بسم الله الرحمن الرحيم

Blood and Lymph Module 2023

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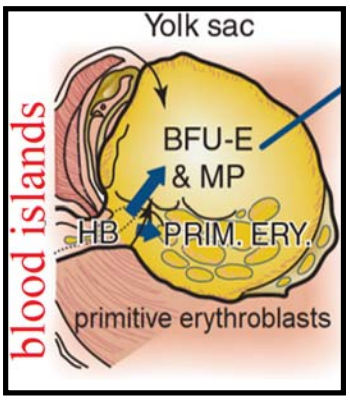
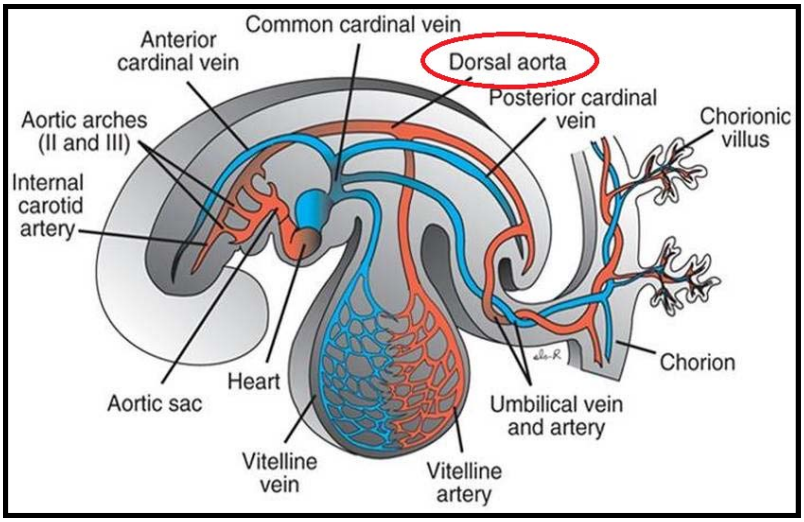
Hemopoiesis

- **Production of blood: Hemopoiesis/ Hematopoiesis/ Hematolymphopoiesis**
- During embryogenesis, hematopoiesis occurs in **spatially and temporally distinct sites**, including the extraembryonic yolk sac, the fetal liver, the thymus, and the preterm marrow.
- Hemopoiesis is first established soon **after implantation of the blastocyst**, with the appearance **of primitive erythroid cells** (transient cohort of embryonic RBC) in blood islands of **the yolk sac** beginning at day 18 of gestation.



- **Definitive Hematopoietic stem cells** (HSC) which **persist throughout fetal and adult life**, containing **erythromyeloid and lymphoid potential**, subsequently arise from the embryonic **the dorsal aorta.**

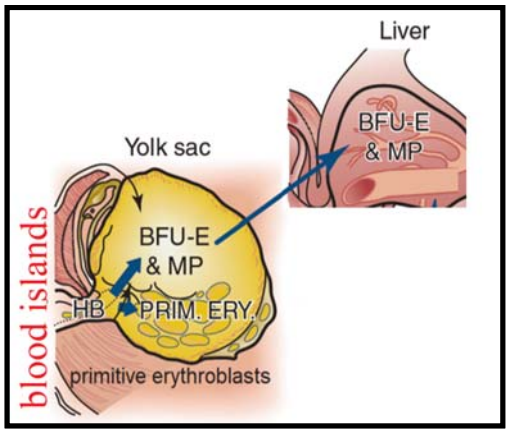
- These **HSC migrate** from the **the dorsal aorta** to **the fetal liver** and, by about 60 days of gestation, the first fetal red blood cells are released into the circulation **to replace primitive embryonic** red blood cells.

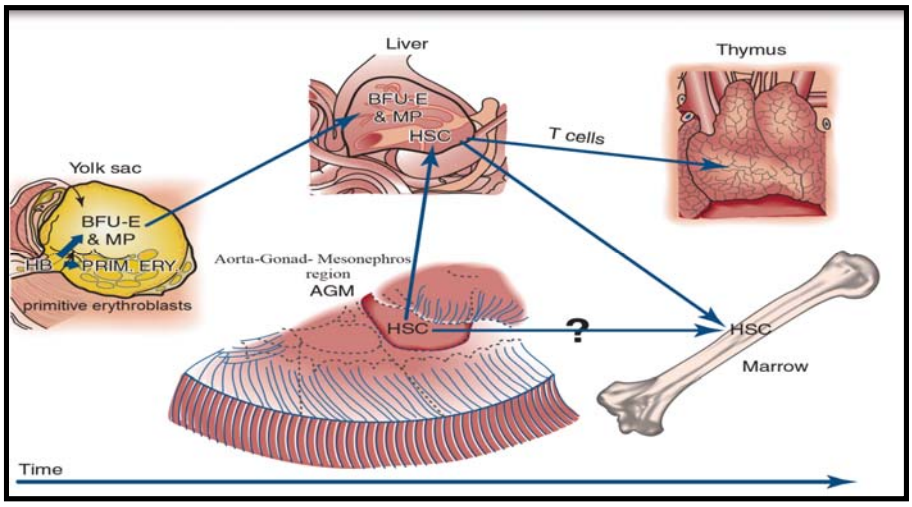


- The **yolk sac** provides two transient populations of committed progenitors:

- The **first wave of progenitors** produces:
  - **primitive erythroblasts** *(PRIM. ERY.)*.

- The **second wave** produces:
  - **Burst-forming unit–erythroid** *(BFU-E)* that seed the liver.
  - several **myeloid progenitors** *(MP)* that seed the liver.





- **Long-term hematopoietic stem cells** *(HSCs)* arise later in the **dorsal aorta**
- The HSC subsequently populate the liver and ultimately the bone marrow to generate the **full line of definitive hematopoiesis**.
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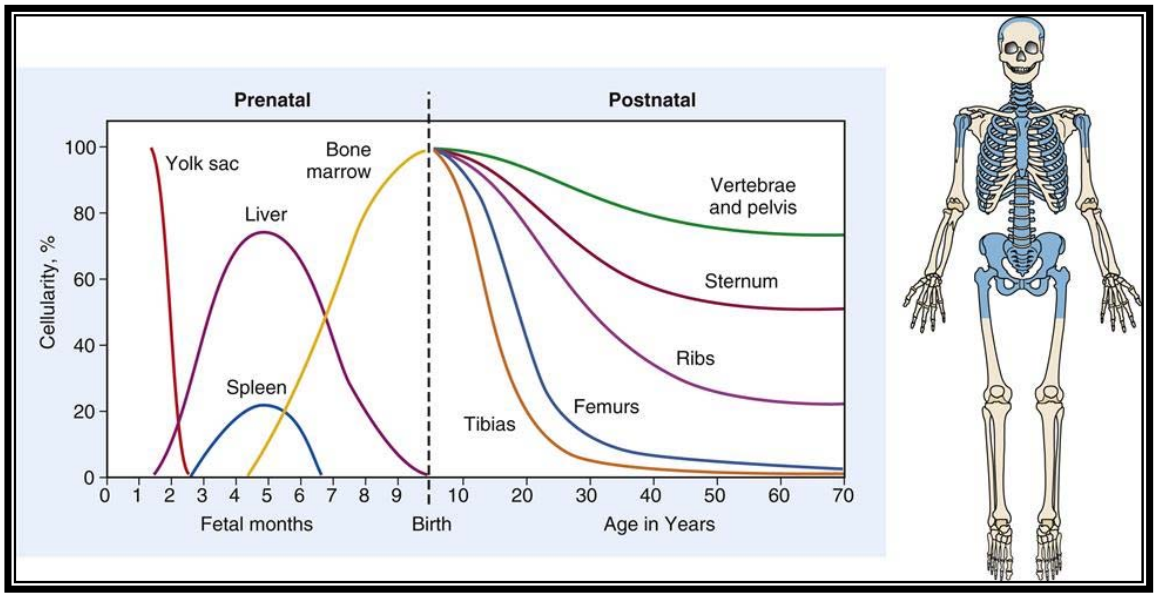
- The HSCs from liver also provide **young lymphoid cells** to the thymus, and T-lineage maturation occurs there.

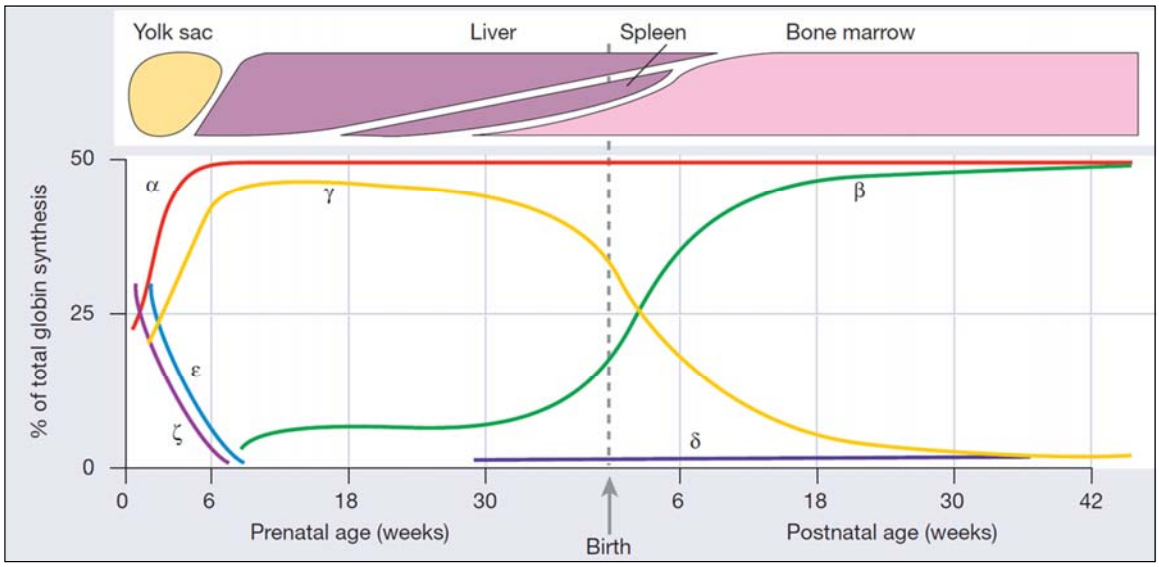
Site of Hemopoiesis

- In the **first few weeks (~ 8 weeks)** of gestation the **yolk sac** is a transient site of hemopoiesis.
- **From about 2–7 months of fetal life**, the **liver** and **spleen** are the major hemopoietic organs and continue to produce blood cells **until about 2 weeks after** birth.
- The **placenta** also contributes to fetal hemopoiesis.
- The **bone marrow** is the most important site from 5-9 months of fetal life.
- During normal childhood and adult life bone marrow is the only source of new blood cells.

	Sites of haemopoiesis.
Fetus	0–2 months (yolk sac)
	2–7 months (liver, spleen)
	5–9 months (bone marrow)
Infants	Bone marrow (practically all bones)
Adults	Vertebrae, ribs, sternum, skull, sacrum and pelvis, proximal ends of femur

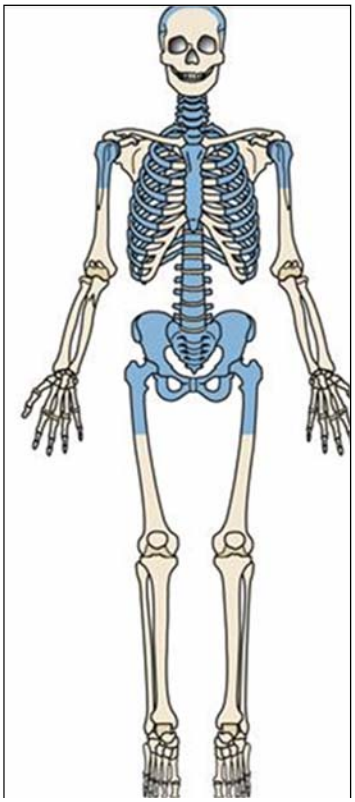
Expansion & decline of hemopoietic activity in extramedullary & medullary sites

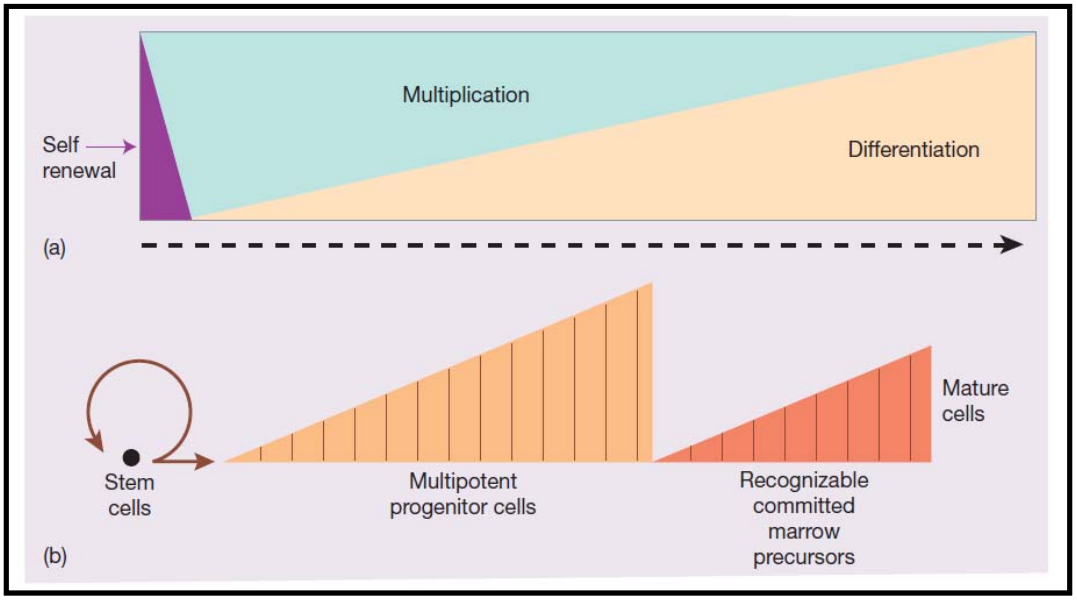




Site of Hemopoiesis

- In **infancy** all the bone marrow is hemopoietic but during **childhood** there is progressive **fatty replacement** of marrow throughout the long bones.
- So that in **adult life** hemopoietic marrow is **limited to the central skeleton and proximal ends of the femur and humerus.**
- Even in these hemopoietic areas, adipocytes approximately constitute (~50-70%) **of the marrow.**
- The remaining fatty marrow is capable of **restoring** to hemopoiesis and in many diseases there is also expansion of hemopoiesis down the long bones.
- Moreover, the liver and spleen can resume their fetal hemopoietic role (‘**extramedullary hemopoiesis**’).

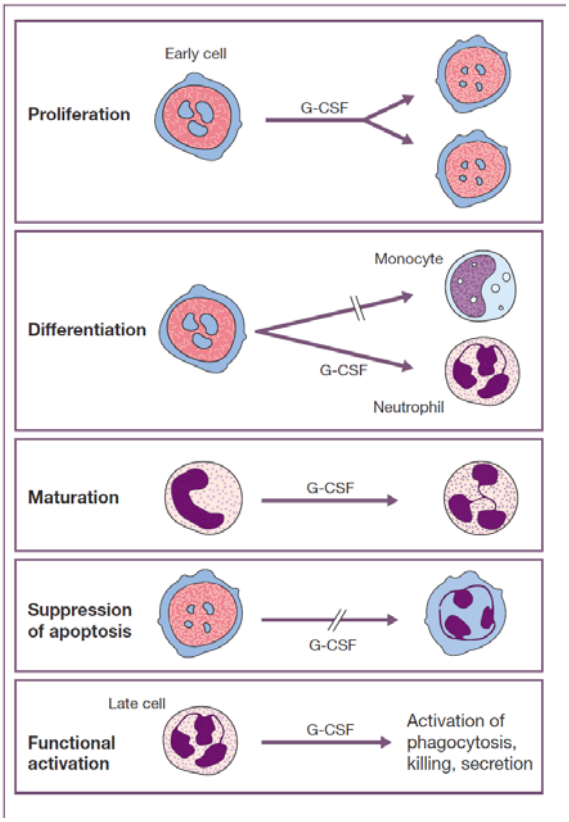


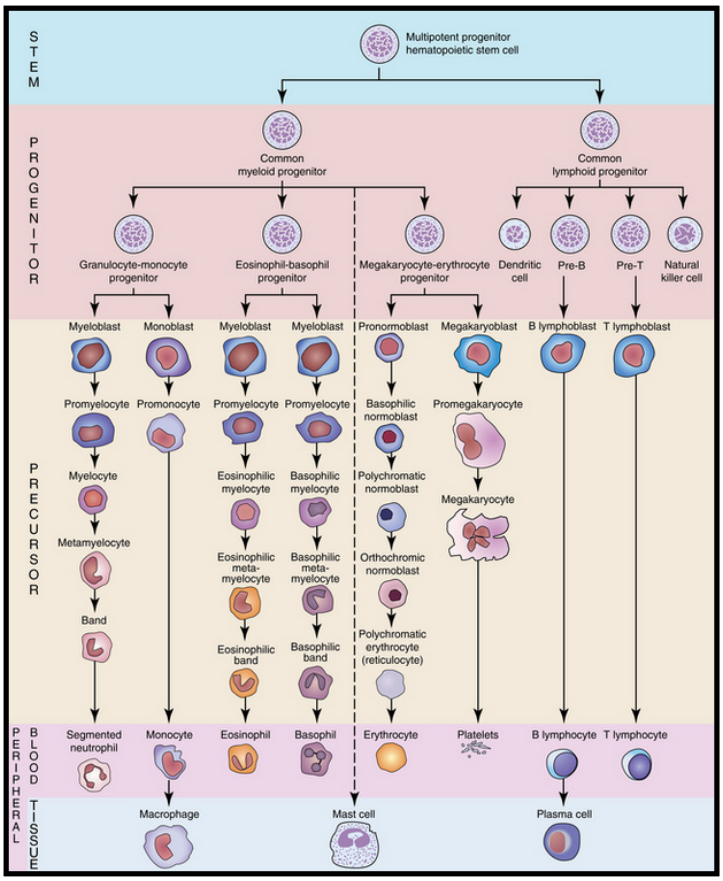


- Hemopoiesis starts with **stem cell division** in which one cell replaces the stem cell (**self-renewal**) and the other is committed to **differentiation**.
- Bone marrow cells are increasingly **differentiated and lose the capacity** for self-renewal as they mature.
- A **single stem cell** gives rise, after multiple cell divisions , **to >10^6 mature cells**.

Hemopoietic growth factors

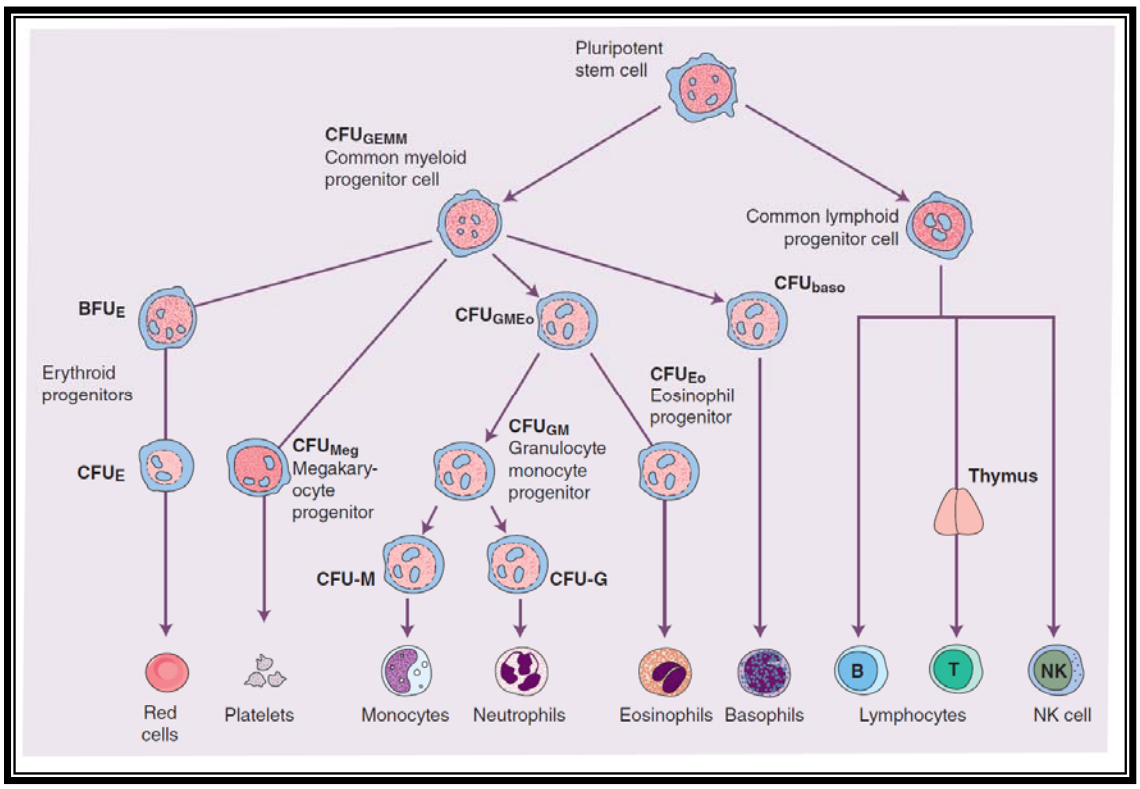
- The hemopoietic growth factors are a group of **glycoprotein hormones** that regulate the **proliferation** and **differentiation** of hemopoietic progenitor cells and the **function** of mature blood cells.
- Hemopoietic growth factors **may act locally** at the site where they are produced by cell–cell contact (e.g. SCF) or **circulate in plasma** (e.g. G-CSF or erythropoietin, EPO).
- hemopoietic growth factors also **bind to the extracellular matrix** to form niches to which stem and progenitor cells adhere.
- The growth factors may cause cell **proliferation**, but can **also stimulate differentiation** and **maturation, prevent apoptosis** and affect the function of mature cells

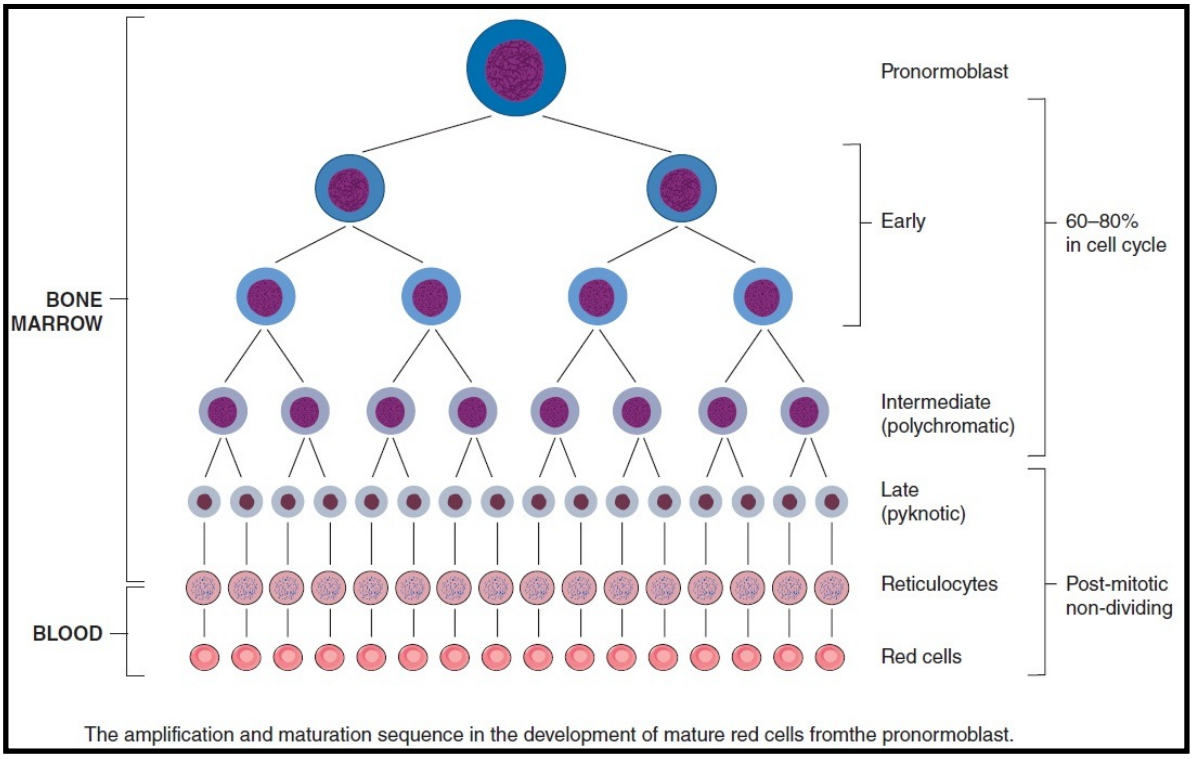




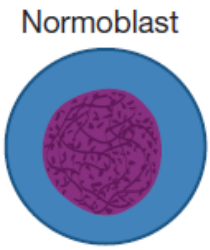
Cellularity of the bone marrow

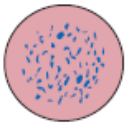
- **Bone marrow cellularity** refers to the amount or percentage of hematopoietic cells relative to marrow fat .
- Overall bone marrow **cellularity approximates 100 percent at birth** and declines with time, paralleling an age-associated reduction in hematopoietic activity.
- Cellularity is age dependent — in newborns, all marrow is hematopoietic (shows 100% cellularity), **with age hematopoiesis diminishes**, and the amount of fat increases.
- Accordingly, bone marrow cellularity in the adult is approximately **50 percen**t, with the remainder of the marrow being composed of adipose tissue.
- The **stem cell** has the capability for **self-renewal**, so that marrow cellularity remains constant in a normal, healthy steady state.
- The bone marrow normally contains **more myeloid cells than erythroid cells** in the ratio of 2 : 1 to 12 : 1, the largest proportion being neutrophils and metamyelocytes.





The amplification and maturation sequence in the development of mature red cells fromthe pronormoblast.





