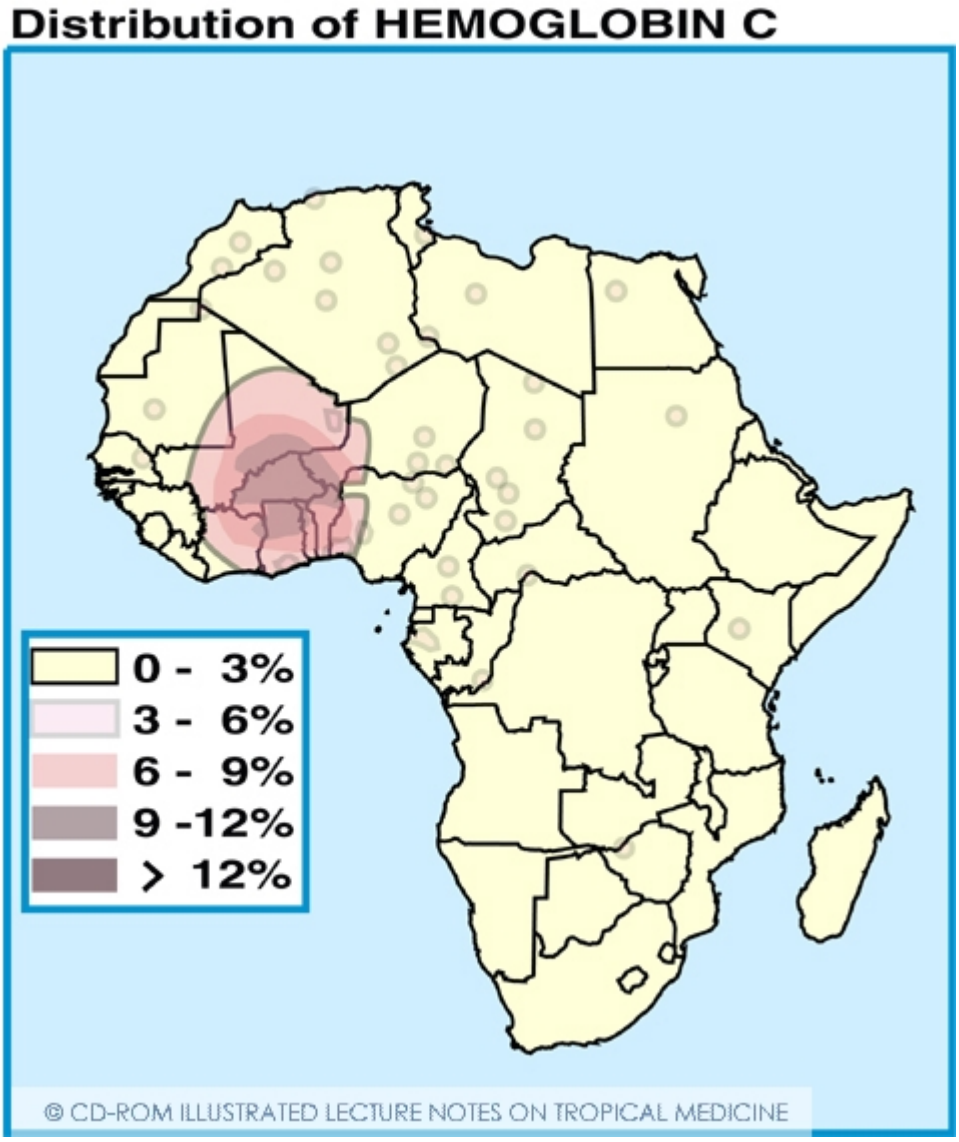
Haemoglobin C

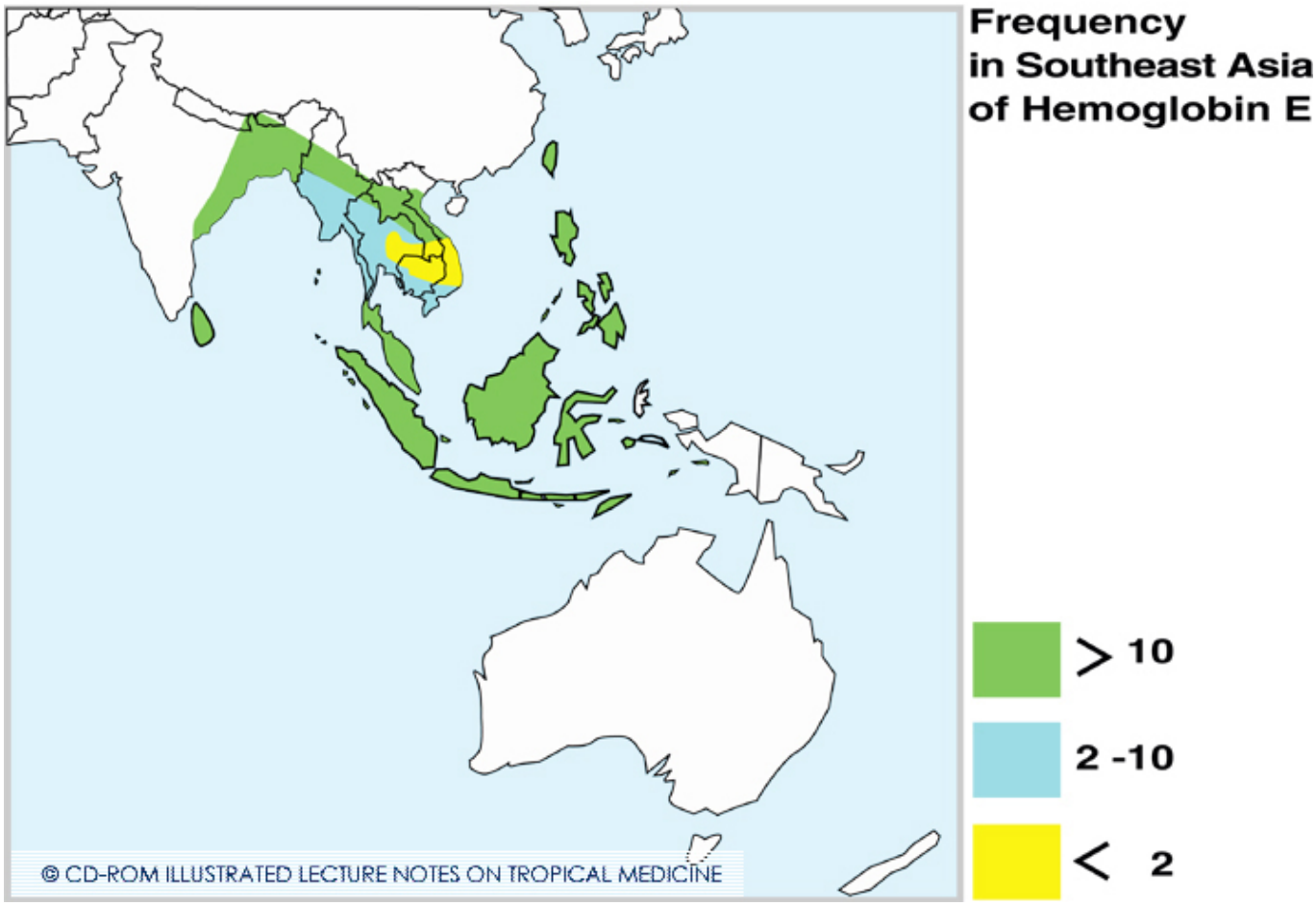


Map, distribution of haemoglobin C. Copyright ITM

Hb C is another haemoglobin variant that is common in West Africa. Here the 6th amino acid of the beta chain (glutamic acid, negative charge) is not replaced by a valine but by a lysine (positive charge, basic amino acid), i.e.: (beta6 Glu -> Lys) mutation. Sickling does not occur. Haemoglobin C has no protective effect on P. falciparum infection. The heterozygote state for Hb C is clinically silent. By electrophoretic analysis, 30-40% of the haemoglobin is Hb C and 50-60% is Hb A. People who are homozygous for Hb C (Hb CC) display mild chronic

haemolysis, mild to moderate anaemia and mild splenomegaly. On electrophoresis Hb A is absent. There is often microcytosis and there are many target cells and some spherocytes. Cholelithiasis is common. There are rarely major complications. Treatment is not necessary.

Haemoglobin E



Above: Map, distribution of haemoglobin E in Southeast Asia. Copyright ITM

Three splice site mutations are known to occur in exon 1 of the beta globin gene. These mutations result in three different abnormal haemoglobins: Malay, E, and Knossos. Haemoglobin E is a very common abnormal haemoglobin in Southeast Asia and India. The mutation GAG to AAG which leads to haemoglobin E, creates an alternate splice site competing with the normal splice site. This results in abnormal haemoglobin production and mild thalassemia in the homozygous state, with a mild microcytic anaemia with a

haemoglobin usually above 10 g%. Clinically the affected persons are not ill, although a mild splenomegaly can develop. Electrophoresis reveals approximately 90% Hb E with varying amounts of Hb F.

The heterozygote has a haemoglobin of about 12 g% with microcytosis and an electrophoretic pattern showing Hb E plus Hb A_2 of 20 to 30%. On standard alkaline electrophoresis haemoglobin E co-migrates with Hb A_2.

