>HbA</sup> or HbS<sup>HbS</sup>.

**Part B:**

<b><u>Tubes</u></b>	<b><u>DNA</u></b>	<b><u>Hb Genotype</u></b>
1 & 2	Control	N/N
3 & 4	Control	M/N
5 & 6	Control	M/M
7 & 8	Fetus	M/M or N/N
9 & 10	Fetus + M (0.25)	?
11 & 12	Fetus + M (0.5)	?
13 & 14	Fetus + M (0.75)	?
15 & 16	Fetus + M (1.0)	?
17 & 18	Control Negative	-

The first PCR tube in each reaction set was filled with the primer for normal allele and the common C primer, while the second tube is loaded with the primer for mutant allele and the common C. The control tubes do not have the DNA sample.

PCR was carried out on the required number of tubes using the following programme:

Total cycles - 28

Denaturation (Initiation) - 93°C/3; 93/1

Annealing (Hybridization) - 67°C/1

Elongation (Extension) -72°C/1; 72/2

In the presence of a complementary allele, amplification proceeds, while the no amplification occurs when the allele is non complementary. The PCR produces multiple copies of the targeted DNA sequences annealed by the primer in each tube. In HbSHbS fetus, the Hb<sup>S</sup> allele is amplified in the tube with mutant primer and subsequently displayed as band, while in the HbAHbA fetus, the Hb<sup>A</sup> allele is amplified in the tube with normal primer and also displayed as band. In HbAHbS fetus, individual alleles is amplified in the respective tubes containing appropriate primers and also displayed as bands.

### **3.9. Gel Documentation/Ultraviolet Translumination**

The PCR products were run on 2% Agarose gel electrophoresis in accordance with manufacturer's instruction. The PCR products were mixed with 1% ethidium bromide and loaded into the wells in the gel matrix. Electrical current was applied. Bands were obtained depending on the allele presents in each tube. In Haemoglobin SS fetus, the tube with mutant primer produces band; In Haemoglobin AA fetus, the tube with normal primer produces band, while in Haemoglobin AS fetus, both tubes produce bands

The gel image was viewed using the ultraviolet transillumination, for the display of the DNA bands. Comparison of the band from the sample DNA was made with the confirmed controls in order to determine the genotype.

### **3.10. Data Management and Analysis**

Data extracted from the questionnaires and the haemoglobin genotype results were collated and entered into the Epi Info version 6.1 statistical software package.

The counts of haemoglobin genotypes that changed with each different concentrations of maternal DNA were made and the statistical significance of the change with each quantity of maternal DNA was determined using  $\chi^2$ , at  $p < 0.05$ .

In each of the population groups (fetal, infancy and adolescence), the actual counts and ratios of different genotypes were done and this was compared with the expected genotype ratio based on Mendelian principle. Statistical significance was determined with  $\chi^2$  test (goodness of fits), and the discrepancy between the observed and the expected ratios was considered significant at  $P < 0.05$ .

Generally, expected genotypic and allelic frequencies were determined from the observed gene frequencies using the Hardy–Weinberg proportions:

$$P^2 + 2pq + q^2 \text{ where}$$

$p$  = frequency of the HbA allele and  $q$  = frequency of HbS allele.

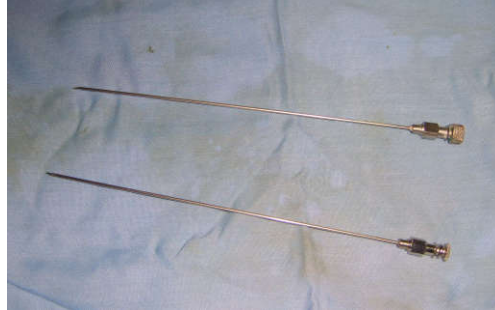
Data from the questionnaire were analysed and presented as simple percentages and frequency tables

## **CHAPTER FOUR**

### **RESULTS**

## 4.1 General

In the study period, fifty three women were counselled on CVS, of which 3 were excluded based on the inclusion and exclusion criteria.



**Figure 15: CVS Needles**

## 4.2 Biosocial and Obstetric Characteristics

The baseline biosocial and obstetric parameters in participants are summarized in table 1. About a third (34.0%) of the subjects were between 26 and 30 years age group, while the least number of 8 (8.0%) were in the  $\geq 40$  years age groups. The maternal age range was 23 – 47 years with a mean of  $30.9 \pm 3.6$  years. Women that have had either three or four children (Para 3 – 4) was highest (52.0%) or those with five and above children ( $\geq$  Para 5) were least (4.0%). Majority of procedures were done between  $11^{+0} - 11^{+6}$  weeks (42.0%), while the least (26.0%) were done between  $13^{+0} - 13^{+6}$  weeks. Christianity was the predominant (80.0%) religion. All of them had either secondary or tertiary education and majority (90.0%) were either middle or high socio economic group.

**Table 1: Baseline Biosocial and Obstetric parameters**

<b>Parameters</b>	<b>Number (N=74)</b>	<b>Percentage (%)</b>
<b>Maternal Age (Years)</b>		
21 – 25	9	12.2
26 – 30	22	29.8

31 – 35	18	24.3
36 – 40	16	21.6
≥ 41	9	12.2

[Mean 30.9: Range 23 – 47]

#### **Parity**

1 – 2	30	40.5
3 – 4	34	45.9
≥ 5	10	13.5

[Mean 3.3: Range 0 – 6]

#### **Gestational Age (Weeks)**

11 <sup>+0</sup> – 11 <sup>+6</sup>	29	39.2
12 <sup>+0</sup> – 12 <sup>+6</sup>	24	32.4
13 <sup>+0</sup> – 13 <sup>+6</sup>	21	28.4

#### **Religion**

Protestant	13	17.6
Catholic	17	22.9
Anglican	25	33.8
Islam	12	16.2
Others	7	9.5

#### **Economic level**

Low	13	17.6
Middle	34	45.9
High	27	36.5

#### **Educational level**

Primary	-	-
Secondary	24	32.4
Tertiary	50	67.6

### **4.3. Maternal DNA in Chorionic Villi**

The gel images in Haemoglobin genotypes AA, AS and SS, as observed under ultraviolet transillumination are shown in Figures 16,17 and 18 respectively.

<<<<<<<<<<

Thirty seven samples that were of haemoglobin genotypes AA or SS were subjected to further molecular analysis to determine the effect of increasing concentrations of maternal DNA (Heterozygous AS; HbAHbS) on the previously established haemoglobin genotypes. These are made up of 22 HbAHbA and 15 HbAHbS respectively.

