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Berndt Zur*

Hemoglobin variants – pathomechanism, symptoms and diagnosis

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Abstract: The diagnosis of hemoglobin variants that are not any of the better-known forms of thalassemia, sickle cell, HbC, HbD, or HbE anomalies is often challenging and requires detailed knowledge of the difference in symptoms and analysis. Experience in laboratory medicine plays an important role as the range of variants is extensive and lack of expertise can result in a wrong diagnosis. Hemoglobin variants with low oxygen affinity may present cyanosis and low oxygen saturation levels, whereas variants with increased oxygen affinity show polyglobulia and concomitant complications. Differential diagnosis of methemoglobin variants requires careful assessment, which can be problematic especially in pediatric medicine. Other variants, due to their instability, can cause more or less distinct hemolysis or thalassemia syndromes depicting serious disease patterns. Clear distinction is not always possible as several symptoms are often present. Many variants are autosomal dominant inherited.

Keywords: autosomal dominant hemoglobinopathy; hemoglobin anomaly; hemoglobinopathy; hemoglobin variants; instability; methemoglobin; oxygen affinity.

Introduction

The most common and originally endemic hemoglobin disorders thalassemia and sickle cell disease have long since spread to Central Europe through migration where they play an increasing role in clinical practice. Rare hemoglobin anomalies represent a diagnostic challenge for the treating and diagnosing physician, and the full range of symptoms is sometimes underestimated (Table 1). Medical laboratories perform hemoglobin chromatography or hemoglobin electrophoresis as standard diagnostic tools. In many cases, however, this is not enough. Further diagnostics should be made available to specialists in order to avoid misinterpretations. Laboratory medicine must play a key role in this context. Hemoglobinopathies are divided into thalassemia and structural abnormalities of hemoglobin. Thalassemia is characterized by an imbalance of globin synthesis. Structural abnormalities of hemoglobin cause the formation of abnormal hemoglobin, the most famous being sickle cell hemoglobin. However, structural defects can also cause thalassemic syndromes, such as hemoglobin E, which is commonly found in Southeast Asia. Therefore, the classification of hemoglobin disorders cannot simply be a static one [1]. Hemoglobins identified by a letter are mostly endemic varieties, such as Hb Lepore. They are often also described by means of a location (e.g. Hb D Punjab or Hb Q Iran). This nomenclature is, however, incomplete and, based on current knowledge about the spread of some hemoglobin variants, it no longer applies in every case. The focus of this review is aimed mainly at the numerous rare structural hemoglobin abnormalities or hemoglobin variants that affect the oxygen binding and stability of hemoglobin and most of which do not occur endemically. While the nomenclature of these variants is based on the discovery site or the origin of the patient, there are no hard and fast rules here and discoverers are free to assign names as they see fit. Therefore, a categorization by designation is impossible. In contrast to the known anomalies involving autosomal recessive inheritance, the variants are frequently characterized by autosomal dominant inheritance. As numerous variants cause several symptoms, a clear definition is not possible in every case. Unstable variants may have a high or low oxygen affinity; variants affecting oxygen affinity can cause hemolysis and methemoglobinemia. They may also appear clinically inconspicuous, while playing a role in the context of other diseases. For the purposes of this review, the variants have been classified based on their

***Correspondence: Privatdozent Dr. med. Berndt Zur,** Institut für Klinische Chemie und Klinische Pharmakologie, Universitätsklinik Bonn, Sigmund-Freud-Str. 25, 53127 Bonn, Germany,
Tel.: +49 228 287 12122, Fax: +49 228 287 12159,
E-Mail: berndt.zur@ukb.uni-bonn.de; and Institute of Clinical Chemistry and Clinical Pharmacology, University Hospital Bonn, Bonn, Germany

Table 1: Symptoms caused by hemoglobin variants.

↓O2 affinity variants	↑O2 affinity variants	Methemoglobin variants	Unstable and thalassemic variants
Cyanosis	Erythrocytosis	Cyanosis	Anemia
O2 saturation↓	Hyperviscosity	O2 saturation↓	Hemolysis
Anemia	Anemia	Anemia	Jaundice
Hemolysis	Hemolysis	Hemolysis	Cyanosis
Dyspnea	Thromboembolism	Dyspnea	O2 saturation↓
Methemoglobinemia	Fatigue	Jaundice	↑O2 affinity
Fatigue	Headache	Hemolysis by ox. substances	↓O2 affinity
	Nosebleeds	Brown blood	Dyspnea
			Methemoglobin
			Thromboembolism
			Priapism
			Splenomegaly
			Hemolysis by ox. substances
			Pigmenturia

primary effects. Given the large number of different hemoglobin anomalies, variants are presented which, in terms of their symptoms, serve as examples for other variants. Grouping them within the classification is rarely possible since an exchange of different amino acids can also have different effects at one and the same mutation site. For better understanding, some variants are presented in more detail.

