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Stepwise diagnostics of hemoglobinopathies

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Abstract: Hemoglobinopathies belong to the most common monogenic hereditary diseases worldwide. A particularly high prevalence is seen in the Mediterranean countries, in parts of Asia, the Middle East and West Africa. Nevertheless, due to migration hemoglobinopathies play an increasingly important role in Germany as well. Basic testing consists of blood count and hemoglobin differentiation. In addition, an iron deficiency should be excluded if necessary. Molecular genetic testing is used for the verification of hematologic findings and serves in the assessment of risk for a severe form of a hemoglobinopathy in offspring. In order to ensure efficient diagnostics, family history and previous findings of the patient should be communicated to the laboratory. This is especially crucial in the case of prenatal diagnostics.

Keywords: abnormal hemoglobins; HbC; HbE; HbS; hemoglobin differentiation; hemoglobinopathy; HPFH; HPLC; hypochromia; iron-deficiency anemia; microcytosis; molecular diagnostics sickle cell disease; stepwise diagnostics; α-thalassemia; β-thalassemia.

Introduction

Hemoglobins are ferrous proteins, found in erythrocytes. They are responsible for transporting oxygen within the human organism. Hemoglobin is a heterotetramer consisting of two kinds of identical polypeptide chains. Each of the chains carries a heme group with an iron molecule to which oxygen can bind reversibly.

The adult hemoglobin (HbA) consists of two α and two β-chains (α2β2). The fetal hemoglobin (HbF), however, consists of two α and two γ chains (α2γ2). HbF has a higher affinity for oxygen, thereby ensuring the placental oxygen transfer from the mother to the child. During the first year of life, the content of fetal hemoglobin decreases continuously and is replaced by the increasing synthesis of adult hemoglobin [1].

Hemoglobinopathies are disorders due to the faulty formation of hemoglobin. They are among the world's most common monogenic hereditary diseases and, depending on the molecular phenotype, are assigned to the thalassemias or to the group of abnormal hemoglobins. Thalassemias are caused by mutations in the various globin genes that reduce the synthesis of one or more normal globin chains. The most common and clinically most significant thalassemias are α- and β-thalassemia. Abnormal hemoglobins are caused by mutations that lead to structural defects and therefore to the production of abnormal globin chains. Among the most common abnormal hemoglobins are HbS, HbC and HbE. A special form of hemoglobinopathy is the hereditary persistence of fetal hemoglobin (HPFH), in which the synthesis of HbF in adulthood is increased moderately to excessively. A carrier of HPFH usually shows no clinical symptoms. An overview of the main hemoglobinopathies can be found in Table 1.

The various forms of hemoglobinopathies can also occur in combination [2]. Combinations of α- with concurrent β-thalassemia are frequently encountered, as are thalassemias combined with abnormal hemoglobins, or the simultaneous presence of two abnormal hemoglobins (e.g. HbSC). These combined forms can partially be detected already in the blood count and hemoglobin differentiation.

Diagnostic procedure

Hemoglobinopathy diagnostics is usually necessary only when microcytic anemia is present. Methemoglobinopathies

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Table 1: Overview of the main hemoglobinopathies [1].

Thalassemia
– α- and β-thalassemia
– δβ-thalassemia
Abnormal hemoglobins
– Anomalies of the β-globin chain (HbS, HbE and HbC)
– Anomalies of the α-globin chain (Hb Constant Spring)
– Fusion hemoglobins (Hb Lepore)
Hereditary persistence of fetal hemoglobin (HPFH)

require a specific diagnosis of MetHb, and the same applies to unclear cyanosis.

The diagnosis of hemoglobinopathies is done in stages. The first stage is the red blood cell count and hemoglobin differentiation. For verification or detailed characterization of a suspicious finding, molecular genetic analysis can be performed in a second stage.

Hemoglobinopathy diagnostics is indicated in these cases: [2–4]

- Hypochromia and/or anemia where iron deficiency was previously ruled out
- Chronic hemolytic anemia
- Anemia caused by drugs
- Vaso-occlusive crises of unknown etiology
- Hematology-related erythrocytosis and/or cyanosis
- Hydrops fetalis syndrome of unknown etiology
- Positive family history
- Prenatal diagnostics

The following parameters of the blood count are diagnostically relevant:[5]

- Erythrocyte count
- Hemoglobin concentration
- Hematocrit
- MCH (mean corpuscular hemoglobin)
- MCV (mean corpuscular volume of erythrocytes)
- MCHC (mean corpuscular hemoglobin concentration)
- RDW (indicates the deviation of the erythrocytes from the normal size)