Table 2 A and B outlines the result of different concentrations of maternal DNA on fixed concentration of foetal DNA with respect to haemoglobin genotype result on individual sample.

The established haemoglobin genotype AA was maintained as AA in all the 22 (100%) samples with 0.25ul of maternal DNA, but changed to HbAHbS in 8 (36.4%) samples with 0.5 and 0.75ul each and in 18 (81.8%) samples with 1.0ul of maternal DNA (Table 2A). The pattern of change in haemoglobin genotype SS in the presence of maternal DNA were as follows: 100% maintained with 0.25ul, changed in 2 (6.7%) with 0.25 and 0.5ul and 12 (80.0%) with 1.0ul (Table 2B)

Summary and statistical inference from the changes in DNA results with varying concentrations of maternal DNA is shown in table 3.

**Table 2: Fetal Haemoglobin Genotype Result with different concentrations of Maternal DNA**

**A: Haemoglobin Genotype AA**

	<b>4.0 + 0.00</b>	<b>4.0 + 0.25</b>	<b>4.0 + 0.50</b>	<b>4.0 + 0.75</b>	<b>4.0 + 1.00</b>
<b>1</b>	AA	AA	AS	AS	AS
<b>2</b>	AA	AA	AS	AS	AS
<b>3</b>	AA	AA	AA	AA	AS
<b>4</b>	AA	AA	AA	AA	AS
<b>5</b>	AA	AA	AA	AA	AS
<b>6</b>	AA	AA	AA	AA	AS

7	AA	AA	AA	AA	AS
8	AA	AA	AA	AA	AA
9	AA	AA	AA	AA	AA
10	AA	AA	AS	AS	AS
11	AA	AA	AA	AA	AS
12	AA	AA	AS	AS	AS
13	AA	AA	AA	AA	AS
14	AA	AA	AA	AA	AA
15	AA	AA	AS	AS	AS
16	AA	AA	AS	AS	AS
17	AA	AA	AA	AA	AS
18	AA	AA	AA	AA	AA
19	AA	AA	AS	AS	AS
20	AA	AA	AA	AA	AA
21	AA	AA	AA	AA	AS
22	AA	AA	AA	AA	AA
23	AA	AA	AA	AA	AS
24	AA	AA	AS	AS	AS
25	AA	AA	AA	AA	AA

#### B: Haemoglobin Genotype SS

	4.0 + 0.00	4.0 + 0.25	4.0 + 0.50	4.0 + 0.75	4.0 + 1.00
1	SS	SS	SS	SS	SS
2	SS	SS	AS	AS	AS
3	SS	SS	SS	SS	AS
4	SS	SS	SS	SS	AS
5	SS	SS	SS	SS	AS
6	SS	SS	SS	SS	AS
7	SS	SS	SS	SS	AS
8	SS	SS	SS	SS	SS
9	SS	SS	SS	SS	AS
10	SS	SS	AS	AS	AS
11	SS	SS	SS	SS	AS
12	SS	SS	SS	SS	AS
13	SS	SS	SS	SS	AS
14	SS	SS	SS	SS	AS
15	SS	SS	SS	SS	AS

**Table 3: Analysis of Change in Fetal Genotypes with Maternal DNA**

**Haemoglobin Genotype      Maintained(%)      Changed(%)    $\chi^2$       p-value**

**AA (N = 22)**

4.00 + 0.00	22 (100.0)	- (0)
4.00 + 0.25	22 (100.0)	- (0.0)



4.00 + 0.50	14 (63.6)	8 (36.4)
4.00 + 0.75	14 (63.6)	8 (36.4)
4.00 + 1.00	4 (18.2)	18(81.8)
<b>SS (N = 15)</b>		
4.00 + 0.00	15 (100.0)	- (0.0)
4.00 + 0.25	15 (100.0)	- (0.0)
4.00 + 0.50	14 (93.3)	1 (6.7)
4.00 + 0.75	14 (93.3)	1 (6.7)
4.00 + 1.00	3 (20.0)	12 (80.0)

#### 4.4. Distribution of Fetal Haemoglobin Genotypes based on Mendelian Ratio

The actual and expected haemoglobin genotype distributions based on the Mendelian ratio expected from heterozygous mating pattern is shown in table 4. There was statistical difference in the HbAHbA and HbSHbS distributions, while non was found with HbAHbS distribution

**Table 4: Fetal Haemoglobin Genotypes: Actual and Expected based on Mendelian Ratio**

<b>Haemoglobin Genotypes</b>	<b>Actual count</b>	<b>Expected Count</b>
HbAHbA	22	18.5
HbAHbS	37	37.0
HbSHbS	15	18.5

$$\chi^2=0.193; P=0.908$$

#### 4.5 Frequency Distribution of Fetal Haemoglobin Genotype and Alleles

The distributions of the three different haemoglobin genotypes and the alleles responsible for the genotypes are shown in Table 5A. The fetal haemoglobin genotype with the highest frequency was HbAHbS (37; 50.0%), while HbSHbS has the lowest frequency (15;20.3%).The allelic frequencies computed from the actual counts of haemoglobin genotypes were found to be Hb<sup>A</sup> (0.570)and Hb<sup>S</sup> (0.430) {Table 5B}.Chi-square analysis in table 5C, revealed that there was nostatistically significant difference ( $\chi^2$ , at  $p < 0.05$ ).

**Table 5: Frequency Distributions of Fetal Haemoglobin Genotypes and Alleles**

**A: Actual counts of Fetal Haemoglobin Genotypes and Alleles in the sample**

<u>HbAHbA</u>	<u>HbAHbS</u>	<u>HbSHbS</u>	<u>Total</u>	
Number of Individuals	22	37	15	74
Number of Alleles:	HbA 44	37	-	81 } 148
	HbS -	37	30	67 }

---

**B: Observed and Expected Fetal Genotypic and Allelic Frequencies**

	<u>Alleles</u>		<u>Genotypes</u>		
<u>HbA</u>	<u>HbS</u>	<u>HbAHbA</u>	<u>HbAHbS</u>	<u>HbSHbS</u>	
Observed	0.810.67	0.22(0.731)	0.37(0.246)	0.15(0.024)	
Expected	0.569 0.431	0.185(0.714)	0.37(0.262)	0.185(0.024)	

---

**C: Difference between Observed Genotypes and Hardy–Weinberg Expectations**

<u>HbAHbA</u>	<u>HbAHbS</u>	<u>HbSHbS</u>	
Observed	22.0	37.0	15.0
Expected	16.2	24.5	9.3