Basics

Hemoglobin is a tetrameric protein, with two α and two β globin chains each, which are bound ionically, non-covalently to hydrophobic contact regions and the heme group, which consists of a porphyrin ring with a central iron atom for oxygen affinity. The heme group is located in a V-shaped pocket and surrounded in the tertiary structure by hydrophobic amino acids for stability. The conformation of the hemoglobin molecule changes with oxygenation, the R (relaxed) state, and deoxygenation, the T (tense) state. This allosteric effect is responsible for the sigmoidal oxygen-hemoglobin binding curve of hemoglobin [2]. The intraerythrocytic 2,3 bisphosphoglycerate (2,3-BPG), being an allosteric effector, serves to reduce the oxygen affinity of hemoglobin by binding to deoxyhemoglobin by means of salt bridges, thus facilitating the release of oxygen. It cannot bind to oxyhemoglobin. These capabilities are necessary to ensure that the hemoglobin can absorb oxygen in the lungs and release it into the tissue. The Bohr effect describes the oxygen saturation curve of hemoglobin as a function of the pH value and the oxygen partial pressure, and causes an increased release of carbon dioxide in the presence of a high oxygen concentration (lungs), as well as a release of oxygen and facilitated uptake of carbon dioxide in the periphery. Hemoglobin-oxygen affinity increases with decreasing temperature, declining 2,3-BPG concentrations and pH increases [3]. The hemoglobin of an adult consists of up to approx. 97% HbA (two α and two β globins each), up to approx. 3.5% HbA2 (two α and two δ-globins), and up to 1% HbF (two α and two γ-globins). Newborns have a high proportion of HbF, which rapidly decreases to approx. 1%, depending on the individual. To the same extent as the HbF percentage drops, the HbA portion increases, reaching adult levels around the age of six months. Therefore, mutations of γ- and α-globin genes can manifest clinically already in newborns, whereas β-globin gene mutations, due to the conversion of HbF to HbA, occur only in the course of the first months of life. On chromosome 11, two genes encode α globin. α-1 represents approximately 30% and α-2 around 70% of the contribution to the protein biosynthesis of α globin. On chromosome 16, one gene each encodes β globin and δ-globin, and two genes encode γ-globin [4]. Here, the oxygen dissociation curve shows a reduced oxygen affinity indicated by a shift to the left, and an increased oxygen affinity by shifting to the right [2]. The p50 value describes the partial pressure of oxygen at 50% hemoglobin saturation, and it is the most important parameter for assessing oxygen affinity [5]. The oxygen affinity of hemoglobin has a direct influence on the release of erythropoietin (EPO), which is predominantly formed in the fibroblasts of the peritubular cells of the kidney. The mechanism of EPO formation is very complex. In simple terms, this is the knowledge to date: Normoxia causes the transcription factor GATA-2 to slow down the EPO promoter. The hypoxia-inducible transcription factors (HIF) are also denatured, so that EPO cannot

be formed. With hypoxia, the inhibition is removed, and HIF, by activating the EPO enhancer, cause the formation of EPO, which eventually binds to specific receptors of the erythrocyte precursor cells "colony-forming units-erythroid (CFU-Es)". Through inhibition of apoptosis and acceleration of the differentiation and proliferation of precursor cells in the bone marrow, EPO thus causes an increase in the red blood cell count. The time period from the formation of EPO to a detectable increase of reticulocytes is approximately 3–4 days [6]. A mutation with increased oxygen affinity can lead to poorer oxygen delivery and cause erythrocytosis.

Mutations

The exact number of hemoglobin variants is currently specified in the Database of Human Hemoglobin Variants and Thalassemias (HbVar) as 1212, or 1635 when thalassemia mutations are added. Mutations in α globin are rarely the cause of severe disorders because of the twofold encoding of α globin. The amino acid exchanged through mutation has different characteristics (hydrophilic, hydrophobic, pH, etc.), which is why it can have very different effects on the protein structure. Mutations of the α1β2 bond, the β1α2 contact region of the C-terminal end, the heme pocket, as well as central and distal histidine, which is directly involved in the heme formation, are regions that can affect oxygen binding and the stability of hemoglobin. There may be deterioration in the changeover from the oxygenated R structure to the deoxygenated T structure, or in the stabilization of the T and R structures of the hemoglobin molecule [2]. Twenty-one amino acids of the globin chain come into direct contact with heme in the quaternary structure (Figure 1) [7]. Mutations of hyper-unstable variants often cause a lengthening or shortening of globin chains, which cannot be used in the hemoglobin synthesis and which become denatured already in the precursor cells [8]. Post-translational modifications that produce discrepancies between the gene sequence and protein structure are sporadically possible [9]. There is no reliable information about the prevalence of rare hemoglobin variants that are not counted among the "endemic hemoglobin variants". Available data from Germany have been obtained from test results of a specialized university contract laboratory over a period of four decades [10]. Traditional endemic areas for a geographical-ethnic predisposition, as in the case of thalassemia, sickle cell anemia, HbC and HbE anomalies, often do not exist, with the exception of hyper-unstable α variants.

