NESTROFT AS A SCREENING TOOL TO IDENTIFY BETA THALASSAEMIA TRAIT IN PAEDIATRIC CASES

Dissertation Submitted

FOR

M.D. DEGREE EXAMINATION

BRANCH - III PATHOLOGY

THE TAMILNADU DR.M.G.R. MEDICAL UNIVERSITY

TIRUNELVELI MEDICAL COLLEGE HOSPITAL

TIRUNELVELI

MARCH – 2010

CERTIFICATE

We hereby certify that the work embodied in the dissertation entitled **"Nestroft As A Screening Tool To Identify Beta Thalassaemia Trait In Paediatric Cases"** is a record of work done by **Dr. T.A. Santhi** in the Department of pathology, Tirunelveli Medical College, Tirunelvelli. During her Post Graduate Degree course in the period 2007 – 2009. This work has not previously formed the basis for the award of my degree or diploma.

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ACKNOWLEDGEMENT

On completion of my dissertation work, I take immense pleasure to acknowledge all those who have helped me to make it possible.

I express my profound sense of gratitude to respected **Dr. Sithy Athiya Munuvarah M.D.,** Professor and Head of Department of Pathology, Tirunelveli Medical College, Tirunelveli for suggesting this work for dissertation and her unstinted guidance, which has been the motive force in bringing forth this piece of work.

It gives me immense pleasure to sincerly thank **Dr. K. Swaminathan, M.D., Dr. K. Shantharaman, M.D., Dr. S. Vallimanalan, M.D., Dr. J. Suresh Durai, M.D., Dr. Arasi Rajesh, M.D., Dr. N. Jeyabalan, M.D.,** Readers in Pathology for their constant support and encouragement. I profusely thank all the Assistant Professors and tutors in the Department of Pathology for their valuable suggestions at every stage of this work.

I would like to place on record my thanks to the Professor, Department of Pediatrics for providing the cases for this study.

I am grateful to the Dean, Tirunelveli Meidcal College for allowing me to undertake this study.

It is my duty to thank my senior and junior colleagues for their help and support throughout the tenure of this work.

I thank all my family members for their encouragement and support during this study.

CONTENTS

INTRODUCTION

In India, there are around 20 million carrier cases of β-thalassaemia and around 8000-10,000 children are born every year with β-thalassaemia major, Lokeshwar M.R. Shah N, Kanakia N. et.al 2006. The birth of a thalassaemia child places considerable health and economic strain not only on the affected child and his/her family but also on the community.

Since, thalassaemia is a severe and incurable disease, emphasis has to be shifted from the treatment of an affected child to the prevention of such births in future. Identifying carriers for β- thalassaemia patients, thus plays an important role in preventing this, Verma IC et.al (1992).

β- thalassaemia trait presents with microcytic hypochromic blood picture similar to the picture of iron deficiency anemia and anemia of chronic diseases. The differentiation between these conditions becomes mandatory which usually relies on the measurement of serum ferritin, serum iron, total iron binding capacity and Hb A_2 levels.

These diagnostic methods are costly and hence constitute a significant burden on public health economy particularly in developing countries like India. This made the way for developing a simple and economic screening test for the detection of carriers of β- thalassemia.

In 1988 Mehta BC et al developed a simple cost effective screening test for the detection of cases of β-thalassemia trait based on reduced

osmotic fragility. This is called NESTROFT (Naked Eye Single Tube Red cell Osmotic Fragility Test). We have tried to assess the usefulness of this screening test in our paediatric cases presenting with anemia.

AIMS AND OBJECTIVES

This study was aimed :

1. To detect cases of β-thalassemia trait in paediatric patients presenting with anaemia using NESTROFT as a screening tool.
2. To compare the various red cell indices and discriminant functions derived from them in identifying thalassemia trait.
3. To evaluate the peripheral smear findings with those cases.

REVIEW OF LITERATURE

Thomas Cooley and Lee (1925) described a form of severe anemia that occurred in early life associated with splenomegaly and bonemarrow changes. George H.Whipple and William L. Brad ford (1932) coined the disease as thalassemia from "the sea" because early patients were all from regions surrounding the Meditteranean area.

Thomas Cooley and Lee loc.cit et al(1925) described the disease is the homozygous state of an autosomal gene for which an heterozygous state is associated with much milder hematologic changes. The severe homozygous condition to be known as thalassemia major, the heterozygous as thalassemia minor.

Thalassemia is a group of disorders, each resulting from an inherited abnormality of globin production a condition collectively known as hemoglobinopathies.

Thalassemias are classified into $\alpha and\ \beta$ by decreased or absent synthesis of $\alpha and\ \beta$ globin chain respectively. β-thalassemia is the most common form of inherited hemoglobinopathy Model B et.al (1984).

Thalassemia is the most common genetic disorder worldwide. It occurs with a particularly high frequency in a broad belt extending from the Mediterranean basin through the Middle East, Indian sub continent, Burma, South East Asia, Melanesia and Islands of Pacific. Angastiniotis .M, Modell .B et al (1998).

Approximately 3% of the world population carry β-thalassemia gene. In India frequencies between 3.5 and 14.9% have been reported ChattergiaJB et al (1976).

In India, β-thalassemia has a high frequency in certain communities like Punjabis, who have migrated from West Pakistan, Lohanas, Sindhis, Bengalis, Gujaratis, Bhanushalis, Gujaroti Khojas and Jains. Verma IC,et al loc cit(1992).

Indian Academy of Paediatrics has reported a frequency of thalassaemia trait of 3 – 18% in Northern India and 1-3% or even less in the Southern part of India.

STRUCTURE OF HEMOGLOBIN

Haemoglobin is a heterogenous mixture of protein consisting of the major component hemoglobin A and the minor component A_2. Each consists of two separate pairs of identical globin chains. In hemoglobin A, α chain combine with β- chain $(\alpha_2 \beta_2)$. In hemoglobin A_2 α chain combine with δ chains $(\alpha_2 \delta_2)$ and in Hemoglobin F α chain combine with γchain $(\alpha_2 \gamma_2)$. The primary structure of hemoglobin is the four polypeptide chains attached to a haem molecule. Each chain is arranged in a series of eight helical segments joined by short non-helical segments, is referred as secondary structure. Each heme molecule is enclosed in a pocket by the folds of the chain.

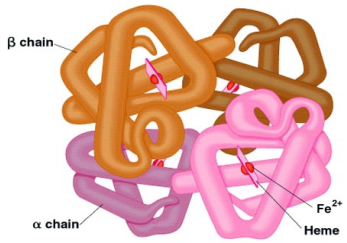


Fig1: Structure of Haemoglobin

Globin gene Clusters:

The α genes cluster usually contains one functional ε gene and two α gene $(\alpha_1 \alpha_2)$. The α gene cluster also contains four pseudogenes $\psi\varepsilon_1$, $\psi\varepsilon_2$, $\psi\alpha_1$ and θ.

Both the genes are located in a region of homology approximately 4 kb long and interrupted by two small non-homologous regions. Homologus regions are due to duplication of gene and non-homologus regions are due to DNA insertions into the non-coding regions around one of the two genes. The α gene cluster is located in the telomeric region of chromosome 16.

The β gene cluster is located in the short arm of chromosome 11, has $G_\gamma and\ A_\gamma$ gene pairs and five functional genes $\varepsilon, G_\gamma, A_\gamma, \delta and\ \beta$.

Different hemoglobins are produced during development and two globin gene switches take place. The embryonic ε gene is found in the embryonic phase and produces hemoglobin - Hb Gower 1 $(\varepsilon_2\xi_2)$ and Hb Gower 2 $(\alpha_2 \varepsilon_2)$. The duplicated fetal γ genes encode the globin chains $J_\gamma and\ A_\gamma$ in the polypeptide chain, the fetal Hemoglobin contains α_2, γ_2 chain and the fetal to adult hemoglobin switch occurs during the perinatal period. Embryonic hemoglobins are expressed in yolk sac and fetal hemoglobins are produced in fetal liver. The embryonic and fetal switch occurs during 6 and 10 weeks of gestation. The fetal to adult switch

occurs at about the time of birth, Karlsson. S et. al (1985) and Terrenato. L. et al (1981).

