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CRITERIA FOR DIFFERENTIAL ANALYSIS OF MAIN LABORATORY MARKERS OF HEMOLYTIC ANEMIA

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Introduction and Relevance of the topic

In the field of modern hematology and clinical laboratory diagnostics, the problem of hemolytic anemia (HA) remains one of the most complex and urgent areas for medical scientists, researchers and practitioners. According to the statistics of the World Health Organization and extensive epidemiological studies conducted in recent years, hemolytic anemia accounts for 5.3% of blood system diseases and 11.5% of all anemia cases recorded worldwide[1]. Pathological rapid destruction (hemolysis) of erythrocytes (red blood cells) before their physiological lifespan (normally about 100-120 days) triggers extremely severe cascade reactions in the body. As a result of premature damage and destruction of erythrocytes, the oxygen-carrying capacity of the blood decreases sharply, which leads to severe hypoxia, ischemia and multiorgan failure of vital organs such as the brain, heart, kidneys[4].

The incidence of autoimmune hemolytic anemia (AIHA) in the adult population is approximately 0.8–3 cases per 100,000 population per year, and without appropriate therapeutic interventions, its mortality rate is estimated to be around 11%. Although it is relatively rare in children and adolescents (0.2 cases per 100,000 population per year), it poses serious clinical challenges for pediatricians and neonatologists, particularly complications such as hyperbilirubinemia and kernicterus[1-5]. If the hemolysis process is rapid and the correct and accurate diagnosis is not made in a timely manner, patients may develop acute renal failure, disseminated intravascular coagulation (DIC) syndrome, and even sudden death[3].

From this perspective, in the diagnosis of hemolytic anemia, it is of great importance not only to assess the patient's clinical symptoms (jaundice, spleen enlargement, weakness, tachycardia), but also to conduct a thorough and differential analysis of laboratory parameters[7]. Laboratory tests are a key tool not only in confirming the state of hemolysis, but also in differentiating its exact mechanism (intravascular or extravascular) and in quantitatively determining the severity of the disease[1,2,3].

Today, fundamental research is actively being conducted in major research centers around the world to study the molecular mechanisms of hemolytic anemia and find the most effective, targeted methods for their treatment. Studies by Shanmuga Priya M. and co-authors (2017), Zangeneh MM et al. (2018), Goorani S. and his research group (2019) have shown the role of oxidative stress, immune dysfunction, and cell membrane defects in the pathogenesis of hemolysis[12]. A critical decrease in hemoglobin concentration and erythrocyte count requires immediate effective therapeutic intervention (steroids, monoclonal antibodies, blood transfusions, or complement inhibitors). The choice of such complex treatment strategies is based on a direct correct diagnosis, a correct assessment of changes in blood parameters, and an objective analysis of laboratory tests. In

this regard, the analysis of key laboratory markers in hemolytic anemias of various etiologies, their significance and limitations are considered a very promising and necessary direction[15].

Pathophysiological Mechanisms and Topographic Classification of Hemolysis

To fully understand the diagnostic value of laboratory markers and the dynamics of changes in serum, it is necessary to analyze in depth where and by what mechanisms the hemolysis process occurs. From a physiological and pathological point of view, erythrocyte destruction occurs mainly in two different anatomical environments: outside the blood vessels (extravascular hemolysis) and directly inside the blood vessels (intravascular hemolysis)[2]. This topographical distinction reveals a completely different biomarker profile in laboratory analyses.

Extravascular Mechanisms of Hemolysis

Extravascular hemolysis is most often carried out by macrophages in the reticuloendothelial system (RET), particularly in the spleen, liver, and, to a lesser extent, in the bone marrow. Normally, approximately 1% of circulating erythrocytes are removed from the bloodstream through this system every day due to aging. However, in pathological conditions, such as warm autoimmune hemolytic anemia (wAIHA) or hereditary spherocytosis, this process is accelerated hundreds of times[1].

In wAIHA, the patient's body produces autoantibodies of the IgG class against antigens on the surface of their own erythrocytes. These immunoglobulins are optimally fixed to the erythrocyte membrane at human body temperature (37°C). These "marked" erythrocytes, passing through the splenic blood vessels (sinusoids), are recognized by splenic macrophages. Macrophages bind to IgG through their surface Fc receptors and phagocytose the entire erythrocyte or "bite off" part of its membrane. The erythrocyte, which has partially lost its membrane, loses its double concave (discoid) shape and becomes a spherical spherocyte[19]. Since spherocytes lose their elasticity, they become clogged and completely disintegrate the next time they pass through the spleen[3-7].

The laboratory peculiarity of extravascular hemolysis is that intracellular hemoglobin is not released directly into the blood plasma. Inside macrophages, hemoglobin is immediately broken down into globin chains and heme groups. The iron is removed from the heme group and transported to the bone marrow for reuse by the transferrin protein, while the remaining porphyrin ring is converted to biliverdin and then to unconjugated (indirect) bilirubin[21]. Unconjugated bilirubin passes from the macrophage into the blood and bound to albumin, reaching the liver. Therefore, in extravascular hemolysis, free hemoglobin is absent or very low in the blood, but the level of unconjugated bilirubin is sharply increased[4].