	Normoblast	Reticulocyte	Mature RBC
Nuclear DNA	Yes	No	No
RNA in cytoplasm	Yes	Yes	No
In marrow	Yes	Yes	Yes
In blood	No	Yes	Yes

The structure, genetic control, and synthesis of normal hemoglobin

- **Synthesis of different structural hemoglobins** at each stage of human development, due to different oxygen requirements.
- **All hemoglobins have the same general tetrameric structure**, consisting of two different pairs of globin chains, each attached to one heme molecule.
- **Adult and fetal hemoglobins** have α chains combined with β chains (Hb A, α2β2), δ chains (Hb A2, α2δ2), and γ chains (Hb F, α2γ2).
- **In embryos, α-like chains** called ζ chains combine with **β-like chains** γ chains to produce **Hb Portland (ζ2γ2),** or with ε chains to make **Hb Gower 1 (ζ2ε2),** while α and ε chains form **Hb Gower 2 (α2ε2).**
- **Fetal hemoglobin is heterogeneous**; there are two varieties of γ chain that differ only in their amino acid composition **at position 136**, which may be occupied by either **glycine or alanine**; γ chains containing glycine at this position are called Gγ chains, those with alanine Aγ chains

Ontogeny of Human Hemoglobin

Human hemoglobins are **heterogeneous** proteins packed inside the RBCs.

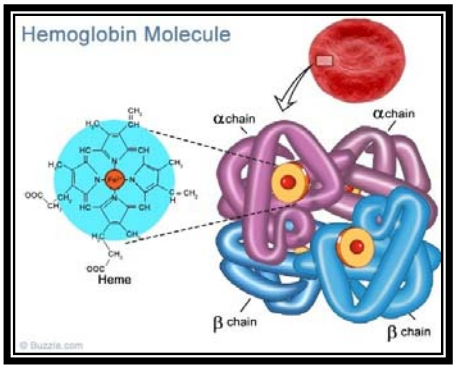
About **270 millions** of molecules of hemoglobin/ RBC.

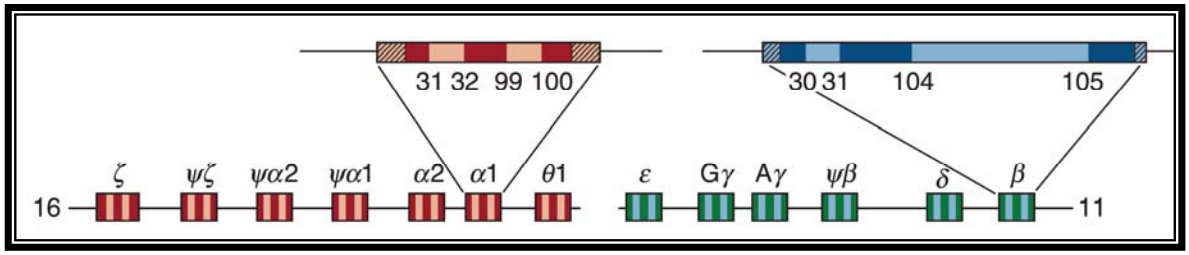
Tetrameric in structure, made up of two different pairs of globin polypeptide chains:

2 α-like (141 a.a) chains, controlled by gene cluster (ζ and α) on chromosome 16.

2 β-like (146 a.a) chains, controlled by gene cluster (ε, γ, δ and β) on chromosome 11.

This **heterogeneity** is expressed at **all stages** of development, different hemoglobin forms are synthesized during human life.





α-gene and β- gene clusters

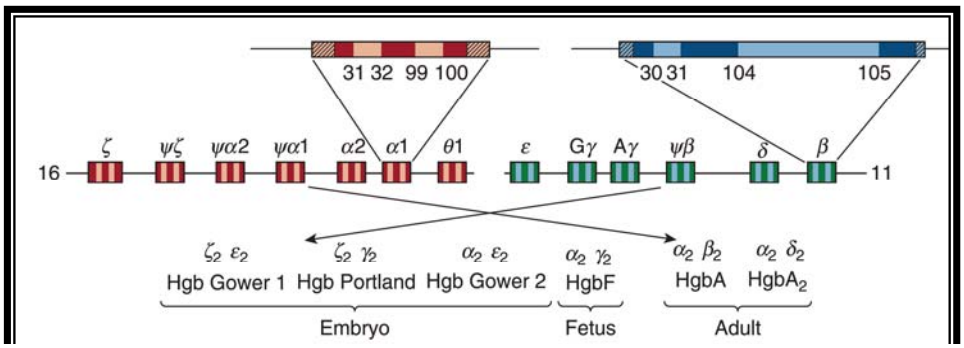
- The **α-gene cluster** contains one functional ζ (Zeta) gene and **two α** genes, designated α2 and α1. It also contains **four pseudogenes**: ψξ1, ψα1, ψα2, and θ (Theta) with unknown function.

5′–ζ–ψζ–ψα1– α2–α1–3′

- The **β-gene cluster** contains **6 genes**, ε (Epsilon), [**^{G}γ , ^{A}γ** (gamma) **136^{Gly} or γ 136^{Ala}**.], ψ β, δ (Delta) , and β of which **5 are functional genes**.

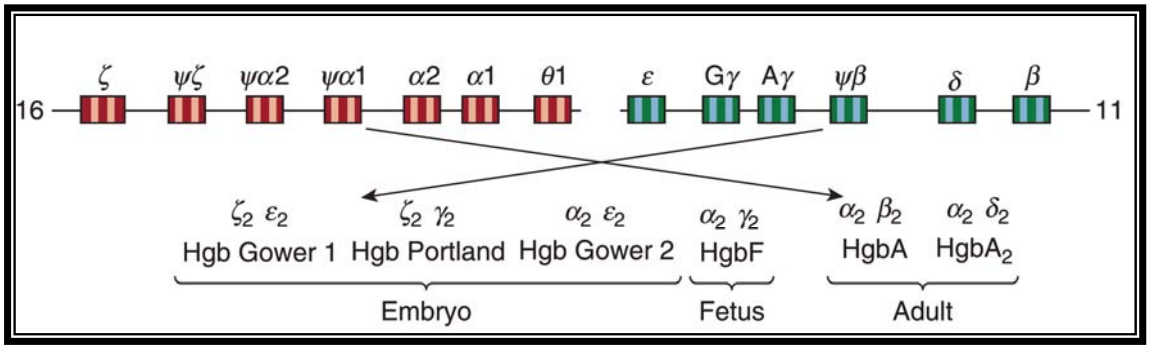
5′–ε–Gγ–Aγ–ψβ–δ–β–3′

- At birth, the ratio of molecules containing **γ 136^{Gly} / γ 136^{Ala}** chains is approximately 3:1, which varies widely in the trace amounts of Hb F present in normal adults.



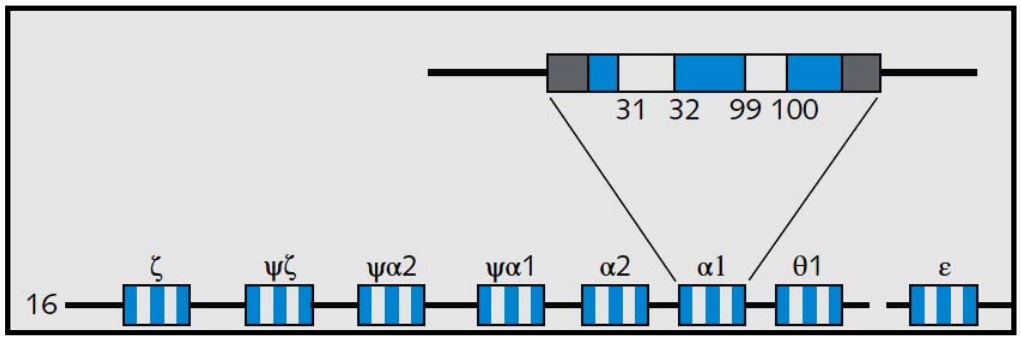
Heterogeneity of Human Hemoglobin

Hemoglobin Type	Structure	Developmental Stage
Hb Gower 1	$\zeta_2 \varepsilon_2$	embryo
Hb Gower 2	$\alpha_2 \varepsilon_2$	embryo
Hb Portland	$\zeta_2 \gamma_2$	embryo
HbF	$\alpha_2 \gamma_2$	fetal and adult
HbA_2	$\alpha_2 \delta_2$	adult
HbA	$\alpha_2 \beta_2$	adult



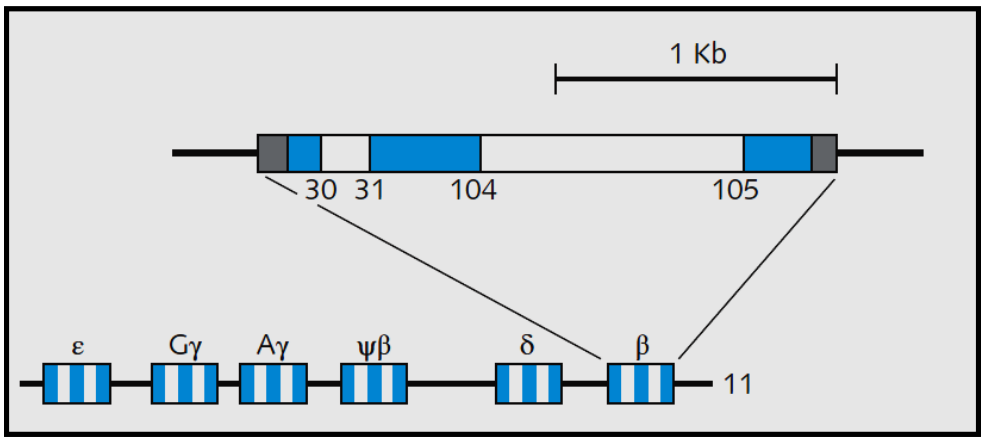
The α-like globin gene cluster: 16p13.3

- The structure of the human globin genes is **similar to that of all mammalian genes**.
- They consist of long strings of nucleotides that are divided into **coding regions (exons)**, and **non-coding** inserts called **intervening sequences (IVSs) or introns**.
- The α-like globin genes **contain two introns and three exons**, one intron between codons 31 and 32 and other intron between codons 99 and 100.
- The introns and exons, together with short non-coding sequences at the 5′UTR and 3′ UTR ends of the genes, represent the **major functional regions** of the particular genes.
- There are also extremely important **regulatory sequences** which subserve these functions that lie outside the genes themselves.



The β-like globin gene cluster: 11p15.5

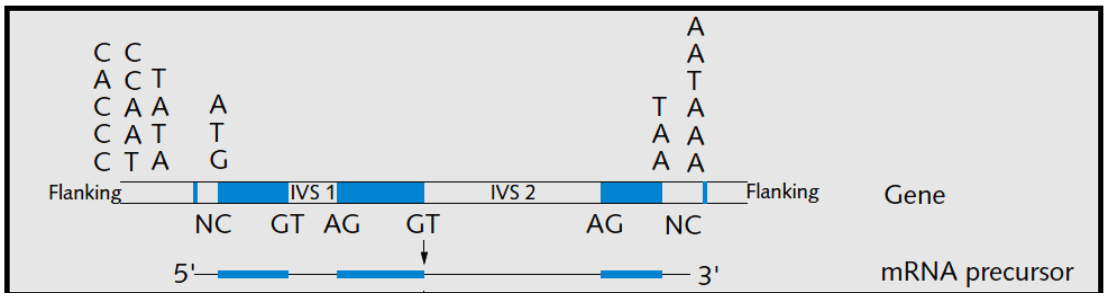
- The β-like globin genes **also contain two introns and three exons**, one between codons 30 and 31 and the other intron between codons 104 and 105



Genetic control of globin chains

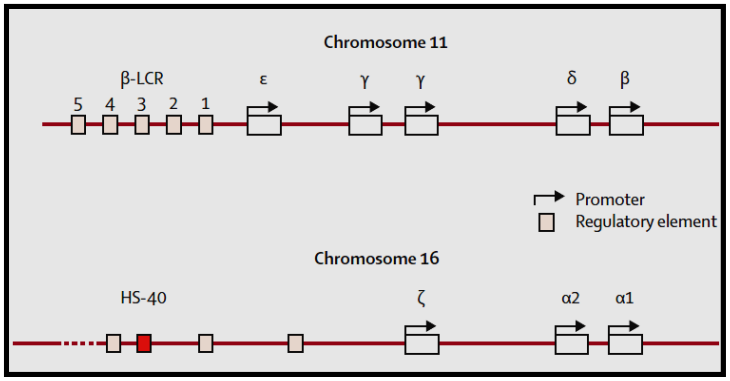
- At the 5′ non-coding (flanking) regions of the globin genes, as in all mammalian genes, there are blocks of nucleotide homology (***promoter elements)***.
- **The first, the ATA box, is about 30 bases upstream(-30)** of the first transcried nuculetide (+1); **The second, the CCAAT box, is about 70 base pairs upstream (-70**).
- About 80–100 bases further upstream there is the sequence **CACCC**,which may be inverted or duplicated.
- These **three highly conserved DNA sequences**, called ***promoter elements***, are involved in the initiation of transcription of the individual genes.

- In the 3′ non-coding region of all the globin genes there is the sequence AATAAA, which is the signal for cleavage and **polyA addition to RNA transcripts**



Regulatory elements of globin genes

- The globin gene clusters also contain **several regulatory sequences**, which interact to **promote** erythroid-specific gene expression and **coordination** of the changes in globin gene activity during development.
- These include the globin genes themselves and their promoter elements: **enhancers** and “master” regulatory sequences called, the **locus control region (LCR)** for β globin gene cl and, in the case of the α genes, **HS40** (a nuclease-hypersensitive site in DNA 40 kb from the α-globin genes).
- LCR and HS40 have a segmental structure made up of an array of short motifs that represent the **binding sites for transcriptional activators or repressors**.

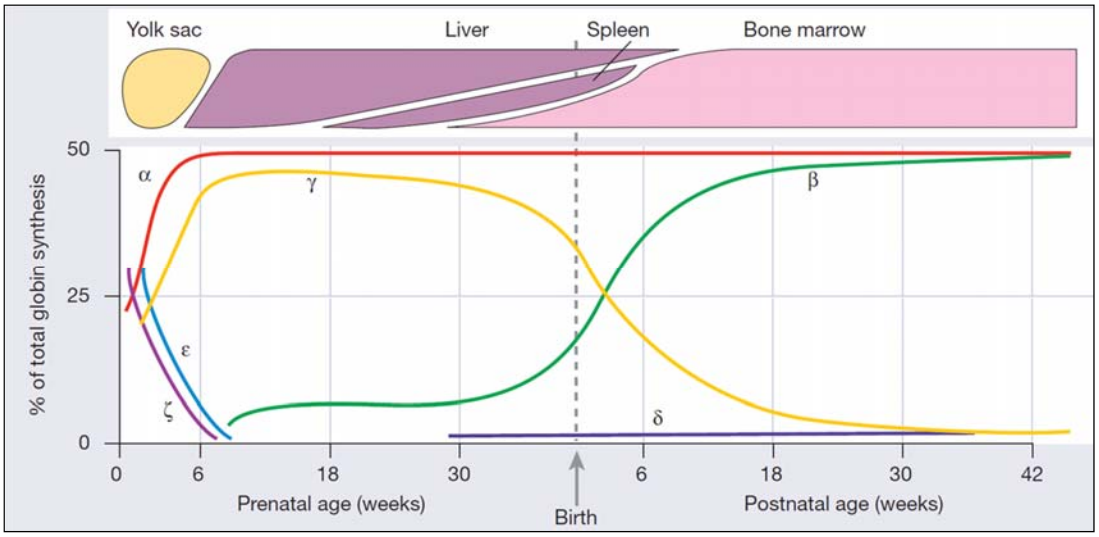


Fetal to Adult Hemoglobin Switch

- After birth, the adult β and δ globin chains begin gradually to replace the γ-globin chain.
- This results in a **major switch** from HbF ($\alpha_2\ \gamma_2$) to the adult hemoglobin HbA ($\alpha_2\ \beta_2$) synthesis.
- This switch **occurs** about the time of birth and **ends** 6 -9 months later.
- After the HbF ($\alpha_2\ \gamma_2$) to HbA ($\alpha_2\ \beta_2$) switch:
- HbA ($\alpha_2\ \beta_2$) ~ 97-98% of the total hemoglobin
- HbA_2 ($\alpha_2\ \delta_2$) accounts for approximately 2%.
- HbF ($\alpha_2\ \gamma_2$) ~(1%) is also found in adults blood.

HbF($\alpha_2\ \gamma_2$) to HbA ($\alpha_2\ \beta_2$) Switch

This switch **occurs** about the time of birth and **ends** 6 -9 months later.



Before week 8 of intrauterine life, three embryonic hemoglobins: Gower 1 ($\xi 2\varepsilon 2$), Gower 2 ($\alpha 2\varepsilon 2$), and Portland ($\xi 2\ \gamma 2$) are present

Hemoglobinopathies

Hemoglobinopathies

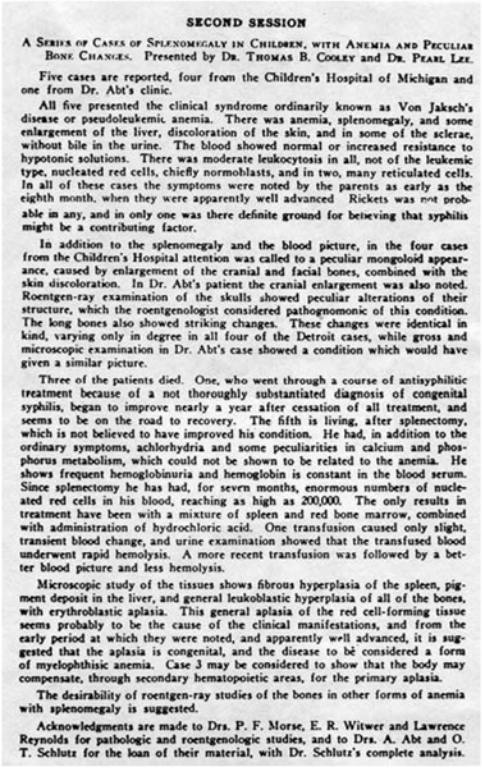
- The **inherited** disorders of hemoglobin are known as **Hemoglobinopathies** that could be due to **Quantitative** or **Qualitative** defects.
- **Quantitative** (defect in the rate of production of one or more of the globin chains) abnormalities: known as Thalassemias (the commonest genetic blood disorders of mankind).
- **Qualitative** (production of different hemoglobin molecules): known as structural hemoglobin variants (HbS, HbC, HbD, HbE, HbH).
- More than 1000 naturally occurring human hemoglobin variants have been described and the majority are resulted from substitution of single amino acid in the hemoglobin tetramer structure.

Hemoglobinopathies

- **Hemoglobinopathies** constitutes a major public health problem in many parts of the world. ~ **5-7%** of the world population being carriers for hemoglobinopathic disorder.
- **300,000 - 400,000 babies** are born each year with a serious hemoglobin disorder
- ~ 90% of these births occur in low- or middle-income countries and pose an increasing burden on global health resources
- **> 1%** of couples are at risk of getting an affected baby.
- Their **high frequency** is a reflection of natural selection (malaria hypothesis) and **consanguineous marriages** in many countries.
- **Hemoglobinopathies** accounts for ~3.5% of deaths in children < 5 years.

The Thalassemias

- Thalassemias are a **heterogeneous** group of genetic disorders (*simply autosomal recessive*), affect men and women equally, and characterized by mutations (monogeneic disorder) in the hemoglobin gene clusters that **impair the rate of synthesis** of one or more of the globin chains (α, β, γ, δ, ε).
- **1925**, the condition was first described by two Detroit pediatricians **Thomas B. Cooley and Pearl Lee**. It was **named** splenic anemia, erythroblastosis, **Mediterranean anemia, or Cooley's anemia**.
- **1936**, the term thalassemia was used after recognizing the disorder in many of the patients came from the **Mediterranean region.**
- The term thalassemia was derived from the Greek, **Thalassa *θαλασσα* (sea)** and **aima *αίμα* (blood)** meaning the sea in the blood or **sea blood**.



SECOND SESSION

A SERIES OF CASES OF SPLENOMEGALY IN CHILDREN, WITH ANEMIA AND PECULIAR BONE CHANGES. Presented by DR. THOMAS B. COOLEY and DR. PEARL LEE.

Five cases are reported, four from the Children's Hospital of Michigan and one from Dr. Abt's clinic.

All five presented the clinical syndrome ordinarily known as Von Jaksch's disease or pseudoleukemic anemia. There was anemia, splenomegaly, and some enlargement of the liver, discoloration of the skin, and in some of the sclerae, without bile in the urine. The blood showed normal or increased resistance to hypotonic solutions. There was moderate leukocytosis in all, not of the leukemic type, nucleated red cells, chiefly normoblasts, and in two, many reticulated cells. In all of these cases the symptoms were noted by the parents as early as the eighth month, when they were apparently well advanced. Rickets was not probable in any, and in only one was there definite ground for believing that syphilis might be a contributing factor.

In addition to the splenomegaly and the blood picture, in the four cases from the Children's Hospital attention was called to a peculiar mongoloid appearance, caused by enlargement of the cranial and facial bones, combined with the skin discoloration. In Dr. Abt's patient the cranial enlargement was also noted. Roentgen-ray examination of the skulls showed peculiar alterations of their structure, which the roentgenologist considered pathognomonic of this condition. The long bones also showed striking changes. These changes were identical in kind, varying only in degree in all four of the Detroit cases, while gross and microscopic examination in Dr. Abt's case showed a condition which would have given a similar picture.

Three of the patients died. One, who went through a course of antisyphilitic treatment because of a not thoroughly substantiated diagnosis of congenital syphilis, began to improve nearly a year after cessation of all treatment, and seems to be on the road to recovery. The fifth is living, after splenectomy, which is not believed to have improved his condition. He had, in addition to the ordinary symptoms, achlorhydria and some peculiarities in calcium and phosphorus metabolism, which could not be shown to be related to the anemia. He shows frequent hemoglobinuria and hemoglobin is constant in the blood serum. Since splenectomy he has had, for seven months, enormous numbers of nucleated red cells in his blood, reaching as high as 200,000. The only results in treatment have been with a mixture of spleen and red bone marrow, combined with administration of hydrochloric acid. One transfusion caused only slight, transient blood change, and urine examination showed that the transfused blood underwent rapid hemolysis. A more recent transfusion was followed by a better blood picture and less hemolysis.

Microscopic study of the tissues shows fibrous hyperplasia of the spleen, pigment deposit in the liver, and general leukoblastic hyperplasia of all of the bones, with erythroblastic aplasia. This general aplasia of the red cell-forming tissue seems probably to be the cause of the clinical manifestations, and from the early period at which they were noted, and apparently well advanced, it is suggested that the aplasia is congenital, and the disease to be considered a form of myelophthisic anemia. Case 3 may be considered to show that the body may compensate, through secondary hematopoietic areas, for the primary aplasia.

The desirability of roentgen-ray studies of the bones in other forms of anemia with splenomegaly is suggested.

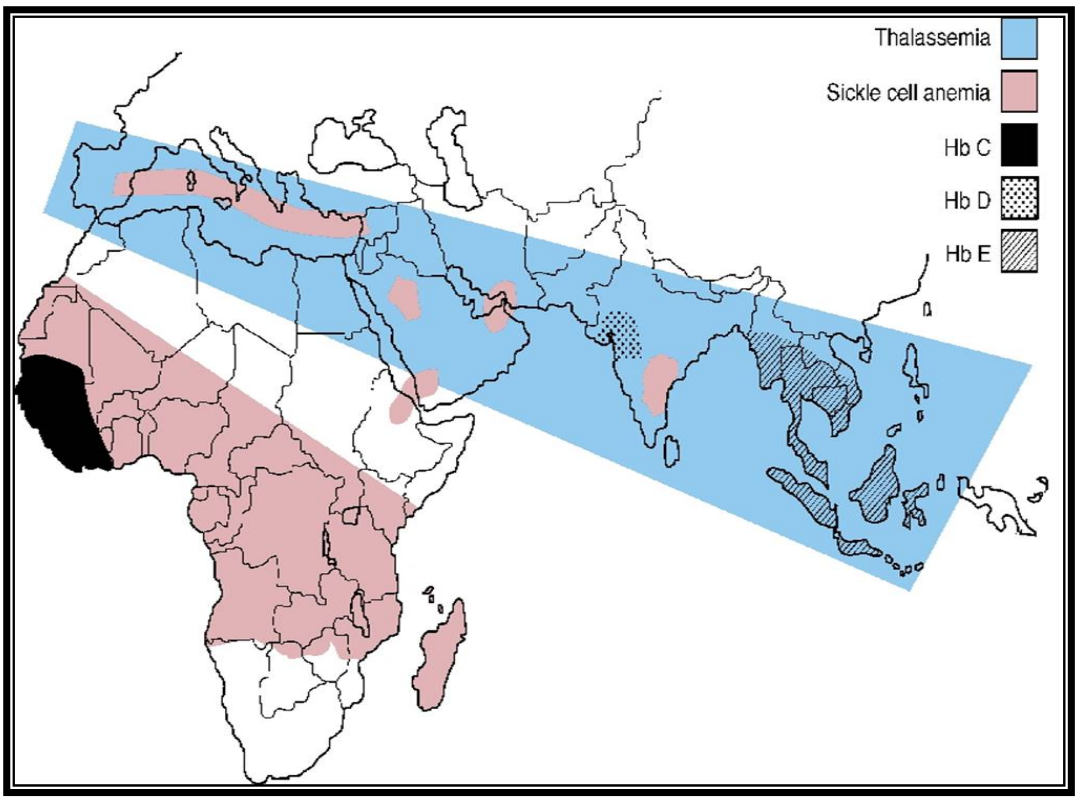
Acknowledgments are made to Drs. P. F. Morse, E. R. Witwer and Lawrence Reynolds for pathologic and roentgenologic studies, and to Drs. A. Abt and O. T. Schlutz for the loan of their material, with Dr. Schlutz's complete analysis.

The first description of Thalassemia (Cooley's anemia), in the Transactions of the American Pediatric Society, 37, 29–30, 1925

Geographic Distribution of Thalassemias

- Thalassemias have been encountered practically in **every racial group and geographic location** of the world.
- Thalassemias are **most common** in the Mediterranean region and equatorial or near equatorial regions of Africa and Asia.
- Thalassemias are found in a broad belt extended from the Mediterranean basin and Arabian peninsula to India and south eastern Asia.
- The **high frequencies** of thalassemia are found in those areas historically affected with **endemic malaria**.