In addition, the iron status should be ascertained, as anemias are often caused by iron deficiency, which may also influence the results of the hemoglobin differentiation. Iron deficiency is the most important differential diagnosis of the clinically asymptomatic thalassemic trait (not of asymptomatic thalassemia). The most important parameters for the detection of an iron deficiency are microcytosis, serum ferritin and RDW. The elevated soluble transferrin receptor in iron deficiency allows to demarcate it from anemia due to chronic disease. The subsequent determination of the hemoglobin pattern is usually made either by acid or alkaline electrophoresis or chromatographically using HPLC. HPLC uses cation- and anion-exchange systems to separate normal and abnormal hemoglobins, allowing for a quantitative analysis of all separable Hb fractions. It yields exact and reproducible HbA_2 values. They play a key role especially in thalassemia diagnostics [6–9]. The material used is EDTA blood as well, which still provides conclusive results even several days after the sample was taken.

Usually β-thalassemia and most abnormal hemoglobins can already be detected based on the hemoglobin differentiation and the blood count. A molecular genetic analysis serves here primarily to verify the hematological findings and determine the type of thalassemia (β^0 or β^+), as well as to clarify ambiguous results. Since the presence of α-thalassemia is not necessarily detectable in hematologic tests, molecular genetic analysis of the α-globin-gene-complex and the α-globin-genes should be performed.

Abnormal hemoglobins

Abnormal hemoglobins are caused by mutations in the globin genes and lead to amino acid substitution in the corresponding globin chain. This results in changes in the secondary and tertiary structures of the Hb tetramer. The majority of known mutations affect the β-globin chain. Some of the abnormal hemoglobins described lead to moderate to severe clinical symptoms; most of them, however, are of little clinical significance. The various forms are classified according to their pathophysiological properties as follows:

- Hemoglobins with a tendency to aggregate (e.g. HbS and HbC)
- Hemoglobins with reduced synthesis of total hemoglobin (e.g. HbE and Hb Lepore)
- Hemoglobins with increased tendency for precipitation (e.g. Hb Köln)
- Hemoglobins with impaired function (e.g. anomalies with altered oxygen affinity, HbM)
- Silent hemoglobin variants

Abnormal hemoglobins are in most cases detected by hemoglobin differentiation. A molecular genetic analysis serves here here to confirm the hematological findings, but, if necessary, may also be applied in the context of prenatal diagnostics [4].

Sickle-cell disease

This term covers both homozygous sickle-cell disease (HbSS) and a series of mixed heterozygous hemoglobinopathies, such as HbS/β-thalassemia, HbSC or HbSD disease. Sickle-cell disease is based on a defect in the β-globin chain, which causes the formation of an abnormal hemoglobin called HbS. The disease originates with the mutation p.Glu7Val (historically Glu6Val) in the β-globin gene (*HBB*). The affected β-globin chain in its deoxygenated state is unstable and has an increased tendency to aggregate. Heterozygous HbS carriers (HbAS) show a normal blood count and are generally symptom-free. The percentage of HbS varies between 35% and 45%. The HbA_2 value may be increased. If the amount of HbS is below 30%, an existing iron deficiency or coexisting α-thalassemia should be considered [2, 4, 6, 10].

In the homozygous state, the mutation results in the so-called homozygous sickle-cell disease, which is characterized by chronic hemolytic anemia, acute crises due to vascular occlusion and the resulting tissue and organ damages. The characteristic sickle-shaped deformation of the red blood cells is due to the modified properties of hemoglobin S when deoxygenated. The blood count shows a markedly reduced hemoglobin level of 6–9 g/dL. The hemoglobin pattern shows >90% HbS, a variable percentage of HbF <10%, and a HbA_2 amount of <3.5%. HbA is undetectable (Table 2) [4, 6].

The HbS anomaly can also occur in combination with other abnormal hemoglobins or thalassemias, from which rarer forms of sickle-cell disease arise. For compound heterozygosities, the combination with β-thalassemia, HbC or HbD plays a particularly important role. The combination of HbS with β^0-thalassemia is characterized by severity similar to the homozygous variant (HbSS). In addition to hypochromic microcytosis, these patients show a percentage of HbS>80%, HbF<20% and HbA_2>3.5%. The hemoglobin concentration is lowered to an extent similar to that in patients with homozygous sickle-cell disease [2, 4].

The hemoglobin pattern of a patient with HbS in combination with β^+-thalassemia shows a percentage of HbS>55%, HbF>20% and HbA_2>3.5%. Unlike homozygous sickle-cell disease or the combination HbS/β^0-thalassemia, the percentage of HbA is approx. 20% in this case [4].