Mechanisms of Intravascular Hemolysis

Intravascular hemolysis is characterized by the occurrence of a pathological process directly in the bloodstream and is often accompanied by clinically severe, acute complications[3]. It is caused by cold agglutinin disease (CAD), paroxysmal nocturnal hemoglobinuria (PNH), disseminated intravascular coagulation (DIC), thrombotic thrombocytopenic purpura (TTP), thrombotic microangiopathies (TMA) such as hemolytic uremic syndrome (HUS), as well as ABO incompatible blood transfusion reactions[18].

In terms of molecular mechanism, for example, in cold agglutinin disease (CAD), the body produces autoantibodies of the IgM class. When the patient's body temperature drops (in acral areas, e.g., fingers, ears, tip of the nose, when it drops to 30°C), IgM antibodies bind strongly to the I/i antigens on the surface of erythrocytes, causing them to stick together (agglutination) and strongly activating the complement system in the classical way. In the final step of the complement system, a "membrane attack complex" (MAC) consisting of C5b-9 proteins is formed, which perforates the erythrocyte membrane[3,20].

As a result, the erythrocyte bursts in the blood vessel itself, and all its components - including large amounts of free hemoglobin, lactate dehydrogenase (LDH), and other enzymes - are released directly into the blood plasma. Free hemoglobin in the plasma immediately binds to the haptoglobin protein, which leads to a sharp and rapid depletion of the haptoglobin reserve [7]. When haptoglobin is depleted, free hemoglobin is filtered by the renal tubules and appears in the urine (hemoglobinuria). The renal tubular cells try to reabsorb hemoglobin and accumulate its iron in the form of hemosiderin, which is excreted in the urine as hemosiderinuria. Free hemoglobin has the property of binding nitric oxide (NO), so intravascular hemolysis causes systemic endothelial dysfunction, such as severe vascular spasms, smooth muscle contraction, esophageal and abdominal pain, and acute renal failure [3].

Table 1

Comparative analysis of pathogenetic, laboratory and clinical features of extravascular and intravascular hemolysis

Feature	Extravascular hemolysis	Intravascular hemolysis
Main mechanism	Phagocytosis by spleen and liver macrophages	Complement or mechanical breakdown in the bloodstream
Free plasma hemoglobin	Normal or slightly increased	Sharply increased
Haptoglobin level	Decreased or normal (in inflammation)	Sharply decreased (often below the measurement limit)
Lactate dehydrogenase (LDH)	Moderately increased	Sharply and highly increased
Unconjugated bilirubin	High (main diagnostic sign)	Increased, but hemoglobinemia predominates
Changes in urine	Increased urobilinogen, no hemoglobinuria	Hemoglobinuria, hemosiderinuria are present.
Peripheral blood morphology	Spherocytes	Schistocytes, fragmented cells
Clinical examples	Warm AIHA (wAIHA), Hereditary spherocytosis	Cold AIGA (CAD), PNH, TMA, TTP, G6PD attack

Differential Analysis and Biochemical Characteristics of Basic Laboratory Markers

The diagnosis of hemolytic anemia and its etiology is not limited to the analysis of a single marker. On the contrary, it is a complex mosaic that requires a synergistic study of the cascade of changes of various biochemical, hematological and immunological markers. No single marker is 100% specific by itself; their correct combination and dynamics provide a diagnostic conclusion. In order to assess the degree of hemolysis and quantify changes in blood parameters, the following basic laboratory criteria are differentially analyzed.

1. Hemoglobin (Hb) and Hematocrit (Hct): Primary Indicators of Anemia

The first indicators to be considered in a blood test are hemoglobin and hematocrit. Although a decrease in these indicators is not specific for hemolysis, their level is the main criterion for determining the presence of anemia, its clinical severity and the need for blood transfusion. In hemolytic anemias, the mean corpuscular volume (MCV) of erythrocytes is usually normocytic (80-100 fL), but against the background of active compensatory reticulocytosis it can often take on a macrocytic appearance (MCV > 100 fL), since reticulocytes have a larger volume than mature erythrocytes.

Analysis of changes in blood parameters shows that a decrease in hemoglobin below 8 g/dL is directly correlated with tissue hypoxia, heart failure, stroke, and increased risk of mortality. Researchers (Barcellini W. et al.) propose to stratify the severity of hemolysis according to hemoglobin level: mild (>10 g/dL), moderate (8-10 g/dL), severe (6-8 g/dL), and extremely severe life-threatening (<6 g/dL) anemia[3,4,9].

