
**“UTILITY OF UMBILICAL CORD BLOOD RED BLOOD CELL INDICES
MEAN CORPUSCULAR VOLUME IN SCREENING FOR ALPHA
THALASSEMIA”**

**BY
(REG NO. BM0121008)**

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
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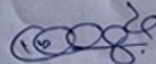
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LIST OF ABBREVIATIONS

Hb	Hemoglobin
MCV	Mean corpuscular volume
RBC	Red blood cells
β LCR	Beta-globin gene cluster
HbF	foetal hemoglobin
TDT	transfusion-dependent thalassemia
NTDT	non-transfusion-dependent thalassemia
HPLC	high performance liquid chromatography
IDA	Iron Deficiency Anemia
RDW	Red cell distribution width
CBC	Complete blood count
MCH	Mean corpuscular hemoglobin
PCV	Packed cell volume
CE-HPLC	Capillary Electrophoresis-High Performance Liquid Chromatography
PCR	polymerase chain reaction
MLPA	multiplex ligation-dependent probe amplification
RPO	right probe oligonucleotides
LPO	Left probe oligonucleotides
UCB	Umbilical Cord Blood
NVD	Normal vaginal delivery
LSCS	Lower segment caesarean section

ABSTRACT

TITLE--UTILITY OF UMBILICAL CORD BLOOD RED BLOOD CELL INDICES MEAN CORPUSCULAR VOLUME IN SCREENING FOR ALPHA THALASSEMIA

BACKGROUND:

Thalassemia's represent a group of inherited hematologic disorders where abnormal hemoglobin production occurs due to autosomal recessive inheritance. The condition is characterized by an imbalance between alpha and non-alpha globin chains, resulting in inefficient erythropoiesis and peripheral hemolysis, ultimately leading to chronic hemolytic anemia. The alpha thalassemia gene [HbA] is located on chromosome 16, with two copies present on each allele. It is thought that carriers of hemoglobinopathies are somewhat protected in a malarial environment, as alpha thalassemia is common in tropical and subtropical regions of the world where malaria is still an epidemic. The goal of newborn hemoglobinopathy screening is to identify thalassemia syndromes and Hb variations that have a substantial negative influence on health. Studies on decreased MCV in cord blood are scarce. Few research from other nations have examined the effectiveness of MCV in alpha thalassemia screening. Therefore, we intended to conduct a screening study for alpha thalassemia identification in newborns based on the MCV value of cord blood and by subjecting the blood samples showing microcytosis to genetic studies for confirmation of alpha thalassemia thus predicting the utility of MCV in screening of alpha thalassemia.

METHODOLOGY:

This was a longitudinal study done on 1000 newborns delivered at a tertiary care centre over a period of one year to find the usefulness of cord blood for screening alpha thalassemia. The cord blood collected was analysed for complete hemogram to

look for red cell indices(MCV, MCH). MCV<95 and/or MCH<30 were subjected for alpha thalassemia gene deletion study by MLPA [Multiplex Ligation Dependent Probe Amplification]. Neonates with negative MLPA were followed up at the age of 6months for repeat complete hemogram, High Performance Liquid Chromatography [HPLC] was done to look for other variants of haemoglobinopathies. Those with negative MLPA and negative HPLC were evaluated for other causes of microcytosis by serum iron studies and bone marrow iron staining accordingly.

RESULTS

Among the study participants, 52.6% (n=526) of the delivery were cesarean section. Majority of the study participant's gestation age was 38 weeks (n=377, 37.9%). Mean birth weight of the newborns was 2.75 ± 0.31 Kg. The minimum and maximum weight of the newborns were 1.5 and 4 Kg respectively. Among the study participants, prevalence of mean corpuscular volume of 95 and below was 3.3%. The overall prevalence of α -thalassemia among the study participants (n=1000) was 1.3%.

CONCLUSION:

Cord blood was found to be useful in screening for α -thalassemia in the present study. Prevalence of microcytosis in the study was 3.3%. Prevalence of α -thalassemia, β -thalassemia trait and $\delta\beta$ -thalassemia trait in our study were 1.3%, 0.1% and 0.1% respectively. Maternal factor fetomaternal haemorrhage was significantly associated with microcytosis. All RBC indices were significantly associated with mode of delivery.

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INTRODUCTION

Thalassemia's are a group of inherited autosomal recessive hematologic diseases characterized with abnormal hemoglobin production¹. They are named from the Greek word "thalassa," which means sea, reflecting their prevalence in populations around the Mediterranean Sea. Thalassemia's are the most common monogenetic disorder of haemoglobin². The ensuing imbalance in the alpha/non-alpha-globin chain, which causes inefficient erythropoiesis and peripheral hemolysis, ultimately leading to chronic hemolytic anemia, is the defining feature of the condition. Depending upon the type of affected globin chain, they are classified into alpha thalassemia or beta thalassemia. Alpha thalassemia gene [Hb A] is present on chromosome 16 with 2 genes present on each allele. Accordingly, four different clinical phenotypes of alpha thalassemia are possible namely; silent carrier-1 gene deleted[-a aa], alpha thalassemia carrier- 2 genes deleted.[-a,-a] or --aa, Hb H disease when 3 genes deleted[--,-a] and Hb Barts /hydrops foetalis[-- --] when all 4 genes deleted.

As against beta thalassemia, there are only few studies of prevalence and molecular characteristics of alpha thalassemia. Prevalence in India varies from 1 to 80% depending on geographical area and ethnicity. From tribal area and in patients with sickle cell anemia, high prevalence of alpha thalassemia has been reported. There are no studies about prevalence of alpha thalassemia from North Karnataka. Alpha thalassemia is prevalent in tropical and subtropical regions of globe where malaria is still epidemic and is believed that carriers of hemoglobinopathies are relatively protected in malarial environment^{3,4}.

It is currently unknown how much thalassemia will ultimately cost the economy and healthcare systems, but it is known to grow in high prevalence nations where more patients are survive and leading longer life, and also in nations in which immigration and demographic shifts will cause the prevalence to rise⁵

Screening of new born for haemoglobinopathies aims to detect thalassemia syndromes and Hb variants with significant impact on health^{6,7}. It could allow prevention of complications, early management and genetic counselling. Alpha thalassemia silent carrier or carrier state usually don't have any significant clinical manifestations. However, HB H disease children can have anemia, splenomegaly and complications of ineffective. They may develop acute hemolysis necessitating blood transfusion when they have infections. Early identification during the new born period will facilitate proper parental education, prevent complications, unnecessary investigations or iron treatment.

Neonatal screening for alpha thalassemia has been described by estimation of Hb Barts using isoelectric focusing method. However, availability of this method makes it difficult for universal screening of newborns. Alternatively, targeted screening of newborn with lower MCV is an attractive option. There are limited number of studies of low MCV in the cord blood. There are few studies from other countries looking at utility for MCV in screening for alpha thalassemia. Hence, we planned to undertake the study to find the role of cord blood MCV in detection of alpha thalassemia.

AIM AND OBJECTIVES

AIM: Utility of umbilical cord blood red blood cell indices mean corpuscular volume in screening for alpha thalassemia

PRIMARY OBJECTIVE

To determine the usefulness of cord blood MCV in screening for alpha thalassemia

SECONDARY OBJECTIVE

1. To estimate the prevalence of alpha thalassemia in newborns delivered at KLE'S Dr Prabhakar Kore Hospital
2. To determine other causes of microcytosis in cord blood.

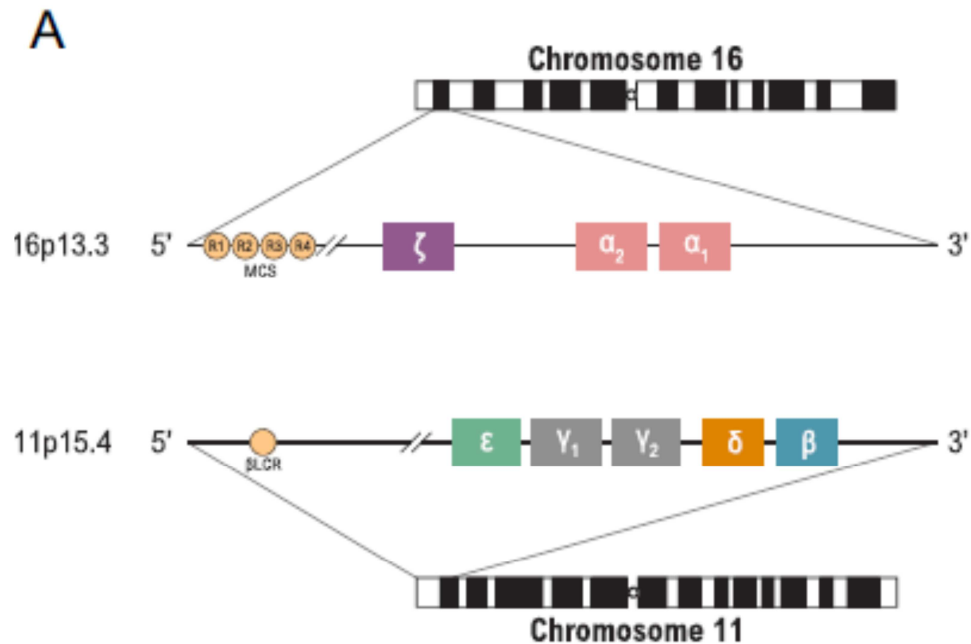
REVIEW OF LITERATURE

Red blood cells (RBCs) are biconcave disk-shaped enucleated cells. A hemoglobin moiety contains 4 globin polypeptide chains of globin protein (two alpha and two beta), The heme molecule, which binds to oxygen, is present in all of them. Red blood cells and hemoglobin molecules cannot operate properly without the stability and equilibrium of their globin chains. Hemolytic anemia occurs when the genes encoding these globin chains (HBB for beta-globins and HBA for alpha-globins) have or develop abnormalities (pathogenic variations) that alter the amount or structure of globins.

Molecular genetics of normal hemoglobin

Two gene clusters are involved in the regulation of human hemoglobin synthesis. An embryonic ζ gene and two fetal/adult α genes are found in the first cluster, which is located on chromosome 16. Situated on chromosome 11, the second cluster consists of the adult δ and β genes, each represented once, as well as an embryonic ϵ gene and two embryonic/fetal γ genes⁸

Fig.1: Molecular genetic pattern of normal hemoglobin



The alpha-globin gene cluster is located on chromosome 16 at position 16p13.3, while the beta-globin gene cluster is positioned on chromosome 11 at location 11p15.4. Within the alpha-globin gene cluster, four well-preserved enhancer elements, called MCS-R1 to MCS-R4, regulate the genes encoding ζ -globin, α_2 -globin, and α_1 -globin. The locus control region (β LCR), on the other hand, is a specific enhancer group that controls the genes that produce ϵ -globin, γ_1 -globin, γ_2 -globin, δ -globin, and β -globin within the beta-globin gene cluster.

The genes that encode embryonic ζ -globin (from the alpha-globin locus) and ϵ -globin (from the beta-globin locus) are activated and expressed during primitive erythropoiesis in the yolk sac during early fetal development.

This expression continues until around 8 weeks of gestation, after which these genes are turned off, leading to a switch in gene expression to α - and γ -globin during foetal life.

There is a change in γ -globin expression to β -globin at birth. Initially, during the stage of yolk sac development, embryonic hemoglobin forms, such as $\zeta_2\gamma_2$ (known as HbPortland), $\zeta_2\varepsilon_2$ (known as HbGower 1), and $\alpha_2\varepsilon_2$ (known as HbGower 2). As development progresses, the production of ζ - and ε -globin chains stops, leading to the formation of foetal hemoglobin ($\alpha_2\gamma_2$, known as HbF).

Towards the end of foetal development and shortly after birth, there is a gradual decrease in γ -globin production and an increase in β -globin production. Eventually, β - and δ -globin production replace γ -globin production, leading to the production of adult hemoglobin ($\alpha_2\beta_2$, known as HbA1, and $\alpha_2\delta_2$, known as HbA2).

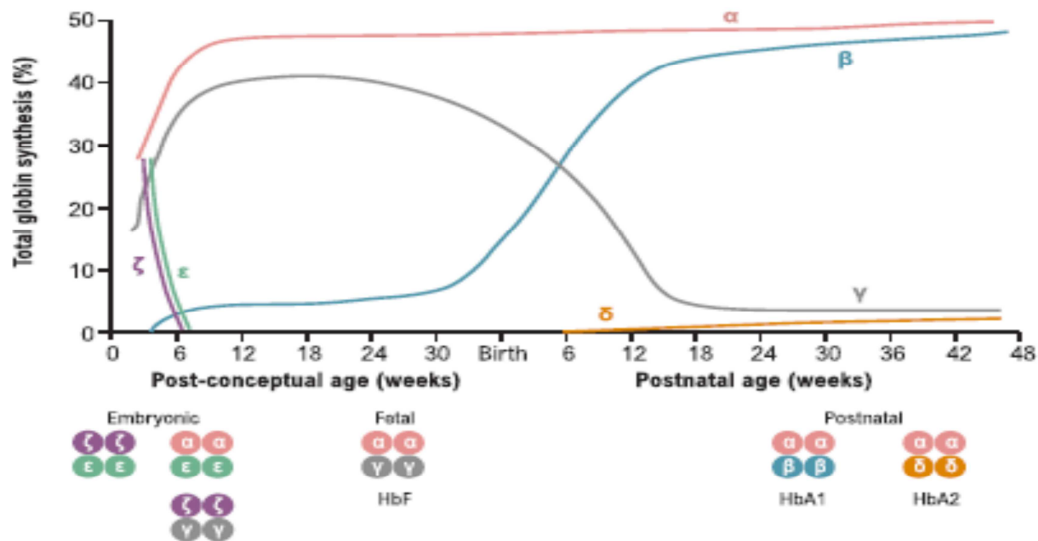


Fig.2: Journey of molecular genetics of hemoglobin

The tetrameric protein known as hemoglobin is made up of two homodimers of globin subunits, each of which is made up of a globin chain conjugated with a heme moiety that has a liganded iron atom in the center and binds one oxygen molecule⁹. As an embryo develops into a foetus and then an adult, the expression of human globin genes changes¹⁰. As a result, the composition of Hb tetramers also varies: from foetal Hb F to adult HbA1 and $\alpha 2\delta 2$ (HbA2), and from embryonic hemoglobins $\zeta 2\eta 2$ (Hb Portland), $\zeta 2\varepsilon 2$ (Hb Gower 1), and $\alpha 2\varepsilon 2$ (Hb Gower 2) to $\alpha 2\eta 2$ (Hb F) foetal Hb. Normal adult proportions of Hb types are typically 95% to 98% HbA1 ($\alpha 2\beta 2$), 2% to 3% HbA2 ($\alpha 2\delta 2$), and less than 2% Hb F ($\alpha 2\gamma 2$)

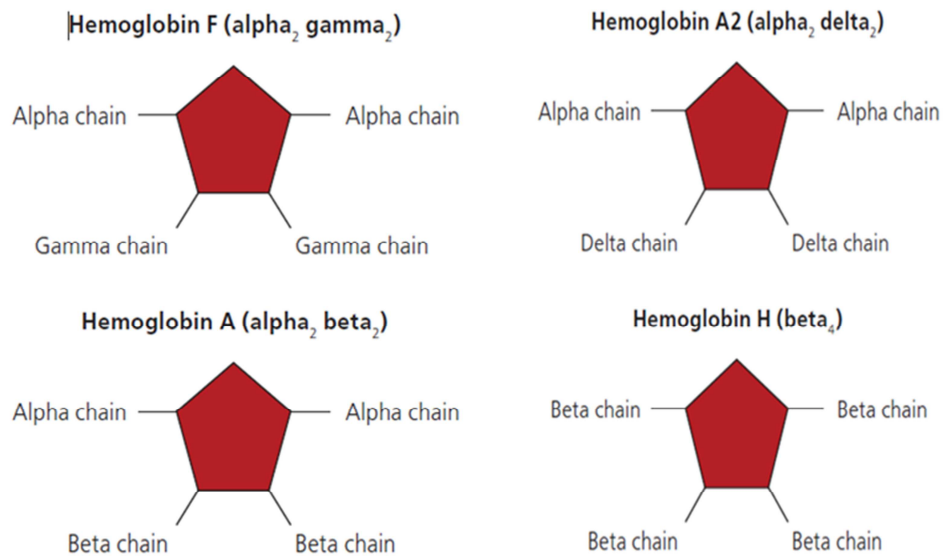


Fig.3: Types of Hemoglobins

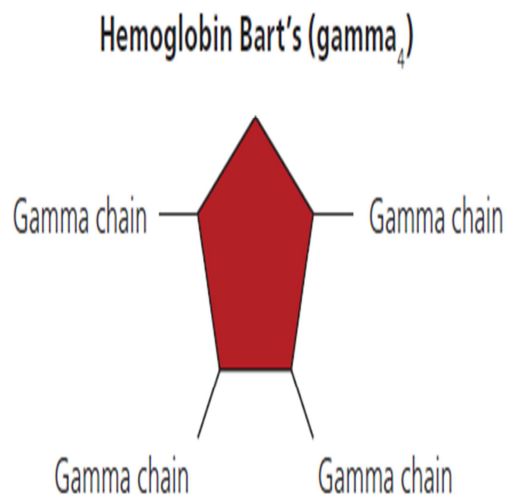


Fig.3: Types of Hemoglobins

A dentist by the name of Cooley noticed in 1925 that newborns were showing signs of iron insufficiency, which included enlarged spleens (splenomegaly) and malformed bones.

Seven years later, in the year 1932, Whipple & Bradford provided the first explanation for the disease pathology. They noted that most patients affected by this condition were from the Mediterranean region. Because of this, they named the condition "thalassemia."

History

Hemoglobinopathies are hereditary hemoglobin abnormalities caused by deletions or mutations in the alpha- or beta-globin genes. They fall into two primary categories: hemoglobin structural variations and thalassemias. Deficits in the synthesis of hemoglobin chains cause thalassemias, which are classified as beta or alpha thalassemias based on whether the globin chain in question is beta or alpha. Reduced synthesis of either the alpha or beta chains of hemoglobin (Hb) characterizes

a broad category of hereditary illnesses known as thalassemias. These disorders result from genetic deletions or mutations that impact important gene regions. The inability of RBCs to carry enough oxygen results in anemia, when there is impaired production of one or both of these two proteins

All genetic disorders related to hemoglobin are collectively known as "hemoglobinopathies," which can be categorized into two main groups. While hemoglobin structural variations include aberrant hemoglobin proteins, thalassemia disorders cause severe anemia and associated problems. These disorders result from changes to the α - or β -globin genes, such as mutations or deletions. The globin genes that cause thalassemia are faulty. These abnormalities interfere with the normal synthesis of hemoglobin (Hb), causing either an excessive or insufficient amount to be produced. The general structure of hemoglobin does not alter in spite of these modifications. Unusual Hb variations cannot arise without structural changes in hemoglobin. These illnesses manifest in many combinations, such as sickle cell disease (HbS), β^0/β^+ -thalassemias, and Hb E/ α - thalassemia¹¹

In 2012, a new classification system for thalassemia (TDT and NTDT) was proposed and subsequently adopted by the Thalassemia International Federation in their updated guidelines and publications. This classification distinguishes patients as either transfusion-dependent thalassemia (TDT) or non-transfusion-dependent thalassemia (NTDT)¹². Differentiating between these categories requires a thorough clinical evaluation using various clinical and hematological parameters, with particular attention to baseline hemoglobin levels.

Thalassemia is an autosomal recessive disorder, which means both parents must be affected with the disease or carriers of disease. **Alpha thalassemia** occurs due to

deletion of alpha-globin gene, which causes decreased or complete lack of production of alpha-globin chains. Alpha thalassemia is characterized by two phenotypes: alpha-thalassemia I, or mild, and alpha-thalassemia II. Typically, thalassemia-related clinical signs are absent in people with alpha thalassemia II. Now that alpha-thalassemia II is known to cause a decrease in alpha globin expression, alpha-thalassemia I, or minor, is recognized by the total lack of alpha globin proteins. Alpha-thalassemia alpha zero (α^0) and alpha-thalassemia alpha plus (α^+) are the names given to these two variations of the disease.

The absence of any alpha-globin gene sets is the defining feature of alpha(0) thalassemia. This deficiency prevents the synthesis of normal alpha-globin, resulting in the inability to generate any A, F, or A2 hemoglobin that is normally functioning. Individuals with this deficiency may develop a condition known as hydrops foetalis or "hemoglobin Bart," where Most babies with this illness die at birth or shortly after birth. On the other hand, alpha (+) thalassemia involves around fifteen reported genetic mutations. Due to the functional loss of at least one alpha-globin gene, these mutations result in a limited production of alpha-globin proteins.