When Hb E trait combines with a beta^0 thalassemia mutation, a severe transfusion-dependent (EBeta^0) anaemia will ensue. EBeta^0 thalassemia patients who undergo splenectomy may stop being dependent on transfusions.

Glucose-6-phosphate dehydrogenase deficiency

General

In 1926 some people who had been given primaquine (an antimalarial) developed dark urine and haemolytic anaemia. The mechanism was not understood until 30 years later. Adult red blood cells have neither mitochondria nor a nucleus. The cells have no Krebs cycle and meet their energy requirements by glycolysis, an anaerobic process (Embden-Meyerhof chain). This is a very inefficient way of producing ATP, but in this way the erythrocytes do not use the oxygen they transport and the cells are effective carriers of oxygen. By glycolysis a molecule of glucose supplies two ATP molecules and two NADH molecules. By a side-reaction, 2,3 diphosphoglycerate is also produced, a substance that has an important effect on the release of oxygen (see oxygen dissociation curve).

Another metabolic pathway in the cytosol of the red blood cell is the hexose monophosphate shunt (also called the pentose phosphate shunt). The first enzyme in this latter chain is G6PD. The hexose monophosphate chain provides two molecules of NADPH per molecule of glucose. It is the only source of NADPH in the red blood cell.

The normal enzyme is called “type B”.

About 20% of Black people in Africa have “type A+”. This variant is functionally normal, but has a different electrophoretic pattern. “Type A-” has the same electrophoretic characteristics as “type A+”, but has lesser activity. This form is common in Central Africa. People with “type A-” are normally not anaemic. Enzymes with little or moderate activity rarely cause clinically serious problems.