2. Reticulocytes: A Criterion of Bone Marrow Compensatory Function

Reticulocytes are immature red blood cells newly released from the bone marrow, free from nuclear debris but still containing polyribosomes and RNA strands. Normally, they constitute 0.5–1.5% of the total number of erythrocytes circulating in the peripheral blood and mature into fully mature erythrocytes within 1–2 days[7].

The massive destruction of erythrocytes due to hemolysis causes tissue hypoxia, which in turn sharply increases the synthesis of the hormone erythropoietin (EPO) by the kidneys[15]. Erythropoietin stimulates the process of erythropoiesis (blood formation) in the bone marrow and causes the release of large numbers of reticulocytes into the bloodstream[4]. Therefore, reticulocytosis (more than 2.5% of the connecting indicator and an absolute number of >100 x 10^9/L) indicates a healthy compensatory response of the body to the hemolytic process.

Diagnostic limitations and risk factors: Reticulocyte counts are not always elevated as expected. Clinical data suggest that 20-40% of patients with autoimmune hemolytic anemia have paradoxical reticulocytopenia (a lower than normal reticulocyte count).[9] This may be due to several factors:

- *The hemolysis is so severe that in response, the bone marrow is depleted of folic acid or vitamin B12 stores (megaloblastic crisis).*
- *Autoantibodies attack and destroy not only mature erythrocytes in the peripheral blood, but also erythroid precursors in the bone marrow.*
- *Concomitant viral infections (e.g., Parvovirus B19) have temporarily suppressed bone marrow function (aplastic crisis).*

If a patient with confirmed active hemolysis has a low reticulocyte count, this is considered a very serious prognostic sign and requires the physician to immediately perform emergency blood transfusion procedures on the patient[3].

3. Lactate Dehydrogenase (LDH) and Its Intracellular Kinetics

Lactate dehydrogenase (LDH) is an oxidoreductase enzyme present in the cytoplasm of all living cells and catalyzes the conversion of pyruvate to lactate (lactic acid) in the final step of anaerobic glycolysis. Since erythrocytes do not contain mitochondria, they are dependent solely on anaerobic glycolysis for energy (ATF). Therefore, they accumulate very high concentrations of the enzyme LDH (especially its isoenzymes LDH-1 and LDH-2)[4].

During hemolysis, the integrity of the cell membrane is disrupted and all intracellular LDH is released into the blood plasma. The increase in the level of LDH in the blood is directly proportional to the volume of erythrocytes destroyed [27]. Its increase by several tens of times above the norm (usually 140-280 U/L) (for example, up to 1000-5000 U/L) is a reliable sign, characteristic mainly of intravascular hemolysis. In extravascular hemolysis, the level of LDH also increases, but this change is often relatively moderate (1.5-2 times higher than normal).

Limitations and variability of LDH assay: Although LDH is a highly sensitive marker of hemolysis, its greatest weakness is its complete nonspecificity. Its levels are elevated in any setting of extensive tissue necrosis or cellular destruction[7]. For example:

- Myocardial infarction or acute heart failure.

- Liver diseases (acute hepatitis, liver cirrhosis), in which the LDH-5 isoenzyme predominates[27].
- Malignant tumor lysis syndrome (leukemia, lymphoma).
- In megaloblastic anemias (vitamin B12 deficiency), cells die as a result of ineffective erythropoiesis in the bone marrow.

Therefore, in clinical practice, LDH is never interpreted in isolation to diagnose hemolysis. If a patient has elevated LDH with reticulocytopenia and normal haptoglobin, it is important to understand that the focus of the pathology is not hemolysis, but ischemia/necrosis of another organ or tissue[28]. To strengthen the differential diagnosis, it is recommended to examine the spectrum of LDH isoenzymes.

4. Haptoglobin: A Specific Bindant of Free Hemoglobin

Haptoglobin is a protein of the alpha-2-globulin group, synthesized mainly by hepatocytes of the liver. Its only evolutionary function in the body is to bind free, toxic hemoglobin dimers released into the bloodstream in a strong and irreversible manner. Free hemoglobin, as a carrier of iron ions in the blood, is a very strong pro-oxidant; it damages the vascular endothelium, destroys nitric oxide (NO), and accumulates in the renal tubules, causing acute renal failure [1,2,4,7].

Haptoglobin combines with free hemoglobin to form a high molecular weight "hemoglobin-haptoglobin" complex. This large complex cannot be filtered through the renal tubules. Instead, it is rapidly recognized by special CD163 receptors (scavenger receptors) on the surface of macrophages in the liver and spleen, and is removed from the bloodstream and degraded within the macrophages.

This process rapidly depletes the serum haptoglobin reserve. In both intravenous hemolysis and prolonged moderate extravascular hemolysis, haptoglobin levels in blood tests are markedly reduced (often <0.1 g/L or undetectable). Studies have shown that low haptoglobin levels are one of the most reliable individual markers for the diagnosis of hemolysis.