Alpha Thalassemia	α genes	Globin Chains	Hemoglobin	Anemia
Normal	$\alpha\alpha/\alpha\alpha$	$\alpha^2\beta^2$	A	None
Silent Carrier	$\alpha\alpha/\alpha-$	$\alpha^2\beta^2$	A	None
Trait	$\alpha-/ \alpha-$ OR $--/\alpha\alpha$	$\alpha^2\beta^2$	A	Mild
Hb H disease	$--/\alpha$	$\alpha^2\beta^2,\beta^4$	A, H	Intermediate
Hydrops fetalis	$--/--$	$\gamma^4,$ $\zeta^2\gamma^2,$	Barts Portland	Lethal

Fig.4: Types of Alpha thalassemia

Alpha-thalassemia ($-\alpha/\alpha$) occurs when an individual inherits a combination resulting in three functional alpha-genes out of a possible four. These individuals are called asymptomatic carriers for alpha-thalassemia. Different names for this condition include "alpha thalassemia minima," "alpha thalassemia-2 trait," & "heterozygosity for alpha plus thalassemia minor." These carriers are usually healthy or might have milder anemia. This disorder is commonly referred to as a "silent carrier" due to its difficulty in being identified using conventional hematological tests. This condition could only be identified by DNA analysis (Camacho et al., 1999). It does not require therapy because of a little alpha protein shortage; as a result, the hemoglobin level seems normal (Leung and Lao, 2012).

Alpha thalassemia trait

When two healthy alpha genes are inherited, one from each of the two chromosomes ($-\alpha/-\alpha$), the condition known as thalassemia ($-\alpha/-\alpha$) is referred to as a trans deletion. However, homozygosity for alpha (+) thalassemia ($\alpha\alpha/--$), which results in two on the same chromosome, is referred to as a "cis deletion" and is associated with "alpha thalassemia minor" or "alpha thalassemia-1 trait." Each time a parent carries the cis deletion, one in four (25%) of the offspring may be born with alpha thalassemia major. People with alpha thalassemia trait have lower RBCs than normal and are moderately anemic, but they do not exhibit any symptoms and may only be identified by routine testing (Leung and Lao, 2012).

Hemoglobin H/ Alpha thalassemia intermedia:

In individuals inheriting only one healthy alpha gene ($-\alpha/--$), Hemoglobin H (Hb H) is produced abundantly from tetramers of excess beta chains, a condition referred to as

"Hb H disease." People with 3 alpha globine gene deletions experience severe anemia and typically cannot survive without blood transfusions. Alpha thalassemia intermedia newborns appear normal at birth, but as they get closer to their second year of life, they generally have anemia and splenomegaly. Although hepatomegaly is not frequently observed, there may be a connection between the condition and mental retardation in those who have it. This kind of anemia raises the risk of developing respiratory tract infections, leg and foot ulcers, gall stones as hemolysis takes place. Hemoglobin H illness typically does not present with bone abnormalities (Lee et al., 2010). The usual discrepancy in alpha and beta chain synthesis leads to the aggregation of beta chains within red blood cells.

Normally, beta chains are exclusively associated with alpha chains. When three genes are deleted from an alpha thalassemia, the beta chains build up into groups of four, resulting in a unique hemoglobin known as "hemoglobin H." The result of this illness is "hemoglobin H disease." This hemoglobin type has two problems. Firstly, the cell receives oxygen from it inefficiently, rendering it nearly ineffective. The damage caused by hemoglobin H protein to red blood cell membranes speeds up the process of cell death. The severe and life-threatening anemia in hemoglobin H disease results from reduced alpha chain synthesis and red blood cell breakdown. Without medical intervention, the majority of affected individuals succumb in teens or earlier (Camacho et al.).

All individuals with HbH disease experience varying degrees of anemia. However, When it comes to clinical symptoms, non-deletional HbH carriers typically have more severe manifestations than deletional carriers. Many patients who were diagnosed with deletional HbH were found after hemolysis episodes brought on by

illness or inflammation, or by routine complete blood counts. Acute hemolytic episodes leading to jaundice or asymptomatic gallstones are uncommon

Effects of α -Globin Genotypes on Severity of Hb H

The clinical characteristics of patients with deletional Hb H are typically consistent, whereas those with non-deletional Hb H show significant variability in their clinical presentation and disease progression¹³. A notable example is seen in patients with HbH-HbCS. Despite sharing the same alpha-globin genotype, some individuals never need medical intervention and are only diagnosed incidentally during investigations for other health concerns. In contrast, a few patients may experience severe fetal anemia, leading to hydropic features before birth. Individuals with less common non-deletional Hb H genotypes, such as HbH-HbQS¹⁴ and HbH-HbAdana¹⁵, were more likely to exhibit the clinical condition described as "Hb H hydrops fetalis" than individuals with the more common HbH-HbCS genotype. Transfusion needs also varied among individuals with HbH-HbCS and HbH-HbPS.

Alpha thalassemia major/ Hemoglobin Bart's disease

Hemoglobin Bart's disease, a life-threatening condition that occurs when all four alpha genes are lost. During fetal development inside the womb, four gamma-globin chains are produced, which combine to form an abnormal type of hemoglobin called "hemoglobin Bart's." According to Lee et al. (2010), a significant number of people afflicted with hemoglobin Bart's disease do not have a chance of survival, and those who do usually die soon after delivery. Some of these kids reportedly had been spared thanks to pregnancy-related blood transfusions (Camacho et al., 1999).

Alpha-thalassemia major manifests clinically as intrauterine anemia, marked hepatosplenomegaly (liver and spleen enlargement), cardiovascular malformations frequently associated with heart failure symptoms, skeletal abnormalities, delayed brain growth, and oedema (fluid retention). Oedema can cause the foetus and placenta to swell, which raises the possibility of intrauterine mortality, which usually happens between 23 and 38 weeks of gestation.

Although this illness is usually fatal, in certain situations survival has been made possible by intrauterine transfusion of the foetus and/or rapid postnatal transfusion. Irreversible anomalies, however, can still happen despite intense, lifelong blood transfusions and supportive treatment. Selective abortion counselling and conversations are necessary due to the serious complications for both the foetus and the obstetric problems¹⁶.

The impact of simultaneous inheritance of β -thalassemia.

Due to its high prevalence in Southeast Asia, Hb E is found in up to 50% of individuals in certain parts of Thailand¹⁷, often interacting with Hb H disease. It is known as AE Bart's illness when hemoglobin H (Hb H) is inherited in combination with heterozygous hemoglobin E (Hb E), and EF Bart's disease when hemoglobin H (Hb H) is inherited in combination with homozygous HbE. AE Bart's and EF Bart's syndromes share similar alpha-globin genotypes and clinical signs similar to hemoglobin illness. Nevertheless, compared to pure Hb H illness, there is a lower incidence of infection or inflammation-induced hemolysis. While homozygous beta-thalassemia heterozygosity alone usually results in less severe anemia than co-inheritance of Hb H¹⁸, it usually causes more severe anemia.

Genetic Modifiers of Hb H

Predicting the clinical severity of Hb H disease after diagnosis remains challenging, particularly among individuals with non-deletional genotypes. Beyond the genetic factors investigated for β thalassemia, there is ongoing research in the scientific community to determine whether other genetic factors influence the varying severity of the condition.¹⁹ ATRX (α -thalassemia/mental retardation syndrome X-linked) and AHSP (α -hemoglobin stabilizing protein)²⁰ are two additional possible disease modifiers in addition to the co-inheritance of Hb E and β -thalassemia heterozygosity. Mutations of the ATRX gene located at chromosome Xq13.3 lead to a very rare condition known as ATRX syndrome. Numerous genes, including those encoding the α - and β -globin chains, are significantly regulated by the ATRX protein. Despite possessing four normal α -globin genes, males with ATRX syndrome have a shortfall in α -globin chains and Hb H production, which is similar to the pattern observed in classical Hb H illness.

EPIDEMIOLOGY

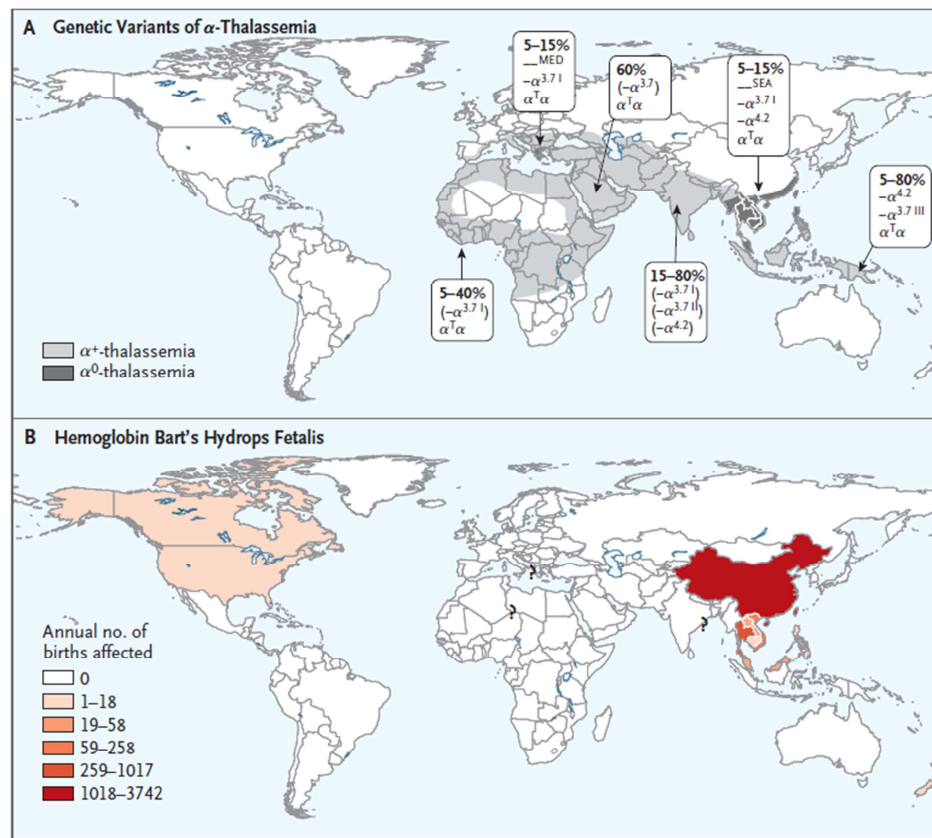
Alpha-thalassemia mutations are present in nearly every population on the planet. They are highly prevalent in Asian individuals. Consanguineous marriages, or unions between closely related persons, account for a sizable fraction of weddings in various societies throughout North Africa, the Middle East, and West Asia, ranging from 20% to over 50%. This is one of the reasons why alpha-thalassemia is so common in certain areas.

Despite the paucity of data available for Southeast Asia, recent systematic reviews have estimated prevalence rates of alpha-thalassemia, encompassing all forms

of mutations, ranging from 17.3% in Malaysia to 51.5% in Vietnam. In general, 22.6% of the Asian countries under analysis had this incidence.

Carrier frequencies have been measured in surveys done throughout the Mediterranean region; they range from 3% to 4% in Turkey and Italy to as high as 60% in Eastern Saudi Arabia. certain African countries like Nigeria and Kenya, nearly half of the population has been observed to carry an alpha-thalassemia mutation. In parts of India, Papua New Guinea, and Nepal, carrier rates are extremely high, nearing fixation at 80% to 90%.

A recent global systematic literature review provided prevalence rates for clinically significant forms of alpha-thalassemia, such as Hb H disease and Hb Barts hydrops fetalis



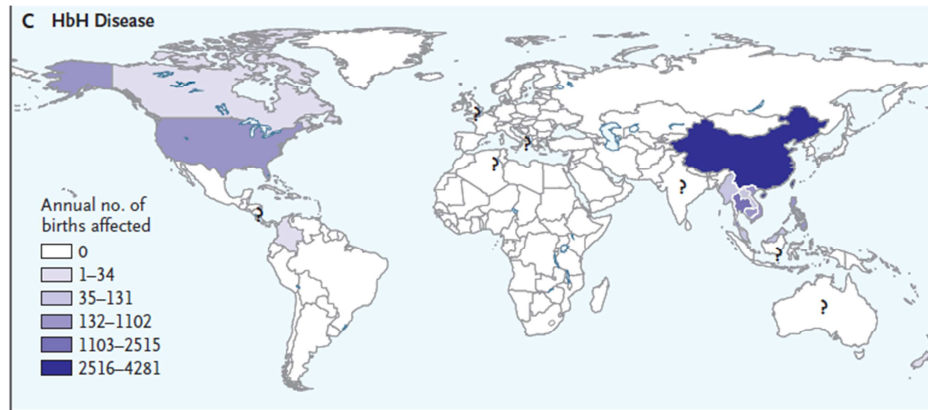


Fig.5:Genetic variants of alpha thalassemia in different parts of globe

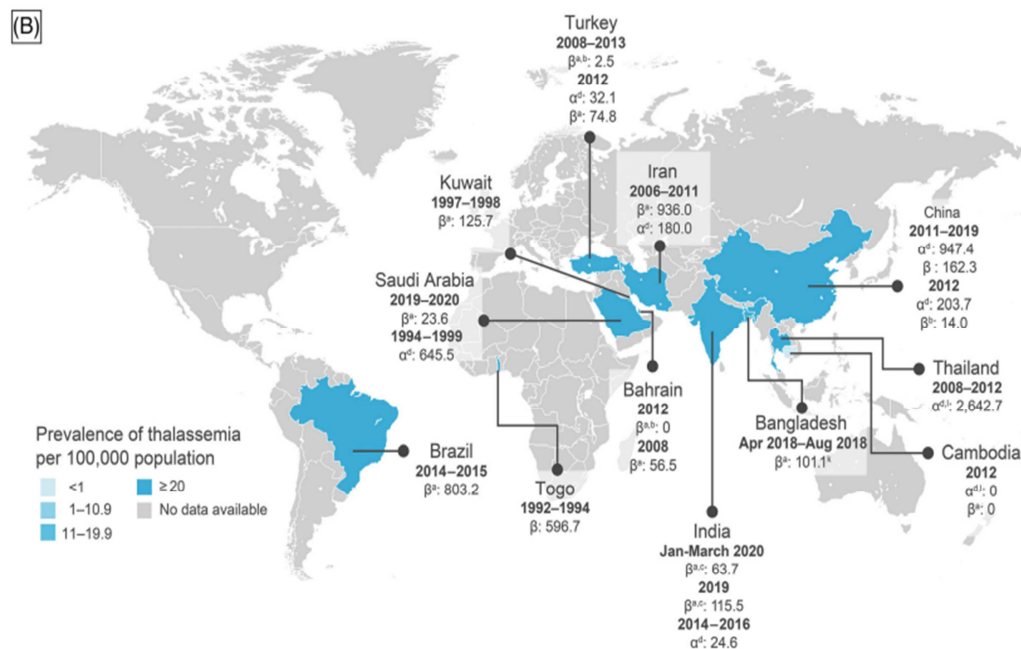


Fig.6:Prevalence of thalassemia

Syndrome. These rates varied widely, ranging from 0.03 cases per 100,000 people in Spain (2014-2017) to 4.5 cases per 100,000 people in Malaysia (2007-2018). In the United States, the prevalence ranged from 0.04 cases per 100,000 people (2001-2004) when considering both the US and Canada, to 0.6 cases per 100,000 people in the US alone during the period 2004-2008.

In recent decades, significant global population movements have occurred, resulting in the spread of alpha-thalassemia to many other parts of the world, including northern Europe and the Americas. This has led to an increased prevalence of alpha-thalassemia in these regions(2004–2008).

According to numerous studies (Lau et al. 1997; Li et al. 2006; Michlitsch et al. 2009; Vichinsky 2009, 2012; Lal et al. 2011), up to 40% of people live in the regions of China and Southeast Asia. According to Weatherall and Clegg (2001), there is also a high incidence in the Mediterranean and Middle Eastern regions. The regional distribution of alpha thalassemia has changed significantly due to immigration patterns (Michlitsch et al. 2009). Once uncommon in North America, it is now posing a serious threat to public health.

The demographic pattern of alpha-thalassemia undergoes changes due to various factors such as population aging, advancements in medical care, and shifts in causes of mortality. Improvements in survival rates, awareness programs, and screening technologies, coupled with reduced pregnancy termination rates due to available treatment options, may have contributed to increases in prevalence after the year 2000.

Conversely, increased premarital screening and prenatal thalassemia detection programs have led to a decrease in prevalence in some parts of the world. These programs help identify carriers of alpha-thalassemia before marriage or pregnancy, thus reducing the likelihood of passing on the condition to offspring.

Pathophysiology

Alpha thalassaemia occurs due to deletions of alpha-globin genes, and β thalassaemia occurs due to point mutation in splice site and promoter regions of beta-globin gene on chromosome²¹. Alpha thalassaemia and beta-thalassaemia differ in their underlying mechanisms. In alpha thalassaemia, the shortage of alpha chains leads to an excess production of gamma or beta chains. These surplus chains form Hb Bart's and Hb H, respectively. Unlike beta-thalassaemia, these soluble tetramers do not accumulate in the bone marrow, making erythropoiesis more efficient. Hb H, however, is erratic and builds up in red blood cells over time. This results in inclusion bodies that are confined in the spleen and other microcirculation organs, decreasing the survival rate of red blood cells. Because of their lack of alpha chains, Hb Bart's and Hb H both show an extremely high affinity for oxygen.

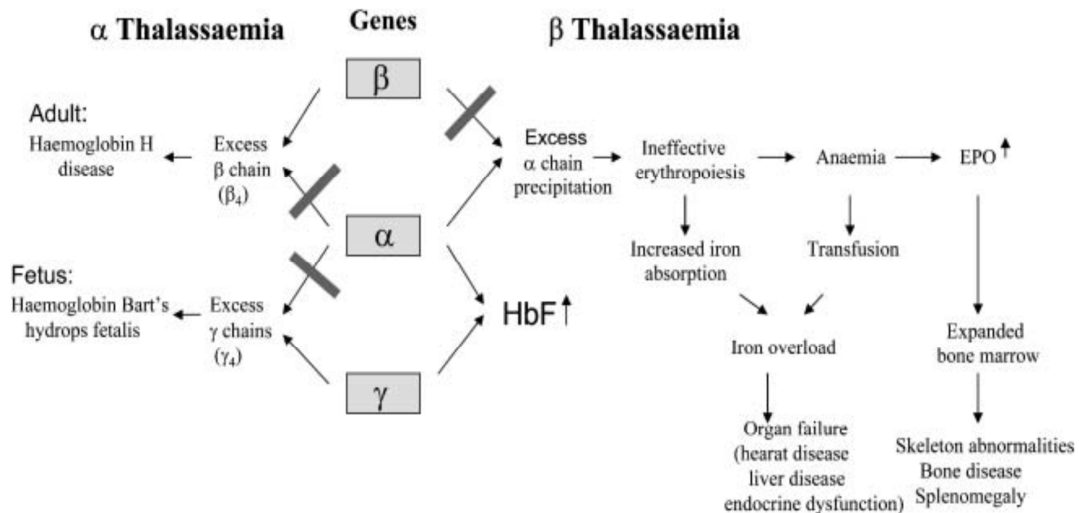


Fig.7:Pathophysiology of thalassaemia

Table 1: Types of hemoglobinopathy and number of chains

<i>Types of hemoglobinopathy</i>	<i>No. of alpha and beta chains present</i>
HemoglobinD-Punjab – ($\alpha 2\beta D 2$):	globin polypeptide chains consist of one delta, two betas, and one alpha.
HemoglobinH - ($\beta 4$)	Different forms of alpha thalassemia may also contain four beta chains.
Hemoglobin Barts – ($\gamma 4$)	α thalassemia variants may also have four γ chains.
Hemoglobin S – ($\alpha 2\beta S 2$)	noticed in people with sickle cell anemia. When the β -chain gene experiences a point mutation, the properties of hemoglobin are altered.
Hemoglobin SC disease	Sickle red blood cells are caused by a complicated heterozygous hemoglobin whose other gene codes for hemoglobin C.
Hemoglobin E ($\alpha 2\beta E 2$)	A mild chronic red cell hemolysis is the cause of anemia in people with a hemoglobin variation carrying a mutant β -chain gene.
Hemoglobin C ($\alpha 2\beta C 2$)	Mild chronic hemolytic anemia is caused by a mutation in the β -chain gene sequence.
Hemoglobin (AS)	typified by sickle cell trait and arises from a hybrid gene made up of an adult and an allele specific to sickle cell illness.