Another important variant is the “Mediterranean type” and is virtually totally inactive. The less active the enzyme, the easier it is for the red blood cell to be damaged by certain chemical substances. Enzymes with very little or no activity are common in people in the Mediterranean basin.

Clinical aspects

People with a very low G6PD activity can lead a normal life. In some situations, problems can arise. A crisis begins acutely and symptoms worsen in the course of a week. Jaundice, renal pain, haemoglobinuria and mild splenomegaly occur. Newborn children with G6PD deficiency are at greater risk of kernicterus and phototherapy is sometimes necessary. In many people the haemolysis is self-limiting, even if primaquine, for example, is continued to be administered. Circumstances that can trigger symptoms include:

The neonatal period (neonatal jaundice). Severe kernicterus due to G6PD-deficiency-related haemolysis is an avoidable cause of mental retardation. It is possible that the icterus is due to haemolysis combined with impairment of the liver function in these neonates.

A short list of drugs and chemicals that should be avoided by persons with G6PD deficiency includes: primaquine, methylene blue, niridazole, nitrofurantoin, sulfacetamide, sulfamethoxazole, sulfanilamide, sulfapyridine, phenylhydrazine, uropyrine. The administration of such medication is followed, after a 1- or 2-day delay, by falling haemoglobin concentration. Heinz bodies (denatured haemoglobin adherent to the RBC membrane), appear in the early stages of drug administration and disappear as haemolysis progresses.

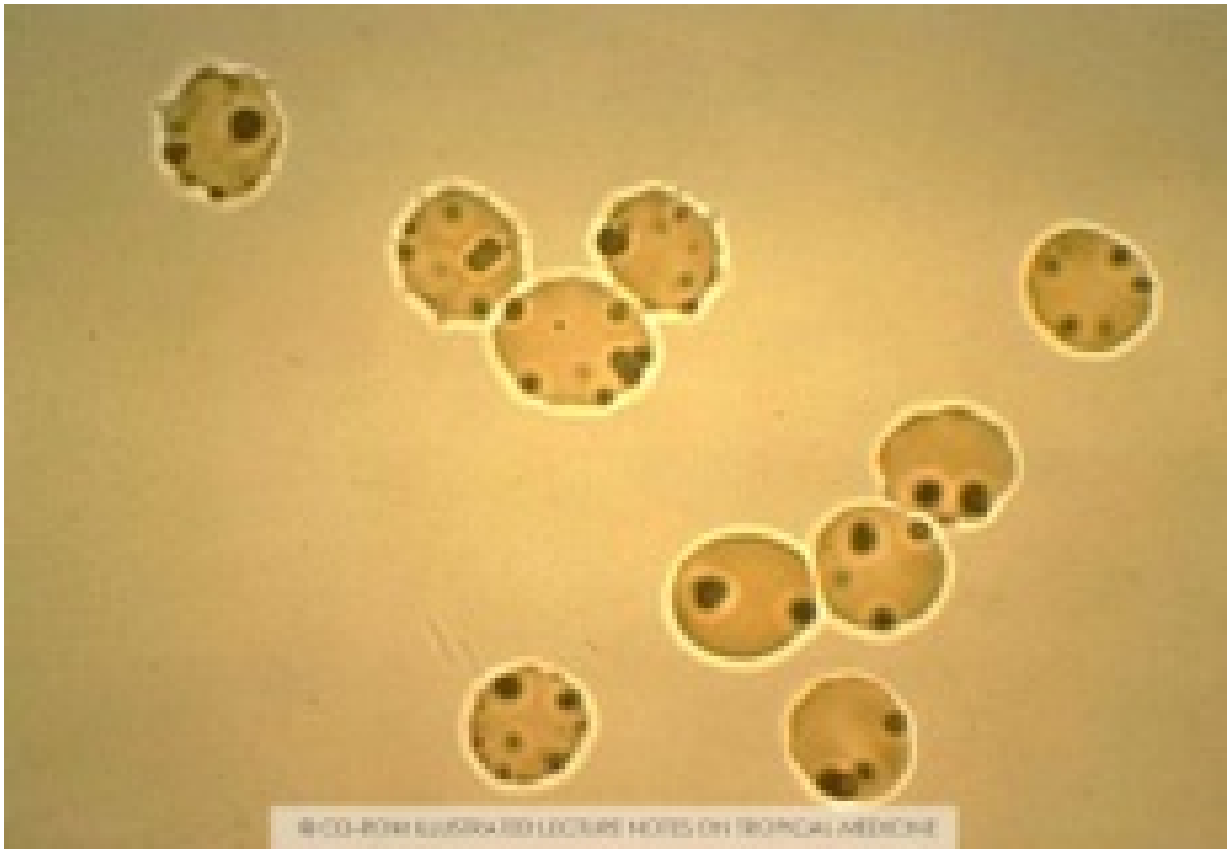
In sub-Saharan Africa there are few clinically relevant problems, but in the Mediterranean basin severe, even life threatening reactions are more common. Serious infections involving

acidosis can cause acute haemolysis. The mechanism by which this occurs is not clear, but leukocytes might damage erythrocytes in their environment by releasing active oxygen species during phagocytosis (cfr production of H2O2 by neutrophils and macrophages).

Favism. In the case of severe deficiency, serious haemolysis can occur if fava beans ["Vicia fava", favism] are eaten or the pollen of the plants are inhaled. The symptoms occur quite quickly after aerogenic exposure but only develop after 5 to 24 hours after eating fava beans. Divicine and isouramil are oxidants present in this plant and they are normally reduced and inactivated by reduced glutathione. Our knowledge of favism is however incomplete. There is no absolute correlation between G6PD activity and the clinical symptoms. Other factors undoubtedly also play a part. People with "type A-" do not suffer from favism.