Geographic distribution of common Hemoglobinopathies



Geographic Distribution of Thalassemias

- The high frequencies of thalassemia in many parts of the world could be due to the **relative protection** of the thalassemia carriers against *Plasmodium falciparum* malaria.
- Protection mechanism of this protection was related mainly to:
- **Delayed disappearance of HbF** in infants heterozygous for thalassemia.
- **Enhanced removal** of parasite-infected red blood cells
- **Reduced invasion and growth** of *P. falciparum* parasites
- **Reduced pathogenicity** through reduced cyto-adherence of *P. falciparum* parasites

Molecular Classification of Thalassemias

- At the molecular level, Thalassemias are classified according to **which particular globin** chain(s) is produced in a reduced amount: (α, β, γ, δ, ε).

- Consequently, thalassemias are classified according to the particular globin gene or genes affected (i.e. **α, β, δ, δβ , γδβ and εγδβ** thalassemias) with **α- and β-thalassemias** being the most commonly reported

- Also, the **severity of thalassemia mutation is** usually sub-classified according to whether the synthesis of the affected globin chain is **totally absent or only partially reduced**.

- Totally absent globin synthesis is designated with an "^{0}" superscript, e.g. **β^{0} -thalassemia** while partially reduced synthesis is designated with "$^{+}$" superscript, e.g. **β^{+} -thalassemia**

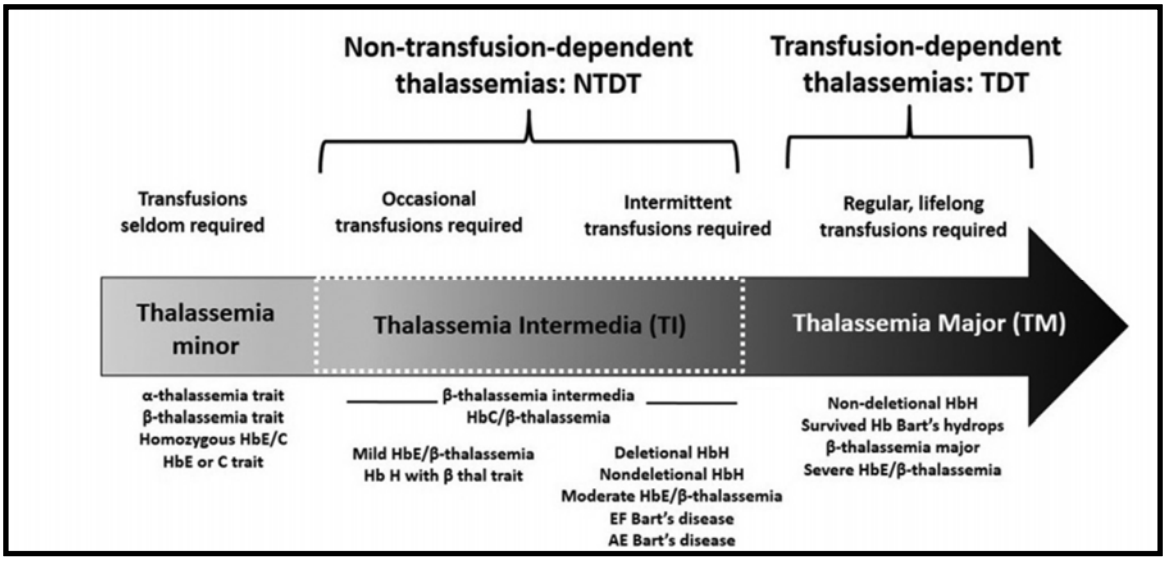
Clinical-management Classification of Thalassemias

- Thalassemias are also classified with a **more simplified categorization** largely based on **clinical-management criteria** (Transfusion therapy: the frequency and magnitude of transfusion requirements) which indirectly reflects the underlying **clinical severity** of the disease.

- **Transfusion therapy** is not only able to control most of the underlying pathophysiological mechanisms but it also contributes significantly in **ameliorating and minimizing secondary morbidities**.

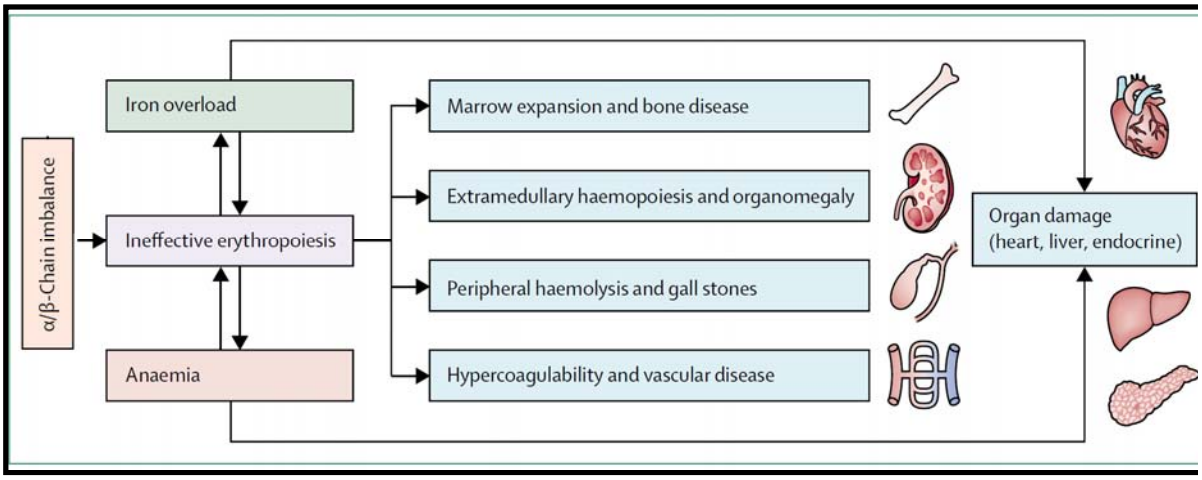
- Therefore, thalassemic patients are commonly **categorized as**:
- **Transfusion-dependent thalassemia (TDT)**
  - (lifelong regularly transfused for survival) patients who are not capable of producing sufficient hemoglobin to survive without blood transfusions
- **Non-transfusion-dependent thalassemia (NTDT).**
  - patients with NTDT can still require transfusion therapy sporadically, but not for their entire lifetime.

The clinical spectrum of thalassemia syndromes based on their requirement of regular blood transfusions into:
transfusion dependent thalassemia (TDT)
and
non–transfusion dependent thalassemia (NTDT)



Pathophysiology and associated morbidity

- The **hallmarks** of the thalassemia disease—with or without treatment—are the **α-globin or β-globin chain imbalance** leading to:
  - *ineffective erythropoiesis*
  - *an array of subsequent pathophysiological mechanisms*
  - *a multimorbidity profile.*



α-Thalassemias

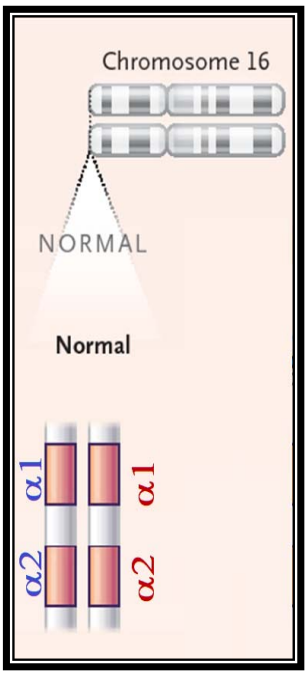
- α thalassemia includes all of those conditions in which there is a **shortage** in the production of the α globin chains of hemoglobin (Hb), so that the production of fetal HbF ($\alpha_2\gamma_2$) and adult HbA ($\alpha_2\beta_2$) are affected.
- In the **fetal life**, a deficiency or underproduction of α-chains leads to the production of an **excess of γ chains** which form **γ4 tetramer known as Hb Bart's.**
- While in **childhood or in early adult** life a deficiency or underproduction of α chains leads to an excess of β chains which form **β4 tetramer known as HbH**.

Hemoglobin Type	Structure	Developmental Stage
Hb Gower 1	$\zeta_2\varepsilon_2$	embryo
Hb Gower 2	$\alpha_2\varepsilon_2$	embryo
Hb Portland	$\zeta_2\gamma_2$	embryo
HbF	$\alpha_2\gamma_2$	fetal and adult
HbA_2	$\alpha_2\delta_2$	adult
HbA	$\alpha_2\beta_2$	adult

Consequently, the presence of Hb Bart's or HbH are the **hallmark of α-thalassemia** person.

Interactions of α-Thalassemia Haplotypes

- As there is **duplication** of the α-globin gene, a significant amount of **globin chain imbalance** is required before detectable amounts of Hb Bart's or HbH appear in the red cells.
- It is important to keep in mind that **the expression of the α2 gene is two to three times greater than expression of the α1 gene**.
- The α-globin gene haplotype (A haplotype is a group of genes or alleles within an organism that are inherited together from a single parent) can be written αα, indicating the α1 and α2 genes, respectively.
- A normal individual has the genotype αα/αα.



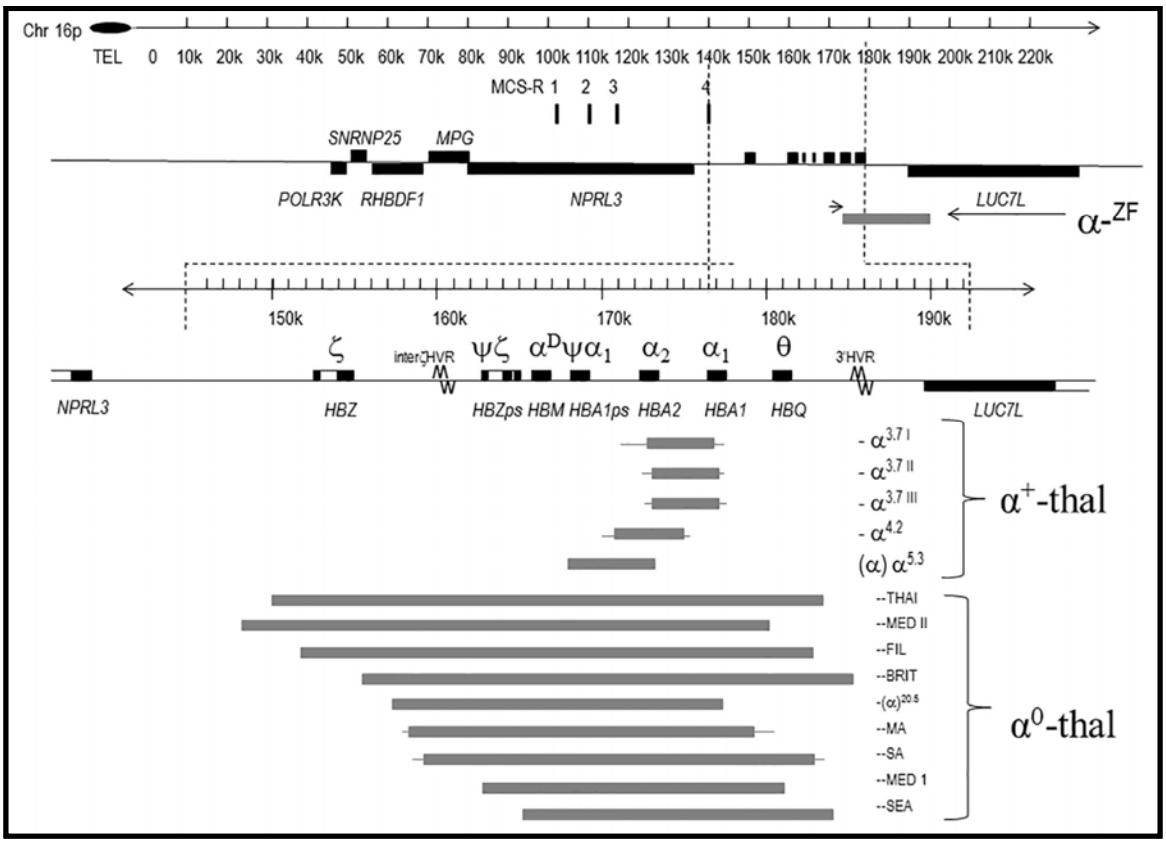
- The **normal production** of four functional α-globin genes may be **decreased** by:
- 1 (-α/ αα)
- 2 (--/αα or -α/-α)
- 3 (--/-α) or
- all 4 (--/--) copies of the genes
- This **explains** the clinical variation and **increasing severity of the disease**.

α-Thalassemias mutations

- α-thalassemia **most frequently** results from **large deletion mutations** in α genes. Non-deletion mutations (**point mutations)** are found **in rare cases.**
- α-thalassemia may be **classified** according to whether the **loss** of α-globin genes is **complete** α^0-thalassemia (--/--) or **partial** α^+-thalassemia (-α/ αα), (--/αα or -α/-α), (--/-α) .
- A deletion involving one (–α) or both (– –) α genes can be further classified based on its size, written as a superscript; thus, **$-\alpha^{3.7}$** indicates a deletion of 3.7 kb including one α gene.

- When the **sizes of the deletions are not established**, a superscript describing their geographic or family origin is useful; thus, **– – ^{MED}** describes a deletion of both α genes first identified in individuals of Mediterranean origin.

- **For nondeletion mutation**, the nomenclature **$\alpha^{ND}\alpha$** is given, with the superscript ND indicating the gene is thalassemic.
- However, when the **precise molecular defect is known**, as in hemoglobin Constant Spring, for example, $\alpha^{ND}\alpha$ can be replaced by the more informative **$\alpha^{CS}\alpha$**.



α^{+}-Thalassemias mutations

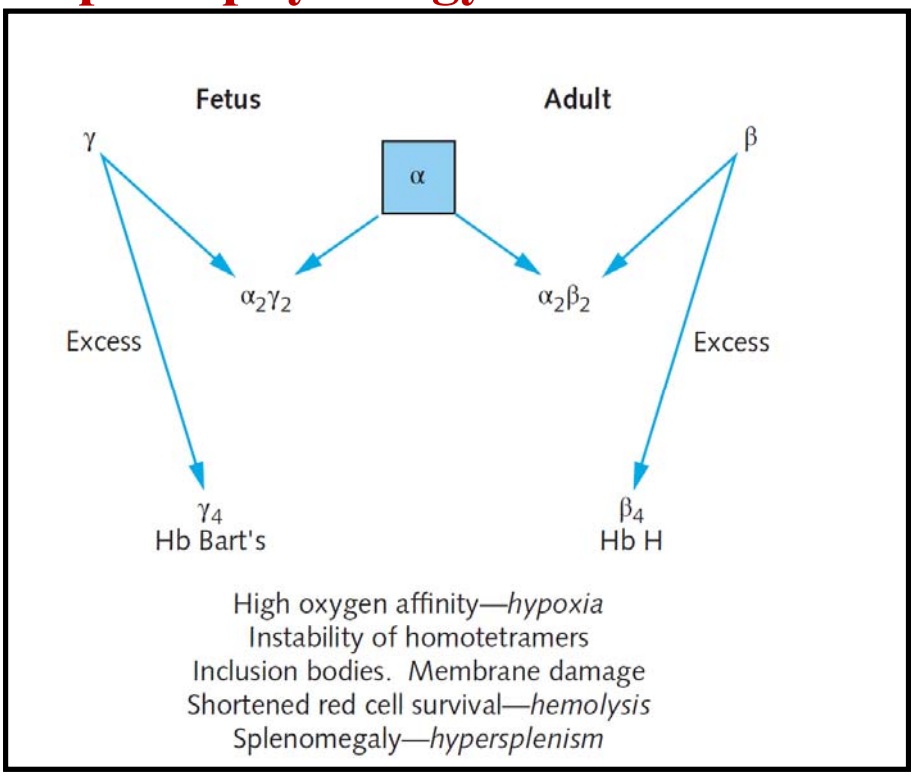
- **Deletions involving *α*2 or *α*1 genes** [The most common forms of *α*+-thalassemia (–*α*3.7 and –*α*4.2) involve deletion of one or the other of the duplicated *α*-globin genes]
- **Nondeletion α-Thalassemia [**Point mutations involving *α*2 or *α*1 genes]. most of the nondeletion mutants discovered to date affect **predominantly α2** gene expression.
- **mRNA processing mutations**
  - Splice site [5′ splice site of IVS-1 of α2-globin gene]
  - Poly(A) signal [poly-A addition signal site (AATAAA→AATAAG) and downregulates the α2 gene by interfering with 3′ end processing]
- **mRNA translation**
  - Initiation the initiation codon is inactivated by a T→C transition
  - Nonsense, frameshift
  - Termination [mutations that affect termination codon (TAA) of translation and give rise to elongated α chains e.g hemoglobin Constant Spring ($\alpha^{142\ STOP \rightarrow Gln}$; TAA → CAA)
- **Posttranslational stability [Unstable α-globin variants]**

Clinical classification of α-Thalassemias

- There are a **variable clinical phenotypes** of α-Thalassemia Syndromes
- Upon the number of α-genes affected, the disease can be **classified** into four clinical subtypes of **increasing severity**:
- **One α gene defect**
  - α-thalassemia **silent carrier** (α-thalassemia-2 trait)
- **Two α genes defect (cis or trans)**
  - α-thalassemia **minor** (α-thalassemia-1-trait)
- **Three α genes defect**

  Hemoglobin **H disease** (HbH) or **α-thalassemia intermedia** is a moderately severe type, usually results from the compound heterozygous state for α^{0}-thalassemia and either deletion or non-deletion α^{+}-thalassemia. It occurs most frequently in **Southeast Asia** ($--^{SEA}/-\alpha^{3.7}$) and the **Mediterranean region** (usually $--^{MED}/-\alpha^{3.7}$).
- **Four α genes defect**
- The most severe form is Hb Bart's **hydrops fetalis or α-thalassemia homozygous or α-thalassemia major:** four α genes defect [usually results from the homozygous state for α^{0}-thalassemia, most commonly $--^{SEA}/--^{SEA}$ or $--^{MED}/--^{MED}$. Hb Bart's **hydrops fetalis (**abnormal accumulation of fluid in 2 or more fetal compartments) with Hb Bart's (**γ4 tetramer)** in which **all four α loci are deleted**.

The pathophysiology of α-thalassemia



α Thalassemia traits

one α gene defect

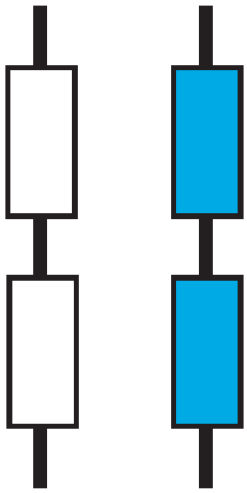
- α^+-thalassemia trait or α-thalassemia-2 trait or α-thalassemia silent carrier

two α genes defect (cis or trans)

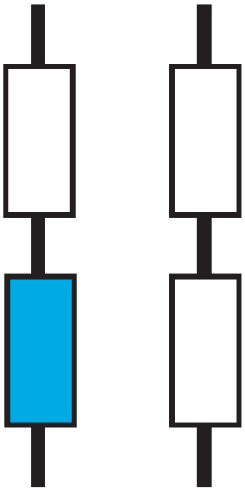
- α^0-thalassemia trait
- α-thalassemia-1-trait

- It does not matter whether the α genes are lost on the same chromosome or on opposite pairs of homologous chromosomes.
- Apart from mild to moderate microcytic hypochromic RBC [anemia] (detected on a routine blood count), carriers (heterozygotes) of α thalassemia, whatever the molecular basis, **are clinically asymptomatic** and the diagnosis (when made) is often established during a **regular health check** or during antenatal screening.
- **Complains** related to severe anemias, such as fatigue, listlessness and shortness of breath are **uncommon** and almost certainly related to other concomitant disorders.

α-thalassemia-1-trait
α^{0}-thalassemia trait

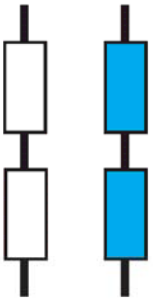


α-thalassemia-2 trait
α-thalassemia silent carrier
α^{+}-thalassemia trait



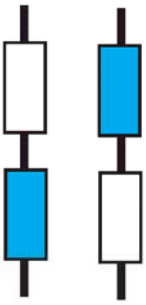
Two α genes defect (cis or trans)

α^{0}-thalassemia trait
α-thalassemia-1-trait



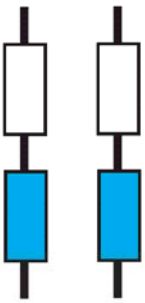
Heterozygous state for α0 thalassemia

(−−/αα)



Compound heterozygous state for α+ thalassemia

(−α/α−)

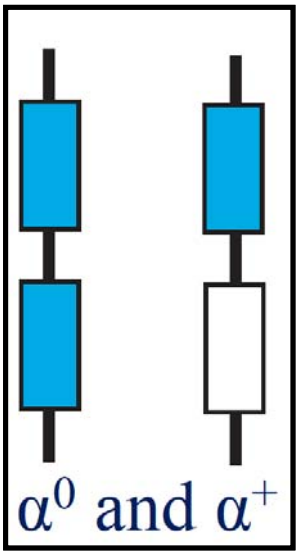


Homozygous state for α+ thalassemia

−α/−α

HbH disease (three α genes defect)

- HbH disease or sometimes known as **α-thalassemia intermedia**
- The loss of three α globin genes, which usually results from the **compound heterozygous states** for α^0 and α^+ thalassemia
- HbH disease is characterized by **a wide range of phenotypic characteristics**. The severity of the clinical features is clearly related to the molecular basis of the disease.
- The form that results from **deletions (−α/−−)** usually follows a **relatively mild course**, with moderate anemia and splenomegaly that does not require regular blood transfusions.
- However the variety that results from the **interactions of a nondeletional** ($\alpha\alpha^{ND}$) α-globin gene mutation(e.g. hemoglobin Constant Spring) together with **α^0-thalassemia** (−−) leading to the genotype ($\alpha\alpha^{ND}$ / −−) follows a much **more severe c**ourse.
- HbH disease patients usually **produce less than 30%** of the normal amount of α globin.



HbH disease (three α genes defect)

- The predominant features in HbH disease are **anemia (2.6-13.3 g/dl)** with variable amounts of **HbH (0.8-40%),** occasionally **accompanied by Hb Bart's** in the peripheral blood.

- **Hb Bart's and Hb H** tetramers have **very high O_2 affinity** and are therefore **useless (**poor carriers of O_2) for effective oxygen delivery.

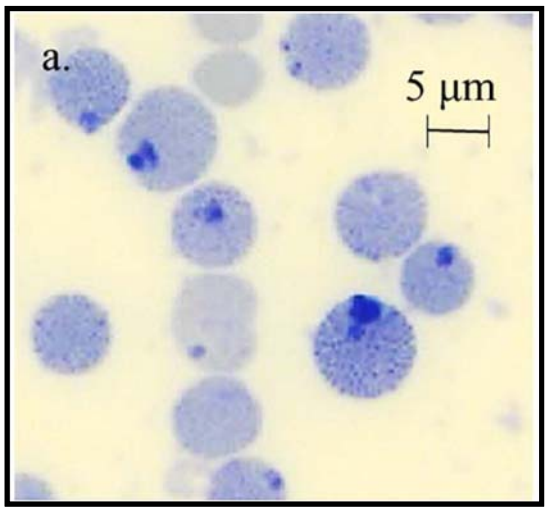
- The HbH patients usually have splenomegaly (which may be severe) and occasionally this is complicated by **hypersplenism (an overactive spleen).**
- **Jaundice** may be present in variable degrees and children may **show growth retardation**.

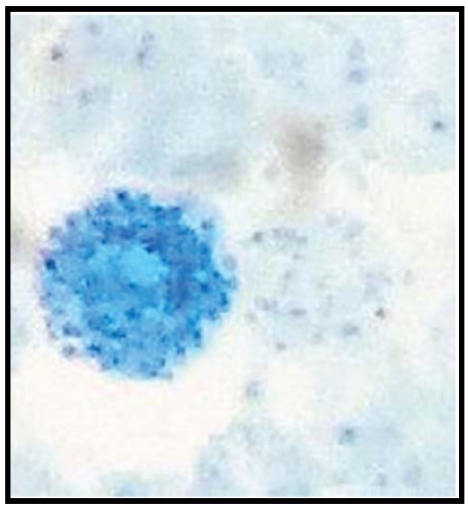
- Older patients often have **some degree of iron overload**.
- Patients with **non-deletional types of HbH** disease are **more severely affected** than those with the **common deletional types** of HbH disease.

HbH disease (three α genes defect)

- The blood film of HbH patients shows hypochromia and anisopoikilocytosis.
- The **reticulocyte** count usually is approximately **5 %.**
- Blood smear with brilliant cresyl blue results in **ragged inclusion bodies (Heinz bodies**) in almost all RBC.
- Hb H constitutes **0.8- 40 %** of the total hemoglobin.
- Traces of Hb Bart's may be present
- **Hb A2** level usually is slightly **subnormal**.

Inclusion Bodies (Heinz bodies) within Red cells of HbH disease patient
Golf ball appearance



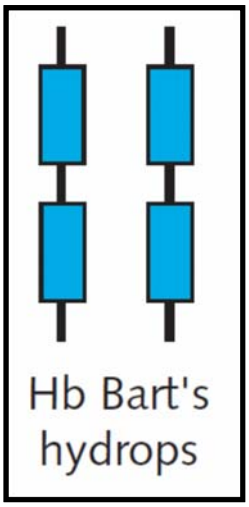


Deletion vs non-deletion HbH disease

- **Nondeletional forms of HbH disease** are characterized by severe anemia, often occurring from early life, and are associated with increasing splenomegaly, iron loading, and a variety of other clinical complications, including infections, leg ulcers, gallstones, and folic acid deficiency
- **The non-deletion** form is characterized by a significantly **more ineffective erythropoiesis** and **erythroid apoptosis** than the deletion types of HbH disease.
- The **more severe forms of HbH disease** are predominantly those involving **non-deletion mutations**, of which **Hb CS is** the most common in South-East Asia.
- **The hemoglobin in the non-deletion** form is lower, but the MCV higher due to **overhydration of cells** containing HbCS.