α -Thalassemia heritability

α -thalassemia has a complicated genetic makeup. Two α -globin genes are inherited by each individual from each parent. Offspring of parents with one or more

missing α -globin genes are susceptible to α -thalassemia trait, hemoglobin related diseases, or hemoglobin related Barts hydrops fetalis syndrome. The specific risk is contingent upon the quantity of deleted or inactivated genes, the α -globin gene(s) ($\alpha 2$ or $\alpha 1$) that is(are) impacted, and whether the deleted or inactivated genes are located on one or both chromosomes²². For instance, compared to people with $-\alpha / \alpha$ (homozygous $\alpha +$ thalassemia), those with $/\alpha \alpha$ ($\alpha 0$ -thalassemia) are more likely to have children who are seriously affected. Molecular diagnosis is therefore a crucial factor in reproductive counselling for both $\alpha +$ - and $\alpha 0$ -thalassemia individuals.

Fig.8: Classification of alpha thalassemia

Genotype		Phenotype	Clinical Presentation		
$-\alpha/\alpha\alpha$; $\alpha^{-}/\alpha\alpha$; $\alpha^{\dagger}\alpha/\alpha\alpha$; $\alpha\alpha^{\dagger}/\alpha\alpha$ (heterozygous α^{-} -thalassemia)	Carrier States	Silent carrier or α -thalassemia minima	<ul style="list-style-type: none"> Normal or mild decrease in MCH/MCV 	Transfusions rarely required	
$--/\alpha\alpha$; $\alpha^{\dagger}\alpha^{\dagger}/\alpha\alpha$; $-\alpha^{\dagger}/\alpha\alpha$; $\alpha^{\dagger}-/\alpha\alpha$ (α^{\dagger} -thalassemia)		α -thalassemia trait or α -thalassemia minor	<ul style="list-style-type: none"> Normal or borderline anemia RBC microcytic and hypochromic 		Occasional transfusions required
$-\alpha/-\alpha$ or α^{-}/α^{-} $\alpha^{\dagger}\alpha^{\dagger}/\alpha^{\dagger}\alpha^{\dagger}$ or $\alpha\alpha^{\dagger}/\alpha\alpha^{\dagger}$ $-\alpha/\alpha^{\dagger}\alpha$ or $\alpha^{-}/\alpha\alpha^{\dagger}$ (homozygous α^{-} -thalassemia)					
$--/-\alpha$ or $--/\alpha^{-}$ (deletional)	Clinically Relevant Forms	HbH disease (α -thalassemia intermedia)	<ul style="list-style-type: none"> Clinical severity is variable Mild to moderate anemia RBC markedly microcytic and hypochromic 	NTDT	
$--/\alpha^{\dagger}\alpha$ or $--/\alpha^{\dagger}\alpha^{\dagger}$ $\alpha^{\dagger}\alpha^{\dagger}/-\alpha$ or $\alpha^{\dagger}\alpha^{\dagger}/\alpha^{-}$ $\alpha\alpha^{\dagger}/-\alpha^{\dagger}$ or $\alpha^{\dagger}\alpha^{\dagger}/-\alpha^{\dagger}$ (nondeletional)			<ul style="list-style-type: none"> More severe anemia RBC markedly microcytic and hypochromic 		Regular transfusions required
Any combination of $-$ or α^{\dagger} resulting in deletion or inactivation of all 4 genes		Hb Barts hydrops fetalis syndrome (α -thalassemia major)	<ul style="list-style-type: none"> Often die in utero or shortly after birth 		

Classification of based on transfusion

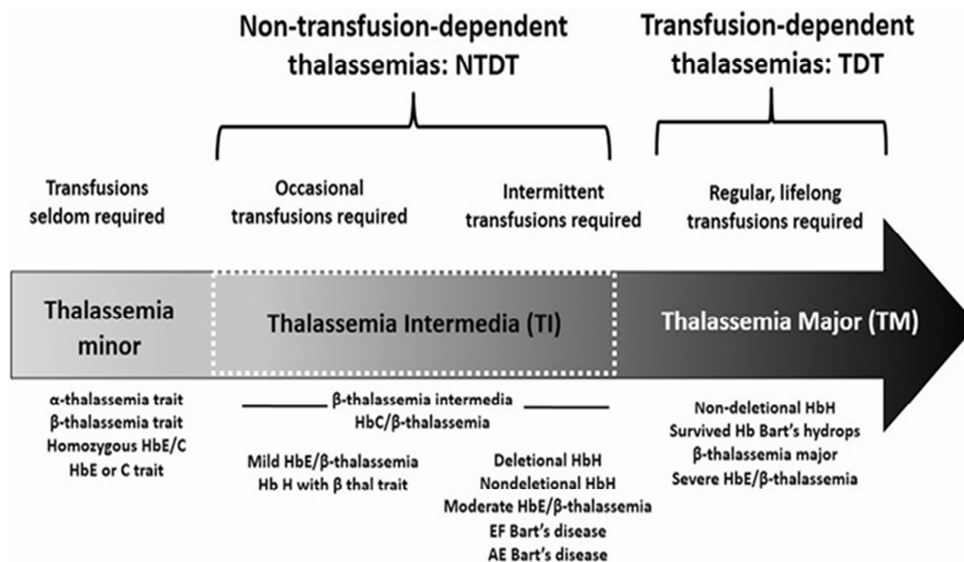
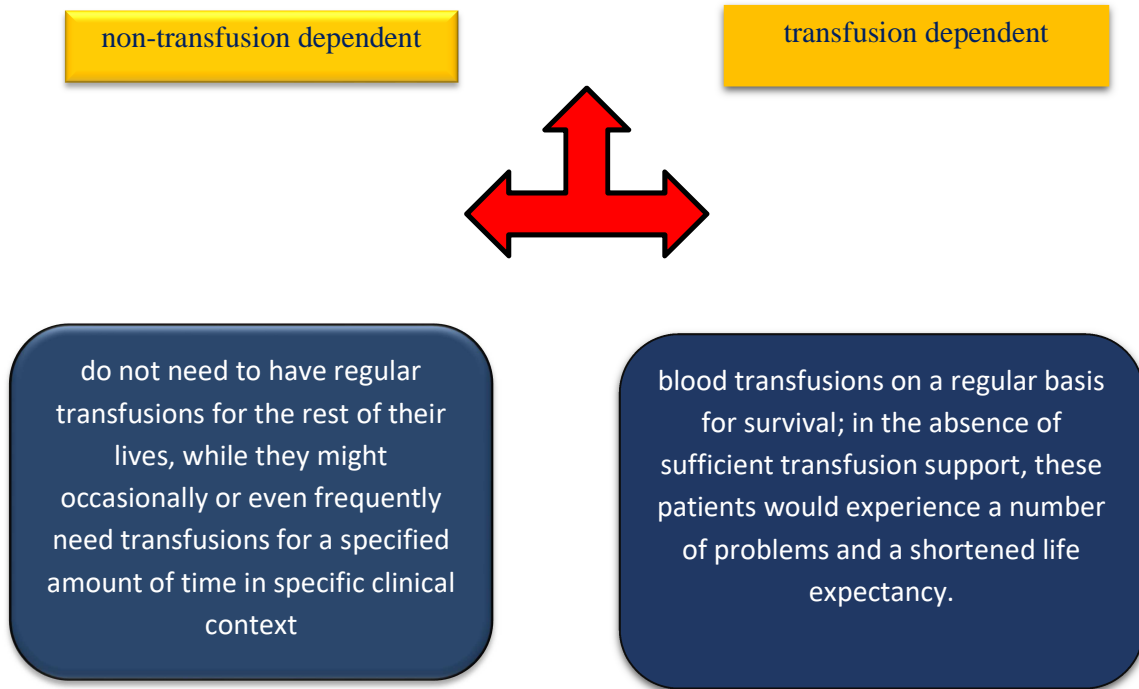


Fig.9: Classification of thalassemia based on transfusion dependency

Alpha-thalassemia Genetics and Clinical Consequences

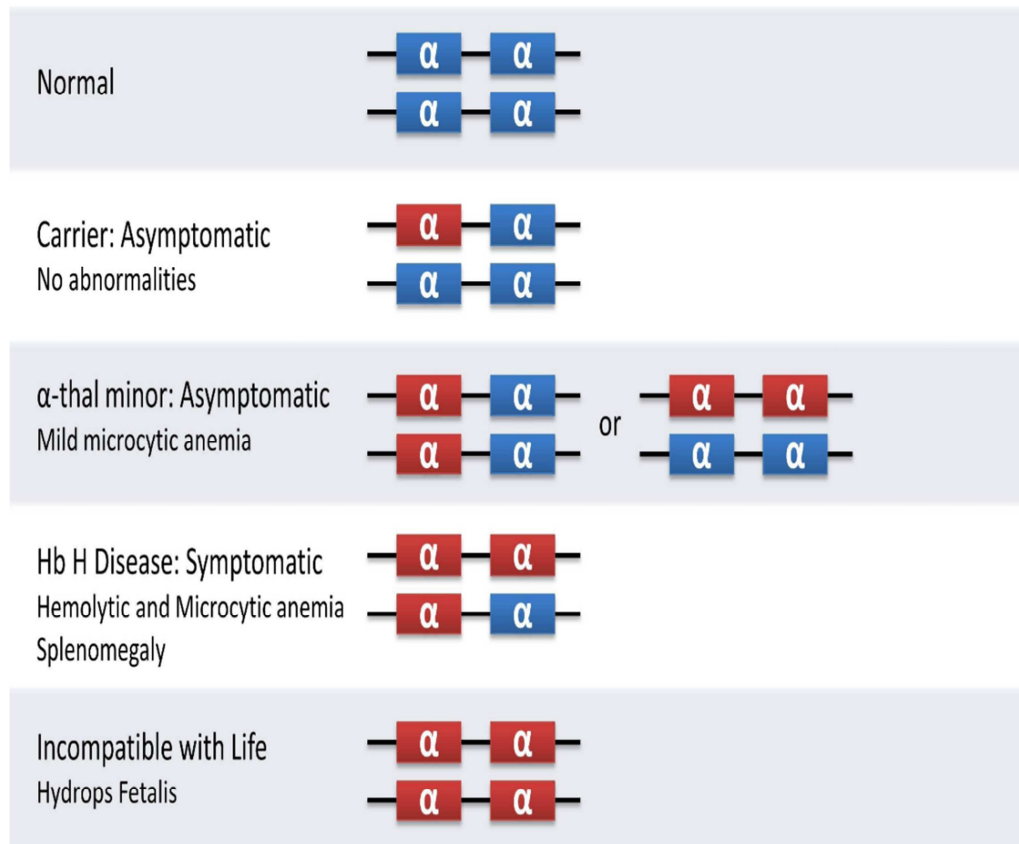


Fig.10:: Alpha Thalassemia genetics

Condition	Deletion of no. of allele genes	Clinical presentation	Clinical features
Silent carrier	1	normal	Clinically and haematologically normal
Thalassemia trait	2	Asymptomatic	Mild anemia, Microcytosis, hypochromia
HbH disease	3	symptomatic	hemolytic anemia, Moderate

			to severe microcytic, hypochromic, mild jaundice, moderate hepatosplenomegaly
Hb Bart hydrops foetalis ^{16,17}	4	severe	Severe anemia, generalized oedema, ascites, marked hepatosplenomegaly, skeletal and cardiovascular malformations, usually death in utero

Individuals with α -thalassemia trait, intermedia, and hydrops fetalis are often found to have Hb Barts. Because of the prevalence of Hb Barts in the hemoglobin profile, a diagnosis of α -thalassemia major is irreversible²³. Babies with α -thalassemia intermedia frequently have Hb A, Hb F, and Hb Barts levels that are higher than 15%.²⁴ Most people with α -thalassemia trait may have normal HbA2 levels if tested six months following diagnosis, even though they have thalassemic red cell indices^{25,26}.

It can be difficult to diagnose this group using Hb electrophoresis or high-performance liquid chromatography (HPLC) because Hb Barts disappears a few weeks after delivery. Performing Hb electrophoresis or HPLC for all neonates, even in regions with a higher frequency of the α -thalassemia gene, is also costly. Certain authors tried for checking some inexpensive sensitive markers. The work was carried out in Thailand²⁷, and in, Saudi Arabia. MCV < 90 and MCH <30 shows high predictive value in Jeddah, Saudi Arabia study²⁸.

Beta thalassemia occurs due to point mutations in beta globin gene. It is classified into 3 categories based on the zygosity of beta-gene mutation. Beta thalassemia minor or beta-plus thalassemia occurs due to heterozygous mutation, in which beta chains are not produced sufficiently. Clinically, disease is mild and asymptomatic. Beta thalassemia major occurs due to homozygous mutation of beta-globin gene, causing total absence of beta chains. Clinical manifestations of patients with beta thalassemia major includes jaundice, hepatosplenomegaly, growth retardation, endocrine abnormalities and anemia requiring blood transfusions. Beta-thalassemia intermedia patients present with mild to moderate clinical symptoms.

Thalassemia major and minor:

Thalassemia minor occurs due to mutation in 1 gene- causing mild clinical features. Thalassemia major or cooley's anemia occurs due to mutation in 2 genes. After six months of life, when adult hemoglobin starts to replace fetal hemoglobin (Hb-gamma), clinical symptoms usually appear in babies. Intravascular hemolysis results from beta-thalassemia variations' excess alpha-globin chains precipitating and damaging red blood cell membranes. Precursor cell death prematurely causes erythropoiesis to be unsuccessful, which in turn causes hematopoiesis to spread outside of the bone marrow.

Southeast Asian populations frequently exhibit hemoglobin E (Hb E), a common hemoglobin variation. Two new terms are starting to appear increasingly frequently in clinical contexts. These classifications include transfusion-dependent and non-transfusion-dependent thalassemia's, categorized based on the need for blood transfusions²⁹.

Hemoglobinopathies, which include sickle cell disease, hemoglobin C (Hb C) disease, and hemoglobin E (Hb E) disease, are hemoglobin variations other than thalassemia's. These disorders result from genetic flaws that change hemoglobin's typical structure. The disorder can become more severe if non-deletional mutations in the α -globin genes combine with deletional variants of α 0-thalassemia. HbH-HbConstant Spring is one of the most prevalent non-deletional mutations and is linked to a syndrome that is commonly seen in Southeast Asia.

Hb H disease is a form of α -thalassaemia resulting from the deletion of three of the four α -globin genes. When this deletion interacts with non deletional mutations such as HbConstant Spring, it can further complicate the clinical picture and increase the severity of symptoms. Hemoglobin Constant Spring (HbCS) is a variant of alpha thalassemia that is associated with more ineffective erythropoiesis and more severe anemia compared to Hb H disease. In individuals with HbCS, the hemoglobin level is typically around 70 g/L. As a result of this pronounced anemia, there is a greater need for blood transfusions, especially during childhood when iron overload becomes an increasing problem.

The co-occurrence of these mutations can lead to a spectrum of clinical manifestations, ranging from mild to severe, depending on the specific genetic makeup of the individual. Understanding the interplay between deletional and non-deletional mutations in α -thalassaemia is crucial for accurate diagnosis, prognosis, and management of affected individuals. The $^{-SEA}+$ deletion is a common reason for two serious conditions in Southeast Asia: Hemoglobin H disease and Hemoglobin Bart's hydrops fetalis syndrome³⁰.

Other mutations

Mutations that affect the d-globin gene are rare and usually don't cause any noticeable health problems. However, they're important because they can make it tricky to diagnose beta thalassaemia. This is because they stop HbA2 levels from going up. When b-thalassaemia and d-thalassaemia (either together or separately) are inherited at the same time, it's the most common reason for having normal HbA2 levels in b-thalassaemia trait.

Delta-beta thalassemia occurs due to the deletion of both delta and beta genes. Individuals who are homozygous for delta-beta thalassemia have 100% foetal hemoglobin (Hb F)³¹, which leads to increased Hb F production. As a result, they may exhibit thalassemia 0 beta-thalassemia trait, but there is typically no increase in the percentage of hemoglobin A2 (HbA2.), and it often remains within normal levels³². However, hemoglobin F (Hb F) is consistently elevated, ranging from 5% to 20%. They also experience microcytic hypochromic anaemia, which means their red blood cells are smaller and paler than normal.

The characteristics of peripheral blood films are similar to those of beta-thalassemia trait, and flow cytometry is the most effective method of visualizing the heterocellular distribution of Hb F. It's critical to differentiate this illness from hereditary fetal hemoglobin persistence (HPFH)³³.

It's important not to mix up delta beta thalassaemia with other conditions like hereditary persistence of foetal haemoglobin (HPFH). In HPFH, there are deletions in the same parts of the b-globin gene cluster, but instead of causing problems, they lead to increased production of Hb F. Also, because there aren't any issues with alpha or

non-alpha globin production, people with HPFH have normal mean cell haemoglobin (MCH) and mean cell volume (MCV). It is well established that hematological parameters in newborns differ from those in infants or adults, and these differences can vary based on the type of blood sample used (umbilical cord blood, venous blood, capillary blood), the timing of blood draw (2, 12, or 24 hours after birth), and fetal nutritional conditions.

Diagnostics

Mean Corpuscular Volume is a laboratory value which measures the mean size and volume of an RBC. It helps in knowing the aetiology of anaemia. Its value can be obtained by multiplying the % hematocrit by 10 divided by erythrocyte count³⁴.

$$\text{MCV (in fl)} = (\text{Hct [in L/L]} / \text{RBC [in } \times 10^{12} / \text{L]}) \times 1000.$$

An MCV below the normal range implies microcytic anaemia and MCV above normal implies macrocytic anaemia. It is also useful in calculating red cell distribution width. A mean corpuscular volume (MCV) of less than 95 fL and/or a mean corpuscular hemoglobin (MCH) value of less than 30 pg are commonly used as cutoff values for thalassemia screening. Based on two standard deviations from the MCV and MCH values in the hematological parameters of umbilical cord blood, these criteria are established. Thalassemia screening using MCV and MCH offers the benefit of obtaining quick, cost-effective, consistent, and precise results using automated hematology analyzers³⁵.

Low MCV readings, however, can also be a symptom of microcytic anemias, such as iron deficiency anemia (IDA). While MCH appears to be similar across

different automated hematology analyzers³⁶, variability in MCV values has been noted among different automated blood cell counters. Furthermore, a low MCV is not appropriate for detecting carriers of Hb E and people with single α -globin gene deletions (-3.7 and -4.2)³⁷, nor for screening non-deletional α -globin gene mutations like Hb Constant Spring (Hb CS) and Hb Quong.

When heterozygous α -thalassemia is combined with either α thalassemia trait alone or with glucose-6-phosphate dehydrogenase deficiency, it might result in normal mean corpuscular volume and a false-negative result when screening for thalassemia³⁸. Therefore, it would be more appropriate to screen for thalassemia with MCV and MCH rather than just MCV; figuring out their threshold values would be essential. Peripheral blood smear interpretation combined with both MCV and MCH would be regarded as a crucial thalassemia screening technique.

Peripheral blood smear interpretation combined with both MCV and MCH would be regarded Thalassaemia patients typically have microcytosis, hypochromia, and an anisopoikilocytosis image in their RBC morphology. A central pallor that takes up more than one-third of the diameter of hypochromic red blood cells (RBCs) is one of their distinguishing features. RBC sizes and the nuclei of tiny lymphocytes can be compared to determine the presence of microcytes. Anisopoikilocytosis can be brought on by a variety of abnormal RBC morphologies, including nucleated RBCs, schistocytes, microspherocytes, target cells, and polychromasia. Target cells and stippling in the blood film are helpful auxiliary findings for thalassaemia diagnosis, however they are not associated with a hemoglobinopathy³⁹.

Peripheral blood smear results may only indicate particular forms of thalassemia's from other causes of anemia, such as IDA or anemia of inflammation. It

is not possible to diagnose a specific type of thalassemia disease based just on RBC morphology.

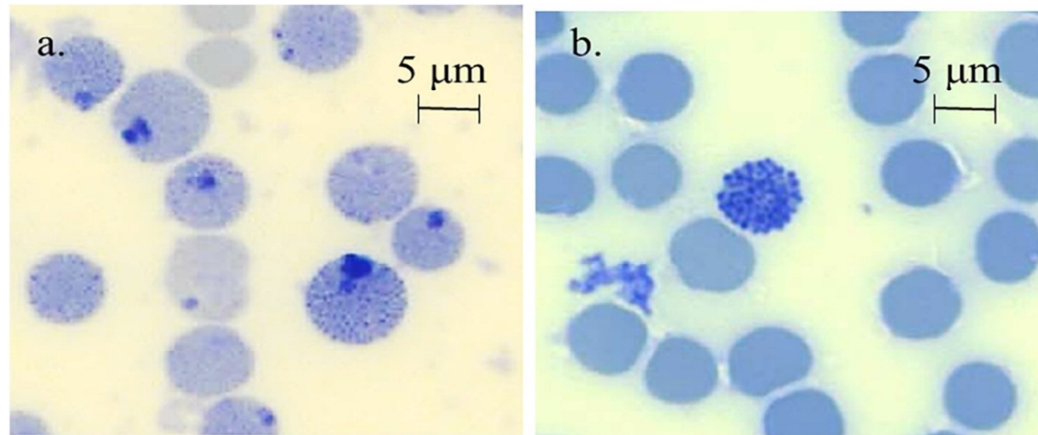


Fig.11: Peripheral smear for iron deficiency anemia

Iron Deficiency Anemia (IDA) is primarily characterized by an elevation in red cell distribution width (RDW), which indicates the severity of microcytic anemia⁴⁰. While all forms of thalassemia result in uniformly high levels of microcytic red blood cells, not all of the thalassemia syndromes exhibit the same level of RDW rise. For example, α -thalassemia minor and Hb H illness show significant increases in RDW⁴¹. As a result, while the RDW may offer data that could be utilized to supplement diagnosis, it is ineffective as a sole screening sign.

