Non-immune hemolytic anemia (NIHA) is characterized by positive routine hemolytic tests but negative anti-human immunoglobulin (Coombs) test. Hereditary non-immune hemolysis includes disorders of erythrocytic enzymes, membrane, hemoglobin (qualitative and quantitative disorders), as well as the rare hereditary forms of thrombotic microangiopathies. Acquired NIHA includes paroxysmal nocturnal hemolysis (PNH), infections, drug and metal intoxications with as a target red blood cells or endothelium of capillaries, the rare acquired forms of thalassemia or erythrocytic membrane disorders, and hemolysis secondary to a dysfunctioning artificial (prosthetic) cardiac valve. Identification of the specific cause of NIHA is sometimes difficult and requires not only a good knowledge of this entity but mainly a qualified specialized hematologic laboratory. An algorithm to be used in every new patient consulting for NIHA is proposed in the last part of this article.

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H emolysis comes from the Greek words haema = blood and lysis = disruption, break. In reality at the time of the introduction of this term, haema was identified by the red blood cells, because of its color. Considering the etymologic point of view we should have said erythrolysis and not hemolysis since by using the term “erythrolysis” we indicate destruction of red blood cells with release of hemoglobin (Hb) while white blood cells and platelets are intact. However, the term of hemolysis is adopted for historical reasons. Hemolysis is distinguished as immune and non-immune.

Immune hemolysis will be treated in the next chapter of this issue of Seminars in Hematology by Barcellini. Here we will deal with the non-immune hemolytic anemia (NIHA). This condition should be considered every time we have anemia associated with high reticulocyte count, slight or marked increase of lactate dehydrogenase (LDH), increase of indirect bilirubin, increase of free Hb in plasma with almost no detection of haptoglobin and negative anti-human immunoglobulin (Coombs) test. Finally in hemolysis we almost always find urobilinogen in urines.

Depending on the degree of increase in LDH, we can separate NIHA into intravascular (very high LDH) and extravascular (slight increase of LDH) forms. It is thus evident that the nonspecific diagnostic tools leading to diagnosis of NIHA will never allow to specify the cause of this type of hemolysis, which includes at least 13 different entities.

CLASSIFICATION OF NON-IMMUNE HEMOLYSIS: ACQUIRED AND HEREDITARY

NIHA can be classified using different bases. We preferred a first division into acquired and hereditary forms. Each form may primarily affect red blood cells (enzymopathies, membrane disorders, and pathologies of Hb) or may be the result of a toxic effect or insult to normal erythrocytes, or a pathology of the environment (drug and metal intoxication, micro- or macro-angiopathy, infections, intravascular coagulopathy, etc). Table 1 depicts this simplified classification, which has the great advantage of being easy to remember.

HEREDITARY NIHA

NIHA Associated With Hereditary Enzymopathies

Because red blood cells have no nucleus and other organelles, they use the Embden-Meyerhof anaerobic glycolysis and its two shunts (Rapport-Luebering and the pentose phosphate) to: (1) assure proper function of K+/Na+ pumps; (2) reduce oxidized Hb—both functions 1 and 2 are involved in maintaining the integrity of RBC membrane; (3) to regulate the affinity of oxygen to Hb; and (4) to provide protection against oxidative stress.1 Figure 1 is a schematic overview of the Embden-Meyerhof pathway as well as the two mentioned shunts.
Mutations in genes coding for red blood cell enzymes implicated in anaerobic glycolysis and in its two shunts lead to NIHA, some of which are associated with extra-erythrocytic symptoms like neurological dysfunction, mental retardation, myopathy, and susceptibility to infections (Table 2). Hemolytic anemia secondary to inherited enzymopathies has no morphologic findings upon microscopic examination of peripheral blood and this together with the negative anti-human immunoglobulin test is the main argument to suspect this entity. In most cases, the anemia is chronic, but the intensity varies enormously depending on the affected enzyme, the nature of the mutation, and the presence or not of poorly defined modifier genes. G-6-PD and glutathionreductase deficiency may be induced by oxidant drugs, infections, or ingestion of some foods and it may result in a severe acute hemolysis.

All enzymopathies but two (G-6-PD and phosphoglycerate kinase deficiencies), have an autosomal recessive transmission. The most prevalent enzyme disorders are G-6-PD, pyruvate kinase (PK), and glucose-6-phosphatase isomerase (G-6-PI) deficiencies. Hexokinase (HK), phosphofructokinase (PFK), aldolase, triose phosphate isomerase (TPI), and phosphoglucokinase (PGK) deficiencies are very rare disorders described in a few families. PK deficiency leads generally to a rather severe chronic hemolysis that usually worsens during infections because

![Diagram](https://example.com/diagram.png)

**Figure 1.** Schematic overview of the Embden-Meyerhof pathway, as well as the Rapoport – Luebering and the pentose phosphate shunts. (Modified from Koralkova et al.)
of lack of one source of adenosine triphosphate (ATP). As there is increased 2,3 DPG levels, affinity of Hb for oxygen is decreased and this explains the relatively good tolerance of the rather severe anemia. Diagnosis is made based on the reduced PK activity. Identification of the mutation on the PKLR gene localized on chromosome 1 is not necessary, at least for clinical purposes. G-6-PI is a homodimer whose structural gene is located on chromosome 19q13.1.\textsuperscript{3,4} Homozygous or double heterozygous G-6-PI–deficient patients have chronic NIHA with acute exacerbations triggered by infections. G-6-PI deficiency in some cases affects non erythroid tissues (see Table 2).

\textbf{G-6-PD deficiency} as well as deficiencies of \textit{enzymes of the glutathione metabolism} (glutathione synthetase, glutathione reductase) have the particularity to cause hemolysis under conditions of increased oxidative stress induced by drugs and infections or foods. G-6-PD is the first enzyme of the hexose monophosphate shunt (see Figure 1) and the most frequent cause of NIHA secondary to an enzymopathy. It is estimated that at least 400 million people carry a mutation in the \textit{G6PD} gene leading to a form of hemolytic anemia.\textsuperscript{5} G-6-PD deficiency has a particular geographic distribution similar to distribution of malaria.

There are at least 140 G-6-PD variants that have been described with different severity of hemolytic disease depending on the level of residual enzymatic activity but also on the coexistence of genetic modifiers (membrane defects, thalassemia, G-6-PI deficiency, PK deficiency). Most variants lead to episodes of acute hemolysis during an oxidative stress without chronic hemolysis. Class I G-6-PD deficiency, frequent in the Mediterranean region and in Middle East including Israel, is associated with chronic hemolysis. Upon an oxidative stress a severe acute hemolytic exacerbation that may be complicated by severe hemoglobinuria and acute renal insufficiency may occur.\textsuperscript{5,6} This acute episode may be triggered by drugs (\textit{www.g6pd.org}) and fava beans. The latter is known as favism. Infections may also be the cause of severe intravascular hemolytic crisis. Generally these patients have a history of neonatal jaundice. G-6-PD deficiency should be considered in patients from Africa, the Mediterranean region, and Asia presenting with a history of an acute hemolytic episode during ingestion of fava beans, or after taking a known oxidative drug, or triggered by infection. This deficiency should be considered in every newborn baby with severe neonatal jaundice from a mother originating from the malaria zone.