HbC disease

HbC disease is based on the mutation p.Glu7Lys (historically Glu6Lys) in the *HBB* gene. The affected β-globin chain in its deoxygenated state is unstable and has an increased tendency to aggregate. Heterozygous HbC carriers (HbAC) are clinically inconspicuous. Only the level of MCHC is increased. The HbC amount of the total hemoglobin is approximately 50% while the rest is made up of HbA [1]. Homozygous HbC mutation carriers (HbCC) and patients with a combination of HbC and β-thalassemia or other abnormal hemoglobins, are refered to as suffering from HbC disease. An exception here is the combination with HbS (HbSC disease). Depending on the molecular background, patients with HbC disease exhibit mild to moderate hemolytic anemia. Table 3 summarizes the most important diagnostic parameters, in cases of homozygosity, for HbC and HbC-β-thalassemia [4].

Table 2: Diagnoses and hematological findings of the main hemoglobin disorders [1, 2, 4, 6].

Diagnosis	Red blood cell count	Hemoglobin pattern[a]
HbS heterozygosity	No abnormalities	HbS: 35%–40% HbA_2 ≥3.5%
HbS homozygosity (sickle-cell anemia)	Hb: 6–9 g/dL	HbS: >90% HbA_2 <3.5% HbF ≤10%
HbC heterozygosity	Normal	HbC≈50% HbA≈50%
HbC disease	Hb: 10–12 g/dL MCV <75 fL MCHC >35 g/dL Target cells	HbC ≥ 95% HbA_2 <4% HbF≈0.5%
HbE heterozygosity	Hb: normal, slightly decreased MCV≈73 fL MCH≈25 pg	HbE=30%–45%
HbE disease	Hb: 10–14 g/dL Increased red blood cell count MCV≈62–67 fL MCH≈20 pg Target cells	HbE >95% HbA_2≈2.5% HbF<3%

[a]The reference ranges are averages and can vary depending on the measuring method used.

HbE disease

HbE disease is based on the mutation p.Glu27Lys (historically Glu26Lys) in the *HBB* gene. On the one hand, this mutation causes an amino acid substitution in the β-globin chain, and on the other hand, a cryptic splice site is activated, whereby the expression of the affected β-globin chain is reduced in the sense of β^+-thalassemia.

Heterozygous carriers of HbE generally show no clinical symptoms, but usually exhibit in their blood count

Table 3: Hematologic data and hemoglobin pattern in three HbC syndromes [4].

Laboratory parameters	HbCC	HbC-β^+-thalassemia	HbC-β^0-thalassemia
Anemia	Mild to moderate	Mild	Moderate to more severe
MCV	70–72 fL	60–70 fL	55–70 fL
Hb variants			
HbA	0%	20%–30%	0%
HbC (incl. HbA_2)	>97%	65%–80%	90%–95%

a light, variable hypochromia (MCH≈25 pg) and microcytosis (MCV approx. 73 fL). The HbE percentage of the total hemoglobin can vary greatly. Generally it is 30%–45%. If the concentration of HbE is low, the possibility of a co-existing iron deficiency or a combination with α-thalassemia should be considered [4].

In the case of homozygous disease, affected patients exhibit hypochromic, microcytic anemia, clearly recognizable by the MCH value (≈20 pg) and MCV (≈62–67 fL), as well as a hemoglobin concentration between 10 and 14 g/dL. The hemoglobin pattern consists of more than 95% HbE, with the balance being HbF and HbA_2. Table 2 summarizes the most important diagnostic parameters [2, 4, 6].

Thalassemia [11–13]

The thalassemias are a clinically and genetically heterogeneous group of diseases that are usually inherited in an autosomal recessive inheritance pattern. They are caused by a reduced globin chain synthesis, which leads to a deficit of the affected globin chain and, thus, a simultaneous surplus of unaffected globin chains. The extent of the imbalance of globin chains in the context of the genetic defect determines the severity of the disease.The hemoglobin molecule consists of 4 globin chains – a heterotetramer made up of two types of chains (HbA: α2β2, HbA_2: α2δ2, HbF: α2γ2). According to the different globin chains, there are four basic forms of thalassemia, of which α- and β-thalassemias, due to the incidence in the population and the clinical relevance, are more important than the rarer δ- and γ-thalassemias. Although most patients suffer from either α- or β-thalassemia, combinations of different forms of thalassemia and hemoglobin abnormalities are possible.