The red blood cell (RBC) count usually decreases in iron deficiency anemia (IDA) and anemia associated with chronic diseases, and this decrease is correlated with the severity of the anemia. On the other hand, thalassemia manifests as microcytic anemia, which is distinguished by an elevated red blood cell count that can facilitate the diagnosis. It is not advised to use RBC count alone as a screening tool for hemoglobinopathies and thalassemia, though.

Considering all of this, several CBC based indices have been developed for hemoglobinopathies and thalassemia screening; nevertheless, none of these indices perform better than the MCV and MCH combination when selecting cases for additional study.

In preterm neonates, Hb is significantly lower ($p < 0.05$) with a remarkable MCV increase ($p < 0.01$) in comparison with full term newborns, but without differences either in RBC or in PCV. It must be pointed out that preterm newborns were not under 30 weeks of age where differences are more remarkable⁴². The MCV is a fascinating metric because it has been suggested that newborn microcytosis serves as an alert for the diagnosis of alpha thalassemia⁴³.

. The proportion of HbA decreases as the number of faulty β -thalassemia genes grows, resulting in an increase in HbF. HbF levels are significantly greater in β -thalassemia groups compared to normal groups, although HbA levels are lower. This implies that HbF and HbA could both function as markers to distinguish the quantity of faulty genes and show the severity of β -thalassemia⁴⁴.

The mild chronic anemia and significantly raised HbA2 levels linked to the heterozygous variant of β -thalassemia serve as the foundation for screening initiatives worldwide⁴⁵. Globally, an estimated 80–90 million individuals carry β -thalassemia¹⁹, with 35–45 million carriers living in India alone⁴⁶. Considering the diagnosis of β -thalassemia, the implications of borderline HbA2 levels are significant.

The main cause of borderline HbA2 levels was determined to be heterozygosity for β -thalassemia. Of the heterozygotes with β -thalassemia, 40% had

iron deficiency anemia (IDA) or abnormalities in the α or δ globin genes concurrently, and the remaining 60% did not have these complicating conditions⁴⁷.

A number of variables, including α -thalassemia, δ -thalassemia, and severe IDA, influence HbA2 levels in addition to the β -thalassemia alleles. It has also been reported that in carriers of β -thalassemia, the co-inheritance of α - or δ -thalassemia lowers/normalizes HbA2 levels^{48,49}.

High performance liquid chromatography

The high-performance liquid chromatography (HPLC) technique separates compounds or molecules based on their chemical properties, making it valuable for diagnosing β -thalassemia trait due to its precise quantification of HbA2⁵⁰. HPLC is an effective method for detecting hemoglobinopathies in infants. It is utilized to measure hemoglobin's S, A2, and F, and can also detect, provisionally identify, and quantify numerous variant hemoglobin's. The analyzers are automated, minimizing the requirement for extensive staff intervention and enabling efficient handling of large volumes of samples. Sample volume (5 μ L) are adequate for examination. Each sample provides quantification of both normal and separated variant haemoglobin's⁵¹. Provisional identification of a broader range of variant hemoglobin's is possible. Detection of δ chain variants, crucial for diagnosing β -thalassemia heterozygosity, is facilitated. As the primary means of identifying any variations found, retention durations are crucial to monitor and maintain when using HPLC for screening⁵². To maintain constant timing of the Hb A2 peak appearance, this procedure frequently involves adjusting the column temperature or flow rate. It's an important process, much like the daily calibration of Hb A2 and Hb F levels, and if possible, it's best to incorporate appropriate controls.

A cord blood sample provides a rapid, simple, and effective method of demonstrating the coexistence of α -thalassaemia by detecting the presence of Hb Barts.

When molecular diagnostic tools were available for the diagnosis presence of HB Barts in newborn is suggestive of α -thalassaemia^{53,54}. There were no direct correlation between the amounts of Hb Barts and the underlying molecular defect. Under reporting of alpha thalassaemia was seen in neonates as surveys showed that assays that detect 0.5% to 1% Hb Barts in cord blood were able to identify major proportion of neonates with thalassaemia detection but many went undetected⁵⁵. Both HbS heterozygous and homozygous HbSS participants had lower levels of HbA when α -thalassaemia was present. Thus, mutation causing α -thalassaemia resulted in smaller RBCs causing it to flow through the narrow capillaries, enhancing the severity of the condition. Also elevated levels of Hb, red blood cell count, Hct, and Hb F with reduced Hb A, Hb D, MCV, and MCH were seen in participants with Hb D. The presence of deletion involving alpha globin gene decrease the Hb A, Hb E, MCV, MCH, Hb, RBC counts, and Hct in participants with Hb E, while only the Hb F increases. When α -thalassaemia is the lone aberration, it appears to have an overall impact on all red cell indices.

Unless the defective hemoglobin was present in a homozygous state, as in people with homozygous Hb S, the influence of abnormal hemoglobin was minimal. Thus, since α -thalassaemia causes a decrease in α -chains, the addition of a structural variant will create a changeable scenario, the outcome of which depends on the rate of net globin chain synthesis.

Starting in the 1970s, Mediterranean countries implemented population screening, genetic counseling, and prenatal diagnosis programs. These programs

involved analyzing foetal blood in second-trimester foetus. As a result of these efforts, there was a significant reduction in the birth rates of affected children.

CE-HPLC, or Capillary Electrophoresis-High Performance Liquid Chromatography, has been demonstrated to be an accurate and straightforward technique for quantifying HbA₂ and Hb F, as well as other hemoglobin subtypes. This method aids in the presumptive identification of various abnormal hemoglobin variants and can also be utilized for newborn screening and prenatal diagnosis of hemoglobinopathies.

Denaturing HPLC is a recently reported strong method for DNA mutation detection (DHPLC). This has been applied to the identification of minor insertions and deletions as well as single nucleotide alterations in the α and β globin genes. PCR is used to amplify certain gene areas, and either partially or totally denaturing HPLC is used for analysis. The technique relies on the difference in the retention times of homoduplexes and heteroduplexes in a blend of PCR products that have been denatured and reannealed. In order to monitor the eluted DNA, UV or fluorescent detectors are used. DHPLC has been demonstrated to be a rapid, sensitive, and specific method of identifying mutations and polymorphisms; yet, DNA sequencing could occasionally be necessary for a conclusive identification of the molecular problem⁵⁶

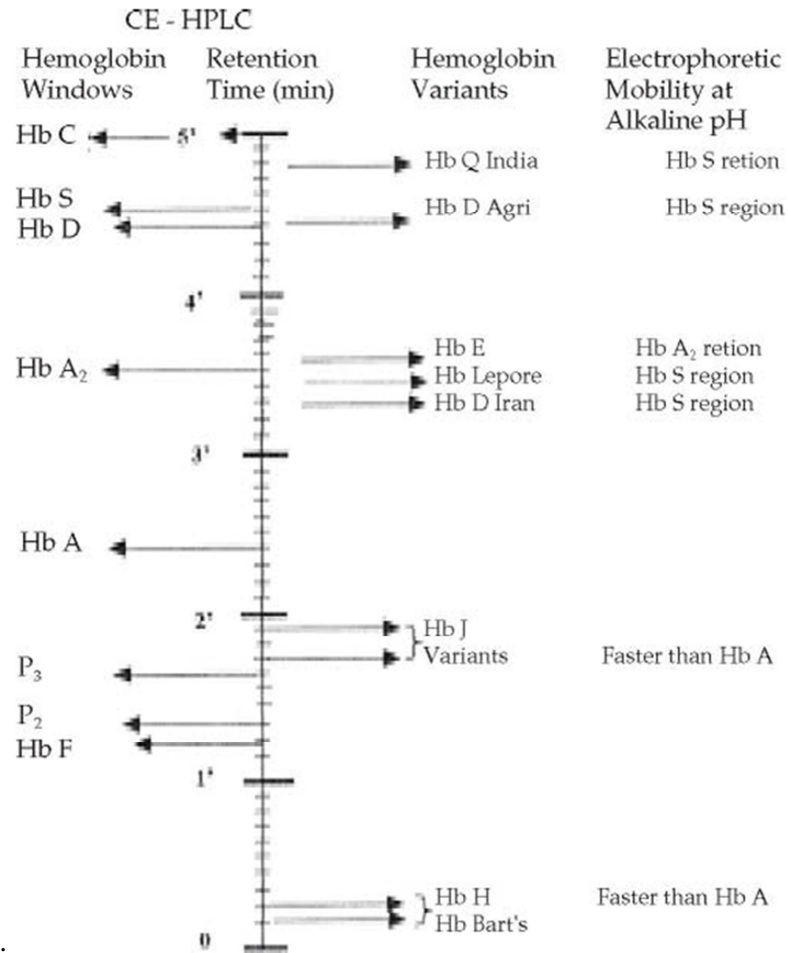


Fig.12: Retention time of hemoglobin variants on CE-HPLC

The figure above displays the electrophoretic mobilities at an alkaline pH coupled with the retention periods of various Hb variations found in India. HbA₂ levels are artificially raised by CE-HPLC in the presence of Hb S because of the presence of Hb S adducts, and falsely decreased in the presence of Hb D because of an integration error.

Hb H and Hb Bart's can only be suspected through visual analysis because they appear as sharp peaks at the beginning of the chromatogram. The software used

for analysis typically does not integrate elution peaks with a retention time of less than 0.63 minutes, so these peaks are not identified in the chromatogram⁵⁷.

Using the VARIANT™ apparatus and the β -Thalassemia Short Program created by Bio-Rad Laboratories, cation exchange HPLC is used to systematically quantify the different fractions of hemoglobin (Hb). This method exposes numerous Hb variants in addition to 1quantifying various Hb fractions. We can standardize the elution periods of variations by comparing the retention times of normal Hb fractions, which adds another identification criterion. Although this method is not used as the primary test for Hb variant screening, it serves as a valuable complementary test for presumptive diagnosis, especially since we have already determined the elution times of over 200 variants.

Hb variant retention periods measured with cation exchange high performance liquid chromatography VARIANT™ system with β -Thalassemia Short Program; Bio-Rad Laboratories). For Hb A2, a reference of 3.8 minutes was used to standardize these data⁵⁸

HUMAN HEMOGLOBIN VARIANTS: LABORATORY METHODS

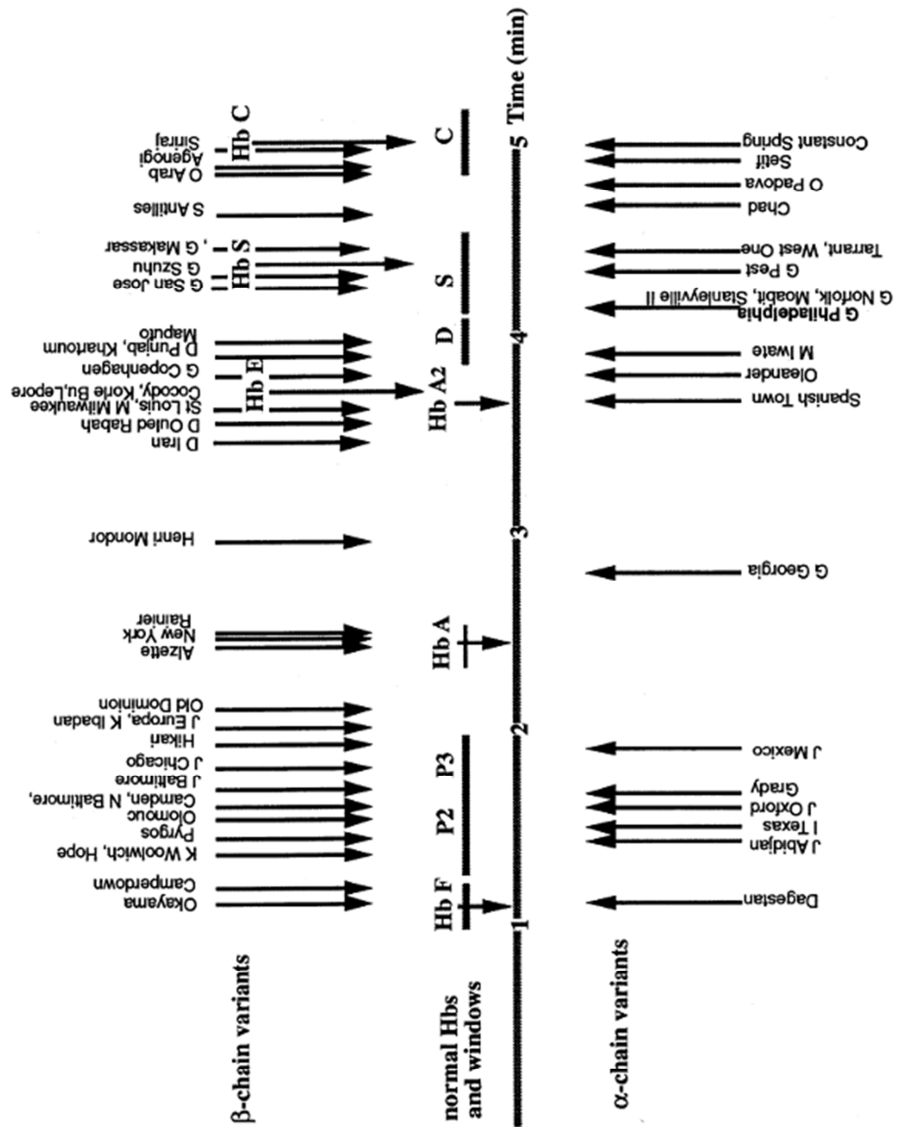


Fig.13: Human hemoglobin variants

Variants that elute inside the window of Hb F

Certain α and β chain polymorphisms have the potential to elute inside the Hb F window. Therefore, any unanticipated rise in Hb F levels—typically greater than 10%—should prompt additional research.

Variants that elute inside the window of P₂

Hemoglobin A1c (Hb A1c), which is elevated in diabetes, elutes in the P₂ window. The only variant that elutes in this window with a percentage of over 40% is Hb Hope.

Variants that elute inside the window of P₃

Together with a few other aberrant hemoglobins like Hb Fannin-Lubbock and Hb N Baltimore, the majority of the α and β chain Hb J variations elute within the P₃ window. Some of these variants can be identified with the use of Hb electrophoresis.

Variants that elute inside the window of A₀

A distinctive hump may be seen in the A₀ peak of one form of Hb Twin Peaks. Hb New York and Hb A₀ have the same retention period.

Variants that elute inside the window of A₂

The HbA₂ window is where hemoglobinopathies Hb E, Hb D Iran, and Hb Lepore all elute in HPLC studies. Hb Lepore's peak has a recognizable hump.

Variants that elute inside the window of C

A few variations elute in the Hb C window, such as Hb O Arab and Hb Constant Spring. A strong peak, 10 Hb Q India, elutes shortly prior to the Hb C window.

MOLECULAR CHARACTERIZATION

Over 90% of incidences of (α)-thalassemia are caused by deletions of certain genes. Sequence variants, including single nucleotide changes, insertions, and short insertions/deletions, are responsible for a small fraction of instances of (α)-thalassemia. Two identical HBA genes are among the closely related genes found in the (α) gene cluster. The most likely cause of deletions is uneven crossing over in these homologous areas during meiosis. There are several breakpoints known to exist, the most frequent deletion extending 3.7 kilobases. Sequence variations account for more than 90% of cases of α -thalassemia compared to cases of (α) -thalassemia. There are reportedly more than 280 sequence variations connected to α -thalassemia. It has been revealed that more than 280 sequence variations are connected to α -thalassemia^{60,61}.

Gene loss involving the HBB gene accounts for some cases of thalassemia. Mutations in the globin gene can be found using a variety of different molecular methods. Molecular techniques can be grouped by mutation type to be targeted as follows:

1. Detection methods for structural variations such as gene deletion, duplication, or triplication and
2. Detection methods for sequence variations such as nucleotide substitution, insertion, or short insertion/deletion

Gap polymerase chain reaction (PCR), which is specifically made for the targeted deletion, can be used to identify identified gene deletions. Southern blotting with labeled complementary gene probes may be used to detect undiscovered gene

deletions. Gene deletions that have been detected as well as those that have not can be found using the multiplex ligation-dependent probe amplification (MLPA) technique. Because of its great sensitivity, ease of use, and capacity to identify a broad variety of deletion mutations, MLPA is preferred. In certain ethnic populations, cost-effective methods such as amplification refractory mutation system (ARMS), reverse dot blotting, denaturing gradient gel electrophoresis (DGGE), and allele-specific PCR (AS-PCR) can be employed to detect prevalent sequence variations.

With the rapid advancement and cost-effectiveness of sequencing technology, labs are now able to sequence the whole globin gene, including its promoter, 3' UTR, exon-intron boundaries, and deep intronic regions. More specifically, targeted genes, exomes, or complete genomes can be sequenced using massively parallel sequencing technology. Fast and quantitative analysis can be achieved with the multiplex ligation-dependent probe amplification (MLPA) approach. It works by hybridizing two nearby oligonucleotides to a section of the genomic DNA, and then measuring changes in the DNA copy number or other variations using quantitative PCR amplification. To apply MLPA, all you need is a thermocycler and capillary electrophoresis (CE) equipment.

In many laboratories, MLPA has replaced southern blotting as the method of choice for finding undiscovered deletions in α -thalassemia due to its higher sensitivity and dependability. Additionally, MLPA does not require radioactive detection, in contrast to southern blotting.

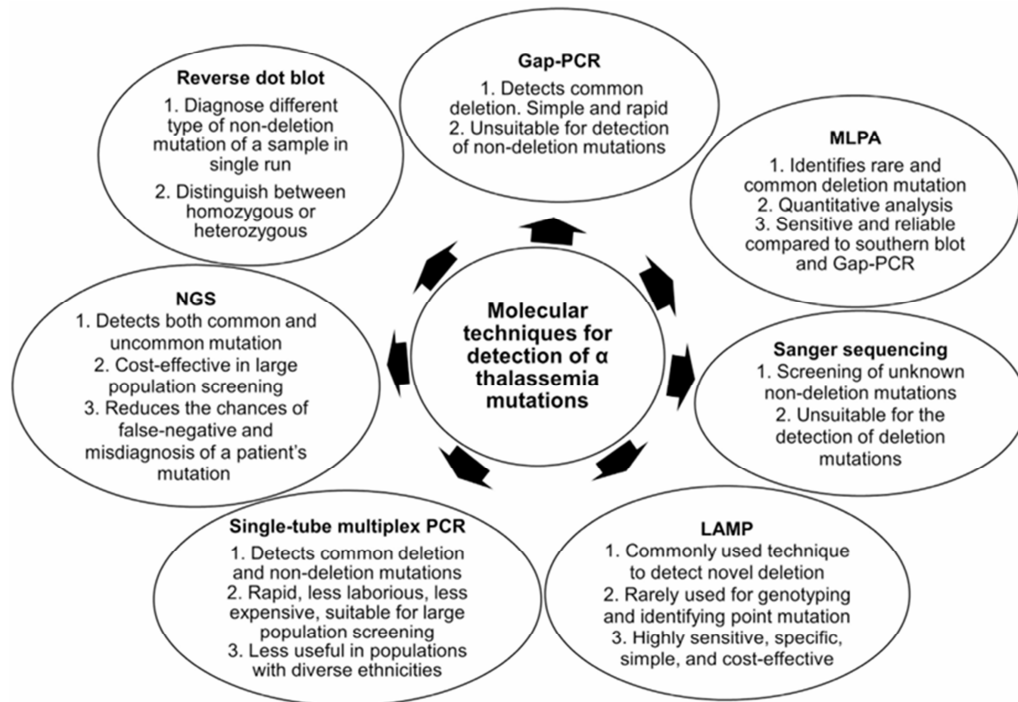


Fig.14: Molecular techniques for diagnosis of alpha thalassemia

Nelida et al showed that maternal smoking habit to have no effect on cord blood Hb concentration in neonates as per their gestational age. Reduced Hb concentration and more MCV were seen in preterm newborns more compared to normal neonates. Peripheral smear showed anisocytosis and many macrocytes, mild poikilocytosis and polychromatophilia. Crenate erythrocytes, spherocytes were also seen. Among preterm neonates, Hb F between 30-34 wk values are more significantly compared to those neonates between 35-37 week ($p < 0.001$). Authors concluded that MCV is an interesting parameter as neonatal microcytosis was proposed to be a major criterion to diagnose alpha thalassemia but it is the need of the hour to apply molecular biology techniques to attain accurate diagnosis

Goh et al did a meta-analysis on the prevalence of alpha thalassemia. The aim of their meta-analysis is to provide update from 2010 to 2020 on the prevalence of α -thalassemia among patients of Southeast Asia. Search was done using PubMed and SCOPUS for various related studies which were published from 2010 to 2020, as per the specified eligibility criteria. 29 studies involving 83,674 subjects were included. Pooled prevalence rates were assessed by random effect models as per the high observed heterogeneity. Results showed that the overall prevalence of α -thalassemia as 22.6%. Prevalence was found to be highest in Vietnam, where the prevalence is around 51.5%. The prevalence in Cambodia was 39.5%. The prevalence was 26.8% in Laos. Prevalence in Thailand was 20.1%, and 17.3% in Malaysia. Their meta-analysis showed high prevalence of α -thalassemia in certain Southeast Asia countries. This meta-analysis helps in designing various screening programs and may help to improve the clinical management of disease.