Diagnosis of G-6-PD deficiency is based by measuring its activity by spectrophotometric analysis of the rate of NADPH production from NADP. Molecular analysis of the gene located at the telomeric region of the long arm of chromosome X is necessary to diagnose females in a heterozygous state or when a new variant is suspected. Peripheral blood smear shows denaturated Hb (Heinz bodies) upon methyl violet staining and in severe hemolytic crisis hemighosts and ghosts (red blood cells with a partially or complete loss of Hb) and the well-described bite cells indicating an extracellular component (in the spleen) of hemolysis, leading to clearance of erythrocytes with Heinz bodies by macrophages are found. When measuring an enzymatic activity, in particular HK, PK, or G-6-PD, we must not forget that it is cell age-related, which means that the youngest cells have the highest enzyme activity. So in the case of a high reticulocyte count, a normal or even slightly increased in vitro activity may correspond to a deficient enzyme. We recommend in such cases (high reticulocyte count) to compare the activity of the tested enzyme with the activity of another enzyme. For example, the PK activity is normal and, at the same time, HK activity is increased; this indicates a PK deficiency.\textsuperscript{6}

Three enzymatic deficiencies of the Embden-Meyerhof pathway either do not create hemolysis (LDH) or a causal relationship to hemolysis has not been established in the few described cases (glyceraldehyde-3-phosphate dehydrogenase, enolase and monophospho-glyceratemutase).

Deficiency of the enzyme of the Rapoport-Luebering shunt (bisphosphoglyceratemutase [BPGM]), does not lead to hemolysis but to erythrocytosis because of increased oxygen affinity of Hb secondary to low levels of 2,3-DPG.\textsuperscript{3,7}

### Table 2. Enzymopathies Associated With Extraerythrocytic Symptoms

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Role in RBCs</th>
<th>Neurological Manifestations</th>
<th>Myopathy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aldolase</td>
<td>E-M pathway</td>
<td>±</td>
<td>±</td>
</tr>
<tr>
<td>Phosphofructo-kinase</td>
<td>E-M pathway</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Phosphoglycerate Kinase</td>
<td>E-M pathway</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Glutathione synthetase, glutathione reductase</td>
<td>Glutathione</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Triose phosphate isomerase</td>
<td>E-M pathway</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Glucosephosphate isomerase</td>
<td>E-M pathway</td>
<td>±</td>
<td>-</td>
</tr>
</tbody>
</table>

All of above-mentioned enzymopathies present chronic non-immune hemolysis. Abbreviation: E-M, Emben-Meyerhof.

### NIHA Associated With Hereditary Defects of the Erythroid Membrane

The red blood cell membrane is organized as a strong spectrin cytoskeleton linked to the lipid bilayer through...
protein complexes involving band 3, ankyrin, protein 4.1, and protein 4.2. This membrane is responsible for the cell ability to undergo extensive reversible deformations during its 120-day lifespan in circulation and for regulation of ion exchange and cell volume homeostasis. Hereditary defects of the erythrocyt membrane are caused by numerous private mutations in genes coding for membrane proteins (Table 3). They include three major entities: hereditary spherocytosis and hereditary elliptocytosis, which affect red blood cell deformability, and hereditary stomatocytosis, which alter membrane ion permeability.

These diseases are reported worldwide. Their clinical severity is highly variable, ranging from compensated hemolysis to severe neonatal anemia. Often diagnosed during childhood, they can be recognized at any time of life. Circumstances of diagnosis vary from neonatal jaundice, anemia, splenomegaly, chronic icterus, and biliary lithiasis, to undiagnosed hyperferritinemia in adulthood. Family history of hemolytic anemia or splenectomy is a useful information since transmission is often but not always dominant. In most cases, the disease presents as a chronic hemolysis, rarely revealed by an acute anemia episode secondary to parvovirus B19 infection. Red blood cell indices show nornocytic regenerative anemia and red blood cell morphology provides important clues. A specialized test is required to confirm the diagnosis. Although osmotic fragility assay is still in use, its sensitivity and specificity are poor, and some laboratories prefer to use modified, more sensitive methods such as the Pink test or the acidified glycerol lysis test. The 5′-esine-maleimide (EMA) binding test, based on flow cytometry, measures the binding of 5′esine-maleimide to band 3 mainly. Developed since year 2000, it is now widely available and has proved high sensitivity and specificity for the diagnosis of hereditary spherocytosis (Figure 2 shows a typical example).

Osmolar gradient ektacytometry and gel electrophoresis of red blood cell membrane proteins are “gold standard” tests, performed in a few reference laboratories that are highly resolutive to identify each disorder and to distinguish between hereditary spherocytosis and congenital dyserythropoiesis type II. Genetic diagnosis is currently not performed in routine practice, it should be considered case by case in specific situations. Hereditary spherocytosis, also known as Minkowski-Chauffard disease, is the most common membrane disorder, present worldwide and most frequent in populations originating from Northern Europe, where its prevalence is estimated to about 1/2,000 births. It is caused by mutations in the ANK, SLC4A1 coding for band 3, SPTA1, SPTB1, or EPB42 genes, which affect coupling of the red blood cell membrane cytoskeleton and the lipid bilayer, causing vesiculation, decreased surface/volume ratio, dehydration, reflected by increased mean corpuscular hemoglobin concentration (MCHS: > 360 g/L), reduced deformability and increased red blood cell splenic destruction. Typical hereditary spherocytosis presents with moderate hemolytic anemia, jaundice, splenomegaly, and gallstones. Early neonatal jaundice is frequent in affected subjects. Hb level, most often normal at birth decreases sharply during the first month of life and may lead to transfusion. Variable clinical severity is observed, sometimes into the same family. About 20% of cases present as a compensated hemolysis; in these cases, the Hb level is normal and the reticulocyte count increased. Less than 10% of cases are severe, requiring frequent transfusions. In these cases, early splenectomy can be discussed. Blood film typically shows spherocytes, but it is important to remember that spherocytes are also observed in a number of other diseases, including immune hemolysis (Figure 3A and B). Diagnosis is simple when a family history is present; however, in 20%–30% of cases, it is lacking because of a recessive transmission pattern or de novo mutations. In these cases, it is important to use a specialized test to discriminate between hereditary spherocytosis, congenital dyserythropoiesis type II, and hereditary stomatocytosis since follow-up and therapeutic issues are different, specifically considering response to splenectomy and the risk of iron overload (Table 3).