$\chi^2=0.193; P=0.908$

---

**Table X: Trend of Genotypes and Alleles in Fetal, Infancy and Adulthood Population**

**A: Genotypes**

<u>Population</u>	<u>HbAHbA</u>	<u>HbAHbS</u>	<u>HbSHbS</u>
Fetal			

Observed	22.0	37.0	15.0
Expected	<i>16.2</i>	<i>24.5</i>	<i>9.3</i>

### Infancy

Observed

Expected

### Adulthood

Observed

Expected

### B: Alleles

<b>Population</b>	<b>HbAHbA</b>	<b>HbAHbS</b>	<b>HbSHbS</b>
<b>Fetal</b>			
Observed	22.0	37.0	15.0
Expected	<i>16.2</i>	<i>24.5</i>	<i>9.3</i>

### Infancy

Observed

Expected

### Adulthood

Observed

Expected

<b>Haemoglobin Genotypes</b>	<b>Actual count</b>	<b>Expected Count</b>
HbAHbA	22	18.5
HbAHbS	37	37.0
HbSHbS	15	18.5

#### 4.6. CVS Complications

Table 6 summarizes the complications recorded. Bleeding from the abdominal needle entry point was the commonest (4.1%). In none of these cases was pregnancy compromised. There was no case of miscarriage recorded, while rupture of membranes occurred in 1 (1.4%) case and pregnancy and delivery remained uneventful.

**Table 6: Complications of Chorionic Villous Sampling**

<u>Complications</u>	<u>Number (N=50)</u>	<u>Percentage (%)</u>
Abortion	0	0.0
Bleeding	3	4.1
Infection	-	-
Rupture Of Membranes	1	1.4

#### 4.7. Awareness and Acceptance of (Early) Prenatal Diagnosis

The women were assessed on their attitude towards prenatal CVS and molecular diagnosis, and the factors that may affect the uptake of diagnosis services. Seventy six percent of the respondents were aware that prenatal diagnosis is available in early pregnancy, and 42% had screening in a previous pregnancy. Some 64% expressed the desire to have a PND in subsequent pregnancies. Ultrasound scan is the most preferred method. Majority (92.0 %) of women prefer first trimester diagnosis. The main factors against a utilisation of prenatal screening are: cost of screening (40.0%), religious barriers (30.0%) and poor public awareness (66.0% (Table 7).

**Table 7: Awareness and Acceptance of (Early) Prenatal Diagnosis**

<u>Parameter</u>	<u>Responses (n=50)</u>	<u>Percentage (%)</u>
Awareness of PND/Molecular Diagnosis		
Yes	38	76.0
No	12	24.0
Previous PND/Molecular Diagnosis		
Yes	21	42.0
No	29	58.0
Desire for PND/Molecular Diagnosis in future pregnancies		

Yes	32	64.0
No	18	36.0
Preferred method		
Scan	677	91.5
Blood	63	8.5
Preferred Gestational Age		
1st trimester	46	92.0
2nd trimester	4	8.0
Perceived barrier against Utilization		
Cost	20	40.0
Religion	15	30.0
Low public awareness	33	66.0

## CHAPTER FIVE

### DISCUSSIONS

Human haemoglobin polymorphism with respect to sickle cell anaemia is of considerable interest in molecular and population genetics, as well as in clinical medicine. Epidemiological studies have shown that about 300,000 infants are born annually in Africa with major haemoglobin disorders. Out of these, sickle cell anaemia is about 200,000 (World Health Organization 2006). Majority of these cases occur in West Africa with Nigeria having the highest incidence (Akinyanju *et al.* 1999). Considering the high perinatal, infant and maternal morbidity, and mortality of sickle cell anaemia, the disease places a significant psychological and financial burden on the patients and their families (Taiwo *et al.*, 2011). This situation is complicated by the high level of poverty and illiteracy in the third world countries like Nigeria. In spite of advances in medical technologies and intervention strategies, available data indicate that the prevalence of infant morbidity and mortality from sickle cell anaemia in

the third world countries is still relatively high when compared with the developed countries (World Health Organization 2006).

These justify the research into aspects of molecular and population genetics that could facilitate effective measures to reduce the burden of the disorder in developing countries. **General Obstetrics and Biosocial Characteristics**

**(from: Influence of Socio-Demographic Characteristics on the Utilisation of a Chorionic Villus Sampling Service in Nigeria).**

It is often reported that the utilization of any health service is a reflection of the socio demographic characteristics of the population in the community (Salako *et al.*, 2006). This study demonstrates a class bias in the utilization of PND molecular service in favour of the younger reproductive age group. This could be a reflection of increasing awareness and acceptance especially among the emerging reproductive age group and also by the increasing educational and economic level. Instructive to observe that multipara made up a significant proportion of the subjects. Various reasons could explain this observation, which include the high fertility rate in Nigeria, being 5.5 children per woman and the low mean age of marriage, being 16.9 years (Oloyede, 2005; **R2 of Influence**). Another explanation is that morbidity and mortality associated with sickle cell disease babies is usually high, thereby encouraging frequent pregnancies in quick succession.

**Majority of procedures were done between 11<sup>+0</sup> – 11<sup>+6</sup> weeks (42.0%), while the least (26.0%) were done between 13<sup>+0</sup> – 13<sup>+6</sup> weeks. Christianity was the predominant (80.0%) religion.**

.....

Contrary to the finding is report from a previous study that the parity in ranged from zero to eleven with the 42.5% of the clients being of para 3 and 4, about a third of the clients were para  $\geq 5$  and 96.6% have at least 2 previously affected children (Oloyede, 2005).

The influence of religion is emphasized by the widespread belief that it is ungodly to unravel the work of God, or attempt to abort the product of such divine creation (Oloyede, 2005). This is unlike what obtains in some parts of the world where there is religious backing for the termination of pregnancy before three months of gestation.

The level of education is known to have a positive influence on the knowledge and attitude to an innovation (**R10 of Influence**). Generally, Christians tend to acquire higher levels of formal education than Moslems in most parts of Nigeria, a practice that could logically translate to positive impact on attendance and utilization pattern.