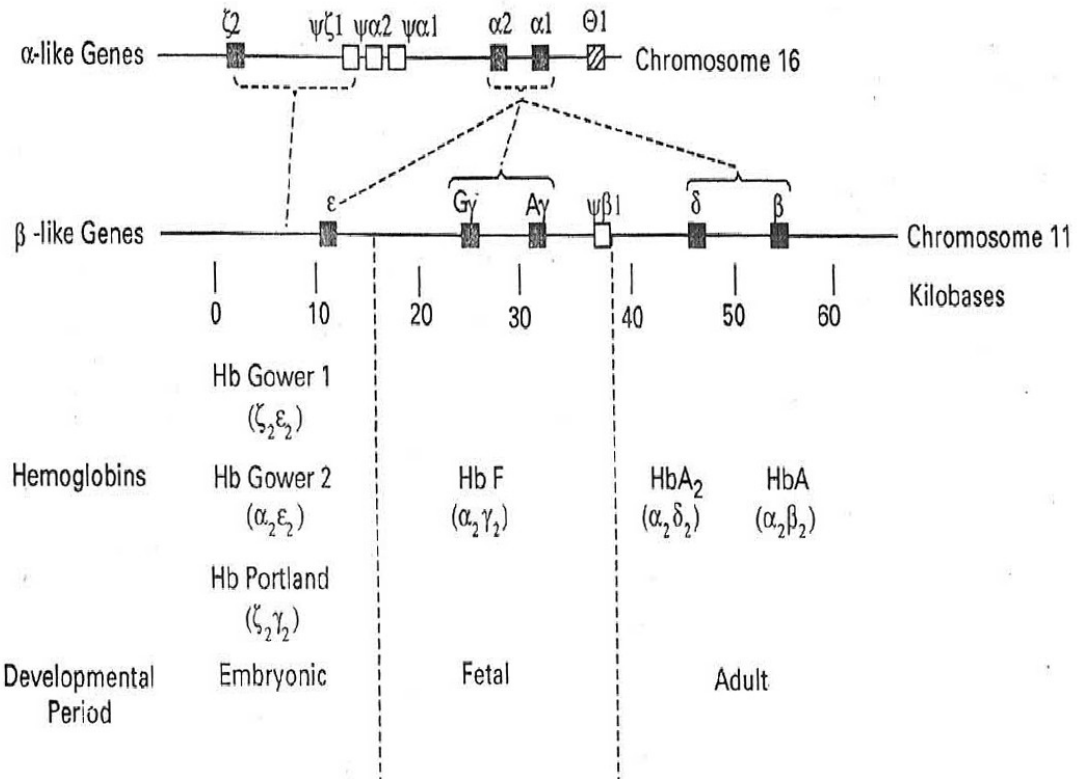


Fig-2 :Chromosomal Organization of the globin genes and their expression during development.

MOLECULAR BIOLOGY OF GLOBIN GENE

Each globin gene is composed of three exons coding for functional domains of the hemoglobin and two intervening sequences (introns). The different globin gene expression is controlled by the action of transcription factors and regulatory elements ie promoters, enhancers and silencers.

The promoters contain sequences that act as binding site for the transcription factors which is responsible for the transcription of the structural genes. The relevant promoter sequences are TATA box located at the initiation site. AAT, CACU boxes located at approximately 70 and 110 basepairs from the initiation site.

Enhancers are regulatory elements that stimulate transcription along with the promoters. Each of these regulatory sequences has binding site for the transcriptional activator and suppressor molecules.

Where a gene is transcribed, mRNA is synthesized from the DNA strand by RNA polymerase. Initially, a large mRNA precursor is formed which contains both introns and exons. The processing of mRNA involves modification at 5' capping site and 3' polyadenylation sites. The introns are subsequently eliminated and the exons are spliced together in nucleus. At this stage the mRNA moves to the cytoplasm to act as a template for the productions of globin chains. Transfer RNA transports aminoacids to the mRNA templates.

Thalassaemia syndromes result from a large number of molecular defects that alter expression of one or more of globin genes.

Molecular Pathology:

β-Thalassaemia is extremely heterogenous at the molecular level. More than 200 different mutations have been described. They fall into two broad categories.

i. Deletion of the β globin gene

ii. Non – deletion mutation affects the transcription processing or translation of β globin messenger. Point mutations are the most commonly involved one in β thalassaemia.

Mutation:

β- thalassaemia mutations result in either a complete absence of β- globin chain (β^0-thalassaemia) or a reduction of β-globin output (β^+-thalassaemia).

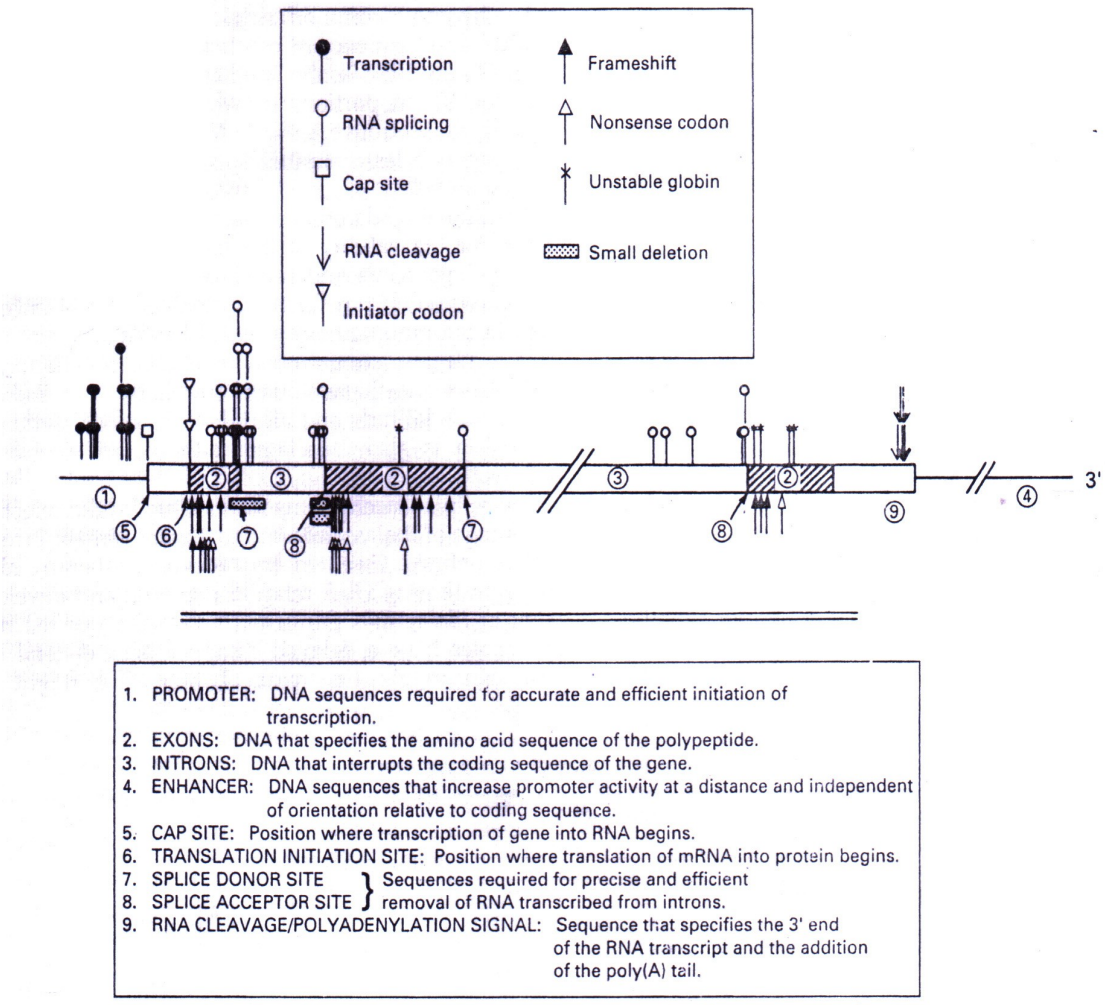


Fig-3: The location of various classes of point mutations that

cause βthalassaemia with respect to important structural

element in the β-globin gene.

Gene Deletion:

Gene deletion is generally rare in β-thalassaemia. One that occurs at 619 basepair deletion at the end of the β gene, is relatively common, in the Sind and Punjab population of India and Pakistan. Thein Sl et. al (1984). In common, the deletion affects the promoter region or a part of the globin gene.

TRANSCRIPTIONAL MUTATIONS

Promoter Mutations:

They reduce the binding of RNA polymerase and the rate of mRNA transcription to 20-30%, resulting in reduced β globin chain output, Gonzales Redondo JM et. al (1989) Maragoudaki .E et al (1999).

5' Untranslated Region mutations – Is single base substitution and minor deletion in 50- nucleotide region. Mutation at β globin gene, mRNA capsite (cap+1A $\rightarrow$ -C) shows thalassemia trait features, Wong C et al (1987).

Mutations Affecting mRNA processing:

RNA processing consists of the removal of intervening sequences and the splicing of the coding regions to produce functional mRNA. This process relies on sequences present at intron / exon boundaries. The invariant dinucleotides GT – at the 5' and AG at the 3' splice junctions and the flanking sequences, are mutation site, Mount SM et al (1982).