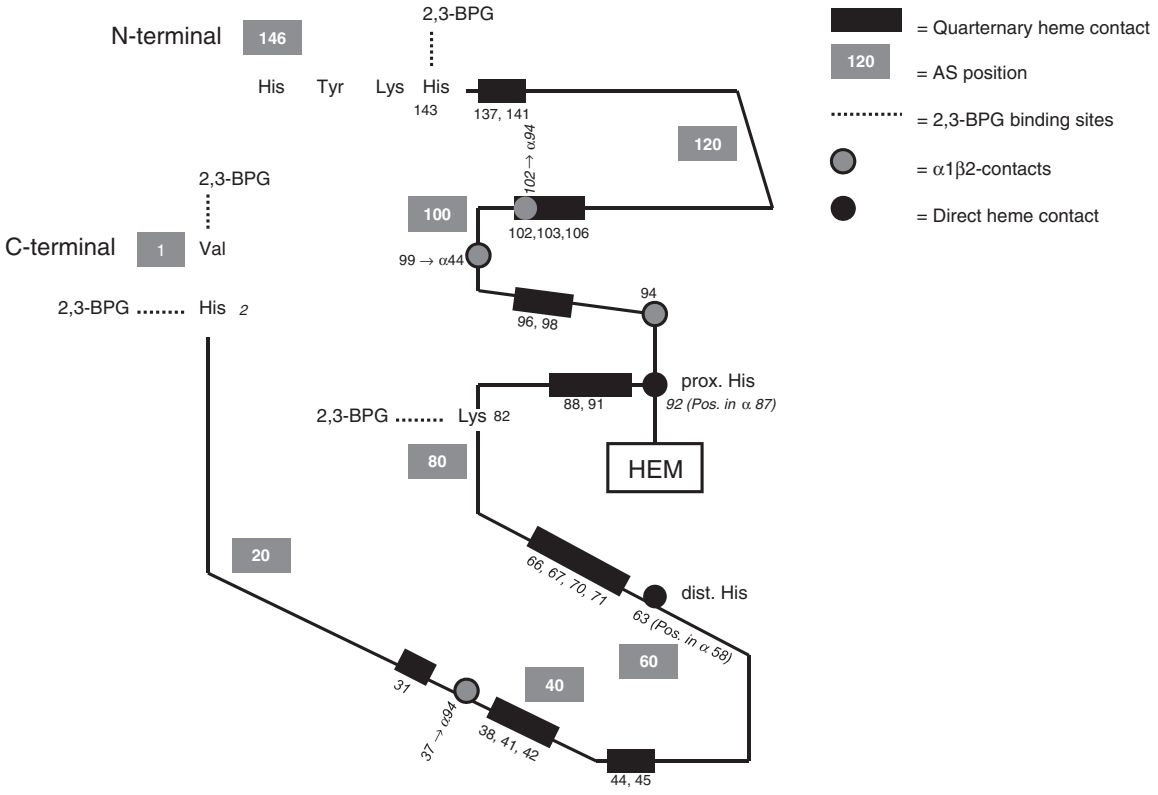

Figure 1: Mutation sites (example of beta globin) that can lead to dysfunction.

The hemoglobin variants described below represent only a selection, but due to their mutation sites and clinical expression, they are representative of other variants.

Variants affecting of oxygen affinity

Variants with increased oxygen affinity

Hemoglobin variants with increased oxygen affinity can be accompanied by erythrocytosis and are characterized by a decreased p50 value [11]. All mutations known so far that affect regions β99 and β146 lead to increased oxygen affinity of hemoglobin. Mutations of the 2,3-BPG binding site at positions β143 and β82 also lead to increased oxygen affinity, as do mutations of the binding site at the n-terminal end that do not cause erythrocytosis, because a lower concentration of 2,3-BPG is observed in this region [12]. While Hb Crete alone does not produce specific symptoms, there are reports of polyglobulia, increased hematocrit and splenomegaly in the case of a combined mutation in the δ-globin gene [13]. Hb Montfermeil may cause slight erythrocytosis as well as pronounced polycythemia with hematocrit levels of 56% and thromboembolic events [14]. In the case of Hb Chesapeake, the mutation in the α globin gene causes only mild erythrocytosis or, in combination with heterozygous α thalassemia, a pronounced polycythemia [15]. Homozygous patients with Hb Saint Nazaire usually have distinct polyglobulia requiring regular bloodletting [16]. A combined heterozygosity of β thalassemia and Hb Malmö causes extreme polycythemia with hemoglobin concentrations up to 23 g/dL and erythrocytes up to 10.5 T/L. But even without concomitant thalassemia, the defect causes polycythemia in affected patients, with symptoms including fatigue, headache and nosebleeds [17]. Hb Headington is characterized by discrete erythrocytosis, concomitant with heterozygous β thalassemia, but also by pronounced polycythemia [18]. There are few known variants in which the 2,3-BPG binding site of α or β globin is mutated, such as Hb Helsinki, Hb Providence and Hb Rahere. Affected patients usually have only mild or no erythrocytosis [19–21]. The extended β globin chain of Hb Saverne causes instability of hemoglobin and produces hemolytic anemia [22], which is even more pronounced in the case of Hb Köln [23]. Although both variants have an increased oxygen affinity, the focus here is on instability; hence they are classified as unstable hemoglobins. Differential diagnoses of polycythemia include a 2,3-BPG mutase deficiency, hemochromatosis, polycythemia vera, an activating mutation of the erythropoietin receptor and secondary erythrocytosis due to other underlying diseases [24]. Physiologically speaking, HbF and HbA2 have an increased oxygen affinity. Then, there is also the pathological β tetramer of the hemoglobin H disease (HbH), which is classified as α thalassemia [25, 26].