Hb Bart's Hydrops Fetalis Syndrome

- **Hb Bart's hydrops fetalis** syndrome is **the homozygous state for α^0 thalassemia** (− −/−−), is characterized by death in utero (stillbirth baby) or just after birth, with the clinical picture of hydrops fetalis. It **most frequently** results from the inheritance of **no α globin genes from either parent,**
- In **some cases** it results from the inheritance of a **severe nondeletion** mutation from one parent and **no α genes** from the other.
- Patients on the **borderline** between severe HbH disease and Hb Bart's hydrops Fetalis syndrome are said to have **HbH hydrops syndrome**.
- Physiologically **non-functional homotetramers γ4 and β4** [unable to release its oxygen] make up most of the hemoglobin in the erythrocytes in infants with the Hb Bart's hydrops Fetalis syndrome. This is reflected in gross intrauterine hypoxia.
- Hb Barts fetuses also have **variable amounts of an embryonic Hb Portland (ζ2γ2),** which is the only functional Hb in these infants and must be the only oxygen carrier keeping these infants alive.



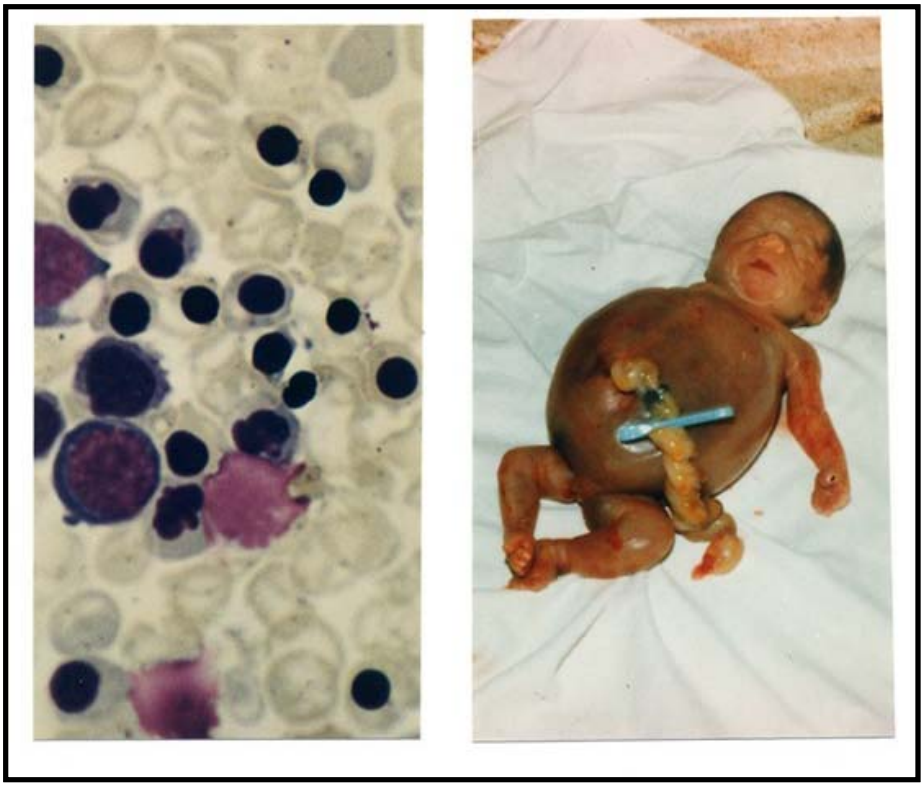
Hb Bart's Hydrops Fetalis Syndrome

- The clinical features are those of a **pale edematous infant** with signs of **cardiac failure** and **prolonged intra-uterine anemia**.

- Pronounced **hepatosplenomegaly**, **retardation in brain growth**, skeletal and cardiovascular deformities and gross enlargement of the placenta are characteristic features.

- Infants with the Hb Bart's hydrops fetalis syndrome **almost always either die in utero (23-38 weeks) or shortly after birth**.

- Although few cases have been described in which the neonate is given intensive life-support therapy and treated with blood transfusion.
- In infants with the hydrops fetalis syndrome, the blood film shows severe thalassemic changes (hypochromia and anisopoikilocytosis) with **many nucleated red cells**.
- The **hemoglobin profile** consists mainly of **hemoglobin Bart's**, with approximately **10 to 20 % hemoglobin Portland**. Usually **no HbA or HbF** is present



Diagnosis and diagnostic methods of α-Thalassemia

- Routine screening of α-thalassemia is essential to **identify couples at risk,** especially severe forms of the disease that occur **in high-frequency areas**.
- Routine screening also helps to **prevent severe maternal complications** in the case of Hb Bart's .
- **Most α-thalassemia traits are asymptomatic** and identified only by chance after routine hematological analyses. However, reduction in Hb level may occur when there is concomitant blood loss, infection, poor nutrition, or other disease that may lead to symptomatic anemia.
- When the level of α globin synthesis falls below ~70% of normal, excess γ globin chains form Hb Bart's which can be detected in the fetal period and excess β globin chains forming β4 tetramers of HbH can be detected in adult life.
- Laboratory testing should include:
- **CBC** with red cell **indices**
- Hb quantification by **HPLC** or capillary/gel Hb **electrophoresis**
- Ultimately, sooner or later, **α/β-globin chain synthesis** ratio measurement.
- **DNA analysis**
  - α/β-globin chain synthesis ratio measurement is sometimes bypassed by DNA analysis as a less complicated method to diagnose α-thalassemia.

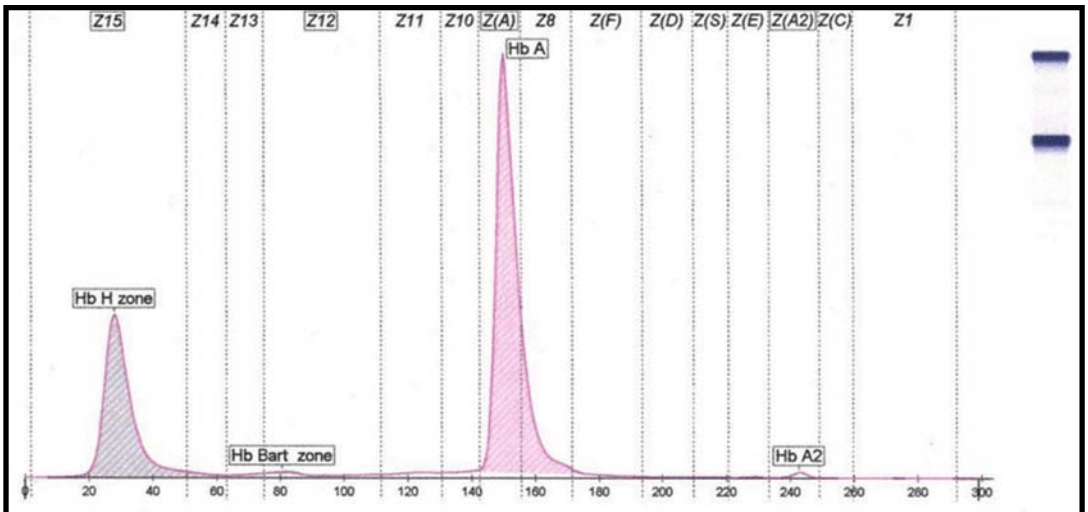
Complete Blood Count and Blood Smear

- In thalassemia screening, **microcytosis** (MCV <80 fL) and **hypochromia** or hypochromasia (MCH <27 pg) [*due to decrease in Hb synthesis*] are used as **cut-off levels** for a further screening methods are is required to confirm the thalassemia disease. (IDA, anemia of chronic disease).
- In thalassemia, RBC morphology showed microcytic, hypochromic, and apparent **poikilocytosis**.
- **All α thalassemia** affected individuals have a **variable degree of anemia** (Hb), **microcytosis, hypochromia** and a **normal or slightly reduced** HbA2 level, which depend roughly on the:
  - **# of mutated α genes**
  - correlates well with the **severity of the mutation**
- Interpretation of blood smear based on RBCs morphology **solely is not possible to define thalassemia** disease as those interpretations can also be detected in IDA.
- A blood smear stained with **brilliant cresyl blue** shows **inclusion bodies (Heinz bodies)** in the RBCs, indicating the presence of HbH.
- The RBC with inclusions **are unstable**, destroyed prematurely in the spleen and result in **moderate to severe hemolysis**.

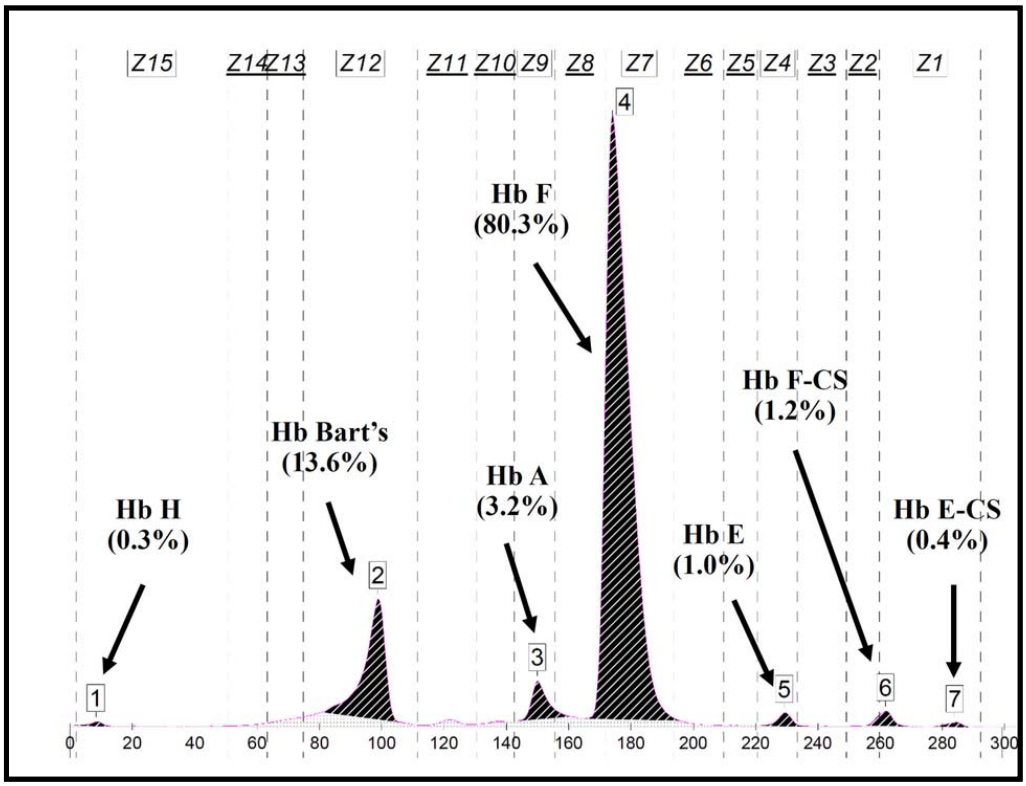
Capillary Electrophoresis (CE)

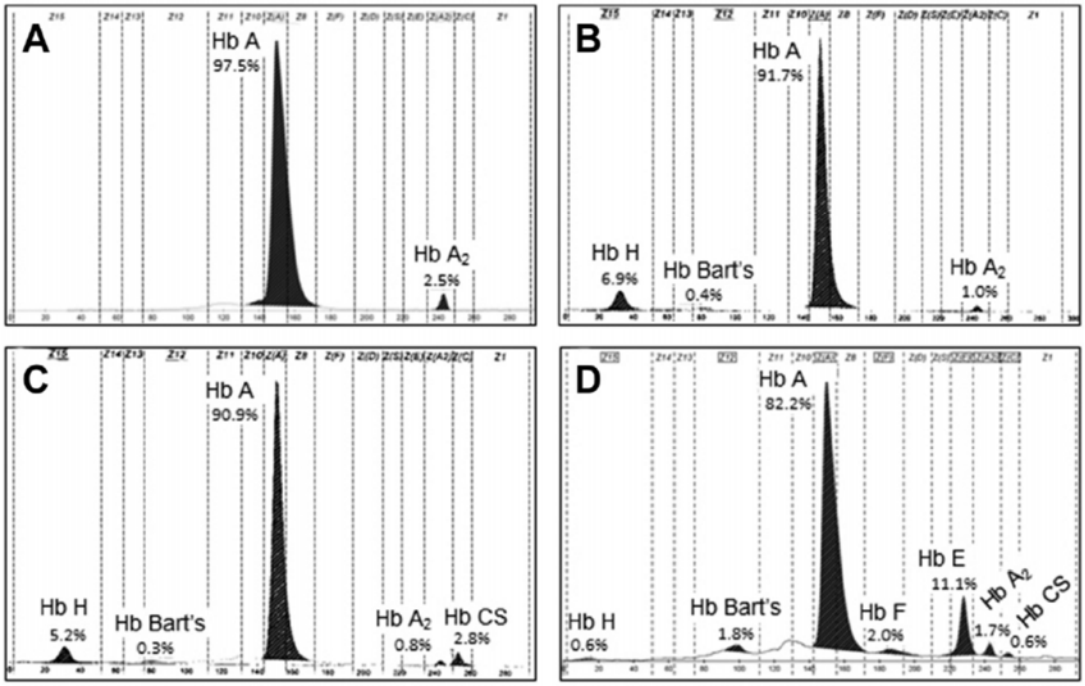
- CE is used to **separate** Hb fractions and **quantify** each fraction level.
- CE separates and quantifies Hb A_2, Hb E, Hb F, **Hb H, Hb Bart's** and some Hb variants. These parameters are required to diagnose α and β-thalassemia, and hemoglobinopathies.
- The Hb fractions are detected directly at a wavelength of 415 nm, which is optimal to Hb, in the following order from cathode to anode: Hb C, A2, E, S, D, F, A, Bart's, J and H.
- **Advantages** of CE include the turn-around time, high throughput, small sample volume requirement, and reasonable cost.
- The CE system can also **quantify Hb H and Hb Bart's levels**, which is a **HPLC limitation**.
- **The levels** of Hb H, Hb Bart's, and Hb CS are **decreased in stored blood** samples, especially at high temperatures as these are unstable hemoglobins.
- The **Hb H has a short life span** of 12 to 19 days as compared to normal 28 to 37 days.
- Factors that lead to unstable Hb H are abnormal RBC membrane with increased rigidity and increased inclusion bodies (**Heinz bodies**). This limitation can be overcome by using fresh blood sample.
- **Molecular analysis is still required** to fully understand the clinical phenotype and the definitive diagnosis. Therefore, molecular diagnosis of DNA sequence is **the most decisive diagnosis.**

Capillary Electrophoresis (CE)



Name	%	Normal Values %
Hb H zone	31.8	
Hb Bart zone	0.4	
Hb A	67.3	
Hb A2	0.5	


Z15
Z14
Z13
Z12
Z11
Z10
Z9
Z8
Z7
Z6
Z5
Z4
Z3
Z2
Z1
Hb H
(0.3%)
Hb Bart's
(13.6%)
Hb A
(3.2%)
Hb F
(80.3%)
Hb E
(1.0%)
Hb F-CS
(1.2%)
Hb E-CS
(0.4%)
1
2
3
4
5
6
7
0
20
40
60
80
100
120
140
160
180
200
220
240
260
280
300


A
Hb A
97.5%
Hb A2
2.5%
B
Hb A
91.7%
Hb H
6.9%
Hb Bart's
0.4%
Hb A2
1.0%
C
Hb A
90.9%
Hb H
5.2%
Hb Bart's
0.3%
Hb A2
0.8%
Hb CS
2.8%
D
Hb A
82.2%
Hb E
11.1%
Hb A2
1.7%
Hb CS
0.6%
Hb H
0.6%
Hb Bart's
1.8%
Hb F
2.0%

Alpha/beta-globin chain synthesis

- Measuring the ratio of α- and β-globin chain synthesis is the most **direct approach (at the protein level)** to diagnose α-thalassemia.

- If the α/β ratio appears **< 0.8 this is indicative** of α-thalassemia
- ~ 0.75 being consistent with a single α gene defect (-α/αα)
- ~ 0.5 for two α genes (--/αα)
- ~ 0.25 for three α-genes (--/-α)

Molecular Diagnosis of α-thalassemia

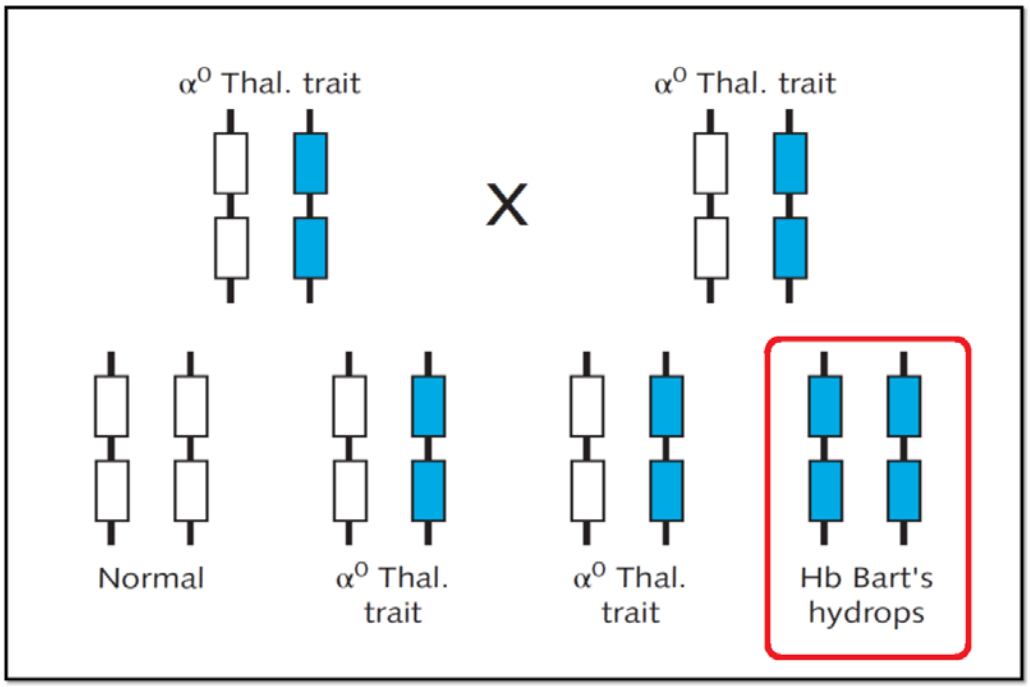
- Molecular genetic testing plays an essential role in the diagnosis of α-thalassemia.
- Genetic testing conducted during the prenatal period will identify fetuses with severe globin phenotypes.
- DNA analysis has greatly evolved as compared to that in the past 40 years.
- Recently, known and unknown mutations can be diagnosed by using many different polymerase chain reaction (PCR) based techniques and DNA sequencing.

Genotype/Phenotype Correlations : α Thalassemia

- Until now **about 130 different molecular defects** known to cause α thalassemia with an **ever increasing number of potential interactions** determining the clinical phenotypes
- The **severity of the clinical phenotype correlates** very well with the degree of α globin chain deficiency (number of affected genes and the severity of the mutation) .
- The non-deletional forms of α^+-thalassemia result in a **more severe phenotype** than in those with deletional forms of α^+-thalassemia.
- It is important to keep in mind that **the expression of the α2 gene is two to three times greater than expression of the α1 gene**.

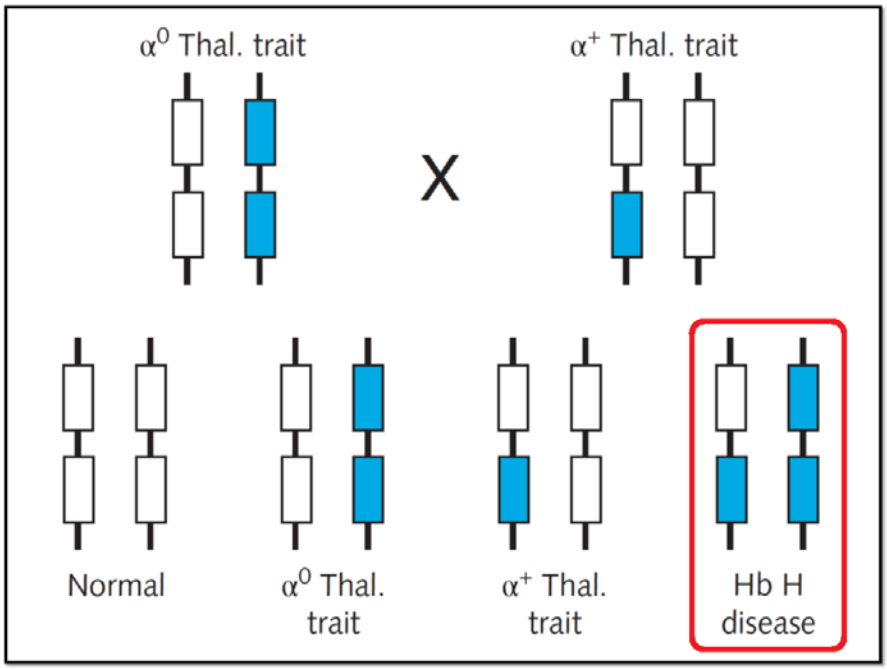
Genetic counselling: Hb Bart's hydrops fetalis genetic risk

- When both parents carry an α^o thalassemia mutation (--/αα) the risk of their offspring having Hb Bart's hydrops fetalis is 1:4 (25%).



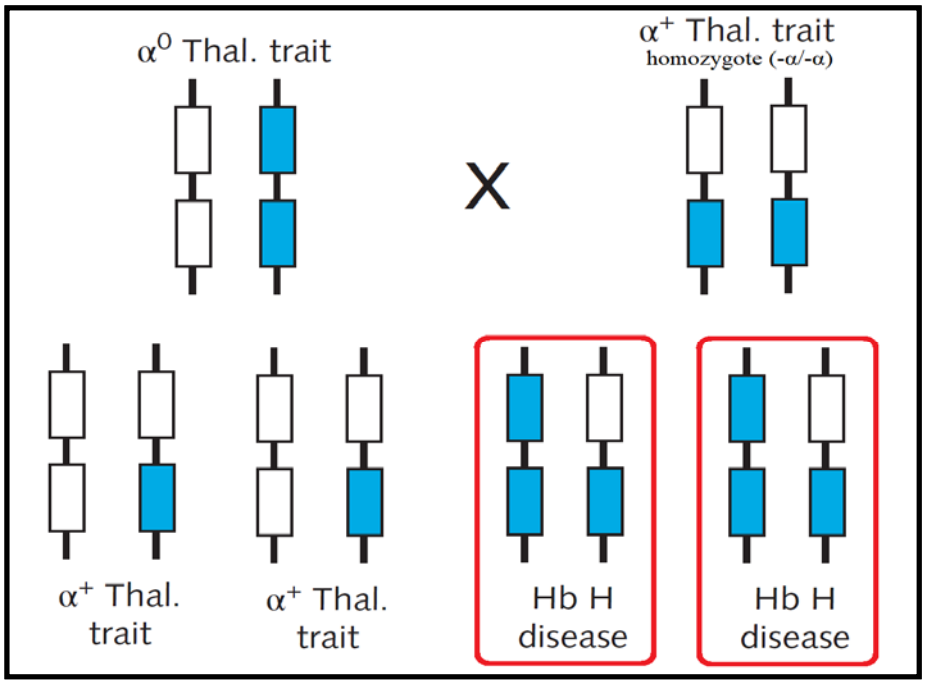
Genetic counselling: HbH genetic risk

- When one parent carries α^{o} thalassemia(--/αα) and the other carries an α^{+} thalassemia (- α/αα) the risk of their offspring having HbH disease is 1:4 (25%).



Genetic counselling : HbH disease genetic risk

- When one parent carries α^{o} thalassemia(--/αα) and the other carrier of α+ thalassemia is a homozygote (-α/-α) clearly the risk of HbH disease is 1:2 (50%).



β-Thalassemias

- The **β-thalassemias** are the most important types of thalassemias. They are so common, widely distributed and result in **severe anemia** in the **homozygous and compound heterozygous states**.
- β-thalassemias have been encountered sporadically in practically every racial group. However, they have a **high incidence** in a broad belt extending from the Mediterranean basin and parts of north and west Africa through the Middle East and Far East.
- β-Thalassemia types includes: **β**-thalassemia, **δβ**-thalassemia, and **γδβ**-thalassemia and **εγδβ** thalassemias.

- β-Thalassemia is caused by mutations resulting in a **single nucleotide substitution**, **small deletions or insertions** within the β-globin (*HHB*) gene or its immediate flanking sequence, or **in rare cases, gross deletions**. These mutations result in reduced production of β-globin chains and HbA.
- **δ β-thalassemia** is due to reduced δ and β chain synthesis resulting usually from **deletions** in the δ and β globin genes, it is a **less common type**. While **γδβ**- and **εγδβ** thalassemia are **rare forms** of thalassemia which results from a long deletion in the β-like globin cluster, and so removing the, ε, γ δ and β globin genes

β-Thalassemia Molecular Pathogenesis

- More than **350 β-thalassemia mutations** have been described, and they are commonly **assigned a severity index**, with **β++ and β+** denoting mild-moderate mutations that cause a relative reduction of β-globin chain synthesis and **β^0 referring to severe mutations** that can lead to a complete absence of β-globin chain product.
- The severity of anemia requiring transfusion, and clinical morbidity in β-thalassemia are **closely tied to the degree of α/β chains imbalance**
- Deficient production of β-globin chains leads to the **accumulation of excess, unstable α-globin tetramers** in erythroid cells.
- Free α-globins are unstable and **generate cytotoxic reactive oxidant species** and **cellular precipitates** that impair the maturation and viability of red-cell precursors, resulting in **ineffective erythropoiesis** and premature hemolysis of circulating red cells.
- Patients who have **severe β-thalassemia mutations** in the homozygous or compound heterozygous state tend to have **more severe clinical manifestations**, whereas patients who **co-inherit α-thalassemia** tend to have a milder disease

Types of β-thalassemia Mutations

- The β-thalassemia **genetic variants or mutations affect every step** in the pathway of β-globin gene expression: transcription, mRNA processing, mRNA translation, and post translational integrity of the β polypeptide chains.
- However only about **forty mutations account for 90%** or more of the β thalassemia worldwide
- These mutations ranging from **silent mutations** (**β^{++}**), to **mild mutations** that cause a relative reduction in β-globin chain production (**β^{+}**), to severe mutations that result in complete absence of β-globin chain synthesis (**β^{0}**).