Splice junction and consensus sequence mutation:

Mutations at position 5q IVS-1 ($G \rightarrow c, G \rightarrow T\ G \rightarrow A$) produce a consistent reduction of β- globin synthesis leading to β^0 thalassaemia where as IVS -1-6, T→C mutation, is quite common in the Mediterranean region and results in a mild thalassaemia picture, Thamagnini G.P et al (1983).

Cryptic site mutation in introns and exons:

A number of nucleotide substitution involving these sequences, transform a cryptic site into a legitimate one. Eg. IVS $1 \rightarrow 110\ G \rightarrow A$ substitution, $IVS\ 1 \rightarrow 116 \rightarrow T \rightarrow G$ substitutions results in β^0, β^+ thalassaemia, Spritz .R.A et al (1981) and Metherall JE et al (1981).

Poly (A) and other 3' untranslated Region mutants:

Polyadenylation is important for determining the stability of mRNA, mutation at the AAUA AA sequence affecting the translation leads to β^+ thalassaemia.

Mutation affecting mRNA translation:

A large group of mutations alter the different steps of mRNA translation. Initiation codon ATG mutation, nonsense codon TAA, TAG or TGA mutation and frame shift mutation affect the mRNA translation. The frame shift resulting from a single base deletion at codon 6 (-A) is relatively common in Mediterranean population where as - 4 nucleotide deletion at codon 41 and 42 common in Indian population, Chang JC et al (1983)and Kimurd A et al (1983).

PATHOPHYSIOLOGY OF BETA- THALASSAEMIA

The basic defect is a reduced or absent production of β-globin chains with relative excess of α-chains. This results in decrease in the hemoglobin production and an imbalance of globin chain synthesis. The former leads to reduction in mean cell hemoglobin (MCH) and mean cell volume (MCV). The latter has dramatic effects on the red cell precursors, resulting in their premature destruction in the bone marrow and extra medullary sites. This is called as ineffective erythropoiesis and is the hallmark of β-thalassaemia, Rachmilewitz EA et al (2001)and Finch CA et al (1970).

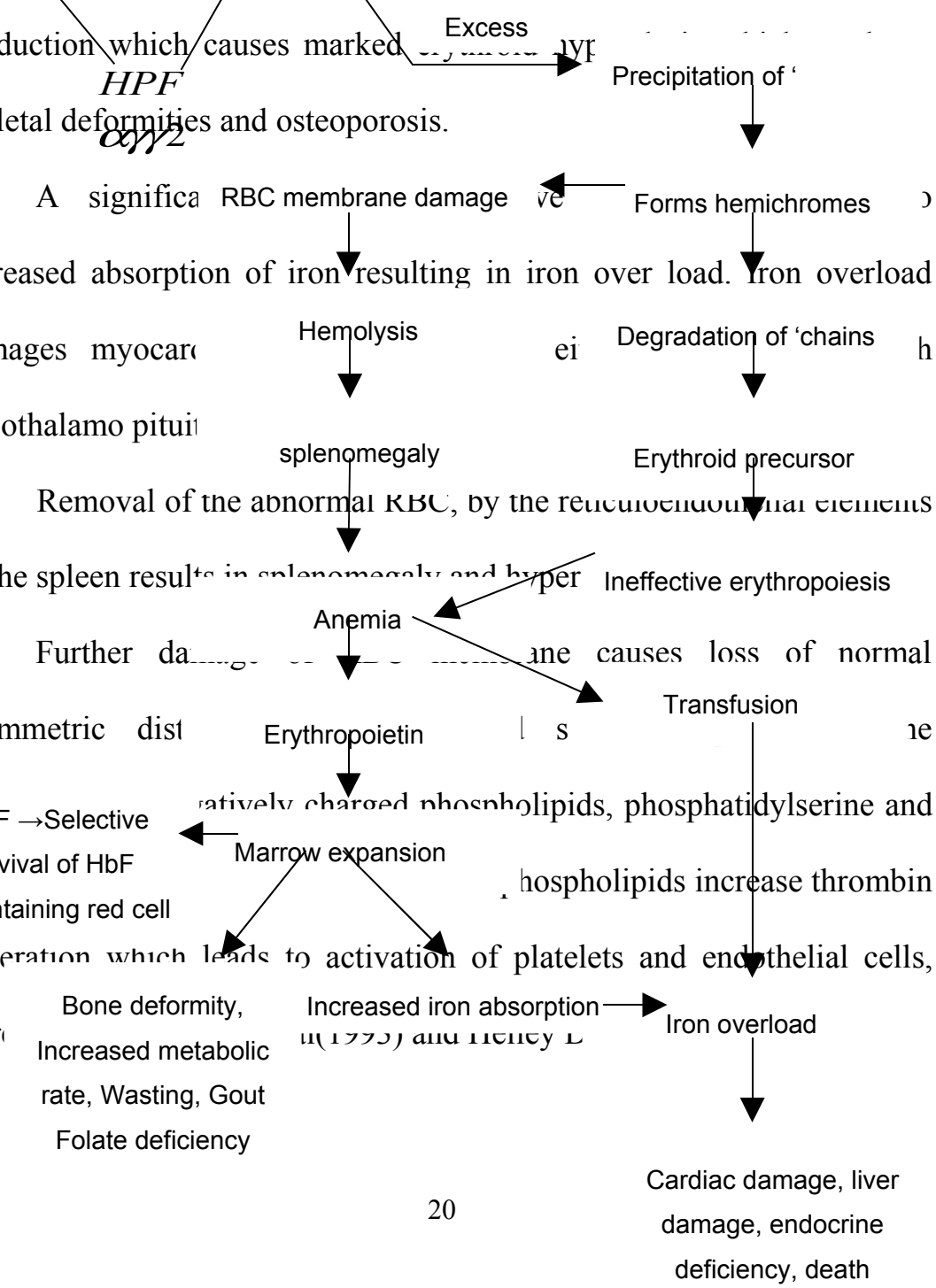
Fessas B et al., (1966) described the presence of inclusion bodies in erythroblasts as precipated α chains. Oxidation of excess α-chains results in the formation of hemichromes, which gives structural damage to red cell membrane. Excess chain precipitation in the red cell membrane causes structural and functional alterations which leads to changes in deformability, stability and hydration of red cells, Rachmileritz EA et al loc cit (2001) Rachmileritz EA et. al (1985). Schrier SL et. al (1989).

Besides oxidation, free α- chain are subjected to dehydration, resulting in the formation of denatured α- globin protein, heme and free iron. These degraded products damage the erythroid precursors and red cell membranes. Free iron, via the Fenton Reaction generates reactive oxygen species which causes lipid and protein peroxidation with

subsequent damage to red cell membranes and intracellular organelles, Hebbel RP et al (1985) and Grinberg LN et al loc cit (1995).

These alterations of erythroid precursors result in an enhanced rate of apoptosis. Apoptosis contributes to ineffective erythropoiesis occurs at the polychromatophilic erythroblast stage, Yuan J et al (1993).

Finch CA et al loc cit (1970) stated that s deficient s ineffective erythropoiesis and anemia leads to increased erythropoietin production which causes marked



skeletal deformities and osteoporosis.

A significa ve increased absorption of iron resulting in iron over load. Iron overload damages myocar ei h hypothalamo pitui

Removal of the abnormal RBC, by the reticuloendothelial elements of the spleen result

Further da ane causes loss of normal asymmetric dist s ne atively charged phospholipids, phosphatidylserine and hospholipids increase thrombin generation which leads to activation of platelets and endothelial cells, Bor (1995) and Heiley E

Fig 4: Pathophysiology of β-thalassaemia

CLASSIFICATION OF BETA – THALASSAEMIA

Silent Carrier:

These patients have no symptoms but have minimal changes in RBC indices. The mutation that causes the thalassaemia is very mild and represents β^+ thalassaemias (β^+/β).

β Thalassaemia Trait:

Patients have mild anaemia, abnormal RBC indices and abnormal hemoglobin electrophoresis with elevated HbA_2, HbF or both. Peripheral blood film usually shows marked hypochromia, microcytosis, target cells and faint basophilic stippling. The production of β- Chains from abnormal allele varies from complete absence to variable degrees of deficiency. (β^+/β) or (β^0/β).

Thalassaemia Intermedia:

This condition is usually due to compound heterozygous state resulting in anaemia of intermediate severity.