Transfusions. Normal red blood cells keep their G6PD activity if they are stored for transfusion. The small amount of activity that G6PD-deficient cells still have, will decrease as time goes by. If this type of blood is transfused into a person who is already ill and who may be receiving potentially haemolysing medication, haemolysis of the transfused blood can occur quickly.

Diagnosis



G6PD-deficiency. Heinz bodies are visible in the erythrocytes and consist of denatured haemoglobin.

The morphology of the red blood cells is normal between crises. During a crisis inclusions can be detected in red blood cells (Heinz bodies) by means of a supravital dye such as crystal violet. Heinz bodies are formed by denatured, damaged haemoglobin. Cells with these inclusions are quickly removed via the spleen. Detection of Heinz bodies is an insensitive test for G6PD deficiency. G6PD activity can be measured directly in a well-equipped laboratory. The simplest quantitative assay measures the reduction of NADP to NADPH in the presence of glucose-6-P and haemolysate. It is important to know that a test might give misleading too high results if performed in less than 2 weeks after a haemolytic episode. Older erythrocytes have less enzyme activity and will be eliminated first after a haemolytic crisis. If the test is then performed on the remaining younger red cells (which have a higher enzyme activity), the activity of the enzyme is overestimated. In a blood smear stained with May-Grünwald Giemsa; Heinz bodies cannot be detected, but one can recognise ‘bite cells’ (keratocytes, blister cells) and dense erythrocytes with irregular outline. In normal people, the activity of

G6PD is reduced by half over 120 days (the normal life span of an erythrocyte). It is therefore mainly the older cell population that is affected. This also explains why most clinical episodes are self-limiting (usually about 25% of the cells are haemolysed). It is precisely because of this limited haemolysis that people with, for example, leprosy, can often continue to take dapsone. Reticulocytosis increases after a few days.

For didactic examples of the more unusual blood smears (including G6PD-pathology), see: http://content.nejm.org/cgi/content/full/353/5/498

G6PD-deficiency, methaemoglobinemia and methylene blue

In haemoglobin, iron in haem is present as Fe^{2+}. If iron in haem becomes oxidised to Fe^{3+} it is called methemoglobin. This cannot carry oxygen. Normally, the unpronounceable enzyme NADH-dependent cytochrome b_5 methemoglobin reductase will reduce methemoglobin to haemoglobin. This is a rather slow process. When methemoglobinemia occurs, one would usually administer methylene blue. However, after administration methylene blue first has to be reduced in the body to its active metabolite leukomethylene blue. It is the leukomethylene blue which will convert Fe^{3+} in haem into Fe^{2+}. The conversion of methylene blue to leukomethylene blue is catalysed by NADPH methemoglobin reductase, a reaction requiring NADPH. Because there is an important NADPH-deficit in G6PD-deficient red blood cells, this conversion will not take place, and treatment with methylene blue will not work. What is more, administration of methylene blue in case of important methemoglobinemia is dangerous in case of G6PD-deficiency, because it will increase haemolysis. Methylene blue is an oxidant which will increase the anaemia and the hypoxemia. If one cannot wait for spontaneous improvement, blood transfusion and oxygen administration are warranted.

G6PD deficiency, hereditary transmission

The activity level of the G6PD enzyme is genetically determined. The G6PD gene is located on the **X** chromosome (a man has XY and a woman has XX). A man with a defective gene (hemizygote) and a woman with 2 defective genes (homozygote) are affected. A woman with just 1 mutant gene (heterozygote) is a carrier, but normally does not display any symptoms. She may well pass the defective gene on to her child. Because in women 1 of the 2 X chromosomes is inactivated in each nucleated cell (Lyons hypothesis), a heterozygous

woman has 2 populations of erythroblasts and therefore also 2 populations of red blood cells: a normal population and a deficient population.

Heterozygous women with a high percentage of deficient cells may become symptomatic. Normal women are therefore genetic chimaeras: some cells contain an active paternal X-chromosome and others contain an active maternal X-chromosome. There are no cells in which both chromosomes are active. Note of course that early precursor cells contain DNA but erythrocytes themselves have no nucleus, and therefore contain no chromosomes or even DNA.

Oxidative stress

Oxidative stress is defined as an imbalance between free-radical production and antioxidant protection. There are many varieties, but important ones include the hydroxyl radical (°OH), hydrogen peroxide (H_2O_2) and the superoxide radical $O_2^{\circ -}$, with the ° symbol indicating an unpaired electron. To give a ballpark idea, it is estimated that an average adult human forms 1.7 kilograms of superoxide each year. Each cell in our body produces about 50 hydroxyl radicals each second, one of the most reactive species which exists. It basically reacts instantly with any other molecule, be it fat, protein or DNA which it encounters, thereby damaging it. If there would be no defence against free-radicals, cellular damage would advance at a very fast pace. The cellular defence against free-radicals include antioxidants such as vitamin C and E, glutathione and catalase.

Hexose monophosphate shunt

In order to have enough reduced glutathione, a supply of NADPH is needed. The first reaction in the hexose monophosphate shunt produces NADPH. This reaction is catalyzed by the enzyme G6PD. In very general terms it can be said that the hexose monophosphate shunt (= pentose phosphate chain), has two main functions:

- the production of ribose, a component of nucleotides, for e.g. DNA and ATP. In summary, this pathway transforms glucose-6-phosphate into ribose-5-phosphate. However the erythrocyte has no nucleus nor ribosomes, there is no need for ribose synthesis in these

cells.