Clinical variability and errors in interpretation of haptoglobin:

Important notes to avoid possible errors in interpreting results:

1. **Masking by inflammation:**Haptoglobin is a member of the "acute-phase reactants" (like S-reactive protein). In the presence of various systemic inflammations, infectious diseases, trauma, or malignant tumors, the liver, under the influence of cytokines (especially IL-6), sharply increases the synthesis of haptoglobin. Therefore, if a patient has hemolysis and severe inflammation at the same time, the level of haptoglobin may be normal or even elevated, despite its "consumption". This situation can lead to erroneous laboratory conclusions.
2. **Effects of liver dysfunction:**Since haptoglobin is synthesized in the liver, in conditions such as severe liver failure, cirrhosis, and hepatitis, its level remains consistently low even in the absence of hemolysis.
3. **Neonatal period:**Since haptoglobin synthesis is not yet sufficiently developed in newborns, its amount is physiologically very low; this makes haptoglobin an unsuitable marker for detecting hemolysis in infants.

5. Unconjugated (Indirect) Bilirubin

One of the final end products of hemoglobin catabolism in macrophages is unconjugated (or indirect) bilirubin. Biliverdin, formed from the heme molecule, is enzymatically converted to bilirubin, which is insoluble in water and transported to the liver as a complex with the albumin protein in the blood plasma. In the liver hepatocyte cells, it is conjugated with glucuronic acid by the enzyme UGT1A1 (Uridine diphosphate glucuronosyltransferase) and converted to water-soluble conjugated (direct) bilirubin, which is excreted into the intestine via the bile ducts[7-21].

The hemolytic process (mainly extravascular hemolysis) occurs so rapidly that the amount of bilirubin produced exceeds the liver's ability to capture and conjugate it. As a result, the fraction of unconjugated bilirubin in the serum increases sharply, and the patient develops a lemon-colored jaundice (icterus) of the skin, mucous membranes, and sclera of the eyes[3].

As a hemolytic marker, unconjugated bilirubin changes later and more slowly than LDH or haptoglobin. Its concentration depends not only on the rate of hemolysis, but also on the metabolic capacity of the liver. If the patient has simultaneously increased levels of direct (conjugated) bilirubin and liver transaminases (AST, ALT), then this indicates that the problem is not in hemolysis, but in liver pathologies such as obstruction of the bile ducts (obstructive cholestasis) or viral hepatitis.

Additional Markers in Plasma and Urine

Additional tests are ordered to fully confirm the clinical diagnosis. In massive intravascular hemolysis, the amount of free hemoglobin in the plasma increases to such an extent that the serum visually acquires a reddish-pink color (hemoglobinemia). Hemoglobin filtered by the kidneys gives the urine the color of "Coca-Cola" or black tea. Although there are no erythrocytes in the urinalysis, the chemical analysis gives a positive result for blood, which indicates definite hemoglobinuria[7]. Hemosiderinuria (the release of iron-storing protein particles in the urine) is a late marker that appears several days or even weeks after the onset of intravascular hemolysis[9].

Table 2

Diagnostic value of laboratory markers in hemolytic processes: pathophysiological basis and limitations

Laboratory Marker	Changes in Hemolysis	Mechanism and Main Features	Limitations (Diagnostic Errors)
Haptoglobin	Sharply decreased (↓)	Binds free hemoglobin and is consumed	Increased in inflammation (false normal); decreased in liver disease
Lactate dehydrogenase (LDH)	Strongly increased (↑↑)	Released from broken down erythrocytes into plasma	Also increases in myocardial infarction, liver damage, and malignant tumors
Reticulocytes	Increased (↑)	Compensatory increase in bone marrow activity	B12 may be decreased in folate deficiency and bone marrow damage in AIHA
Unconjugated Bilirubin	Increased (↑)	The end product of hemoglobin catabolism	Nonspecific increase in liver pathologies and Gilbert's syndrome (UGT1A1 defect)
Urobilinogen in urine	Increased (↑)	Excessive absorption of bilirubin from the intestine	May be low due to loss of gut flora when taking antibiotics

Peripheral Blood Smear: Morphological Analysis and Modern Technologies

Differential analysis of the main laboratory biomarkers must be supplemented by morphological examination of the peripheral blood smear. Examination of the shape, size and staining of erythrocytes under a light microscope not only confirms the fact of hemolysis, but often provides visual evidence that directly indicates its underlying cause (etiology). This method of examination is still one of the most inexpensive and informative tools in hematology.

The main morphological forms and their pathogenesis:

1. **Spherocytes:**These are perfectly spherical, reduced-sized, and densely stained erythrocytes that have lost the traditional central pallor. Spherocytes are characteristic of two groups of diseases: warm antibody autoimmune hemolytic anemia (wAIHA) and hereditary spherocytosis. During circulation, red blood cells must be compressed and folded to pass through the splenic sinusoids, which are less than 3 microns in diameter. However, when macrophages partially gnaw through their membrane (due to IgG fixation) or due to hereditary defects in cytoskeletal proteins such as spectrin and ankyrin, the cell loses its volume-to-diameter ratio, loses its elastic discoid shape, and becomes a spherocyte. Since they are inelastic, they become compressed and disintegrate during subsequent passage through the spleen[3,7].