β Thalassaemia associated with β^+ chain structural variants:

The most significant condition in this group of thalassaemic syndromes is Hb S/β thalassaemia which may vary in clinical severity from as mild as thalassaemia intermedia to as severe as β thalassaemia major. When a person inherits one sickle cell gene (β^s) and one β thalassaemia gene the resulting disease is Hb S/β thalassaemia disease. The severity of disease, clinical and laboratory findings of heterozygous Hb S/β state varies from asymptomatic to moderately severe disease. In

this condition the proportion of various hemoglobin are as follows HbS 50 - 90% HbA : 0 - 50% Hb F: 2 - 30% and Hb A_2 > 3.5%

Thalassaemia major $(\beta^+/\beta^+):(\beta^o/\beta^o);(\beta^o/\beta^+)$ **:**

This condition is characterized by transfusion dependent anemia, massive splenomegaly, bone deformities, growth retardation and peculiar facies in untreated individuals. HbF levels range from 10 to 100% and Hb A_2 levels may be normal or increased upto 5 to 7%. Peripheral smear shows microcytic hypochromic red cells, target cells, tear drop cells and nucleated RBC's.

THALASSAEMIA TRAIT

Clinical Features:

The classic heterozygous carrier of β- thalassaemia is usually asymptomatic. The diagnosis is made through evaluation of a positive family history or during population screening, Gardileas C et al (1968), Pootralcul UP et al (1973). During pregnancy anemia is more severe than normal women, Cooley JR et al loccit (1984) White JM et al (1985).

Malaria Hypothesis:

Maccanti A et.al (1942) observed that the children affected by thalassaemia came from malaria endemic areas. Haldane JBS (1949) suggested the heterozygote carrier state patients have a selective advantage for survival where malaria is endemic.

The cellular mechanism responsible for the selective advantage of thalassaemia heterozygotes remain incompletely defined. Erythrocytes containing high Hb F retard the growth and development of Plasmodium falciparam. Pasvol G et. al (1977).

Infants with β-thalassaemia trait have a high Hb F in their first year of life that prevents fatal cerebral malaria, Weathirall DJ et.al (1981).

Using modified tissue culture Brockelman et al (1987) demonstrated decreased parasitic multiplication in β-thalassaemia trait red cells. They theorized that Plasmodium falciparam resistance was

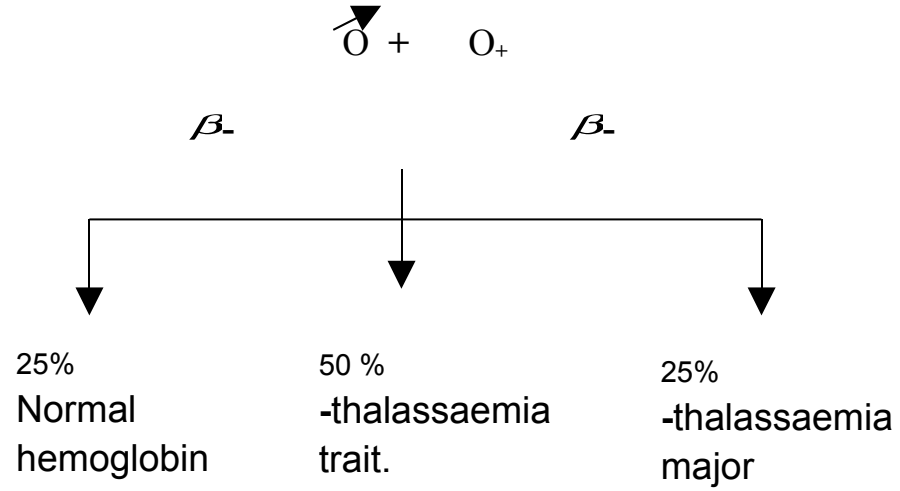
consequence of the inability of the parasite to acquire nutrients through the digestion of hemoglobin in thalassaemic red cells.

Laboratory features:

1. Hemoglobin of value usually ranges from 9 to 11 gm/dl.
2. MCH values of 20 to 22 pg
3. MCV values of 50 to 70 fl, Miliard DP et al (1977).
4. Red cell count is elevated.
5. MCV is directly related to the degree of reduction in β- globin production. MCHC is normal, Rund D et al (1992).
6. Red cell distribution width (RDW) is normal or increased.
7. Peripheral smear shows microcytosis, hypochromic, anisopoikilocytosis, basophilic stippling and reticulocytosis, Pootralcul P et al loc cit (1973). Weatherall DJ et al (1964).
8. Bone marrow study shows mild to moderate erythroid hyperplasia and rare red cell inclusions and mild ineffective erythropoiesis. Pearson HA et al (1960). Megaloblastic transformation as a result of folic acid deficiency and it occurs occasionally, during pregnancy.
9. Osmotic fragility in reduced.
10. Free erythrocyte protoporphyrin is normal or slightly increased .

11. Serum Hb electrophoresis shows high HbA_2 levels ranges from 3.5 to 7 % , HbF is increased in half the patient ie 1-3% Mazza is et al , (1976) and Pootralcul P et al loc cit (1973).

Inheritance of β-thalassaemia:



Burden of Thalassaemia in India:

Almost 25 million people in India are carriers of β - thalassaemia gene. The prevalence of this gene varies from 1-17 with mean prevalence of 3.3%, Verma IC et al loc cit (1992). Over 9000 thalassaemia children are born in every year, IAP text of Paediatrics 5th edition 2006 these children suffer from chronic anemia which results in gross facial deformity, organomegaly and poor growth. Only 10 – 15% of these children receive optimal treatment, Choudhry VP et al (1991). The cost of ideal treatment for each patient is 1 lakh per anum, Chaudhry VP et al loc cit (1991).

Necessity of identifying β - thalassaemia trait

The birth of a thalassaemia child places considerable health and economic strain not only on the affected child, but also to the community and nation. Since it is a severe and incurable disease, emphasis must shift from treatment of the affected child to prevention of such birth in future, Raghavan K etal (1990).

Prevention of the β - thalassaemia major:

Advances in molecular diagnostic technique have made it possible to diagnose β - thalassaemia major at 6 weeks of gestation. Early antenatal diagnosis of β - thalassaemia major followed by medical termination of pregrancy can prevent birth of children with β-thalassaemia major. Prevention of birth of children with β-thalassaemia major would thus spare a lot of distress, effort and expenses for the families involved and for the society. The key requirement to prevent this disease is identification of couples at risk of giving birth to children with β- thalassaemia major. This can be done in two ways, Raghavan K et al loc cit (1990) and Manglani .M et al (1997).

(a) **A population based screening for β thalassaemia trait:**

Mehta BC et al (2002) stated that screening of the general population would be the best way if resources were unlimited and the population manageable. In a populous country such as India screening of communities known to have high prevalence of β- thalassaemia trait would be more costeffective. While conventional counselling would be to

advise an individual with β - thalassaemia trait not to marry another individual with β- thalassaemia trait. The study found "positive counselling" to be more acceptable and extremely useful. Couples at risk of having children with β- thalassaemia major can be advised about the need for prenatal diagnosis at an early stage of pregnancy.

(b) Antenatal Screening:

All pregnant women attending antenatal clinic can be screened for β thalassaemia trait at the time of their first antenatal visit. The spouse of women found to be carriers after confirmatory tests should be tested for β - thalassaemia trait to identify pregnancies at risk of producing children with β thalassaemia major. Women identified to have β thalassaemia trait should avoid routine antenatal iron therapy, Mehta BC et al loc cit (2002).

(c)Avoidance of excessive iron therapy:

Mild to moderate degree of hypochromic microcytic anemia is encountered in about 65 – 85 % of carriers of β- thalassaemia trait. In the absence of definitive diagnosis, many of these patients receive oral or parenteral iron therapy. This may go on intermittently or continuously for prolonged periods of time, as the anaemia persists. This iron therapy may result in iron overload, Mehta BC et al loc cit (2002).

d) The Need for a screening test for β thalassaemia trait:

The diagnostic test for β thalassaemia trait is estimation of hemoglobin A_2 by electrophoresis or chromatography. Both these techniques are expensive and time consuming and need expensive equipment and laboratory expertise, Raghavan K etal loc cit (1990).

Cost effectiveness can be improved if definitive tests were to be performed only on samples with high chances of yielding positive results. The availability of an initial screening test which is inexpensive and easy to perform even under field conditions and can identify positive samples with reasonable sensitivity and specificity would be technically and financially attractive.

Naked Eye single Tube Red cell Osmotic fragility test:

A simple test based on the lowered osmotic fragility of RBCS in thalassaemia has been applied in India by Mehta .BC et al (1988), who first used the term NESTROFT as quoted by Verma IC et al loc cit (1992). The acronym **NESTROFT** stand for **Naked Eye Single Tube Redcell Osmotic Fragility Test**. NESTROFT used to assess osmotic fragility of red cells at a single concentration of buffered saline (0.36% in single tube) visually without a spectrophotometer.