Nadkarni undertook a study to determine the prevalence and molecular genetic bases of alpha-thalassemia among Indians and implications in prenatal diagnosis. 1253 samples were screened for hemoglobinopathies. HbA₂ and HbF levels were measured using HPLC. Alpha-Globin gene mapping was performed by using Southern blot hybridization of BamHI and BglII digests. Results showed that among 1253 patients, 132 had single alpha-gene deletion and 29 had 2 alpha-gene deletions. 15 patients showed presence of alpha-gene triplication. One patient showed the presence of 1 alpha-gene deletion and alpha-gene triplication. The overall prevalence of alpha-thalassemia was found to be 12.9%. Alpha-thalassemia was found to be highest in Punjabi population. The sensitivity for detecting HB Barts by IEF was 75.5%.

Authors concluded that alpha-Thalassemia is the commonest hemoglobinopathy till now, in India, but it is not a reason for serious genetic risk. Milder form that is alpha or alpha-alpha of alpha-thalassemia was found to be predominant. Knowing alpha-genotype helps in genetic counselling for prenatal diagnosis among partners, where 1 of the parents may have decreased along with an increased RBC count and normal HbA2 levels.

Purohit et al. did a study on the of Inherited hemoglobin disorders like alpha thalassemia and sickle gene. In the view of lack of knowledge on the epidemiology malaria resistance genes among tribal dominated malaria endemic areas, their study was conducted. Their prevalence, cross sectional study was done among 594 subjects among 5 populations in Sahara, Kutia, Kuda, Gond and Oraon. The prevalence was found to be highest-42.4% in Sahara, followed by Kutia, Kandha, Kuda, Gond and least in Oraon (2.0%). Sickling test, HPLC, Hb electrophoresis, and molecular studies were done to check for the prevalence of sickle allele, and thalassemia. Sickle and β thalassemia alleles were seen among 13.1% and 3.4% of patients respectively. Sickle allele was seen in heterozygous and homozygous state, though it is common in heterozygous state. The prevalence of alpha thalassemia was found to be 50.84% with an allelic frequency of 0.37. Both $\alpha^{-3.7}$ and $\alpha^{-4.2}$ alpha thalassemia was seen with the frequency of 0.33 and 0.04. Authors concluded that more prevalence of alpha thalassemia and sickle gene among Orissa population could be due to selection pressure of endemic malaria in that part of India.

In a review by Higgs (2013), various types of alpha-thalassemia are discussed. Two common types involve deletions of parts of the alpha-globin genes, called $-\alpha3.7$ and $-\alpha4.2$. There are also many other forms of alpha-thalassemia caused by different-

sized deletions of these genes. These forms are often named after the place where they were first discovered. The most widespread form is called the Southeast Asian deletion (-SEA). This deletion affects both alpha genes but not the embryonic globin genes. When non deletional mutations interact ($-\alpha\text{ND}\alpha$), the clinical symptoms tend to be more severe but less common compared to deletional forms ($-\alpha/-$). The most common interaction involves a mutation in the alpha-globin chain known as Hb Constant Spring ($-\alpha\text{CS}\alpha$). Studies by Lau et al. (1997), Lorey et al. (2001a), Nelida et al. (2008), Michlitsch et al. (2009), Singer et al. (2009), Kidd et al. (2010), Lal et al. (2011), and Vichinsky (2012) have contributed to understanding these interactions and their effects.

METHODOLOGY

Materials and methods

Source of Data:

Umbilical cord blood obtained from the newborn delivered in labor rooms and operation theatre of KLE's DR. Prabhakar Kore hospital and MRC who are more than 36weeks of period of gestation.

Study Design:

Longitudinal study

Study setting:

Obstetrics, Paediatrics and pathology Department of KLE's Dr Prabhakar Kore Hospital, Belagavi, Karnataka

Study Period:

One year

Ethical considerations:

- Prior approval from the Institutional Ethics Committee has been obtained.
- All the participants in this study are Voluntarily involved
- Informed consent was taken from every participant's guardian.
- Participant confidentiality will be maintained.

Participants were not subjected to any potential harm

Sample Size:

Sample size was calculated assuming the proportion of alpha-Thalassemia as 12.9% as per the study by Nadkarni A et al.⁶³. The other parameters considered for sample size calculation were 1.859% absolute precision and 95% confidence level. Based on the previous hospital records, the approximate number of potential Eligible subjects to be attending the study setting during the data collection period was considered as 4000. Hence a finite population correction was applied for 4000. The following formula was used for sample size as per the study by Daniel WW et al.⁶⁴

$$n' = \frac{NZ^2P(1 - P)}{d^2(N - 1) + Z^2P(1 - P)}$$

Where n = Sample size

N = Population Size = 4000

Z = Z statistic for a level of confidence level = 1.960

P = Expected prevalence/proportion of outcome = 0.129

d = Precision = 0.01859

The required sample size as per the above-mentioned calculation was 952. To account for a non-participation rate/ loss to follow up rate of a about 5%, another 48, subjects will be added to the sample size. Hence the final required sample size would be 1000

Sampling Method:

Purposive sampling

Inclusion Criteria:

Newborns of more than 36 weeks period of gestation

Exclusion Criteria:

1. Mother with history of intrauterine viral infection
2. Chronic intrauterine bleeding

Tools used

K2 EDTA vacutainer

Sysmex XN 1000 - 5part analyzer

Plain vacutainer

BIORAD D10 HPLC machine

COBAS PRO and PURE

Study protocol

The parents of new born, who fulfilled the eligibility criteria were briefed about the nature of the study and written informed consent was taken. The UCB (Umbilical Cord Blood) samples were obtained using K2-EDTA vacutainers by skilled midwives and obstetricians. They collected the samples with the placenta still in the uterus right after clamping the cord and separating the infant. After extraction, the samples were gently mixed by inversion around 8-10 times. 2ml of Umbilical cord blood was collected in K 2-EDTA bulb and complete Hemogram was processed on Sysmex XN 100 0 - 5part analyzer. Mean corpuscular volume, Mean Corpuscular

Hemoglobin, Red blood cell distribution width(RDW) and Reticulocyte count was noted. Neonates with red blood cell indices; MCV<95 and/or MCH<30 will be subjected for alpha thalassemia gene deletion study by MLPA[Multiplex Ligation Dependent Probe Amplification].

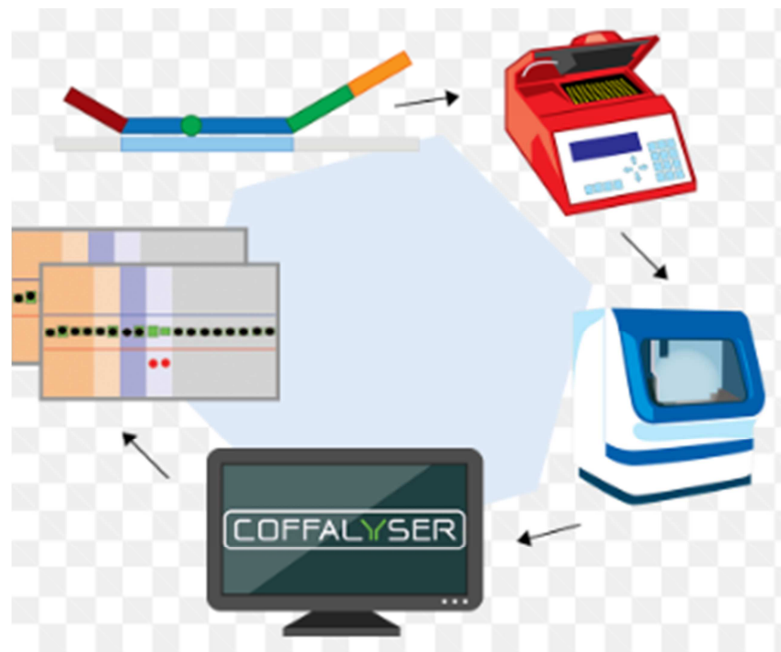


BIO RAD D10 HPLC analyzer for HPLC SYSMEX XN 1000 for RBC Indices

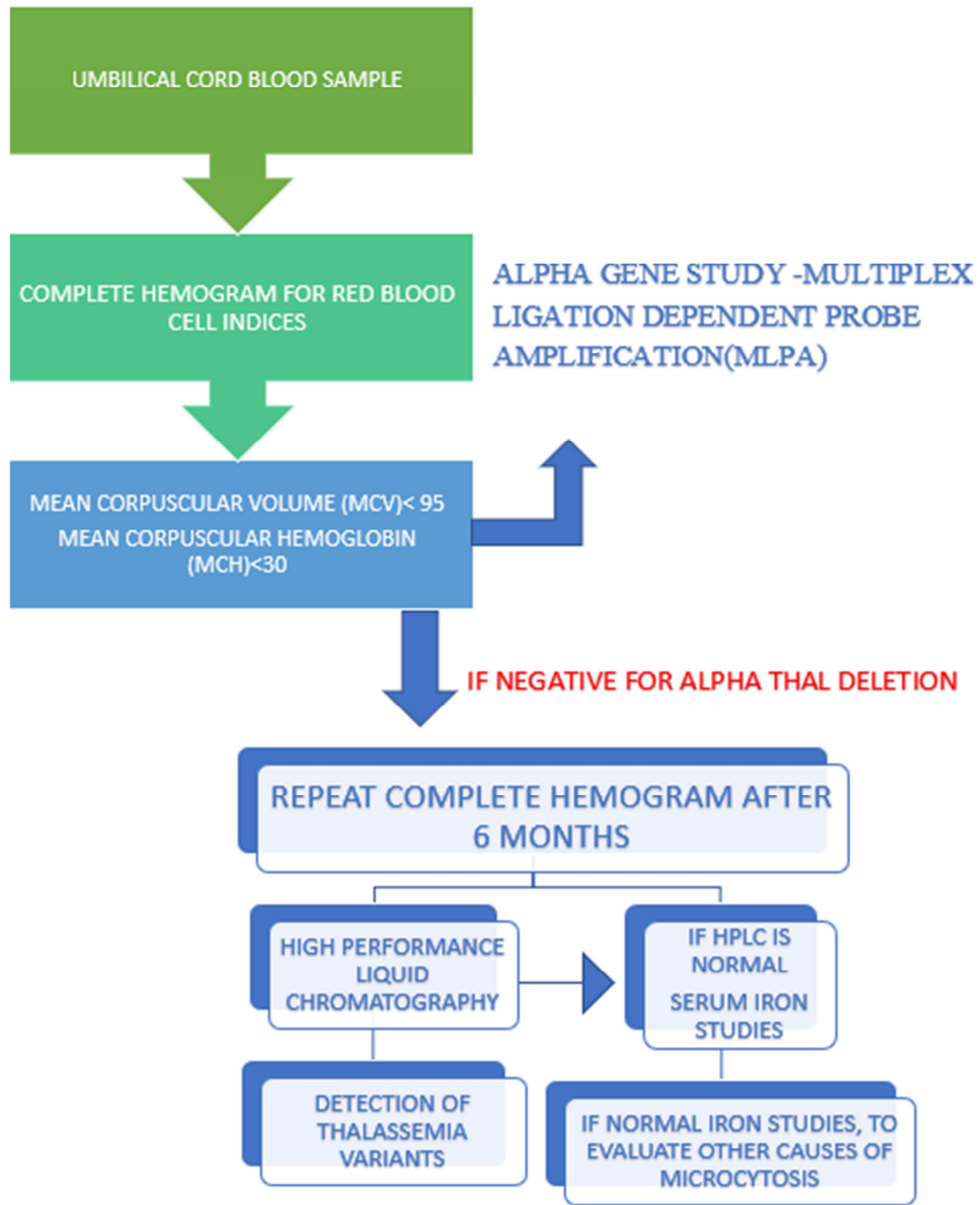
Neonates with negative MLPA were followed up at the age of 6months for repeat complete hemogram, High Performance Liquid Chromatography [HPLC] to look for other variants of haemoglobinopathies. Those with negative MLPA and negative HPLC were evaluated for other causes of microcytosis by serum iron studies and bone marrow iron staining accordingly.



COBAS PRO for Serum iron studies and Iron profile



Multiple Ligation Probe Amplification



Data processing and analysis/statistical analysis:

Alpha-Thalassemia was considered as primary outcome of interest. Descriptive analysis was carried out by mean and standard deviation for quantitative variables, frequency and proportion for categorical variables. The association between categorical explanatory variables and quantitative outcome was assessed by comparing the mean values. The mean differences along with their 95% CI was presented. Independent sample t-test/ ANOVA was used to assess statistical significance. The association between explanatory variables and categorical outcomes was assessed by cross tabulation and comparison of percentages. Odds ratio along with 95% CI was presented. Chi square test/ Fisher's test was used to test statistical significance. Data was analyzed using statistical software IBM SPSS Version 25⁶⁵ and Microsoft Excel. Categorical variables are given in the form of frequency tables. Continuous variables are given as Mean \pm SD/Median (range). To compare normally distributed continuous variables over a group, t-test was used. To compare non-normally distributed continuous variables over a group, Mann-Whitney test was applied. To check the association between categorical variables, Chi-square test was used. P-value less than equal to 0.05 was taken as statistically significant.

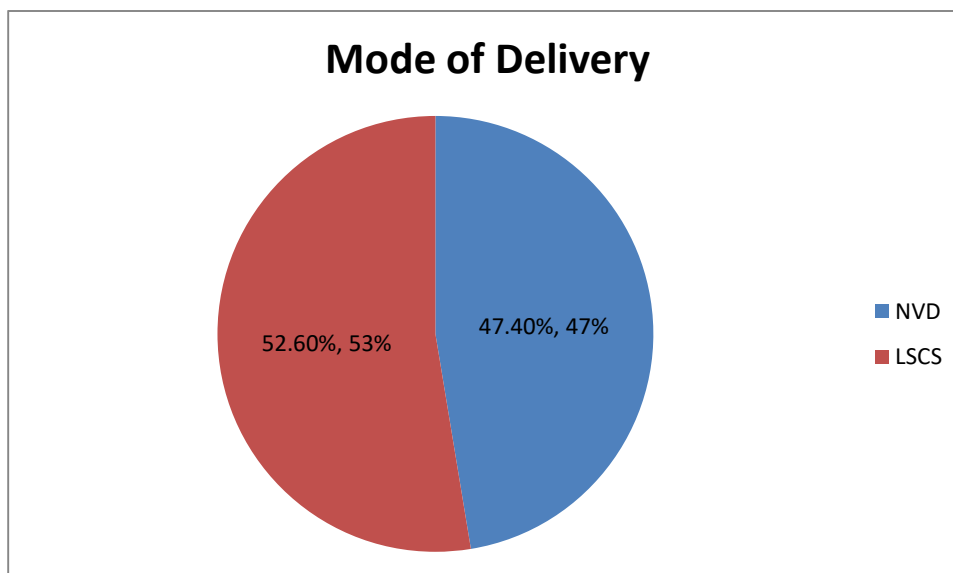
RESULTS

Mode of Delivery

Among the study participants, 52.6% (n=526) of the delivery were cesarean section and the rest 47.4% (n=474) were normal vaginal delivery (Table 1) (Figure 1).

Table 2: Mode of Delivery of study participants

MODE OF DELIVERY	NUMBER (n=1000)	PERCENTAGE
Normal Vaginal Delivery	474	47.4%
LSCS	526	52.6%



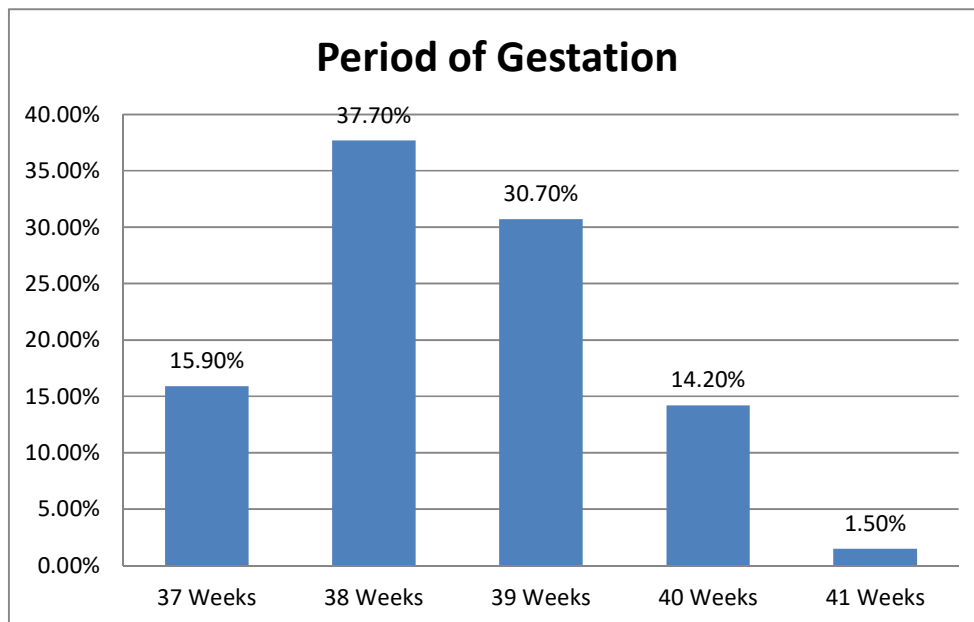
Graph 1: Mode of Delivery of study participants

Period of Gestation

Majority of the study participant’s gestation age was 38 weeks (n=377, 37.7%), followed by 30.7% (n=307) at 39 weeks, 15.9% (n=159) at 37 weeks, 14.2% (n=142) at 40 weeks and 1.5% (n=15) at 40 weeks (Table 2) (Figure 2).

Table 3: Distribution of study participants based on gestational age

PERIOD OF GESTATION	NUMBER (n=1000)	PERCENTAGE
37 Weeks	159	15.9%
38 Weeks	377	37.7%
39 Weeks	307	30.7%
40 Weeks	142	14.2%
41 Weeks	15	1.5%



Graph 2: Period of Gestation

Birth weight

Mean birth weight of the newborns was 2.75 ± 0.31 Kg. The minimum and maximum weight of the newborns were 1.5 and 4 Kg respectively (Table 3).

Table 4: Birth weight of newborns

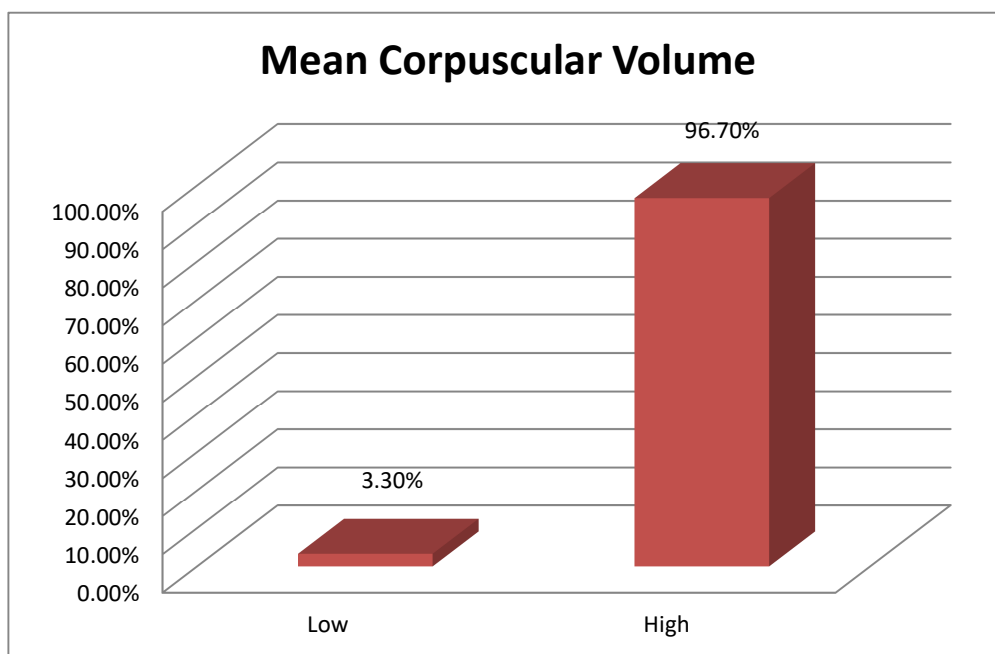
BIRTH WEIGHT	NUMBER (n=1000)
Mean	2.75 Kg
Standard Deviation	0.31 Kg
Minimum	1.5 Kg
Maximum	4 Kg

Mean Corpuscular volume

Among the study participants, prevalence of mean corpuscular volume of 95 and below was 3.3% (n=33) (Table 4) (Figure 3).