Variants with decreased oxygen affinity

Hemoglobin variants with reduced oxygen affinity often go hand-in-hand with mild anemia due to a decreased erythropoietin response, and are characterized by an increased p50 value and decreased oxygen saturation. Many variants also exhibit minor instability and slightly elevated methemoglobin levels. Clinical signs include cyanosis with mild anemia, which, however, can vary greatly in their characteristics [27]. The mutation of Hb Kansas favors the T-structure of hemoglobin, producing low oxygen affinity as well as discrete anemia [28]. Hb Beth Israel, a mutation at the same position, but involving a different amino acid substitution, causes cyanosis and low oxygen saturation values up to 63% [29]. Hb Louisville causes hypochromic, microcytic, hemolytic anemia, and dyspnea, often triggered by infections or oxidative substances (Table 2). However, a phenotypically different expression has been found in family studies despite identical genetics [30]. Hb Bassett is characterized by low oxygen saturation of up to 85%, without additional abnormalities [31]. The mutation of Hb Denver concerns the heme binding site; clinically, mild hemolytic anemia with cyanosis has been observed [32]. The mutation of Hb Rothschild destabilizes the globin subunits. The hemoglobin tetramer has low oxygen affinity, while the dissociated dimers have an increased one. In clinical terms, only decreased oxygen saturation values are observed [33]. Using the example of Hb Venusberg, it is possible to illustrate the problem of differential diagnostic assessment and diagnostics. Hb Venusberg was first discovered in a 14-year-old boy with unexplained intermittent low oxygen saturations with cyanosis who had undergone regular cardio-pulmonary examinations since birth. His 49-year-old mother had suffered since childhood significant performance limitations with

Table 2: Oxidative drugs.

Antibiotics:	Sulfonamides, nalidixic acid, nitrofurantoin, niridazole
Antimalarials:	Pamaquine, primaquine
Painkillers:	Phenazone
Methb therapy:	Methylene blue, toluidine blue
Anti-inflammatories:	Sulfasalazine

intermittent cyanosis of the lips, which saw her hospitalized regularly for diagnostic purposes. As both patients had inconspicuous examination results (hemoglobin electrophoresis), hemoglobin variants were ruled out as possible suspects. In the end, it took 10 years before a diagnosis was made [34]. However, oxygen affinity can be reduced also in connection with widespread hemoglobin anomalies, such as sickle cell disease. Also, patients may sometimes have decreased oxygen saturation values. However, this can vary, depending on the HbF portion, as HbF has a high oxygen affinity. While this is only a secondary aspect in the care of sickle cell patients, it possibly plays a role in the differential diagnosis [35].

Variants with pulse-oximetrically false values

Pulse oximetry is a non-invasive photometric method for the continuous measurement of arterial oxygen saturation by means of a finger clip. Interference may occur due to motion artifacts, hypoperfusion of the application site, venous pulsation, dyshemoglobins, optical and electrical interference, intravascularly applied dyes, nail polish and, in individual cases, due to anatomic-histologic circumstances [36]. For some hemoglobin variants, pulse oximetry produces false low oxygen saturation readings. The behavior of pulse oximetry in the context of hemoglobin variants has been described in the literature only rarely [37]. An example of false low oxygen saturation values in pulse oximetry is Hb Bonn, which was discovered in a 4-year-old boy and his father. The discrepant saturation values produced by blood gas analysis and pulse oximetry had to be followed up through numerous cardiopulmonary examinations. The father was incorrectly diagnosed with sleep apnea on the basis of the pulse-oximetry results. It took years before a diagnosis was made. In spectrophotometry, Hb Bonn causes an additional absorption peak at 668 nm of oxyhemoglobin, which ultimately led to false low oxygen saturation values in pulse oximetry [38].