β-Thalassemia

Beta thalassemia is based on a quantitative defect of β-globin chain synthesis, which in large part is caused by point mutations in the β-globin gene (*HBB*). These mutations trigger a reduced expression of structurally normal β-globin. Increased erythropoiesis produces an imbalance in the synthesis of globin chains and thus a surplus of free α-globin chains that denature rapidly due to their instability. Depending on the molecular phenotype of a partial or complete loss of β-chain synthesis, one can distinguish between β^+-or β^0-thalassemia. Relative to the severity of the disease, β-thalassemia can be divided into the clinical forms thalassemia minor, thalassemia intermedia and thalassemia major, which are each based on a different genetic constellation [1]. A conspicuous blood count (hypochromia, microcytosis) in combination with a conspicuous hemoglobin differentiation (elevated HbA_2) represents the typical laboratory diagnosis of heterozygous β-thalassemia.

Hypochromic, microcytic anemia is diagnosed in most of these cases. A MCH<25 pg (usually between 19 and 23 pg), combined with elevated HbA_2 of 3.5% and higher (generally, between 4.0 and 6.0 percent), gives rise to the suspicion of an underlying heterozygous β-thalassemia (thalassemia minor). However, the HbA_2 fraction may also be between 6.5% and 8.0% in individual cases. The HbF fraction varies. HbF is elevated up to 3% in approx. 30% of patients. In rare cases, the HbF level may be as high as 3%–15%. Cave: the presence of iron deficiency may lead to reduced HbA_2 and thereby could mask an existing heterozygous β-thalassemia because of the missing typical increase in HbA_2 (>4%) [4].

A more pronounced microcytic, hypochromic blood count emerges in connection with β-thalassemia major. Usually, MCH is at 14–20 pg, and MCV at 50–60 fL. In addition, those patients typically suffer from anemia requiring transfusions, which is reflected in a hemoglobin concentration of less than 7 g/dL. The resulting hemoglobin pattern after chromatographic separation shows a significant increase in HbF of approximately 70%–90% and variable HbA_2 [2, 5].

A diagnosis of β-thalassemia intermedia and/or its differentiation from β-thalassemia major is difficult due to the hematologic phenotype and hemoglobin pattern that may potentially be similar [5]. The anemia in β-thalassemia intermedia (Hb of 6–10 g/dL) is generally

not as pronounced as in the case of thalassemia major, but MCH (15–23 pg) and MCV (55–70 fL) are similarly reduced. The hemoglobin differentiation shows elevated HbF (up to 100%) with a variable HbA_2 fraction [2, 4].

A pathogenic mutation can be identified by molecular genetic analysis. The result of the genetic test, however, does not allow for an assessment of the clinical progression.

Molecular genetic diagnostics of β-thalassemia

The molecular genetic diagnostics regarding the β-globin-gene complex is carried out in stages – first, a sequence analysis is performed to locate a mutation in the *HBB* gene. If no mutation is detected deletion diagnostics of the β-globin-gene complex is performed. The β-globin-gene-complex can also be tested for gross deletions first when preliminary findings hint to a deletional genotype (for example, when δβ-thalassemia is suspected). The molecular genetic testing is used to verify hematological findings and to assess the risk to offspring to develop a severe form of hemoglobinopathy.

α-Thalassemia

α-Thalassemia is based on a quantitative defect of α-globin chain synthesis. The deficit of α-globin chains leads to the formation of excess hemoglobins that play a significant role in the clinical picture of α-thalassemia. The most common molecular genetic cause of α-thalassemia are large deletions in the α-globin gene cluster, which result in the loss of one or more α-globin genes. Since the α-globin gene cluster in a normal human cell consists of four α-globin genes (one *HBA1* and one *HBA2* gene on each chromosome 16), the severity of the disease depends on the number of deleted α-globin genes. The most common deletions are $-\alpha^{3.7}$, $-\alpha^{4.2}$, $--^{SEA}$, $--^{FIL}$, $--^{MED}$, $-(\alpha)^{20.5}$ and $--^{THAI}$. In rare cases, point mutations, small deletions or insertions in α-globin-genes can also be responsible for α-thalassemia. Triplication of the α-globin-gene leads to the phenotype of thalassemia intermedia or thalassemia major only in combination with concurrent heterozygous β-thalassemia.

Symptoms can be assigned four levels of severity in clinical terms [14]:

- α-thalassemia minima (asymptomatic form): only one α-globin gene is deleted (-α/αα); this form is usually discovered only by chance, because all hematological parameters are normal.
- α-thalassemia minor: deletion of two α-globin-genes (--/αα, -α/-α), minor hematologic changes (hypochromia, microcytosis)
- HbH disease: only one functional α-globin-gene is left (--/-α), An essential feature is the varying degrees of hypochromic, microcytic anemia and the detection of hemoglobin H (HbH), a tetramer of four β-globin chains (β_4).
- Hb Bart's/hydrops-fetalis-syndrome: complete loss of α-globin-genes (--/--), generally incompatible with life. Affected children usually die in utero or shortly after birth.