.....If one reflects on this situation in Lagos, a metropolitan mega city, the situation in other parts of the country especially in the rural areas are likely to be worse. There were more subjects within the reproductive age group and over two-thirds below 50 years. This may be accounted for by the source of subject recruitment. Being hospital based, the majority of those who attend are middle aged females and children. Literacy level is relatively high compared to other places in Nigeria. Many people received tertiary or at least secondary education. It is pertinent to note that education has a major influence in access and utilization of healthcare facilities in southwestern Nigeria (Salako et al. 2006). The implication of this is that the frequency of sickle cell haemoglobin allele might be gradually reducing in the population. Several factors may contribute to this: first, the high level of education in the study population could positively influence awareness of the condition and improve socioeconomic levels might have led to a better utilization of reproductive health options such as the use of contraception in AS/AS marriages. Secondly, it is possible that the availability and utilization of preventive options such as antenatal chorionic villus sampling or amniocentesis for early pregnancy diagnosis could make parents opt for abortion of affected fetuses (Oloyede 2005). Previous study has shown that the majority of those who utilize such services are in Lagos, being a centre of excellence for the service (Lewis 1970). Thirdly, there is an increasing societal discouragement of marital union of heterozygous (HbA/HbS) individuals as a way of reducing the incidence of sickle cell disease. Moreover, as implied by the low inbreeding coefficient obtained in the study, Yorubas are strictly outbreeding ethnic group that strongly discourage consanguineous matings. The implication of such practice is that autozygosity or homozygosity by common descent for HbS allele would be rare or absent in the population.

## **Molecular Study**

RFLP remains a major tool in molecular of diagnosis. Its most important drawback is the requirement for large quantity sample from which DNA will be extracted (**R**). In addition to this, restriction enzyme is needed to digest genomic DNA and further analysis involves Southern blotting technique. Consequently, it has not been a favoured tool for analysis involving fetal samples. The chorionic villus being the major fetal source of DNA is included in this category. Procedure safety requires that sample size is limited to avoid complications such as miscarriage.

A more innovative technique developed to reduce some of the drawbacks of RFLP is RFLP PCR. This technique was used in the analysis of 50 samples in Nigeria at inception of molecular diagnosis (Adewole *et al.*,1999). Although, involves PCR, it is however not completed without restriction enzymes, while it also time consuming. **Other studies on it**

The amplification refractory mutation system polymerase chain reaction using only a minute amount of CV, and primers is one of the recent molecular diagnosis tool ( **R**).

## **Maternal DNA Contamination**

Misdiagnosis remains the most significant challenge to PCR based molecular diagnosis. It may occur for several reasons: failure to amplify the target DNA fragment, mispaternity, maternal contamination, and sample exchange( **R**). To limit the possibility,Misdiagnosis from failure of DNA amplification, chorionic villous DNA analysis was recommended to be carried out with two different procedures: i.e. RDB hybridisation and primer-specific amplification (ARMS) (**R** ).

Misdiagnosis from inadequate isolation of the fetal sample from contaminating maternal tissues, which can lead to misinterpretation, particularly with PCR amplified DNA is the most serious challenge (Cao and Rosatelli, 1993).Several steps are recommended to avoid misdiagnosis resulting from mispaternity or maternal contamination. These includecareful



dissection of the maternal decidua from the fetal trophoblast under the inverted microscope, using minimal amount (about 3-4 microg) of chorionic villi DNA, to reduce the chances of co-amplifying the DNA from the maternal decidua, limiting the number of PCR cycles to minimize undesirable co-amplification of traces of maternal DNA and carrying out DNA polymorphism analysis parallelly with mutation analysis(Cao and Rosatelli, 1993; **R**).

A previous study examined the tolerable degree of maternal DNA contamination in prenatal diagnosis of sickle cell anemia was investigated by mixing various amounts of DNA from HbS carriers with DNA from normal individuals and sickle cell anemia patients. The results indicate that lower rate of admixed maternal DNA does not prevent an exact direct DNA-diagnosis of sickle cell anemia ( Oehme *et al.*, XXX)([Renate Oehme](#), [W. -D. Jonatha](#), [J. Horst](#); **R**). Our study however showed that.....

Precautions was taken to avoid sample mix-up in the laboratory.

### **Population Genetic**

Study of population genetics is the bed rock for an effective premarital counselling and screening programme.

### **Genotype and Allelic Frequencies**

In this study, the ratio of distribution of AA, AS and SS was roughly 1: 1.6: 0.6. This is in sharp contrast to statistical prediction for an autosomal recessive gene based on Mendelian principle 1:2:1.

.....

### **There are two possibilities. Either enhanced survival of homozygous HbA or reduced survival of homozygous HbS**

The implication of the frequency distribution is that of HbAHbS and HbSHbS do not survive as expected based on the Mendelian ratio.

A possible postulate is that the effect of malaria endemicity is not transferred into the fetal population and consequently heterozygote advantage is not maintained, with reduced survival of foetuses with the genotype. *in pregnancy and also in line with the gene frequency finding in a 7, 12previous study*(**Influence**)

The reduced survival in HbAHbS is more pronounced in HbSHbS fetuses, where about reduction of about 50% was found below the expected Mendelian ratio.

Common to the two haemoglobin genotype group is the presence of the haemoglobin S allele. The final implication of these two statistics is that HbS allele may have a lesser survival potential in utero than even in the adult population. On the contrary, HbAHbA genotype maintains its expected Mendelian ratio, thus supporting the postulation that haemoglobin A allele has superior survival.

.....

Studies in adult population with respect to haemoglobin genotype distribution have been varied. HbAHbA found in this study was established as the most common haemoglobin genotype in some parts of Nigeria (*Bakare et al. 2006; Zacccheaus 2006; WHO 2006*)

Although, the allelic frequency was studied in the fetal population, with bias in favour of assortative mating, the allelic frequencies could however be adoptable because of previous studies that have shown persistence of heterozygote such mating pattern in the adult population (*Taiwo et al., 2011*).

The superior survival advantage of Hb<sup>A</sup> allele over Hb<sup>S</sup> allele could be explained by the presence and frequency of HbF.

.....

It is of interest that despite the seriousness of the sickle cell anaemia and the selective pressure against HbSHbS genotype, the disease is still present in many populations especially in Nigeria and other tropical countries where malaria is endemic (*Moormann et al. 2003; WHO 2006*). This observation had been attributed to balancing selection referred to as heterozygote superiority, whereby heterozygotes are at a selective advantage over the two homozygotes. Apparently, the abnormal haemoglobin mixture in the heterozygote provides an unfavourable environment for the growth and survival of the malaria parasite in the red cell. Natural selection cannot therefore eliminate the detrimental allele from the population because of its beneficial effects in the heterozygote state. It is noteworthy that HbC allele was not detected in this study despite the fact that individuals carrying this allele are occasionally encountered clinically in Nigeria.////////

Of special significance was the finding that the Hardy–Weinberg equilibrium was not disturbed in the study population, similar to the findings in adult population that displayed absence of assortative mating pattern with respect to the HbA/HbS gene locus (*Taiwo et al., 2011*). This may imply inadequacy of genetic counselling and the need for more aggressive

awareness campaign aimed at reducing the incidence of sickle cell disease. The occurrence of HbSS was low in agreement with the results of other studies (Bakare *et al.* 2006; Zacheaus 2006). The overall implication of the allelic distribution is that the frequency of sickle cell haemoglobin allele might be gradually reducing in the population.