Methemoglobin variants

Methemoglobin is also known as ferrohemoglobin or hemiglobin. It is produced by oxidation of the heme's central Fe^{2+} to $+Fe^{3+}$. MetHb is formed in vivo at any time, but it is immediately reduced to hemoglobin by the NADH-dependent enzyme methemoglobin reductase. A MetHb share of 1% is considered normal. A share of more than 15% manifests itself clinically in the form of cyanosis; over 30% causes dyspnea and confusion; more than 50%, metabolic acidosis, cardiac arrhythmias, and coma. Death occurs with a share of over 70% [39]. The correct determination of the methemoglobin (HbM methemoglobin) produced by hemoglobin variants is problematic. Commonly used pulse oximeters measure the hemoglobin spectrum at a wavelength of 660 nm (oxyhemoglobin) and 940 nm (deoxyhemoglobin). Methemoglobins not caused by hemoglobin variants have an absorption peak at 635 nm; those caused by hemoglobin M-variants, at 600 nm. This leads to false low values when analyzing HbM-methemoglobin. More recent multi-wavelength pulse oximeters can capture methemoglobins at these wavelengths [40]. Photometric measurement methods with a cyanide additive also produce false low readings for many HbM variants due to a slow and incomplete response. Differential diagnoses of methemoglobinemia include NADH-methemoglobin reductase deficiency, poisoning by detergents and nitrites, etc. The hemoglobin variants that cause MetHb formation cannot be treated by redox dyes, such as methylene blue. Depending on the variant, hemolysis may be induced. The mutations often affect proximal histidine, which immediately and covalently binds to the heme group, as well as distal histidine, which binds non-covalently to and is in direct contact with heme, through tyrosine substitution. Mutations at other positions are also possible. There is usually close contact with the heme pocket and distal histidine. The substituted amino acids can form a covalent bond to heme iron and thus bring about a stabilization of MetHb. What hemoglobin M-variants have in common is instability, which is characterized by varying degrees of hemolysis. A visible sign of methemoglobinemia is the chocolate-brown color of blood [41, 42]. The variants account for between 20 and 40 percent of total hemoglobin. The mutations of HbM Saskatoon and HbM Hyde Park relate to proximal and distal histidine in β globin. In clinical terms, both variants exhibit cyanosis and slight hemolytic anemia. HbM Iwate and HbM Boston with mutations of the proximal and distal histidine of the α globin gene are low-oxygen-affinity variants that may occur already in newborns. There is no other hematological abnormality except for cyanosis. The MetHb concentration in these variants is approximately 10%–20%, but the methods of measurement and the exact concentrations described in the literature are not conclusive [43, 44]. HbM Dothan affects distal histidine, which leads to a slight instability with cyanosis; the MetHb concentration is 20% [45]. Hb Chile is an unstable high-oxygen-affinity variant. Patients develop cyanosis and slight hemolytic anemia, which is aggravated by administration of methylene blue and sulfonamides. Methemoglobin fluctuates between 11% and

18% [46]. Mutations in the same place are high-oxygen-affinity Hb St. Louis and Hb Genova. Patients with Hb St. Louis also develop MetHb cyanosis and mild hemolytic anemia, those with Hb Genova, only chronic hemolytic anemia [47, 48]. Hb Tübingen and Hb Southampton are characterized by pronounced hemolysis. The amino acid substitutions create hydrophilic residues that, through water ingress into the heme pocket, are conducive to auto-oxidation. However, this generally involves a mild form of cyanosis. The meth-Hb concentration is 13%, which is why they are considered unstable variants [49, 50].

HbF variants

Methemoglobinemia (mutations of the γ-globin gene) caused by hemoglobin F-variants only affects newborns up to 4 months of age and are self-limiting, but they can result in unnecessary or hasty action on the physician's part. These variants are also referred to as HbFM variants. Not all mutations of the γ-globin gene cause methemoglobinemia. The pathophysiological explanations must be seen as analogous to the positions of β globin gene mutations, with the difference being that two genes on chromosome 16 encode for γ-globin and that HbF generally has a higher oxygen affinity [4]. HbFM Fort Ripley, with a pathophysiology analogous to HbM Hyde Park, and HbFM Osaka, with a pathophysiology analogous to HbM Saskatoon, manifest themselves in affected newborns through cyanosis caused by methemoglobin and hemolytic anemia. The clinical presentation differs greatly from individual to individual [51, 52]. In the case of HbFM Circleville, by contrast, which has the same mutation site as HbFM Osaka, but different amino acid substitution, no hemolysis is observed [53]. As for HbF Viseu, MetHb concentrations fluctuate between 16% and 21%; newborns are diagnosed with cyanosis, but no other abnormalities [54]. HbF Cincinnati and HbF Sarajevo cause neonatal cyanosis, but not methemoglobinemia. Newborns with HbF Sarajevo exhibit pronounced cyanosis. The analogous mutation site of the β globin gene causes Hb Kansas, a variant with low oxygen affinity. Here, too, many variants cannot be detected by means of the standard diagnostics of HPLC or electrophoresis [55, 56].

Unstable variants

These variants are characterized by the instability of the hemoglobin tetramer. Some mutations cause the denaturation of the hemoglobin tetramers already in the bone marrow and, as protein in the periphery, can often not be detected. The mutations, mostly de novo mutations, frequently affect the hydrophobic, water-repellent, groups of hemoglobin. A hydrophilic amino acid substitution produces a leak, and water can destabilize the molecule. An amino acid replacement by proline causes instability in the secondary structure by influencing the helical structure and the interhelical bonds. The mutations may also cause a dissociation of heme and globin. Heme with central Fe^{3+} (MetHb) is dissolved out of the heme pocket and connects in further steps with denatured globin residues. This creates irreversible hemichromes that connect to the cytosolic portion of the band-3 protein of the erythrocyte membrane. This connection causes a decreased deformability of the red blood cells and leads to the removal of affected erythrocytes in the spleen. The precipitates are referred to as Heinz bodies and trigger an attachment of IgG antibodies, which additionally triggers the elimination of erythrocytes. Due to the presence of free heme and the separation of iron, a Fenton reaction–a catalyzed oxidation of iron salts with hydrogen peroxide-occurs. This leads to the denaturation of hemoglobin, formation of methemoglobin and membrane damage. Therefore, Heinz bodies are often detected only in a blood smear after splenectomy. Erythrocytes with Heinz bodies also have a high affinity to attach to the vascular endothelium. In some variants, oxidatively acting substances, drugs (Table 2), and infections trigger hemolytic crises. A classic sign is pigmenturia (brown urine), which is caused by dipyrromethene produced in the degradation of heme. Unstable variants with increased oxygen affinity exhibit less pronounced anemia as a result of the induced release of erythropoietin. Diagnostically speaking, many variations are undetectable with the conventional methods of HPLC or electrophoresis. Hyper-unstable variants show no pathological erythrocytes in the peripheral blood due to early demise in the precursor cells [8, 57–60].