Procedure:

A stock solution of 10% buffered saline of pH 7.4 is prepared noted in (Appendix 1), from this, 1% buffered saline was prepared by

1:10 dilution with distilled water. 0.36% buffered saline was prepared from 1% buffered saline.

2 ml of 0.36% buffered saline was taken in one tube (10cm x 1 cm) and 2ml of distilled water was taken in another tube as control. A drop of well mixed EDTA blood was added to each of the tubes which were left undisturbed for 30 minutes at room temperature.

Interpretation:

After 30 minutes, the contents of both the tubes were shaken and the tubes held against a white paper on which a thick black line was drawn. The line would be clearly visible through the contents of the tube containing distilled water due to complete lysis. If the line was similarly visible through the contents of the tube with buffered saline the test was considered negative, where as if the line was not visible the test was taken to be positive.

The tubes were then left undisturbed for two hours. The content of the control tube shows uniformly pink, the tube containing buffered saline shows a sediment at the bottom and top part of saline is clear, the test is positive. If the tube containing buffered saline is having similar to the control tube, it is taken as negative.

Indication of positive NESTROFT:

A positive NESTROFT indicates that all the red cells in tested sample have not undergone complete lysis in 0.36% of buffered saline.

These unlysed red cells gives haziness of the contents of the tube and render the line on paper indistinct. These red cells also sediment as a button at the bottom of test tube when it is left undisturbed for sometime. Thus a positive NESTROFT indicates decreased red cell osmotic fragility and increased resistance to osmotic fragility.

Utility of NESTROFT:

Decreased osmotic fragility in β-Thalassaemia trait, is indicated by a positive NESTROFT. It is also positive in Iron deficiency anaemia, anaemia due to liver diseases, HbE disorder.

Confirmation of β-Thalassaemia Trait:

β-Thalassaemia trait can be confirmed by estimating the Hb A_2 level. The common method employed for estimating Hb A_2 are Haemoglobin electrophoresis on cellulose acetate at alkaline pH.

Haemoglobin A_2 can be quantified by cellulose acetate electrophoresis followed by elution and spectrometry but this is a labour intensive technique of large numbers of samples require testing. Quantification of haemoglobin by scanning densitometry is not recommended as the precision is not good enough for detection of β-Thalassaemia trait, Clarke MG et al (2000) and Schmidt MR et al (1975) found that quantification of Hb A_2 by densitometry produces unreliable results and does not allow differentiation of β-Thalassaemia trait carriers from persons with normal levels of Hb A_2.

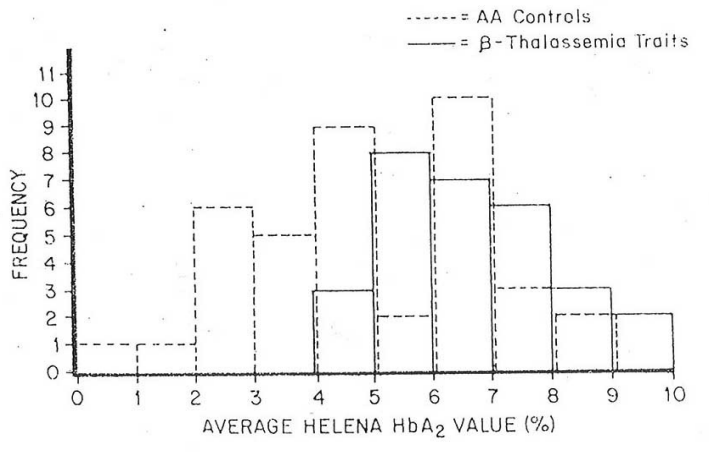


Fig .5

the other hand quantitation of Hb A_2 by elution from cellulose acetate membranes or by column chromatography yielded satisfactory results.

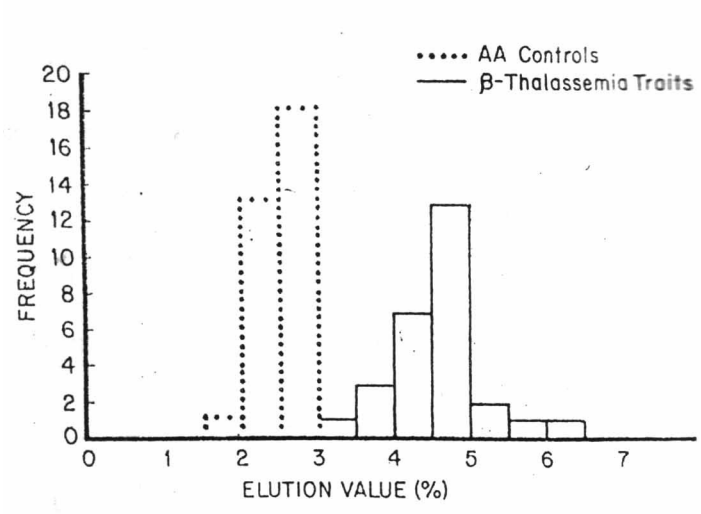


Fig – 6

Frequency histogram of average scanning denstiometry (with Helena) values for HbA_2 from AA controls and β-Thalassaemia trait patients show an overlap which means that this method cannot reliably differentiate β-Thalassaemia trait from normal.

ElutionValue (%):

Schmidt MR et al loc cit (1975), frequency histogram obtained by elution from cellulose acetate membranes for HbA_2 from AA controls and β-Thalassaemia trait showed good separation and is superior to densitometric analysis.

At alkaline p^H electrophoretic migration of HbC, HbE, HbA_2 and HbO are similar HbS, HbD and HbG also comigrate, Clarke MG etal loc cit (2000). At acid pH (on citrate agar or agarose gel) electophoretic separation of HbC from Hb E and HbO and HbS from HbD and HbG is accomplished. It is not possible to differentiate HbE from HbO and HbD from HbG using electrophoretic methods. This is usually used as a supplement to cellulose acetate electrophoresis, Bain BJ et al loc cit (1998) and Cotton F et al (1999).

Electrophoresis at acid pH is indicated in investigation of suspected high affinity haemoglobins even when electrophoresis at alkaline pH is normal, since certain high affinity haemoglobins have abnormal mobility in acid pH but normal mobility in alkaline pH.

Capillary Zone Electrophoresis (CZE)

This is a relatively new technique for the measurement of Hb A_2 and HbF, Fredric Cotton et al (1999) compared CZE with commonly used quantitative methods like cation exchange HPLC and anion exchange chromatography in micro columns (MAEC) CZE compared well with these methods and the advantage of CZE was that, presence of

HbS does not interfere with the estimation of HbA_2, Shibabi ZK et al (1999). Measurement of Hb A_2 using cation – exchange HPLC is complicated in individuals with HbS because Hb A_2 is spuriously increased by the presence of HbS adducts. Which coelute with HbA_2 in this system. The different patterns obtained with CZE is shown in figure (A,B,C and D).