*.....The low inbreeding coefficient obtained in the study and the genotypic frequencies which were not significantly different from Hardy–Weinberg frequencies may indicate absence of assortative mating with respect to HbA/HbS gene locus among the Yorubas in Lagos. This may imply inadequacy of genetic counselling and the need for more aggressive awareness campaign aimed at reducing the incidence of sickle cell disease and other haemoglobin disorders.*

### **Procedure Related Miscarriage**

Procedure related abortion is the most important complication of chorionic villous sampling (Oloyede, *et al.*, 2004). The risk is however known to be influenced by factors such as the route of sampling, number of aspirations, and skill of the obstetrician. All procedures were transabdominal because of its reported risk of lower miscarriage (Oloyede, *et al.*, 2009). Maximum number of aspiration was less than thrice in the study. There was no case in this study series. This can be attributed to improvement in experience and skill of the operator, which has been reported in studies to have a positive effect on outcome of procedure and reflects the presence of a learning curve (Oloyede, 2011).

### **Awareness and Acceptance of Molecular Prenatal Diagnosis**

Apart from educational influence, the pattern of utilization may also be a reflection of the convenience, cost of travel from other states to Lagos, as well as availability of information. The Igbo tribe also utilize the service fairly well. When we analyze the influence of occupation on utilization, it became obvious that majority of the clients belong to the high-income group or top cadre workers and civil servants. CVS, like most other recent innovation in medicine attract a high cost and only those who can afford the service have access to

5,8,11it . Better financial status has been adduced as one of 4the reasons why most of the clients are from Lagos .

.....Acceptance of prenatal diagnosis for SCD differs from that reported for thalassemia. Influences include the mode and time of carrier identification; the age, education, religion, and national background of the prospective parents; the experience of having cared for or lost a child with SCD; the timing and effectiveness of genetic counseling; the attitude of the community; and the available medical resources. Reliable obstetric services are paramount. However, the key factor to increasing acceptance of prenatal diagnosis has been the early timing of the procedure. Women who were tested in the second trimester had difficulty with pregnancy termination in the 20<sup>th</sup> week or later. Earlier fetal diagnosis and selective abortion have become simple, reliable, and safe and have contributed to the wide acceptance of prenatal diagnosis of SCD.

Another decisive factor is the experience of having a child with SCD, which often convinces couples at risk to seek prenatal diagnosis. In the UK, prenatal diagnosis was offered to a group of unselected women attending the obstetric clinic of a hospital in London after detection of the trait. The option was also presented to another group of women at the clinic who had an affected child. The unselected women were not sufficiently motivated to present for testing in a timely fashion, to bring their partners for testing, and were less likely to proceed to prenatal diagnosis (22%). In contrast, almost all women in the second group had their partners tested and requested prenatal diagnosis if their partners bore the beta<sup>S</sup> gene.<sup>32</sup> In Guadeloupe, where most beta<sup>S</sup> carriers are identified after examination of the cord blood, 64 out of 144 mothers at risk for having beta<sup>S</sup>/beta<sup>S</sup> children (44%) elected to have prenatal diagnosis. Of these, only 16 out of 27 given unfavorable news (59%) proceeded to termination of pregnancy. The respective percentages for 41 women at risk for the beta<sup>S</sup>/beta<sup>C</sup> combination were 34% and 60%.<sup>22</sup> In Nigeria, where identification of carriers is limited to women who have had one or more affected children (retrospective screening), almost all 263 women at risk requested prenatal diagnosis. However, only 63% of those given an unfavorable diagnosis chose to terminate pregnancy, due most often to religious convictions or fear of the obstetrical procedure.<sup>32</sup> Greece has a national program to identify carriers of inherited hemoglobin disorders at school age or before marriage. Women screened in this program are twice as likely to have a child with beta<sup>S</sup>/beta-thalassemia as the beta<sup>S</sup>/beta<sup>S</sup> combination. Each year nearly all 60-80 women at risk for SCD seek prenatal diagnosis, and undergo selective abortion in the case of unfavorable results.<sup>18,33</sup>

.....

A major goal of good antenatal care service is the delivery of the baby without congenital malformation. Worldwide congenital malformations contribute to an incidence of between 2% and 5% of live births and 20% of stillbirths and neonatal deaths (Ajayi 2003; Oloyede et al. 2006). While it is best detected in early pregnancy, the situation in most obstetrics unit in Nigeria is that of late pregnancy diagnosis or even diagnosis at birth as a chance event (Oloyede et al. 2006). In order to derive the maximum benefit of such services, the optimum period for screening is in early pregnancy. This allows for enough time to decide on the management options for an affected fetus. Such decisions include: intrauterine fetal therapy

where feasible, termination of an affected fetus or the opportunity to prepare to live with an abnormal child (Ajayi 2003). Women are directly affected by abnormalities in the fetus, while the family and the society are indirectly affected. Such effects range from psychological depression to financial demands for treatment and rehabilitation of affected fetuses. The attitude of women towards prenatal screening is therefore vital to the success of any screening service.

.....

Nigeria is a heterogeneous population with over 70% of the population living in the rural areas. Poor communication and inadequacy of orthodox health care facilities and poverty in the rural areas makes the utilization of this type of service to remain poor. This can therefore explain the restricted utilization to mainly the urban dwellers, in the high socioeconomic group. Prenatal diagnosis was however introduced for the benefit of the population that lives in both the rural and urban areas.

This was a retrospective study that was conducted on 79.7% of the total women sampled, leaving out 21.1% who for various reasons did not complete the clinical data cards satisfactorily. This is not unexpected, as counseling for the procedure is non directive, thus allowing a woman to opt out of any aspect of the procedure at any time. Also the location of the study centre could create a bias in the population studied as well as the small sample size, which appear small to be fully representative of the entire population. Nevertheless, the majority of the women reside in Lagos, which is often said to be fairly representative of the entire nation. Our findings from the study could therefore reflect those of other Nigerian women who should utilize the service.