The phenotypic expression of Hb Köln is very different from one individual to another. The disease occurs due to the change of HbF to HbA in children during the second to the third month of life, at the earliest, manifesting as jaundice and hemolytic anemia [23]. Pulse-oximetrically measured oxygen saturation values are false low [38]. A splenectomy in childhood due to severe hemolytic anemia and splenomegaly is rarely indicated, but there are also reports of relatively mild cases with occasional hemolytic crises, especially after infections. After splenectomy, patients exhibit a tendency towards thromboembolic events due to the increase of erythrocyte inclusions [61, 62]. The mutation of Hb Mainz affects the same locus and is

similar to Hb Köln in terms of the clinical expression. One patient was also reported to have exhibited pulmonary hypertension, secondary to chronic hemolysis [63]. Hb Olmsted causes severe hemolytic anemia, splenomegaly and gallstones in childhood. Thromboembolic events and priapism have been described in splenectomized patients [62]. A special case is the high-oxygen-affinity Hb Zürich. The substitution of proximal histidine for arginine causes a wide opening of the heme pocket. Heme is oxidized to methemoglobin, which, in contrast to the HbM variants, destabilizes the hemoglobin molecule. Through the wide opening, reactive components, such as sulfonamides or methylene blue, can bind directly to heme and contribute to the formation of methemoglobin, resulting in severe hemolysis. The carbon monoxide binding capacity (CO) is significantly enhanced by the steric conditions in the molecule and stabilizes the hemoglobin molecule by preventing the formation of methemoglobin. Therefore, smokers with high COHb values exhibit significantly less pronounced hemolytic anemia. The clinical expression of Hb Zürich varies; severe hemolytic anemia, which improved after a splenectomy, has been described [64, 65]. The slightly high-oxygen-affinity Hb Hasharon shows no hematological abnormalities in many sufferers. What occurs is a slightly reduced α globin production and imbalance of the globin chains, without causing thalassemia. However, cases of hemolytic anemia with splenomegaly have been reported. Oxidative substances (Table 2) and infections can trigger hemolysis. This variant is frequently encountered in Ashkenazi Jews, but it also occurs in other populations. Newborns with Hb Hasharon may exhibit Hb H (β tetramers) and Hb Bart's (γ tetramers), which can lead to an incorrect diagnosis [66–68]. Hb Leiden and Hb Seattle Evans are variants in which oxidative substances or infections can also trigger hemolysis [69, 70]. An unstable γ globin variant that manifests clinically only in the first few months of life, Hb Poole, is detected on the basis of jaundice and a slightly pronounced hemolytic anemia [71].

Hyper-unstable and dominant thalassemic variants

Hyper-unstable variants are characterized by proteolysis of the globin chains in the precursor cells of erythrocyte development. The mutations frequently cause shortened or lengthened globin chains that prevent a stable hemoglobin synthesis. An excess of normal globin chains can produce a globin synthesis imbalance and manifest as thalassemia [58, 60].

Hyper-unstable α globin gene mutations

In the best-case scenario, hyper-unstable α globin chains are, due to the duplicity of the genes, clinically detectable via hematologic changes. In connection with heterozygous α thalassemia, a more or less severe HbH disease may dominate. Some hyper-unstable α variants frequently occur in areas where α thalassemia is endemic. Hb Constant Spring lengthens the α globin. A mRNA instability results in a reduced translation of the globin gene. The surplus of the hydrophobic α chain results in precipitation in the erythrocytes, and β globin precipitates cause additional cell damage [72, 73]. This variant and the variants Hb Icaria and Hb Koya Dora produce an α thalassemic clinical picture [74, 75]. Hb Taybe is particularly widespread among Arabs in Israel. The phenotype varies greatly in combination with α thalassemia [76]. With combined heterozygosity, Hb Heraklion produces an atypical, mild HbH disease [77]. Hb Agrinio is extremely unstable and can only be detected by means of DNA sequencing. The mutation is relatively common in Greece. In conjunction with α thalassemia, it leads to severe forms of HbH disease, as does Hb Quong Sze, which occurs mainly in China [78, 79]. Hb Petah Tikva, by contrast, leads, when combined, to a mild form of hemoglobin H disease, while Hb Questembert is responsible only for mild chronic hemolytic anemia, which amplifies under oxidative stress [80, 81].