- the generation of reducing power in the form of NADPH. The pentose phosphate chain reduces NADP+ to NADPH. By oxidising NADPH to NADP+ again, other substances are reduced via a redox reaction. NADPH is an important electron donor (= reducing capacity). The main function of NADPH is to reduce oxidised substances such as glutathione and to allow reductive biosyntheses to take place.
  The NADPH/NADP ratio controls the rate of reaction in an autoregulatory manner. In a quiescent state, this ratio is very high and G6PD is nearly completely inhibited. When NADPH is oxidized, as when glutathione is reduced in the glutathione reductase reaction, NADPH is converted to NADP+ and G6PD becomes active, reconverting NADP+ to NADPH.

Red blood cells and NADPH

Why does G6PD deficiency seem to affect red blood cells especially? Erythrocytes do not have mitochondria therefore red blood cells do not have a back-up system for NADPH production. They have no alternative source of NADPH, as opposed to other cells which have mitochondria. Acetyl-CoA in the mitochondria (entry point for the Krebs cycle) cannot pass through the mitochondrial membrane by itself. If it is bound to citrate it can pass through the membrane. In cells with mitochondria some citrate bound to acetyl-CoA shifts from the mitochondrial matrix to the cytosol, after which the compound is divided again. The citrate therefore acts as a carrier. Citrate is then converted to oxaloacetate and then to malate. Afterwards (malate + $NADP^+$) is converted to (pyruvate + CO_2 + NADPH). As a result, even if the hexose monophosphate shunt is functioning poorly, cells with mitochondria can still produce NADPH. The effects of G6PD deficiency are therefore most apparent in the red blood cells (cells without mitochondria).

Glutathione

Why do we need glutathione? Haemoglobin and many other biological molecules contain many sulphur groups (SH groups =sulfhydryl groups). These are necessary for the molecule to function properly. If these are oxidized, haemoglobin can no longer function as it should. Glutathione is a tripeptide containing cysteine as the second amino acid. This amino acid

has a SH group. The reduced glutathione (i.e. with a SH group), converts non-functional, oxidized cysteine disulphide groups (S-S) in other molecules such as haemoglobin into functional SH groups via the enzyme glutathione peroxidase. In this process glutathione itself is oxidized (two glutathione molecules are then bound by a disulphide bridge). Glutathione also reacts with hydrogen peroxide (H_2O_2) and corrosive organic peroxides. In this way it has an important protective role as an anti-oxidant. If the G6PD enzyme is deficient, no NADPH is formed, neither is any protective reducing glutathione formed and haemoglobin molecules and red blood cell membrane molecules that contain SH groups may be permanently damaged by oxidizing substances. The non-functional, denatured haemoglobin is precipitated in the form of Heinz bodies and the resulting damage to the membrane then leads to haemolysis resulting in moderate, but acute anaemia.

Note on *P. vivax* eradication: In countries attempting to eliminate *P. vivax* infection, the existence of G6PD deficiency is driving the development of a simple, user-friendly point-of-care test for its detection. Today, primaquine and tafenoquine are the only drugs capable to eliminate the hypnozoites in *P. vivax* infections. However, both drugs can provoke a severe haemolytic crisis in a person with G6PD deficiency. Therefore, testing for G6PD deficiency is imperative before these drugs can be administered safely.

Beta thalassemia

General

In addition to the Mediterranean basin the disease also occurs in Africa, the Middle East, India and Myanmar, Southeast Asia including southern China, Malaysia and Indonesia. There are indications that the high frequency of heterozygous beta thalassemia carriers in the tropics can be explained by a relative protection against the fatal *P. falciparum* malaria (compare with sickle cell trait and G6PD deficiency), but this is controversial.

Embryonal Hb	Fetal Hb	Adult Hb

Hb Gower 1: $zeta_2$ $epsilon_2$ Hb Gower 2: $alpha_2$ $epsilon_2$ Hb Portland: $zeta_2$ $gamma_2$	Hb F: $alpha_2$ $gamma_2$	Hb A: $alpha_2$ $beta_2$ Hb A_2: $alpha_2$ $delta_2$

An average normal adult has Hb A 97%, Hb A_2 2%, Hb F 1%.

About 150 different mutations have been reported in people with beta thalassemia. About 20 mutations are responsible for 80% of the beta thalassemias. Some are simple nucleotide substitutions, with missense or nonsense consequences, multiple substitutions, deletions with frameshifts or abnormalities in the promoter. Sometimes something goes wrong with the splicing of mRNA. Within each geographic population there are unique mutations. Individuals who have beta thalassemia major are usually homozygous for one of the common mutations, or heterozygous for one of the common mutations and one of the geographically-unique mutations. All result in reduced synthesis of beta globin chains ($beta^+$-thalassemia) or the absence of synthesis of beta globin chains ($beta^0$-thalassaemia). Clinically mild forms of beta thalassemia are called thalassemia intermedia, whereas minor forms are non-symptomatic. If the production of both beta and delta chains is diminished, there is delta-beta-thalassemia (a consequence of gene fusion). The imbalance in globin chain synthesis (there are more alpha chains than beta chains) leads to precipitation of alpha chains in the red cell (= inclusion bodies or α-hemichromes), which leads to premature destruction of the cell in the bone marrow or the peripheral blood.