The forms minima and minor in the blood count. The hemoglobin level is in the normal range. It may, however, be slightly reduced in the case of heterozygous α^0-thalassemia and homozygous α^+-thalassemia. MCV and MCH vary depending on the form. In the case of thalassemia minima, MCH is within the normal range, but usually below 27 pg. With thalassemia minor, the value is significantly below the normal range (see Tables 4 and 5). Patients with hemoglobin H disease have Hb levels of 8–10 g/dL and an MCV of less than 22 pg [1, 2, 6, 15]. In the blood count of carriers of thalassemia minima, the key parameters (hemoglobin, MCV, MCH, MCHC) are barely decreased or are within the normal range (Table 4).

MCV and MCH are significantly reduced in patients with α-thalassemia minor (Table 5) [1, 4].

The hemoglobin differentiation by means of HPLC is normal in all three patients. This largely rules out the presence of β-thalassemia, and there is a suspicion of α-thalassemia or iron deficiency anemia. For the differentiation of thalassemia and iron deficiency, one can determine the iron status as well as use the Huber-Herklotz formula (only applies to MCH<27 pg): HH=(MCH×RDW)/(10×Ec)+RDW Readings below 21 indicate α-thalassemia, while those over 23 point to iron deficiency. Molecular genetic testing is necessary to confirm a suspected diagnosis of α-thalassemia [16].

Molecular genetic diagnostics of α-thalassemia

The molecular genetic testing of the α-globin-gene complex is carried out in stages. The first step is performed to detect frequent deletions by means of Gap-PCR or MLPA. If necessary, this can be followed up with a sequence analysis to detect mutations in the α-globin-genes. Diagnostic testing plays an important role for

Table 4: Typical hematological findings and hemoglobin pattern in heterozygous α^+-thalassemia.

Laboratory parameters	Reference range (man)	Results (man)	Reference range (woman)	Results (woman)
Hemoglobin	13.7–17.5 g/dL	15	11.2–15.7 g/dL	11.7
Hematocrit	40.1–51.0%	43.4	34.1–44.9%	37.5
MCV	79.0–92.2 fL	**77 ↓**	79.4–94.8 fL	88.7
MCH	25.7–32.2 pg	26.6	25.6–32.2 pg	27.7
MCHC	32.3–36.5 g/dL	34.6	32.2–35.5 g/dL	**31.2 ↓**
Hb variants (HPLC)				
HbF	<2.0%	<0.1	<2.0%	<0.1
HbA_2	1.5%–3.5%	3.2	1.5%–3.5%	2.1

Bold values indicate values which differ from the normal value.

Table 5: Typical hematological findings and hemoglobin pattern in homozygous α^+-thalassemia (thalassemia minor).

Laboratory parameters	Reference range (woman)	Results (woman)
Hemoglobin	11.2–15.7 g/dL	12
Hematocrit	34.1–44.9%	37.4
MCV	79.4–94.8 fL	**76.0 ↓**
MCH	25.6–32.2 pg	**24.4 ↓**
MCHC	32.2–35.5 g/dL	32.1
Hb variants		
HbF	<2.0%	0.4
HbA_2	1.5%–3.5%	2.8

Bold values indicate values which differ from the normal value.

carriers wishing to have children in order to assess the risk of severe hemoglobinopathies in their offspring. Only the heterozygous alpha-zero deletion has clinical significance and may cause the hydrops fetalis syndrome if both parents are affected.

Conclusions

Hemoglobinopathies are among the most common monogenic hereditary diseases worldwide. A particularly high prevalence exists in Mediterranean countries, in parts of Asia, the Middle East and West Africa. Due to migration, also physicians in middle and northern europe are increasingly faced with hemoglobinopathies. Basic diagnostics consists of blood count and hemoglobin differentiation. In addition, where appropriate, iron deficiency should be ruled out. Molecular genetic testing is used to verify hematological findings and to assess the risk to offspring with respect to developing a severe form of hemoglobinopathy. Prior to any molecular genetic testing, existing preliminary findings and a family history should be shared with the examining laboratory to ensure targeted and cost-effective diagnostics. This is especially true for time-critical prenatal diagnostics. The introduction of hemoglobinopathy screening for at-risk populations makes sense also in Germany and is currently underway.

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