..... This study showed that health workers and clinics still constitute an important route for the dissemination of information on CVS in Nigeria. This is contrary to another study, which reported that the mass media is the most popular source of health information. Our observation is however explainable. In Nigeria, the first time most people get information about sickle cell disorder is usually when an affected child manifests the symptoms of the disease or its complications or a laboratory diagnosis is made. This is because most doctors, specialists and health workers live and work in urban areas, where such children are referred to in conditions of emergencies. On such occasions, part of the counseling may be on PND. Next to that is the information obtained from friends and relations who usually have experienced the trauma of a SCD or have done CVS. Unlike in other disease conditions especially HIV/AIDS, which goes with a lot of media supports, the media support for SCD and CVS is very negligible. The media as a source of information in this study is low.

Conclusion In conclusion, this study has reported on the socio demographic characteristics of PND clients in Nigeria and also shown that a particular class of Nigerians is enjoying the service. Women in this class are mostly Christians, from the Yoruba or Igbo tribe with at least

119

secondary level of education. Usually they would have an affected child and obtained the information in the course of caring for the child during medical crises.

The above information should help clinicians and policy makers to decide on the best ways of reaching out to the population at risk. Some of the recommended steps include the following:

### **(Influence)**

## **Conclusion**

The greatest burden of SCA has been reported in Nigeria. Hence, the justification for study into premarital screening and counselling, as well as molecular prenatal diagnosis.

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There are several ways to minimize contaminants in a PCR amplification: PCR optimization (Innis and Gelfand, 1990):



- Amount of starting DNA
- Careful primer design
- Primer concentration
- Enzyme concentration – Magnesium ion ( $Mg^{2+}$ ) concentration
- Nucleotide concentration
- Buffer composition
- Number of cycles
- pH

Manual Hot Start method

AmpliTaq Gold® DNA Polymerase as an automatic Hot Start

Limiting dNTPs and primers

All of these methods increase the specificity of the PCR amplification and decrease the amount of contaminants that can interfere with a sequencing reaction.

.....

### Determining DNA **Quality**

The following methods can be used to examine DNA quality:

Agarose gel electrophoresis

Purified DNA should run as a single band on an agarose gel.

Note Uncut plasmid DNA can run as three bands: supercoiled, nicked, and linear.

**Spectrophotometry** The A260/A280 ratio should be 1.7–1.9. Smaller ratios usually indicate contamination by protein or organic chemicals.

Agarose gels reveal the presence of contaminating DNAs and RNAs, but not proteins.

Spectrophotometry can indicate the presence of protein contamination, but not DNA and RNA contamination.

These methods should be used together to get the most information about your DNA template before sequencing.

Note RNA contamination up to 1 µg can be tolerated, but it will affect DNA quantitation greatly.

Neither of these methods shows the presence of contaminating salts that can cause noisy data.

If you suspect that your DNA is contaminated with salt, remove the salt before sequencing.

The most efficient method for salt removal is ultrafiltration with a Centricon-100 column (see page 3-12). Spin columns and ethanol precipitation can also be used (see page 3-33).

''''''''''''''''

## DNA Template Quantity

### Quantitating DNA

DNA template quantitation is critical for successful sequencing reactions. The most common way to determine DNA quantity is to measure the absorbance (optical density or O.D.) of a sample at 260 nm in a spectrophotometer.

One O.D. unit is the amount of a substance dissolved in 1.0 mL that gives an absorbance reading of 1.00 in a spectrophotometer with a 1-cm path length. The wavelength is assumed to be 260 nm unless stated otherwise. A<sub>260</sub> values can be converted into ng/μL using Beer's

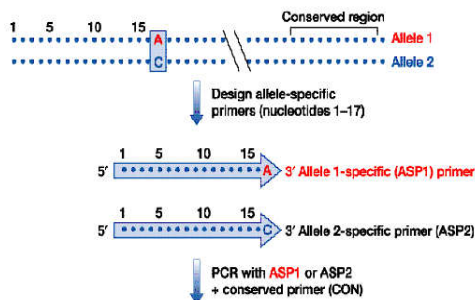
Law:

The following formulas are derived from Beer's Law (Ausubel et al., 1998): One A<sub>260</sub> unit of single-stranded DNA contains 33 ng/μL. One A<sub>260</sub> unit of double-stranded DNA contains 50 ng/μL.

Note Absorbance measurements of highly concentrated (O.D.>1.0) or very dilute (O.D.<0.05) DNA samples can be inaccurate.

DNA can also be quantitated by fluorometric analysis employing either Hoechst dye #33258 (Hoefer, Inc., 1993) or Picogreen (Molecular Probes, Inc., 1995).

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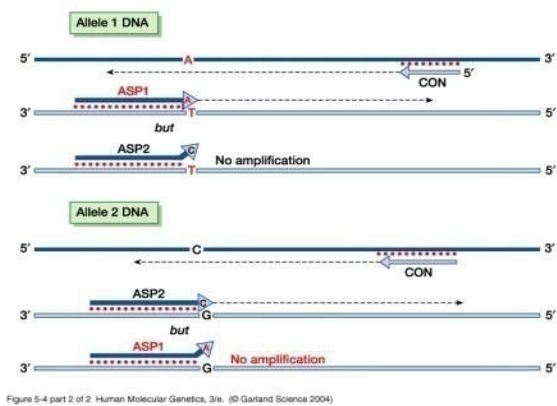


Figure 13: Amplification Refractory Mutation System (ARMS): Allele Specific PCR Amplification

## Counselling

When both partners carry the beta<sup>S</sup> gene, genetic counseling can offer the following alternatives: (a) abstinence from childbearing, (b) assumption of the risks, (c) pregnancy with the option of prenatal diagnosis, and (d) in recent years - [selection of a non-beta<sup>S</sup> embryo ex vivo](#) that is implanted into the mother's uterus. The importance of proper counseling cannot be overemphasized. Ideally, counselling should be non-directive, enabling the partners to understand the probabilities, limitations, and potential consequences of the options. The final decision rests with the couple at risk.