- Most types of β-thalassemias are due to **point mutations**, and large deletion mutations are found in rare cases.

- Deletions of the β globin genes are not commonly found in patients with β thalassemia, with **one exception: a 619-bp deletion** involving the 3′ end of the gene is found frequently in the **Sind populations of India and Pakistan**, where it constitutes about **30%** of the β thalassemia alleles. Other deletions are extremely rare.

Mild forms β^{++} & β^{+} thalassemia: Defective β globin gene transcription

- A **much more common** group of mutations, which results **in mild to moderate** decrease in the **rate of transcription** of the β globin genes, involves single-nucleotide substitutions **in or near the TATA box** at about −30 nucleotides (nt) from the transcription start site (+1), or **in the proximal or distal** promoter elements e.g −90 , -101, −105 nt.

- These mutations result in **decreased (10-25%) β globin mRNA production** of the normal output. Thus, they are usually associated with the **mild or extremely mild (β^{++})** thalassemia or **moderate β^{+} thalassemia** forms . They are particularly common in African populations, an observation which explains the unusual mildness of β thalassemia in this racial group.

-

Mutations cause abnormal processing of mRNA

- Mutations that affect **splicing sites** completely **abolish normal splicing** and produce the phenotype of β^0 thalassemia.
- The transcription of genes carrying **splicing sites** mutations appears to be normal, but there is complete inactivation of splicing at the altered junction leading to **β^0**.
- Mutations within the **Splice site consensus sequences** can reduce the efficiency of splicing to varying degrees. For example, mutations of the nucleotide at position 5 of **IVS-1-5** (the first intervening sequence), G→C or T, result in a **marked reduction of β** chain production and in the phenotype of **severe β+ thalassemia**.
- On the other hand, the substitution of C for T at position **6 (IVS-1-6)** leads to **only a mild** reduction in the output of β chains, leading to **β^{++} thalassemia.**

- Mutations that involve the sequence **AAUAAA in the 3′ untranslated regions**, which is the **signal for cleavage and polyadenylation** of the β globin gene transcript. These **mutations destabilize the transcript**. For example, a T→C substitution in this sequence leads to production of only **one-tenth** of the normal amount of β globin mRNA transcript, and hence to the phenotype of a **moderately severe β+ thalassemia**

Mutations that result in abnormal translation of β globin mRNA

- ***There are 3 main classes of mutations :***
- **Nonesense (** Stop codon and premature termination of a protein):
- Base substitutions that change an **amino acid codon to termination codon** prevent the translation of β globin mRNA and result in the phenotype of **β^0 thalassemia.**

- **Frameshift**
- Insertion or deletion of **one, two, or four nucleotides** in the **coding region** of HHB that disrupt the **normal reading frame, cause a frameshift**, and hence interfere with the translation of β globin mRNA. This type of mutation always leads to the phenotype of β^0 thalassemia.

- **Initiation codon**
- These mutations affect the initiation codon of HHB thus reduce the efficiency of translation and lead to the phenotype of β^0 thalassemia.

Mutations that result in Unstable β globin chain variants

- Some forms of β thalassemia result from the synthesis of highly unstable β globin chains which are **incapable of forming hemoglobin** tetramers and which **are rapidly degraded**, leading to the phenotype of **β^0 thalassemia**.

Dominantly inherited β thalassemia

- Some forms of **β thalassemia are dominantly inherited**, in that inheritance of a single copy of the β thalassemia allele results in clinical disease
- **Heterozygous** have **moderate to severe anemia**, **splenomegaly** and the hematological hallmarks are **elevated HbA_2** and imbalanced globin chain synthesis
- More than **30 dominantly inherited** β thalassemia variants have now been described; they include **single base missense mutations (**different amino acids) **and minor insertions/deletions** that result in **truncated or abnormally elongated** β globin
- The common feature of these variants is the production of **highly unstable and non-functional β globin variants** that are not able to form viable tetramers with α globin.
- **These highly unstable and non-functional β globin variants** precipitate in the erythroid precursors and **together with the redundant α chains**, overload the proteolytic mechanism causing **premature death of these** erythroid precursors, leading to ineffective erythropoiesis.
- The dominantly inherited β thalassemia variants **are rare**, and found in dispersed geographical regions where the gene frequency for **β thalassemia is very low**.

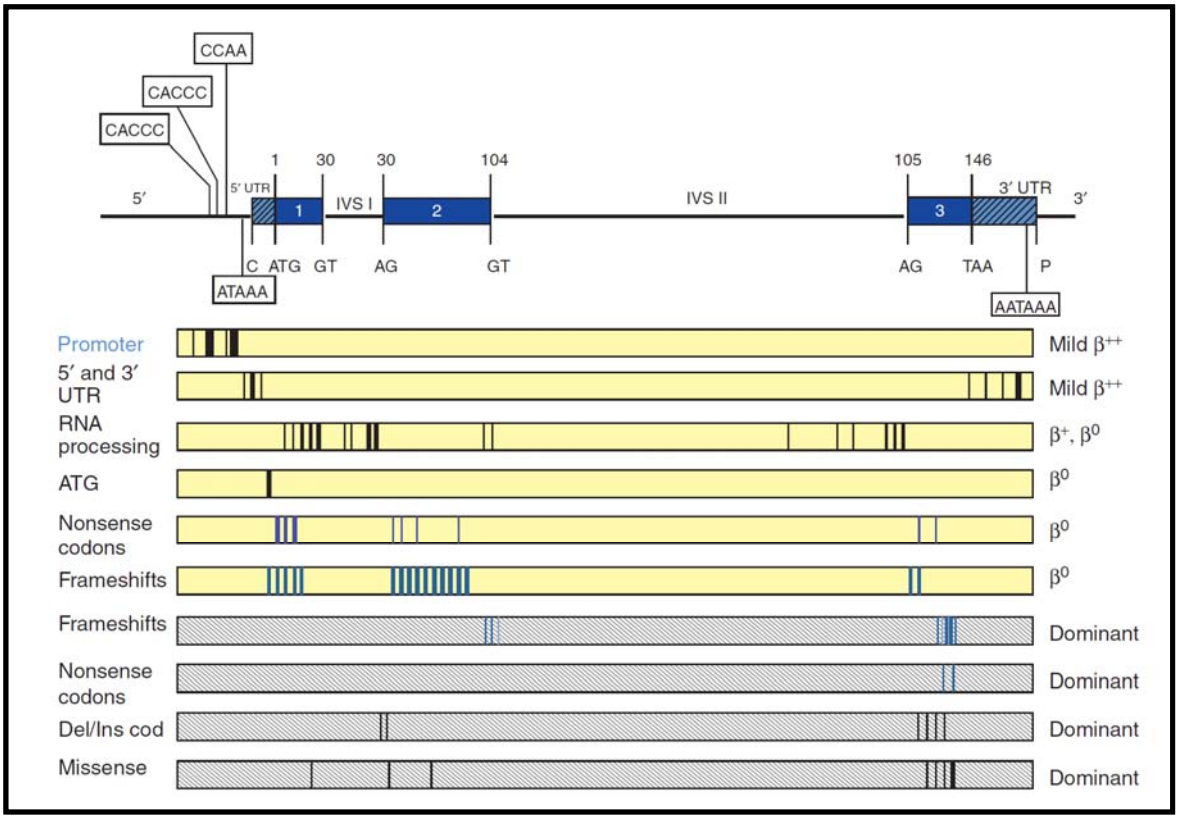


Table 1. Point mutations that cause β-thalassemia

Mutation	Type
I. Transcriptional mutations	
Promoter regulatory elements	
1) −101 (C → T)	β^{++} (silent)
2) −101 (C → G)	β^{++} (silent)
3) −92 (C → T)	β^{++} (silent)
4) −90 (C → T)	β^{+}
5) −88 (C → T)	β^{++}
6) −88 (C → A)	β^{+}
7) −87 (C → G)	β^{++}
8) −87 (C → T)	β^{++}
9) −87 (C → A)	β^{++}
10) −86 (C → G)	β^{+}
11) −86 (C → A)	β^{++}
12) −73 (A → T)	β^{++}
13) −32 (C → A)	β^{+}
14) −32 (C → T)	β^{+}
15) −31 (A → G)	β^{+}
16) −31 (A → C)	β^{+}
17) −30 (T → A)	β^{+}
18) −30 (T → C)	β^{+}
19) −29 (A → G)	β^{+}
20) −29 (A → C)	β^{+}
21) −29 (G → A)	β^{+}
22) −28 (A → C)	β^{+}
23) −28 (A → G)	β^{+}
24) −27 (A → T)	β^{+}
25) −27 to −26 (−AA)	β^{+}
26) −25 (G → C)	β^{+}
5′ UTR	
27) CAP +1 (A → C)	β^{++} (silent)
28) CAP +8 (C → T)	β^{++} (silent)
29) CAP +10 (−T)	β^{++} (silent)
30) CAP +20 (C → T)[a]	?
31) CAP +22 (G → A)	β^{++}
32) CAP +33 (C → G)	β^{++} (silent)
33) CAP +40 to +43 (−AAAC)	β^{+}
34) CAP +45 (G → C)	β^{+}
II. RNA processing	
Splice junction	
1) IVS1-(−2) CD30 (AGG → GGG)	β^{0}
2) IVS1-(−2) CD30 (AGG → CGG)	β^{0}
3) IVS1-(−1) CD30 (AGG → ACG) (Arg → Thr)	β^{0}
4) IVS1-(−1) CD30 (AGG → AAG)	β^{0}
5) IVS1-1 (G → A)	β^{0}
6) IVS1-1 (G → T)	β^{0}
7) IVS1-1 (G → C)	β^{0}
8) IVS1-2 (T → G)	β^{0}
9) IVS1-2 (T → C)	β^{0}

Consensus splice sites	
28) IVS1-5 (G → C)	β^0
29) IVS1-5 (G → T)	β^+
30) IVS1-5 (G → A)[b]	β^+
31) IVS1-6 (T → C)	β^{++}
32) IVS1- (−3) CD29 (GG**C** → GG**T**)	β^+
33) IVS1-128 (T → G)	β^+
34) IVS1-129 (A → G)	
35) IVS2-5 (G → C)	β^+
36) IVS2-843 (T → G)	β^+
37) IVS2-844 (C → G)	β^{++} (silent)
38) IVS2-844 (C → A)	β^{++} (silent)
39) IVS2-848 (C → A)	β^+
40) IVS2-848 (C → G)	β^+
Cryptic splice sites	
41) IVS1-110 (G → A)	β^+
42) IVS1-116 (T → G)	β^0
43) IVS2-654 (C → T)	β^0/β^+
44) IVS2-705 (T → G)	β^+
45) IVS2-745 (C → G)	β^+
46) IVS2-837 (T → G)	?
47) CD10 (GC**C** → GC**A**)	
48) CD19 (A**A**C → A**G**C) Hb Malay (Asn → Ser)	β^{++}
49) CD24 (GG**T** → GG**A**)	β^{++}
50) CD26 (**G**AG → **A**AG) (Glu → Lys, Hb E)	β^+
51) CD26 (G**A**G → G**C**G) (Glu → Ala, Hb Tripoli)	β^+

Mutation	Type
10) IVS1-2 (T → A)	β^0
11) IVS2-1 (G → A)	β^0
12) IVS2-1 (G → C)	β^0
13) IVS2-2 (T → A)	? β^0
14) IVS2-2 (−T)	β^0
15) IVS1-3′ del 17 bp	β^0
16) IVS1-3′ end del 25 bp	β^0
17) IVS1-3′ end del 44 bp	β^0
18) IVS1-3′ end duplication 22 bp	β^0
19) IVS1-130 (G → C)	β^0
20) IVS1-130 G → A	β^0
21) IVS1-130 (+1) CD30 (AG**G** → AG**C**) (Arg → Ser)	β^0
22) IVS2-849 (A → G)	β^0
23) IVS2-849 (A → C)	β^0
24) IVS2-850 (G → C)	β^0
25) IVS2-850 (G → A)	β^0
26) IVS2-850 (G → T)	β^0
27) IVS2-850 (−G)	β^0

52) CD27 (GCC → TCC) (Ala → Ser, Knossos)[c]	β^+
RNA cleavage—Poly A signal	
53) AATAAA → AACAAA	β^{++}
54) AATAAA → AATGAA	β^{++}
55) AATAAA → AATAGA	β^{++}
56) AATAAA → AATAA**G**	β^{++}
57) AATAAA → AA−AA	β^+
58) AATAAA → A——	β^+
59) AATAAA → AAAAAA	β^+
60) AATAAA → **C**ATAAA	β^{++} (silent)
61) AATAAA → **G**ATAAA	β^+
62) AATAAA → ——	β^+
Others in 3′ UTR	
63) Term CD +6, C → G	β^{++} (silent)
64) Term CD +90, del 13 bp	β^{++} (silent)
65) Term CD +47 (C → G	β^{++}
III. RNA translation	
Initiation codon	
1) ATG → **G**TG	β^0
2) ATG → **C**TG	β^0
3) ATG → **A**CG	β^0
4) ATG → **A**GG	β^0
5) ATG → **A**AG	β^0
6) ATG → AT**C**	β^0
7) ATG → AT**A**	β^0
8) ATG → AT**T**	β^0
9) 45 bp insertion (−22 to +23)	?
Nonsense codons	
1) CD6 GAG → TAG	β^0
2) CD7 GAG → TAG	β^0
3) CD15 TGG → TAG	β^0
4) CD15 TGG → TGA	β^0
5) CD17 AAG → TAG	β^0
6) CD22 GAA → TAA	β^0
7) CD26 GAG → TAG	β^0
8) CD35 TAC → TAA	β^0
9) CD37 TGG → TGA	β^0
10) CD39 CAG → TAG	β^0
11) CD43 GAG → TAG	β^0
12) CD59 AAG → TAG	β^0
13) CD61 AAG → TAG	β^0
14) CD90 GAG → TAG	β^0
15) CD112 TG**T** → TGA	β^0
16) CD121 GAA → TAA	β^0

Mutation	Type
Frameshift	
1) CD1 −G	β^0
2) CD2/3/4 (−9 bp, +31 bp)	β^0
3) CD2−4, 5−9, 7, 10	β^0
4) CD5-CT	β^0
5) CD6 −A	β^0
6) CD8 −AA	β^0
7) CD8/9 +G	β^0
8) CD9 +TA	? β^0
9) CD9/10 +T	β^0
10) CD11 −T	β^0
11) CD14/15 +G	β^0
12) CD15 −T	β^0
13) CD15/16 −G	β^0
14) CD15/16 +G	β^0
15) CD16 −C	β^0
16) CD22/23/24 −7 bp (−AAGTTGG)	β^0
17) CD24 −G; +CAC	β^0
18) CD24/25 −GGT	?
19) CD25/26 +T	β^0
20) CD26 +T	β^0
21) CD27/28 +C	β^0
22) CD28 −C	β^0
23) CD28/29 −G	β^0
24) CD31 −C	β^0
25) CD35 −C	β^0
26) CD36/37 −T	β^0
27) CD37/38/39 del 7 bp (−GACCCAG)	β^0
28) CD38/39 −C	β^0
29) CD38/39 −CC	β^0
30) CD40 −G	β^0
31) CD40/41 +T	β^0
32) CD41 −C	β^0
33) CD41/42 −TTCT	β^0
34) CD42/43 +T	β^0
35) CD42/43 +G	β^0
36) CD44 −C	β^0
37) CD45 −T	β^0
38) CD45 +T	β^0
39) CD47 +A	β^0
40) CD47/48 +ATCT	β^0
41) CD49 −C	β^0
42) CD51 −C	β^0
43) CD53/54 +G	β^0
44) CD54 −T	β^0
45) CD54/58 (−T ATG GGC AAC CCT)	β^0
46) CD55 −A	β^0

Mutation	Type
47) CD54/55 +A	β^0
48) CD56-60 +14 bp	β^0
49) CD57/58 +C	β^0
50) CD59 −A	β^0
51) CD62/63/64 del 7 bp (−TCATGGC)	β^0
52) CD64 −G	β^0
53) CD67 −TG	β^0
54) CD71/72 +T	β^0
55) CD71/72 +A	β^0
56) CD72/73 −AGTGA, +T	β^0
57) CD74/75 −C	β^0
58) CD76 GCT → −T	β^0
59) CD76 −C	β^0
60) CD82/83 −G	β^0
61) CD81-87 (−22 bp)	β^0
62) CD83-86 del 8 bp (−CACCTTTG)	β^0
63) CD84/85 +C	β^0
64) CD84/85/86 +T	β^0
65) CD88 +T	β^0
66) CD88 −TG	β^0
67) CD89/90 −GT	β^0
68) CD95 +A	β^0
69) CD106/107 +G	β^0

Clinical Classification

- Individuals with β-thalassemia have been typically categorized as minor, major, or intermedia on the basis of their α-globin or β-globin chain imbalance, severity of anemia, and clinical picture at presentation
  - **major (**homozygotes or compound heterozygotes)
  - **intermedia (**compound heterozygosity of mild and severe alleles)
  - **Minor** mostly heterozygotes

- β-Thalassemia **major** (also known as Cooley's anemia or homozygous β-thalassemia) is a **severe, transfusion dependent disorder**.

- The term β-thalassemia **intermedia** is applied to a **less severe clinical** phenotype in which significant anemia occurs but regular transfusion therapy is not required (only occasionally require transfusion).

- While β- thalassemia **minor** (also known as β-thalassemia trait or heterozygous β-thalassemia) is the **symptomless carrier state**.

Genotype–Phenotype Association: major/intermedia

- Patients who are **homozygous or compound heterozygous** for β-thalassemia mutations **can have β-thalassemia major or intermedia.**
- Patients with **β-thalassemia major** generally present **early in life**, with severe anemia and symptoms.
- Patients with **β-thalassemia intermedia** tend to present **later in life**, with mild-to-moderate anemia and symptoms.

- The *HBB* mutation **severity**, **coinheritance** of α-thalassemia, and genetic capacity for **continued or increased HbF** production are some of the **factors** that can **determine whether** β-thalassemia is manifested as the **intermedia or major phenotype.**

- β-Thalassemia **intermedia may also result from a heterozygous state** associated with increased production of α-globin chains by a **triplicated or quadruplicated α-globin genotype**, or from **dominant** (inclusion-body) β-thalassemia, or from **deletion forms of δβ-thalassemia**

Transfusion-dependent vs non–transfusion-dependent β-thalassemia

- **Phenotype classification** according to transfusion requirements are now commonly used as transfusion requirement has major effects on associated pathophysiological features and practical management.

- Patients with **transfusion-dependent** β-thalassemia (β-thalassemia major or severe HbE–β-thalassemia) **require lifelong, regular transfusions for survival**.

- Patients with **non–transfusion-dependent** β-thalassemia (β-thalassemia intermedia or mild-to-moderate HbE–β-thalassemia) require **no transfusions, occasional /infrequent** transfusions, but for a limited period, because of specific circumstances (e.g., pregnancy, surgery, or acute infection to support a growth spurt during childhood or manage a clinical complication).

The pathophysiology of β-Thalassemia

- The basis of β-thalassemia is the **Imbalance in α-/non-α-globin chains**.
- **Excess α-globin tetramers** accumulate and precipitate in the erythroid precursors forming **inclusion bodies** that, bound to the RBC **membrane skeleton**, cause **oxidative membrane damage** and extensive premature destruction by apoptosis (**intramedullary hemolysis**) of the RBC precursors in the bone marrow **(ineffective erythropoiesis).**
- Severe ineffective erythropoiesis results in **erythroid marrow expansion (**Hypertrophy of erythroid marrow) to as much as **30 times the normal level**, and consequently causes massive bone marrow expansion and hyperplasia that lead not only to **serious deformities of the skull and long bones** and may cause cortical thinning and pathological **fractures of long bones**.

- Increased erythropoietin synthesis may also **stimulate extramedullary erythropoiesis (**primarily in spleen, & liver). Extramedullary erythropoiesis results in hepatomegaly and splenomegaly (**heptosplenomegaly**) ..
- The **increase in plasma volume** as a result of shunting through expanded marrow and progressive splenomegaly **intensify the anemia** and worsen the condition

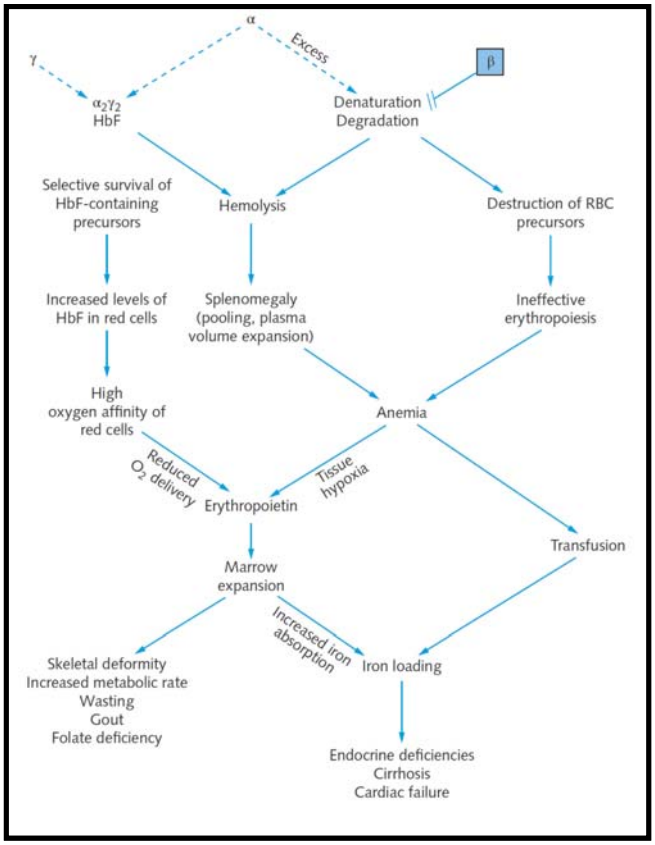
The pathophysiology of β-Thalassemia

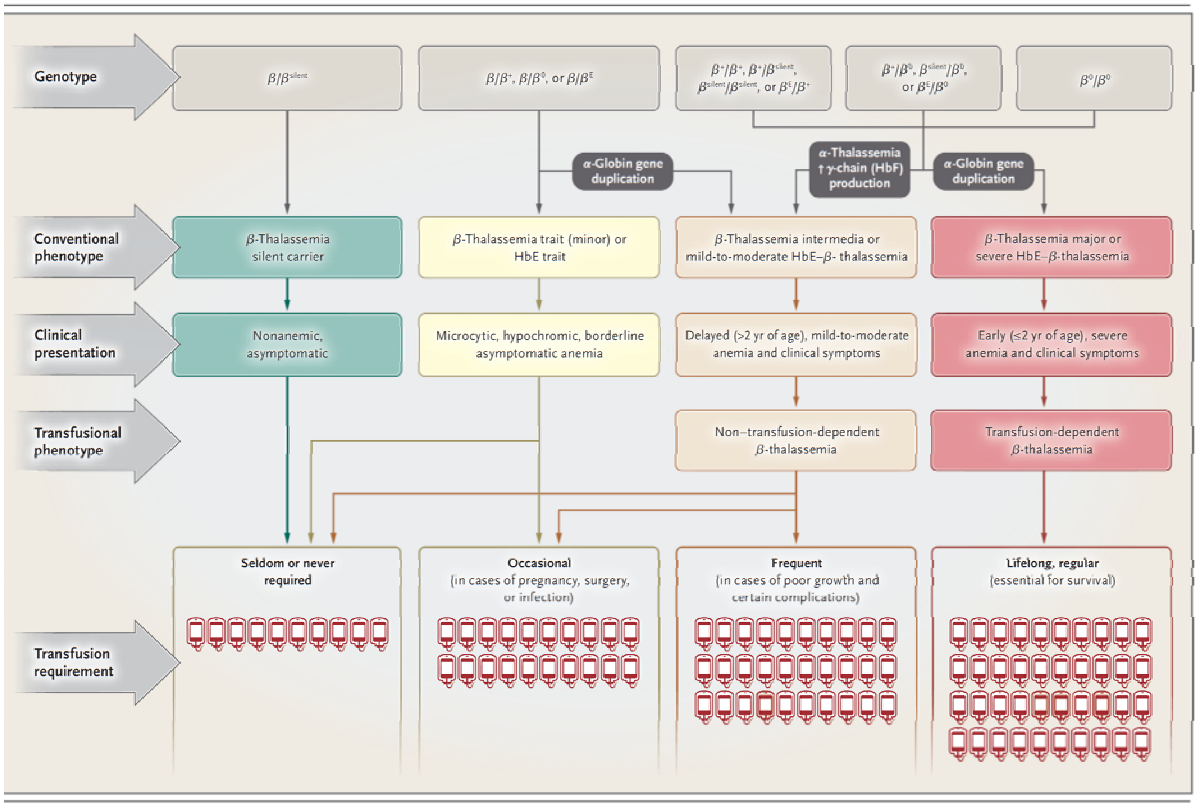
- **The RBCs lipid membrane composition** in β-thalassemia express **prothrombotic markers** on their surface, leading to **a hypercoagulable state**, which is further promoted by **platelet activation,** leading to venous and arterial **thrombosis**, pulmonary hypertension, and cerebrovascular events, including silent infarcts, which increase with aging

- In **non-transfused patients**, erythropoiesis, anemia, and hypoxia **downregulate hepcidin,** the master regulator of iron homeostasis, leading to **excessive duodenal iron absorption** and development of systemic iron overload.

- In **regularly transfused patients**, iron overload is due mostly to **red cell breakdown** of the transfused blood, leading to progressive iron loading of the tissues, with subsequent liver, cardiac, and endocrine damage.
- When the **iron-binding capacity of transferrin is saturated**, iron can appear in the serum as non-transferrin bound **iron**, which is a powerful catalyst for the **formation of free radicals** capable of causing **oxidative stress and damage** to mitochondria, lysosomes, lipid membranes, protein, and DNA, consequently leading to organ compromise typical of thalassemia.