Hereditary elliptocytosis is most prevalent in African malaria endemic regions and typically shows 20%–100% red blood cells with an oblong or elliptic shape. It is caused by mutations in the SPTA, SPTB, or EPB4.1 genes impairing spectrin polymerization and inducing a fragility of the red blood cell cytoskeleton. Transmission is autosomal-dominant. Although striking on a blood film (Figure 3C), this condition is asymptomatic in most affected subjects, who present no anemia, no hemolysis, no splenomegaly, and normal red blood cell indices after the firsts months of life. Elliptocytosis is often expressive in the neonatal period, presenting as a marked neonatal jaundice and anemia. Blood film is typical showing elliptocytes and a high level of red blood cell fragmentation, sometimes causing a false microcytosis (Figure 3D). Symptoms usually regress in several weeks or months to the typical pauci or asymptomatic condition. In rare cases of homozygosity or compound heterozygosity, a severe hemolytic anemia persists. These forms, also known as “poikilocytosis” or “pyropoikilocytosis,” respond well to splenectomy. A distinct type of elliptocytosis named ovalocytosis is described in populations originating from Southeast Asia; it is caused by a unique deletion in SLC4A1 coding for band 3. Red blood cells appear as typical ovalocytes or ovalostomatocytes (Figure 3E). This condition is asymptomatic in heterozygotes and lethal in homozygotes. Hereditary stomatocytosis are rare disorders caused by ion transport defects. Several genes have been recently elucidated as a cause of these heterogeneous diseases. Transmission is autosomal-dominant. Two main clinical phenotypes are observed; in both cases, a cation leak results in altered intracellular cation content and red blood cell volume. Red blood cell indices, blood film, ektacytometry, and red blood cell membrane electrophoresis lead to the diagnosis. The rarest form is overhydrated stomatocytosis, caused by mutation in RHAG, which presents as an expressive hemolytic anemia with a large increase of
| Characteristics of Red Blood Cell Hereditary Membrane Disorders in Their Typical Presentation |
|-----------------------------------------------|-------------------------------|-------------------------------|-------------------------------|-------------------------------|
| **Hereditary Spherocytosis**                  | **Hereditary Elliptocytosis in Adults, Neonates, or Homozygotes** | **Hereditary Dehydrated Stomatocytosis (xerocytosis)** | **Southeast Asian Ovalocytosis** | **Congenital Dyserythropoiesis Type II** |
| Transmission                                  | Dominant                       | Dominant                      | Dominant                      | Recessive                     |
| Geographic distribution                       | Worldwide; frequent in Northern Europe | Africa; rare in Europe        | Frequent in Northern Europe   | Southeast Asian origin        |
| Genes                                         | ANK1, SPTA1, SPTB1, EPB41, SLC4A1 | SPTA1 (95%)                   | PIEZO1                        | SLC4A1                        |
| Hemolysis                                     | + or ++                        | -                             | +                             | -                             |
| Hemoglobin                                    | N or \_                        | N                             | N                             | N                             |
| MCV                                           | N or \_                        | \_                            | N or \_                       | N                             |
| MCHC                                           | N or \_                        | \_                            | N or \_                       | N                             |
| Reticulocyte                                  | ↑ or ↑↑                         | ↑                             | ↑ or ↑↑                       | N or \_                       |
| Red cell morphology                           | ± elliptocytes, acanthocytes, mushroom cells | 20%– Elliptocytes             | <10%                          | Ovalocytes                    |
| Ferritin                                      | N                             | N                             | ↑                             | ↑                             |
| Osmotic fragility                             | ↑ or N (30%)                   | N                             | ↑ or N (30%)                  | ↑ or N (30%)                  |
| EMA test relevance                            | ↑↑                             | +                             | +                             | +                             |
| Ektacytometry                                 | Typical profile                | Typical profile               | Typical profile               | Abnormal but atypical profile |
| SDS PAGE                                      | Increase in ankyrin, band 3, spectrin, or protein 4.1 | +                             | +                             | Decrease in protein 4.1       |
| Congenital dyserythropoiesis type II is added as a differential diagnosis for hereditary spherocytosis.
MCV (110 to 140 fL) and decreased MCHC, reflecting red blood cell overhydration. The most prevalent form, dehydrated hereditary stomatocytosis (also known as xerocytosis), is caused by mutations in PIEZO1, which codes for a recently discovered mechanically activated cation channel. Typical dehydrated hereditary stomatocytosis is characterized by loss of K⁺ and water causing cell dehydration, as evidenced by increased MCHC. It is usually associated with a normal Hb level, normal to high MCV, high reticulocytes, and mild hemolysis (the haptoglobin level is weakly decreased). A variable proportion of the red blood cells appear as stomatocytes on blood films (Figure 3F). Pseudohyperkalemia, loss of K⁺ from red blood cells on storage at room temperature with no clinical consequences, can be observed in affected subjects, as well as frequent, spontaneously resolutive perinatal edemas for unknown reasons. In addition to usual splenomegaly and cholelithiasis, the course of dehydrated hereditary stomatocytosis is frequently associated with iron overload that may lead to hepatosiderosis. Importantly, splenectomy is contraindicated here since it is associated with a very high rate of thromboembolic events. It is therefore important to confirm the diagnosis of a membrane disease before considering splenectomy.

NIHA Associated With Unstable HBs and Sickle Cell Disorders

The unstable HBs vary in their degree of instability and in their clinical manifestations, with some causing a chronic hemolytic anemia in the heterozygous state, and others presenting as hemolytic crisis precipitated by a triggering factor (see later) or when associated with β-thalassemia. An example of the later is the unstable Hb Saki, which is very mild in the heterozygous state but when inherited with a β⁰-thalassemia creates a hemolytic form of thalassemia intermedia. Although more than 1,200 variant globin chains have been described, only

**Figure 2.** The EMA binding test showing the decreased binding of the 5’ eosine maleimide to hereditary spherocytosis red blood cells as compared with control red blood cells. MFI, mean fluorescence intensity.