Table 5: Level of mean corpuscular volume among study participants

MEAN CORPUSCULAR VOLUME	NUMBER (n=1000)	PERCENTAGE
Low (95 and below)	33	3.3%
High (More than 95)	967	96.7%



Graph 3: Incidence of low and high Mean Corpuscular Volume among study participants

Low MCV Subset

Prevalence of low MCV was 3.3% (n=33). However, a total of 4 study participants were lost to follow up with one mortality among them. Hence the number of study participants with low MCV for further analysis was n=29.

Prevalence of α -thalassemia

Among the low MCV subset (n=29), the prevalence of α -thalassemia was 44.8% (n=13).

The overall prevalence of α -thalassemia among the study participants (n=1000) was 1.3%.

Comparison of microcytosis with maternal factors

Among the study participants, maternal factors like fetomaternal hemorrhage, jaundice, history of blood transfusion, gall stones, splenectomy, and hemoglobinopathies were compared with the microcytosis. Microcytosis was significantly associated with fetomaternal hemorrhage (p=0.033) (Table 5).

Table 6: Comparison of microcytosis with maternal factors

MATERNAL FACTORS	MICROCYTOSIS		P VALUE
	YES (n=33)	NO (n=967)	
Feto-maternal hemorrhage			
Yes	1 (3%)	0 (0%)	0.033
No	32 (97%)	967 (100%)	
Jaundice			
Yes	4 (12.1%)	157 (16.2%)	0.365
No	29 (87.9%)	810 (83.8%)	
H/o Blood transfusion			
Yes	0 (0%)	4 (0.4%)	0.874
No	33 (100%)	963 (99.6%)	
Gall Stones			
No	33 (100%)	967 (100%)	NA
Splenectomy			
No	33 (100%)	967 (100%)	NA
Hemoglobinopathies			
Yes	0 (0%)	1 (0.1%)	0.967
No	33 (100%)	966 (99.9%)	

HPLC results

Among the individuals who were not diagnosed with α -thalassemia (n=16), a high-performance liquid chromatography was done (No α -thalassemia subset). High-performance liquid chromatography identified one study participant with β -thalassemia trait (6.25%) and one study participant with $\delta\beta$ -thalassemia trait (6.25%) (Table 6) (Figure 4).

Table 7: HPLC results

HPLC	NUMBER (n=16)	PERCENTAGE
β -thalassemia trait	1	6.25%
$\delta\beta$ -thalassemia trait	1	6.25%
Normal	14	87.5%

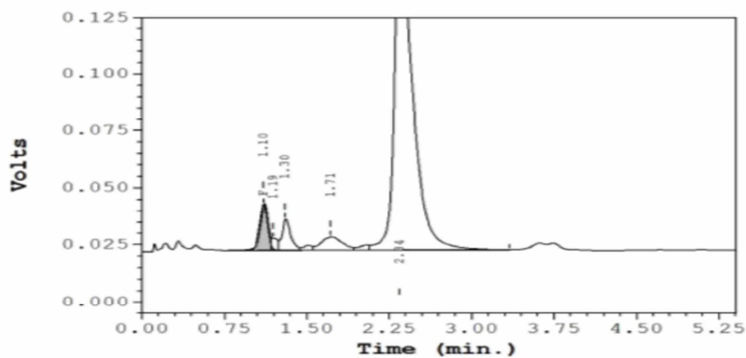
Peak Name	Calibrated Area %	Area %	Retention Time (min)	Peak Area
F	5.6*	---	1.10	102297
Unknown	---	0.9	1.19	17601
P2	---	4.0	1.30	74317
P3	---	4.1	1.71	77199
Ao	---	85.6	2.34	1606890

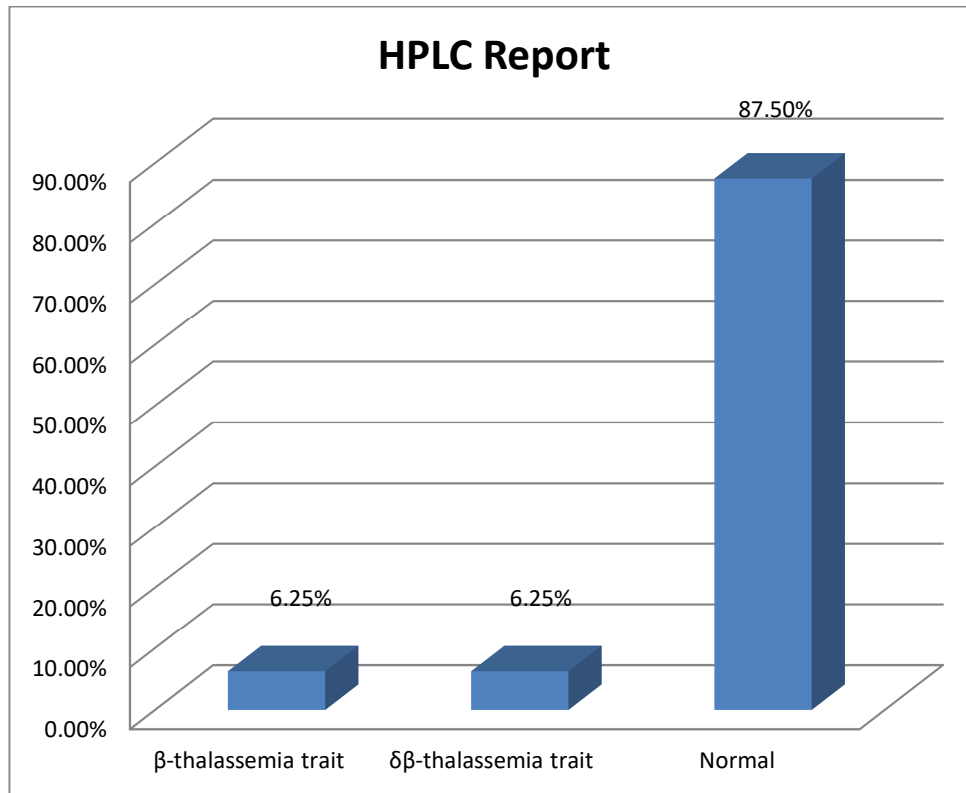
Total Area: 1,878,304

F Concentration = 5.6* %
A2 Concentration = %

*Values outside of expected ranges

Analysis comments:





Graph 4: HPLC Report

Correlation of RDW with iron deficiency anemia and iron profile

Red cell distribution width (RDW) was correlated with iron deficiency anemia and iron profile (serum iron, serum ferritin, transferrin saturation and total iron binding capacity). Among the study participants with no thalassemia identified, RDW was investigated. Normal RDW was reported in 15 (88.2%) individuals and 2 (11.8%) had high RDW values. RDW was not statistically significant with any of the variables in iron profile and iron deficiency anemia (Table 7).

Table 8: Correlation of RDW with iron deficiency anemia and iron profile

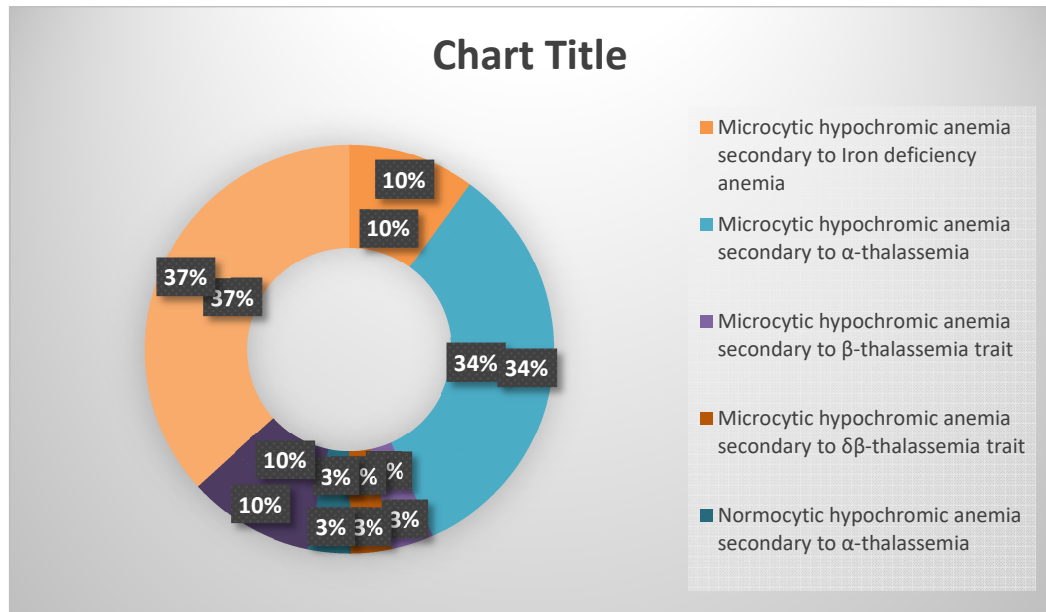
STUDY VARIABLE	RDW		P VALUE
	NORMAL (n=15)	HIGH (n=2)	
Iron Deficiency Anemia			
Yes	3 (20%)	0 (0%)	0.669
No	12 (80%)	2 (100%)	
Serum Iron			
Low	9 (60%)	1 (50%)	0.669
Normal	6 (40%)	1 (50%)	
TIBC			
Normal	5 (33.3%)	1 (50%)	0.596
Abnormal	10 (66.7%)	1 (50%)	
Transferrin Saturation	6 (40%)	0 (0%)	0.404
Normal	9 (60%)	2 (100%)	
Abnormal			
Serum Ferritin			
Normal	11 (73.3%)	2 (100%)	0.574
Abnormal	4 (26.7%)	0 (0%)	

Type of anemia as per MCV and final diagnosis

Among the study participants, microcytic hypochromic anemia due to iron deficiency anemia was diagnosed based on MCV and final diagnosis in 10% (n=3). Microcytic hypochromic anemia due to α -thalassemia based on MCV and final diagnosis in 33.3% (n=10) of the study participants. Microcytic hypochromic anemia due to β -thalassemia trait based on MCV and final diagnosis in 3.3% (n=1) of the study participants. Microcytic hypochromic anemia due to $\delta\beta$ -thalassemia trait based on MCV and final diagnosis in 3.3% (n=1) of the study participants. Normocytic hypochromic anemia due to α -thalassemia based on MCV and final diagnosis in 3.3% (n=1) of the study participants. Normocytic normochromic anemia due to α -thalassemia based on MCV and final diagnosis in 3.3% (n=1) of the study participants (Table 8).

Table 9: Association between type of anemia as per MCV and final diagnosis

Type	Number (n=30)
Microcytic hypochromic anemia secondary to Iron deficiency anemia	3 (10%)
Microcytic hypochromic anemia secondary to α -thalassemia	10 (33.3%)
Microcytic hypochromic anemia secondary to β -thalassemia trait	1 (3.3%)
Microcytic hypochromic anemia secondary to $\delta\beta$ -thalassemia trait	1 (3.3%)
Normocytic hypochromic anemia secondary to α -thalassemia	1 (3.3%)
Normocytic normochromic anemia secondary to α -thalassemia	3 (10%)
Normal	11 (36.7%)



Graph 5: Association between type of anemia as per MCV and final diagnosis

Comparison of any external congenital anomalies with MCV

Among the overall study participants (n=1000), prevalence of bilateral congenital talipes equinovarus (CTEV), left lower limb CTEV, preauricular sinus, and preauricular tags were found in 0.1% each (n=1 each). Prevalence of sacral pit among the study participants was 0.7% (n=7) (Table 9).

Table 10: Prevalence of congenital anomalies among study participants

Congenital anomalies	Number (n=1000)
Bilateral CTEV	1 (0.1%)
Left lower limb CTEV	1 (0.1%)
Preauricular sinus	1 (0.1%)
Preauricular tags	1 (0.1%)
Sacral pit	7 (0.7%)

Congenital anomalies and MCV

Among the study participants with low MCV, there were no congenital anomalies. Among high MCV, 1.1% (n=11) of the study participants had congenital anomalies (Table 10).

Table 11: Congenital anomalies in MCV

Congenital anomalies	MCV		P value
	Low (n=33)	High (n=967)	
Yes	0 (0%)	11 (1.1%)	0.690
No	33 (100%)	956 (98.9%)	

Comparison of jaundice at birth and MCV

Low and high MCV was compared with the status of jaundice at birth. MVC was found significant with the presence of jaundice at birth (p=0.028) (Table 11).

Table 12: Comparison of jaundice at birth and MCV

Jaundice at birth	MCV		P value
	Low (n=33)	High (n=967)	
Yes	10 (30.3%)	151 (15.6%)	0.028
No	23 (69.7%)	815 (84.4%)	

Association of jaundice at birth and α -thalassemia

No statistical significance was found when the presence of α -thalassemia was compared with jaundice at birth ($p=0.493$) (Table 12).

Table 13: Association of jaundice at birth and α -thalassemia

Jaundice at birth	α -thalassemia		P value
	Yes (13)	No (16)	
Yes	5 (38.5%)	5 (31.3%)	0.493
No	8 (61.5%)	11 (68.8%)	

Comparison of RBC Indices at birth (from cord blood) and after 6 months

RBC indices were investigated at birth using cord blood. RBC indices were repeated after 6 months for the infant. The results were compared at birth and after 6 months. MCV, MCHC (mean corpuscular hemoglobin concentration), and reticulocyte count were found to be statistically significant ($p=0.000$, 0.000 , and 0.002 respectively) (Table 13).

Table 14: Comparison of RBC indices at birth and 6 months

Variable	At birth	After 6 months	P value
MCV	90.03 \pm 5	71.96 \pm 9.9	0.000
MCH	30.36 \pm 2.67	29.36 \pm 4.01	0.232
MCHC	31.21 \pm 1.45	21.32 \pm 3.5	0.000
RDW	17.31 \pm 2.57	16.5 \pm 3.13	0.299
Reticulocyte count	2.0 \pm 1.0	1.24 \pm 0.53	0.002

Comparison of α -thalassemia with gestational age

Status of α -thalassemia was compared with the gestational age in the study participants. α -thalassemia of 38.5% (n=5) reported in 38 weeks, 46.2% in 39 weeks and 15.4% in 40 weeks of gestational age ($p=0.109$). (Table 14).

Table 15: Comparison of α -thalassemia with gestational age

Gestational age	α -thalassemia		P value
	Yes (13)	No (16)	
38 Weeks	5 (38.5%)	8 (50%)	0.109
39 Weeks	6 (46.2%)	2 (12.5%)	
40 Weeks	2 (15.4%)	6 (37.5%)	

Comparison of α -thalassemia with birth weight

α -thalassemia was compared with birth weight of the newborns. Mean birth weight of newborns with and without α -thalassemia were 2.91 ± 0.36 and 2.97 ± 0.28 respectively (Table 15).

Table 16: Comparison of α -thalassemia with birth weight of newborns

Birth weight	α -thalassemia		P value
	Yes (13)	No (16)	
Mean	2.91 ± 0.36	2.97 ± 0.28	0.316

RBC indices of cord blood between healthy neonates and α -thalassemia babies

RBC indices of cord blood were compared between α -thalassemia and healthy newborns. Mean value of MCV of α -thalassemia and healthy newborns were 90.95 ± 2.56 and 89.28 ± 6.58 respectively. Mean value of MCH of α -thalassemia and healthy newborns were 30.12 ± 1.60 and 30.82 ± 3.48 respectively. Mean value of MCHC of α -thalassemia and healthy newborns were 31.03 ± 1.13 and 31.26 ± 1.85 respectively. Mean value of RDW of α -thalassemia and healthy newborns were 17.80 ± 3.11 and 16.74 ± 1.25 respectively. Mean value of reticulocyte of α -thalassemia and healthy newborns were 1.95 ± 1.45 and 1.92 ± 0.60 respectively (Table 16).

Table 17: Comparison of RBC indices among α -thalassemia and healthy newborns using at birth

RBC Indices	α-thalassemia (n=13)	Healthy neonates (n=14)	P value
MCV	90.95 ± 2.56	89.28 ± 6.58	0.350
MCH	30.12 ± 1.60	30.82 ± 3.48	0.496
MCHC	31.03 ± 1.13	31.26 ± 1.85	0.745
RDW	17.80 ± 3.11	16.74 ± 1.25	0.290
Reticulocyte	1.95 ± 1.45	1.92 ± 0.60	0.949

Comparison of RBC indices between healthy neonates and α -thalassemia at 6 months

RBC indices were compared between α -thalassemia and healthy newborns at 6 months. Mean value of MCV of α -thalassemia and healthy newborns were 77.70 ± 7.98 and 71.57 ± 7.53 respectively. Mean value of MCH of α -thalassemia and healthy newborns were 29.99 ± 2.55 and 29.27 ± 4.26 respectively. Mean value of MCHC of α -thalassemia and healthy newborns were 23.12 ± 3.17 and 21.80 ± 2.97 respectively. Mean value of RDW of α -thalassemia and healthy newborns were 16.20 ± 2.35 and 15.56 ± 2.12 respectively. Mean value of reticulocyte of α -thalassemia and healthy newborns were 1.16 ± 0.44 and 1.07 ± 0.44 respectively (Table 17).

Table 18: Comparison of RBC indices among α -thalassemia and healthy newborns using at 6 months

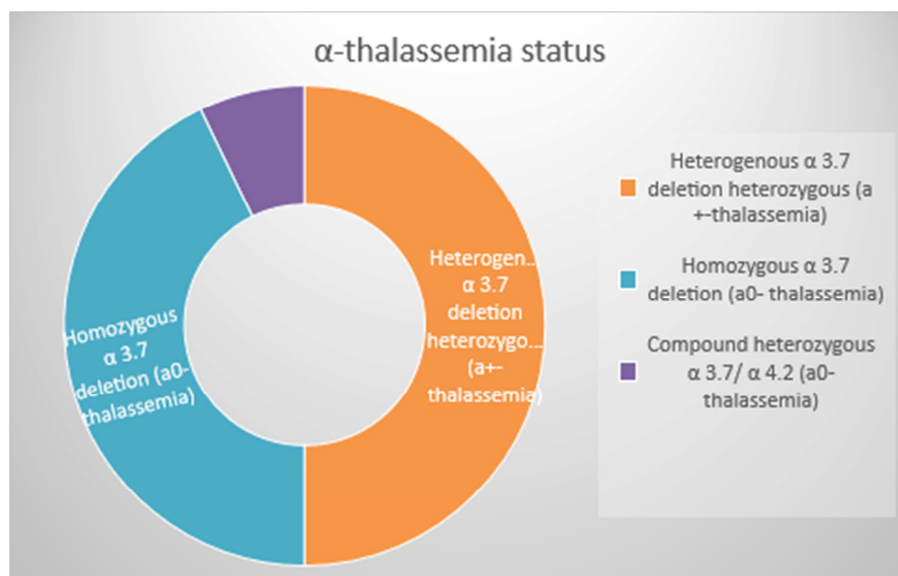
RBC Indices (at 6 months)	α-thalassemia (n=13)	Healthy neonates (n=11)	P value
MCV	77.70 ± 7.98	71.57 ± 7.53	0.074
MCH	29.99 ± 2.55	29.27 ± 4.26	0.362
MCHC	23.12 ± 3.17	21.80 ± 2.97	0.654
RDW	16.20 ± 2.35	15.56 ± 2.12	0.594
Reticulocyte	1.16 ± 0.44	1.07 ± 0.44	0.637

Prevalence of different α -globin status

Heterogenous $\alpha^{3.7}$ deletion heterozygous (a+-thalassemia) was present in 53.8% of the study participants. Homozygous $\alpha^{3.7}$ deletion (a0- thalassaemia) was present in 46.2% of the study population. Compound heterozygous $\alpha^{3.7}/\alpha^{4.2}$ (a0- thalassaemia) was present in 7.7% of the study population (Table 18).