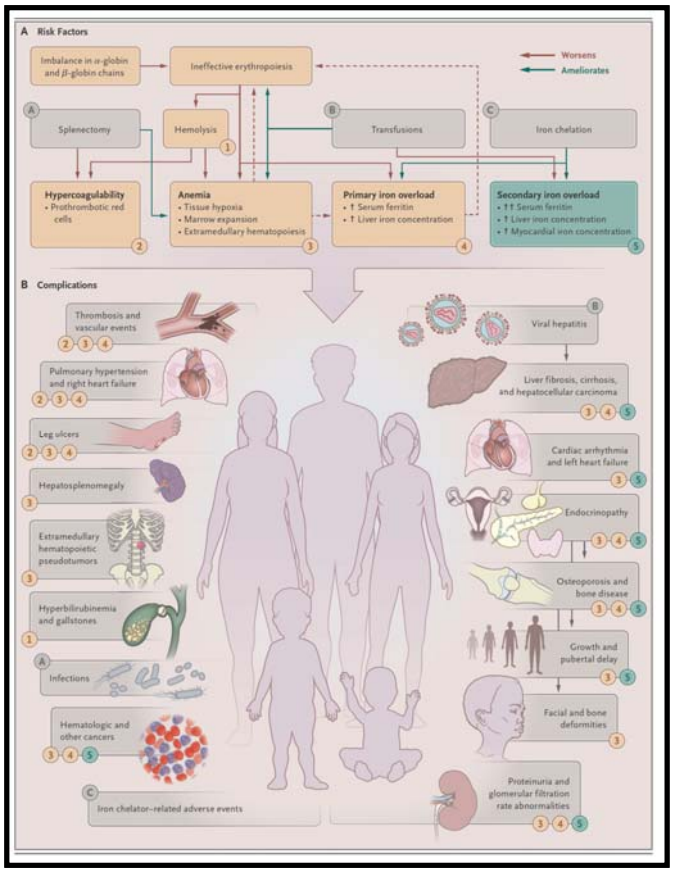
- There is **an increase of γ chain production** a highly stimulated erythroid bone marrow. A large proportion of hemoglobin in the blood of β thalassemics is HbF. However, it has a **higher oxygen affinity** than Hb A.





Clinical Implications / complications and Iron overload

- **Iron overload** remains one of the most relevant clinical considerations in β-thalassemia.
- **Transfusional iron intake** saturates the capacity of serum transferrin and leads to the emergence of **non–transferrin-bound iron species** that can readily accumulate in body tissues (commonly the liver, followed by the heart and endocrine organs), causing **damage to vital organs**.
- **Serum ferritin > 2500 ng/ ml** is associated with an increased **risk of heart disease and death**, whereas levels < 1000 ng per milliliter are associated with prolonged survival.
- **Liver iron** concentrations > 7 mg /gm are associated with an increased risk of **liver disease**, and concentrations **above 15 mg/ gm** are associated with an increased **risk of heart disease**.
- The risk of **death due to cardiac iron overload** has decreased since the introduction of iron chelation in 1967 and was further reduced with the introduction of oral chelators .
- **Low survival rates** continue to be reported in some **resource limited countries**, especially for patients who have received inadequate blood transfusion and iron-chelation therapy, with less than half of patients reaching their **fourth decade of life**.



Clinical Diagnosis of β-thalassemia

- **β-thalassemia major** is suspected in infants or children less than 2 years old with **severe microcytic anemia, mild jaundice, and hepatosplenomegaly.**
- **β-thalassemia intermedia** is suspected in individuals who present at a **later age** with similar but milder clinical findings.
- **β-thalassemia minor/trait/carrier/heterozygous** is clinically asymptomatic

Hematologic diagnosis

- **β-thalassemia major** is characterized by reduced Hb level (<7 g/dl), MCV> 50 < 70 fl, and MCH > 12< 20 pg. **HbA2 is variable**.

- **β-thalassemia intermedia** is characterized by Hb levels between 7 and 10 g/dl, MCV > 50 < 80 fl, and MCH > 16 < 24 pg. **HbA2 is variable**

- The **peripheral blood smear** in affected individuals demonstrates RBC morphologic changes with **microcytosis, hypochromia, anisocytosis, poikilocytosis, and nucleated RBCs.** The number of erythroblasts is related to the degree of anemia and is markedly increased following splenectomy.
- **β^0**-Thalassemia homozygotes show complete absence of globin β-chain production and **HbF constitutes 92–95%** of the total Hb.
- In **β^+**- homozygotes and **β+/ β0** compound heterozygous , HbF is 70–90% and HbA 10–30% according to the variable degree of reduction of β-globin chain synthesis.

- **β-thalassemia minor** is characterized by reduced MCV and MCH, with increased **HbA2 level**. Erythroblasts are normally not seen.

Molecular diagnosis of β-thalassemia

- Commonly occurring mutations of *HBB* are detected by **PCR-based procedures**.
- The most commonly used methods are: reverse dot blot analysis and primer-specific amplification, with a set of probes or primers complementary to the most common mutations in the population from which the affected individual originated.
- If targeted mutation analysis fails to detect the mutation, ***HBB* sequence analysis** can be used to detect mutations in *HBB*.
- Sequence analysis may be considered first if the affected individual is not of an ancestry at high risk or if targeted analysis reveals only one or no pathogenic variant.
-

Management of β-thalassemia patients

- **Transfusion and Iron Chelation**
- **Regular transfusions** are administered in transfusion-dependent patients to achieve target pretransfusion hemoglobin levels **of 9 to 10.5 g /dL** (**11 to 12 g/dL** in patients with heart disease).
- Advances in donor blood screening and preparation have decreased the rates of alloimmunization and blood borne infections in most countries.
- Transfusion-dependent patients should be **monitored for iron overload** with the use of serum ferritin measurements and hepatic and myocardial MRI, and **iron chelation should be administered** shortly after transfusion of **10 packed red-cell units** or when the **serum ferritin > 1000 ng/ml**.
- **Three iron chelators**, used alone or in combination, are currently available for managing iron overload:
- ***Subcutaneous deferoxamine (Desferal)***
- **The oral agents**: deferiprone (Ferriprox) and deferasirox (Exjade).

- **Desferal** (Deferoxamine) and (**Exjade**) deferasirox are approved for treatment in patients **> 2 years of age**, whereas **Ferriprox** (deferiprone) is approved as second-line therapy in patients **> 6 years of age**

Structural hemoglobin variants

- **Hb variants (qualitative Hemoglobinopathies)** are characterized by **structural abnormalities** in the globin chains, including α-, β-, δ-, and γ-globins.

- The vast majority of Hb variants arise as a result of **single nucleotide mutations** (substitution missense mutations) , leading to an amino acid change in either α-, β-, δ-, or γ-globin subunits of the Hb tetramer.
- **Most Hb variants** have **no meaningful clinical** significance (**silent or benign or harmless**) and are occasionally detected during **routine electrophoresis testing**, such as pre-pregnancy examinations and genetic counseling.

- Since the molecular characterization of sickle cell hemoglobin (HbS) in the mid of last century (1950's) , there has been a rapid and exponential increase in the number **(>1000)** of Hb variant or " structurally abnormal" hemoglobins

- Hemoglobin variants were labeled by letters of the alphabet (e.g., HbC, HbD, HbE, HbJ).

- When the letters of the alphabet were used, the practice of naming the variant after the **geographic location** where they were first described was adapted (e.g., HbKoln, HbZurich).

Structural hemoglobin variants

- Hb variants that significantly **alter** the **structure, stability, synthesis, or function** of the Hb molecule have **hematologic and/or clinical consequences**.
- **HbS and HbC** are two examples of mutations that alter both the charge and the physical/chemical properties of the Hb leading to **polymerization** in (HbS) or **crystallization** in (HbC) with profound effects on the function, morphology, rheology, and life span of the red cells.
- **Except** for the commonly occurring variants (HbS, HbC, HbE, and HbD), **very few** abnormal Hbs have been observed in the **homozygous** state.

- Several **mechanisms** account for the pathogenesis of **unstable Hb variants**.
  - The common mechanism involves the **precipitation** of the **unstable Hb** molecule within RBC and attachment to its inner layer the membrane (“**Heinz body” formation**), leading to impaired **deformability** then premature destruction of RBC precursors.
  - Mutations in certain residues alter (**increase or decrease**) the **oxygen affinity of the Hb molecule.**
  - Mutations of heme binding site, lead to oxidation of iron atom in heme from ferrous **(Fe^{2+}) to ferric (Fe^{3+}) state** with resultant methemoglobinemia (Hb M).

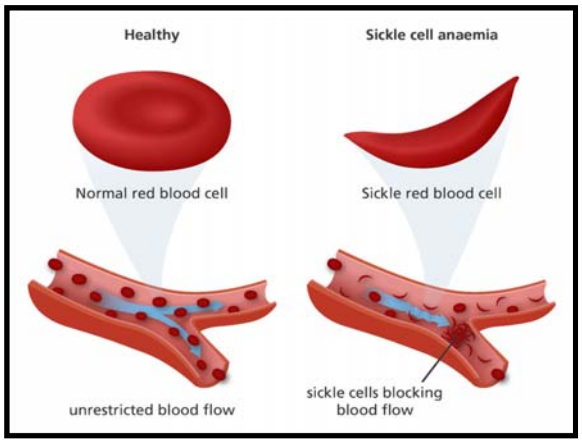
Sickle Cell hemoglobin (HbS)

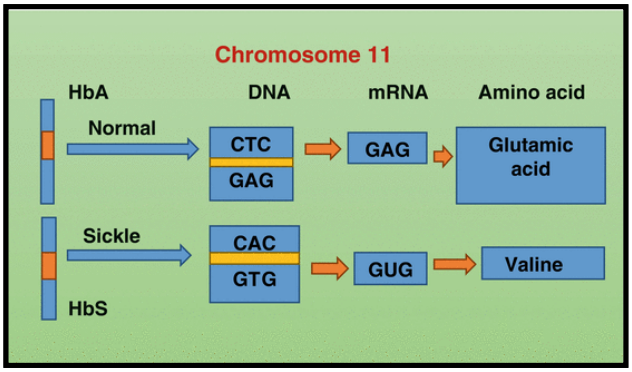
- In the **late 1950s**, **globin polypeptides** from Sickle Cell anemia (**SCA**) patients were **sequenced** and linked the abnormality to a change in the amino acid composition of the β-globin chain (replacement of glutamic acid[Glu] by valine[Val] at residue **6**).
- **In 1977**, the corresponding change in codon 6 of the β-globin gene was identified as **missense mutation (GAG→GTG)** A>T substitution .

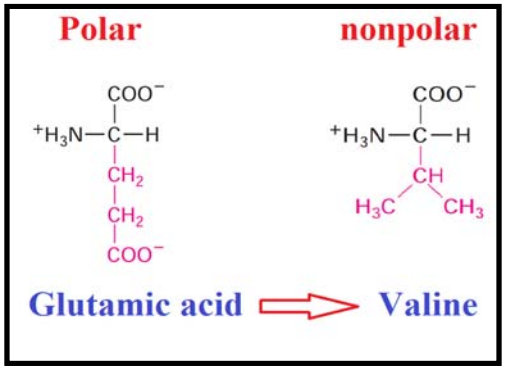
- Inheritance of only **one HbS allele** is termed sickle cell **trait (HbAS)**, that affected > 300 million people worldwide.
- Sickle cell **trait (HbAS) is** considered a generally **asymptomatic** state with HbA ($\alpha_2\beta_2$) in the cell preventing sickling **except** in the **most unusual circumstances**.

- In **sickle cell trait HbAS** (heterozygous) the percentage of **HbA is always higher** (~60 percent) than HbS (~40 percent) .

- **HbAS cells sickle at low O_2 tension** (partial pressure of O_2) of **~15 mmHg**.
- (O_2 tension in venous blood 30 - 40 mmHg, O_2 in arterial blood 75 -100 mmHg. (atmospheric 160 mm Hg for oxygen).







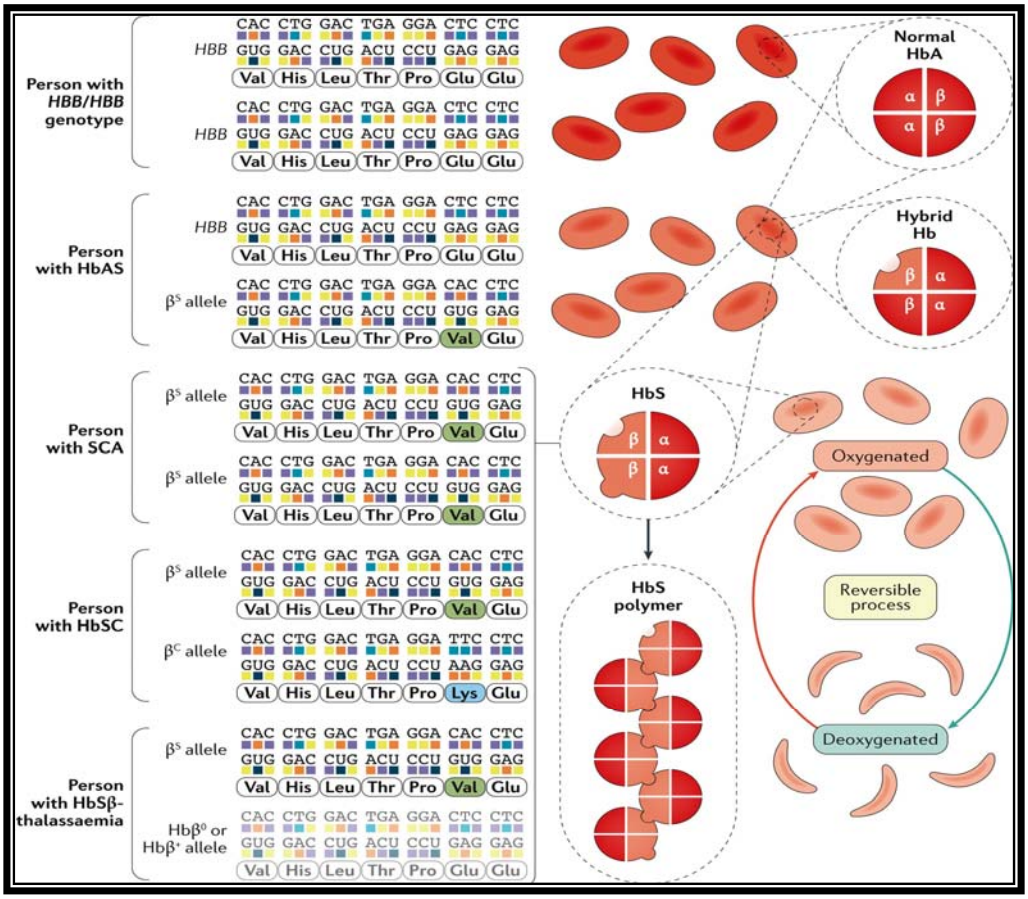
Sickle Cell hemoglobin (HbS)

- **RBC sickling in HbAS (heterozygous)** have been reported to increase **during exercise** leading to microcirculatory dysfunction.
- There is a **30-fold increased risk** of sudden death in HbAS heterozygous subjects, therefore many sport associations recommended **mandatory testing for HbAS** for all athletes.
- The risk of **venous thromboembolism (**obstruction of a blood vessel by a blood clot) is increased **twofold** in HbAS heterozygous subjects compared to those without the trait.
- The risk of venous thromboembolism is **most likely greater for pulmonary** embolism than for **deep vein thrombosis**.
- HbAS heterozygous subjects usually **do not have increased** perioperative morbidity or mortality.
- The **life span** of patients with HbAS heterozygous is **normal**.

Sickle Cell Disease (SCD)

- **Sickle cell disease (SCD)** is broad term that defines a **group of inherited diseases** (including sickle cell anemia (SCA), Hemoglobin SC disease (HbSC) and HbSβ-thalassemia) characterized by mutations in the gene encoding the hemoglobin subunit β (***HBB***).
- **In sickle cell anemia (SCA) or [Hemoglobin SS disease],** individuals inherit two copies of the sickle variant (HBSS) and **under deoxygenation** (Hb is not bound to oxygen) and **hypoxic conditions**, HbSS tetramers can polymerize and cause the erythrocytes to take **a crescent or sickled shape** from which the disease takes its name.

- **Polymerization** of Hb molecules results in **stiffened /thickened mature RBC** as well as **reticulocytes** and changes their **shape and physical- mechanical** properties, resulting in **hemolytic anemia and blockage of blood flow**, particularly in small (and some large) vessels, which can **damage any organ**.
- Changes in RBC **physical-mechanical properties or deformability** lead to increased blood apparent **viscosity** and along with increased cellular adhesion and inflammation, contribute to **vaso-occlusion (**microcirculation is obstructed by sickled RBCs) and **ischemic injury**, the primary driver of morbidity in SCA.
- Vaso-occlusion and its downstream pathologies contribute to a **mortality rate** for SCA as high as **7.3%** in children under 5-years old and a significantly reduced overall life expectancy.

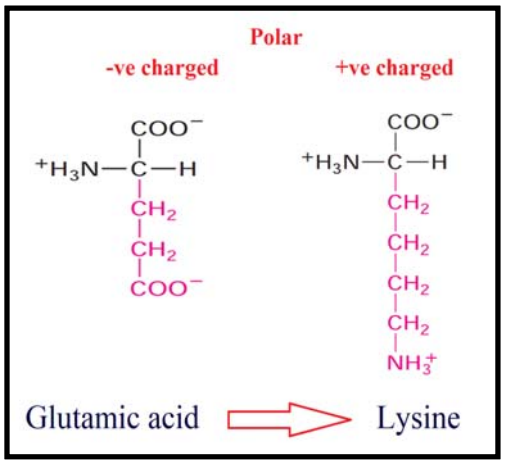


Sickle cell trait is protective against malaria

- Sickle cell trait (HbAS) is **probably protective** against severe malaria.
- Reasonable **protective** mechanisms include:
- Selective **sickling of parasitized RBC**, resulting in more effective removal if infected RBC by the monocyte-macrophage system
- **Inhibitory effect on parasite growth** by increased RBC potassium loss (dehydrated spherocytic with increased hemoglobin concentration), decreased RBC pH, and increased endothelial adherence of parasitized sickle red cells.

Hemoglobin C (HbC) disease

- HbC was the **second Hb** variant described after HbS
- HbC trait is found in **2 % of African-Americans**
- ~ 1/6000 have homozygous HbC
- **Coinheritance** of HbC with HbS results in **HbSC disease**, which is the second most common form in the United States.
- HbC is the result of a **G**AG→**A**AG missense mutation in codon 6 of the *β*-globin gene, leading to glutamic acid substituted by lysine (**Glu→Lys**).



The Hb in RBC from **homozygous HbC** individuals form **crystals.**

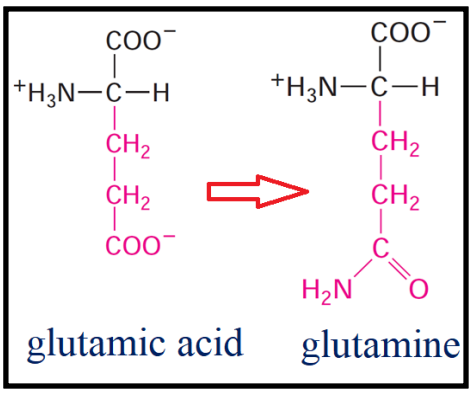
Crystal-containing HbCC RBC have **impaired deformability and filterability** and result in impaired **rheological** properties of HbCC red cells; their **life span is reduced to 40 days**.

Clinical and laboratory Features of HbCC

- Hemoglobin C disease (Hb CC) may have a mild degree of hemolytic anemia, splenomegaly, and borderline anemia.
- **Chronic hemolytic** states with mild to moderate **splenomegaly associated with abdominal pain** is a common feature of homozygous patients [HbCC].
- Although the clinical complications of hemoglobin C disease are not severe, inheritance with other hemoglobinopathies such as hemoglobin S may have significant consequences,
- **Life expectancy** of HbCC individuals is **comparable to non-HbC**
- ***Laboratory Features***
- HbCC individuals have a **mild to moderate hemolytic anemia (**10 -11 g/dL) associated with reticulocytosis (3-4) %.
- There usually is **mild microcytosis** (MCV 70-75 fL) and, often, an **elevated MCHC**.
- The blood film is characteristic, showing an **abundance of target cells**, **microspherocytes**, and HbC RBC **crystals**.
- **Differential** diagnosis is usually achieved by **Hb electrophoresis**.

Hemoglobin D (HbD) disease

- **HbD** was the **third** Hb variant identified after HbS and Hb C.
- The substitution (GAA→CAA) in HbD occurs at **a.a # 121:** glutamic acid to glutamine (*β*121Glu→Gln).
- The genes for Hemoglobin D have the highest frequency among people of **Asiatic Indian heritage**. However, they are also found in people of European descent, especially British and Irish.



- **HbD heterozygotes are asymptomatic**, are not anemic, and have normal RBC indices.
- **Homozygotes** for HbD are **asymptomatic** and are hematologically normal with normal RBC indices, **but** blood films may show (**poikilocytosis : target cells) .**
- **Coinheritance** of HbD with HbS heterozygous results in **a severe SCD** phenotype not different from homozygous HbS.
- HbD should be **distinguished** from HbS. This can be done by a combination of routine alkaline and acid Hb electrophoretic methods **and capillary electrophoresis**
- Such methods allow accurate diagnosis of SCA from compound heterozygosity for HbS and HbD.

Hemoglobin E (HbE) disease

- HbE was the **4th** abnormal Hb described. It is most commonly found in **Southeast Asia**.
- HbE is due to missense **G**AG→**A**AG mutation (***β*26Glu→Lys**).
- The **coinheritance** of HbE with other globin mutants (*α*-thalassemia, *β*-thalassemia, HbS, other Hb variants), which are also common in the populations where HbE is prevalent, results in a **wide spectrum of Hemoglobinopathies** with varying degrees of severity (**HbE disorders or HbE syndromes**).
- ***Clinical Features***
- Individuals with **homozygous HbE are clinically asymptomatic, usually** do not have hepatosplenomegaly or jaundice. They are usually diagnosed during screening programs or family studies.
- ***Laboratory Features***
- **HbE-trait** individuals have a **borderline microcytosis** (MCV in the lower 80s) but are not anemic.
- **Homozygotes for HbE** are usually **only borderline or mildly anemic** (Hb 11 to 13 g/dL), but they are **microcytic hypochromic with some** target cells,.
- **Hb electrophoresis** shows > 90 % HbE and 5 to 10 % HbF and elevated levels of HbA2.

Hb Electrophorasis

- Alkaline electrophoresis
- Acidic (citrate agar gel) electrophoresis
- Capillary electrophoresis

Electrophoresis

- **Electrophoresis** is one of the efficient and most frequently applied separation technique for the analysis of **protein, glycan, and nucleic acid mixtures**.

- Electrophoresis uses an **electrical current to separate** normal and abnormal types of hemoglobin in the blood that can be **visualized and measured (**electropherogram).

- The **rate** of migration/movement is dependent on:
  - Net electrical charge of the molecule
  - Electric field strength
  - Properties of the supporting medium
  - Size and shape of the molecule
  - Temperature of the operating system
  - Buffers and its ionic strength.

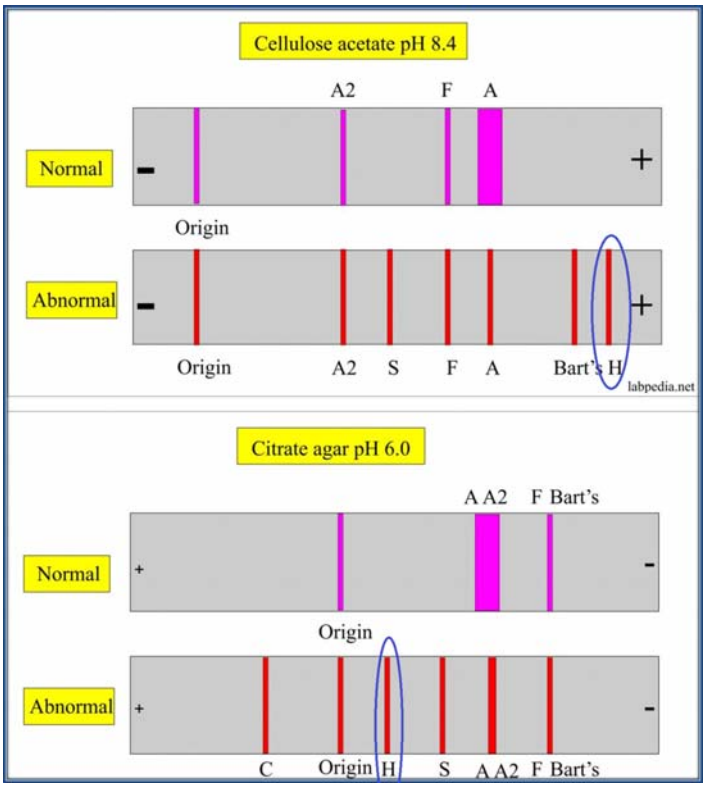
Hemoglobin electrophoresis

- Hemoglobin electrophoresis is the **movement /migration** of hemoglobin (protein molecule) in an electric field at a fixed pH. The **different subtypes of hemoglobin** molecules are composed of various combinations of globin chains whether the normal chains or the abnormal ones, therefore **they will migrate at different rat**es toward the opposite pole of the electric filed.

- In hemoglobin electrophoresis **red cell lysates (hemolysate)** are subjected to electric fields under **alkaline** (alkaline gel) or **acidic** (acid gel) pH. This can be **carried out** on filter paper, a cellulose acetate membrane, a citrate agar gel, or an agarose gel.