Figure 1: The Placenta and Fetus

## **Salting Method**

### **Reagents**

#### **Preparation of Cell lysis buffer**

PH-8

Material -2 litres conical flask, Magnetic stirrer

Method: ( Add to the measuring cylinder)

1. Add Tris HCL 1.57gram                      10mm/l
2. Add sucrose 110gram                      11% w/v
3. Add MgCL<sub>2</sub> 1.01gram                      5mm/L
4. Add Triton 10ml                      x -100(1%u/v)
5. Add 1000ml of dH<sub>2</sub>O  
5.5 placed on magnetic stirred till the solution dissolve
6. check the PH
7. Add 37% HCL to bring the PH to 8 at least (600-700) of HCL

#### **Preparation of Nucleic lysis buffer**

1. Tris HCL 0.785gram                      10mm/l
2. SDS 5gram                      1%w/v
3. Na<sub>2</sub>EDTA 1.875GRAM                      10mm/l
4. Add dH<sub>2</sub>O 500ml  
1.5 placed on magnetic stirred till you have a clear contain solution
2. measure the PH

### **PREPARATION OR NaCL**

1. To a conical flask add 17.5g of NaCL
2. Add dH<sub>2</sub>O 50ml
3. Shake vigorously and pour in sterile universal container and vortex  
NaCL company: merch : k39301104842 Germany.
4. Dispense in appropriate bottle



**Figure 3: Micro pipette**



**Figure 4: Chorionic villi in Eppendorf tube**

**Figure 5: Centrifuge**



**Figure 6: Vortex mixer**



**Figure 7: Sterilizing unit**



**Figure 8: DNA sample after Solution 1**



**Figure 9: Extracted DNA after Solution 2**

## Results

Table 1 summarizes the characteristics of the women. Ten pregnant women underwent chorionic villous sampling.

**Table 1: Characteristics of Women/Procedure**

<u>Patient</u>	<u>Gestational Age(Wks)</u>	<u>Sample Weight (mg)</u>	<u>Numberof Aspirations</u>
1	11	25	2
2	15	30	1
3	13	25	2
4	12	25	3
5	11	35	1
6	11	40	2
7	11	35	2
8	13	45	2
9	12	20	1
10	14	40	3

### **Gestational Age**

The lowest gestational age was 11 weeks and the highest was 15 weeks. The majority (80%) were within the recommended 11- 13 weeks of pregnancy.

### **Weight of Sample**

The minimum wet weight os villi aspirated was 20mg and the maximum was 45mg. The mean weight was 25mg.

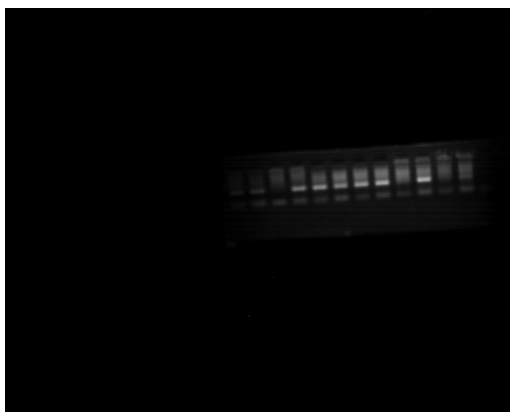
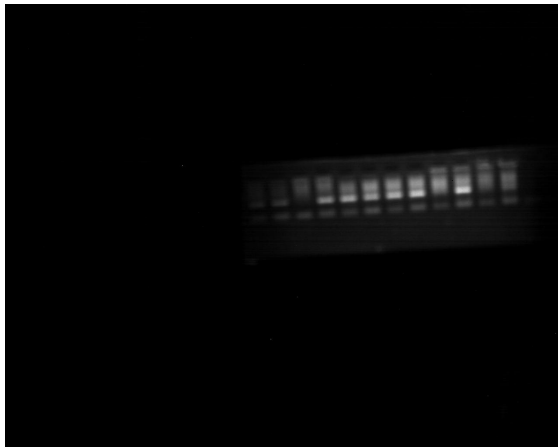
### **Number of Aspirations**

Thirty percent (3) of the women had only one needle aspiration to obtain adequate sample. Fifty percent (5) had twice aspiration and 20% (2) had three aspiration attempts. There was no statistical correlation between the number of aspirations and the weight of sample obtained.

There was no clear statistical relationship between the gestational age and the number of aspiration attempts.

Quality of the DNA from agarose gel electrophoresis showed clear and clean bands.

Figure 10 is a typical electrophoretic pattern of the DNA after completion of analysis. Pattern were those of AA, AS and SS.



### **DISCUSSION**



Ethical considerations

### **Contribution to knowledge**

The ASO ARMS technique now demonstrated to be a reliable method of PND of SCD using boiling method. It is relatively cheaper and free from the influence of maternal contamination

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## **Early Prenatal Molecular diagnosis of Sickel Cell Anaemia and association with Sex Ratio and common Aneuploidies**

## **Prenatal Molecular and Cytogenetic Diagnosis in Fetuses at risk of Sickel Cell Anaemia using Chorionic Villi**

## **Prenatal Molecular and Cytogenetic studies in Fetuses at risk of Sickel Cell Anaemia using Chorionic Villi**

## **Early Prenatal Molecular diagnosis of Sickel Cell Anaemia and association with Sex Ratio and common Aneuploidies**

### **Discussions**

The Y chromosome- in about 10% of Y, the long arm is either shorter or longer than usual. This trait is hereditary. Apparently the length of y has little significance on fertility, though in one study, long y seemed to be associated with an increased risk of abortion (R in Human genetics)

**(India)**Chromosomal analysis of aborted foetus is important for prognostication<sup>7</sup> because it gives information on frequency and type of chromosomal abnormality, on aetiology and risk assessment for future pregnancies<sup>3,8</sup>. Cytogenetic evaluation of chromosomally normal spontaneous abortions had given conflicting results about sex ratio<sup>3,9</sup>. However, most studies indicated greater number of female<sup>10-13</sup> particularly with alloimmune recurrent spontaneous abortion<sup>14,15</sup> and complete hydatidiform mole<sup>16</sup>. Most significant factor influencing the sex ratio is the presence of different frequency of contamination and overgrowth of tissue cultures by maternal cells<sup>3,17-20</sup>. In this study we tried to overcome difficulties of conventional cytogenetics *viz.*, selected successful cases, clonal selection, restriction to dividing cell populations and maternal cell contaminations by selecting chorionic tissue under microscope by single trained person and using molecular methods, in particular interphase fluorescent *in situ* hybridization (FISH), and attempted to find out true sex ratio and frequency and types of common aneuploidy through interphase FISH onto uncultured chorionic tissue.