2. **Schistocytes:**They are truncated, fragmented, helmet-shaped, or triangular-shaped erythrocyte fragments[9]. They indicate that hemolysis is caused by purely mechanical damage to small blood vessels covered with microthrombi (fibrin threads). This condition is manifested in Thrombotic Microangiopathies (TMA) - including Hemolytic Uremic Syndrome (HUS), Thrombotic Thrombocytopenic Purpura (TTP), and DVS syndrome. If a patient is diagnosed with thrombocytopenia and schistocytes together, it is necessary to immediately consider the risk of TTP and check the activity of the ADAMTS13 enzyme. Schistocytes should not be found in autoimmune hemolysis; if they are detected in the setting of wAIHA, another serious complication should be sought immediately.

3. **Bite cells (Degmacytes) and Blister cells:**These forms are mainly erythrocytes damaged by severe oxidative stress. They are most common in glucose-6-phosphate dehydrogenase (G6PD) deficiency and in immune hemolysis caused by certain drugs (e.g., dapsone). When the body's antioxidant capacity is reduced, the hemoglobin molecule is oxidized and deposited on the cell membrane in the form of "Heinz bodies." When these deposits are broken off (pitted) by splenic macrophages, crescent-shaped bite marks (bite cells) are left on the cell [6].

4. **Polychromasia and Macrocytes:**This sign represents reticulocytes in a blood smear. Young cells that have prematurely left the bone marrow due to accelerated erythropoiesis stain differently and appear as pale blue/pink colors. These indicate that the bone marrow is functioning normally and is responding to hemolysis in a compensatory manner[7].

Artificial Intelligence and Automation in Morphological Diagnostics

In recent years, hematology laboratories have undergone a major revolution. While traditional microscopic analysis is a subjective approach dependent on human factors and requires a lot of time, today automated blood morphology analyzers based on Artificial Intelligence (AI) and Deep Learning algorithms are widely used in practice to perform morphological analyses objectively and without errors[32].

New generation machine vision technologies (e.g. MC-80 flow cytometry and morphological analyzer systems) developed in 2024-2025 will be able to scan each sample and analyze hundreds of thousands of individual cells in real time[33]. In a recent large-scale study (2024) led by Dr. Ahmed Elnady Elsafty, neural networks were trained on more than 1 million red blood cells[34]. The system accurately identifies and counts spherocytes, schistocytes, ovalocytes, and teardrop cells with a sensitivity and specificity of more than 99%. These digital analyzers will not only significantly simplify the diagnosis of hemolytic anemia for the physician, but will also automatically distinguish and alert with “red flags” even small morphological changes that are too small for the “human eye” to detect, such as early-stage microspherocytosis[32]. These technologies are expected to bring diagnostic accuracy to an absolute high level in the future.

Coombs Test (DAT) Criteria in the Differential Diagnosis of Immune Hemolytic Anemias

Once all biochemical and morphological markers have confirmed the presence of hemolysis, the main task is to differentiate its cause - in particular, immunological and non-immunological causes. For this purpose, the Direct Antiglobulin Test (DAT), traditionally known as the Coombs test, is accepted as the "gold standard" worldwide.

The basic principle of the Coombs test is that a suspension of washed red blood cells taken from the patient's blood is added to Coombs' reagent (a special antiserum obtained in rabbits or mice against human immunoglobulins and complement components). If the patient's red blood cells are coated in vivo (in the body itself) with autoantibodies (IgG, IgA, IgM) or complement components (C3d) due to the disease, the Coombs reagent causes them to stick together, forming agglutination (precipitation) that can be seen with the naked eye.

Screening with polyspecific reagents is usually performed first. If this is positive, the laboratory must perform a detailed differential analysis (DAT panel) with monospecific antisera (anti-IgG and anti-C3d)[24]. It is based on the reactions in this panel that autoimmune hemolytic anemias are divided into strict clinical categories.