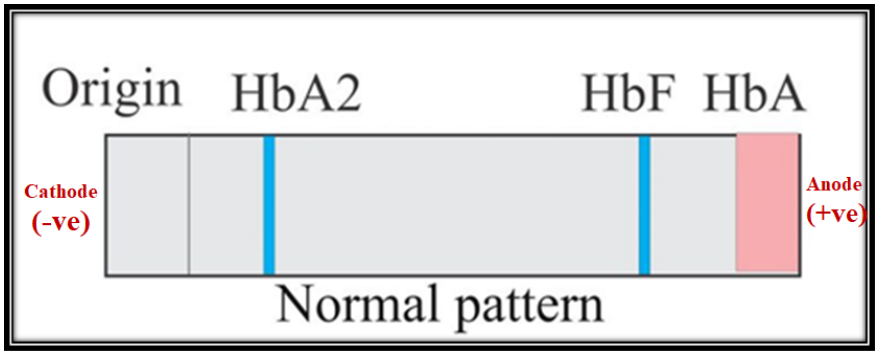
- Separation of different hemoglobins **is largely** dependent on the **net charge** of the hemoglobin molecule.

- **Changes in a.a composition** of the globin chains (**Hb variants**) results in **alteration** hemoglobin molecule **net charge**, causing a **change of the speed of migration** or separation.
- On **alkaline g**el in hemoglobin electrophoresis, the **HbH is fast-migrating** Hb
- On the acid gel, the **HbH** migrates **between** the S and C hemoglobins.



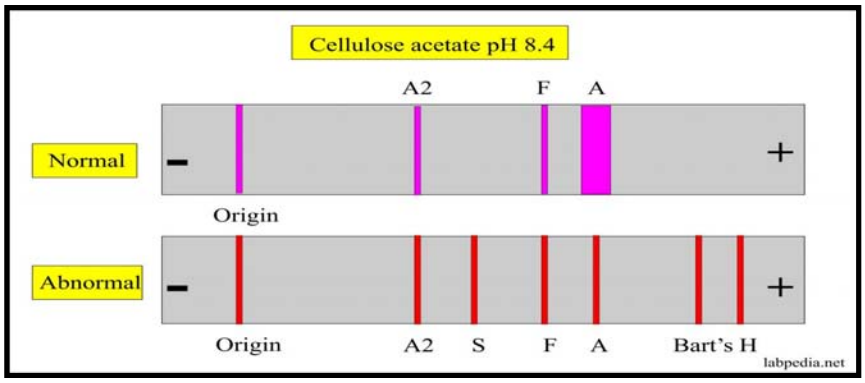
Hemoglobin electrophoresis (alkaline electrophoresis)

- In alkaline electrophoresis a hemolysate of RBC is applied to cellulose acetate in a buffer solution at **pH 8.4-8.6**.
- At this **alkaline pH (8.4-8.6)** all hemoglobins are **carrying a negative** net charge therefore move **from the cathode (-ve)** toward the **anode (+ve)** and after application of **specific dye**, the different hemoglobins can be visualized and quantified by densitometry.
- In **normal** adults, **HbA is the fastest**, followed by HbF. HbA2 moves only slightly from the point of origin near the cathode.



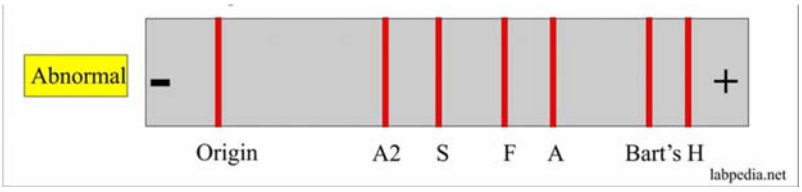
Hemoglobin electrophoresis (alkaline electrophoresis)

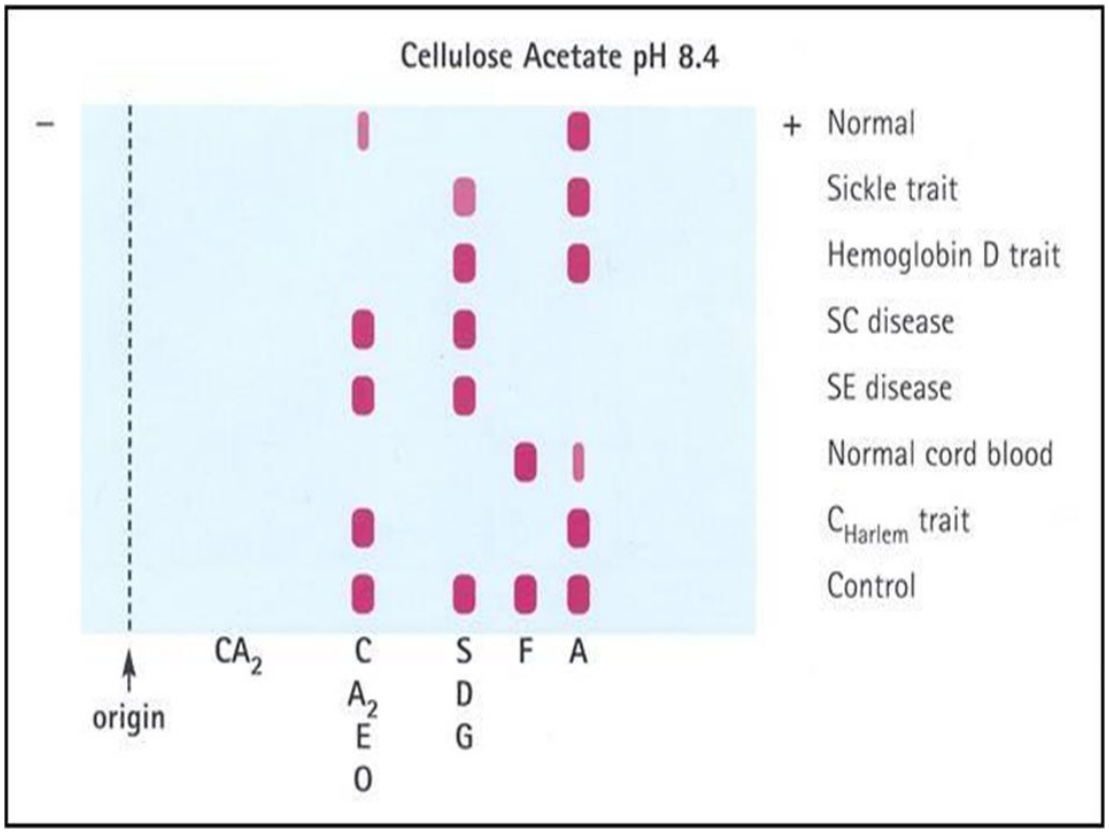
- For **abnormal hemoglobins**, **HbC migrates with HbA2** near the cathode, while HbS lies between hemoglobin A2 and Hemoglobin F.
- **HbH and Hb Bart's are unstable and very fast moving** placing them past HbA and near the anode with HbH being the fastest of the two. Therefore, if any **of HbH or Hb Bart's is expected a shorter electrophoresis time** is recommended to ensure that HbH remained on the acetate paper.
- **Disadvantage** that many abnormal **hemoglobins overlap**. In addition Quantitative densitometry of abnormal hemoglobins is **inaccurate** at low concentrations (e.g., HbA2 and HbF in adults).



Migration of Various Hemoglobin Bands in Alkaline Gel and Acid Gel Electrophoresis

Region	Hemoglobin Present
Alkaline Gel Electrophoresis	
Top band (farthest from origin: H Lane)	HbH, HbI
J Lane	HbJ
	Hb Bart's and HbN are between HbJ and HbH lane
Fast hemoglobin	Hb Hope
A Lane	HbA
F Lane	HbF
S Lane	HbS, HbD, HbG, Hb Lepore
C Lane	HbC, HbE, HbO, HbA_2, HbS/G hybrid
Carbonic anhydrase band (faint)	HbG_2, HbA_2', HbCS

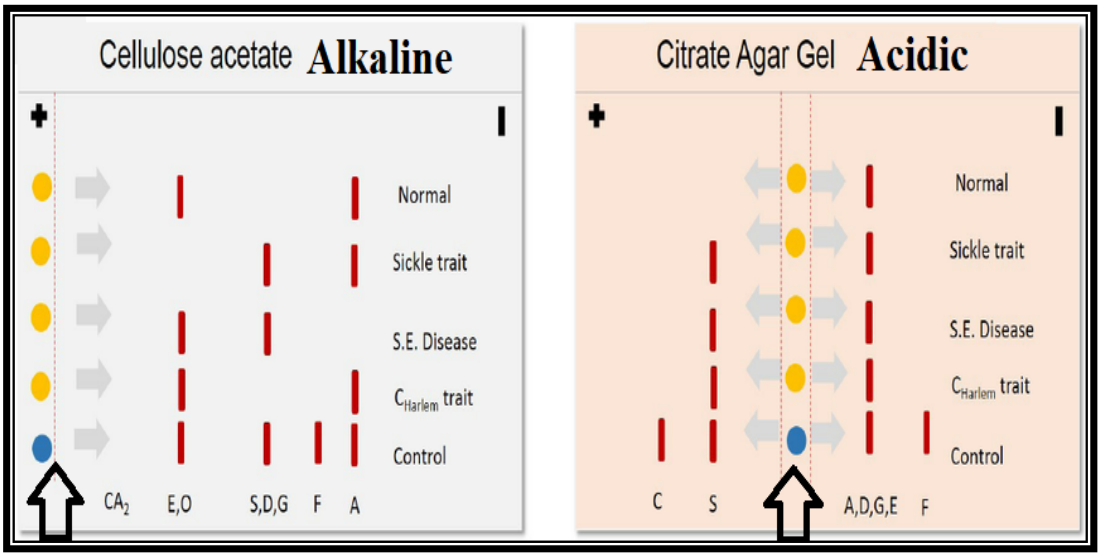




Hemoglobin Electrophoresis on acidic gels

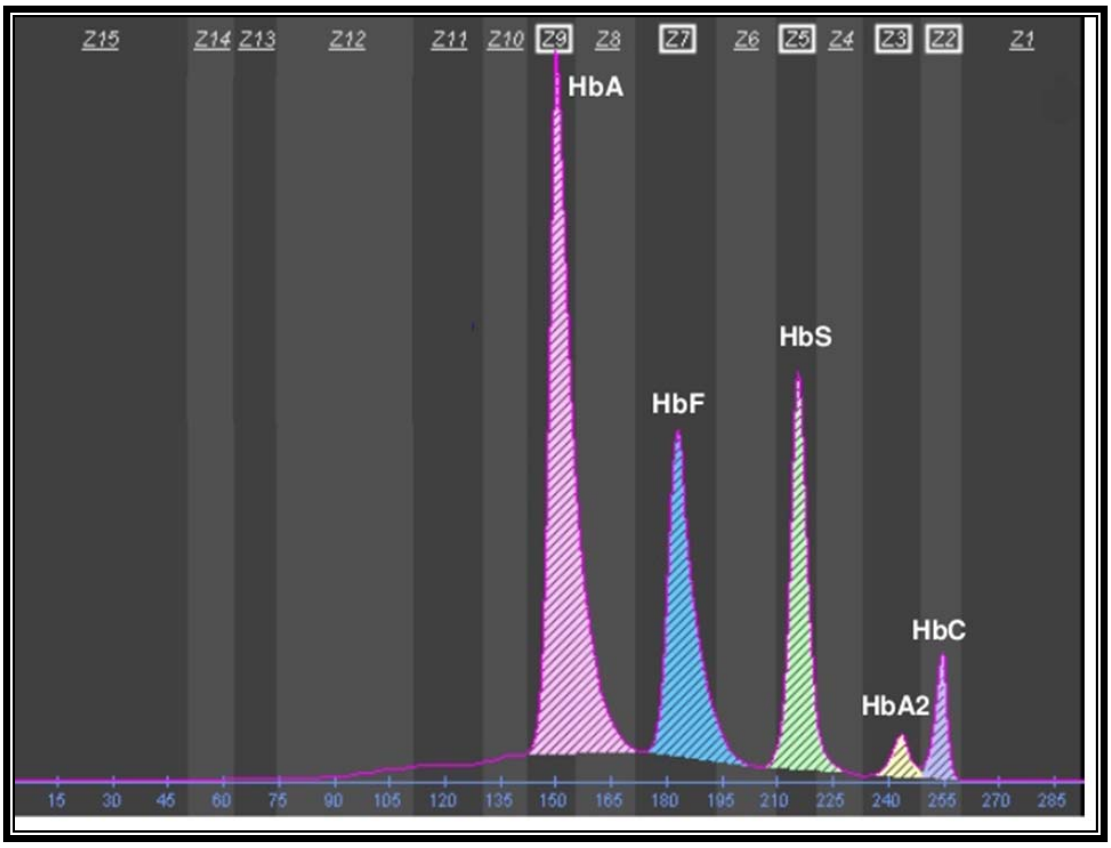
- As of the **limitation** of hemoglobin alkaline electrophoresis, electrophoresis on acidic medium (citrate agar gel), **should follow to confirm the identification** of hemoglobin variants, in particular, to differentiate Hb S from HbG, HbD, Hb Lepore.

- Citrate Agar Gels are suitable for:
- the separation of HbF
- for the differentiation of HbS from HbG, D, Lepore
- to differentiate and HbC from HbA2, E,O.



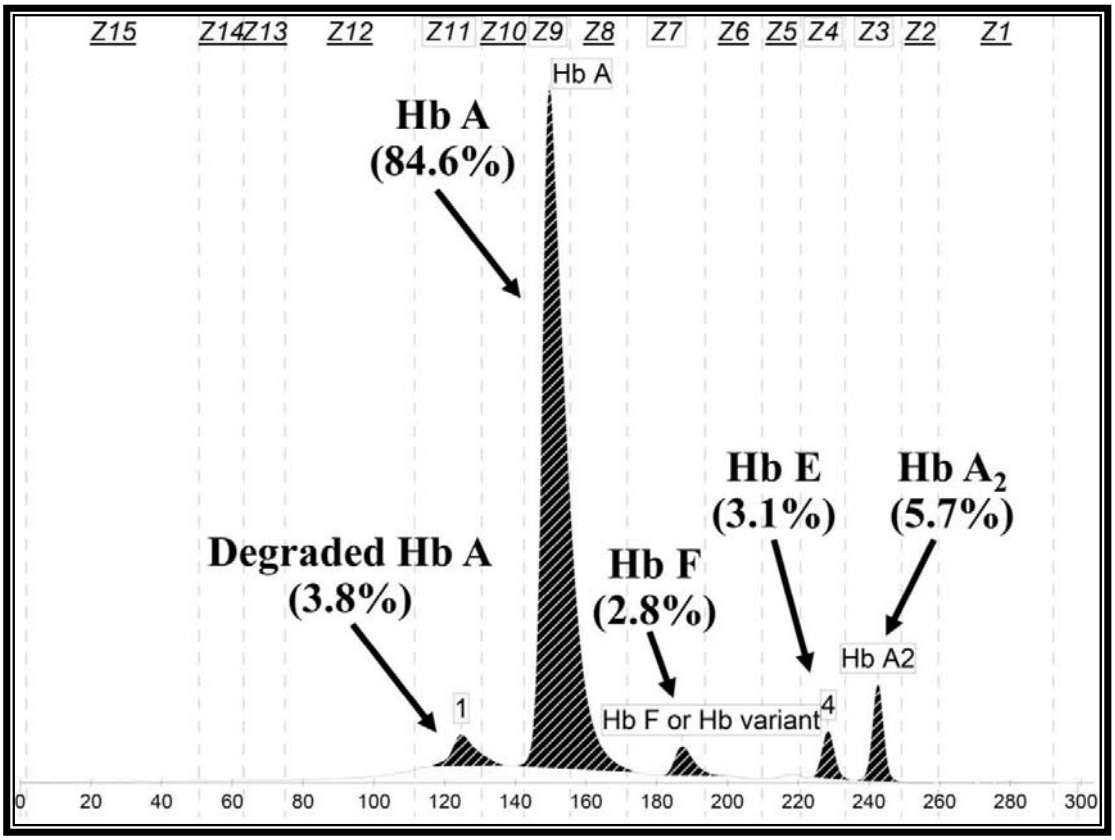
Capillary electrophoresis

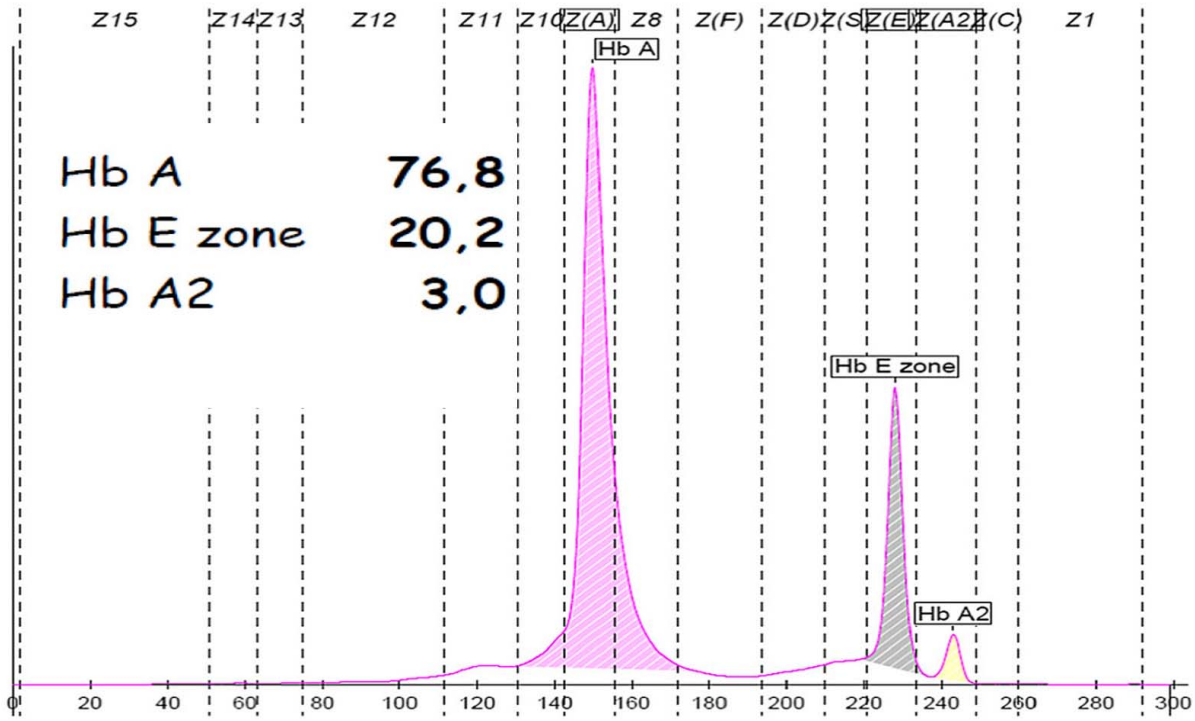
- **Capillary electrophoresis** CE is becoming routinely used in clinical laboratories as **a reliable method** for the detection of Hemoglobinopathies
- The CE assay **separates the Hb fractions in silica capillary** tubes by their electrophoretic mobility and electro-osmotic flow at a **high voltage in an alkaline buffer**.
- The Hb fractions are directly detected at wavelength of 415 nm.
- A grid divides the Hb migration into **15 zones on the x-axis** from 0 to 300, with HbA standardized to migrate at position 150 **(zone #9)**

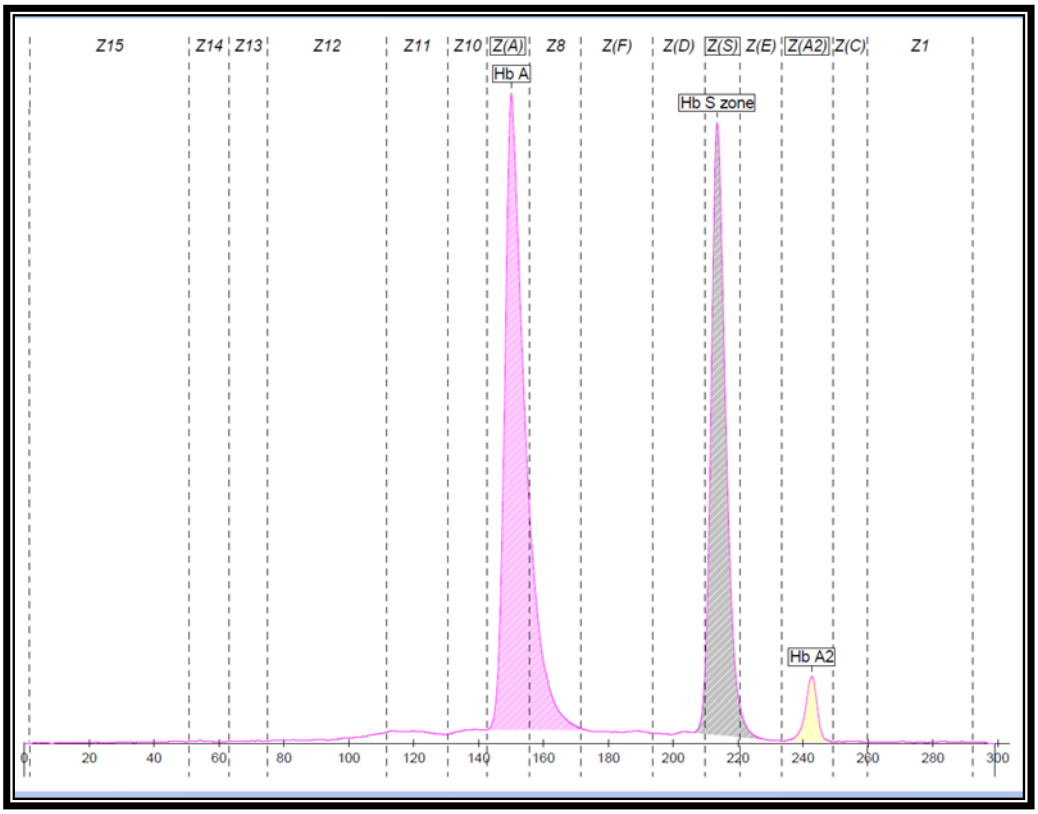


Zones in Which Common Hemoglobins Appear on Capillary Electrophoresis

Zone	Hemoglobin
Zone 1	HbA_2'
Zone 2	HbC, HbCS
Zone 3	HbA_2, Hb-O-Arab
Zone 4	HbE, Hb Koln
Zone 5	HbS
Zone 6	HbD-Punjab/Los Angeles/Iran, HbG-Philadelphia
Zone 7	HbF
Zone 8	Acetylated HbF
Zone 9	HbA
Zone 10	Hb Hope
Zone 11	Denatured HbA
Zone 12	Hb Bart's
Zone 13	
Zone 14	
Zone 15	HbH


Z15
Z14
Z13
Z12
Z11
Z10
Z9
Z8
Z7
Z6
Z5
Z4
Z3
Z2
Z1
Hb A
Hb A
(84.6%)
Degraded Hb A
(3.8%)
Hb F
(2.8%)
Hb E
(3.1%)
Hb A2
(5.7%)
Hb A2
1
Hb F or Hb variant
4
0
20
40
60
80
100
120
140
160
180
200
220
240
260
280
300


Z15
Z14
Z13
Z12
Z11
Z10
Z(A)
Z8
Z(F)
Z(D)
Z(S)
Z(E)
Z(A2)
Z(C)
Z1
Hb A
Hb A 76,8
Hb E zone 20,2
Hb A2 3,0
Hb E zone
Hb A2
0
20
40
60
80
100
120
140
160
180
200
220
240
260
280
300

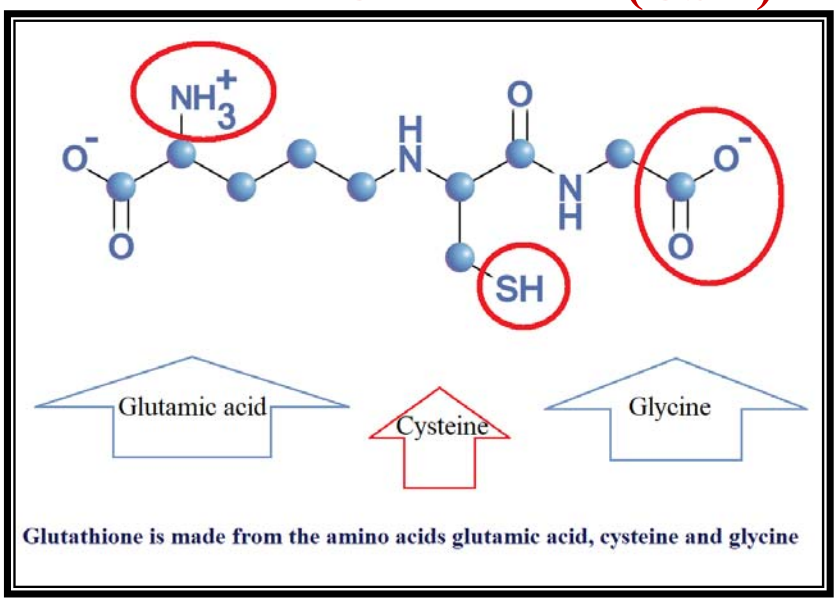

Z15
Z14
Z13
Z12
Z11
Z10
Z(A)
Z8
Z(F)
Z(D)
Z(S)
Z(E)
Z(A2)
Z(C)
Z1
Hb A
Hb S zone
Hb A2
0
20
40
60
80
100
120
140
160
180
200
220
240
260
280
300

Biochemistry of Glucose-6-phosphate dehydrogenase (G6PD)

G6PD Enzyme

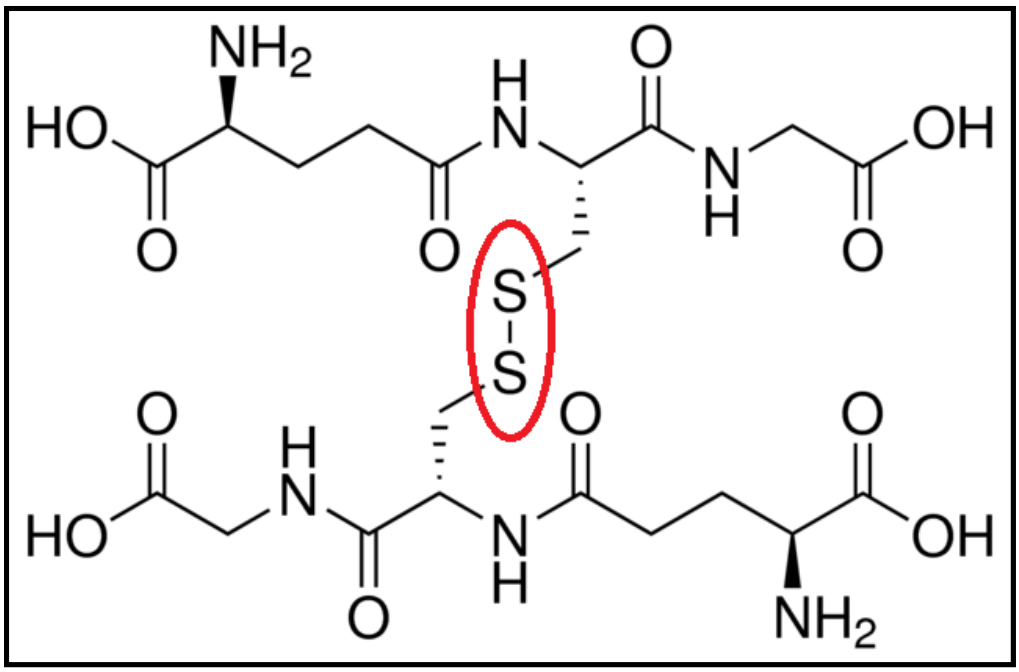
- Glucose-6-phosphate dehydrogenase (G6PD) enzyme is a housekeeping enzyme of RBC.
- It is the **first and rate-controlling enzyme** in the hexose-monophosphate shunt of the ***Embden-Meyerhof pathway*** (anaerobic glycolysis pathway inside RBC), which reduces NADP+ to NADPH that **maintains the reduced state of glutathione (GSH).**
- In RBCs, the hexose-monophosphate shunt is the **only source** of NADPH, for maintaining high cellular levels of GSH (**reduced glutathione)** to protect RBCs from **oxidative stress-induced damage** and thus RBC destruction and hemolysis.
-
- **NADPH levels are maintained by G6PD**; thus, in **G6PD-deficient** RBCs, GSH is not restored to adequate levels under oxidative stress, leading to a buildup of **free radicals** and **oxidation of hemoglobin that precipitates (denatured) as Heinz bodies** within RBC.
- Heinz bodies are clumps of denatured hemoglobin resulting from exposure to high oxidant levels that oxidize **sulfhydryl groups** on adjacent molecules, joining them with a **covalent disulfide S-S bond**
- Precipitated /**denatured hemoglobin** is **disruptive** to the structure and function (**membrane integrity**) of the RBC membrane, RBC deformability, filterability and **leads to hemolysis**.