Table 19: Prevalence of different α -globin status in the study population

α-thalassaemia status	Number (n=13)	Percentage
Heterogenous $\alpha^{3.7}$ deletion heterozygous (a+-thalassaemia)	7	53.8%
Homozygous $\alpha^{3.7}$ deletion (a0- thalassaemia)	6	46.2%
Compound heterozygous $\alpha^{3.7}/\alpha^{4.2}$ (a0- thalassaemia)	1	7.7%



Graph 6: Prevalence of different α -globin status in the study population

Comparison of mode of delivery and RBC indices at birth

Mean values of MCV in NVD and LSCS groups were 111.96 ± 5.94 and 111.34 ± 6.33 respectively. Mean values of MCH in NVD and LSCS groups were 36.64 ± 19.50 and 35.51 ± 2.76 respectively. Mean values of MCHC in NVD and LSCS groups were 31.63 ± 1.56 and 31.64 ± 2.31 respectively. Mean values of RDW in NVD and LSCS groups were 17.54 ± 2.17 and 18.24 ± 9.67 respectively. Mean values of RDW in NVD and LSCS groups were 2.24 ± 2.05 and 2.59 ± 1.62 respectively. All RBC indices were significantly associated with mode of delivery (Table 19).

Table 20: Association of mode of delivery and RBC indices at birth

RBC Indices	NVD (n=473)	LSCS (n=526)	P value
MCV	111.96 ± 5.94	111.34 ± 6.33	0.000
MCH	36.64 ± 19.50	35.51 ± 2.76	0.000
MCHC	31.63 ± 1.56	31.64 ± 2.31	0.000
RDW	17.54 ± 2.17	18.24 ± 9.67	0.000
Reticulocyte	2.24 ± 2.05	2.59 ± 1.62	0.000

DISCUSSION

A class of single-gene haemoglobin diseases with differing clinical severity is known as thalassemia. The majority of discussions centre on haemoglobin E (Hb E) and beta thalassemia's among the several forms of thalassemia's. Despite being the most prevalent genetic disorders, alpha thalassemia's are frequently overlooked due to the low number of clinically symptomatic individuals. Nevertheless, alpha thalassemia's with clinical symptoms may be lethal (Anwar S et al)⁶⁶.

Overall prevalence of thalassemia in our study was 1.5%. Prevalence of α -thalassemia, β -thalassemia trait and $\delta\beta$ -thalassemia trait in our study were 1.3%, 0.1% and 0.1% respectively. In a study conducted in South eastern part of China, the overall prevalence of thalassemia was 6.8%. Prevalence of α -thalassemia, β -thalassemia, concurrent α - and β -thalassemia's were 4.84%, 1.87% and 0.1% respectively. In a study conducted by Huang H et al⁶⁷, the prevalence of hemoglobinopathies was 0.26%. A retrospective study conducted by Zhan W et al⁴⁶, among pregnant women who are at risk of giving birth to new born with thalassemia's had gone through cordocentesis for diagnosis were included in the study. The foetal blood samples were tested. The distribution of α -thalassemia, β -thalassemia and healthy individuals were 67.87%, 19.68% and 12.45% respectively.

A study conducted from Central India by Upadhye D et al⁵⁶ stated the prevalence of single alpha gene deletion was 28.3% and Prevalence of deletion of two alpha genes was 21.5%. A study from USA conducted by Eldibany MM et al⁷⁰ showed a prevalence of α -thalassemia and β -thalassemia of 27.7% and 35% respectively. In a study conducted by Sa-Nga Pootrakul et al⁷³ in Thailand, prevalence

of β -thalassemia was 55.6%. Prevalence of α -thalassemia in a study conducted by Trent RJ et al⁷² in Sydney was 5.2%. Prevalence of α -thalassemia of 0.97% was reported in a study conducted in Pakistan by Malik SA et al⁷³.

Huang H et al⁶⁶ in a study described both hemoglobinopathy and thalassemia are hereditary illnesses resulting from abnormal haemoglobin; however, hemoglobinopathy is produced by a change in the shape of the globin peptide chain, which typically does not result in anaemia, and thalassemia is caused by decreased or absent synthesis of globin peptide chains.

Among α -thalassemia, β -thalassemia and healthy individuals, haematological parameters like hemoglobin, MCV, MCH, MCHC and RDW were significant (Zhang W et al).

Prevalence of low MCV in our study was 3.3%. In a study conducted by Guo R et al⁶⁸ in USA on infants had a prevalence of low MCV of 22.2%. Prevalence of low MCV in another study from USA by Schmaier AH et al⁶⁹ showed 4.5%. In a study conducted by Khalesi N et al⁴⁷ in Iran, low MCV was found in 2.3% of the neonates.

Although MCV is frequently used to assess anaemia in adults and children, it is not as often employed in the NICU. Because foetal haemoglobin lacks beta chains, beta thalassemia, a major cause of microcytosis in adults and children, does not present during the neonatal stage. Because a lack of iron during this crucial stage of brain development could result in long-term neurodevelopmental issues, congenital iron insufficiency is significant. Maternal history of iron deficiency, utero-placental insufficiency, diabetes, obesity, cigarette smoking, and psychological stress are all

risk factors for iron deficiency. Up to 17% of premature babies are known to have had iron insufficiency at delivery, making premature birth another significant risk factor for iron deficiency. A study conducted by Guo R et al⁶⁸ described significantly greater rate of microcytosis and potential iron shortage than other studies.

A study conducted by Upadhye D et al⁸⁵ in newborn stated alpha thalassemia majorly impacts the haemoglobin profile and RBC indices. Therefore, MCV and MCH levels could still be utilised to suspect the existence of alpha thalassemia in infants in laboratories without the capability for IEF or HPLC to identify cases with alpha thalassemia at birth. Checking for the alpha gene abnormalities in our community is crucial since, in India, where sickle cell disease is more widespread and alpha thalassemia is also common and a major ameliorating factor for the severity of the condition.

Our study compared the microcytosis with maternal factors like foeto-maternal hemorrhage, jaundice, history of blood transfusion, gall stones, splenectomy and hemoglobinopathies. Microcytosis was significantly associated with foeto-maternal hemorrhage ($p=0.033$). To our knowledge, no studies showed this comparison.

Neonatal low MCV with comorbidities

Mean values of MCV in NVD and LSCS groups were 111.96 ± 5.94 and 111.34 ± 6.33 respectively. Mean values of MCH in NVD and LSCS groups were 36.64 ± 19.50 and 35.51 ± 2.76 respectively. Mean values of MCHC in NVD and LSCS groups were 31.63 ± 1.56 and 31.64 ± 2.31 respectively. Mean values of RDW in NVD and LSCS groups were 17.54 ± 2.17 and 18.24 ± 9.67 respectively. Mean

values of RDW in NVD and LSCS groups were 2.24 ± 2.05 and 2.59 ± 1.62 respectively. All RBC indices were significantly associated with mode of delivery. In a study done by Samantaray et al⁷⁴, RBC indices value were significantly higher value in normal vaginal delivery groups on comparison with cesarean section groups. Only reticulocyte and normoblast were significantly higher in cesarean section group when compared to the normal vaginal delivery group. A study done by Redzko et al⁷⁵ found that Mode of delivery had an impact on WBC, hemoglobin, hematocrit, RBC distribution, platelets and nucleated RBCs.

A study done by Nwannadi et al⁷⁶ stated that Umbilical cord blood serves as a valuable source of hematopoietic stem cells for transportation. It also provides an excellent specimen for neonatal investigations and can be used as an alternative to blood transfusions, especially in children. The analysis of umbilical cord blood is widely accepted for obtaining information on genetic defects, hematological disorders, and infections in new born. These disorders include sickle cell disease, thalassemia's, thrombocytopenia, and leukocytosis.

Prevalence of α -thalassemia in our study was 1.3%. Prevalence of β -thalassemia trait and $\delta\beta$ -thalassemia trait were 0.1% in each group. The overall prevalence of α -thalassemia, β -thalassemia and $\delta\beta$ -thalassemia were 7.88%, 2.21% and 0.48% respectively in a study done by Lai K et al⁷⁷. A study conducted by Fathi et al. stated prevalence of α -thalassemia was 3.3%.

Types of α -thalassemia

In our study, $-\alpha^{3.7}$ (with deletion heterozygous (α^+ -thalassemia)), $-\alpha^{3.7}$ (deletion (α^0 - thalassemia)) and $-\alpha^{4.2}$ were studied. Prevalence was 53.8%, 46.2% and 7.7%

respectively. In another study done by Lai K et al⁷⁷, the prevalence of these groups was 1.59%, 0.54% for $-\alpha^{3.7}$ and $-\alpha^{4.2}$ groups (Lai K et al). Common gene deletion of 3.7 single gene deletion was reported as 42.4% in a study conducted by Fathi et al⁷⁸ in Iran.

In a study done by Alauddin H et al⁸¹ tested with umbilical cord blood of newborn showed 43.4% of α -thalassemia with 36 positives among 83 study participants. Among the study participants, one $\alpha\alpha/\alpha^{4.2}$ genotype and two $\alpha\alpha/ -\alpha^{3.7}$ was found (Alauddin H et al). In a study conducted in Bangladesh by Anwar S et al⁶⁶ showed 4.16% (n=16) of the study population with homozygous deletions ($-\alpha^{3.7}$). A total of 11.77% (n=48) of the study participants had a heterogenous deletions. Among the heterogenous deletions, prevalence of $-\alpha^{3.7}$, $--^{SEA}$, $-\alpha^{4.2}$, $--^{MED}$, $--^{THAI}$ were 4.41%, 4.41%, 1.25% 0.98% and 0.74% respectively. Two individuals (0.59%) had a compound heterogenous deletion. The compound heterogenous deletion was $-\alpha^{3.7}/ -\alpha^{4.2}$.

Single α -globin gene defect was found in 17.7% of the study participants. Among the single α -globin gene defect, $\alpha\alpha/ -\alpha^{3.7}$ was found in 14.1% (n=80), $\alpha\alpha/ -\alpha^{4.2}$ in 0.9% (n=5) and $\alpha\alpha/ \alpha^{CS}\alpha$ in 2.7% (n=15) of the study participants. Two α -globin gene defect was found in 5.8% of the study participants. Among them, $-\alpha^{3.7}/ -\alpha^{3.7}$ was found in 0.7% (n=4), $-\alpha^{3.7}/ \alpha^{CS}\alpha$ in 0.2% (n=1) and $\alpha\alpha/--^{SEA}$ in 4.9% (n=28) of the study participants. Hb H disease with 0.9% (n=5) with $--^{SEA}/ -\alpha^{3.7}$ and 0.4% (n=2) with $--^{SEA}/ \alpha^{CS}\alpha$ was found. Two patients (0.4%) with $\alpha\alpha/ -\alpha^{Q-Thailand}$ was reported in a study conducted by Charoenkwan P et al⁸².

A retrospective analysis study from Malaysia by Ahmad et al⁸³ across different ethnic groups of the country showed 51.2% of α -thalassemia. In this study, eight study participants reported with deletions and mutations. Three double gene deletions (with -SEA, -THAI and -FIL), single gene deletions in two study participants ($\alpha^{-3.7}$, $-\alpha^{-4.2}$).

In a study conducted by Upadhye D et al⁸⁵ in Central India, among 265 newborns, 102 normal newborns, 57 sickle homozygous newborns and 106 sickle heterozygous newborns were identified. Among the study participants, 28.3% had single α -gene deletion ($-\alpha/\alpha$). In this, 21.5% had two α -gene deletions ($-\alpha/-\alpha$). Among the study participants, 33% had sickle heterozygous and 71.9% sickle homozygous newborns had normal genotype. Deletion of $-\alpha/-\alpha$ was found in 28.3% of sickle heterozygous and 7% sickle homozygous individuals.

Homozygous α^+ thalassemia ($-\alpha^{3.7}$ deletion) was found in 60% (n=118) of the study participants in Omani neonates. $\alpha^{\text{T-Saudi}}$ in heterozygous state was found in 12 study participants. Others were α^+ thalassemia heterozygotes (with majority of $-\alpha^{3.7}$ deletions with few $-\alpha^{4.2}$ deletions) were found in a study conducted by Alkindi S et al⁸⁴.

In a study conducted by Kyriacou K et al²⁷, among 485 study participants, 10.6% had α -globin gene abnormalities. $-\alpha^{3.7}$ deletions was found in 7.7% (n=75) of the study population accounting to most common type of abnormality. Prevalence of $\alpha^{\text{PA-2}}\alpha$ was 0.2% (n=2). Prevalence of $\alpha^{-5\text{nt}}\alpha$ was 0.8% (n=8). Prevalence of $-\alpha^{\text{MED-1}}$ was 0.5% (n=5) and $-\alpha^{20.5}$ was 0.3% (n=3). Prevalence of compound $\alpha\alpha\alpha$ was 1% (n=10).

Comparison of MCH between maternal and cord blood showed significant difference (Timilsina et al⁷⁹). In our study, mean value of MCV of α -thalassemia and healthy newborns were 77.70 ± 7.98 and 71.57 ± 7.53 respectively. Mean value of MCH of α -thalassemia and healthy newborns were 29.99 ± 2.55 and 29.27 ± 4.26 respectively. Mean value of MCHC of α -thalassemia and healthy newborns were 23.12 ± 3.17 and 21.80 ± 2.97 respectively. Mean value of RDW of α -thalassemia and healthy newborns were 16.20 ± 2.35 and 15.56 ± 2.12 respectively. Mean value of reticulocyte of α -thalassemia and healthy newborns were 1.16 ± 0.44 and 1.07 ± 0.44 respectively. No significant difference among cord blood and blood at 6 months reports.

Literature on congenital anomalies in low MCV of cord blood in newborns were limited.

A study done by Morton⁸⁰ from New Zealand found that prevalence of Iron deficiency was 7% of the study population. In our study, 1.5% of the study population had iron deficiency anemia.

LIMITATIONS

There are a few limitations in our study. Lower sample size of the subset groups. This study is a single center study. A bigger cohort study with multiple centers should be planned to get a better result which can be generalized in the population

CONCLUSION

Cord blood was useful in screening for α -thalassemia in the present study. Prevalence of microcytosis in the study was 3.3% in our study. Prevalence of α -thalassemia was 1.3% in the study. Prevalence of homozygous, heterozygous and compound heterozygous mutations of α -thalassemia were 53.8%, 46.2%, and 7.7% respectively. Prevalence of β -thalassemia trait and $\delta\beta$ -thalassemia trait in our study were 0.1% and 0.1% respectively. Prevalence of iron deficiency anaemia in our study was 10%. Maternal factor fetomaternal haemorrhage was significantly associated with microcytosis. All RBC indices were significantly associated with mode of delivery.

SUMMARY

The study was conducted over a period of 1 year between November 2022 and October 2023 at KLES Dr Prabhakar Kore Hospital. All newborn delivered in Labor rooms of DR.KLE's Prabhakar kore Charitable and MRC Hospital are taken in the study after taking informed consent. The UCB (Umbilical Cord Blood) samples were obtained using K2-EDTA vacutainers with the placenta still in the uterus right after clamping the cord and separating the infant. After extraction, the samples were tested for complete hemogram. Mean corpuscular volume, Mean Corpuscular Hemoglobin, Red blood cell distribution width(RDW) and Reticulocyte count was noted. Neonates with red blood cell indices; MCV<95 and/or MCH<30 were subjected for alpha thalassemia gene deletion study by MLPA[Multiplex Ligation Dependent Probe Amplification]. Neonates with negative MLPA were followed up at the age of 6months for repeat complete hemogram, High Performance Liquid Chromatography [HPLC] to look for other variants of haemoglobinopathies. Those with negative MLPA and negative HPLC were evaluated for other causes of microcytosis by serum iron studies and bone marrow iron staining accordingly.

- The data obtained was tabulated into Microsoft Excel sheets and processed using Data was be analysed by using coGuide software, V.1.0⁸⁶
- The study included 1000 newborn whose cord blood was subjected to complete hemogram
- 33 out of 1000 were screened MCV of less than 95
- 29 out of 33 are analysed for alpha thalassemia gene analysis by MLPA method

- 13 out of 29 are tested positive for gene analysis(44.8%).
- 7 out of 13 are Heterogenous $\alpha^{3.7}$ deletion heterozygous (α^+ -thalassemia) was present in 53.8% of the study participants
- 6 out of 13 are Homozygous $\alpha^{3.7}$ deletion (α^0 - thalassemia) was present in 46.2% of the study population.
- 1 out of 13 are compound heterozygous Compound heterozygous $\alpha^{3.7}/\alpha^{4.2}$ (α^0 - thalassemia) was present in 7.7% of the study population
- 1 out of 16 was resulted beta thalassemia trait (6.25%)
- 1 out of 16 was resulted delta beta thalassemia trait(6.25%)
- 4 out of 29 are resulted iron deficiency anemia
- 3 were lost to follow up and 1 expired.
- Prevalence of alpha thalassemia among low MCV was 44.8%
- Overall prevalence of alpha thalassemia among study subjects was 1.3%
- Microcytosis was significantly associated with fetomaternal hemorrhage
- RDW was not statistically significant with any of the variables in iron profile and iron deficiency anemia
- MVC was found significant with the presence of jaundice at birth
- No statistical significance was found when the presence of α -thalassemia was compared with jaundice at birth
- A statistical significance was noted when MCV, MCHC and reticulocyte count were compared at birth and after 6 months.
- No association between alpha thalassemia and birth weight
- A Statistical significance was found when RBC indices of cord blood were compared between α -thalassemia and healthy newborns

- A Statistical significance was found RBC indices were compared between α -thalassemia and healthy newborns at 6 months
- RBC indices were significantly associated with mode of delivery

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ANNEXURE-I

KAHERs JNMC BELAGAVI

INFORMED CONSENT FORM

**“TO STUDY UTILITY OF UMBILICAL CORD BLOOD RED BLOOD CELL
MEAN CORPUSCULAR VOLUME AS A SCREENING FOR ALPHA
THALASSEMIA”**

Name of Student/Principal Investigator: Dr.

Name of Guide/Co Investigators : Dr.

Mr./Mrs./Ms. _____, you are being requested to enroll your baby as a subject in a research study titled **TO STUDY THE UTILITY OF UMBILICAL CORD BLOOD RED BLOOD CELL MEAN CORPUSCULAR VOLUME AS A SCREENING FOR ALPHA THALASSEMIA** to be conducted by Dr _____, a post graduate student in Department of Paediatrics at J.N. Medical College, Belgaum, is the principal investigator of this study, under the guidance of Dr. _____ Professor, Department of Paediatrics, and Dr. _____ Paediatric haemato-oncologist, J.N. Medical College, Belagavi.

Objective: To determine the usefulness of cord blood MCV in screening for alpha thalassemia

Introduction: : Haemoglobinopathies are inherited disorders of red blood cells that possess significant cause of morbidity and mortality, impose a heavy burden on family. The study aims to assess the utility of umbilical cord blood red blood cell MCV for screening of alpha thalassemia

Explanation of procedure: In this study, we will need to give umbilical cord blood sample of 3ml in EDTA vacutainer. If you agree to participate, then only cord blood sample is taken. Sample will be sent for complete hemogram investigation to look for RBC Mean Corpuscular Volume indices. If the values are below the normal range, they will be educated regarding the importance of further confirmative test for alpha Thalassemia (alpha gene study). Newborn who follow-up for alpha gene study and result negative for the test should be followed up after 6 months. After 6 months, Babies who is on follow up, blood sample of 3ml in EDTA to be taken for complete hemogram investigation. If MCV is below the normal range, baby will be followed up for further test i.e., High Performance liquid chromatography [HPLC] and serum ferritin level test.

Withdrawal from participation in the study: Participation in this study is voluntary. You will be free to decide whether to participate in this study or continue participation once enrolled. In case you decide to withdraw your participation, you are free to do so. However, please convey the decision to the principal investigator.

Possible benefits from participating in the study: You will/will not have nor get any benefits by participating in this study. The data gathered will help the population at large.

Possible risks from participating in the study: There are no risks involved in participating in this study.

Privacy and confidentiality: The information collected from you will be coded, to prevent any person from identifying you. Your identity will never be revealed. The data collected from you will be kept confidential and only processed or aggregated data will be used for publication.

Financial incentives: You will not receive any payment for participating in this study.

Authorization for publication of aggregated data: Results obtained after processing of the aggregated data will be published for scientific purposes and or presented to scientific groups. However, your identity will never be revealed.

Questions: In case of any questions with regard to this study, you are free to contact “Dr. _____” If you have any question or complaints with regard to your right as study participant you may contact Dr Harsha Hegde, Chairperson, Ethical committee of JNMC, 0831-2473777 Extension 4052.

Legal rights: By signing this consent form, we are not waiving any of your legal rights.