Clinical Classification Based on Coombs Test and Thermal Amplitude

1. **Warm antibody AIGA (wAIHA):**The most common of all types of AIGA (up to 60-70%). In this disease, autoantibodies are mainly of the IgG class, which actively bind to Rhesus or common protein antigens on erythrocytes at normal human body temperature (37°C - warm temperature)[1].
   - *DAT Result:*Usually strongly positive for anti-IgG. Occasionally, positive for IgG and C3d simultaneously[7].
   - *Type of hemolysis:*Pure extravascular hemolysis with the involvement of macrophages.
2. **Cold Agglutinin Disease (CAD/CAS):**In this case, antibodies of the IgM class predominate. These antibodies are activated at cold temperatures (0-4°C, practically at <30°C in the extremities) and bind to the I/i antigens of erythrocytes[3]. When the temperature returns to normal, IgM is released into the central bloodstream, but it leaves behind traces of the complement system (the C3d protein).
   - *DAT Result:*Only positive for anti-C3d (absolutely negative for IgG).
   - *Type of hemolysis:*Severe complement-mediated intravascular hemolysis.
3. **Mixed AIGA:**A very serious condition in which the patient has both warm IgG and cold IgM autoantibodies at the same time.
   - *DAT Result:*Both anti-IgG and anti-C3d are positive. The disease usually progresses rapidly and aggressively, with very low hemoglobin levels[23].
4. **Paroxysmal Cold Hemoglobinuria (PCH):**A rare pathology that occurs acutely, often in children, after viral infections. It involves biphasic Donat-Landsteiner antibodies (which are IgG in nature). They activate complement in the cold, and destroy the cell in the heat.
   - *DAT Result:*During the active phase of the disease, it is predominantly positive for C3d. Diagnosis requires a specific Donat-Landsteiner test.
5. **DAT-negative AIGA:**One of the most challenging diagnostic challenges is the negative result of a standard Coombs test, despite the presence of all clinical signs of hemolysis.[23] Approximately 5–10% of hospitalized patients with AIGA fall into this category.[38] This may be due to a variety of reasons: the amount of immunoglobulins on the erythrocyte surface is below the sensitivity limit of the test (e.g., less than 200 IgG molecules per cell), the fact that the antibodies belong to a rare class such as IgA (most commercial reagents do not detect IgA), or their affinity (binding strength) is too low to be removed during the washing step.[7] In such cases, more sensitive tests such as microgel column agglutination or elution methods must be used.[25]

Drug-Induced Immune Hemolytic Anemia (DIIHA)

Drug-induced immune hemolytic anemia (DIIHA) is always a risk factor in clinical practice in the pathogenesis of hemolysis. Although DIIHA occurs in 1 case per million people, based on statistical and epidemiological data, it is often misdiagnosed as conventional AIGA and leads to the selection of incorrect treatment tactics. More than 140 drugs known to science have been fully proven to cause hemolysis[15]. The most dangerous of these include cephalosporin antibiotics (e.g. cefotetan, ceftriaxone, cefmetazole), penicillin groups, non-steroidal anti-inflammatory drugs (diclofenac), and platinum-based antineoplastic agents used in oncological diseases, such as fludarabine. In addition, recent studies have confirmed that modern immune checkpoint inhibitors (ICIs) (such as Nivolumab), which are widely used in the treatment of cancer, can additionally cause severe, potentially fatal autoimmune hemolysis by taking the patient's immune system off the "brakes" [3].

Clinical differentiation criteria and laboratory features of DIIHA:

In DIIHA, the drug acts as an intermediary between erythrocytes and the immune system, and two main biochemical mechanisms have been described in its functioning:

1. **Mechanism of drug adsorption (Hapten model):**It is mainly observed in high-dose penicillin groups. The drug substance is directly and covalently attached to proteins on the erythrocyte membrane (adsorbed), transforming it into a specific neoantigen[40]. The immune system now sees this complex as "foreign" and produces IgG. In this model, the Coombs (DAT) test is usually positive for strong IgG. How is it different from AIGA? In AIGA, antibodies are produced against the erythrocyte itself and react when they are eluted (washed) and added to another donor's blood. In DIIHA, antibodies bind only in the presence of the drug. Therefore, laboratory tests of the eluate give a completely negative result when mixed with regular test blood, since the drug is not present in the test tube[7]. It is this subtle differential factor that reveals the direct effect of the drug.

2. **Immune complex (Neoantigen/Trinity) mechanism:**This mechanism is characteristic mainly of cephalosporins and other drugs, and is often characterized by very strong and tragic consequences. The drug combines with some proteins in the blood plasma or with antibodies to form an "immune complex", which then binds nonspecifically to circulating erythrocytes. This "ternary complex" activates the complement system very quickly and massively. As a result, the Coombs test is usually positive only for C3d, and the patient develops fulminant acute intravenous hemolysis, severe hemoglobinuria, and, in a short time, acute renal failure[40]. The only priority in treatment is immediate discontinuation of the drug.

Diagnostic Markers of Hereditary and Congenital Anemias

In patients with negative immunological tests, including polyspecific and monospecific DAT assays, but active intravascular or extravascular hemolysis, etiological investigations should be directed towards hereditary and enzymatic defects[7].

1. Glucose-6-phosphate dehydrogenase (G6PD) Enzyme Deficiency

G6PD deficiency is the most common enzymatic deficiency in the world, and is inherited as an X-linked recessive trait (hence the majority of patients are males, while females are carriers)[43]. The G6PD enzyme is a key enzyme in the pentose phosphate cycle, converting NADP+ to NADPH in the cell. NADPH, in turn, is involved in the regeneration of glutathione, which is the only powerful shield that protects erythrocytes from oxidative stress, hydrogen peroxide, and free radicals. What happens when G6PD deficiency occurs? Patients can be completely healthy in their daily lives. However, when they take certain medications (such as the antimalarial drugs primaxine,

sulfonamides, and dapsone)[44], have viral/bacterial infections, or consume fava beans (legumes) (favism), oxidants in the blood oxidize the unprotected hemoglobin[6].