Reduced Glutathione (GSH)

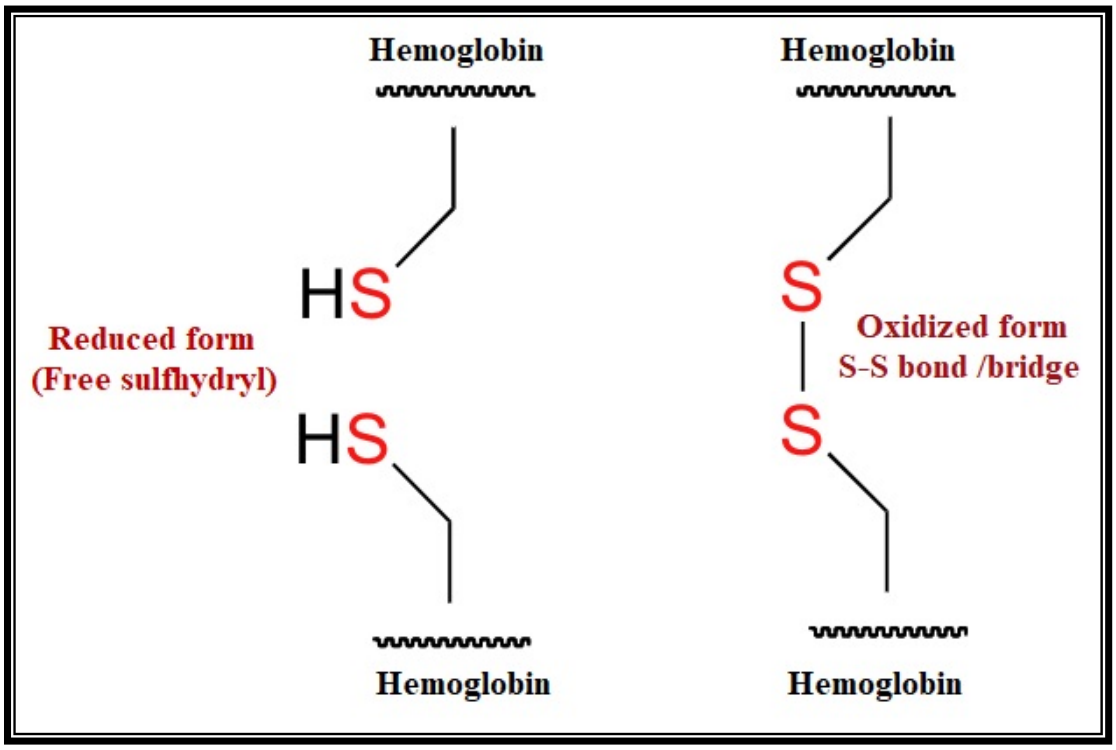


Glutathione is a **powerful antioxidant** produced by the liver to protect the body against free radicals, peroxides, and heavy metals. It is found in **most of the tissues**, especially in high concentrations in the liver, and plays an extremely important role in protecting **hepatocytes, erythrocytes, and other cells against toxic injury**. It also **eliminates poisons** such as drugs and pollutants from our bodies

Oxidized Glutathione (GS-SG)

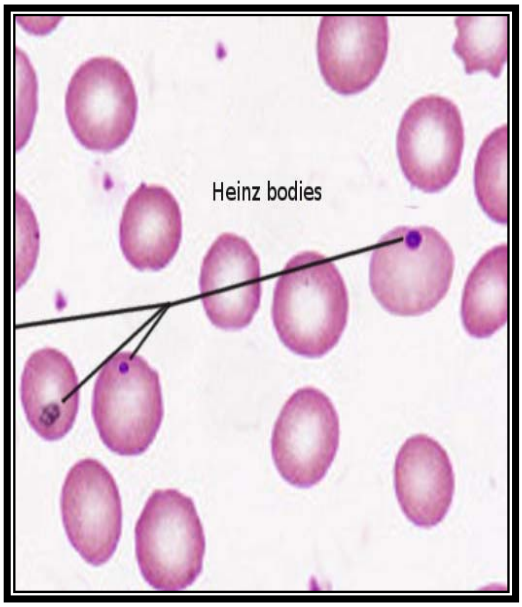


Reduced & oxidized sulfhydryl groups in hemoglobin



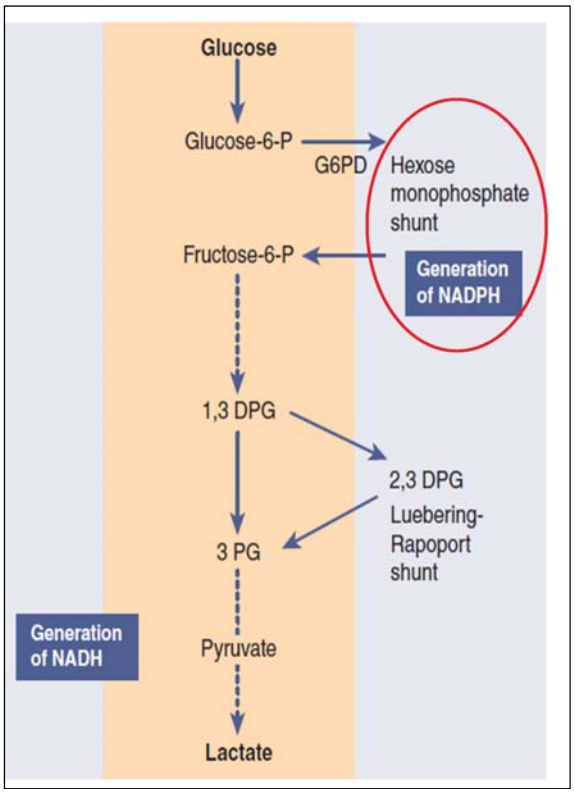
Formation of Heinz bodies

- Within the RBCs, **oxidative stress** (high reactive oxygen species) leads to the **oxidation of sulfhydryl (SH)** groups on neighboring hemoglobin molecules, resulting in the formation of **disulfide bridges** (S-S), which in turn leads to decreased hemoglobin solubility and ultimately the **irreversible precipitation** of oxidized hemoglobin (**Heinz bodies**).



RBC metabolic pathways

- Mature RBC has no mitochondria.
- ATP is generated by **anaerobic** glycolysis.
- **Glucose** is catabolize to **lactate**.
- This pathway is known as
- ***(Embden-Meyerhof pathway).***
- Other shunts are also directly connected to Embden-Meyerhof pathway:
  - ***Hexose-Monophosphate Shunt***
  - ***Rapoport-Luebering shunt***



Embden-Meyerhof pathway

- In this ***Embden–Meyerhof pathway,*** glucose that enters the red cell from plasma by facilitated transport (passive) is metabolized to **lactate**.
- For each molecule of glucose used:
  - **2 ATP** are generated, used for maintenance of red cell volume, shape and flexibility.
  - **NADH**, which is needed by the enzyme methemoglobin reductase to reduce functionally dead methemoglobin containing Fe^{3+} to functionally active, hemoglobin containing Fe^{2+}.
  - **2,3-DPG**, through the Luebering–Rapoport shunt, 2,3-DPG important in the regulation of hemoglobin's oxygen affinity.
  - **NADPH** through **Hexose monophosphate shunt**

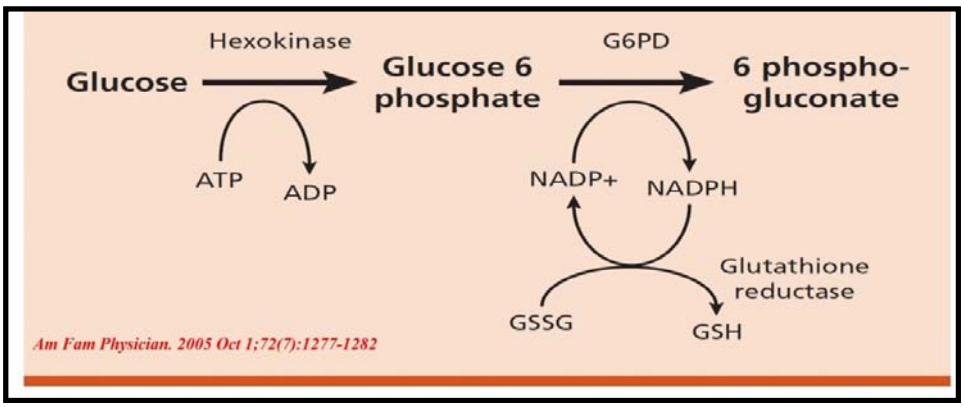
Importance of Hexose monophosphate shunt

•Under normal conditions, about **10% of the glucose** utilized by RBC is catabolized via the hexose-monophosphate shunt.

• This shunt provides a critical protection against oxidative damage through generation of the **NADPH** which enables cells to **counterbalance oxidative stress**.

• Since RBCs do not contain mitochondria, **the HMS is their only source of NADPH**; therefore, defense against oxidative damage is dependent on G6PD.

•G6PD deficient red cells are highly susceptible to oxidative damage.



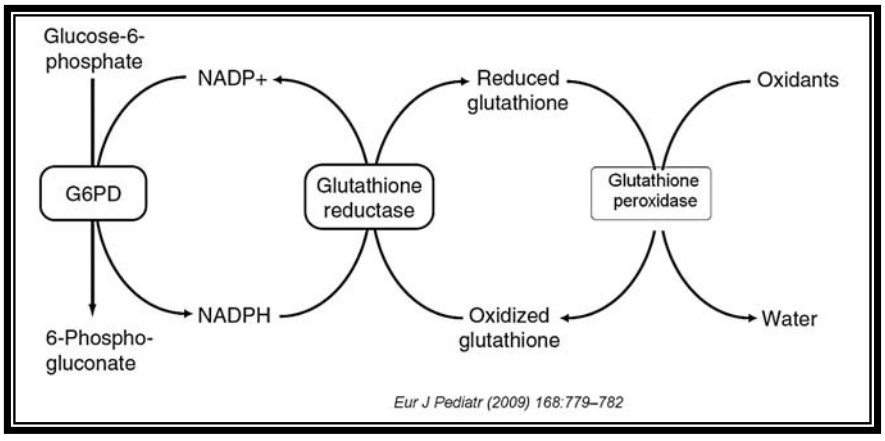
Importance of the Hexose-Monophosphate Shunt

• G6PD is the first and rate-controlling enzyme in the **Hexose-Monophosphate Shunt**

• G6PD oxidizes glucose-6-phosphate **to** 6-phosphogluconate. **NADP+**, which serves as a **cofactor** in this reaction, is reduced to **NADPH**.

• **NADPH** is a **cofactor/ substrate** for **glutathione reductase**, which then reduces the oxidized glutathione (GSSG) to the reduced form **(GSH) of glutathione**.

• **Reduced GSH** serves as a **cofactor/substrate** for the enzyme **glutathione peroxidase**, which reduces various peroxides to water.



Glucose-6-phosphate dehydrogenase (G6PD) deficiency

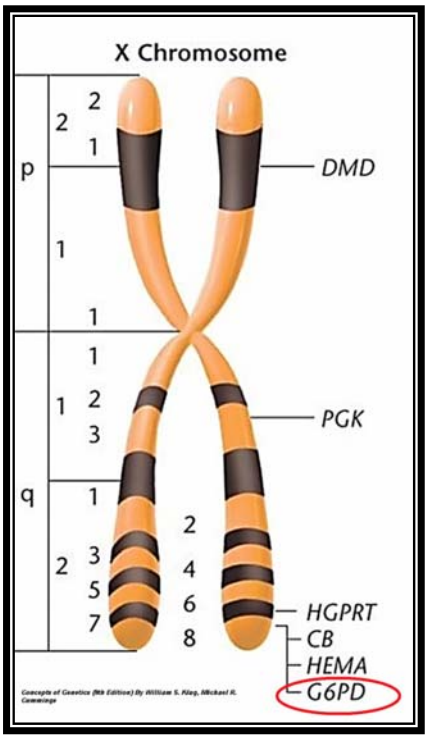
- G6PD deficiency is a common inherited blood enzymopathy affecting > **500 million** people worldwide.
- G6PD deficiency is found in almost **all geographic locations**, but much more frequently in areas where *Plasmodium falciparum* **malaria** was or is endemic, with highest incidences in Africa, South-East Asia, Middle East and the Mediterranean basin.
- Deficiency of G6PD has so far been linked **to 216 distinct genetic variants**, giving rise not only to a **wide range** of **biochemical** heterogeneity, but also to different **clinical manifestations.**
- Some **genetic variants** occur at **relatively high frequencies** within defined ethnic groups, and often more than one variant may be observed within a given population.

G6PD deficiency and Favism

- **Favism** was recognized for over a century as a form of **acute hemolytic anemia (AHA)** that is life-threatening especially in children.
- It became clear that G6PD deficiency was an essential inherited predisposition **to develop favism**.
- **Favism** is seen more **frequently among children than in adults**, and particularly in young children **morbidity can be substantial**.
- In a region where the frequency of G6PD deficiency in the population is sizable and **fava beans** are a dietary staple, the **risk of acute hemolytic crisis (Favism)** in young children is **high**.
- With **appropriate management**, which often must include blood transfusion, **full recovery** from favism without complications is the rule; however, if **not promptly** diagnosed and managed it is still a **life-threatening condition**

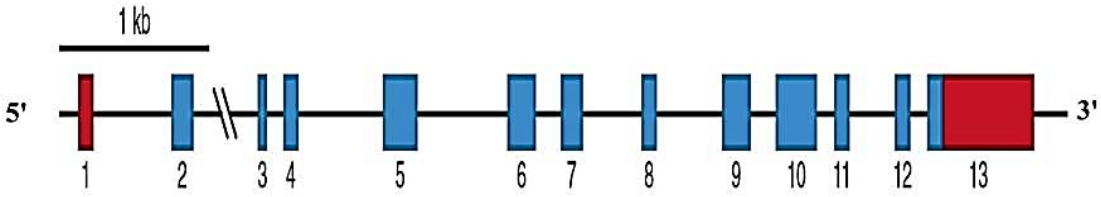
Molecular genetics of G6PD deficiency

- The G6PD gene maps to the **sub-telomeric** region of the **long (q)arm** of the X chromosome (Xq28).
- As it is a **X-linked gene**, **males** are more frequently affected than females.
- However, **heterozygous females** for G6PD variation can be affected due to genetic mosaicism, which results from **X-chromosome inactivation (Lyonization)** in somatic cells.
- **Most G6PD-deficient** subjects are usually **not anemic**; nevertheless, occurrences of **acute hemolysis, jaundice** and moderate to severe life-threatening **anemia** are common clinical presentations of G6PD deficiency.
- The symptoms are often **triggered by exogenous oxidizing agents**, through ingestion of particular nutriments (e.g. fava beans and other legumes), through medicines that induce oxidative stresses (e.g. quinolones and sulfa drugs), or viral infections.



The G6PD gene

- Consists of 13 **Exons** and 12 **Introns**, **Exon 1 is noncoding**.
- Encodes **515 amino acids**, About 18.5 kilobases (kb) in length.
- The enzymatically active form of G6PD enzyme is either a **dimer** or a **tetramer** of a single polypeptide subunit of about 59 kd.
- **Almost all variants** of the G6PD gene that result in enzyme deficiency affect the coding sequence.
- most of the 216 variants are **single-base substitutions** leading to **amino acid replacement**.



Luzzatto & Poggi, Glucose-6-Phosphate Dehydrogenase Deficiency. In: Nathan &Oski's Hematology of Infancy and Childhood, 2009

Sex-Linked Inheritance

- **Sex-linked inheritance** refers to the pattern of inheritance shown by genes that are located on either of the **sex chromosomes (X or Y)**.

- Genes carried on the **X chromosome** are referred to as being **X-linked**, and those carried on the **Y chromosome** are referred to as exhibiting **Y-linked or holandric inheritance.**

- Because ***G6PD*** *deficiency* is X-linked,
- Males can be only:
    - normal hemizygotes
    - or deficient hemizygotes.
- Females can be:
    - normal homozygotes,
    - deficient homozygotes
    - heterozygotes

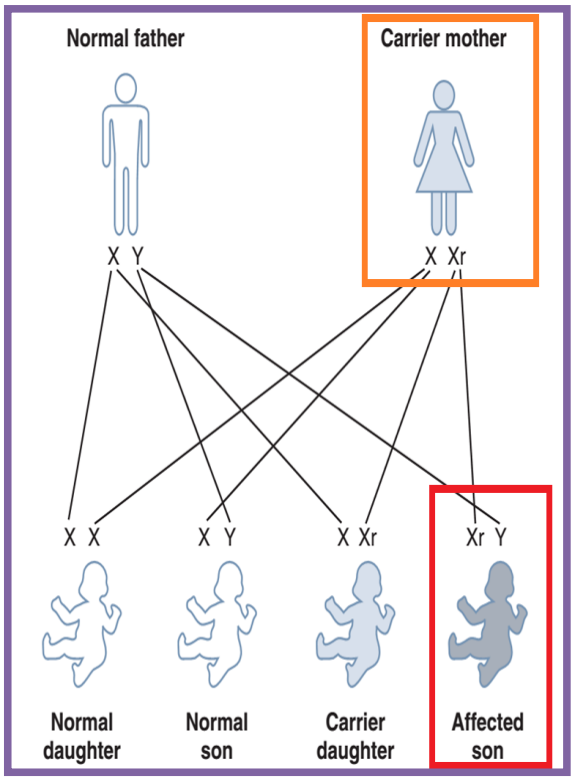
X-Linked Recessive Inheritance and Genetic Risks

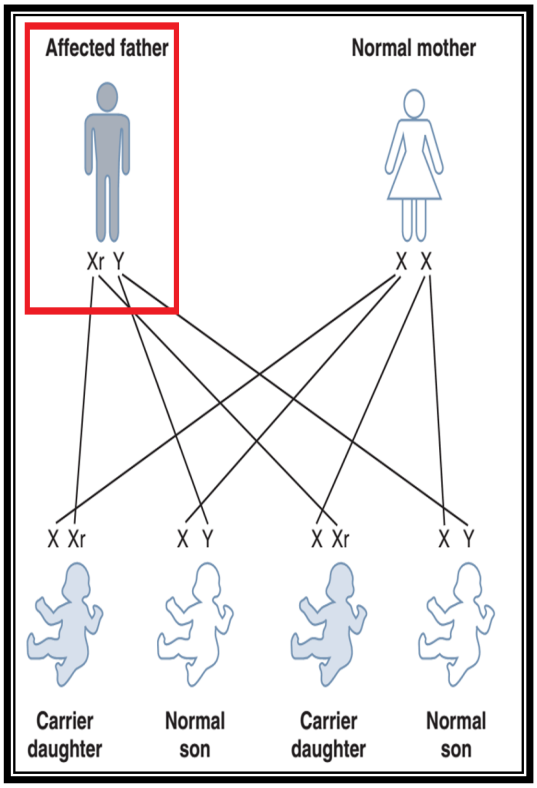
- An X-linked recessive trait is one determined by a gene carried on the X chromosome and **usually manifests only in males**.

- A male with a mutant allele on his single X chromosome is said to be **hemizygous** for that allele.

- Diseases inherited in an X-linked manner are transmitted by healthy **heterozygous female carriers (mothers) to affected males (50%)**, as well as by affected males (fathers) to their **obligate carrier daughters (100%)**, but none of their sons will be affected

- **Normally male cannot transmit an X-linked trait to his son**,





X-Chromosome Inactivation (Lyonization)

- The X chromosome contains many important protein-coding genes, and it has long been known that human females have two X chromosomes and males have only one. Thus, females have two copies of each X-linked gene, and males have only one copy. **Yet males and females do not differ in terms of the amounts of protein products** (e.g., enzyme levels) encoded by most of these genes. What could account for this?.

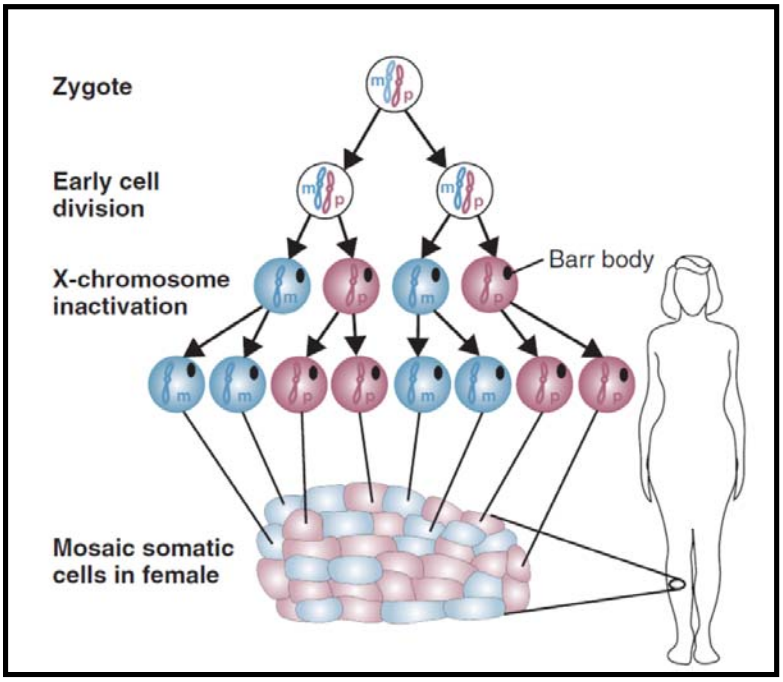
- In the early **1960s Mary Lyon hypothesized** that one X chromosome in each somatic cell of the female is **inactivated**. This would result in dosage compensation, an equalization of the amount of X-linked gene products in males and females.

- The Lyon hypothesis stated that X inactivation **(Lyonization)** occurs early in **female embryonic development** and that the X chromosome contributed by the **father is inactivated** in some cells, whereas in other cells the X chromosome contributed by the **mother is inactivated**.

X-Chromosome Inactivation (Lyonization)

- In each cell, one of the **two X chromosomes is chosen at random for inactivation,** so the maternally and paternally transmitted X chromosomes are each inactivated in about half of the embryo's cells. Thus, **inactivation**, like gamete transmission, is analogous to a coin-tossing experiment

- Once an X chromosome is inactivated in a cell, it will **remain inactive in all progenies of that cell.**
- X inactivation is therefore a randomly determined, but fixed (or permanent) process.
- As a result of X inactivation, **all normal females** have **two distinct populations of cells**: one population has an **active paternally** derived X chromosome, and the other has an **active maternally** derived X chromosome.
- Because they have two populations of cells, **females are mosaics for X chromosome activity**.
- **Males**, having only one copy of the X chromosome, are not mosaics but are **hemizygous** for the X chromosome (hemi means "half").



The X inactivation process. The maternal (m) and paternal (p) **X chromosomes are both active in the zygote and in early embryonic cells**. X inactivation then takes place, resulting in cells having either an active paternal X or an active maternal X chromosome. Females are thus X chromosome mosaics.

Skewed X-Inactivation

- The process of **X-inactivation** usually occurs **randomly**, there being an **equal chance** of either of the two X chromosomes in a heterozygous female being inactivated in any one cell.

- After **X-inactivation in embryogenesis**, therefore, in roughly half the cells one of the X chromosomes is active, whereas in the other half it is the other X chromosome that is active.

- Sometimes **this process is not random (Skewed)**, allowing for the possibility that the active X chromosome in most of the cells of a heterozygous female carrier is the one bearing the mutant allele. If this happens, **a carrier female would exhibit some of the symptoms** and signs of the disease and be a so-called manifesting heterozygote or carrier.

WHO Classification of G6PD deficiency

Class I
Severely deficient, associated with chronic non-spherocytic hemolytic anemia

Class II
Severely deficient (1–10% residual activity), associated with acute hemolytic anemia

Class III
Moderately deficient (10–60% residual activity)

Class IV
Normal activity (60–150%)

Class V
Increased activity (>150%)

World map distribution of G6PD deficiency