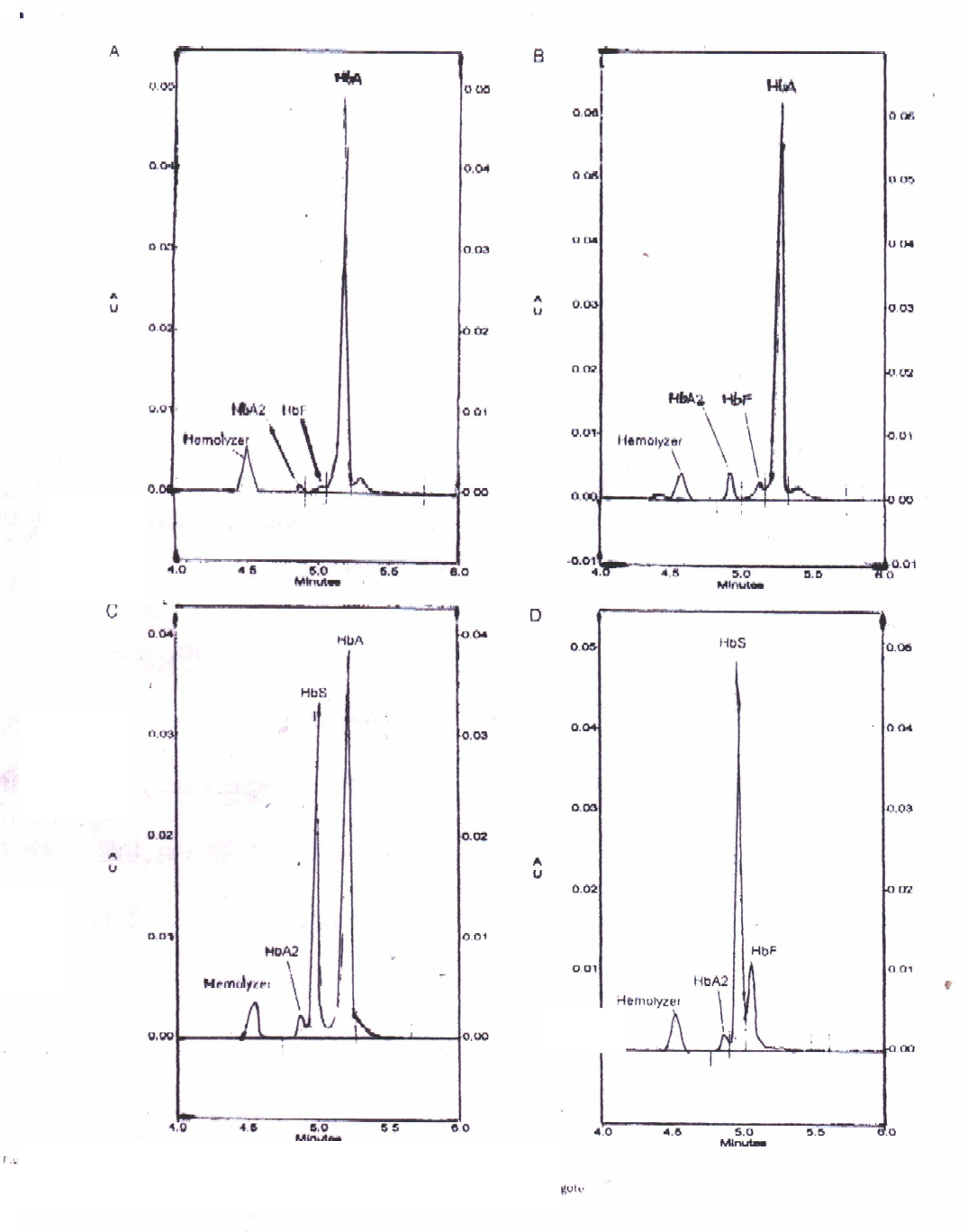


Fig -7 Typical electrophenograms obtained by CZE,

In the above mentioned study separation of Hb fractions was achieved in 6 mins. The hemolyzer marker, HbA_2, HbS, HbF, and HbA displayed migration times of 4.59, 4.92, 4.99, 5.12, 5.27 min respectively.

The imprecision by CZE technique ranged from 3% to 6% for Hb A_2 and HbF at physiological and pathological concentrations. The imprecision was always <2% for migration times. The CV HbF quantification was 5 % for concentrations ranging from 1.8 to 18.9% The CV for Hb A_2 was 3-6% for concentration ranging from 2.0 to 5.6% . Lenkin MA et al (1997).

Microcytic hypochromic red cells with mild anisocytosis also seen in the following conditions.

1. Iron deficiency anemia
2. Anemia of chronic diseases
3. Sideroblastic anemia
4. β- thalassaemia major

Of these, Iron deficiency anemia, β thalassaemia trait and anemia of chronic disease account for more than 80% cases of microcytic hypochronic anemia.

Iron deficiency anaemia:

The causes of iron deficiency anaemia are

1. Decresed dietary intake
2. Impaired absorption
3. Increased requirement
4. Chronic blood loss

There is no regulated pathway for iron excretion, which is limited

to the 1-2 mg / day lost by shedding of mucosal and skin epithelial cells. The normal non – vegetarian diet contains 10 – 20 mg of iron mostly in the form of heme iron, about 20% of which is absorbed, (in content to 1 to 2 % of non heme iron). The total body iron content is normally 2 gm in women and upto 6 gm in men. Approximately 80% of functional iron is bound in hemoglobin: rest 20% in myoglobin, iron binding enzymes and cytochromes. The storage pool of hemosiderin and ferritin has approximately 15 to 20% of total body iron. Plasma iron bound to transferrin is transported to the marrow, where it is transferred to developing red cells and is incorporated into hemoglobin. Mature red cells are released into the circulation and after 120 days are ingested by macrophages in the reticuloendothelial system. Hence iron is extracted from hemoglobin and returned to the plasma, completing the cycle.

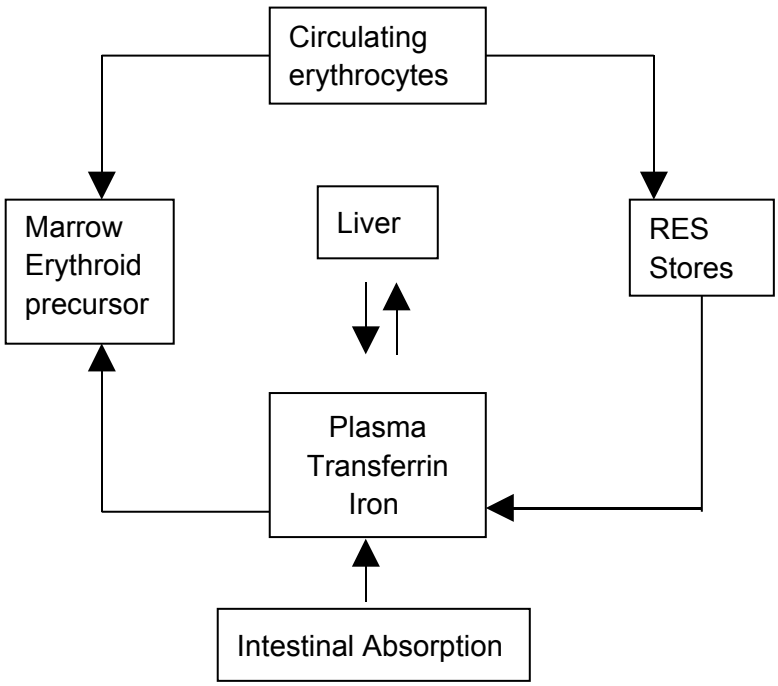


Fig – 8: Internal iron cycle

Clinical features of Iron deficiency anaemia:

Weakness, lethargy, koilonychia, glossitis, pharyngeal webs, muscle dysfunction and gastritis, pica are other features.

Laboratory features:

Peripheral smear shows microcytic hypochromic red cells with anisopoilkilocytosis most frequently target cells, elliptocytes and reticulocytosis, White blood cells and platelets are usually normal but may be thrombocytosis seen in case of severe bleeding.

Bone marrow shows mild to moderate increase in erythroid progenitors predominantly polychromatic normoblasts and micronormoblasts and disappearance of stainable iron. Granulopoietic cells and megakaryocytes are present in normal numbers and appearance.

Serum iron is decreased to less than 30 μg/dl. Total iron binding capacity is increased to 420 μq/dl. Transferrin saturation is decreased to less than 15%. Serum ferritin is decreased to less than 30 μg/dl.

Table–2: Iron studies in differentiating thalassaemia from iron defeciency anaemia.

Disease	**Serum Iron**	**Serum Ferritin**	**TIBC**	**% of saturation**	**Storage Iron**
BTT	N or ↑	N to ↑	N to ↓	↑	↑
IDA	N / ↓	↓	↑	↓	↓

Nathan and Oskis Hematology of infancy and childhood 2009.

Anaemia of chronic diseases:

It is the most common cause of anaemia in hospitalized patients. In chronic diseases, the red cell production is impaired leading to anaemia that mimics iron deficiency anaemia the signs and symptoms of these patient depends upon the underlying disorder.

Laboratory findings:

Peripheral smear shows normocytic normochromic red cells, in long standing cases show microcytic hypochromic blood picture, reticulocytes counts are normal.

Iron profile shows decreased serum iron, decreased total iron binding capacity, low transferrin saturation and normal or increased serum ferritin.

RBC Indices and β thalassaemia trait, Eldibany MM et. al (1999) and Telmissani OA et al (1992).

RBC indices were first introduced by Wintrobe et al (1940). These include MCV, MCH and MCHC. In βthalassaemia trait MCV is normal or rarely decreased. MCHC is also normal or decreased RDW is usually normal, Kanavakis E et al (1982).

Table – 3: Red cell Indices in βthalassaemia and Iron deficiency anaemia.

	RDW	RBC Count	MCV	MCH	MCHC
BTT	Usually Normal	N/or ↑	↓↓	↓↓	↓
IDA	↑ed	↓↓	↓	↓	↓↓

DISCRIMINANT FUNCTIONS:

Mentzer index is an useful screening test, which is derived from MCV and RBC count if it is less than 13 considered as β-thalassaemia trait, Mentzer WC et al loc cit (1973) England and Fraser proposed a linear discriminant function (DF) derived from MCV, RBC count and Hb concentration, positive value indicates iron deficiency anemia and negative value indicate β- thalassaemia trait, England JM et al loc cit (1979) . Green and King proposed the formula, $\frac{MCV^2 \times RDW}{Hb \times 100}$ with values less than 65 indicates β-thalassaemia trait. Shine and Lal introduced the formula $\frac{MCV^2 \times MCA}{100}$ with values less than 1530 was considered to β- thalassaemia trait, Shine I et al loc cit (1977).