**Figure 3.** Red blood cell morphology in hereditary membrane disorders. (A) Hereditary spherocytosis: anisocytosis and numerous spherocytes, lacking central pallor. (B) Hereditary spherocytosis caused by band 3 deficiency: focus on mushroom-shaped red blood cells (arrows). (C) Typical hereditary elliptocytosis: smooth, regular oval shaped erythrocytes. (D) Hemolytic elliptocytosis: elliptocytes and poikilocytes caused by red blood cell fragmentation. (E) Southeast Asian ovalocytosis: typical cigar-shaped red blood cells with one or two “horizontal” slits. (F) Hereditary stomatocytosis: numerous stomatocytes (with slit-like stomata).
150 cause hemolytic anemia because of instability (the great majority) of the $\alpha\beta$ contact. The mechanism of hemolysis varies and includes methemoglobin, hemichromes, or Heinz bodies formation and membrane damage. Hemolytic crises are often precipitated by the administration of redox-active drugs or by infections. Affected patients, in addition to anemia, present jaundice, splenomegaly of various degrees, and potential complications such as cholelithiasis, leg ulcers, and pulmonary hypertension. Most often, transmission is autosomal-recessive and “de novo” mutations are frequent. Peripheral blood smear in case of NIHA secondary to an unstable Hb shows marked polychromatophilia, basophilic stippling, and moderate aniso-poikilocytosis (Figure 4A and B). Heinz bodies are seen upon brilliant cresyl blue staining (Figure 4C). Heat stability and isopropanol test confirm the diagnosis (Figure 4D). Investigation of a possible NIHA secondary to an unstable Hb includes Hb studies by isoelectric focusing (IEF) and high-performance liquid chromatography (HPLC), as well as sequencing of the $\alpha$- and $\beta$-globin genes. Often the unstable Hb gives a false increase in HbA$_2$, creating diagnostic problems with $\beta$-thalassemia. Unstable Hb affecting $\gamma$-globin genes with HbF instability as a consequence should be sought whenever a neonatal anemia with jaundice and no enzymopathy or membranopathy is present.

Recently an $\alpha$-Hb–stabilizing protein (AHSP) has been described that is synthesized at a high concentration in the erythroid precursors. AHSP protects the free $\alpha$-Hb chains in maintaining it in its soluble state. It is early to foresee a possible involvement of AHSP in the case of NIHA secondary to an unstable Hb. Preliminary results however suggest a regulatory role in the $\alpha/\beta$-globin chain in case of $\alpha$- or $\beta$-thalassemia.

Sickle cell disorders (S/S, S/$\beta$-thalassemia, S/C, S/E, S/ Hb Lepore to mention the most frequent), whenever the sickle phenomenon occurs, present as chronic NIHA worsening during crisis. Diagnosis is made by the clinical picture and peripheral blood smear (Figure 5A) and confirmed by sickling test and separation of Hbs by electrophoresis or HPLC. Pathophysiology of the disease is beyond the scope of this review.

**NIHA Associated With Thalassemia Syndromes**

There are two forms of thalassemia associated with overt non-immune hemolysis: HbH disease (three out of four genes $\alpha$ absent or nonfunctional) and $\beta$-thalassemia intermedia. Some investigators include under thalassemia intermedia both HbH disease and the classical form of $\beta$-thalassemia intermedia (see below).

*HbH disease* may result from the association of a gene with complete loss of $\alpha$-globin production ($\alpha^0$-thal) with a gene with reduced but not eliminated $\alpha$-globin gene expression ($\alpha^+$/thal). $\alpha^0$-thalassemic chromosome is the result of an interstitial deletion or an upstream deletional $\alpha^0$/thal with both $\alpha$-globin genes intact. The interstitial deletion of $\alpha$-globin genes ($\alpha^0$/thal) is mainly found in the far East (China, Thailand, Malaysian peninsula, and Philippines with carrier rates of 15%, 2.2%–9%, 4.5%, and 5%, respectively). Reduced but not eliminated

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**Figure 4.** Red blood cell morphology in unstable Hb showing anisopoikilocytosis and basophilic stippling on Wright stain (A, B), and Heinz bodies on brilliant cresyl blue stain (C). Heat stability test (D) for a control (left) and unstable Hb Mizuho CTC -> CCC (Leu -> Pro) $\beta$-chain variant codon 68 (right).
α-globin gene expression (α⁺-thal) may be of deletional (-α) or non-deletional form (α⁺α). Seventy different α variants, mainly in the HBA₂ gene, have been described, with Hb Constant Spring being the most prevalent.\(^{26}\) Clinical manifestations of HbH disease include a NIHA with jaundice and splenomegaly. Acute hemolytic episodes are frequent and generally triggered by fever and infection.\(^{27}\) It is worth mentioning that association of α₀-thal with non-deletional α⁺-thal could result in a severe phenotype requiring transfusions. This is due to the fact that α-globin variants leading to α-thalassemia are frequently unstable and this results in the precipitation both of the excess of β-globin chains (=HbH) and of the unstable α-globin variant. A classic example of this situation is the compound heterozygote of α₀-thal with Hb Suan-Dok (alpha 2, 109 Leu → Arg).\(^{28}\) Furthermore, there are cases of homozygosity for non-deletional α⁺-thal resulting in HbH disease. An example is the homozygous poly(A) mutation in the Middle East.\(^{24}\) Diagnosis of NIHA due to HbH disease is based on blood count and red blood cell indices and morphology (hypochromic anisopoikilocytosis with numerous target cells, Figure 5B), observation of inclusion bodies after exposure to supravital staining (cresyl brilliant blue for instance), separation of Hbs by HPLC and electrophoresis and finally DNA studies of the α-globin cluster using different techniques: polymerase chain reaction (PCR) amplification of the deleted α-globin gene, analysis of a larger or no common deletion by multiplex ligation-dependent probe amplification (MLPA), sequencing of HBA₂ and HBA₁ genes.

Beta-thalassemia intermedia is characterized by a moderate to severe microcytic anemia with splenomegaly and varying degrees of non-immune hemolysis. Described since 1940, it was rapidly characterized by an important clinical heterogeneity, which anticipated its complex molecular pathology.\(^{29}\) Typically these cases show microcytic/normocytic anemia with high reticulocyte count, high levels of Hbs A₂ and F, hyperbilirubinemia, and high LDH. Peripheral blood smears show marked anisocytosis, poikilocytosis, rare to numerous erythroblasts, and numerous erythrocytes with basophilic stippling (Figure 5C). Molecular studies have revealed a double pathology one being a classic β₀- or a severe β⁺-thalassemia the other a silent or mild β-thalassemic mutation or the association of a β-thalassemic chromosome with a chromosome carrying more than two α-globin genes, often triplicated, rarely quadruplicated, α-globin gene rearrangement.\(^{30,31}\) In 1988 we described that the exon III mutations of the β-globin gene lead to β-thalassemia intermedia inherited in a dominant manner, which means a single β-globin mutation.\(^{32}\) About 20 different β-globin exon III mutants have been identified so far, and interestingly not all of them are associated with β-thalassemia intermedia.\(^{29,33}\) This suggests that mRNA and protein stability play an important role in determining the phenotype of the exon III β-globin mutation. This form of β-thalassemia intermedia is characterized by the presence of numerous erythroblasts with large inclusion bodies upon staining of bone marrow smears with brilliant cresyl blue (Figure 5D). For this

**Figure 5.** Red blood cell morphology in (A) sickle cell disease (A), (B) HbH disease, and (C) thalassemia intermedia, showing numerous target cells on Wright staining, and (D) large inclusion bodies (bone marrow, brilliant cresyl blue staining) in a case of β-globin exon III mutation.
reason this type of β-thalassemia intermedia it is also called “inclusion body β-thalassemia.” For a complete description of the molecular pathology as well as the clinical manifestations of β-thalassemia intermedia, we refer the reader to the recent publication by Musallam et al.