(India)Ashutosh Halder & Ashish Fauzdar. Skewed sex ratio & low aneuploidy in recurrent early missed abortion. Indian J Med Res 124, July 2006, pp 41-50

### **Justification/Significance**

### **Aims and Objectives**

### **Significance**

**(India)***Background & objectives:* Conventional cytogenetic studies have revealed more number of females in spontaneous abortion and it has been assumed that a large proportion of those were resulted from maternal contamination and overgrowth

of maternal decidua in long term culture. In this study we have attempted to overcome difficulties of conventional cytogenetics by using meticulous tissue dissection and molecular methods onto uncultured chorionic villous tissue thus bypassing long term culture to find out true sex ratio and frequency as well as type of common aneuploidy in early missed abortions.

## **Conception**

Male: Female = 115:100

Males experience more of spontaneous abortions and still birth than females

Males also has higher neonatal and infant mortality

About 30 years

M:F = 50:50

At 65 years

M:F = 70:84

*Maleness seems to carry intrinsic risk.*

*Study of Amish families with and without long arm of Y chromosome*

*In families with: women died in mid-70's and men 5 or 6 years earlier*

*In families without: women died at ave. Age*

## **Aims/Objectives**

To demonstrate the application of polymerase chain reaction (PCR) using ASO – ARM technique in early prenatal diagnosis of SCA

To establish the haemoglobin genotype distribution among couples at risk of producing fetuses with SCA

To demonstrate the acceptability of prenatal diagnosis using PCR based technology

To determine the prevalence of procedure related abortion

To compare the influence of extraction method on the quantity and quality of extracted DNA using boiling and salting methods

To determine the minimum quantity of chorionic villi necessary to undertake molecular diagnosis

To establish the (true) primary sex ratio in SCA fetuses

To confirm if there is any association/correlation between haemoglobin genotype, primary sex ratio and pregnancy outcome

To characterize and determine the frequency of common aneuploidies in fetuses with SCA

## **Rationale**

RFLP is the first method used and was abandoned because of logistic and cost reasons

Procedure related abortion is around 1%. What is it in our environment?

Failure of amplification due to inadequate sample previously reported

Important to establish the best method that can be used in low resource settings

Multiple aspirations is an important contributor to abortions and other complications. The weight of sample correlates with no of aspirations and is important to limit aspiration to the no that will give a result

Males experience more spontaneous abortions, still births and neonatal death than females

Males seems to carry an intrinsic problem and survive less

## Sex distribution

HbS is transmitted as an autosomal codominant characteristic. The male-to-female ratio is 1:1. No sex predilection exists, since sickle cell anemia is not an X-linked disease.

Although no particular gender predilection has been shown in most series, analysis of the data from the US Renal Data System demonstrated marked male predominance of sickle cell nephropathy in affected patients. [\[13\]](#)

**Sex ratio** is the ratio of males to females in a population.

The primary sex ratio is the ratio at the time of conception, secondary sex ratio is the ratio at time of birth, and tertiary sex ratio is the ratio of mature organisms. In humans the secondary sex ratio is commonly assumed to be 105 boys to 100 girls (which sometimes is shortened to "a ratio of 105"). In human societies, however, sex ratios at birth or among infants may be considerably skewed by sex-selective abortion and infanticide (see [missing women](#)).

.....

The common aneuploidies that are found in liveborn could either be of autosomal or sex chromosomes abnormality. The common reported autosomal abnormalities include trisomy 21, 18 and 13. Although they also contribute significantly to spontaneous abortion, a reasonable proportion is also found in surviving fetuses. Down syndrome, named after Lagdon Down in 1866 (R) is the most common autosomal disorder, with a worldwide incidence of about 1/850 at birth. This figure is also influenced by maternal age, rising as the age of mother rises. It is however known that the majority of cases are in women below the traditional cut off age of 35 years (R). In Nigeria, Adeyokunu reported an incidence of 1 in 865, thereby signifying the importance of the condition in the community (R). The commonest mechanism for DS is non disjunction in meiosis (95%), occurring in maternal meiosis I in about 85% of cases and paternal meiosis II in 15% of cases. In another 4% of cases, it is due to translocation, which could be both reciprocal or Robertsonian (centric) involving the acrocentric chromosomes 13 - 15 (D group) and 21 (G group) t(14; 21). It follows unbalanced translocation. Mosaicism occurs in about 1% of cases (R). Other common aneuploidies include Trisomy 13 and 18. All these are also produced by similar mechanism as in DS. Trisomy 18 accounts for % of spontaneous abortions and found in 1 in X liveborn (R). Trisomy 18 contributes X to spontaneous abortion and has a prevalence of X among livebirth (R). Their incidences in fetuses with SCA have not been widely reported in literature.

Sex chromosomal abnormalities are usually of the X chromosome. The most prevalent is XO constitution, referred to as Turner syndrome. While it is the commonest contributor to spontaneous abortion from chromosomal disorder, survivors are also reported to be relatively stable after birth. More than half of patients are monosomic for X (45 XO) and are chromatin negative, while the others are due to structural abnormalities of X chromosome such as deletion and ring chromosome (ref 2,3 pg 189 of med genetics)

HbS is transmitted as an autosomal codominant characteristic. The male-to-female ratio is 1:1. No sex predilection exists, since sickle cell anemia is not an X-linked disease.

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"a ratio of 105"). In human societies, however, sex ratios at birth or among infants may be considerably skewed by sex-selective abortion and infanticide (see [missing women](#)).

**Table 2: The actual counts of Fetal Hemoglobin Genotypes and Alleles in the sample**

		HbAHbA	HbAHbS	HbSHbS	Total
<b>Number of Individuals</b>		366	123	12	501
<b>Number of Alleles:</b>	HbA	732	123	-	855
	HbS	-	123	24	147

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**Table 3 The observed and the expected genotypic and allelic frequencies**

	Alleles	Genotypes		
	HbAHbS	HbAHbA	HbAHbS	HbSHbS
<b>Observed</b>	0.853 0.147	0.731	0.246	0.024
<b>Expected</b>	0.845 0.155	0.714	0.262	0.024

**Table 4 Discrepancy between observed number of individuals in different genotypic classes and Hardy–Weinberg expectations**

	HbAHbA	HbAHbS	HbSHbS
<b>Observed</b>	366	123	12
<b>Expected</b>	364.5	125.6	10.8

$\chi^2=0.193$ ;  $P=0.908$

**Table 3: Fetal Haemoglobin Genotype result**

<b>Fetal Haemoglobin Genotype</b>	<b>Number</b>	<b>Percentage</b>
	N= 50	100.0
Haemoglobin genotype AA	16	30.0
Haemoglobin genotype AS	25	50.0
Haemoglobin genotype SS	9	20.0

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