Clinical aspects

Beta thalassemia minor

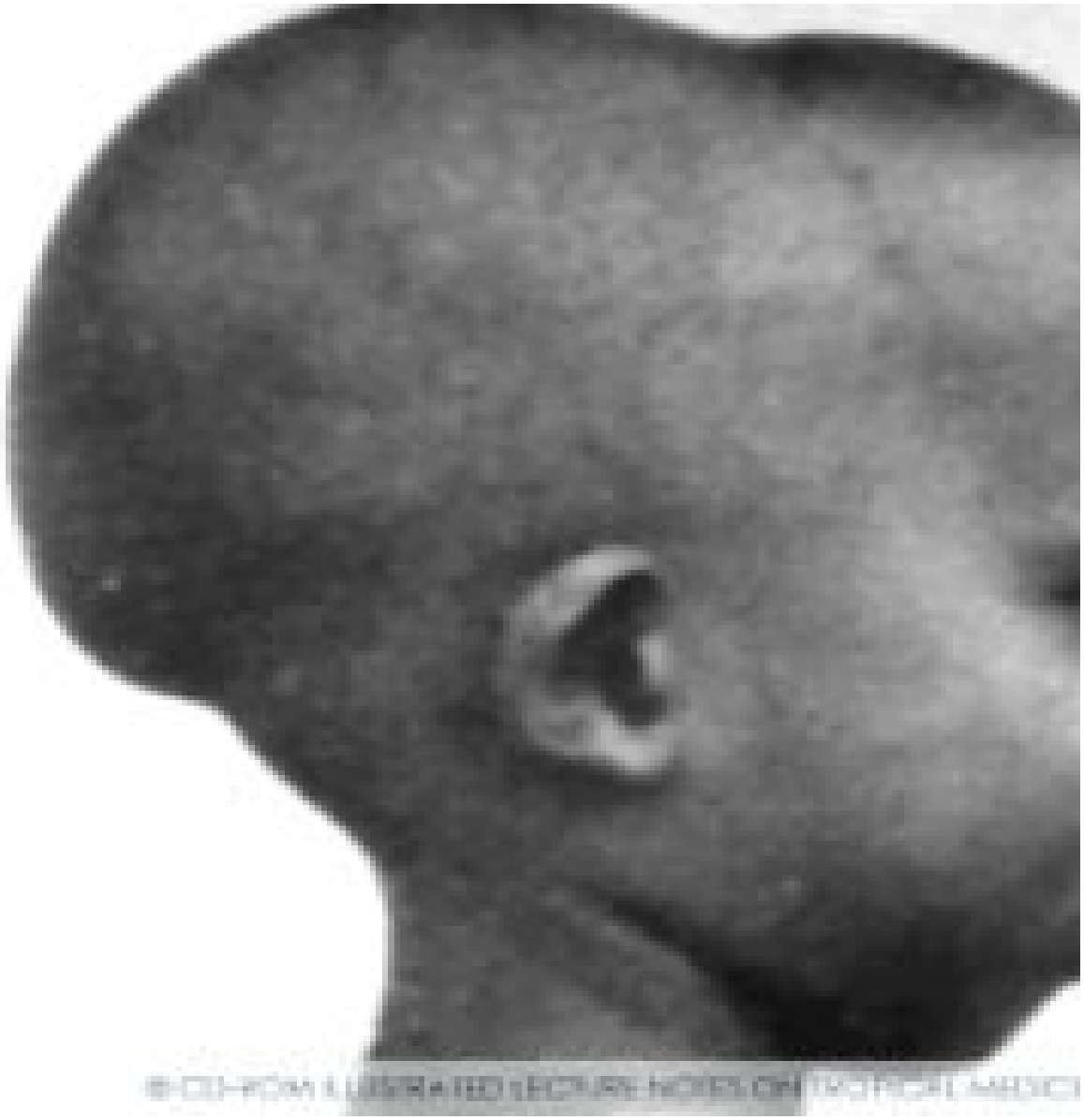
The heterozygous condition is known as beta thalassemia minor. One beta gene is defective, the other is normal. Fewer beta globin chains than normal are produced but the healthy gene largely compensates for this. There is a typical microcytosis but rarely anaemia. This form is often found by chance and can wrongly be regarded as an iron deficiency.

There is a diagnostic problem for patients suspected to be double heterozygous for Hb E and

beta-thalassemia. Hb E and Hb A2 cannot be distinguished in alkaline gel, but diffuse differently in an acid gel.