Thomas S srivastava A et al (1996) derived the formula from MCH and RBC Count if it is > 3.80 it denotes iron defeciency anaemia,

< 3.80 denotes β-thalassaemia trait. Bessman JB et al loc cit (1983) derived the RDW index from MCV, RDW and RBC count if the results are >220 it denotes iron deficiency anaemia if <220 it signifies β-thalassaemia trait.

MATERIALS AND METHODS

In my study i have included 100 patients in the age group of 0-12yrs, all of whom presented with features and symptoms of anemia to the outpatient and inpatient ward, Department of Paediatrics, Tirunelveli Medical College Hospital, Tirunelveli during the period of August 2008 to March 2009.

All these patients were subjected to basic hematologic investigations and those who presented with Hb less than 11.5gm% and MCV of less than 80fl, were included in the study. Peripheral smear examination were done to exclude any hematological malignancy and β-thalassaemia major.

Selected patients were subjected to the following investigations:

1. Complete blood hemogram - Total RBC count, MCV, MCH, MCHC and RDW were calculated in all selected patients using automated cell counter Sysmax – K21,22 (Transasia biomedicals).
2. Peripheral smear analysis was done to look for the degree of anisocytosis and poikilocytosis and they were graded as mild (+), moderate (++) and severe (+++), as graded in US standard reference.
3. NESTROFT was carried out in these cases with fresh working solution. (Appendix No:1) Raghavan .K et. al (1998) Mehta BC et. al Ind. J. Hemat (1990)

4. Discriminant functions like Mentzer's index, England and Fraser index, Green and King Index, Shrivastava's formula, Shine and Lal Index, RDW index were carried out with the data obtained from the cell counter.

Mentzer Index - Mentzer et al (1973).

MCV /RBC count

>13 Iron Deficiency Anemia

<13 β- thalassemia trait

England Fraser Index - England Fraser PM Lancet (1973).

MCV – 5 Hb – RBC – K. where K =8.4

Positive value – Iron deficiency anemia.

Negative value - β- thalassemia trait.

Green and King Index

MCV^2 x RDW/Hb x 100

>65 Iron deficiency anemia

<65 β-thalassaemia trait

Srivastava's formula -Thomas S. Srivastava A et.al (1996)

MCH / RBC

>3.80 – Iron deficiency anemia

<3.80 - β- thalassaemia trait

Shine and Lal Index - Shine I, Lal S.et al Lancet (1977)

MCV2 x MCH/100

>1530 Iron deficiency anemia

<1530 β-thalassaemia trait

RDW Index **-** Bessman JB, Gilmer Pri et.al (1983)

MCV x RDW/RBC count

>220 – Iron deficiency anemia

<220 - β- thalassaemia trait

5. NESTROFT positive samples were sent to Microbiological Laboratory, Coimbatore for Hb electrophoresis by HPLC (D10-BIORAD) method. Hb A2 level >3.5% was taken as the gold standard to diagnose β-thalassemia trait.

RESULTS

The evaluated patients were divided into two groups i.e beta thalassaemia trait and non beta thalassaemia trait. Beta thalassaemia group includes positive NESTROFT and the standard reference values for various discriminant functions and peripheral smear showed microcytic hypochromic anemias. Non beta thalassaemia trait group includes iron deficiency anaemias, anaemia of chronic diseases and sideroblastic anaemias.

Sensitivity, specificity, positive predictive value and negative predictive value of NESTROFT and the various discriminant functions were evaluated and their significant difference made out between them and tabulated.

Haemoglobin electrophoresis was done in all NESTROFT positive cases and three cases showed positive for beta thalassaemia trait.

Comparison of NESTROFT with other discrimiant functions:

β - thalasaemia trait was evaluated by Mentzers index and the same was screened by NESTROFT.

Table – 1: Mentzer's index and NESTROFT

Nestroft	Mentzer's inex			Sensitivity	Specificity	Positive predictive value	Negative predicative value
	BTT	Non BTT	Total				
Positive	14(a) (25%)	40(b) (75%)	54				
Negative	1(c) (2.1%)	45(a) (97.9%)	46	93.3%	52.9%	25.9%	97.8%
Total	15	85	100				

*BTT :- Beta Thalassaemia Trait.

Sensitivity : $a/(a+c)^{\times 100}$ =93.3%

Specificity : $d/(b+d)^{\times 100}$ =52.9%

Positive predictive value = $a/(a+b)^{\times 100}$ =25.9%

Negative predictive value = $\frac{d}{(c+d)} \times 100$ =97.8%

Table 1 shows sensitivity and specificity and positive predictive value and negative predictive value of Mentzers index as calculated and the result showed 93.3% of sensitivity and 52.9% of specificity which were statistically shows significant difference ($p<0.01$) postive predictive value and negative predictive value also showed statistically significant difference ($p<0.01$).

Table – 2: England Fraser index with NESTROFT

Nestroft	England transfer Index			Sensitivity	Specificity	Positive predictive value	Negative predicative value
	BTT	Non-BTT	Total				
Positive	50 (92%)	4 (8%)	54				
Negative	9 (20%)	37 (80%)	46	84.7%	90.2%	92.6%	80.4%
Total	59	41	100				

*BTT :- Beta Thalassaemia Trait.

Table 2 shows the percentage of sensitivity and specificity of England Fraser index were 84.7% and 90.2% which were statistically insignificant $p>0.05$ and similarly positive predictive value and negative predictive value were showed the insignificant difference of $p>0.05$.

Table – 3: NESTROFT with Green and King index

Nestroft	Green and king index			Sensitivity	Specificity	Positive predictive value	Negative predicative value
	BTT	Non BTT	Total				
Positive	47 (87%)	7 (13%)	54				
Negative	4 (8%)	42 (92%)	46	92.2%	85.7%	87.0%	91.3%
Total	51	449	100				

*BTT :- Beta Thalassaemia Trait.

Table 3 shows sensitivity and specificity of Green and King index with NESTROFT positive cases were 92.2% and 85.7%. The difference for the specificity and sensitivity was statistically insignificant $p>0.05$. The positive and negative predictive value also did not differ significantly $p>0.05$.

Table – 4: NESTROFT with Srivastava's formula

Nestroft	Srivastavas			Sensitivity	Specificity	Positive predictive value	Negative predicative value
	BTT	Non BTT	Total				
Positive	33 (62%)	21 (38%)	54				
Negative	4 (8.6%)	42 (91.4%)	46	89.2%	66.7%	61.1%	91.3%
Total	37	53	100				

*BTT :- Beta Thalassaemia Trait.

Table 4 shows the sensitivity of 89.2% and specificity of 66.7% of Srivastava's formula which showed statistically significant difference of $p<0.05$. The positive and negative predictive value also showed the significant difference of $p<0.05$.

Table – 5: NESTROFT with Shine and Lal index

Nestroft	Shine and car index			Sensitivity	Specificity	Positive predictive value	Negative predicative value
	BTT	Non BTT	Total				
Positive	53 (98%)	1 (2%)	54				
Negative	30 (65%)	16 (35%)	46	63.8%	94.1%	98.2%	34.9%
Total	83	17	100				

*BTT :- Beta Thalassaemia Trait.

Table 5 shows the sensitivity of 63.8% specificity of 94.1% of Shine and Lal index showed statistically significant difference of $p<0.01$. The positive predictive value and negative predictive value also showed the significant difference of $p<0.01$.

Table – 6: NESTROFT with RDW index

Nestroft	RDW index			Sensitivity	Specificity	Positive predictive value	Negative predicative value
	BTT	Non BTT	Total				
Positive	48 (88%)	6 (12%)	54				
Negative	3 (6.5%)	43 (93.5%)	46	94.1%	87.8%	88.9%	93.5%
Total	51	49	100				

*BTT :- Beta Thalassaemia Trait.

Table 6 illustrates the sensitivity (94.1%), the specificity (87.7%) positive predictive value (88.9%) negative predictive value (93.5%) of RDW index was statistically showed the insignificant difference $p>0.05$.