NIHA Associated With the Hereditary Forms of Thrombotic Microangiopathies

Thrombotic microangiopathy (TMA) is defined by the presence of fibrin and/or platelet thrombi in the microcirculation. It is typically seen in kidney biopsies of patients diagnosed with haemolytic uremic syndrome (HUS) and thrombotic thrombocytopenic purpura (TTP). TMA is characterized by NIHA, thrombocytopenia, and related organ damage (kidney, brain, and pancreas). With the discovery of an inherited deficiency of the plasma protein ADAMTS13 in TTP and very recently of mutations in the specific complement control proteins (factor H, factor I, MCP [membrane cofactor protein or CD46], and thrombomodulin [THBD]) in up to 66% of patients with atypical HUS (aHUS = cases of HUS not associated with Shiga toxin-producing Escherichia coli), these two entities are now well separated clinically and pathophysiologically with different treatment approaches. The acquired forms of TMA are described later.

Differentiation of TTP from HUS is done on the basis of clinical laboratory parameters. In general, both entities have NIHA with thrombocytopenia and schizocytes in peripheral blood smears (Figure 6). TTP is characterized by neurological symptoms, absence of severe renal failure, and fever. Usually there is no prodromal phase of diarrhea. Atypical HUS is mainly seen in children; neurological symptoms are rare, while severe renal injury sometimes requiring hemodialysis is a prominent finding. Specific laboratory tools include measurement of ADAMTS13 activity and search of antibodies against ADAMTS13 (low and absence, respectively, in the congenital form of TTP). In such cases mutation analysis of ADAMTS13 gene is necessary to confirm the diagnosis of congenital TTP. When aHUS is suspected then DNA mutation analysis of CFH, CFI, MCP, CFB, and C3, as well mutations in the THBD (thrombomodulin) gene, is done. Not all patients with a mutation in these genes will develop an aHUS. It is suggested that environmental triggers like hypertension, infection, pregnancy, drugs, surgery, or hematopoietic stem cell transplantation play an important role.

ACQUIRED NIHA

NIHA Secondary to Paroxysmal Nocturnal Hemoglobinuria

Some basic notions concerning complement: The term “complement” was introduced by Paul Ehrlich in 1890. Complement is part of our innate defense. Complement’s 35 proteins are produced in the liver. Complement needs activation to participate in our innate immune response. There is a network of plasma and cellular membrane proteins that regulate complement’s activation. Deficit of certain complement’s factors or its “hyperactivation” leads to diverse pathologic situations. In general, deficits of various factors of the complement lead to autoimmune disorders or to infections. However, there are two NIHAs associated with hereditary or acquired deficits of complement: aHUS and paroxysmal nocturnal hemoglobinuria (PNH).

The defect in PNH consists in the acquired somatic mutation in the PIG-A gene that prevents all glycosylphosphatidylinositol (GPI)-anchored proteins from binding to cell surface. Decay-accelerating factor (DAF or CD55) and CD59 (also called protectine or MIRL) need a GPI anchor to bind to erythrocytic membrane. CD55 prevents formation and augments instability of the C3 convertases, attenuating the complement cascade. CD59 forms a defensive shield for red blood cells from complement-mediated lysis as it inhibits the assembly of the membrane attack complex. Without this protective complement inhibitor shield, PNH red blood cells are destroyed in circulation, explaining the hemolytic anemia with increased free Hb. This leads to hemoglobinuria and to NO consumption, the later resulting in abdominal pain and in the late complication of pulmonary hypertension. Thrombosis is another clinical sign and symptom of PNH. PNH is presenting as an intravascular NIHA; it may be associated with iron deficiency because of hemoglobinuria.

Figure 6. Numerous schizocytes observed on blood smears in a case of TMA.
Flow cytometry is the gold standard for the diagnosis of PNH (Figures 7 and Figure 8). The following case is a typical PNH and illustrates difficulty of early diagnosis: a female patient born in 1971 suffered since 1 year from episodes of abdominal pain and jaundice. An iron deficiency state has motivated complete investigations of her gastrointestinal tube to identify the source of bleeding, but without any result. Hemolysis with slight leukothrombocytopenia brought the patient to the hematologist. Laboratory tests showed: Hb 91 g/L; white blood cell count 1.9 g/L; platelets 158 g/L; free Hb 36 µmol/L; Coombs test negative; bilirubin 36 µmol/L; LDH 1,212 U/L; haptoglobin < 0.008 g/L; ferritin 9 µg/L; C-reactive protein 0.5 mg/L; liver function tests normal; amylase normal; d-dimers normal; protein electrophoresis: slight hyper-γ-globulinemia; renal function tests normal. Flow cytometry was done. In neutrophils, a PNH clone was found at 92%; in monocytes, the PNH clone was at 91%; and in red blood cells, the PNH I clone was 54%, PNH II clone 12%, and PNH III clone 34% (see Figures 7 and Figure 8; nonpublished observation of P.B.).

NIHA Secondary to Drugs and to Metal Intoxication in a Normal Erythroid Setting

Hemolysis induced by drugs may be mediated by an immune mechanism, possibly by a toxic effect on the microcirculation resulting in microangiopathic hemolytic anemia (discussed later), or may represent a toxic insult to the normal erythrocyte by the drug. In a majority of cases, the latter effect results in an oxidative damage of Hb or of the erythroid membrane. Here we report the case of ribavirin-induced hemolysis as an example. Ribavirin is one of the major agents used in combination therapy with interferon for the treatment of chronic hepatitis C. One of the serious adverse events associated with this therapy is hemolytic anaemia induced by ribavirin. The anemia may appear 2 weeks after start of ribavirin administration.

PNH : Fania test

Neutrophiles and monocytes

Figure 7. Diagnosis of PNH by fluocytometry on red blood cells. Figure kindly provided by Dr G. Georgiou.

Figure 8. Diagnosis of PNH by flow cytometry on leukocytes. Figure kindly provided by Dr G. Georgiou.
A recent study showed a reduction of ATP in erythrocytes of patients presenting this complication. Using metabolomic analysis of erythrocytes of those patients, Karasawa et al recently demonstrated that concentrations of intermediate metabolites produced by glycolysis and the pentose phosphate cycle were significantly decreased after ribavirin administration. Thus, acquired NIHA triggered by ribavirin is the result of an oxidative damage of erythrocytes. As not all patients receiving ribavirin will present this side effect, increased sensitivity of some patients to ribavirin-induced NIHA is currently investigated by analysis of single-nucleotide polymorphisms (SNPs) of the inosinotriphosphatase (ITPA) gene.