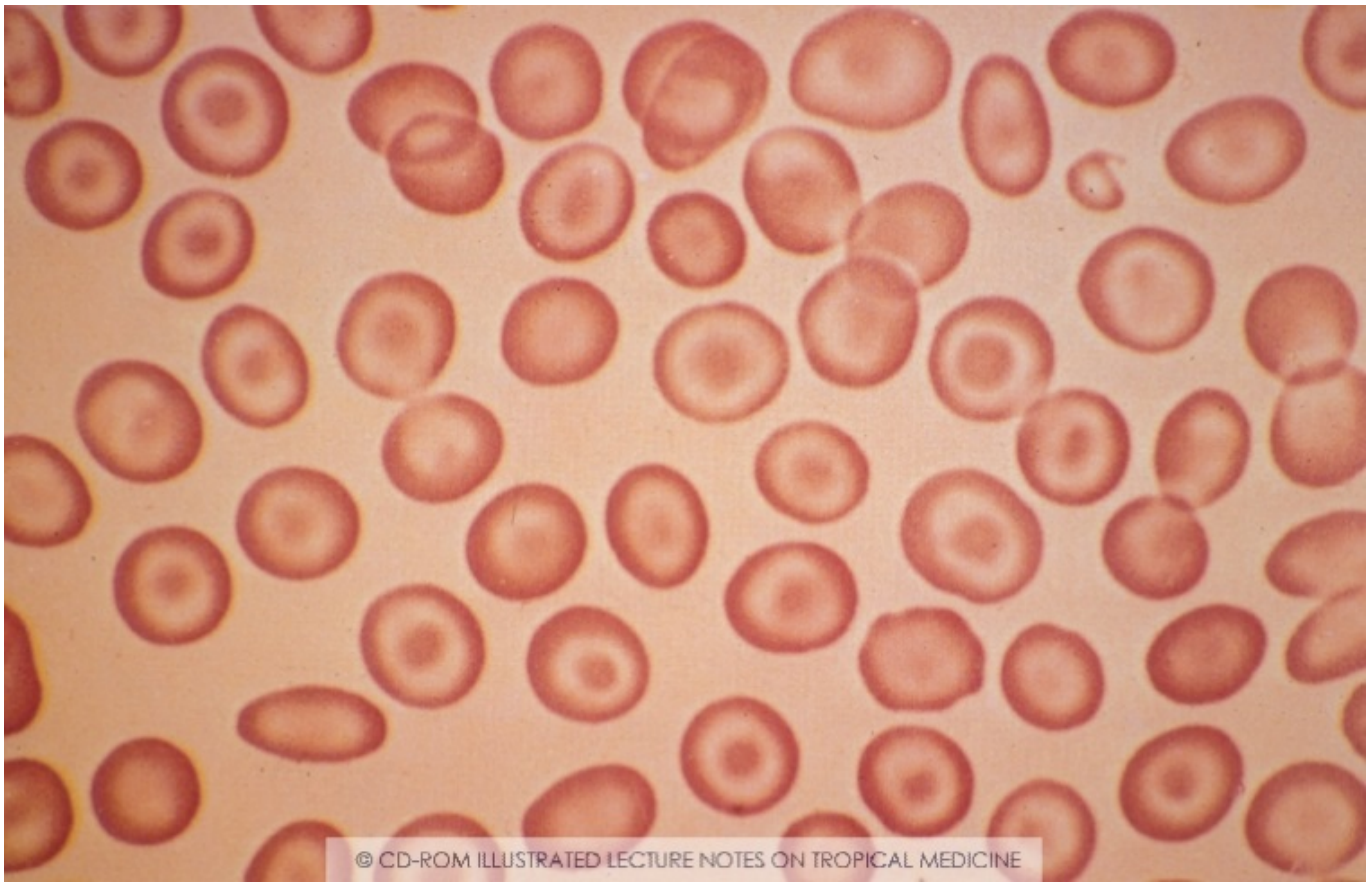
But Hb A and E cannot be distinguished in acid gel. This occurs mainly in people from Southeast Asian origin.

Beta thalassemia major



Beta-thalassemia major. Skull bossing due to expansion of the diploë (red bone marrow in

skull). See also “hair-on-ends” aspect by Rx



Beta-thalassemia minor with microcytes and target cells

The homozygous or doubly heterozygous condition is much more serious. The severity depends on which mutation(s) causes or cause the disorder and how many beta globin chains can still be produced.

There is therefore a spectrum of clinical severity: thalassemia major or thalassemia intermedia. Patients with thalassemia major are by definition transfusion dependent. The affected infants are normal at first. Newborns still have Hb F, which is not affected in this condition. By the age of 6 to 9 months, the children develop faulty erythropoiesis with anaemia and hypertrophy of the bone marrow, spleen and liver with hepatosplenomegaly. In severe beta thalassemia, erythropoiesis can increase up to 10-fold. The relative excess of alpha globin chains interferes with the normal maturation of the cells in the bone marrow.

Ineffective erythropoiesis occurs. There is pronounced haemolysis with considerable splenomegaly. Enlargement of the liver always occurs. Sometimes there are gallstones (bilirubin stones due to the haemolysis). The red bone marrow increases in volume, with swelling of the diplok in the cranial bones, osteopenia and a lowering of the fracture threshold and often microfractures around the main joints. The diploë is the central layer of spongy bone between the two layers of compact bone of the flat cranial bones. The face is often deformed somewhat due to cranial bossing and hypertrophy of the maxillae resulting in a mongoloid appearance. Bone marrow expansion can lead to compression of the spinal cord. Extramedullary haematopoiesis can occur, not only in spleen and liver, but also in the posterior mediastinum and even kidneys, leading to local masses which can resemble lymphoma.

There is haemolytic anaemia; microcytosis with normoblasts in the peripheral blood and an increase in the minor haemoglobins (Hb F, Hb A2). Children can survive only with regular blood transfusions and folic acid supplements. Iron overload and infections due to the repeated transfusions are a very real risk. The abnormal accumulation of iron results in dilated cardiomyopathy, endocrine disorders (destruction of the pituitary gland and hypogonadism with impaired sexual development) diabetes, liver disease (often together with hepatitis B and C). Later, restrictive lung disease and pulmonary hypertension can occur (pulmonary hypertension tends to occur in all chronic severe haemolytic diseases).