Hyper-unstable β globin gene mutations

Mutations producing hyper-unstable β globin chains are classified as dominant β thalassemia. Here, missense, frameshift, and nonsense mutations frequently cause the α globin to be shortened or lengthened. Mutations affecting Exon 3 generally have a greater impact, as the modified mRNA is not degraded in the nucleus and triggers a protein biosynthesis that creates globin fragments that undergo proteolytic degradation [58]. In contrast to hyper-unstable α globins, this phenotype is very similar interindividually. The phenotype of Hb Indianapolis in a heterozygous setup has been described in the literature in contradictory terms. On the one hand, there is a report of one family with Thalassemia major who required regular transfusions and suffered organ damage due to an iron overload. On the other hand, patients do not exhibit any hematological changes [82, 83]. Hb Stara Zagora is a variant with a shortened globin chain, triggering an immediate proteolysis by preventing dimer formation. Hemolytic anemia, with the need for transfusions, clinically

appears as the result of thalassemia intermedia/major [84]. In the case of Hb Jambol, the globin chain extension leads to the clinical picture of thalassemia intermedia/major from the second month of life [85]. Hb Genova is the counterpart to Hb Agrinio (see above), causing a pronounced thalassemic syndrome in combination with heterozygous β thalassemia, an extremely severe form of thalassemia major [48]. Hb Florida lengthens globin. Affected patients need occasional blood transfusions and develop hepatosplenomegaly, the clinical picture of β thalassemia intermedia [86]. Hb Grand Junction, however, causes only a mild thalassemic phenotype [87]. A consistent classification of variants does not make sense in many cases. Hemolysis and the clinical picture of thalassemia can be equally important.

HbA_{1c} analysis

Many variants are described in the literature as clinically inconspicuous. With the same mutation, however, a variant may generally develop a very different phenotype. Most certainly, some variants impair the HbA_{1c} diagnosis on account of their instability (shortened erythrocyte survival), or the quantification process by interfering with the analysis method [88]. Hb Venusberg and Hb Bonn (see above) also fall into this category. For example, Hb UKB is not detectable by chromatography, but by electrophoresis where it is observed with a fraction of 50.9%. The HbA_{1c} value is at 5.2% with HPLC. Yet, immunological measurement yielded an HbA_{1c} value of 6.5%. The 73-year-old patient in whom this variant was first described had suffered for some years from diabetes type II, including diabetic nephropathy requiring dialysis. Possibly, false low HbA_{1c} values negatively impacted on the treatment of diabetes mellitus in this case [89]. The same mutation site of Hb Hafnia also causes false low HbA_{1c} readings, like some other so-called "silent variants" [90, 91].

Diagnosis of variants

The indication for diagnosis of hemoglobin variants may be varied and requires knowledge of the broad spectrum of clinical manifestations. These can include methemoglobinemia, cyanosis, hemolysis, polyglobulia, etc. (Figure 2). Hence, for the diagnosis of hemoglobin variants, the laboratory must be well informed and communication with the treating physician must take place. Laboratory specialist should also be able to give advice on the clinical symptoms, consequences and treatments of variants. The investigator's experience with specific test

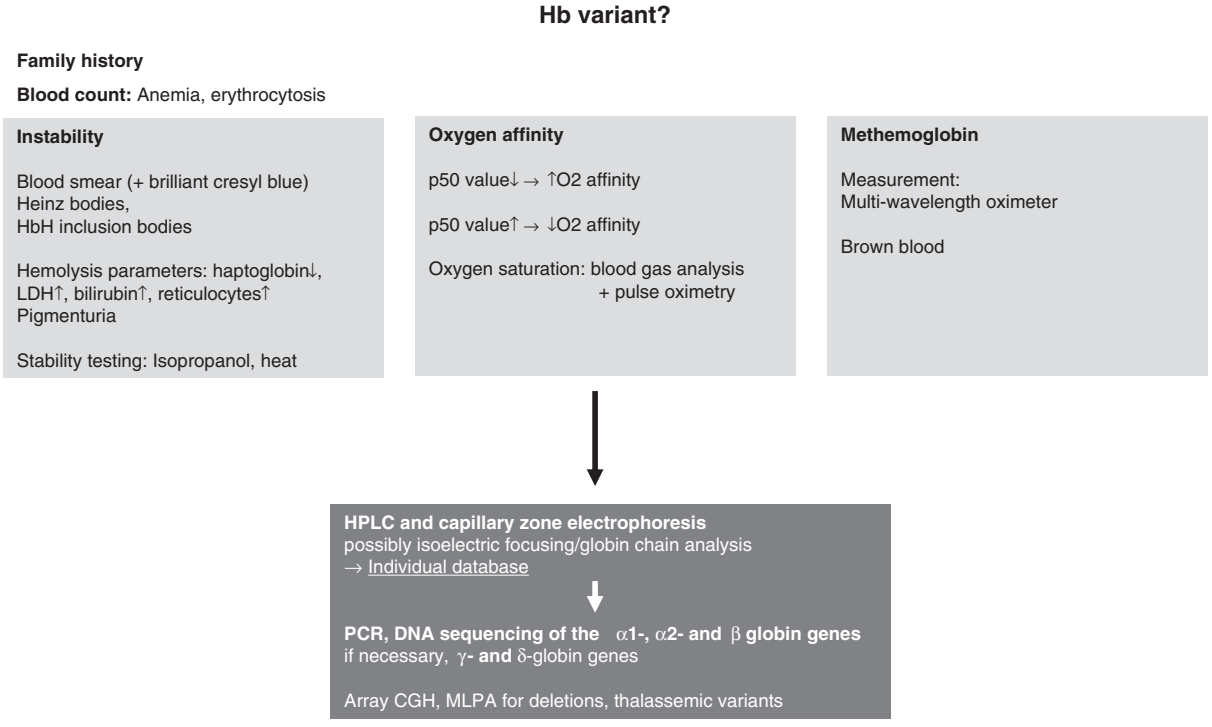

Figure 2: Staged diagnosis of hemoglobin variants.