Wilson's disease is an autosomal-recessive disorder characterized by the progressive accumulation of copper in the body. The gene responsible for Wilson’s disease is located on chromosome 13 and encodes a protein belonging to the cation-transporting ATPase family. Typical symptoms of the disease are hepatic disorders, neuropsychiatric abnormalities, and non-immune hemolysis in association with acute liver failure. Erythrocytic damage in Wilson’s disease is believed to be caused directly by the oxidant effects of copper on Hb, by a direct damage to the membrane phospholipids, or by inhibition of glycolytic enzymes. Hemolytic anemia may be the initial manifestation of Wilson’s disease and often antedates symptomatic liver. Peripheral blood smear besides reticulocytosis may show Heinz bodies, stomatocytes, and blister cells. One should think of copper intoxication in the presence of oxidant-induced hemolysis, in a young patient, without G-6-PD deficiency or an unstable Hb and without history of exposure to an oxidative drug.

Concerning intoxication of other heavy metals, zinc creates an acquired myelodysplastic syndrome of sideroblastic anemia type. Acute lead intoxication is able to induce oxidative damage in circulating blood cells and cause non-immune hemolysis. However, chronic lead poisoning leads to a microcytic anemia of central origin with numerous basophilic stippling and a mild β-thalassemic phenotype. This is due to impair haem synthesis and to inhibition of intracellular iron delivery to ferrochelatase. Finally, lead induces inhibition of 5′-pyrimidine nucleotidase, explaining the aforementioned basophilic stippling.

**NIHA Secondary to Infections**

Infections are often a triggering or aggravating factor for hemolysis in subjects carrying a hereditary or acquired red blood cell disease. These situations, as well as hemolysis related to microangiopathy or immune reaction, are discussed elsewhere in this issue of *Seminars.* Parasitic or bacterial infection may also cause hemolysis because of physical invasion of the red blood cell or toxin production.

**Malaria,** caused by invasion of red blood cells by several species of *Plasmodium,* is probably the world’s most common cause of hemolytic anemia. Detailed description of malaria is beyond the scope of this review. The spectrum of clinical presentation and severity is broad, and depends on the *Plasmodium* species, on parasitemia, and on the host immune state. The majority of morbidity and mortality from malaria is caused by *Plasmodium falciparum,* the only species that can be lethal. The most common symptoms are cyclic fever, splenomegaly, hemolytic anemia, and thrombocytopenia. Because of the potential severity, specifically in non-immune subjects, malaria should always be searched for in a febrile subject coming from an endemic area. Pathophysiology of malarial hemolysis is complex and includes (1) rupture of parasitized red blood cells in the circulation, which occurs during acute malaria and correlates with parasitemia; (2) phagocytosis of parasited and unparasited red blood cells by macrophages and hyperplenism, which is frequent in endemic areas, since the spleen has an essential role in clearance of infected red blood cells; (3) rare autoimmune reaction; and (4) rare Blackwater fever, which is a severe intravascular hemolysis related to irregular use of anti-malarial drugs, mostly quinine.

Anemia may not be present in the early stage of the disease. It is typically normocytic, normochromic, with reticulocytes more often normal than elevated because of erythropoietic suppression by the *Plasmodium* and/or inflammation reaction. Thrombocytopenia is frequent. Diagnosis is made by parasitological diagnosis including observation of the parasite on blood films. Since the parasite is not always present in the peripheral blood, a negative blood film does not exclude the diagnosis.

Finally, it is important to recognize post-artesunate hemolysis. Artesunate has replaced quinine as the recommended first-line treatment of severe malaria. Delayed hemolysis has been reported in 7%–21% of patients 2–3 weeks after initiation of the treatment, and can be severe enough to require blood transfusion. Pathophysiology is not yet fully elucidated but may include delayed splenic destruction of parasited red blood cells, as well as immune mechanisms. Patients treated by artesunate should be monitored for at least 4 weeks in order to detect signs of hemolysis and to allow appropriate symptomatic treatment.

*Babesia* are intraerythrocytic protozoa transmitted by ticks. Babesiosis is a common infection in wild or domestic animals caused by *Babesia microti* in North America and *Babesia divergens* in Europe. Rarely, humans can be affected, causing a malaria-like disease. Severe hemolysis can occur in splenectomized subjects. Diagnosis is made by examination of blood smear and serology.

*Bartonella bacilliformis,* a bacteria that invades red blood cells and causes a severe acute hemolytic anemia, the Oroya fever, is endemic in South America. This is the first stage of the infection, which then progresses to a chronic granulomatous disorder called verruca peruana. Oroya fever responds well to antibiotherapy.

*Type A Clostridium perfringens* sepsis should be monitored for acute hemolysis. The bacteria produces a lecinthase causing a severe intravascular hemolysis associated with disseminated intravascular coagulation (DIC)
Table 4. Infection With Microorganisms That May Be Associated With Hemolysis

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Infection Type</th>
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<tbody>
<tr>
<td>Aspergillus</td>
<td>Epstein-Barr virus</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>Atypical pneumonia virus</td>
</tr>
<tr>
<td>Haemophilus influenzae</td>
<td>Coxsackie virus</td>
</tr>
<tr>
<td>Leishmania donovani</td>
<td>Cytomegalovirus</td>
</tr>
<tr>
<td>Leptospirosis interrogans</td>
<td>Herpes simplex virus</td>
</tr>
<tr>
<td>Mycobacterium tuberculosis</td>
<td>Influenza A virus</td>
</tr>
<tr>
<td>Mycoplasma pneumoniae</td>
<td>Rubeola virus</td>
</tr>
<tr>
<td>Neisseria meningitidis</td>
<td>Varicella virus</td>
</tr>
<tr>
<td>Salmonella</td>
<td></td>
</tr>
<tr>
<td>Shigella dysenteriae</td>
<td></td>
</tr>
<tr>
<td>Streptococcus pneumonia</td>
<td></td>
</tr>
<tr>
<td>Toxoplasma</td>
<td></td>
</tr>
<tr>
<td>Vibrio cholera</td>
<td></td>
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<tr>
<td>Yersinia enterocolitica</td>
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</table>

and shock. Diagnosis is made by usual bacteriological methods and PCR tests.64

Other microorganisms may be associated with hemolysis through different mechanisms, including immune reaction.63 They are listed in Table 4.63