*Laboratory and differential signs:*Oxidized globin chains deposit on the cell surface as solid masses—the well-known "Heinz bodies" that are visible under a microscope. Splenic macrophages gnaw at these deposits, resulting in the appearance of "bite cells" in blood smears. During an attack, the levels of unconjugated bilirubin and LDH in the blood immediately increase. The main laboratory problem and limitation is that a direct test for G6PD levels can give falsely normal results, as the dead, deficient cells are rapidly destroyed during an attack and replaced by young, more enzymatically active reticulocytes released into the blood.[46] Therefore, the test should only be performed a few weeks after the attack has passed and the hemolysis has subsided. According to genetic research (Next Generation Sequencing - NGS technologies) over the past 10 years, hundreds of mutations of this enzyme have been identified, and specific population variants (e.g. Canton, Kaiping) are being identified, and this genetic screening method is considered more reliable than the classic enzyme test[43].

2. Hereditary Spherocytosis

This type of anemia is mainly caused by defects in the genes encoding the synthesis of proteins (spectrin, ankyrin, pallidum and band 3) involved in the cytoskeleton of the erythrocyte cell membrane. Due to the weakening of the internal support of the cell, it takes on the form of a spherocyte. As a result of prolonged chronic extravascular hemolysis in the spleen [2], the patient often develops anemia, splenomegaly and cholesterol gallstones (bilirubin-rich gallstones) from childhood. In the laboratory differential analysis: in contrast to the spherocytes in AIGA, the DAT (Coombs) test is completely negative here [7]. Although the MCV indicator in the blood test is variable, the Mean Corpuscular Hemoglobin Concentration (MCHC) is higher than normal (>36 g/dL) due to cellular dehydration. As a specific diagnostic criterion, the Osmotic Fragility Test - measuring how quickly erythrocytes rupture in hypotonic solutions of different concentrations, as well as the EMA-binding (eosin-5-maleimide) test - directly counting the amount of membrane proteins in flow cytometry have been introduced into practice[3].

Experimental Biomarkers, In-Vivo Models, and Modern Therapeutic Approaches

The analysis of laboratory markers is not limited to the diagnosis of the disease. They serve to study the effectiveness of drugs and the antioxidant defense process in the body. Today, extensive animal models and research are being conducted in the world's medical sciences, including in Central Asia, to study the pharmacological capabilities of medicinal plants and nanotechnologies against hemolytic anemia[10].

Researchers (Zangeneh, Goorani, Gheith) developed in-vivo models of hemolytic anemia in experimental white rats by inducing a strong toxic and oxidizing agent such as phenylhydrazine (PHZ). Phenylhydrazine directly oxidizes hemoglobin in the bloodstream, causing its massive breakdown, pathological increase in spleen and liver weight, and lipid peroxidation. Against the background of such hemolysis, pro-inflammatory cytokines (such as IL-6) increase sharply, while red blood cell parameters (RBC, HGB, HCT) decrease to a minimum. Experimental analyses have shown that various plant extracts (Beta vulgaris – red beet leaves, Allium saralicum – garlic varieties, Ziziphora clinopodioides – deer grass) are able to inhibit oxidative stress directly as antioxidant agents[11]. Among the most innovative advances, bioactive nanoparticles obtained through "green synthesis": iron (FeNPs), silver (AgNPs) and gold (AuNPs) nanoparticles are being tested in the treatment of anemia. They significantly restored the body weight loss caused by hemolysis, red blood cell parameters, and platelet and white blood cell (WBC) profiles to a stable state[10]. This approach suggests that the parallel study of oxidative stress markers, in particular

enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GPX) and malondialdehyde, alongside standard hemolysis biomarkers, may be promising in the future for predicting anemia attacks[46].

The Impact of Biological and Targeted Therapy on Markers

Laboratory indicators are also used to monitor the effectiveness of the most modern targeted therapies. Today, treatment of AIGA is not limited to blood transfusions or splenectomy.