Laboratory

Severe haemolytic anaemia is present, which is accompanied by microcytosis (low MCV), target cells, a high RBC count with a relatively low reticulocyte count considering the severity of anaemia. The high RBC count is a compensation for the low amount of normal Hb in each red blood cell (contrary in iron deficiency where the marrow cannot produce as many RBCs). The Mentzer index is helpful in differentiating iron deficiency anaemia from thalassemia: it is the quotient of the MCV (in fl) divided by the red blood cell count (in millions per µl). If the Mentzer index is less than 13, thalassemia is more likely, if the result is greater than 13, iron-deficiency is more plausible.
On hemoglobinophoresis, a higher level of HbA2 ($alpha_2delta_2$) is usually found in beta thalassemia patients: the excess alpha globin chains bind to the delta globin chains. Diagnostic confirmation by globin gene testing will be rarely available in the tropics.

Prevention

A child born of two heterozygous parents has a 25% probability of being homozygous. There are screening programmes for detecting carriers in Italy, Sardinia, Cyprus and Greece. These are based on MCV and the concentration of Hb A_2. Prenatal diagnosis can be carried out with various techniques (e.g. villous chorion sampling carried out in weeks 9-13).

Therapy

Non-transfused thalassemia intermedia patients are encouraged to avoid high-iron and iron supplemented foods and are encouraged to drink tea with meals, which decreases iron absorption.

Folic acid is usually given. With beta thalassemia major there is a great need for transfusion. Because of the repeated transfusions, iron overload occurs after a number of years (the time varies). Iron chelation is carried out with deferoxamine (Desferal).

Bone marrow transplantation can be carried out as curative therapy and at present is the only definite treatment. Of course, the bone marrow of an identical twin cannot be used but that of a HLA-DR matched relative can be used.

Alpha thalassemia

Alpha genes can be lost through deletion or inactivated by point mutations. If insufficient alpha chains are produced, the condition is known as alpha thalassemia. This condition is very frequent in Asia (from India to China, including Southeast Asia). The disease also occurs in Africa. Since 4 genes code for alpha chains, there are a number of possibilities:

All 4 alpha genes functional: normal. Genetic alpha alpha/alpha alpha

Only 3 alpha genes functional: silent carrier with no symptoms or signs (thalassemia minima). Genetic alpha-/alpha alpha

Only 2 alpha genes functional: silent carrier, often microcytosis (alpha thalassemia minor

or alpha thalassemia trait). Genetic alpha alpha/– (= alpha^{0} thalassemia) or alpha-/alpha- (= alpha^{+} thalassemia). The two genes can either occur on the same chromosome (cis-type) or on each of the pairs (trans-type). Cis-type alpha^{0} thalassemia trait tends to be found in individuals of Asian descent, while trans-type alpha^{+} tends to run in individuals of African descent. Expert laboratory tests help to distinguish between these two conditions, which is important. If a mother is a carrier of alpha^{0} thalassemia, her pregnancy is at risk for Bart's hydrops fetalis syndrome (worst case scenario), while the worst possible outcome of a pregnancy of a mother with alpha^{+} thalassemia is a much milder condition, haemoglobin H disease.

Only 1 alpha gene functional: excess of beta globin chains. Genetic alpha-/–. The excess beta chains form tetramers and are deposited: ß$_4$ (haemoglobin H). Haemoglobin H is not stable and thermally labile. It contains two reactive SH groups per beta chain. The beta chains in Hb A have only one SH group. This may explain the susceptibility of Hb H to oxidation. The red blood cell inclusions (Heinz bodies = β-hemichromes) can be seen readily with brilliant cresyl blue staining (the same dye as for reticulocytes). The patient is anaemic and there is splenomegaly.

No alpha genes functional: the excess of gamma chains leads to the depositing of tetramers composed of four gamma chains: gamma$_4$ (Barts haemoglobin). Without the alpha globin chains, there can be no fetal or adult haemoglobin which means the red blood cells cannot carry oxygen efficiently throughout the body. Hydrops fetalis with stillbirth is the result. There is an increased risk of toxaemia of pregnancy and of post-partum haemorrhage (hypertrophy of the placenta). The only haemoglobins found in these infants are: Hb Portland (delta$_2$gamma$_2$), Hb H (ß$_4$), and Hb Bart's (gamma$_4$), and no Hb A, Hb A$_2$ or Hb F. Electrophoresis of fetal haemoglobins shows about 80% Barts haemoglobin and about 20% Portland haemoglobin (normally only present in the embryo in the first trimester).

Onyalai

General

Onyalai is a rather mysterious disease, which only seems to occur in central southern Africa

(southern Angola and northern Namibia; Kavango and Ovambo territories). Onyalai means “blood blister” in the language of the Kimbundu, an Angolan tribe. Onyalai is a disease of unknown aetiology. Defective nutrition may be the cause. One hypothesis is that a toxin, possibly acting as a hapten, is responsible for this form of thrombocytopenia. The possible etiological role of mycotoxins from contaminated millet, sorghum and/or maize requires further investigation.

Clinical aspects

The disease differs clinically, epidemiologically and immunologically from immune (previously idiopathic) thrombocytopenic purpura (ITP) It is an acute disease, characterized by the formation of haemorrhagic vesicles and blisters on the palatal and buccal mucous membranes, together with severe thrombocytopenia. This acquired form of thrombocytopenic purpura can lead to haematuria and melena. Epistaxis, petechiae and ecchymoses are common, as are subconjunctival bleeding and menorrhagia. Haemorrhage from ruptured bullae, epistaxis or gastrointestinal bleeding can be severe and may cause shock and even death.

Treatment

Transfusion of blood and of platelets can be lifesaving. High dose intravenous gammaglobulin may be followed by a rise in the platelet count and cessation of haemorrhage but in general this treatment is disappointing (and expensive). Splenectomy can be considered for patients with severe uncontrollable bleeding, although splenectomy does not always control the disease.