Acquired HbH Disease

Patients with several hematologic malignancies (myeloproliferative disorders, myelodysplastic syndrome [MDS], acute leukemia)65-67 may develop signs of non-immune hemolysis with concomitant association of microcytosis, which is not found in previous hemograms or in other family members. Iron studies are normal. This triad (microcytic hemolysis with normal iron status in a context of an hematologic malignancy) should orient to acquired HbH disease. In fact in such cases, Hb studies by HPLC and IEF reveal a significant level of HbH in association with high HbF. Upon blue Cresyl staining, numerous inclusions bodies are seen in microscopic analysis (Figure 9). This entity, studied in extent by Higgs’ group, was proven in the great majority of cases to be secondary to acquired mutations in ATRX,68 a chromatin modeling gene, while α-globin cluster is intact. There is, however, one case of acquired α-thalassemia in a MDS patient, in which a 1.9-Mb deletion, including both α-globin genes, was characterized in one chromosome 16.69 For a detailed description of the molecular basis of this entity (=acquired HbH disease), we suggest an excellent review by Gibbons.70

NIHA Secondary to Acquired Microangiopathies

Drug-induced thrombotic microangiopathy (TMA) may be the result of two mechanisms: immune-mediated and dose- or duration-related toxic reactions.71 An immune reaction is suspected when the TMA is a late event after initial drug administration (within 21 days) or the result of re-exposition to the drug.71 Dose or duration toxicity may be acute gradually developing TMA, often manifested as kidney failure.71,72 A systemic review of published data described 78 drugs suspected to cause TMA. However, for only 22 drugs (seven with an immune mechanism; 15 with a toxic one) is there evidence of a definite association with TMA.71 Cyclosporine, sirolimus, and tacrolimus are the most common drugs that induce toxic TMA. Failure to recognize a drug as the cause of TMA is deleterious for the patient if the drug continues to be administrated or, in case of an immune mechanism, if the patient is re-exposed to the drug.

Tumor-related TMA has been described since 1980 and may be secondary to chemotherapy (as above) or directly related to the neoplastic disease. In most cases, patients suffered from metastatic adenocarcinoma.73 Possible sites of the primary tumor include stomach, lung, breast, colon, liver, urinary bladder, ovary, and prostate.74 Gastric and mammary origin seem to be the most frequent tumors.75 Clinically there is no hypertensive crisis nor fever. The patients develop a Coombs negative hemolytic anemia and thrombocytopenia, and there are numerous fragmentocytes in peripheral smear. Very often the hematologic picture is complicated by DIC. Renal insufficiency is not the primary event but appears later. Autopsy reveals a metastatic tumor with microvascular anomalies in the lungs. Lung, bone marrow, liver, lymph nodes, pancreas, and spleen represent the main sites of metastasis.76 Pulmonary tumor TMA may create severe pulmonary hypertension, which overwhelms the clinical picture and is usually rapidly fatal. Gastric carcinoma is the most frequent primary origin in such cases.76 In spite of aggressive antitumor treatment, the course is disastrous with an average survival of less than 15 days except for rare

Figure 9. Specific “golf ball” inclusions revealed by brilliant cresyl blue staining in red blood cells in a case of acquired HbH disease.
cases where surgical intervention can remove the totality of the primary tumor or in cases the tumor is hormone-dependent.76

Pregnancy-associated TMAs are separated into those associated with preeclampsia and those belonging to the HELLP syndrome (Hemolysis, Elevated Liver enzymes, Low Platelet count). When HELLP occurs in a woman with the anti-phospholipid syndrome (APLS), and there is extensive microangiopathy and multiorgan failure, the condition is termed “catastrophic APLS.”77 Finally, pregnancy may be a trigger for acquired or congenital TTP or atypical HUS.78

In preeclampsia, renal involvement (proteinuria) is constant, as well as hypertension, edema, and hyperuricemia. TMA appears in severe cases.79 Preeclampsia is associated with a characteristic glomerular lesion, known as glomerular capillary endotheliopathy. The pathophysiology of preeclampsia is the excess of placental soluble vascular endothelial growth factor (VEGF) receptor (sFlt-1) or sVEGFR-1) that binds circulating VEGF and placenta growth factor (PIGF). Increased sFlt-1 in preeclampsia leads to highly decreased VEGF with as a consequences arterial hypertension, endotheliopathy, proteinuria, edema, and renal TMA.77

HELLP is defined by the presence of NIHA, low platelets (<100 g/L), the presence of numerous schizocytes in peripheral blood, and elevated liver enzymes (more than twice the upper normal value). Hypertension is generally mild but present in most cases. Signs of preeclampsia are missing or are subtle. Preeclampsia is 10 times more frequent than HELLP. The placental lesion is probably similar in both conditions and delivery is at present their only efficient treatment.

Although pathogenesis is similar in HELLP and preeclampsia, findings support the hypothesis of more profound damage to the syncytiotrophoblast membrane in HELLP with higher levels of sFlt1 and sEndoglin values in HELLP than in preeclampsia. Furthermore, the inflammatory response is more enhanced in HELLP than in preeclampsia.80 TMA is believed to be multifactorial: the result of damage of the vascular endothelium by antiangiogenic substances produced by the placenta, the exposure to tumor necrosis factor (TNFα) resulting from the enhanced inflammatory response, and the high levels of active von Willebrand factor (vWF) combined with decreased levels of ADAMTS13.77,81 Another difference between preeclampsia and HELLP is the higher concentration of FasL in maternal blood in patients with HELLP as compared to those with preeclampsia. FasL is toxic to human hepatocytes and further triggers the production of TNFα, which induces hepatocyte apoptosis and necrosis.82 Kidney dysfunction is moderate in HELLP and when there is severe renal failure in HELLP, biopsy revealed a different mechanism from preeclampsia, that of TMA and acute tubular necrosis. Finally, DIC is more frequent in HELLP and may lead to multiple organ failure.

In conclusion, although bioactive substances emitted from the oxidatively stressed placenta in the maternal blood are the common factors for the development of both conditions, some specific cytokines (neurokinin B) are responsible for the HTA and the glomerular endotheliopathy in preeclampsia, while inflammatory cytokines and FasL are responsible for the TMA and liver damage, the main characteristics of HELLP.77

HIV-associated thrombotic microangiopathy was first described in 1984 and the incidence was relatively high before the introduction of highly active antiretroviral therapy (HAART) in the mid-1990s.83,84 In the post-HAART era, TMA is rare (estimated incidence 0.3%)85 and is mainly associated with advanced human immunodeficiency virus (HIV) disease. Two clinical patterns are described, both show hemolytic anemia, presence of schistocytosis, and thrombocytopenia, but differ by pathophysiology, prognosis, and treatment. The first one is characterized by a sudden onset in patients with a moderate immune deficiency and a few events of opportunistic diseases. ADAMTS13 is profoundly decreased; the prognosis is good with a response rate and an overall survival comparable to that of HIV-negative TTP.86 It is believed to be an auto-immune disorder secondary to anti-ADAMTS13 autoantibody developed in the early phase of the HIV infection. The second clinical pattern is a NIHA with microangiopathy and multiple organ failure, with progressive onset, occurring in profoundly immunocompromised patients with a history of multiple opportunistic diseases. ADAMTS13 activity is detectable and the prognosis is reserved. This form has dramatically decreased after introduction of HAART.80 It reflects an endothelial dysfunction with excessive liberation of vWF, hyperexpression of adhesion molecules, and, as a consequence, formation of platelet thrombi. Furthermore, HIV directly infects endothelial cells and increases their activation and liberation of vWF, leading to apoptosis. These huge quantities of high-molecular-weight multimers of vWF saturate cleavage capacity.86 Endothelial dysfunction is also aggravated by concomitant DIC and the effect of inflammatory cytokines like TNFα and transforming growth factor (TGF)-β1 liberated by mononuclear cells during opportunistic infections. Any patient with hemolytic anemia with microangiopathy should be tested for HIV infection.