Table – 7: COMPARISON OF THE VARIOUS DISCRIMINANT FUNCTIONS

Sl.No.	Discriminant function	Specificity	Sensitivity	Positive predictive value	Negative predictive value
1	Mentzers Index	52.9%	93.3%	25.9%	97.8%
2	England Fraser index	90.2%	84.7%	92.6%	80.4%
3	Green and King index	85.75	92.2%	87.0%	91.3%
4	Srivastava's formula	66.7%	89.2%	61.1%	91.3%
5	Shine and Lal Index	94.1%	63.8%	98.2%	34.9%
6	RDW index	87.8%	94.1%	88.9%	93.5%

Table 7 shows various discriminant functions Mentzer index, Shine & Lal index and Srivastava's formula were statistically shows significant difference and were ideal for screening tools to identify the β -thalassemia trait cases. From the above results the association of NESTROFT was evaluated with chi-square test (χ^2) as below.

Table 8: Association between NESTROFT with other indices

NESTROFT with	Chi-square test (χ^2)	Significance

Mentzer's Index	9.21	P<0.01
Srivastava's Formula	27.1	P<0.01
Shine and Lal Index	16.83	P<0.01
England Fraser Index	51.786	P<0.001
Green & King Index	57.910	P<0.001
RDW Index	64.180	P<0.001

Table 8 shows the analysis of chi-square test. The NESTROFT as a screening tool was positively associated with other indices.

Table 9 : Comparison of red cell indices

Red cell indices	β-thalassemia trait cases		Non β- thalassemia trait cases	
	Mean	**SD**	**Mean**	**SD**
MCV(fl)	59.6	4.2	72.8	7.3
MCH(pg)	19.1	1.9	24.1	5.4
RDW	14.3	0.8	15.3	2.5
RBC count	5.39	0.6	3.88	0.7
Hb level g/dl	10.6	0.6	8.3	1.9

In the present study by employing the independent t-test it was assessed that the mean MCV, mean MCH were lower than non β-thalassemia trait cases which is statistically significant with. P value of less than 0.001.

Mean of the RDW values of the thalassemia trait cases in the study was 15.23. Optimum cut off value of RDW to differentiate between B-TT and IDA was found to be 15.23. The mean RDW of β- thalassemia trait was significantly lower than non β-thalassemia trait. Since the difference of mean 2 is statistically significant $p < 0.01$

Mean RBC count is higher in β- thalassemia trait as compared to non β -thalassemia trait cases.

Table 10: Evaluation of peripheral smears.

Peripheral smears were evaluated for microcytosis, anisocytosis & hypochromias.

	β- thalassemia trait		
	Mild	Moderate	Severe
Microcytosis	12 (22%)	40 (74%)	2 (4%)
Hypochromia	36 (66%)	17 (34%)	
Anisocytosis	46 (85%)	9 (15%)	

Table 10 shows, peripheral smears of β-thalassaemia trait cases were evaluated and showed mild to moderate microcytosis, hypochromic red cells with mild anisocytosis, few target cell seen in minimal cases 5% and very few cases 3% show occasional basophilic stipplings.

DISCUSSION

This study was done to evaluate the usefulness of NESTROFT along with various other RBC indices and discriminant function in detecting cases of beta thalassaemia trait and to differentiate them from non beta thalassaemia trait cases. We have also tried to evaluate the peripheral smear findings with the RBC indices.

Regarding the usefulness of the NESTROFT as a screening test for the detection of beta thalassaemia trait our observation was almost similar to that of Manglani et al (1997) who have screened 830 cases from general population from various region of our country. The NESTROFT positive cases were confirmed with Hb electrophoresis.

The sensitivity, specificity, positive predictive value and negative predictive value were 64.2%, 94.4%, 97.6% and 35.3% respectively. We also had a similar observation and 3 cases were positive for beta thalassaemia trait by Hb electrophoresis. Our positive predictive value was lower than that of the other similar studies due to the small size of the study group included. We have also tried to correlate the RBC indices and other discriminant function with other similar studies.

The mean hemoglobin value of our study group was 10.68m/dl which was lower than that of similar observation made by Madhan N et al (1994) with a hemoglobin of 11.6gm/dl, Das Gupta et al (1994) with a

mean hemoglobin of 11.2gm/dl, and Mohamed et al (1999) with 11.3gm/dl.

The mean red blood cell count values of our study group was almost similar to that of the observation of Madhan N et al loc cit (1994) with $5.67x10^6$cells/cumm and Das Gupta et al loc cit (1994) with $5.6x10^6$cells/cumm.

The mean MCV of our study group was 62.8fl. This was similar to the observation Madhan N et al (1994) (or) slightly lower than the observation made by Mohamed et al (1999).

The mean RDW of our study was 14.3%. This was slightly lower than the values of Madhan N et al loc cit (1994) with 16.3% Das Gupta et al loc cit (1994) with 15% of Mohamed et al loc cit (1999) with 16.6%.

Comparing the sensitivity, specificity, positive and negative predictive value of various discriminant functions were found. Mentzers index and Shrivastava's formula were the most useful screening test than the others. This goes in hand with the observation of Manglani et al (1994) study, the sensitivity and specificity positive predictive value and negative predictive value of Mentzer's Index were respectively 66.2%, 82.8%, 89.5% and 44.3%. Ntaios et al found that sensitivity specificity, positive predictive value and negative predictive value were respectively 59.78%, 98.3%, 99.11% and 44.02%. In our present study sensitivity specificity, positive predictive value and negative predictive value were

respectively 73.3%, 59.9%, 25.9% and 97.8% which compared to the other studies and found that sensitivity is higher than other studies.

Manglani et al loc cit (1994) found that sensitivity specificity, positive predictive value and negative predictive value of Srivastava's formula respectively, 55.6%, 79.7%, 85.0% and 36.1%. In our present study showed sensitivity, specificity, positive predictive value and negative predictive value of 89.2%, 66.7%, 61.1% and 91.3% which shows comparatively higher sensitivity value.

In beta thalassaemia trait cases by England Fraser (1979) after evaluation of 1500 peripheral smears, suggested that microcytic hypochromic red cells, significant target cells and basophilic stippling in RBCS. In the present study, showed mild to moderate microcytic hypochromic red cells, with mild anisocytosis and few cases (5%) showed target cells and very few cases (3%) showed basophilic stippling.

SUMMARY AND CONCLUSION

In this study we have analysed the usefulness of NESTROFT along with other discriminant function and peripheral smear evaluation to detect cases of beta thalssaemia trait in the paediatric cases presenting with anemia.

A positive test of NESTROFT indicates lowered red cell osmotic fragilities which is suggestive of beta thalssaemia trait . In our study on 100 cases of anaemia, we had 54 cases with positive NESTROFT. Those cases were further subjected to hemoglobin electrophoresis in which we had 3 positive cases. The NESTROFT positively was correlated with other discriminant function and we found Mentzers Index, Shine and Lal Index and Shrivastava's formula were ideal for screening cases of beta thalssaemia trait as they showed stastiscally significant results. The other discriminant function were of not much use in our study.

Analysis of the various red cell indices has shown that the mean MCV value was 59.6fl in our cases much lower than that of non beta thalssaemia trait cases. The mean hemoglobin level was 10.6 gm/dl much higher than the mean hemoglobin value in non beta thalssaemia trait cases.

The mean RDW of beta thalssaemia trait was 14.3 whereas of the non beta thalssaemia trait was 15.3.

Evaluation of the peripheral smear showed very mild anisocytosis with microcytic hypochromic red cells in our cases of beta thalssaemia trait. There was significant anisocytosis in cases of non beta thalssaemia trait. The degree of Poikilocytosis was very mild and even absent in most of our cases of beta thalssaemia trait. Moderate degree of polychromasia was more common in case of beta thalssaemia trait than non beta thalssaemia trait. So we conclude a major screening programme including various red cells indices, discriminant function and NESTORFT will be useful to detect cases of beta thalssaemia trait in the general population. The present data will form a basic database for the forthcoming study.

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PROFORMA

Name : Age/Sex:

Hospital No : Address : Occupation:

Chief Complaints:

C/o

- Breathlessness
- Palpitation
- Chest pain
- Cough.
- Fever
- Generalized weakness/fatigue
- Bleeding tendencies
- H/o Blood transfusion

General examination of:

- Pallor
- Jaundice
- Cyanosis
- Clubbing
- Oedema

- Lymphadenopathy

Systemic Examination:

- Hepatomegaly
- Splenomegaly
- Respiratory system
- Cardiovascular system.

Investigation:

1. Complete Hemogram:
    - Hb%
    - TC
    - DC
    - RBC Count
    - WBC Count
    - PCV
    - MCV
    - MCH
    - MCHC

- RDW

2. Peripheral Smear Examination:

3. NESTROFT

4. Discriminant functions.

    - Mentzer's Index
    - England Fraser Index
    - Green and King Index
    - Srivastava's formula
    - Shine and Lal Index
    - RDW Index

5. Hemoglobin Electrophoresis.

6. Radiological examination – X – ray.