- **Hot AIGA approach:**The first line is glucocorticoids (prednisolone), which suppress the activity of the immune system. They normalize hemoglobin levels in 70-85% of patients and lower markers (reticulocytes, LDH). However, in severe or refractory cases, secondary therapy is used. Rituximab (a monoclonal antibody directed against the CD20 antigen on B lymphocytes) destroys the source of immunoglobulin production at the cellular level. In severe and rapidly deteriorating attacks, a combination of cyclophosphamide, iptacapone, and prednisolone (CPI regimen) is also effective[54]. Also, a special drug called Nipokalimab, an FcRn receptor blocker that reduces the amount of toxic IgG autoantibodies in the bloodstream and shortens their half-life, is undergoing successful trials under the supervision of the FDA (ENERGY studies) and is expected to change the treatment paradigm[24].
- **Special pathway in cold agglutinin disease (CAD):**Unlike warm AIGA, neither steroids nor splenectomy are of any benefit (since hemolysis occurs mainly in the liver and intravascularly)[24]. The only effective mechanism here is B-cell depletion (with Rituximab) or inhibition of the complement cascade. Sutimlimab (a C1s inhibitor), which has recently entered phase 3 clinical trials, directly blocks the activation of proteins in the blood system, eliminates C3d fixation on the surface, and directly preserves the life of the erythrocyte[26]. Patients receiving such drugs recover haptoglobin levels very quickly.

The criteria for monitoring the effectiveness of treatment in laboratory analysis are clear: if hemoglobin increases to >12 g/dL and all hemolytic markers (LDH, bilirubin, haptoglobin) return to full normal, this is called Complete Response. If, despite maintaining hemoglobin, Coombs' test (DAT) remains positive for a long time, this indicates clinical stability, but the presence of active autoantibodies in the blood indicates the possibility of relapse of the disease at any time [9].

Marker Variability and Limitations in Interpreting Results (Laboratory Error Analysis)

In clinical practice, it is often the case that a single marker change in isolation leads to a completely incorrect diagnosis.

1. **Negative interference of transfusion history with markers:**Serological and morphological analyses performed in patients who have received large or incorrect blood transfusions (red blood cell transfusions) give fundamentally misleading results. As a result of the body's collision with foreign blood antigens, the patient begins to form alloantibodies, which leads to false positive results of the Coombs test (DAT and IAT), regardless of the hemolytic process in general [3]. Therefore, according to clinical guidelines, it is strictly required that samples for reticulocyte count, Coombs test and all plasma analyses be collected before the blood transfusion procedure.
2. **The masking effect of liver, kidney and chronic pathologies:**As discussed above, lactate dehydrogenase and unconjugated bilirubin may be unreasonably elevated in the absence of hemolysis, in cases of hepatitis or B12 deficiency (ineffective erythropoiesis), and haptoglobin may be elevated due to inflammation, tumor, or trauma, which can completely confuse the clinical picture.[27] To differentiate between these, focusing diagnostic attention on the reticulocyte index and the amount of the specific isoenzyme of LDH (LDH-1) in the blood adequately resolves the problem.

3. **Immune function and the acute cytokine cascade:**Recent studies have suggested that cytokines such as IL-6 and IL-8 play a role in the pathogenesis of the disease, which is characterized by hyperactivation of B lymphocytes, and that these markers may directly predict future hemolytic attacks or disease aggressiveness[37]. In this regard, in chronic processes, the analysis of a cytokine panel, mainly in conjunction with hemolytic biomarkers, may allow specialists to reduce errors[58].

Conclusion

Hemolytic anemia is a group of diseases that are molecularly and genetically diverse and pathophysiologically very complex. Through a comprehensive and in-depth analysis of the theoretical, fundamental biochemical and morphological data presented in the study, firm clinical conclusions were formed that serve to correctly assess the severity of the disease in practice:

1. The presence of hemolysis in the patient's bloodstream and its clinical severity cannot be determined by a single marker in isolation. The combination of processes such as a sharp decrease in haptoglobin, a sharp increase in lactate dehydrogenase activity, the accumulation of unconjugated bilirubin, and a compensatory increase in the number of reticulocytes in the peripheral blood due to hypoxia is the main formula that definitively confirms the disease. It is a crucial diagnostic need for physicians to take into account the nonspecific properties of dynamically changing markers such as LDH and haptoglobin and the possibility of their masking by concomitant liver diseases or acute inflammatory processes.

2. The correct interpretation of human blood morphology relies not only on traditional microscopic examination (looking for forms such as spherocytes, schistocytes), but also the practice of using digital artificial intelligence analyzers based on deep neural networks, which accurately and without human subjectivity reveal whether the nature of hemolysis is mechanical (microangiopathy) or autoimmune (IgG fixation). Morphology is an indispensable tool for differential analysis.

3. The direct antiglobulin (Coombs) test is the "gold standard" for classifying the underlying hemolytic process. The fact that the test responds differently to IgG or C3d antisera or is completely false negative provides direct support for physicians in choosing the type of medication to prescribe (glucocorticoids, complement inhibitors, or neoantigen removal).

In Uzbekistan and the world, hematology and laboratory medicine are increasingly improving the criteria for the differentiation of autoimmune, hereditary and iatrogenic (drug-induced) hemolytic anemias. Due to the widespread introduction of cytokine panels, next-generation sequencing (NGS) genetic tests and molecular flow cytometry into practice, laboratory tests will provide the basis for the rapid prediction of severe complications such as ischemia, stroke and multi-organ hypoxia that are expected to occur in patients in the future, and for the correction of specific treatment tactics.

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