Microangiopathy related to DIC is characterized by massive systemic intravascular activation of coagulation leading to widespread deposition of fibrin in the circulation. This results to the consumption of platelets and coagulation proteins. Furthermore, the thrombotic complications lead to microangiopathic anemia (presence of numerous schizocytes in peripheral blood smears).87 A variety of clinical conditions can cause DIC, infections by Gram-positive and Gram-negative bacteria being the most frequent. DIC is also associated with polytrauma, fat embolism, and extensive burns. Amniotic fluid embolism and placenta previa are among the most common obstetric
complications associated with DIC. Other causes are large vascular aneurysm, severe pancreatitis, snake bites, and hemolytic transfusion reaction. A special cause of DIC is acute promyelocytic leukemia. Diagnosis of DIC is based on clinical manifestations (bleeding, thrombosis, dysfunction of one or more organs), plus hemolytic microangiopathic anemia, plus coagulation abnormalities: increased activated partial thromboplastin time, prolonged prothrombin time, increased fibrin degradation products, and low plasma levels of coagulation factors (V, VII). Hypofibrinogenemia is seen in severe cases as fibrinogen is increased since it is an acute-phase protein.

**NIHA Associated With Mechanical Valves**

Mild compensated hemolysis associated with mechanical valves is observed in a large proportion of patients. With the improved valve models, it is now observed in less than 1% of patients with prosthetic heart valves. Diagnosis is made by the appearance of hemolysis (weakness, hemoglobinuria, congestive heart failure) associated with the presence of reticulocytosis, high LDH with low haptoglobin levels, indirect hyperbilirubinemia, and fragmented erythrocytes on the peripheral blood smear while the number of platelets is normal. The main mechanism for valve-associated hemolysis—usually severe in the clinical setting—is paravalvular leak. This is visualized by echocardiography, particularly transesophageal echocardiography. Hemolysis should raise the suspicion of structural valve deterioration and it is usually caused by suture dehiscence because of heavy annular calcification, or localized infection. The recently published European guidelines on the management of valvular heart disease recommend reoperation in case of endocarditis or if the hemolysis is severe enough to warrant repeated blood transfusions. Although it is not the aim of the present review, we mention that during the last years, percutaneous closure of paravalvular leaks has been described (not in case of active infection, vegetations, or thrombi), with the aim to avoid surgical replacement of the dysfunctional valve. The procedure is technically demanding and time-consuming, and its effects on hemolysis are inconsistent.

**NIHA Secondary to Acquired Membrane Disorders**

In addition to immune hemolytic anemia, a number of acquired conditions can affect the red blood cell membrane and cause secondary hemolytic anemia. These situations lead to normocytic regenerative anemia or worsening of pre-existing anemia associated with elevated hemolysis markers. They include liver disease, thermal injury, and macro- and microangiopathies, clostridial sepsis, and poisoning with some snake or spider venoms. Additionally, rare cases of acquired elliptocytosis secondary to protein 4.1 deficiency have been described in the context of MDS or myeloproliferative diseases. This situation is characterized by a hemolytic phenotype, including elevated reticulocyte count, quite unusual in these disorders, and the presence of elliptocytes on blood smears. Diagnosis can be made by ektactometry and/or electrophoresis of red blood cell membrane proteins.

**Can Hypersplenism Be Considered as NIHA?**

Hypersplenism represents the increased pooling and/or destruction of the corpuscular elements of the blood by the enlarged spleen. Splenomegaly is usually associated with hypersplenism which may be the consequence of abnormal blood flow (cirrhosis, splenic vein obstruction), infiltration (metabolic diseases, ex Gaucher’s disease; lymphomas), or increased workload. Hypersplenism is characterized by a significant reduction in one or more of the cellular elements of the blood in the presence of normocellular or hypercellular bone marrow. In the majority of cases hypersplenism is asymptomatic; however, in rare cases it may manifest with bleeding disorders or hemolytic anemia. Here we must say that in hemolytic anemias, for example, hereditary spherocytosis, hypersplenism with developed splenomegaly is a response to hyperfunction of the normal spleen and is not a primary event.

So the answer to the question is yes, in some cases hypersplenism may be considered in the differential diagnosis of NIHA. Finally, we must not forget the maxim that “in hypersplenism when anemia is present is multifactorial in its origin.”

**CONCLUSIONS AND PROPOSED DIAGNOSTIC ALGORITHM**

If it is easy to diagnose NIHA (association of hemolysis with negative anti-human immunoglobulin test), it may be difficult to identify the exact clinical entity. This is because causes of NIHA are extremely heterogeneous and sometimes two or more entities may have the same clinical and hematological presentation. Here, we propose a simplified classification where the basis is to distinguish hereditary from acquired forms of NIHA. For this reason, it is mandatory to begin with an exhaustive personal and familial anamnesis of the patient.

Then we suggest to consider if hemolysis, including clinical symptoms, is restricted to red blood cells (this happens in most cases of hereditary disorders), or if it is associated with other signs, such as thrombocytopenia, or an environmental pathology. Once hereditary or acquired form of NIHA is established, accurate diagnosis of NIHA requires good knowledge of this entity and a qualified specialized hematology laboratory. Indeed, diagnostic methods/laboratory investigations are diverse and include microscopy for red blood cell morphology observation, biochemical methods such as HPLC or electrophoresis for hemoglobin analysis, enzymatic activity measurement,
Non-immune hemolysis

analysis of red blood cells by flow cytometry, studies of the erythrocyte membrane, and finally analysis of erythroid genes at a molecular level.

Figure 10 shows the proposed algorithm to be used in a new patient consulting the clinician for NIHA. We are convinced that accurate diagnosis is crucial since there is no symptomatic therapeutic approach that does not risk aggravating the clinical situation. Furthermore, genetic counseling is impossible to offer without a precise diagnosis.

REFERENCES


64. Shindo Y, Dobashi Y, Sakai T, Monma C, Miyatani H, Yoshida Y. Epidemiological and pathobiological profiles of
Non-immune hemolysis

...and other secondary hemolytic processes... 