CONSENT STATEMENT

I am making a voluntary decision to participate in the study “**TO STUDY UTILITY OF UMBILICAL CORD BLOOD RED BLOOD CELL MEAN CORPUSCULAR VOLUME AS A SCREENING FOR ALPHA THALASSEMIA**”. My signature below indicates that I have decided to participate and I have read the information provided above or the information provided above has been read to me in the language that I understand best. I was given the opportunity to ask questions and that they have been answered to my satisfaction.

Name of the parent:

Signature or left thumb impression of the parent:

Name of the baby:

Name of the investigator:

Signature of the investigator:

ANNEXURE-II

PROFORMA

Baby of

Address

Ph No.

IP No.

Date of Delivery

Mode of Delivery

Period of Gestation

birth wt.

Foeto maternal Haemorrhage	Yes	No
Jaundice	Yes	No
Any family h/o Blood transfusion	Yes	No
gallstones	Yes	No
splenectomy	Yes	No
hemoglobinopathies	Yes	No

	at birth	6 months of age
Jaundice		
any external congenital anomaly		
MCV		
MCH		
MCHC		
RDW		
Reticulocyte count		
Peripheral smear		
Golf ball inclusions@6months		

HPLC report at 6months of age

HbA1	HbA2	Hbf	Other

final impression of HPLC report

alpha thalassemia gene deletion present/absent

any h/o blood transfusion or other ailments from birth to 6months of age.

S.ferritin	
S.Iron	
TIBC	
Transferrin saturation	

mode of delivery	period of gestation	birth weight	jaundice at birth	any external congenital anomaly in baby	FOETOMATERNAL-HAEMORRHAGE	jaundice in mother	family H/o blood transfusion	gall stones	splenectomy	hemoglobinopathies	MCV of cord blood	MCH of cord blood	MCHC of cord blood	RDW of cord blood	Reticulocyte count of cord blood	peripheral smear of cord blood	MCV after 6months of age	MCHC after 6months of age	MCH after 6months of age	RDW after 6months of age	Reticulocyte count after 6months of age	golf ball inclusion bodies	Peripheral smear after 6months of age	HbA1	HbA2
LSCS	38weeks	3.4kg	no	nil	no	no	no	no	no	no	94.2	34.2	34.1	15.8	1.4	lymphocytosis with eosinophilia	60	14.9	24.8	20	1.2	absent	microcytic hypochromic anemia with pencil cells+	96.2	1.6
LSCS	39weeks	2.8kg	no	nil	no	no	no	no	no	no	95	29.4	30.7	17.4	0.5	leucopenia	74.2	22.6	30.4	13.7	0.9	absent	normocytic normochromic	95.1	2.5
nvd	38weeks	2.8kg	no	nil	no	no	no	no	no	no	75.5	36.1	28.1	18.3	1.2	normal	71.2	25.5	35.9	17.5	1.5	absent	microcytic hypochromic anemia	95.6	2.7
LSCS	38weeks	3.5kg	yes	nil	no	no	no	no	no	no	87	28.8	33.1	18	4.6	normal	82	24	29.3	14.8	1.1	absent	normocytic hypochromic		
LSCS	38weeks	2.8kg	no	nil	no	no	no	no	no	no	92.3	31.4	30.8	17.5	1.8	microcytic hypochromic anemia	84.5	26.1	30.2	15.2	1.2	absent	normocytic normochromic	94.1	2.4
nvd	38weeks	3.3kg	yes	nil	no	no	no	no	no	no	91.7	32.8	32.9	14.3	1	normal	74.7	23.5	28.8	15.8	0.8	absent	normocytic normochromic		
nvd	39weeks	2.9kg	yes	nil	no	no	no	no	no	no	92.2	28.2	29.3	17.4	1.2	normocytic hypochromic anemia with leucopenia	81	25.3	31.2	19.9	0.9	absent	normocytic normochromic		
LSCS	40weeks	3.3kg	no	nil	no	no	no	no	no	no	88.4	27.4	31	25.7	1.4	thrombocytopenia	61.2	19.2	31.4	17	1.2	absent	microcytic hypochromic		
LSCS	40weeks	2.7kg	yes	nil	no	no	no	no	no	no	92.7	31.5	32.5	14.2	2.5	normocytic hypochromic anemia with leucopenia	78	20.2	25.9	13.2	2.6	absent	microcytic hypochromic	93.7	non quantifiable
nvd	40weeks	3.4kg	no	nil	no	no	no	no	no	no	88.1	27.4	31.9	16.2	1.9	microcytic hypochromic anemia	86.2	27.9	32.3	13.2	1.2	absent	normocytic normochromic	94.2	2.1
LSCS	40weeks	3.1kg	no	nil	no	no	no	no	no	no	91	30.9	34	15.6	1.2	microcytic hypochromic anemia	82.5	21.5	28.3	14.8	0.9	absent	normocytic hypochromic	94.1	2.5
LSCS	38weeks	2.9kg	no	nil	no	no	no	no	no	yes- mother- beta thalassemia trait	90	29.8	30.1	14.9	1.9	normocytic normochromic anemia	76.9	21.1	27.4	14.2	0.8	absent	normocytic hypochromic	95.7	2.5
LSCS	38weeks	2.8kg	no	nil	no	no	no	no	no	no	93.3	31.2	31.8	17.8	1.5	normal	81.9	22.9	28	16	1.2	absent	normocytic normochromic	93.7	2.7
LSCS	39weeks	2.6kg	no	nil	no	no	no	no	no	no	95	31.5	32	15.6	2.2	normal	84	24.7	29.4	16.5	0.9	absent	normochromic normocytic	95	2.7
LSCS	38weeks	2.9kg	yes	nil	no	no	no	no	no	no	91.2	32.1	29.6	17.1	3.2	normocytic hypochromic	80.7	25	32.2	15.2	1	absent	normocytic normochromic	94.8	2.2
LSCS	38weeks	2.8kg	no	nil	no	no	no	no	no	no	91	31.5	30.6	18.7	1.8	normocytic hypochromic anemia	74.6	21.9	29.3	16.7	0.5	absent	normocytic hypochromic	94.8	2.7

LSCS	38weeks	2.8kg	yes	nil	no	no	no	no	no	no	106.2	33.9	32	17.9	3.5	normal							
nvd	40weeks	2.7kg	no	nil	no	no	no	no	no	no	112.5	35.6	31.6	16.2	3	normal							
LSCS	38weeks	2.8kg	no	nil	no	no	no	no	no	no	114.3	36.1	31.6	22.4	2.8	leucopenia							
LSCS	40weeks	3.1kg	no	nil	no	no	no	no	no	no	110.8	34.3	31	16.8	4.9	microcytic normochromic anemia							
nvd	40weeks	2.6kg	no	nil	no	no	no	no	no	no	106.1	33.8	31.9	18.7	2.6	normal							
nvd	38weeks	2.4kg	no	nil	no	no	no	no	no	no	120.3	37.5	31.2	18.7	3.4	normal							
nvd	37weeks	2.2kg	no	nil	no	no	no	no	no	no	111.9	32.9	29.4	20.5	3	normal							
LSCS	39weeks	2.9kg	no	nil	no	no	no	no	no	no	104	35	33.7	16.5	3.3	normal							
LSCS	40weeks	2.7kg	no	nil	no	no	no	no	no	no	119.6	37.2	31.1	21.2	3.4	neutrophilia							
LSCS	40weeks	2.9kg	no	nil	no	no	no	no	no	no	115.1	36.2	31.4	17.4	2.9	neutrophilia							
LSCS	39Weeks	3.3kg	no	nil	no	no	no	no	no	no	106.6	33.5	31.4	16.6	0.7	neutrophilia							
LSCS	40weeks	2.7kg	no	nil	no	no	no	no	no	no	113.2	38.1	33.7	19.8	5.8	normal							
LSCS	39weeks	2.5kg	no	nil	no	no	no	no	no	no	108.1	34.8	32.2	16	3.5	normal							
LSCS	40weeks	3kg	no	nil	no	no	no	no	no	no	112.3	36	29.7	17	4.3	normal							
LSCS	38weeks	2.7kg	no	nil	no	no	no	no	no	no	104.2	34.2	32.8	18.2	2.9	leucopenia							
nvd	40weeks	3kg	no	nil	no	no	no	no	no	no	118	36.1	30.6	15.9	3.6	normal							
LSCS	39weeks	2.8kg	no	nil	no	no	no	no	no	no	114.6	34.7	30.3	18.2	4.2	normal							
nvd	38weeks	2.5kg	no	nil	no	no	no	no	no	no	114.7	35.8	31.2	17	3.9	normal							
LSCS	38weeks	2.9kg	no	nil	no	no	no	no	no	no	109	34.6	31.7	19.3	4	normal							
LSCS	39Weeks	3kg	no	nil	no	no	no	no	no	no	116.1	37	31.9	19.8	2.8	normal							
LSCS	37weeks	2.7kg	no	nil	no	no	no	no	no	no	116.2	36.8	31.6	19.3	3.1	leucopenia							
LSCS	39Weeks	3kg	no	nil	no	no	no	no	no	no	113.8	36.9	32.4	19.8	3.2	normal							
LSCS	38weeks	3.1kg	no	nil	no	no	no	no	no	no	119.7	36.7	30.7	18.8	3.5	normocytic normochromic anemia							
LSCS	38weeks	2.7kg	no	nil	no	no	no	no	no	no	111.4	34.5	31	18	3.1	normal							
nvd	39Weeks	2.6kg	no	nil	no	no	no	no	no	no	115	36	31.2	18.3	3	normal							
nvd	40weeks	3.1kg	no	nil	no	no	no	no	no	no	110.8	34.3	31	16.8	4.9	microcytic normochromic anemia							
nvd	39Weeks	2.6kg	no	nil	no	no	no	no	no	no	104.6	35	33.4	17.6	3.5	normal							
nvd	38weeks	2.9kg	no	nil	no	no	no	no	no	no	108.1	32.9	30.4	23.2	2.8	normal							
LSCS	38weeks	2.9kg	no	nil	no	no	no	no	no	no	116	38.9	30.9	18.2	4.7	normocytic normochromic anemia							
LSCS	39Weeks	2.7kg	no	nil	no	no	no	no	no	no	115.1	36.2	31.5	16.9	5	normocytic normochromic anemia							
LSCS	38weeks	2.5kg	yes	nil	no	no	no	no	no	no	111.5	35.4	31.7	17.4	3.2	leucopenia							
LSCS	37weeks	2.1kg	no	nil	no	no	no	no	no	no	106.8	34	31.8	18	3.4	esinophilia with thrombocytopenia							
LSCS	38weeks	3.3kg	no	nil	no	no	no	no	no	no	111.5	35.5	31.9	17.6	3.3	normocytic normochromic anemia							
LSCS	38weeks	2.2kg	no	nil	no	no	no	no	no	no	108.8	35.8	30.9	15.2	2.8	microcytic normochromic anemia							
LSCS	41weeks	2.5kg	no	nil	no	no	no	no	no	no	115.1	36.6	31.8	16	3.7	normal							
LSCS	40weeks	2.5kg	no	nil	no	no	no	no	no	no	107.2	33.2	30.9	16.9	2.2	normal							
LSCS	37weeks	2.9kg	no	nil	no	no	no	no	no	no	110.5	34.5	31.3	19.1	4	normocytic normochrmic anemia							
nvd	38weeks	2.7kg	no	nil	no	no	no	no	no	no	113.7	35.3	31	22.3	4.7	normocytic normochromic anemia							
LSCS	39weeks	2.7kg	no	nil	no	no	no	no	no	no	109.6	30.5	30.8	24.2	4.6	normal							
LSCS	37weeks	2.6kg	no	sacral pit present	no	no	no	no	no	no	106.7	34.6	32.4	17.4	4.4	normal							
nvd	40weeks	2.5kg	no	nil	no	no	no	no	no	no	102.8	32	31.2	14.5	3.1	normal							
nvd	39weeks	2.8kg	no	nil	no	no	no	no	no	no	113.3	35.6	31.4	18.5	2.8	neutrophilia							
nvd	39weeks	3.1kg	no	nil	no	no	no	no	no	no	104.1	32.8	31.5	19.2	4	normal							
LSCS	38weeks	2.9kg	no	nil	no	no	no	no	no	no	108.5	35.9	30.3	18.9	3.1	normal							
LSCS	38weeks	2.6kg	no	nil	no	no	no	no	no	no	118.2	35.9	30.3	18.9	5.1	normal							

nvd	39weeks	2.8kg	no	nil	no	no	no	no	no	no	102.5	34.8	33.9	17.4	2	normal						
LSCS	37weeks	2.9kg	no	nil	no	no	no	no	no	no	112.9	39.7	35.2	14.6	1	pancytopenia						
LSCS	37weeks	2.8kg	no	nil	no	no	no	no	no	no	115.2	39	33.9	14.9	0.8	lymphocyt						
LSCS	40weeks	2.5kg	no	nil	no	no	no	no	no	no	116	37.1	32	16.1	0.8	normal						
LSCS	39Weeks	3kg	no	nil	no	no	no	no	no	no	115.8	37.2	32.1	15.8	0.6	normal						
nvd	38weeks	2.9kg	no	nil	no	no	no	no	no	no	103.5	34.8	33.6	15.4	0.8	normal						
LSCS	40weeks	2.8kg	no	nil	no	no	no	no	no	no	113.3	36.3	32.1	17.3	1.5	normocytic hypochromic anemia						
nvd	40weeks	2.9kg	no	nil	no	no	no	no	no	no	116.6	36.6	31.4	17	1.9	normal						
nvd	38weeks	2.5kg	no	nil	no	no	no	no	no	no	114	33.6	31.5	16.1	1.5	normal						
nvd	40weeks	2.5kg	no	nil	no	no	no	no	no	no	112.4	32.5	31.6	14.8	3.4	normal						
nvd	38weeks	2.7kg	no	nil	no	no	no	no	no	no	113.9	35.6	31.2	16.8	1.7	normal						
nvd	38weeks	2.9kg	no	nil	no	no	no	no	no	no	103	36	29.3	17.1	1.8	normal						
nvd	38weeks	3.3kg	no	nil	no	no	no	no	no	no	101.5	33.5	29.6	16.5	1.6	normal						
nvd	40weeks	3.4kg	no	nil	no	no	no	no	no	no	106.4	33.9	31.8	19.2	0.7	normal						
nvd	37weeks	3kg	no	nil	no	no	no	no	no	no	111.3	35.2	31.6	17.6	0.6	normocytic normochromic anemia						
LSCS	39Weeks	3.1kg	no	nil	no	no	no	no	no	no	115	35.6	30.9	16.3	1.3	normal						
nvd	41weeks	2.9kg	no	nil	no	no	no	no	no	no	111	31.2	31.5	14.5	1.2	normal						
nvd	41weeks	2.9kg	no	nil	no	no	no	no	no	no	112	333.5	30.6	15.7	1.5	normal						
nvd	39Weeks	3.1kg	no	nil	no	no	no	no	no	no	116	35.1	30.3	17.4	1.4	normal						
nvd	38weeks	2.5kg	no	nil	no	no	no	no	no	no	107.5	31.4	29.2	23.5	1.5	normocytic hypochromic anemia						
LSCS	38weeks	1.9kg	no	nil	no	no	no	no	no	no	105.6	34.1	31.3	18.9	2	normal						
LSCS	38weeks	1.5kg	no	nil	no	no	no	no	no	no	108.6	34.1	31.4	15.4	4.7	normal						
LSCS	38weeks	2.7kg	no	nil	no	no	no	no	no	no	109.9	34.6	31.5	18.6	3.9	normocytic normochromic anemia						
LSCS	40weeks	3kg	no	nil	no	no	no	no	no	no	109.4	35	32	16.8	1.4	normal						
LSCS	39weeks	2.5kg	yes	nil	no	no	no	no	no	no	109.9	34.9	31.8	16.7	1.9	normal						
nvd	40weeks	2.7kg	no	nil	no	no	no	no	no	no	116.2	35.9	30.9	18.6	1.8	normal						
nvd	40weeks	2.5kg	no	nil	no	no	no	no	no	no	111	32.1	31.7	18	2.2	normal						
nvd	37weeks	2.7kg	no	nil	no	no	no	no	no	no	114.6	36.3	31.7	19	0.5	normal						
nvd	38weeks	2.6kg	no	nil	no	no	no	no	no	no	110.5	32.9	29.8	17.4	1.5	normocytic normochromic anemia						
nvd	39Weeks	2.6kg	no	nil	no	no	no	no	no	no	104.1	34.1	32.7	16.8	1.2	normal						
LSCS	38weeks	2.8kg	no	nil	no	no	no	no	no	no	95	31.2	32.6	17	2.1	microcytic hypochromic anemia						
LSCS	39weeks	3.6kg	no	nil	no	no	no	no	no	no	110.7	37.5	33.9	15.5	1.2	normal						
nvd	39weeks	2.5kg	no	nil	no	no	no	no	no	no	118.7	38.1	32.1	16.7	1.3	normal						
LSCS	38weeks	2.75kg	no	nil	no	no	no	no	no	no	118.6	38.6	32.5	16.7	1.5	normal						
nvd	41weeks	2.8kg	no	nil	no	no	no	no	no	no	117.8	38.8	32.9	14.6	1.2	normocytic normochromic anemia						
LSCS	37weeks	2.5kg	no	nil	no	no	no	no	no	no	107.4	35.5	33	15.8	1	leucopenia						
nvd	37weeks	2.6kg	no	nil	no	no	no	no	no	no	109.1	36.1	33.1	15.1	1	leucopenia						
LSCS	39Weeks	3.2kg	no	nil	no	no	no	no	no	no	115.3	38.4	33.3	14.5	1	normal						
nvd	38weeks	2.9kg	no	nil	no	no	no	no	no	no	111.8	37.8	33.8	14.1	0.5	normal						
LSCS	37weeks	2.6kg	no	nil	no	no	no	no	no	no	119.1	38.5	32.3	19.3	3.6	normal						
nvd	40weekd	2.8kg	no	nil	no	no	no	no	no	no	98.3	30.7	31.3	22.9	3.8	normal						
nvd	38weeks	2.6kg	yes	nil	no	no	no	no	no	no	109.4	34.6	31.7	16.2	4	normal						
nvd	38weeks	2.3kg	no	nil	no	no	no	no	no	no	112.6	37.5	33.5	19.7	5	leucopenia						
nvd	40weekd	2.9kg	no	nil	no	no	no	no	no	no	108.6	33.6	32.7	16.7	2.7	normal						
LSCS	40weekd	2.7kg	no	nil	no	no	no	no	no	no	108.6	36.6	33.7	16.9	2.4	esinophilia with thrombocytopenia						
LSCS	39Weeks	2.7kg	no	nil	no	no	no	no	no	no	110.8	36.2	32.7	18.1	4.8	normal						
nvd	39Weeks	2.4kg	no	nil	no	no	no	no	no	no	111.6	37.3	33.5	18.6	4.6	neutrophilia with leucocytosis						

HbF		final HPLC report	gene	mutation	type of alpha thalassaemia	s.ferritin	s.iron	TIBC	Transferrin saturation	any loss to follow up or death	any H/o blood transfusions or other ailments from birth to 6months of age	final result
	Other											
0.9	1.3	normal	negative	aa/aa		6.01	46	548	8	nil	nil	iron deficiency anemia
0.9	1.5	normal	positive	a ^{3,7} deletion heterozygous	a+-thalassaemia	69.1	42	310	14	nil	nil	alpha thalassaemia
0.6	0	normal	negative	aa/aa		34.9	83	414	20	nil	nil	normal
			positive	a ^{3,7} deletion homozygous	a0- thalassaemia					nil	nil	alpha thalassaemia
2.5	1	normal	negative	aa/aa		76.4	82	372	22	nil	nil	normal
			positive	a ^{3,7} deletion heterozygous	a+-thalassaemia					nil	nil	alpha thalassaemia
			positive	a ^{3,7} deletion heterozygous	a+-thalassaemia					nil	nil	alpha thalassaemia
			positive	a ^{3,7} deletion homozygous	a0- thalassaemia					nil	nil	alpha thalassaemia
5.6	0.9	delta beta thalassaemia trait	negative	aa/aa		82.2	59	331	18	nil	nil	delta beta thalassaemia
2.5	1.2	normal	negative	aa/aa		72	81	372	22	nil	nil	normal
1.2	1.2	normal	negative	aa/aa		43.9	34	467	7	nil	nil	normal
0.6	1.2	normal	negative	aa/aa		67	77	324	24	nil	nil	normal
2.5	1.1	normal	negative	aa/aa		64	82	372	22	nil	nil	normal
2.3	nil	normal	negative	aa/aa		67.87	64	407	16	nil	nil	normal
1.8	1.2	normal	negative	aa/aa		78	50	432	12	nil	nil	normal
1.3	1.2	normal	negative	aa/aa		52	80	494	16	nil	nil	normal