procedures plays a major role here. Separate databases of hemoglobin variants are frequently set up relative to the methods employed, filled with information gleaned from many years of routine practice [92]. Some manufacturers of analysis systems also provide data, but this should be used for guidance only and not for diagnosis. There is a risk of misdiagnosis here, as different variants may manifest with the same running time and in the same zone with each Hb separation method. Even inconspicuous Hb chromatography or electrophoresis findings cannot be used to rule out an Hb variant if clinical symptoms (see above) cannot be explained by other diseases. Where rare variants are suspected, two different Hb separation methods should be used to confirm the diagnosis, such as chromatographic and electrophoretic methods, which are the most useful in this context. Some variants are detected only with one method, or different variant portions may be observed. Ultimately, though, most cases require a molecular-genetic analysis by way of globin gene sequencing in order to arrive at a reliable diagnosis. At any rate, the test methods used in the diagnostic process must be listed precisely. Some patients with hemoglobin variants have a long history of costly and burdensome tests before a diagnosis is finally reached.

The complete diagnostic process includes complete blood count with red cell indices and reticulocyte counts for the classification of anemia, serum ferritin as a marker for the iron stored, and serum haptoglobin as a hemolysis marker. Most variants have a certain instability. They are often borderline normocytic and normochromic at elevated reticulocyte counts and decreased haptoglobin levels. In variants with increased oxygen affinity, increased ferritin levels in combination with the hemolysis parameters may indicate an iron overload. The decision whether to pursue further diagnostic testing should be left to the specialist. Important information can be obtained from hemoglobin chromatography and/or hemoglobin electrophoresis/hemoglobin capillary zone electrophoresis, but in some cases the hemoglobin variants can be detected only in one method, or not at all. Isoelectric focusing, due to the separation of the hemoglobin fractions based on a pH gradient, allows for additional identification options when HPLC or electrophoresis does not yield any conspicuous findings. A globin chain analysis using HPLC or mass spectrometry can provide necessary information in some cases, particularly in the case of a globin chain imbalance. The diagnosis is generally difficult when dealing with many unstable and hyperunstable variants. The hemoglobin stability test is at best a guide, but not a reliable analysis, because the variants can behave differently in vitro and in vivo. Variants that are denatured in the bone marrow by proteolysis can often only be identified through genetic analysis [92–94]. Blood smears with Pappenheim and brilliant cresyl blue staining should be available for a microscopic evaluation of internal bodies and inclusion bodies. The assessment should be done by an expert. Oxygen affinity measurements by blood gas analysis are necessary to classify the hemoglobin variant. The most meaningful is the p50 value, as it reveals a variant with increased oxygen affinity when there is a reduction, and a variant with low oxygen affinity when there is an increase [95]. Of note, the p50 value may also change with elevated or reduced carbon dioxide partial pressure due to underlying respiratory, pulmonary and cardiac diseases. Pulse oximetry may contribute to the diagnosis due to decreased oxygen saturations. Discrepant blood gas analysis and pulse oximetry saturation values are a clear indication of an Hb variant, as is the case for Hb Bonn (see above). Here, a spectrophotometric hemoglobin analysis may be useful too. If methemoglobin variants are suspected, conventional measurement methods of methemoglobin can fail, and measurements should not be taken with conventional 2-wavelength oximeters, but with the more appropriate multi-wavelength pulse oximeters. Even when a variant has been detected unambiguously, one should perform further differential diagnostic tests, since it is not only the variant alone that can cause the respective symptoms.

Therapy

There is no therapeutic follow-up for many variants. However, in some cases, unnecessary medical examinations are performed, which are a burden on those affected and can incur considerable costs. With some variants, the administration of oxidizing substances or infections may trigger hemolytic crises (Table 2). This concerns, for example, the methemoglobin variants where the administration of methylene blue may cause hemolysis. While methemoglobin cannot be reduced here, this also serves the differential diagnosis, as methemoglobin can be reduced in connection with a methemoglobinemia that has other causes [96]. Low-oxygen-affinity variants can lead to overreactions due to low oxygen saturation values. HbA_{1c} analyses may provide false results, which can lead to false therapeutic consequences in the treatment of diabetes mellitus [91]. Many hemoglobin variants do not require any specific therapy. For variants with low oxygen affinity, therapy usually is not possible. Whether occasional oxygen therapy improves the symptoms is unclear. Variants with increased oxygen affinity and polycythemia

require some regular bloodletting in order to avoid hyperviscosity syndrome [97]. The unstable variants may require occasional or regular blood transfusions, depending on severity. Splenectomy is frequently indicated, but increases the risk of infections and thromboembolism caused by erythrocyte inclusions. Hydroxyurea therapies have occasionally been performed [98]. Regular blood transfusions with simultaneous chelation therapy may be necessary for thalassemic variants [57]. Regular administration of folic acid may be useful in connection with variants involving an increase in cell turnover.

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